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A study of the links between the ASPPs and the Wnt/β-catenin signalling pathway

By

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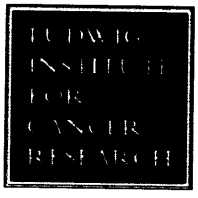

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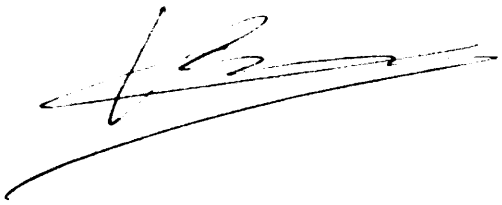

Abstract

The identification of the ASPP family of proteins has helped towards a better understanding of how the function of the tumour suppressor p53 could be specifically regulated. Hence, ASPP1 and ASPP2 bind to- and specifically enhance the transactivation function of p53 on the promoters of proapoptotic genes, whereas iASPP acts as an inhibitor. However, yeast-two-hybrid experiments have shown that the ASPPs, and in particular ASPP2, also interacts with a large number of proteins involved in other major signalling pathways, suggesting that they could be a multi-function family of proteins.

One of ASPP2-interacting partners is Adenomatous Polyposis Coli 2/L (APC2/APCL). APCL is a central nervous system-specific homologue of APC, a key factor in the Wnt signalling pathway. As the biological significance of this interaction remains unknown, we explored the interplay between the ASPPs and the APC family. We show that APC and APCL can affect the subcellular localization of ASPP1 and ASPP2 as well as positively regulate their function on p53.

As APCL, like APC, is believed to be able to target β-catenin for degradation, we investigated whether ASPP2 could play a role in the regulation of β-catenin. Herein, we show that ASPP2 is a negative regulator of β-catenin transcriptional activity and that they can form a complex at the level of the *adherens* junctions.

In vivo, ASPP2-null mice exhibit severe anomalies in the central nervous system (CNS) formation during embryonic development. Hence, the eyes and the brain were characterized by increased number of cells, poor cellular differentiation and tissue disorganization. Cells expressing nuclear β-catenin were found in a very limited and organized apical region of the developing cortex in the wild-type, whereas in the ASPP2-deficient embryos, they were disseminated throughout the overgrown regions of the brain, suggesting that ASPP2 may regulate β-catenin function during CNS development.

Finally, the involvement of ASPP2 in the regulation of β-catenin transcriptional activity could contribute to its function as a tumour suppressor and, importantly, its role in epithelial integrity suggests that it could be determinant in preventing cellular invasion and metastasis.

To my family

Table of contents

Title.....1

Abstract.....3

Table of contents.....5

Figures and Tables.....11

Acknowledgements.....13

Chapter I: Introduction.....14

1.1 Cancer.....14

1.2 Apoptosis.....20

1.2.1 Introduction.....20

1.2.2 Caspases.....23

1.2.3 Extrinsic apoptotic pathway.....25

1.2.4 Intrinsic apoptotic pathway.....28

1.3 The tumour suppressor p53.....31

1.3.1 P53 is a tumour suppressor gene.....31

1.3.2 The p53 full length protein is a transcription factor.....32

1.3.3 P53 levels and activity are tightly controlled.....34

1.3.4 P53 stabilization and activation.....38

Activation of p53 by phosphorylation at its N- and C-terminus.....38

Activating post-translational modification of p53 in its C-terminus.....40

1.3.5 The p53 response.....41

Overview....41
Cell cycle arrest....42
Apoptosis....42
1.4 New p53 co-factors: the ASPP family of proteins....45
1.4.1 Definition and terminology....45
1.4.2 Function....48
1.4.3 Regulation....50
1.4.4 ASPP interacting partners....51
1.5 The APC family of proteins....54
1.5.1 Overview....54
1.5.2 The APC genes....55
1.5.3 The domains in APC proteins....56
The oligomerization domain....56
The Armadillo region....58
The binding sites for β-catenin: the 15- and the 20-amino acid repeats....59
Axin binding site....60
The basic domain in APC....61
The EB1 binding domain....61
The HDLG binding site....62
1.5.4 The association between APC localization and function...63
APC, component of the β-catenin degradation complex...63
Nuclear-cytoplasmic shuttling of APC....64
APC at the membrane....65
APC at the microtubule tips....66
1.6 The Wnt/β-catenin signalling pathway....67
1.6.1 Brief overview....67
1.6.2 The canonical Wnt/β-catenin signalling pathway....68
1.6.2.1 At the cell membrane....68
The Wnt ligands....68
The Wnt coreceptor: the Fz/LRP complex....70
1.6.2.2 In the cytoplasm....71

The cytoplasmic destruction complex....72
The E3 ligase complex....75
1.6.2.3 In the nucleus....76
How does β-catenin translocate to the nucleus?....76
How does β-catenin activate gene transcription?...77
β-catenin target genes....79
1.6.3 The plasma membrane pool of β-catenin....80
E-cadherin....80
β-catenin....82
α-catenin....83
p120 catenin....84
Dissociation of β-catenin from the adherens junctions....86
1.6.4 The role of the Wnt/β-catenin signalling pathway in development and cancer....87
1.7 Aim of study....90

Chapter II: Materials and Methods....92

2.1 Materials....92
2.1.1 Reagents....92
2.1.2 SDS-polyacrylamide gels....103
2.1.3 Antibodies....104
2.1.4 Plasmids....109
2.1.5 Cell lines....112
2.2 Methods....114
2.2.1 Tissue culture....114
2.2.2 DNA techniques....116
2.2.3 Protein manipulation....120
2.2.4 Cell-based assays....124
2.2.5 *In vitro* assays....127
2.2.6 Mouse work....129

2.2.7 Data analysis.....131

Chapter III: APCL and APC positively regulate the function of ASPP1 and ASPP2 in p53 mediated apoptosis.....132

3.1 Introduction.....132
3.2 Results.....136
3.2.1 APCL induces the translocation of ASPP1 and ASPP2 to a perinuclear region.....136
3.2.2 APCL enhances the ability of ASPP1 and ASPP2 to increase p53 transactivation on the PIG3 promoter.....140
3.2.3 APCL specifically enhances the synergy between p53 and ASPP2 on the promoters of proapoptotic genes.....143
3.2.4 The C-terminal ASPP2 binding region of APCL is partially required for the effect of APCL on the synergy between ASPP1&2 and p53.....145
3.2.5 APC colocalizes with and induces the accumulation of ASPP1 and ASPP2 at the cell extremities.....147
3.2.6 APC positively modulates the synergy between ASPP1&2 and p53 on the PIG3 promoter.....149
3.2.7 Inhibition of GSK3β modulates the function of the ASPPs in the regulation of p53.....153
3.3 Discussion.....157
3.3.1 APCL and APC differentially affect the subcellular localization of ASPP1 and ASPP2.....158
3.3.2 APC and APCL positively regulate the action of ASPP1 and ASPP2 on p53 transactivation of proapoptotic genes.....160
3.3.3 The function of APC and APCL as down-regulators of β-catenin may play a role in the regulation of ASPP1 and ASPP2.....163

Chapter IV: ASPP2: a new negative regulator of β-catenin during the embryonic development of the CNS.....166

4.1 Introduction.....166

4.2 Results.....169

4.2.1 ASPP2 is required to inhibit the Wnt/β-catenin pathway in MEFs.....169

4.2.2 ASPP2 delays the accumulation of β-catenin in the nucleus.....173

4.2.3 ASPP2 favours phosphorylation of β-catenin at serine 45 in MEFs.....176

4.2.4 The binding between ASPP2 and PP1 may play a role in the regulation of β-catenin.....178

4.2.5 ASPP1 and ASPP2 can colocalize with β-catenin where cells form contacts.....183

4.2.6 ASPP2 can bind to β-catenin.....189

4.2.7 The interaction between ASPP2 and β-catenin is cell-cell contact dependent.....192

4.2.8 ASPP2 interacts with β-catenin in the *adherens* junction complex.....196

4.2.9 ASPP2 is essential fir central nervous system development.....201

4.2.10 The role of ASPP2 in CNS development involves its function as a regulator of β-catenin.....206

4.3 Discussion.....211

4.3.1 ASPP2 negatively regulates β-catenin.....212

4.3.2 ASPP2, a new component of the adherens junction complex.....214

4.3.3 ASPP2 is essential to ensure the proper function of β-catenin during the formation of the neuroepithelium and the eye.....215

4.3.4 Summary.....217

Chapter V: APP-BP1 and ubiquitin-like modifications play a role in the regulation of the ASPP family.....218

5.1 Introduction.....218
5.2 Results.....221
5.2.1 APP-BP1 positively regulates the function of ASPP1 and ASPP2.....221
5.2.2 ASPP1 family is highly modified by ubiquitin-like proteins.....224
First indication that the ASPPs are modified.....224
ASPP1 is a better candidate than ASPP2 for ubiquitin-like modifications.....226
5.3 Discussion.....230

Chapter VI: Final discussion and perspectives.....233

6.1 Overview.....233
6.2 ASPP2, a connection between distinct pools of β-catenin.....235
6.3 More binding partners, a role for iASPP mediated regulation of β-catenin.....242
6.4 Concluding remarks and perspectives.....244

References.....246

Figures

Figure 1.1: Apoptosis in the *C. elegans* system....22

Figure 1.2: Classification of mammalian apoptotic caspases....24

Figure 1.3: Simplified scheme of the intrinsic and the extrinsic apoptotic pathways....26

Figure 1.4: p53 structure and post-translational modifications....33

Figure 1.5: Regulation of p53 levels....36

Figure 1.6: p53 mediated apoptosis....44

Figure 1.7: The ASPP family of proteins....46

Figure 1.8: Structure of APC and APCL and binding partners....57

Figure 1.9: Canonical Wnt signalling pathway....69

Figure 1.10: Components of the *adherens* junctions and binding domains....81

Figure 3.1: Structure of APC, APCL and APCLΔ....134

Figure 3.2: immunofluorescence shows that exogenously expressed APCL protein can colocalize in a perinuclear region with the different members of the ASPP family of proteins to different degrees....137

Figure 3.3: APCL stimulates the synergy between p53 and ASPP2 or ASPP1 on PIG3 promoter....141

Figure 3.4: APCL specifically enhances the synergy between p53 and ASPP2 on the promoter of the proapoptotic gene *PIG3*....144

Figure 3.5: the C-terminal truncated form of APCL, APCLΔ, is less potent in its ability to stimulate the synergy between p53 and ASPP1 (A) or ASPP2 (B) on PIG3 promoter....146

Figure 3.6: Exogenously expressed APC protein can colocalize with the ASPP family of proteins....148

Figure 3.7: APC stimulates the synergy between p53 and ASPP2 or ASPP1 on PIG3 promoter....150

Figure 3.8: Effect of GSK3β inhibition on p53 dependant transactivation of *PIG3* together with the ASPPs....154

Figure 3.9: Model of the regulation of p53 mediated apoptosis by the ASPP family of proteins and by the ASPP2-interacting protein APCL....165
Figure 4.1: β-catenin transcriptional activity is upregulated in the absence of ASPP2 in MEFs....170
Figure 4.2: ASPP2-null MEFs are more sensitive than wild-type MEFs to Wnt signalling....174
Figure 4.3: β-catenin phosphorylation status in wild-type and *ASPP2* -/- MEFs....177
Figure 4.4: The binding of PP1 to ASPP2 is important in ASPP2 mediated regulation of β-catenin....180
Figure 4.5: The ASPP family of proteins can colocalize with β-catenin in close relation with cell-cell contacts....184
Figure 4.6: ASPP2 can bind β-catenin....190
Figure 4.7: the colocalization between ASPP2 and β-catenin is cell-cell contact dependant....193
Figure 4.8: ASPP2 interacts with β-catenin within the *adherens* junctions...197
Figure 4.9: Overgrowth in the brain and retina of ASPP2-null embryos is due to overproliferation....202
Figure 4.10: β-catenin and cyclin D1 expression in the overgrown regions of the ASPP2 knock-out neural tube....209
Figure 5.1: APP-BP1enhances the synergy between p53 and ASPP2 or ASPP1 on *PIG3* promoter....222
Figure 5.2: APP-BP1 is involved in ASPP1 stabilization....223
Figure 5.3: The ASPPs are potentially modified by ubiquitin-like proteins..225
Figure 5.4: ASPP1 is strongly modified *in vivo* compared to ASPP2....228
Figure 6 Model illustrating the roles of ASPP2 in p53 mediated apoptosis and in the regulation of β-catenin....236

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Chapter I: Introduction

1.1 Cancer

The last quarter of a century has seen a surge in research directed towards cancer and as a result, our understanding of the disease has greatly increased. It is now widely accepted that dynamic changes in the genome, which may arise due to, for example, lesions in our DNA known as somatic mutations or gene silencing by epigenetic changes such as gene methylation, are fundamental to the development of cancer. Most evidence indicates that most cancers arise from a single cell that has acquired malignant characteristics through the accumulation of genetic mutations (Vogelstein and Kinzler 1993). Cellular clones of the variants resulting from these mutations are then thought to be selected according to their ability to proliferate, regardless of the normal growth-control mechanisms, and their ability to invade and destroy normal tissues. These changes in cell behaviour are used as hallmarks of cancer cells and can be classified into six categories: self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of programmed cell death (apoptosis), limitless replicative

potential, sustained angiogenesis, and tissue invasion and metastasis (Hanahan and Weinberg 2000). Furthermore, insights into the molecular basis of cancer have been enhanced by the notion that the genes involved in these different pathways can be classified in two distinct groups: proto-oncogenes and tumour suppressor genes.

By definition, a proto-oncogene is a normal gene that can become oncogenic due to mutation or increased expression (Todd and Wong 1999). The field of virology has greatly contributed to the discovery and the understanding of oncogenes. *Src* (*c-src*) was the first proto-oncogene to be discovered in the 1970s by Bishop and Varmus (Bishop et al. 1978). Interestingly, *c-src* is closely related to the *v-src* gene, which is expressed by the tumour inducing retrovirus, Rous sarcoma virus (RSV). It is believed that at one point an ancestral virus mistakenly incorporated the *c-src* gene of its cellular host. The normal gene became mutated into an abnormally-functioning oncogene by losing its C-terminal inhibitory phosphorylation site, as it is now observed in RSV (Kmiecik and Shalloway 1987). In this case, *v-src* is known as an instructive example of an oncogene, whereas *c-src* is a protooncogene. Crucially, once the oncogene is transfected back into a normal host, it can lead to cancer.

The products of proto-oncogenes are often proteins involved in the regulation of cellular processes such as cell-cycle, proliferation, survival or angiogenesis and their expression and activity are tightly regulated. As a result of small changes of their original function, proto-oncogenes can become tumour

inducing agents, oncogenes. There are three different types of alterations that can occur, leading to the activation of proto-oncogenes into oncogenes.

Firstly, random point mutations within a proto-oncogene can induce, for example, conformational changes in the protein or loss of consensus recognition sites by other regulatory units, resulting in an increase in the activity of the protein or a loss of regulation. As a typical example, a point mutation in codon 12 of the *Ras* proto-oncogene, from a glycine to a valine, is sufficient to drive abnormal proliferation in some cell lines due to constitutive Ras activation (Bos 1989).

Secondly, a proto-oncogene can become an oncogene when its concentration reaches abnormal levels. This can be achieved in different ways: through an increase of the levels because of lack of regulation, through an increase of protein stability in the case of a mutation impinging on the half-life of the protein, or through gene duplication. The latter is the result of aberrant replication which can result in gene amplification with several copies of a gene in the genome. This can often be observed in the case of the proto-oncogene c-Myc which, as a consequence, is overexpressed and drives cell proliferation (Wong et al. 1986).

Lastly, chromosomal translocation causing either an increase in gene expression or the expression of a constitutively active hybrid protein can also be responsible for the activation of proto-oncogenes into oncogenes. This can typically be observed in leukaemia, where the translocation of *Abl* (chromosome 9) and *Bcr* (chromosome 22) results in a fusion protein called Bcr-Abl which is constitutively active. The highly oncogenic aspect of Bcr-Abl is due to its ability to activate a number of cell-cycle controlling proteins (Ren 2005).

In contrast, tumour suppressor genes play a crucial role in preventing the formation of tumours. Unlike oncogenes, tumour suppressors are recessive and have been described to follow the "two hit" theory (Knudson 1971), as tumourigenesis requires inactivation of both alleles. However, some non classical tumour suppressors are haploinsufficient, meaning that the loss of one allele alone is sufficient to confer a selective advantage towards tumour growth (Cook and McCaw 2000). Furthermore, the number of genes belonging to this category is probably underestimated due to the difficulty in finding them using the traditional approach of identifying tumour suppressor genes. This approach has been to pinpoint small chromosomal regions of loss of heterozygocity (LOH), to narrow the critical region by deletion mapping and finally to search the intact homologous chromosome segment for mutated genes whose function can be demonstrated to protect against cancer development (Sherr 2004). This strategy will, therefore, fail to identify those target genes, which are haploinsufficient for tumour suppression.

Tumour suppressors include a wide range of genes generally involved, directly or indirectly, in the repression of cell cycle or in the promotion of apoptosis. However, it is possible to roughly classify them according to three different main subgroups (Kinzler and Vogelstein 1998): the "gatekeepers", the "caretakers" and "landscapers". The former are genes whose function is directly involved in the control of cell growth, such as p53, Rb, VHL and APC. Their failure to function correctly creates an imbalance of cell division over cell death. The "caretakers" of the genome are genes that can indirectly suppress neoplasia

by sensing anomalies in the DNA, and they typically encode proteins involved in DNA repair. When these genes are inactivated, this leads to an increase in mutation rate and, therefore, the chances of cancer development are increased. The "landscapers" genes are genes which, upon mutation, can increase the risk of creating an altered stromal environment. This is seen in patients with juvenile polyposis syndromes that have an increased risk of the adjacent epithelia becoming malignant.

The past few decades of research have proved that cancer is an extremely complex disease, in the sense that each cancer is unique and involves a wide variety of genes and pathways. As a consequence, finding a cure against cancer is a complex challenge and more innovative and specific therapies are being used to treat particular types of cancer. At present, there is an increasing body of evidence indicating that the cellular and molecular events leading to tumour initiation are driven by cancer stem cell-like cells. For instance, characteristics such as self-renewal, resistance to apoptosis-inducing drugs and cell migration are all stem cell-like features that suggest their involvement in tumour progression. Therefore, eradication of the stem-cell compartment of a tumour may be the essential and most effective way of curing cancer and allowing long-lasting remission. As the ability of tumour cell populations to expand in number is determined not only by the rate of cell proliferation but also by the rate at which cells are eliminated, programmed cell death or apoptosis represents a major brake to cellular expansion; a fact which is highlighted by the increasing number of reports

demonstrating that acquired resistance towards apoptosis is a hallmark of most, and perhaps all, types of cancer.

1.2 Apoptosis

1.2.1 Introduction

Apoptosis is one of the main types of programmed cell death and it was described for the first time in 1972 (Kerr et al. 1972). Apoptosis plays a vital role in the maintenance of tissue homeostasis and in controlling cell number during development (Prindull 1995). Therefore, the integrity of apoptotic mechanisms is 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 diseases. Apoptosis is characterised by cell membrane blebbing, cell shrinkage, chromatin condensation, DNA fragmentation (Wyllie 1980; Wyllie et al. 1980) and randomization of the distribution of phosphatidyl serine between the inner and the outer leaflets of the plasma membrane (Nicoletti et al. 1991). Ultimately cells disintegrate into membrane bound apoptotic bodies and this is followed by the intervention of macrophages or neighbouring cells, thereby avoiding any inflammatory response in the surrounding environment. The absence of the inflammatory response is one of the major differences with another form of cell death, necrosis, as during this process, cellular content is released in the direct environment (Savill and Fadok 2000). In addition to the morphological and molecular changes involved, apoptosis differs

in regard to necrosis by the fact that the genetic programme activated in response to apoptosis is tightly regulated.

The basis of the knowledge on programmed cell death has been provided by studies in *C. elegans*, since many of the molecular actors of apoptosis have their homologues in human. *C. elegans* has been a particularly good model to study as it has a less complex apoptotic pathway in comparison to what can be observed in humans and extensive apoptosis takes place during its development, where 131 of the 1090 somatic cells generated are eliminated this way (Brenner 1974). The 4 important genes involved in the apoptotic pathway in *C. elegans* are EGL-1, CED-9, CED-4 and CED-3 (Figure 1.1). In healthy cells, the anti-apoptotic protein CED-9 binds to CED-4 and inactivates it. EGL-1 is able to initiate cell death by binding to CED-9, releasing CED-4 from the CED-4/CED-9 complex and allowing it to activate CED-3 (caspase homologue).

In mammals, two main apoptotic pathways are commonly described: the extrinsic (or the death receptor pathway) and the intrinsic (the mitochondrial pathway) pathways. These two pathways, although distinct from each other, converge on caspase-3 and subsequently on other proteases and nucleases that drive the terminal event of programmed cell death. Thus, caspases are key regulators of apoptosis.

C. elegans

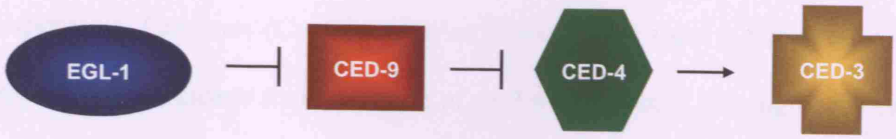


Figure 1.1: Apoptosis in the *C. elegans* system. EGL-1 initiates cell death by binding to CED-9 thus removing its inhibitory effect on CED-4. CED-4, in turn, then activates CED-3 which execute apoptosis by cleaving multiple key cellular proteins.

1.2.2 Caspases

Caspases are a family of proteins that represent one of the main effectors of apoptosis. Caspases (Cysteine ASPartyl proteASE) form a family of cysteine proteases which cleave their substrate at an Asp-Xxx bond, although each caspase has its distinct substrate specificity. The activity of caspases is tightly regulated in order to prevent unwanted cell death and therefore, they are synthesised as inactive precursors, zymogens, made of an N-terminal prodomain, a large subunit (p20) and a small subunit (p10). Upon apoptosis activation, the three parts of the zymogens are cleaved and two large and two small subunits assemble to form an active heterotetramer (Walker et al. 1994). There are currently around 14 mammalian caspases that have been identified (Alnemri et al. 1996) and it is possible to regroup them in three different categories according to their functions: inflammatory caspases, apoptotic initiator caspases and apoptotic effector caspases (Figure 1.2). The former category contains caspases involved in inflammation rather than apoptosis. Initiator caspases are upstream in the activation cascade of caspases. Their prodomain is longer and contains either a death effector domain (DED) (caspase-8 and -10) or a caspase activation and recruitment domain (CARD) (caspase-2, -9), allowing them to interact with upstream adaptator molecules. Upon activation, the initiator caspases are able to process effector caspases and activate them (Degterev et al. 2003). The effector caspases are characterized by a short prodomain and they execute apoptosis by cleaving multiple key cellular proteins, including proteins involved in DNA repair

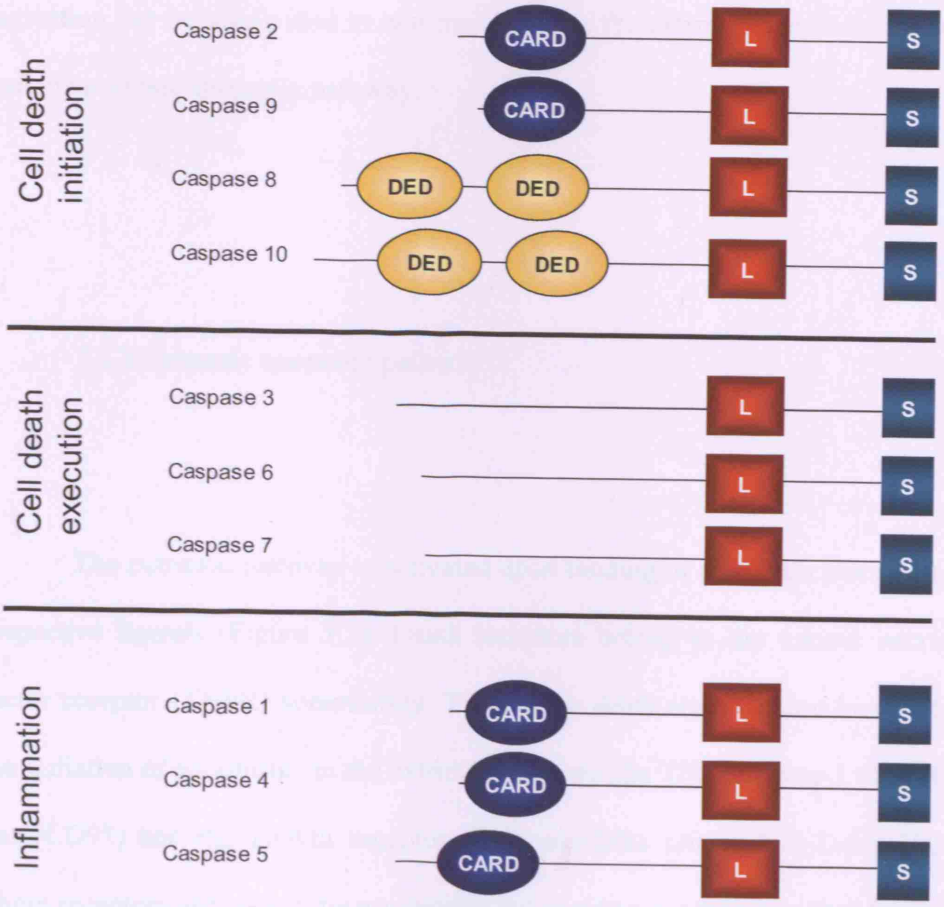


Figure 1.2: Classification of mammalian apoptotic caspases. Above is a schematic representation of the inactive precursors of the caspase family. Each precursor is made up of an N-terminal domain and a large (L) and small (S) subunit. CARD = caspase activation and recruitment domain ; DED death effector domain.

(DNA-PK, PARP), cell cycle proteins and structural proteins (nuclear laminis, fodrin, gelsolin, β-catenin, etc), thus explaining the change of cellular morphology during apoptosis. The vast array of proteins targeted makes caspases the final effectors of apoptosis and the apoptotic signalling pathways that lead to their activation can be subdivided in two major routes: the extrinsic apoptotic pathway and the intrinsic apoptotic pathway.

1.2.3 Extrinsic apoptotic pathway

The extrinsic pathway is activated upon binding of death receptors to their respective ligands (Figure 1.3). Death receptors belong to the tumour necrosis factor receptor (TNFR) superfamily. Three main death receptors are involved in the initiation of apoptosis via the extrinsic pathway: the TNF receptor-1 (TNFR1), Fas (CD95) and the TRAIL receptors DR4 and DR5 (Jin and El-Deiry 2005). These receptors not only induce apoptosis but can also trigger a variety of cellular responses depending on the cell type. As the Fas pathway plays a central role in the physiological regulation of apoptosis and as it has been studied in detail, it will be taken as an example to illustrate the extrinsic apoptosis pathway.

Binding of Fas ligand to its receptor induces the formation of the death-inducing signalling complex (DISC) (Budihardjo et al. 1999). The first step of the complex formation consists of the trimerization of the receptors, which allows

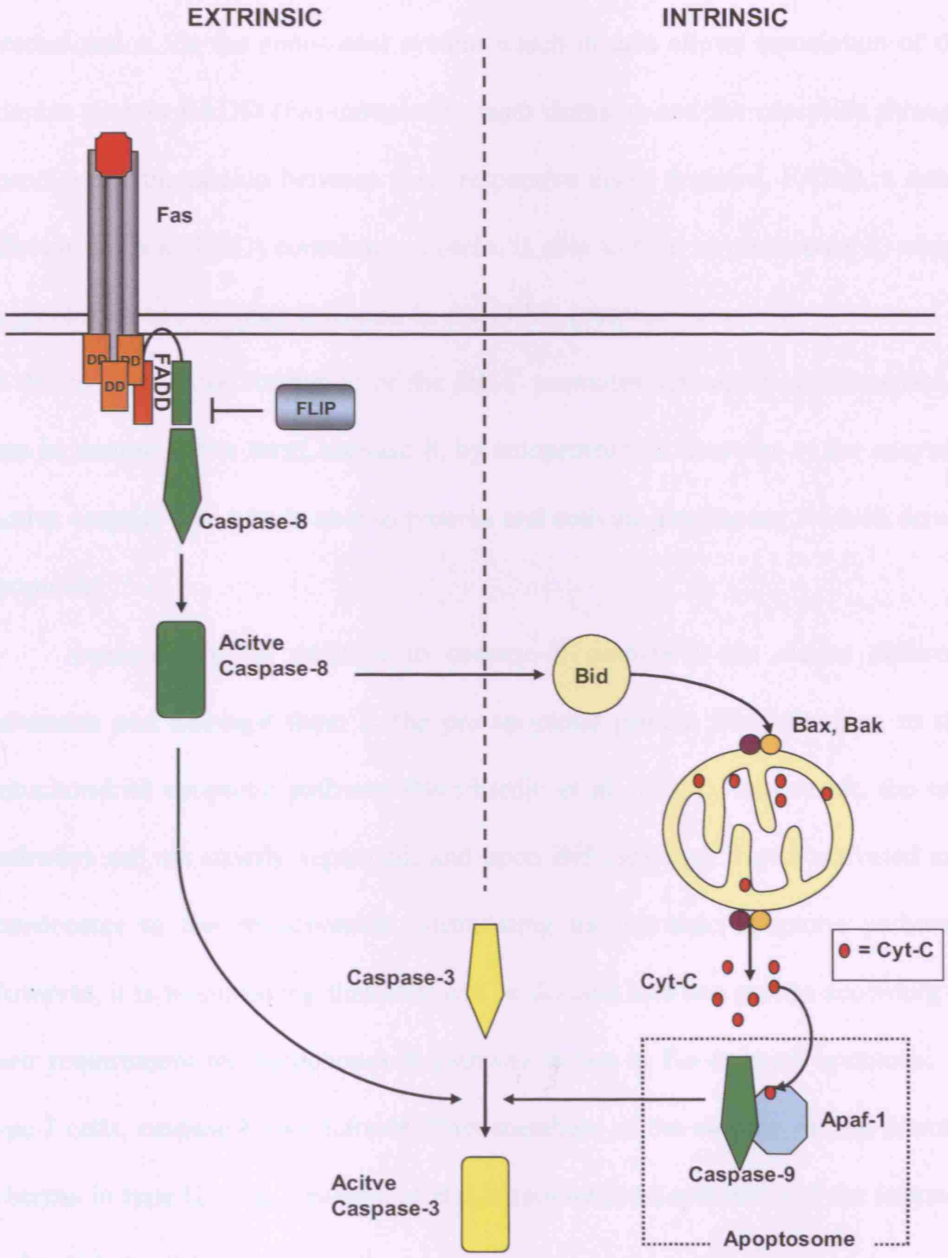


Figure 1.3: Simplified scheme of the intrinsic and the extrinsic apoptotic pathways. Extrinsic pathway: Fas-ligand (in red) induces the trimerization of its receptor Fas which eventually leads to the recruitment of the other components of the death-inducing complex. The adaptator protein FADD (Fas associated death domain) interacts with Fas through its death domain (DD) and allows the recruitment and the activation of caspase-8. Intrinsic pathway: as a result of the activation of members of the Bcl-2 family (Bid, Bax, Bak) the permeabilization of the outer membrane of the mitochondria allows the release of cytochrome C (Cyt-C) and the formation of the Apoptosome. Both pathways ultimately trigger the activation of caspase-3.

intracellular clustering of their death domains (DD). This is followed by internalization via the endosomal system which in turn allows association of the adaptor protein FADD (Fas-associated death domain) and the receptors through homologous interaction between their respective death domains. FADD, a death effector domain (DED) containing protein, is able to bind to procaspase 8, which is present as two distinct isoforms in the DISC (caspase-8a and -8b) (Alnemri et al. 1996). Hence the formation of the DISC promotes activation of procaspase 8 into its mature active form, capsase-8, by autoproteolytic cleavage of the enzyme. Active caspase-8 in turn is able to process and activate procaspase 3 which drives apoptosis.

Interestingly, in addition to caspase-3, caspase-8 can cleave different substrates and amongst them is the pro-apoptotic protein Bid belonging to the mitochondrial apoptotic pathway (Budihardjo et al. 1999). As a result, the two pathways are not strictly separated, and upon Bid cleavage, it gets activated and translocates to the mitochondria, stimulating the intrinsic apoptotic pathway. However, it is worth noting that cells can be divided into two groups according to their requirement for mitochondrial pathway or not in Fas-induced apoptosis. In type I cells, caspase-8 can activate other members of the caspase family directly whereas in type II cells, cleavage of Bid is required and activation of the intrinsic pathway is required.

There are many ways by which the death receptor pathway is negatively regulated. FLIP, which can be found in the DISC, is an important regulator of the Fas pathway. Both the viral protein v-FLIP and its mammalian homologue contain tandems of death effector domains and can therefore compete with caspase-8

within the DISC to inhibit Fas-mediated apoptosis. However, the exact role of FLIP is still controversial as some studies have suggested that it might be able to activate caspase-8. The production of decoy receptors can also be used to regulate this pathway as they compete for ligands and lack a functional death domain, thereby being unable to elicit a downstream response. In the case of Fas, there is a soluble decoy receptor called DcR3. This form of negative regulation is preponderant in the lung and colon carcinomas, where more than 50% of these carcinomas overexpress the DcR3 decoy receptor for Fas-L (Budihardjo et al. 1999). Lastly, proteins like the viral protein CrmA can inhibit the death receptor pathway by inhibiting the proteolytic activation of caspase-8.

1.2.4 Intrinsic apoptotic pathway

In addition to the death receptor pathway which is activated upon external stimuli such as cytokines, apoptosis can also be triggered via the intrinsic or mitochondrial apoptotic pathway, which relies on internal stimuli. Mitochondria are the central component of this pathway, but as mentioned later, the endoplasmic reticulum can also play a role (Figure 1.3).

The pivotal event in mitochondrial apoptosis occurs when the outer membrane of the mitochondria is permeabilized as a result of the activation of members of the Bcl-2 family. Once this is achieved, cell death is perpetrated through either the

release of proapoptotic molecules, such as cytochrome C, or through the loss of mitochondrial function (Green and Kroemer 2004).

Members of the Bcl-2 family of proteins play a central role in the intrinsic apoptosis pathway. There are about 20 Bcl-2 family members which all contain at least one of four Bcl-2 homology (BH) domains and can be subdivided into three groups. The first category consists of anti-apoptotic members including Bcl-2 (whose homologue in *C. elegans* is the anti-apoptotic protein CED-9) and four other relatives which promote cell survival (Bcl-XL and Bcl-w are the closest relatives and Mcl-1 and A1 are the most divergent). Structurally, they are made of three or four BH domains and a transmembrane domain. The pro-apoptotic family members are spread across two other groups: The multi-BH domain, such as Bax, Bak and Bok, which share three domains with Bcl-2 (BH1, BH2 and BH3) and the BH3-only proteins (corresponding to EGL-1 in *C. elegans*), such as Bid, Bad, Noxa and Bim. Both pro-apoptotic groups seem to be required to trigger apoptosis, but the damage-sensing BH3-only proteins seem to be upstream of Bax and Bak as they cannot induce apoptosis in cells deficient for both Bax and Bak (Zong et al. 2001).

When cytochrome C is released into the cytoplasm from the inter-membrane space of mitochondria, it is able to bind to and activate the Apaf-1 adaptator protein (the homologue of the *C. elegans* protein CED-9). The complex formed, with the use of ATP, is called the apoptosome and this is then able to process pro-caspase 9 which in turn will be able to activate effector caspases such as caspase-3 (Li et al. 1997). The second group of proteins released from the

mitochondria upon permeabilization by the BH123 proteins, are the inhibitors of apoptosis proteins (IAPs) antagonists, including Smac/DIABLO, HtrA2/Omi and GSPT1/eRF3. IAPs can normally slow down apoptosis by binding to activated caspases. The antagonists of IAPs, such as Smac for example, favour apoptosis by binding to IAPs and preventing them to efficiently bind to caspases (Du et al. 2000).

The endoplasmic reticulum (ER) compartment is the second organelle to take part in the intrinsic apoptotic pathway. The ER plays a role in controlling the proper folding of proteins and their glycosylation. Stresses such as oxidative stress or chemical toxicity can result in the misfolding of proteins and disturbed calcium homeostasis, resulting in activation of the unfolded proteins response. However, if stress persists, apoptosis can be triggered. ER represents the main stock of Ca^{2+} ions and during ER induced apoptosis, these are released. Mitochondria are able to sense this influx of ions and as a consequence release cytochrome C which can bind to $InsP_3$ receptors on the adjacent ER in a positive regulatory loop and amplify the release of Ca^{2+} ions (Rao et al. 2004). This eventually leads to the activation of caspases and apoptosis.

1.3 The tumour suppressor p53

The balance between pro- and anti-apoptotic molecules is a fragile equilibrium that decides between life and death of the cell. Transcription plays an important role in the regulation of the levels of the different actors of life and death. P53, as a transcription factor mainly, has a crucial role in the regulation of this equilibrium, as it can modulate the expression of proteins of both the intrinsic and the extrinsic apoptotic pathways. As a result, one of its major functions is to trigger apoptosis.

1.3.1 P53 is a tumour suppressor gene

P53 was the first tumour suppressor gene to be discovered in 1979 (DeLeo et al. 1979; Kress et al. 1979; Lane and Crawford 1979; Linzer and Levine 1979) but its function as so was only discovered almost a decade later (for reviews see (May and May 1999; Vogelstein et al. 2000)). P53 was found to be lost or inactivated by mutations in about half of all human cancers and is the most commonly altered gene in human tumours (see (May and May 1999) and references within). Studies in mice lacking functional p53 further demonstrated

the role of p53 as a tumour suppressor, as they proved to be predisposed to early development of tumours (Donehower et al. 1992; Jacks et al. 1994). In agreement with its tumour suppressor role, p53 controls a wide range of cellular processes such as cell cycle control, DNA repair, genome stability, programmed cell death or apoptosis, differentiation, senescence and angiogenesis (May and May 1995).

1.3.2 The p53 full length protein is a transcription factor

P53 is 393 amino acids long and contains four functional domains: a transcription activation domain, a DNA-binding or core domain, an oligomerization domain and finally a regulatory domain (Figure 1.4). Additionally, p53 contains a proline rich domain, which is recognized by SH3 domains, located between the transcriptional activation domain and the core domain.

Within its N-terminus, P53 contains an acidic domain resembling those previously described in well-characterized transcription factors. Further studies showed that the transactivation domain of p53 is contained between amino acids 1 to 42 (Unger et al. 1993). P53 has the ability to interact with double stranded DNA sequences via its DNA binding domain (amino acids 102 to 292) and it is thought to bind as a tetramer to specific consensus sequences of DNA (Kern et al. 1991; el-Deiry et al. 1992; Bargonetti et al. 1993; Clore et al. 1994). As a transcription

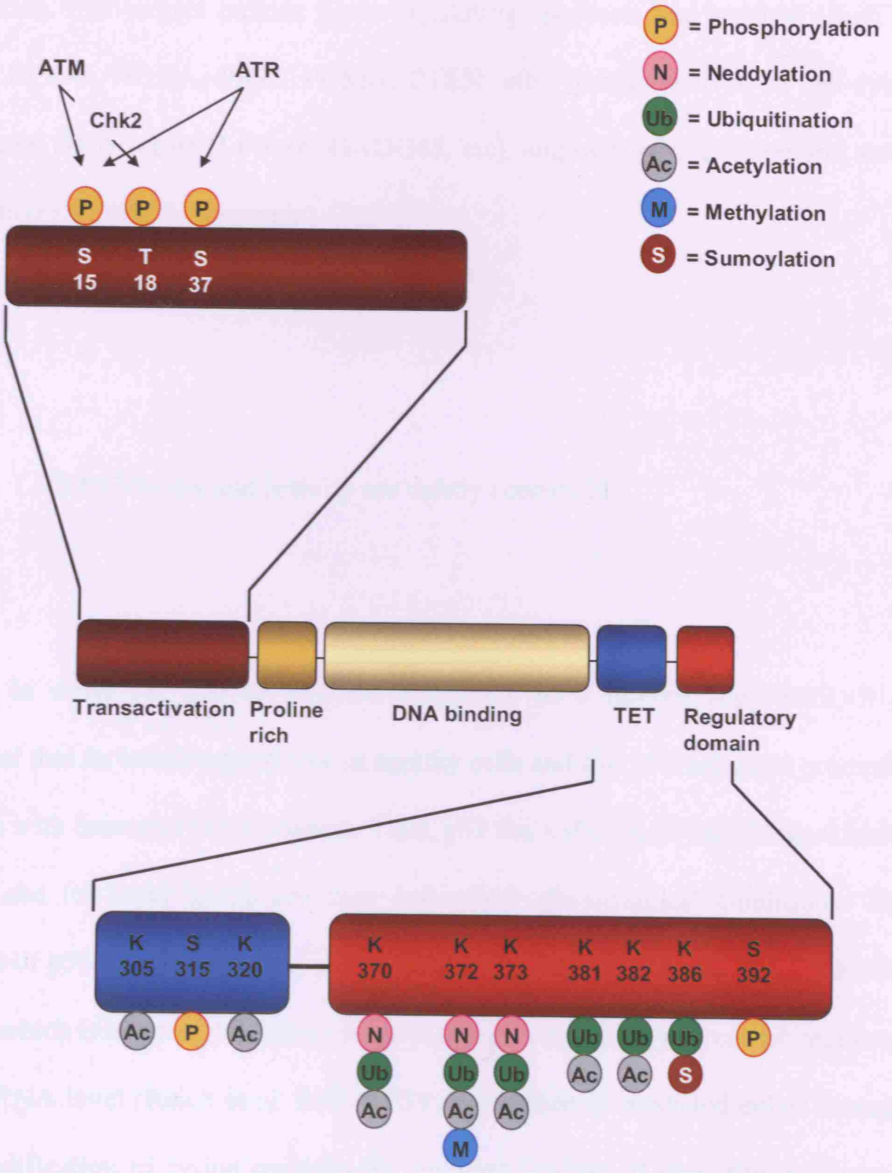


Figure 1.4: p53 structure and post-translational modifications. P53's four functional domains (TET = tetramerisation domain) and the Proline rich region are represented, together with a list of the ways it can be post-translationally modified. A non-exhaustive list of the residues that these modifications take place on is also shown.

factor, p53 can regulate a vast number of target genes either by transactivation or repression. P53 targets include genes regulating apoptosis and survival (such as BAX, BCL-2, NOXA, PIG3, PUMA, PTEN, etc), genes involved in cell-cycle arrest and DNA repair (14-3-3σ, GADD45, etc), angiogenesis, invasion and auto-regulation (MDM2 for example).

1.3.3 P53 levels and activity are tightly controlled

In order for p53 to function efficiently as a tumour suppressor, it is essential that its levels remain low in healthy cells and that it is activated promptly in cells with damaged DNA content. Thus, p53 has a short half-life (Blagosklonny 1997) and its basal levels are kept low under physiological conditions. The amount of p53 in cells is mainly determined by its degradation rate rather than the rate at which it is made. However, there is also evidence that p53 can be regulated at the RNA level (Reich et al. 1983). P53 degradation is mediated either through the modification of lysine residues by covalent linkage of ubiquitin or through various ubiquitin-independent pathways. P53 ubiquitination occurs on lysine residues located at the C-terminus (Yang et al. 2004) (Figure 1.4). The ubiquitination process is generally the result of a cascade of reactions involving a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2) and a ubiquitin-ligase (E3). Several ubiquitin-ligases, like Mdm2, COP1 or Pihr2, have

been shown to be involved in the ubiquitination of p53 (Figure 1.5) (Haupt et al. 1997; Leng et al. 2003; Dornan et al. 2004). The array of E3 ligases available to regulate p53 levels suggest that both Mdm2-dependent and -independent mechanisms are used cooperatively by the cells. The circumstances under which these ubiquitin-ligases might be differentially activated and how they are specifically regulated remains poorly understood

The importance of Mdm2-mediated regulation of p53 has been highlighted by studies in transgenic mice; *Mdm2* knock-out mice are embryonic lethal whereas *Mdm2* and *p53* double knock-out are viable (Jones et al. 1995). Mdm2 binds to p53 in its amino terminal transactivation domain and blocks its transcriptional activity (Momand et al. 1992; Oliner et al. 1993). However, the principal way in which Mdm2 inactivates p53 function is through its function as an E3 ubiquitin-ligase, thus, targeting p53 for degradation by the proteasome (Haupt et al. 1997). It has been shown that depending on the Mdm2:p53 ratio, Mdm2 can either trigger monoubiquitination and proteasomal degradation of p53 in the cytoplasm at low ratio or induce polyubiquitination and nuclear degradation at high ratio (Li et al. 2003). Mdm2 can also promote p53 degradation by facilitating its nuclear-cytoplasmic export (Roth et al. 1998).

Recently, Mdm2 was shown to have an E3 NEDD8-ligase activity towards p53, which results in the inhibition of p53 transcriptional activity, suggesting that the neddylation pathway might play an important role in the regulation of p53 activity (Xirodimas et al. 2004) (Figure 1.4). The canonical neddylation pathway, which is a ubiquitin-like pathway, involves an E1-like-activating enzyme composed of APP-BP1 and hUba3 and an E2-like conjugating enzyme called

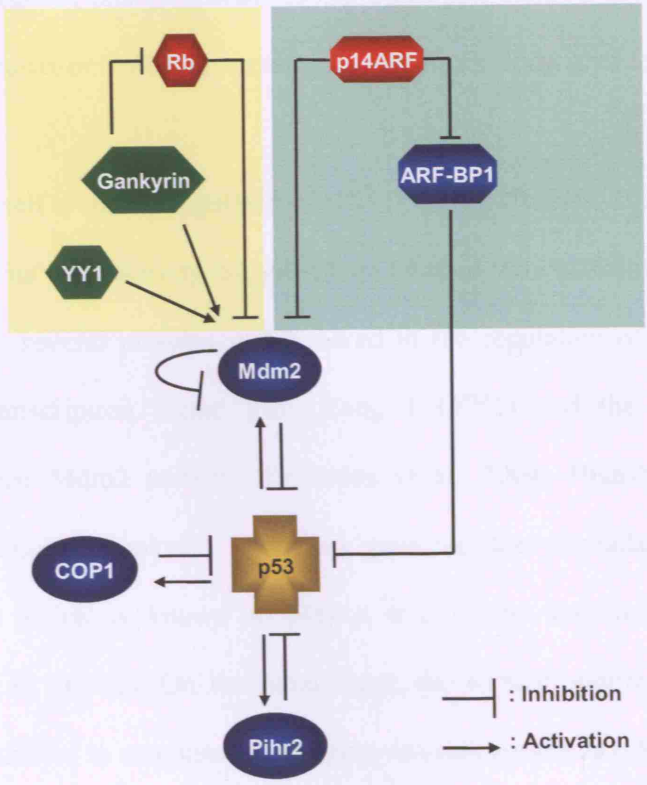


Figure 1.5: Regulation of p53 levels. P53 cellular levels are tightly controlled in a ubiquitin-dependant manner by E3-ligases such as Mdm2, Pirh2, COP1 or ARF-BP1. Mdm2 itself is tightly regulated, in an auto-regulatory manner or by other proteins, such as Rb and p14ARF.

hUbc12. In the case of p53, Mdm2 plays the role of the E3-like-ligase and induces its neddylation.

Interestingly, p53 is found to regulate Mdm2 expression, since Mdm2 is a transcriptional target of p53 (Wu et al. 1993). Thus, p53 controls the levels of its own negative regulator (Mdm2), creating a feedback loop that controls p53 activity.

Mdm2 activity itself is tightly regulated in cells (Figure 1.5). First of all, Mdm2 is able to regulate its own activity by self-ubiquitination which maintains its low levels. However, several proteins are involved in the regulation of Mdm2. For instance, the transcription factor Ying Yang 1 (YY1) and the oncoprotein Gankyrin enhance Mdm2 activity (Gronroos et al. 2004; Higashitsuji et al. 2005a). Interestingly, Gankyrin can also promote the degradation of the oncoprotein Rb which is known to play a role in the stabilization of p53 (Higashitsuji et al. 2005b). On the other hand, the tumour suppressor $p14^{ARF}$, which can be activated in response to oncogene activation such as c-Myc and Ras, can negatively regulate Mdm2 and consequently activate p53 (Gallagher et al. 2006). Interestingly, $p14^{ARF}$ has been shown to bind to ARF-BP1 (also known as MULE or HectH9) and inhibit its function as an E3 ubiquitin-ligase of p53.

Finally, it has been suggested that p53 levels can also be regulated via ubiquitin-independent pathways; for instance, the NAD(P)H:quinone oxidoreductase 1 (NQO1) interacts and stabilizes p53 (Anwar et al. 2003). When p53 is released from the complex, it gets degraded through a ubiquitin and Mdm2 independent pathway.

Several major routes are considered to activate p53. Firstly, two kinases, Ataxia Telangiestasia (ATM) protein kinase and Checkpoint kinase 2 (Chk2), are sequentially activated following exposure to radiation or the presence of high doses of chemotherapeutic drugs, and they phosphorylate p53 on several residues including serine 15 and threonine 18 respectively (Canman et al. 1998). Secondly, in response to ultraviolet light exposure or chemotherapy drugs, p53 is stabilized in an Ataxia Telangiectasia Related (ATR) protein kinase- and caseine kinase II-dependent manner. These two kinases are involved in the phosphorylation of p53 on several residues, including serine 392 and serine 15 (Tibbetts et al. 1999; Keller et al. 2001). Interestingly, it is not uncommon for one particular site to be phosphorylated by various kinases. This redundancy of protein kinases can be explained as an increased level of security, since the inability to phosphorylate these key residues would result in the alteration of p53 tumour suppressor function.

It is believed that several of these phosphorylation events can play a role in the change of affinity between p53 and its various interacting partners occurring after cellular stresses. For instance, phosphorylation of serine 15 plays an important role in the alleviation of the binding between p53 and Mdm2 on one hand, and promotes the binding of p53 to the histone acetyltransferase CBP (CREB-binding protein) on the other hand (Lambert et al. 1998). Other phosphorylations are involved in the conformational changes leading to p53 activation. Namely phosphorylation at serines 315 and 392 may regulate the formation of tetrameric p53 and, therefore, increase the DNA binding ability of p53 (Sakaguchi et al. 1997).

Activating post-translational modification of p53 in its C-terminus

Various types of post-translational modifications, such as acetylation, ubiquitination, sumoylation or methylation can take place in the C-terminal region of p53 (Figure 1.4). As mentioned previously, Mdm2 can, not only ubiquitinate, but also neddylate particular residues in the C-terminus of p53. Ubiquitination of p53 is involved in its degradation by the proteasome, whereas neddylation modulates its activity. SUMO-1, another ubiquitin-like molecule has been reported to modify p53 at lysine 386, but the role of this modification is not fully understood (Gostissa et al. 1999). In addition to Mdm2, several histone acetyltransferases (HATs) such as CBP, p300 and p300/CBP-associated factor (pCAF) have been shown to be able to modify p53 by acetylation of specific residues in its C-terminus (Gu et al. 1997; Lill et al. 1997; Scolnick et al. 1997). The general consensus is that these HATs are co-activators of p53. Finally, Methylation of the C-terminal region of p53 by Set9 methyltransferase has been shown to increase the stabilization of p53 in the nucleus (Chuikov et al. 2004).

1.3.5 The p53 response

Overview

The tumour suppression function of p53 is mainly driven by its ability to regulate the transcription of particular genes, and this is highlighted by the nature of p53 mutations in cancer, which mainly affect the DNA binding domain (80% of all mutations, www-p53.iarc.fr). Moreover, the ability of p53 to act as a transcription factor is evolutionary conserved.

To activate gene transcription, p53 interacts with the basal transcription machinery via its N-terminal transactivation domain, and recruits HATs in order to acetylate histones, thus making chromatin more accessible. In a cooperative fashion, P53 can also induce histone methylation by binding to methyltransferases proteins such as CARM1 and PRTM1 (An et al. 2004). Importantly, p53 can also repress gene transcription in some cases, which is thought to be achieved by the sequestration of the TATA-binding protein (TBP), a component of the TFIID complex, by direct binding to p53 (Seto et al. 1992). As mentioned previously, p53 recognizes consensus binding sites in the promoters of its target genes and more recently, a study based on the whole human genome predicted that p53 would be able to bind to 542 different loci (Wei et al. 2006). As a result, p53 is involved in the regulation of numerous pathways and cellular processes, among which cell cycle arrest and apoptosis are the most studied functions of p53.

Cell cycle arrest

Treatment of cells with DNA-damaging agents can induce p53-dependent arrest at both the G1 and G2 cell cycle stages. This is driven by the induction of the cyclin-dependent kinase (CDK) inhibitor $p21^{WAF1/CIP1}$, which is a crucial target gene of p53 in this process (Figure 1.6) (el-Deiry et al. 1993). CDKs and their corresponding cyclin proteins are key regulators of the progression through the different phases of the cell cycle. $P21^{WAF1/CIP1}$ inhibits the activity of several CDKs, including cyclin E-CDK2 and cyclin A-CDK2 and has been shown to be needed for the activation of cyclin D-CDK4/6 (Sugimoto et al. 2002). In addition, G2 cell cycle arrest is also mediated by two other p53 target genes *Reprimo* and *14-3-3σ* (Hermeking et al. 1997; Ohki et al. 2000).

Apoptosis

P53 mediated apoptosis is thought to be the most conserved function of p53, as organisms such as C. elegans and D. melanogaster are capable of p53-induced apoptosis, but not cell cycle arrest (Ollmann et al. 2000; Schumacher et al. 2001). P53 triggers apoptosis via modulating the expression of genes involved in both the intrinsic and extrinsic apoptotic pathways at almost every level (Figure 1.6). For instance, p53 can induce the expression of death receptor Fas and the TRAIL receptor, DR5 (Wu et al. 1997; Muller et al. 1998). Moreover, the expression of many of the Bcl-2 family of proteins can be regulated by p53. Consequently, p53 represses the expression of anti-apoptotic Bcl-2 proteins, such

as Bcl-2 itself and Bcl-X_L (Ryan et al. 2001) and activates the expression of proapoptotic Bcl-2 genes, such as Bax and BH3-only proteins, such as NOXA and PUMA (p53 upregulated modulator of apoptosis) (Oda et al. 2000; Yu et al. 2001). Interestingly, the latter has been shown to be essential for p53 mediated apoptosis, as PUMA deficient mice can reproduce almost all the apoptotic deficiencies observed in *p53* knockout mice (Yu and Zhang 2003). P53 can also influence apoptosis at the level of the apotosome, by inducing the expression of APAF-1 (Robles et al. 2001). Finally, p53 has been shown to be able to impinge on mitochondrial apoptosis by inducing the expression of a series of p53 induced genes (PIG) (Polyak et al. 1997). These genes encode cytoplasmic proteins which generate reactive oxygen species (ROS) that ultimately lead to mitochondrial mediated death. Among them, PIG3, a quinine oxidoreductase homologue, is the best characterized (Contente et al. 2002). It is worth noting that p53 is also believed to modulate the expression of genes involved in ER mediated cell death, such as the apoptotic protein Scotin (Bourdon et al. 2002).

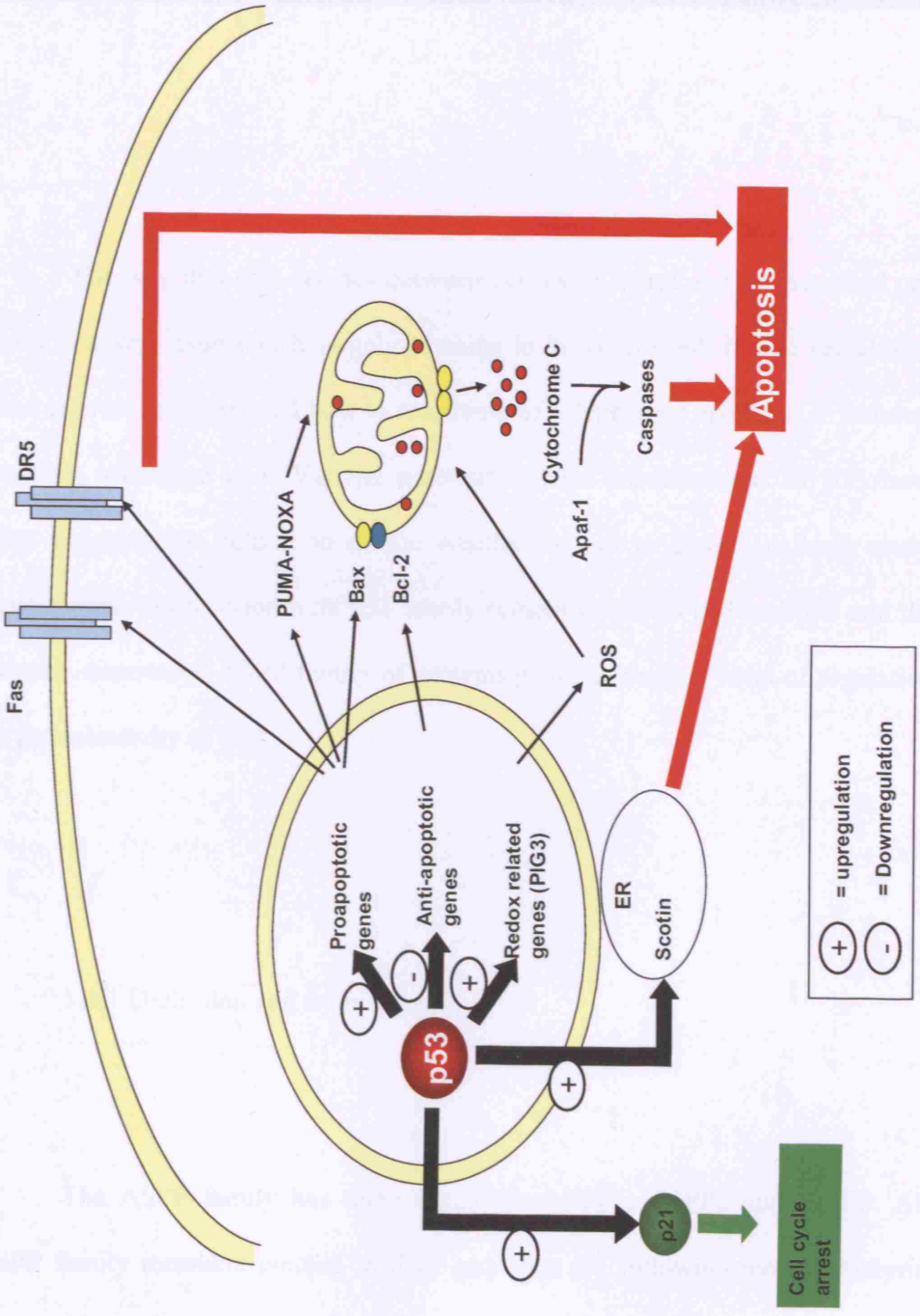


Figure 1.6: p53 mediated apoptosis. Upon stabilization and activation, p53 can induce cell cycle arrest or apoptosis. This diagram depicts how p53 can drive apoptosis at various levels by regulating the expression of groups of genes involved in the intrinsic (PUMA, NOXA, Bax, Bcl-2) or the extrinsic apoptotic pathways (Fas, DR5), redox related genes (PIG3) and genes involved in endoplasmic reticulum (ER) mediated apoptosis (Scotin).

1.4 New p53 co-factors: The ASPP family of proteins

The way that p53 decides between cell cycle arrest and programmed cell death is a key issue which largely remains to be elucidated. It also remains of great interest to understand how to reactivate p53 dependent apoptosis in tumours retaining wild-type p53. Various post-translational modifications of p53 have been suggested to help it to decide whether or not to choose a death route. Furthermore, cooperation with p53 family protein members p63 and p73 and the recently discovered ASPP family of proteins provides another level of regulation for the selectivity of p53.

1.4.1 Definition and terminology

The ASPP family has three members: ASPP1, ASPP2 and iASPP. All ASPP family members contain in their sequence the following motifs: Ankyrin repeat, SH3 domain and Proline rich region. ASPP also stands for Apoptosis Stimulating Protein of p53 to reflect its biological function identified so far (Figure 1.7).

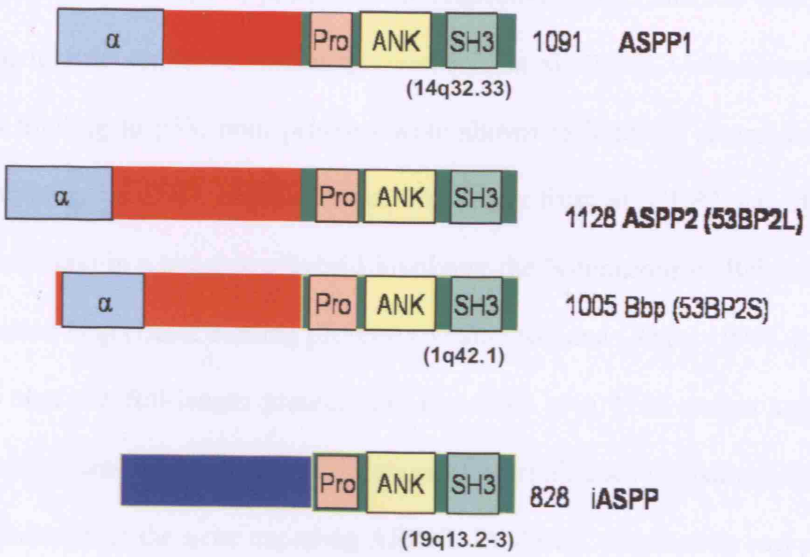


Figure 1.7: The ASPP family of proteins. The ASPP family consists of three members: ASPP1, ASPP2 and iASPP. ASPP2 can be spliced to generate 53BP2S. The ASPP family is characterized by three conserved domains in its C-terminus: a proline rich region (Pro), ankyrin repeats (ANK) and an SH3 domain. Additionally, ASPP1 and ASPP2 have an alpha-helical domain in their N-terminus (α). The chromosomal locus for each gene is indicated in brackets and the length in amino acids of each protein is indicated on the right hand side.

In a yeast-two hybrid, using a mouse p53 bait (residues 73-390) to screen a transformed B-cell cDNA library, two p53 binding proteins were identified: 53BP1 and 53BP2. The two proteins were respectively 1027 and 529 amino acids long and had no sequence similarity (Iwabuchi et al. 1994). Unlike many other proteins binding to p53, both proteins were shown to bind the central region of p53 containing the DNA binding domain. A longer form of 53BP2 was identified (1005 residues) in a yeast two-hybrid involving the N-terminus of Bcl-2 and was hence called Bbp (Bcl-2 binding protein) (Naumovski and Cleary 1996). It is only recently that the full-length protein was identified as a 1128 amino acids long protein and it was renamed ASPP2 (Samuels-Lev et al. 2001). Takahashi et al., in 2004, showed that the gene encoding ASPP2, TP53BP2, can encode two proteins by alternative splicing of exon 3, 53BP2S (Bbp) and 53BP2L (ASPP2), of 1005 and 1128 amino acids, respectively. Thus in the recent literature, the full-length protein could equally be referred to as ASPP2 or 53BP2L. The remaining two members of the family, ASPP1 and iASPP were identified by database searching using the Ankyrin repeats, SH3 domain and proline rich region of ASPP2. The latter was already known as a p65RelA inhibitor (RAI) of 315 amino acids (Yang et al. 1999) but again, the coding region was extended to span 828 amino acids and was re-named iASPP (Slee et al. 2004). More recently, iASPP-SV, a new iASPP isoform of 407 amino acids was discovered (Zhang et al. 2007). Surprisingly it was able to inhibit p53 transcriptional activity on both Bax and p21 promoters. iASPP is encoded by *PP1R13L* on chromosome 19 in humans and by *ape-1* in *C. elegans*. *ASPP1* and *ASPP2* are located on chromosome 14 and chromosome 1 respectively (Trigiante and Lu 2006).

1.4.2 Function

ASPP1 and ASPP2 interact with p53 and stimulate p53-induced apoptosis but not cell-cycle arrest, by specifically enhancing the DNA binding and transactivation function of p53 on the promoters of proapoptotic genes (Samuels-Lev et al. 2001). The crystal structure of the 53BP2-p53 complex showed that 53BP2 binds p53 in its DNA binding domain and allowed identification of the contact residues between ASPP2 and p53 (Gorina and Pavletich 1996). ASPP1 and ASPP2 have also recently been shown to be common activators of the p53 family members p63 and p73 by enhancing their apoptotic function, suggesting that they could suppress tumour growth in tumours lacking p53 or expressing mutant p53 (Bergamaschi et al. 2004). Further studies using mouse models revealed that ASPP2 knockout mice die around birth (Vives et al. 2006a; Vives et al. 2006b). As a result, tumour formation was monitored in *ASPP2* heterozygous mice which demonstrated that the absence of one allele alone was sufficient to significantly increase the occurrence of tumours. Moreover, this discrepancy with the wild-type was further enhanced when mice were γ-irradiated. The early onset of tumour development in ASPP2 heterozygous mice was not accompanied by a loss of heterozygosity, suggesting that ASPP2 behaves like a haploinsufficient tumour suppressor gene. This is supported by the fact that ASPP2 has been found to be down-regulated in human tumours, but has not been found deleted or mutated (Samuels-Lev et al. 2001; Liu et al. 2005). Instead the ASPP2 promoter was found to be methylated on CpG islands in tumour cell lines harbouring wild-

type p53 (Liu et al. 2005). Similar observations were made regarding the expression of ASPP1 in lymphoblastic leukaemia (Agirre et al. 2006). Altogether these data indicate that ASPP1 and ASPP2 are tumour suppressors.

In contrast, the short form of iASPP (RAI), which corresponds to the C-terminal part of full-length iASPP as well as Ce-iASPP (*C. elegans* iASPP), was shown to have an inhibitory effect on p53-mediated apoptosis (Bergamaschi et al. 2003). Full-length iASPP was also shown to have the ability to inhibit p53 (Slee et al. 2004). Nevertheless, it was less active than the short form of iASPP, RAI. This was probably due to its predominantly cytoplasmic localization as opposed to the almost exclusive nuclear localization of RAI. This suggests that the N-terminal part of the protein acts as a regulatory domain and that it might be involved in its retention in the cytoplasm. iASPP appears to be the most conserved member of the family since it shares 38% amino acid identity with iASPP from *Caenorhabditis elegans* (Ce-iASPP). Like ASPP1 and ASPP2, iASPP binds to p53 through its ankyrin repeats and its SH3 domain. Most of the p53 contact residues of ASPP2 are identical in iASPP and some of these are conserved in Ce-iASPP. The similarity between the p53-binding regions of ASPP1&2 and iASPP implies that they compete to interact with p53, which suggests an important biological mechanism in the activation or the inhibition of the p53-dependent apoptotic pathway. In support of this, it has been shown that co-expression of iASPP stimulated Ras-mediated transformation by 15 fold (Bergamaschi et al. 2003). Moreover from 40 human breast-tumour samples expressing wild type p53, iASPP was found to be overexpressed in eight tumours among which seven had

normal levels of ASPP1 and ASPP2 (Bergamaschi et al. 2003). Taken together these data suggest that iASPP is an oncoprotein.

1.4.3 Regulation

The history of how the full-length ASPP family were discovered from the initial identification of smaller proteins, suggests that ASPP1, ASPP2 and iASPP expression is regulated by alternative splicing. Analysis of TP53BP2 revealed that two different forms of ASPP2 could be expressed as a result of alternative slicing of exon 3, namely 53BP2L (the full length ASPP2) without exon 3 and 53BP2S (Bbp) with exon 3 (Takahashi et al. 2004). However, nothing is known about the mechanisms regulating the choice between the expression of one or the other. Recently a novel open reading frame of human iASPP encoding a 407 amino acids long protein was identified. The new splicing variant was named iASPP-SV (iASPP splice variant) (Zhang et al. 2007).

It has been shown that different members of the E2F family of proteins can bind to the promoters of *ASPP1* and *ASPP2* and regulate their expression to varying degrees (Chen et al. 2005; Fogal et al. 2005; Hershko et al. 2005). E2F1 expression can be induced by various DNA damaging agents, which could explain how ASPP2 expression would be induced in certain cell types.

At the protein level, one study showed that ASPP2 can be ubiquitinated and subsequently targeted for degradation by the proteasome (Zhu et al. 2005). Overall, the mechanisms related to the regulation of the ASPP family are poorly understood, and it will be of vital interest in the future, since data so far indicates that they have important roles in cancer. The numerous ASPP family binding partners identified in past research might provide some better insights into how the expression and the function of the ASPPs are regulated.

1.4.4 ASPP interacting partners

The binding between the ASPP proteins and p53 was first demonstrated to occur via the DNA binding domain of p53. However, p53 also binds to the ASPP proteins through its proline rich domain, which contains the most common p53 polymorphism at codon 72 (Bergamaschi et al. 2006). Interestingly, iASPP in particular was shown to bind with higher affinity to p53 containing a proline at codon 72 instead of an arginine. As a result, the inhibitory effect of iASPP is more pronounced on p53Pro72 compared to p53Arg72. These data provide a new mechanism that explains why p53Arg72 is more active in inducing apoptosis (Sullivan et al. 2004).

Furthermore, ASPP proteins have been shown to interact with a number of proteins other than p53 and its family members. Most of the literature regarding

these interactions concerned Bbp (53BP2S), which was first shown to bind Bcl-2 (Naumovski and Cleary 1996). Around the same time, it was also identified as a protein phosphatase 1 (PP1) interacting protein (Helps et al. 1995). The discovery of other interacting proteins followed: p65relA (Yang et al. 1999), APCL (Adenomatous Polyposis Coli Like) (Nakagawa et al. 2000a), APP-BP1 (amyloid precursor protein-binding protein 1) (Chen et al. 2003), Yes-associated protein (YAP) (Espanel and Sudol 2001) and Hepatitis C core virus (Cao et al. 2004). Interestingly, all of these interactions implicate the Ankyrin repeats and the SH3 domain, apart from APP-BP1 which also interacts with the N-terminus of Bbp. More recently, the nuclear adaptor/RNA-binding protein SAM68 and the E3 ubiquitin ligase Siah-1 were identified as new binding partners for ASPP1 (Thornton et al. 2006); personal communication from Dr D Elliott). It would appear that the interaction between ASPP1 and SAM68 might play a specific role in human germ cells, where ASPP1 is involved in the pre-mRNA splicing of a CD44 minigene. RAI has also been reported to bind and inhibit p65relA (Yang et al. 1999). Also, *Drosophila* ASPP (dASPP) was recently shown to bind to *Drosophila* C-terminal Src-kinase and to regulate it positively (dCsk) (Langton et al. 2007). Csk is involved in the phosphorylation of the C-terminal region of Src family kinases (SFKs), and as a result, maintains them in an inactive state. Interestingly, the authors suggest a new role for the ASPP family in epithelial integrity.

Intriguingly, several of ASPPs interacting proteins, such as APCL, Siah-1 or APP-BP1, are directly or indirectly linked to the regulation of β-catenin. For example, APCL, the homologue of the adenomatous polyposis coli (APC) tumour

suppressor, has been shown to down-regulate β-catenin (van Es et al. 1999). Moreover, Siah-1, whose expression can be induced by p53, is involved in the proteasomal degradation of β-catenin in collaboration with APC (Liu et al. 2001; Matsuzawa and Reed 2001). As a result, the ASPPs might be involved in the regulation of β-catenin by interacting with these proteins.

1.5 The APC family of proteins

1.5.1 Overview

The adenomatous polyposis coli (APC) is an important tumour suppressor, particularly in the human colon where it is mutated early on in colorectal tumourigenesis, with more than 80% of colorectal cancers containing inactivating mutations in the *APC* gene. APC is defective in familial adenomatous polyposis (FAP), an autosomal dominant inherited disease characterized by the presence of adenomatous polyps in the colon and in the rectum. In 1991, positional cloning led to the identification of the APC, as a large protein of about 310 kDa (Groden et al. 1991; Kinzler et al. 1991). APC contains many domains for various binding partners and it therefore implicated in multiple cellular functions, including roles in signal transduction in the wnt/β-catenin signalling pathway principally, but also in the mediation of cellular adhesion, the structure of cytoskeleton, regulation of cell cycle and apoptosis (Hanson and Miller 2005).

1.5.2 The APC genes

A patient with colorectal polyposis together with mental retardation and other anomalies was the first clue in localizing the *APC* gene, as a deletion of the chromosomal band 5q21 was observed (Herrera et al. 1986). A study by Bodmer et al. demonstrated the localization of the *APC* gene on chromosome 5 and its close association with bands 5q21-q22 (Bodmer et al. 1987). *APC* was finally cloned and characterized in 1991 and found to consist of 21 exons and be 8535 bp in length (Groden et al. 1991; Kinzler et al. 1991). Several isoforms exist and the most common encodes a 2843 amino acids protein (Horii et al. 1993). Exon 10A is subject to alternative splicing and when it is expressed it results in a 18 amino acids longer protein (Xia et al. 1995).

More recently, a new APC gene was discovered in human and mice (Nakagawa et al. 1998; van Es et al. 1999) and, therefore, the APC family was born. This new member was named APC2 or APCL (for like), referred to as APCL here on in. *APCL* localizes to chromosome band 19p13.3 and encodes a ~245 kDa protein. The *APCL* gene is made up of 14 exons and, like APC, it contains one large exon at the 3' end.

Drosophila encodes two *APC* genes, *dAPC* and E-*APC/dAPC2* which is not to be confused with *APC2/APCL*. There is also an APC-like gene in C. elegans, however not much is known about its function.

1.5.3 The domains in the APC proteins

The APC protein has often been described as a scaffold protein due to its ability to interact with many other proteins through protein-protein interaction domains. Each domain and its role will be discussed (Figure 1.8).

The oligomerization domain

APC molecules are believed to have the ability to interact together and form multimers (at least homodimers), due to an oligomerization domain in their C-terminus. It was shown that the first 171 residues of APC were sufficient for complex formation and furthermore, that the first 45 residues are essential (Su et al. 1993a). Another study showed that within this 171-residue region, there are two regions theoretically capable of forming coiled-coil structures, corresponding to two heptad-repeats, however, amino acids 6-57 are essential for APC oligomerization, suggesting that the first heptad-repeat is critical (Joslyn et al. 1993). It has been proposed that wild-type APC can interact with truncated mutant APC via the oligomerization domain and that the latter can therefore act as a dominant negative by reducing the activity of APC (Su et al. 1993a).

Interestingly, the oligomerization domain is conserved in APCL (45% identical) (Nakagawa et al. 1998; van Es et al. 1999) and it has been suggested

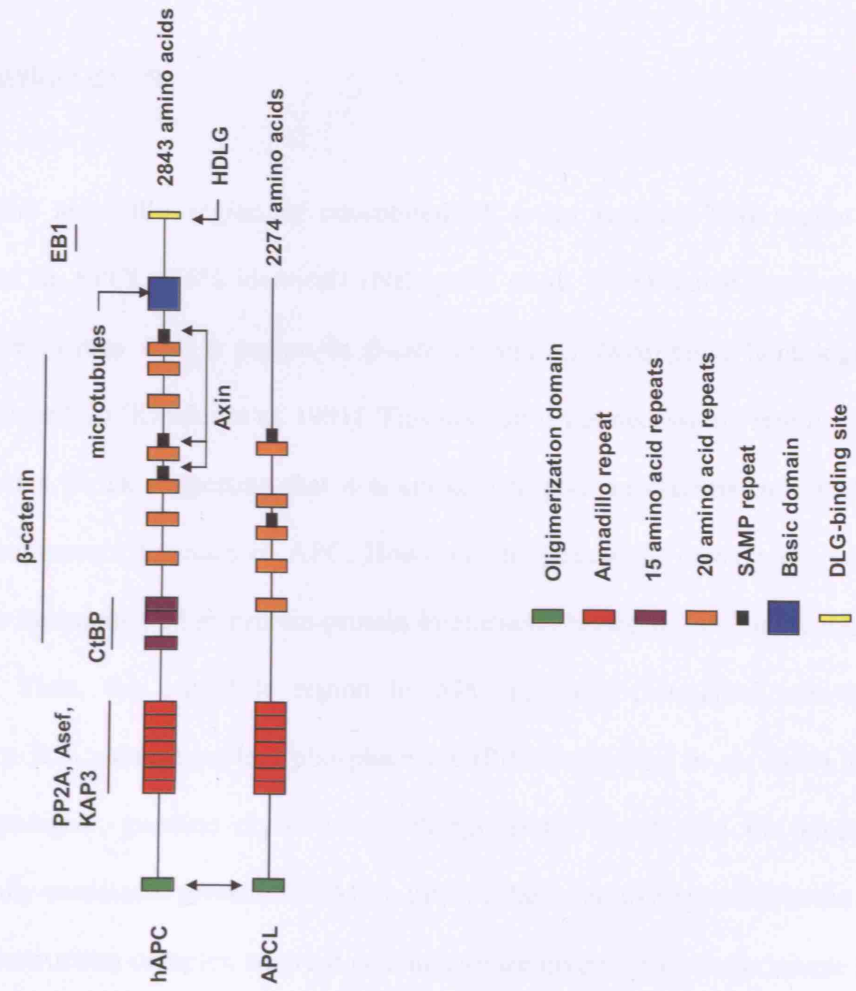


Figure 1.8: Structure of APC and APCL and binding partners. The principal domains of the APC and APCL proteins are shown, together with the areas where the binding partners of APC bind to, which are represented by black lines or black arrows.

that it can form homodimers with itself and heterodimers with APC (Jarrett et al. 2001). However the biological significance of this association is not yet understood.

The Armadillo region

The armadillo region is constituted of seven repeats. This region is conserved in APCL (76% identical) (Nakagawa et al. 1998) and it bears some strong similarities with a region in β-catenin and its *Drosophila* homologue, namely armadillo (Kinzler et al. 1991). This domain is not necessarily removed in APC mutant forms suggesting that it is unlikely to play an essential role in the tumour suppressor function of APC. However, the Armadillo domain has been shown to be implicated in protein-protein interactions between APC and various partners. Thus, the armadillo region of APC mediates interaction with the regulatory B56 subunit protein phosphate 2A (PP2A) (Seeling et al. 1999), the APC-stimulated guanine nucleotide exchange factor (Asef) and the kinesin superfamily-associated protein 3 (KAP3). PP2A is believed to play a role in the β-catenin destruction complex where it can antagonize glycogen synthase kinase 3β function (GSK3β). Asef, on the other hand, acts as a guanine nucleotide exchange factor (GEF) for the Rac and Rho GTP binding proteins, suggesting that APC can play a role in actin cytoskeleton remodelling (Kawasaki et al. 2000). Interestingly, upon binding, wild-type APC is able to relieve the autoinhibition of Asef which in turn can activate cdc42 to suppress anchorage-independent growth. This

mechanism has recently been suggested to explain how Asef could play a tumour suppressor role when APC remains intact (Mitin et al. 2007). Conversely, truncated APC retaining the armadillo region has been proposed to be unable to activate Asef and as a result promote tumourigenesis (Mitin et al. 2007). Finally, KAP3 was shown to be required to allow clustering of APC at tips of membrane protrusions, which may be important in APC's role in cell migration (Jimbo et al. 2002).

The binding sites for β-catenin: the 15- and the 20-amino acid repeats

Both the 15- and 20-amino acid repeats have been shown to be involved in the binding of β-catenin to APC. In APCL, the 15-amino acid repeats are missing and only five 20-amino acid repeats, compared to 7 in APC, are present, but it is still able to bind to β-catenin *in vitro* (Nakagawa et al. 1998; van Es et al. 1999). Interestingly, the 15-amino acid repeats remain present in most APC mutants and as a result they can still bind β-catenin (Rubinfeld et al. 1993; Su et al. 1993b). The crystal structure of a 15-amino acid repeat associated with the armadillo repeat region of β-catenin was resolved (Eklof Spink et al. 2001). β-catenin binding to the 20-amino acid repeats occurs when they are phosphorylated by GSK3β, as with the 15-amino acid repeats (Rubinfeld et al. 1996). The phosphorylation of these repeats is essential to increase their affinity for β-catenin and, in the case of the 20-amino acid repeats, their phosphorylation increases their affinity for β-catenin by 300 to 500 fold (Xing et al. 2004). More recently the

third 20-amino acid repeat was shown to be the tightest binding site for β-catenin (Liu et al. 2006), which correlates with the fact that most APC mutants in colon cancer have lost this repeat.

It is worth noting that the 15-amino acid repeats were also shown to be involved in the binding between APC and CtBP. Mechanistically, the two proteins are thought to cooperate to sequester nuclear β-catenin and thus reduce the levels of free β-catenin available to interact with TCF (Hamada and Bienz 2004).

Axin binding site

Both Axin, which is involved in the β-catenin degradation complex, and its homologue Axin2 or conductin bind to APC via their regulator of the G protein signalling (RGS) domain (Hart et al. 1998; Kishida et al. 1998). Their binding sites on APC are located between the third and fourth, fourth and fifth and after the seventh 20-amino acid repeat, and each binding site contains the characteristic SAMP amino acid sequences (Behrens et al. 1998). The crystal structure of the RGS domain of Axin, together with the 25-amino acid SAMP3 repeat, was resolved revealing that the APC/Axin interaction occurs at a conserved groove on a face of Axin which is distinct from the G-protein interface of classical RGS proteins (Spink et al. 2000).

SAMP repeats are conserved in APCL, but only two are present and both are required for the interaction with conductin, which contrasts with the fact that one SAMP domain in APC is sufficient (van Es et al. 1999).

The basic domain in APC

The basic domain is a highly positively charged domain due to its abundance in lysine and arginine residues, but it also contains a high number of proline residues. It lies within the C-terminus of APC and a fragment corresponding to amino acids 2219–2580 has been shown to allow the binding to microtubules and promote their assembly (Munemitsu et al. 1994; Smith et al. 1994; Deka et al. 1998). The interaction between APC and microtubules has been suggested to be negatively regulated by the phosphorylation of APC by GSK3β (Zumbrunn et al. 2001).

Although not mentioned by Van Es and Nakagawa, the basic region of APC appears to be present in APCL (amino acids 1784-2124) according to http://scansite.mit.edu/ and it has been suggested that exogenous APCL can also bind microtubules (Nakagawa et al. 2000b; Jarrett et al. 2001).

The EB1 binding domain

The C-terminus of APC also contains a binding site for the end-binding protein EB1 (Su et al. 1995). EB1 is a member of a highly conserved and ubiquitously expressed family of +TIP proteins (protein belonging to complexes that specifically track growing MT plus ends) and it plays a fundamental role in the modulation of microtubules dynamics. APC interacts with the C-terminus of EB1 and it is thought that their transient binding can play a role in spindle

positioning and in the fidelity of chromosome segregation (Fodde et al. 2001). It has been suggested that the APC/EB1 interaction promotes microtubules stabilization thus impinging on cell migration (Wen et al. 2004) but more recently a study showed that APC can promote microtubules stabilization and growth without EB1 (Kita et al. 2006).

APCL was shown not only to bind to EB1 but also to one of the EB1 family members, EB3, which is expressed preferentially in brain tissues. As a result the authors proposed that the 8-amino acid motif SESPSRLP within the EB-binding domain, which is found in both APC and APCL, is essential for the interaction between APC and EB1 family members. APCL and EB3 were shown to co-localize in the perinucleus and the cytoplasmic microtubule network in overexpression experiments but it is unclear what the function of this interaction is (Nakagawa et al. 2000b).

The HDLG binding site

The C-terminus of APC contains a S/TXV motif, which has been shown to be an important motif for binding to PDZ domain-containing proteins. The human homologue of the *drosophila* discs large tumour suppressor protein (HDLG) binds this domain via its PDZ domain (Matsumine et al. 1996). This complex has been suggested to be implicated in the negative regulation of cell cycle progression from the G0/G1 to S phase (Ishidate et al. 2000).

1.5.4 The association between APC localization and function

The numerous interaction domains contained in APC ensure that it is a multifunctional protein that plays different roles depending on the cellular context. As a result, it is thought that APC is a highly motile protein and that it can shuttle between different cellular locations in order to fulfil its different functions.

APC, component of the β-catenin degradation complex

In the cytoplasm, APC acts as a negative regulator of the Wnt/β-catenin pathway. It is thought to do so by playing the role of a scaffold protein within the β-catenin destruction complex due to its ability to bind both β-catenin (Rubinfeld et al. 1993; Su et al. 1993b) and axin (Behrens et al. 1998; Hart et al. 1998). Axin also acts as a scaffold protein and it facilitates the phosphorylation of β-catenin at specific residues in its N-terminus by GSK3β (Ikeda et al. 1998), resulting in β-catenin being targeted for degradation by the proteasome. The way APC promotes axin-mediated degradation of β-catenin is still unclear as it has been shown that overexpression of axin alone is enough to promote the destruction of β-catenin in APC mutant cancer cells (Behrens et al. 1998; Hart et al. 1998). As a consequence, APC is thought to have a kinetic role on the complex and facilitate β-catenin degradation.

Nuclear-cytoplasmic shuttling of APC

There have been discrepancies in the literature regarding the nuclear localization of APC. Some articles, depending on the reagents or the techniques used, have described APC as being able to locate to the nucleus, whereas others have found that it was excluded from nuclei. However, the general consensus is that APC can be found in the nucleus, although the majority of the protein is expressed in the cytoplasm (Brocardo et al. 2005). Supporting the idea that APC can shuttle between the cytoplasm and the nucleus, APC is known to contain both functional nuclear export signals (NES) (Rosin-Arbesfeld et al. 2000) and nuclear localization signals (NLS) (Zhang et al. 2000). Moreover, inhibiting the nuclear export receptor CRM1 with leptomycin B promotes the accumulation of nuclear APC (Henderson 2000; Neufeld et al. 2000a; Rosin-Arbesfeld et al. 2000). The Armadillo region is also believed to be involved in the shuttling of APC towards the nucleus (Rosin-Arbesfeld et al. 2000; Galea et al. 2001). Several mechanisms have been proposed to explain the functional relevance of APC being able to localize either in the cytoplasm or the nucleus. One possibility is that APC plays a role in the export of β-catenin out of the nucleus towards the cytoplasm where it can get degraded. Another possibility is that APC, simply by its ability to bind to β-catenin, balances the pools of nuclear and cytoplasmic β-catenin, thus sequestering β-catenin away from TCF in the nucleus and regulating β-catenin degradation in the cytoplasm (Neufeld et al. 2000b). More recent data seem to indicate that APC does not accelerate β-catenin nucleo-cytoplasmic shuttling, arguing against the hypothesis that APC is involved in the active β-catenin export

from the nucleus to the cytoplasm but rather in the retention of β-catenin in a defined compartment (Krieghoff et al. 2006).

APC at the membrane

The majority of the evidence that APC can localize at the membrane comes from studies in *Drosophila*. In *Drosophila*, E-APC/dAPC2 is concentrated at the plasma membrane of epithelial cells, in the apicolateral zones that contain the adherent junctions (Bienz and Hamada 2004). In mammalian cells, APC has also been described at the membrane of epithelial cells (Miyashiro et al. 1995). Recently, AMER1 was identified as being able to control the subcellular distribution of APC between membrane and microtubule associated pools (Grohmann et al. 2007). This membrane pool of APC is believed to have a role in cellular adhesion and recent evidence supports this idea. Studies in colorectal cancer cells show that APC can promote cellular adhesion and induce translocation of β-catenin and E-cadherin to the membrane (Faux et al. 2004). However, it remains unclear how APC can favour cellular adhesion and achieve the relocation of E-cadherin and β-catenin at the level of adherens junctions.

APC at the microtubule tips

As mentioned earlier, APC can bind to and form clusters at the plus end of some microtubules to promote their growth (Kita et al. 2006). This indicates that APC may play a role in microtubule related functions such as cell migration and spindle formation (Nathke 2004; Nathke 2006).

1.6 The Wnt β-catenin signalling pathway

1.6.1 Brief overview

The Wnt signalling pathway, named after its most upstream ligands, the Wnts, is implicated in almost every aspect of embryonic development and it also controls homeostatic cell-renewal in most adult tissues. Somatic mutations in the Wnt signalling pathway can result in its constitutive activation and the development of cancer, in particular cancer of the intestine. In addition, germline mutations in the Wnt signalling pathway are thought to be responsible of several hereditary diseases, for example, germline APC mutation is the genetic cause of hereditary cancer syndrome Familiar Adenomatous Polyposis (Kinzler et al. 1991).

β-catenin is one of the principal components of this pathway, as it is the molecule which integrates the message from the initial activation by Wnt ligands to the translation of this message at the level of gene transcription. In addition to its function in the Wnt signalling pathway, β-catenin also plays a determinant role in cellular adhesion, as it is a major component of the *adherens junctions*.

1.6.2 The canonical Wnt/β-catenin signalling pathway

1.6.2.1 At the cell membrane

The Wnt ligands

The name Wnt comes from the fusion of *Int-1* and *wg*. The former is the mouse *wnt1* gene which was identified in 1982 (Nusse and Varmus 1982) and the latter, the *Drosophila wingless* gene, a fly homologue of *Int-1*, which controls segment polarity during larval development (Nusslein-Volhard and Wieschaus 1980). The Wnts are a large family of secreted glycoproteins and genome sequencing in mammals has allowed the identification of about 20 of them which can be divided in 12 conserved subfamilies. *Wnt* genes are evolutionarily conserved as far as cnidarians, which have eleven subfamilies, but only 6 of these are conserved in *Drosophila* for example, illustrating that some subfamilies have been lost during evolution, but also underlining the complexity and the variety of the *wnt* family. Wnt proteins are characterized by a high number of conserved cysteine residues. Several proteins or complexes are thought to be implicated in Wnt secretion such as porcupine, which is responsible for Wnt palmitoylation. Wntless (Wls/Evi) and the retromer also take part in the trafficking and the secretion of Wnt proteins.

Upon Wnt receptor activation, three different pathways have been described to be activated: the canonical Wnt/β-catenin signalling pathway (Figure 1.9) and two non canonical pathways, the planar cell polarity pathway and the Wnt/Ca^{2+}

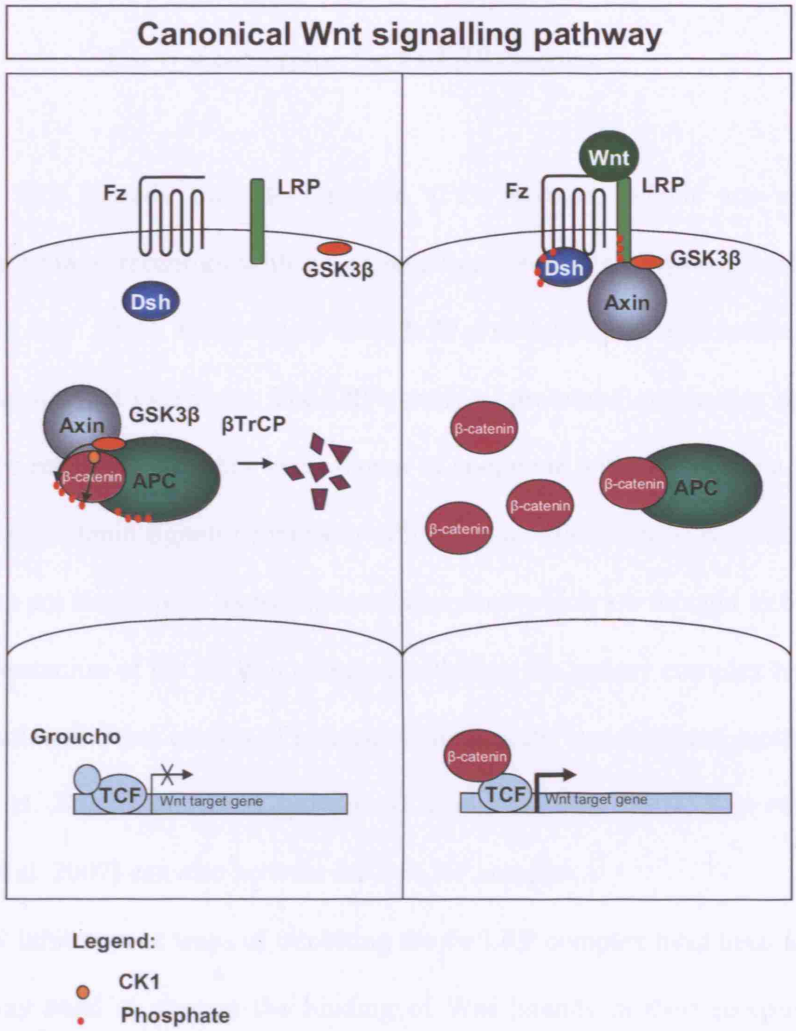


Figure 1.9: Canonical Wnt signalling pathway. In the absence of Wnt ligand bound to its receptor, the cytoplasmic destruction complex is functional and cytoplasmic and nuclear levels of β-catenin are kept low (left panel). Therefore, Wnt target genes are in a repressed state due to the presence of factors such as Groucho. Once bound to Wnt, the Fz/LRP coreceptor activates the Wnt signalling pathway. Eventually, the recruitment away from the destruction complex leads to the stabilization of β-catenin. In the nucleus, β-catenin associates with TCF and displaces Groucho to transactivate Wnt target genes.

pathway (Clevers 2006).

The Wnt coreceptor: the Fz/LRP complex

Wnt ligands bind to Frizzled (Fz) proteins, which are seven-pass transmembrane receptors with an extracellular N-terminal cysteine rich-domain (Bhanot et al. 1996). Interestingly a single Wnt molecule can bind several types of Fz receptors and vice versa. The LRP family of proteins (low-density lipoprotein receptor-related family) has been shown to cooperate with Wnt and Fz to trigger the Wnt/β-catenin signalling pathway (Pinson et al. 2000; Tamai et al. 2000). LRP proteins are single-pass transmembrane receptors which are thought to bind to Fz upon formation of the Fz/Wnt complex, although the ternary complex has not yet been isolated. Other classes of molecules, such as the cysteine-knot protein Norrin (Xu et al. 2004b) and the R-spondins (Kazanskaya et al. 2004; Kim et al. 2005; Nam et al. 2007) can also activate the Fz/LRP complex.

Several inhibitors or ways of inhibiting the Fz/LRP complex have been identified. One way used to prevent the binding of Wnt ligands to their receptors is the secretion of soluble Frizzle related proteins (SFRPs), which resemble the ligand-binding CRD domain of the Fz receptors and as a result can sequester Wnt (Hoang et al. 1996; Finch et al. 1997; Leyns et al. 1997). However, depending on the context this might result in the stabilisation of Wnt. Some other proteins, structurally distinct from Wnt, inhibit the activity of Wnt by directly binding to LRP5/6, such as the secreted Dickkopf (Dkk) (Glinka et al. 1998; Fedi et al.

1999). Upon Dkk binding to LRP6, Kremens transmembrane protein is recruited and the complex is subsequently internalized (Mao et al. 2002).

1.6.2.2 In the cytoplasm

Upon formation of the trimeric complex between Wnt, Fz and LRP, the canonical Wnt/β-catenin signalling pathway is activated. One of the important actors of the Wnt signalling cascade, Dishevelled (Dsh), gets recruited and evidence suggest that it physically interacts with the Frizzled receptor through its PDZ domains (Wong et al. 2003). A key step after Wnt stimulation is the phosphorylation of the cytoplasmic tail of LRP6 by casein kinase 1γ (CK1 γ) and GSK3 to allow the recruitment of Axin (Davidson et al. 2005; Zeng et al. 2005). Axin interacts with LRP5/6 through five phosphorylated PPP(S/T)P repeats in the cytoplasmic tail of LRP. With this model, Axin would be taken away from the β-catenin degradation complex therefore stabilizing β-catenin (He et al. 2004). It has been suggested that, upon Wnt activation, GSK3 is involved in the phosphorylation of LRP6 at the PPP(S/T)P motifs (Zeng et al. 2005), whereas CK1 γ phosphorylates multiple motifs close to the GSK3 sites (Davidson et al. 2005). However it remains inconclusive which of these kinases is the kinase activated by Wnt.

Dsh is essential for this pathway as it binds the Fz/LRP complex, together with Axin, where they interact through their respective DIX (Dishevelled-Axin) domains (Kishida et al. 1999). By interacting with axin, Dsh is believed to be able

to block its function in the phosphorylation and the subsequent degradation of β-catenin (Smalley et al. 1999; Penton et al. 2002; Cliffe et al. 2003). It is also known that Wnt can regulate the phosphorylation of Dsh, but it remains unclear how phosphorylated Dsh functions in signal transduction. Recent results suggest that upon Wnt activation, Dsh forms aggregates at the level of the plasma membrane and as a result triggers the co-clustering of LRP6 and pathway components, such as Axin, GSK3 and Fz, in so-called LRP6-signalosome (Bilic et al. 2007). In this model, Wnt would permit the association of LRP6 and Fz at the level of the Dsh aggregates, thus providing an important concentration of receptors, allowing the phosphorylation of LRP6 by CK1 γ and the recruitment of Axin. Moreover, another recent set of data demonstrates the ability of the DIX domain of Dvl2 (Dishevelled 2) to polymerize gradually and reversibly *in vitro* corresponding to dynamic polymerization *in vivo* (Schwarz-Romond et al. 2007). The authors suggest that this polymerization plays a role in the creation of a dynamic recruitment platform which could act as a clustering factor between Wnt receptors and transducers.

The cytoplasmic destruction complex

β-catenin is not only a major component of the *adherens* junctions, but it also plays a central role in Wnt signalling as a transcriptional coactivator of the pathway. In the absence of activation of the Wnt signalling pathway, the levels and the stability of β-catenin are kept under control by the cytoplasmic destruction complex which is constituted of the tumour suppressors Axin and APC, and the

enzymes GSK3β and Casein kinase 1 (CK1) (Kimelman and Xu 2006). The role of this complex is to perpetrate the phosphorylation of β-catenin on key residues in its N-terminus which will be used as a binding recognition surface for a dedicated E3 ligase complex. β-catenin is then polyubiquitinated and degraded by the proteasome (Aberle et al. 1997).

Axin acts as a scaffold protein in the complex, where it binds directly to most of the other components (β-catenin, APC, and the two kinases CK1 and GSK3β). Its key function is to bring the kinases CK1 and GSK3β to the complex, in order to promote the phosphorylation of β-catenin. The role of Axin as a scaffold protein in the complex is underlined by the fact that the phosphorylation of β-catenin and APC is greatly enhanced in the presence of an axin fragment containing the β-catenin and CK1 binding sites *in vitro* (Ha et al. 2004).

GSK3β phosphorylates other targets in the complex in addition to β-catenin, including APC and Axin, and it has been shown to be important for the stability of the latter (Yamamoto et al. 1999). As mentioned previously, GSK3β, together with CK1, plays a determinant role in the complex since it mediates phosphorylation of β-catenin within its N-terminus. Many GSK3β targets require to be primed by a phospho-serine or phospho-threonine at the n + 4 position (S/T-X-X-X-S/Tp). Although it was initially thought that GSK3β could phosphorylate residues T41, S37 and S33 of unprimed β-catenin, it was later shown that priming phosphorylation by CK1 at residue S45 is required (Amit et al. 2002; Liu et al. 2002). While both CK1α and CK1ε have been shown to be able to phosphorylate

β-catenin at S45 *in vitro*, several lines of evidence indicate that CK1α is the fundamental kinase *in vivo* (Liu et al. 2002; Yanagawa et al. 2002). Phosphorylated β-catenin is then recognized by the F box/WD repeat protein β-TrCP (β-transducin-repeat-containing protein), a component of a dedicated E3 ubiquitin ligase complex (Winston et al. 1999). As a consequence, β-catenin is polyubiquitinated and targeted for degradation by the proteasome (Aberle et al. 1997).

Protein phosphatase 2A (PP2A), which can also be found in the cytoplasmic destruction complex via its interaction with axin, has been shown to negatively regulate the Wnt/β-catenin signalling pathway by dephosphorylating an unknown target (Hsu et al. 1999; Seeling et al. 1999). Interestingly, more recently, protein phosphatase 1 (PP1) has been suggested as a potential positive regulator of the cytoplasmic destruction complex by interacting with axin and by modulating its phosphorylation status (Luo et al. 2007). Thus PP1 might impinge on the stability of the complex.

APC has been shown to be part of the cytoplasmic destruction complex and to be essential in order to properly regulate the levels of β-catenin. However, this evidence is based on genetic observations and there is no consensus on the specific molecular activity of APC. This lack of understanding, coupled to the fact that APC contains many domains for protein-protein interaction, is probably why it has often been described as a scaffold protein. Crystallographic studies are starting to provide more insights into how the destruction complex is structured

and progressively provide a better understanding of its dynamics (for a working model for the destruction complex, see (Kimelman and Xu 2006)).

The E3 ligase complex

As previously stated, the F box/WD repeat protein β-TrCP can recognize β-catenin once phosphorylated by GSK3β, and thus it serves as an intracellular receptor (Hart et al. 1999; Kitagawa et al. 1999; Winston et al. 1999). β-TrCP interacts with a number of proteins, including Skp1 and Cullin, to form a functional E3 ligase complex, which is then able to induce the polyubiquitination of β-catenin. The β-TrCP/Cullin/Skp1 complex constitutes one of the multisubunit, cullin-containing E3-ligases, called SCF (Skp1/Cullin/F box protein) (Weissman 2001).

Interestingly, another E3 ligase complex has been described in the literature as being able to promote the ubiquitination of β-catenin and its subsequent degradation by the proteasome in a p53 response dependent manner (Liu et al. 2001; Matsuzawa and Reed 2001). In this complex, The F box protein Ebi replaces β-TrCP as the molecule which interacts with β-catenin. In contrast to β-TrCP, it can apparently bind unphosphorylated β-catenin at the residues normally targeted by GSK3β. Cullin is absent from this complex but the RING protein Siah-1, the mammalian homologue of *Drosophila* Sina (seven *in absentia*), interacts with the complex as a result of its binding partner SIP. Endogenous Siah-1 levels are kept relatively low due to the interaction of their RING domain with

E2s, thus promoting their own degradation. However, it has been shown that Siah-1b is a direct p53 target (Fiucci et al. 2004) and that, generally speaking, p53 can induce the expression of Siah-family genes in mammals (Amson et al. 1996; Liu et al. 2001).

1.6.2.3 In the nucleus

How does β-catenin translocate to the nucleus?

Upon Wnt activation, the cytoplasmic degradation complex is inhibited and β-catenin is therefore stabilized (Figure 1.9). It is not clear which mechanisms are necessary for the accumulation of β-catenin in the nucleus. It has been proposed that β-catenin can simply passively translocate to the nucleus when its cytoplasmic concentration reaches a certain level or that, as it is often the case, nuclear accumulation of β-catenin upon Wnt activation is the result of an overall increase in its levels. Additionally, the nuclear import of β-catenin is independent of the nuclear import signal and import machinery (Fagotto et al. 1998; Yokoya et al. 1999). Several other mechanisms have been suggested to explain how β-catenin accumulates in the nucleus. For example, The *Drosophila* T-cell factor (TCF), Pan, can function as a nuclear anchor for Arm (Armadillo/β-catenin) (Tolwinski and Wieschaus 2001). However, it has been shown that an Arm mutant missing the Pan binding region can still localize to the nucleus. Other factors like Legless/BCL9 (Lgs) and Pygopus (Pygo) have been shown to

cooperate to anchor Arm in the nucleus (Townsley et al. 2004). The binding regions of β-catenin to these factors probably contribute to its retention in the nucleus; however, the tenth armadillo repeat of β-catenin is required and sufficient for its nuclear import (Koike et al. 2004).

β-catenin also contains an intrinsic nuclear export activity, as well as other binding partners that favour its cytoplasmic localization, so it would seem that its localization is the sum of many variables influencing the equilibrium between nuclear and cytoplasmic β-catenin.

How does β-catenin activate gene transcription?

In the absence of β-catenin in the nucleus, β-catenin target genes are in a repressed state which is characterized by a complex in which TCF binds the transcriptional repressors Groucho (Cavallo et al. 1998; Roose et al. 1998) and the Carboxy-terminal binding protein (CtBP) (Brannon et al. 1999; Cuilliere-Dartigues et al. 2006). The Groucho family of proteins are general long-range transcriptional co-repressors, which have been shown to bind to histone deacetylases (Chen et al. 1999). Upon activation of the Wnt/β-catenin signalling pathway, β-catenin accumulates in the nucleus and binds to the amino-terminal part of TCF family members, displacing Groucho from the complex, in order to activate the expression of Wnt target genes (Daniels and Weis 2005). CtBP on the other hand is a general short-range transcriptional co-repressor and it also interacts with histone deacetylases. However it is not clear how or whether β-catenin plays

a role in the removal of CtBP from the complex. After the removal of both Groucho and CtBP, β-catenin can fully activate the transcription of its targets.

β-catenin interacts with TCF through its central region (armadillo repeats 3 to 11), but, in the nucleus, it can also bind other partners thereby playing a role in gene expression through two of its domains; one encompassing armadillo repeats 1 to 5 and the other armadillo repeat 10 and the C-terminus of β-catenin. They can be referred to as the amino-terminal activating arm (NTAA) and the carboxy-terminal activating arm (CTAA) respectively (Stadeli et al. 2006). Proteins interacting at the CTAA are mainly involved in the activation of transcription. Among these different partners, there are proteins such as TBP, Brahman/Brg-1, CBP/p300, MED12, and Hyrax/Parafibromin.

Two homologous proteins, Pontin and Reptin, bind to the NTAA, and have opposite effects on β-catenin transcriptional activity. Lgs binds to a region in β-catenin overlapping with that of Pontin and Reptin. However, it is not known whether they can bind simultaneously to β-catenin or whether they compete with each other. Lgs binds to the plant homology domain of Pygopus through its homology domain 1 (HD1) and allows its recruitment to β-catenin. Some studies have suggested that Pygopus and Lgs play a role in the import or retention of β-catenin in the nucleus (Tolwinski and Wieschaus 2004; Townsley et al. 2004), but others have demonstrated their essential role in the activation of β-catenin transcriptional function (Hoffmans et al. 2005; Stadeli and Basler 2005).

β-catenin target genes

Following Wnt activation, β-catenin goes to the nucleus leading to the activation of a transcriptional program. The genes activated within the program depend very much on the organ and the cell type in which it has been activated, and it can result in a vast array of biological responses. However, an increasing body of data suggests that Wnt signalling is associated with maintenance or activation of stem cells (Reya and Clevers 2005).

Numerous β-catenin target genes, in various biological systems, have been identified so far. Many of these genes, such as Cyclin D1 and Myc have implications in cancer development. A comprehensive list of Wnt target genes can be found at http://www.stanford.edu/%7ernusse/pathways/targets.html. There does not appear to be a universal array of genes that are transcribed in response to Wnt signalling, rather a cell-type specific response is evident. However, interestingly the transcription of genes involved in the auto-regulation of the pathway can be triggered by Wnt. Hence, genes involved in the negative regulation of the pathway, such as Axin2/conductin (Jho et al. 2002), are generally up-regulated by β-catenin whereas some of the genes involved in the mediation of the Wnt/β-catenin signalling pathway, like Fz and LEF1, have been shown to be down-regulated (Hovanes et al. 2001).

1.6.3 The plasma membrane pool of β-catenin

The fundamental features of β-catenin as a transcription factor and as a cell-cell adhesion molecule make it a very versatile protein. Furthermore, it raises the question whether these two pools of β-catenin are distinct in vivo, or whether the pool of β-catenin contained in the *adherens* junctions can somehow become available, and contribute to Wnt signalling. The adherens junctions themselves play a role in multiple cellular processes, including initiation and stabilization of cell-cell adhesion, regulation of the actin cytoskeleton, intracellular signalling and transcription regulation. The major components involved in the formation of the adherens junctions are the classical cadherin superfamily, and the catenin family members including p120, β-catenin and α-catenin.

E-cadherin

Adherens junctions are built around the cadherin family of single-pass transmembrane Ca^{2+}-dependent cell-cell adhesion proteins (Figure 1.10). Cadherins, such as E-cadherin, form through their extra-cellular domain homo- and heterophilic bonds with cadherins on adjacent cells; other members of this family of proteins include N-, P- and R-cadherin. Cadherins have five characteristic extracellular cadherin (EC) repeat domains allowing the formation of trans-cadherin interactions. This binding is relatively weak, but it may be strengthened by lateral clustering of cadherins due to protein linkage between the

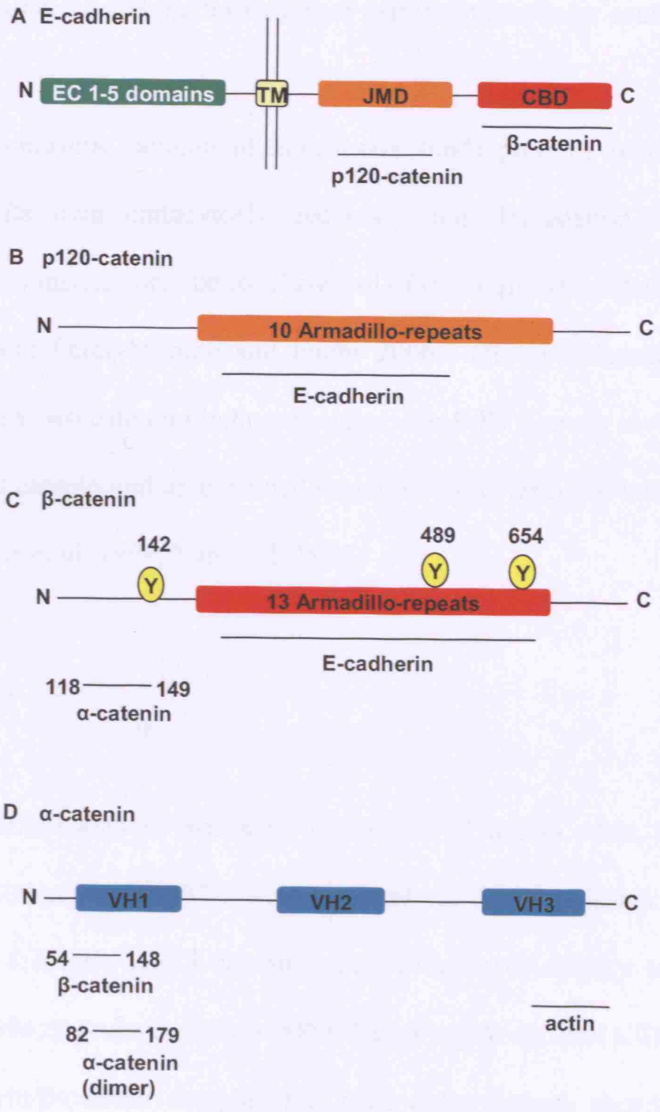


Figure 1.10: Components of the *adherens* junctions and binding domains. A. E-cadherin. EC: extracellular cadherin repeat domain. TM: transmembrane domain. JMD: juxtamembrane domain. CBD: catenin-binding domain. **B.** p120-catenin. **C.** β-catenin. Tyrosine phosphorylation sites are indicated. **D.** α-catenin. VH1-3: vinculin homology domain 1-3. The binding regions of the different proteins involved in the adheres junctions are represented with black lines and when available, the precise binding region is delimited by the position of the amino acids.

cadherin cytoplasmic domain and the actin cytoskeleton (Jamora and Fuchs 2002). Additionally, the juxtamembrane (JMD) domain is required for the proper clustering of cadherins and the formation of mature intercellular contacts (Yap et al. 1998).

The cytoplasmic domain of E-cadherin binds proteins involved in the regulation of its own endocytosis, recycling and degradation, intracellular signalling, gene transcription, and local control of the actin cytoskeleton (Halbleib and Nelson 2006; Perez-Moreno and Fuchs 2006). The cytoplasmic tail of E-cadherin contains two catenin binding domains; the JMD domain involved in the binding to p120 catenin and an extended region in the C-terminus which binds to β-catenin (Aberle et al. 1994; Yap et al. 1998).

β-catenin

β-catenin contains 13 armadillo repeats of 42 amino acids, that form a triple α-helix (Huber et al. 1997). β-catenin binds to the cytoplasmic domain of cadherins in a 1:1 ratio and β-catenin binds with lower affinity to α-catenin (Aberle et al. 1994; Pokutta and Weis 2000; Huber and Weis 2001). The structure of the E-cadherin/β-catenin complex has been characterized, showing that E-cadherin is unstructured in the absence of β-catenin and binds in an extended conformation that forms a large interface with the armadillo repeats of β-catenin, thus allowing different degrees of regulation via phosphorylation for example (Huber and Weis 2001). Interestingly, there are striking similarities in the way TCF and APC binds to β-catenin as compared to E-cadherin. Recently, a detailed

analysis of the thermodynamics of the β-catenin/E-cadherin interaction suggests that the C-terminal tail (located after the armadillo repeats) can modulate the binding affinity of β-catenin for its different binding partners (Choi et al. 2006).

β-catenin has been shown to bind other proteins in the *adherens* junction complex, such as actin, IQGAP and fascin. The association of IQGAP by the small GTPases Rac1 and Cdc42 results in its disassociation from β-catenin, which consequently binds to α-catenin (Fukata et al. 1999; Kuroda et al. 1999). These events are believed to take part in the initiation of the assembly of the *adherens* junction complex and cell-cell adhesion.

α-catenin

Adherens Junctions have been described as being able to physically connect to the actin cytoskeleton through α-catenin which binds to β-catenin (Aberle et al. 1994; Rimm et al. 1995). However, it has not been shown that α-catenin can bind simultaneously to β-catenin and actin, and recent studies have demonstrated that the E-cadherin/β-catenin/α-catenin complex at the adherens junctions forms a highly dynamic link to the cytoskeleton (Drees et al. 2005; Yamada et al. 2005). Therefore they show that the simultaneous interaction between the adherens junctions and the actin cytoskeleton through α-catenin does not take place. Alpha-catenin can form homodimers, which compete with the Arp2/3 complex for binding to actin (Drees et al. 2005; Yamada et al. 2005). These homodimers cannot bind to β-catenin as the β-catenin binding domain of α-catenin overlaps with that of its dimerization domain (Figure 1.8 D) (Pokutta and

Weis 2000). As a result, monomeric α-catenin binds β-catenin but not actin. Furthermore, the formation of α-catenin homodimers occurs at concentrations 10-fold higher than that of the monomeric pool of α-catenin in the cytoplasm of epithelial cells. Therefore, it has been suggested that the increase in local concentration of α-catenin at the membrane during clustering of the cadherin/catenin complex at cell-cell contacts provides a sufficient local increase in α-catenin concentration to induce α-catenin dimerization in the cytoplasm (Drees et al. 2005). Consequently, these dimers can bind to and regulate actin filament organization.

P120 catenin

P120 catenin was first identified as a substrate for Src-tyrosine receptor kinase and was later identified as a member of the catenin family of proteins, based on sequence homology with an armadillo domain of β-catenin (Reynolds et al. 1989; Reynolds et al. 1992). As previously mentioned, p120 catenin binds to the JMD of E-cadherin where it recognizes a highly conserved octapeptide sequence (YDEEGGGE) (Ferber et al. 2002). P120 catenin has been proposed to stabilize E-cadherin at the plasma membrane and therefore initiate cell-cell contacts. It has been suggested that this is achieved by preventing E-cadherin from being internalized or by recycling internalized E-cadherin back to the membrane (Davis et al. 2003; Xiao et al. 2005). Therefore, p120 catenin potentially plays a role in tumour progression and invasion (Berx and Van Roy 2001).

Furthermore, p120 activity has been linked to regulation of the cytoskeleton through Rho family GTPases and as a result, p120 catenin may play a role in cell migration and invasiveness (Noren et al. 2000; Yanagisawa and Anastasiadis 2006). P120 catenin has been shown to induce cell migration and the formation of membrane extensions. This may be accompanied by an increase in Rac and Cdc42 activity although this might depend on the cell type (Noren et al. 2000). Importantly, Rac activation enhances spreading, migration and membrane ruffling via actin polymerization around the cell periphery. Additionally, it has been shown that p120 can inhibit Rho whose activity has been shown to induce focal adhesion formation and cell contractility (Noren et al. 2000). The question remains as to whether the roles of p120 in cadherin stabilization and Rho inhibition were interconnected. Recently, it has been shown that Rac can activate a protein called p190RhoGAP which then gets recruited at the *adherens* junctions where it interacts with p120 and participates in the inhibition of Rho (Wildenberg et al. 2006).

Finally, an increasing body of evidence suggests that, like β-catenin for instance, p120 catenin plays a role in the nucleus and in gene transcription. Most work in this area has focused on the p120/Kaiso complex (Daniel and Reynolds 1999). Thus, it has been shown that p120 has the ability to enter the nucleus where it can interact and displace the repressor kaiso, resulting in gene activation (Kim et al. 2004; Park et al. 2005). Intriguingly, p120 and Kaiso were recently shown to modulate genes known to be regulated by the Wnt/β-catenin signalling pathway (Park et al. 2005).

Dissociation of β-catenin from the *adherens* junctions

It is unclear whether the adhesive and signalling properties of β-catenin are interconnected, but several lines of evidence suggest that this could be the case. Indeed, during carcinoma development for example, cells undergo epithelial-mesenchymal transition (EMT), which is characterized by the loss of the cadherin/β-catenin/α-catenin complex at the membrane (Perl et al. 1998) and it has been shown that loss of E-cadherin mediated adhesion correlates with an increase in β-catenin transcriptional activity (Orsulic et al. 1999).

β-catenin association/disassociation from the *adherens* junction complex appears to be mainly regulated through phosphorylations on key residues. Thus, three residues in the cadherin cytoplasmic domain (S684, S686 and S692) are phosphorylated by CKII and GSK3β kinases which create additional interactions between β-catenin and E-cadherin. In contrast, phosphorylations on specific tyrosine residues in β-catenin are of particular importance in negatively regulating this interaction: phosphorylation of Y142, in the first armadillo repeat, Y489, and Y654, in the last armadillo repeat, have been shown to trigger the disassembly of β-catenin from the *adherens* junction complex and to increase β-catenin dependent transcription (Aberle et al. 1996; Roura et al. 1999; Pokutta and Weis 2000; Piedra et al. 2001; Piedra et al. 2003).

Several kinases involved in these phosphorylations have been identified. For example, Src has been shown to be involved in the phosphorylation of β-catenin at Y654 and to lead to loss of E-cadherin mediated cell-cell adhesion (Roura et al. 1999; Piedra et al. 2001). It is worth noting that phosphorylation at Y654 does not

interfere with TCF binding, as it does not require the last armadillo repeat of β-catenin. Abl has been shown to be responsible for phosphorylating Y489 (Rhee et al. 2002). Phosphorylation of β-catenin at Y142 is thought to be mediated by the kinases Fer and Fyn (Piedra et al. 2003; Xu et al. 2004a). Tyrosine 142, which is present in the first armadillo repeat, plays a role in the interaction between β-catenin and α-catenin. Phosphorylation of Y142 abolishes the binding of these two partners but promotes the binding of β-catenin to BCL9, the human ortholog of the Drosophila segment polarity gene product Lgs (Brembeck et al. 2004). These results provide some insights into the mechanisms by which the membrane pool of β-catenin might become available to ensure its transcription factor function.

1.6.4 The role of the Wnt/β-catenin signalling pathway in development and cancer

Over the past few years, the Wnt/β-catenin signalling pathway has emerged as one of the crucial regulators of the stem cell population. Stem cells are cells that have the ability to self renew, as well as to differentiate to give rise to new lineages. Stem cells play a role throughout development as they are present at both the earliest stages of life, as embryonic stem cells, and within adult tissues, since stem cells are involved in homeostatic self-renewal processes and tissue repair. Not surprisingly, upon its deregulation, the Wnt/β-catenin signalling

pathway is also involved in the development of cancer, in the same tissues where it regulates the stem cell population. One of the most famous examples in which cancer cells acquire stem cell characteristics by hijacking this pathway, is that of colon cancer. In the small intestine, the epithelium consists of villi, where most differentiated cells can be found, and crypts, where the stem cell population resides. It is now accepted that the Wnt/β-catenin signalling pathway is required for the maintenance of he crypt progenitor phenotype. In colon cancer, the Wnt/β-catenin signalling pathway is constitutively active, mainly through stabilization of β-catenin as a result of mutational inactivation of APC, implying that APC transforms epithelial cells through activation of the Wnt cascade. A parallel can be made between colorectal cancer cells and crypt stem cells, as Tcf4 drives the same genetic programme in both (van de Wetering et al. 2002). A study using a system in which the Wnt pathway could be induced at will by inducible deletion of an *APC* allele (Sansom et al. 2004) shows that the absence of APC results in the activation of the Wnt signalling pathway through accumulation of nuclear β-catenin and in the perturbation of differentiation, migration, proliferation and apoptosis. Similarly to colorectal cancer cells, APC-deficient cells maintain a crypt progenitor-like phenotype.

The Wnt/β-catenin signalling pathway is involved, at the same time, in the regulation of the stem cell population and cancer in other tissues. The same model as the one described above in the small intestine, the rapprochement can be made between haematopoietic stem cells and leukaemia. Recent data also suggest that activating Wnt signalling through β-catenin can increase cycling and expansion of neural progenitors, and that its loss causes a reduction in the progenitor

compartment (Chenn and Walsh 2002; Zechner et al. 2003). Just as in the examples above, it is possible to speculate that Wnt signalling might also be involved in the development of tumours in the central nervous system which is supported by the fact that medulloblastoma contain mutations in β-catenin and axin (Dahmen et al. 2001; Baeza et al. 2003).

1.7 Aim of study

The aim of this thesis is to elucidate the links that might exist between the ASPP family of proteins and the Wnt/β-catenin signalling pathway.

Firstly the inter-relationship between the ASPPs and ASPP2's binding partner, APCL, the homologue of APC, will be explored. By principally employing an exogenous approach, we will try to understand the influence that these proteins have on each other's localization and function, in light of the observation that APCL interacts with and induces the translocation of 53BP2 to a perinuclear region (Nakagawa et al. 2000a). The interaction between the ASPPs and APCL could potentially lead to the regulation of either p53 mediated apoptosis or β-catenin stability and activity, thus affecting the Wnt-signalling pathway.

Secondly, as APCL, like its homologue APC, can regulate the levels of β-catenin, the role of the ASPP family of proteins in the regulation of β-catenin and, in a broader term, the Wnt signalling pathway will be investigated (Nakagawa et al. 1998; van Es et al. 1999). The role of the ASPPs, and in particular ASPP2, will be investigated in various cellular models. Interestingly, ASPP2 is mainly expressed in the central nervous system (CNS) during mouse embryonic development, and ACPL is expressed at high levels in the brain, suggesting that they might play a

role together in the Wnt/β-catenin signalling pathway. As a result, the role of ASPP2 on β-catenin will be investigated in vivo, during CNS development.

Finally, as APP-BP1 was shown to bind to ASPP2, the effect that APP-BP1 might have on the regulation of p53 by the ASPPs will be briefly investigated (Chen et al. 2003). APP-BP1 is one-half of a bipartite activating enzyme for the ubiquitin-like protein NEDD8. Furthermore, as the ASPPs interact with other proteins involved in ubiquitin-like modifications such as Siah-1, preliminary data on ubiquitin-like modifications of the ASPPs will be presented.

Chapter II: Materials and Methods

2.1 Materials

2.1.1 Reagents

All chemicals, unless otherwise stated, were obtained from BDH Chemicals, UK. All radio-isotopes, autoradiography films (Hyperfilm), ECL (Enhanced Chemi-Luminescence) reagents were purchased from Amersham Pharmacia Biotech (UK). Nitrocellulose membrane was purchased from Whatman, Germany. All restriction enzymes, and their buffers, were purchased from New England Biolabs (UK). All tissue culture dishes and flasks were obtained from Greiner (UK).

The Luciferase Assay System Kit and the TNT® T7 Quick coupled Transcription/Translation System kits were purchased from Promega (WI, USA) and the QIAGEN Plasmid Mega and RNeasy midi kits were purchased from Qiagen (UK)

5x Agarose Sample Buffer

50% (w/v)	Sucrose
100 mM	EDTA, pH 8.0
0.1% (w/v)	Bromophenol blue
0.1% (w/v)	Xylene cyanol

Agarose gel for DNA electrophoresis

Agarose powder (GibcoBRL, UK) was weighed and dissolved in 1X TAE buffer at an appropriate concentration. The mixture was heated in a microwave oven to dissolve the agarose and the solution allowed to cool to 40°C. Ethidium bromide was added to a final concentration of 20μg/ml. The agarose solution was poured into a casting tray with the required comb and was left to solidify at room temperature.

Ammonium Persulphate (APS)

10% (w/v) stock solution was prepared in water and stored at -20 °C in single-use aliquots.

Ampicillin Stock

0.5g of the antibiotic was dissolved in 10 ml sterile distilled water, creating a 50mg/ml solution. This was stored at -20°C as aliquots.

Blocking Solution

10% (w/v) fat-free milk (Marvel, UK) was prepared in 1X TBS-T.

Calcium chloride

A 2.5M solution was made by dissolving 36.75g $CaCl_2.2H_20$ in distilled water. The solution was then filter-sterilised and stored at room temperature, to be used for calcium phosphate transfection

COmplete™ protein inhibitor cocktail

One COmplete™ protein inhibitor (Boehringer Mannheim, UK) tablet was dissolved in 2.0ml of sterilised distilled water as a 25x stock solution that is stable at -20 °C for 12 weeks.

Cycloheximide

A stock solution of 50mg/ml was prepared by dissolving the powder in ethanol. This was stored at -20°C, and used at a working concentration of 50µg/ml.

DNA Marker

100bp and 1kb DNA ladders were purchased from New England Biolabs and were used as a size standard for agarose gel electrophoresis.

EDTA Solution

A 0.5M $C_{10}H_{14}N_2O_8Na_2.2H_2O$ (EDTA) stock solution was made by dissolving 186 g of EDTA in 700ml distilled water. The pH was adjusted to pH 8.0 with NaOH and the volume made up to 1l with distilled water.

Ethidium Bromide

A 10mg/ml stock solution was prepared by dissolving 0.2g ethidium bromide in 20ml of distilled water and then stored at 4°C in the dark.

G-418 (Geneticin® or Neomycin)

A stock solution was prepared under sterile conditions by dissolving 5mg of the powered G-418 Geneticin® (G-418 Sulphate; Life Technologies Ltd, UK) in 50ml of DMEM to make a 100mg/ml stock which was stored at 4 °C.

20X HBS (HEPES buffered saline) for 100ml (in water):

9.29g	HEPES
0.745g	KCl
16.36g	NaCl
0.213g	Na_2HPO_4 (anhydrous)
2.16g	Glucose

Four different 2X solutions were made and their pH was adjusted with NaOH to 6.7, 6.9, 7.1 and 7.3 respectively. Each solution was filter sterilised for transfections and their efficiency was tested. These were stored in 50ml aliquots at -20 °C for long term storage and 4°C for short term storage.

Iodoacetamide

Iodoacetamide was purchased from Sigma-Aldrich and dissolved in water at a stock concentration of 0.5M. Iodoacetamide was added to lysis buffers when required at a final concentration of 5mM.

Luciferase Assay System

This assay system was purchased from Promega (WI, USA). The solutions were made up according to the manufacturer's directions and stored at -20°C. All solutions were allowed to equilibrate to room temperature before use.

Lipotransfection reagents

Lipotransfections were performed with Fugene 6 (Roche), following the manufacturer's recommendations or as otherwise stated.

Phosphate Buffered Saline (PBS)

12.5 mM	NaCl
1 mM	Sodium dihydrogen phosphate, NaH_2PO_4
1.6 mM	Disodium dihydrogen phosphate, Na_2HPO_4

The pH was adjusted to 7.0 and autoclaved.

Ponceau S Staining Solution (10 X)

5% (v/v)	Acetic acid
2% (v/v)	Ponceau S (sodium salt) (Sigma, MO, USA)
30% (w/v)	Trichloroacetic acid $CCl_3.COOH$
30% (w/v)	5-sulfosalicyclic acid $C_7H_6O_6S.2H_2O$ (Sigma, MO, USA)

The solution was dissolved in water to a 1x dilution before use.

Proteasome inhibitors

MG132 was purchased from Calbiochem and dissolved in DMSO at a stock concentration of 10mM. A final concentration of 30μM was used or as otherwise stated.

Protein G Sepharose

Stored in 20% ethanol at 4°C (Pharmacia Biotech). Protein G Sepharose beads were washed 3 times with cold PBS and resuspended in one volume of PBS before use.

Protein Molecular weight markers

The prestained protein markers were purchased from New England Biolabs and were used as a size standard for SDS-polyacrylamide and tris-acetate gel electrophoresis.

Qiagen Solution-1-Resusupension solution for plasmid preparations

50 mM	Tris-HCl pH 8.0
10 mM	EDTA
100 μg/ml	RNase A (stored at 4 °C after addition of RNase)

Qiagen Solution-2-Lysis solution for plasmid preparations

200 mM	NaOH
1% (w/v)	SDS

Qiagen Solution-3-Neutralizing solution for plasmid preparations

3 M	CH_3COOH, pH 5.5

Qiagen Elution Buffer

1.25 M	NaCl
50 mM	Tris-HCl pH8.5
15%	Isopropanol

Qiagen Equilibration Buffer

750 mM	NaCl
50 mM	MOPS pH 7.0
15 %	Isopropanol
0.15%	Triton® X-100

Qiagen Wash Buffer

1 M	NaCl
50 mM	MOPS pH 7.0
15%	Isopropanol

Reporter Lysis 5x Buffer

The reporter lysis buffer was purchased from Promega, UK as part of the luciferase assay kit. The buffer was diluted with distilled water to make a 1x solution before use.

RIPA Lysis Buffer

150	mM	NaCl
1%	(v/v)	NP40 (or equivalent)
0.1%	(w/v)	SDS
50	mM	Tris-HCl (pH 8.0)
1/25		COmplete™ Protein Inhibitor Cocktail

Ribonuclease A (RNase A)

50mg of ribonuclease A (Sigma) was dissolved in 1ml of 10mM Tris-HCl pH7.5, 15mM NaCl to make a 10mg/ml stock solution which was stored in single-use aliquots at -20°C. (Boiling to remove DNase was not recommended by the manufacturer).

SDS Solution

A 10% (w/v) solution of sodium dodecyl sulphate (SDS) was dissolved in water and stored at room temperature.

6X SDS-PAGE sample buffer (Laemmli buffer)

750 mM	0.5M Tris pH 6.8 (33.3ml in 50ml total)
30% (v/v)	Glycerol (15ml in 50ml total)
6% (w/v)	10% SDS (3g in 50ml total)
0.03% (w/v)	Bromophenol blue
60 mM	2-mercaptoethanol (210µl in 50ml total)

10x SDS-PAGE Running Buffer

720 g	Glycine
150 g	Tris
50 g	SDS

Final volume adjusted to 5l with distilled water

10x SDS-PAGE Transfer Buffer

725 g	Glycine
145 g	Tris

Final volume adjusted to 5l with distilled water. The buffer was then used with 20% ethanol as 1X solution.

Stripping Buffer, commercial

Provided by Chemicon International, USA

50x TAE (Tris-Acetate-EDTA) buffer

242 g	Tris base
57.1 ml	Glacial acetic acid
100 ml	0.5 M EDTA

The final volume was adjusted to 1l with distilled water and pH adjusted to 8.5, before using at a 1X concentration. For making an agarose gel, 2µl of 10mg/ml Ethidium bromide solution per 100ml was added to the 1X TAE.

TE Buffer

10 mM	5 ml of 1 M Tris-HCl pH 8.0
1 mM	1 ml of 0.5 M EDTA, pH 8.0

Final volume adjusted to 500ml with distilled water

Tris Stock solutions

Tris base was dissolved in water to provide 0.5M, 1M and 1.5M solutions which were pH adjusted with concentrated HCl.

10x Tris Buffered Saline Tween (TBS-T)

121 g	Tris base
36.53 g	NaCl
250 ml	Tween-20 (Sigma)

pH adjusted to 7.6 with around 60ml HCl in a total volume of 5 litres. Used at 1x concentration.

2x TYE Medium (per litre)

1.6% (w/v)	16 g Bacto-tryptone (Difco, USA)
1% (w/v)	10 g Bacto-yeast extract (Difco, USA)
85 mM	5 g NaCl

The pH was adjusted to 7.4 and the solution autoclaved.

TYE Ampicillin Plates (per litre)

1% (w/v)	10 g Bactotryptone (Difco, USA)
0.5% (w/v)	5 g Bacto-yeast extract (Difco, USA)
140 mM	8 g NaCl
1.5% (w/v)	15 g Agar (Difco, USA)
50 µg/ml	Ampicillin

The solution was adjusted to pH 7.4 and autoclaved. At 50°C, ampicillin was added to a final concentration of 75µg/ml and the plates poured. The solidified plates were stored at 4°C, for up to one month.

Urea buffer

8 M	urea
1 M	thiourea
0.5% (w/v)	CHAPS (3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate)
50 mM	DTT (Dithiothreitol)
24 mM	spermine

Primers used in PCR

All primers were synthesized by Operon, Germany.

Water

Nanopure water (Type I) generated from the MilliQ water system was used for all procedures.

2.1.2 SDS-polyacrylamide Gels

	Resolving Gels				**Stacking**
	6%	**8%**	**10%**	**12%**	**4 %**
Acryl/Bis	2 ml	2.7 ml	3.3 ml	4.0 ml	1.3 ml
1.5 M Tris-HCl pH 8.8	2.5 ml	2.5 ml	2.5 ml	2.5 ml	--
1.0 M Tris-HCl pH 6.8	--	--	--	--	2.5 ml
10% SDS	100 µl	100 µl	100 µl	100 µl	100 µl
10% APS	100 µl	100 µl	100 µl	100 µl	50 µl
TEMED	10 µl	8 µl	5 µl	5 µl	10 µl
Distilled Water	5.3 ml	4.6 ml	4.0 ml	3.3 ml	6.1 ml
Total volume	10 ml	10 ml	10 ml	10 ml	10 ml

All resolving and stacking gels were prepared using 30% acrylamide/bis-acrylamide (Acryl/Bis) 29:1 (NBL, UK or BioRad, UK). Values given are per 10ml of gel required. Abbreviations: Ammonium Persulphate (APS); N,N,N',N',-tetramethy-ethylenediamine (TEMED), Tris (Tris(hydroxymethyl) aminomethane), sodium dodecyl sulphate (SDS).

Tris-acetate gels and gradient gels were purchased from Invitrogen.

2.1.3 Antibodies

Antigen	Antibody name or catalogue number	Species	Source
p53	DO-1	Mouse mAb	Hybridoma
p300	RW128	Rabbit pAb	Upstate
Actin	C-2	Mouse mAb	Santa Cruz Biotechnology
APC	Ali 12-28	Mouse mAb	abcam
APC	Ab-1 (clone FE9)	Mouse mAb	Calbiochem
APC	F-3	Mouse mAb	Santa Cruz Biotechnology
APC		Mouse mAb	Hybridoma
ASPP2	LX054.2	Mouse mAb	Hybridoma
Mouse ASPP2	LX50.13	Mouse mAb	Hybridoma

ASPP1	N8	Rabbit pAb	Serum
ASPP1&2	LX54.1	Mouse mAb	Hybridoma
ASPP2	DX54.10	Mouse mAb	Hybridoma
ASPP2	BP77	Rabbit pAb	Serum
E-cadherin	#610181 (clone 36)	Mouse mAb	BD transduction laboratories
E-cadherin	24E10	Rabbit mAb	Cell Signaling
N-cadherin	ab12221	Rabbit pAb	abcam
Cleaved-caspase3	#9664	Rabbit mAb	Cell Signaling
α-catenin	#610193 (clone 5)	Mouse mAb	BD Transduction Laboratories
β-catenin	E-5	Mouse mAb	Santa Cruz Biotechnology
β-catenin	#610153 (clone 14)	Mouse mAb	BD Transduction Laboratories

Active-β-catenin	anti-ABC (clone 8E7)	Mouse mAb	Upstate
Phospho- β-catenin (S33/37T41)	#9561	Rabbit pAb	Cell Signaling
Phospho- β-catenin (S45)	#9564	Rabbit pAb	Cell Signaling
Phospho- β-catenin (T41/S45)	#9565	Rabbit pAb	Cell Signaling
Cyclin D1	#2926	Mouse mAb	Cell Signaling
iASPP	LX49.3	Mouse mAb	Hybridoma
iASPP	B18P	Rabbit pAb	Serum
iASPP	N2	Rabbit pAb	Serum
Phospho-histone H3		Rabbit pAb	Upstate
Ki67		Mouse mAb	Novocastra
Nestin		Mouse mAb	DSHB

Gal4	sc-46680	Mouse mAb	Santa Cruz Biotechnology
Gal4	GAL4-TA (768)	Rabbit pAb	Santa Cruz Biotechnology
β-tubulin	TUB 2.1	Mouse mAb	abcam
βIII-tubulin		Mouse mAb	Covance
Ku80	Ab-2 (clone 111)	Mouse mAb	Neomarker
Myc-tag	9E10	Mouse mAb	Hybridoma
PCNA	PC-10	Mouse mAb	Hybridoma
V5	MCA1360	Mouse mAb	Serotec
V5	Ab9116	Rabbit pAb	abcam
Mouse-IgG	Mouse secondary	Rabbit-HRP	Dako
Rabbit-IgG	Rabbit secondary	Goat-HRP	Dako

Alexa Fluor® 488 $F(ab')_2$ fragment of gat anti-rabbit IgG	A11070	Goat	Molecular Probes
Alexa Fluor® 488 $F(ab')_2$ fragment of gat anti-mouse IgG	A11017	Goat	Molecular Probes
Alexa Fluor® 546 $F(ab')_2$ fragment of gat anti-rabbit IgG	A11071	Goat	Molecular Probes
Alexa Fluor® 546 $F(ab')_2$ fragment of gat anti-mouse IgG	A11018	Goat	Molecular Probes
Goat Anti-Mouse IgG_1 (γ_1 chain specific)	1070-03	Goat	SouthernBiotech
Goat Anti-Mouse IgG_{2a} (γ_{2a} chain specific)	1080-02	Goat	SouthernBiotech

Abbreviations – monoclonal antibody (mAb), polyclonal antibody (pAb), horse-radish peroxidase (HRP).

2.1.4 Plasmids

Plasmid Name	Relevant Information	Source / Reference:
p21Luciferase	p53-responsive p21 promoter linked to luciferase reporter	Bert Vogelstein
PIG3Luciferase 17mer	p53-responsive PIG3 promoter containing 17 repeats of p53 binding sites linked to luciferase reporter	Matthias Dobelstein (Contente et al. 2002)
Mdm2-Luc	p53-responsive Mdm2 promoter linked to luciferase reporter	
renilla	pRL-CMV Vector	Promega
TOPFLASH	LEF/TCF reporter plasmid	Upstate
APC	Wild-type APC in pCMV-Neo-Bam	Bert Vogelstein
APCL	pcDNA3.1(+)-APCL(full)-Myc-His	(Nakagawa et al. 2000a)
APCLΔ	pcDNA3.1(+)-APCL(1-1675, del C region)-Myc-His	(Nakagawa et al. 2000a)
β-catenin	Wild-type β-catenin in pCI-neo Vector	Bert Vogelstein (Morin et al. 1997)
β-catenin (MT1)	full-length *Xenopus* β-catenin	(Zhu and Watt 1999)
PP1α	Cloned in TOPO vector	Susana Llanos

SN3 (CMV p53)	Human p53 driven by the CMV promoter	Bert Vogelstein (Baker et al., 1990)
pcDNA3.1/p53	Human p53 driven by the CMV promoter	Gordon Peters (Cancer Research UK, London, UK)
T7-p53	Human p53, driven by the T7 promoter	Previously obtained by Xin Lu (re-cloned from the original SN3)
pcDNA3.1/6KR p53	Human p53 mutated at the C terminal lysine residues 370, 372, 373, 381, 382 and 386 into arginine residues	Ronald T Hay (Rodriguez MS et al., MCB, 2000)
pcDNA3.1/V5-His-TOPO/ASPP1	Human ASPP1 driven by the CMV promoter and V5 tagged at the C terminus	Susana Llanos
pcDNA3.1/V5-His-TOPO/ASPP2	Human ASPP2 driven by the CMV promoter and V5 tagged at the C terminus	Susana Llanos
pcDNA3.1/V5-His-TOPO/ASPP2 PP1binding mutant	Mutant of human ASPP2 which cannot bind to PP1	Susana Llanos
pcDNA3.1/His-TOPO/iASPP-V5	Human iASPP driven by the CMV promoter and V5 tagged at the C terminus	Susana Llanos

pSuper-iASPP RNAi	Expression of iASPP-RNAi in a hairpin loop driven by CMV promoter	Giuseppe Trigiante (Slee EA et al., Oncogene, 2004)
pcDNA3.1/poly-ubiquitin	Expression of multi copies of ubiquitin cDNA cloned downstream of the CMV promoter	Ronald T Hay (University of St Andrews, UK)
PCDNA3.1/V5-His-TOPO	Empty vector containing CMV and T7 promoter sites	Invitrogen

All plasmids constructed by our laboratory or received from other laboratories were sent to MWG for sequencing to confirm their identity. All cDNA encode human wild type proteins unless otherwise stated.

2.1.5 Cell lines

Established Cell lines

Name	Tissue Type/Origin
DU145	Human prostate carcinoma (derived from metastatic site: brain)
H1299	Human lung carcinoma; p53 null
MCF7	Human breast adenocarcinoma, wild type p53
MDCK	Dog kidney (normal)
SaOS-2	Human osteosarcoma; p53 null
U-2 OS	Human osteosarcoma, wild type p53

All cells were obtained from American Type Culture Collection (ATCC) and stored in liquid nitrogen until required.

Constructed Cell lines

Name	Description
L Wnt-3A (ATCC)	L-M(TK-) cells were transfected with Wnt-3A expression vector and stable clones selected in medium containing G418; produces Wnt-3A protein; Mouse
L Wnt-5A (ATCC)	L-M(TK-) cells were transfected with Wnt-5A expression vector and stable clones selected in medium containing G418; produces Wnt-5A protein; Mouse

2.2 Methods

2.2.1 Tissue culture

Basic Media

RPMI 1640 and Dulbecco's Modified Eagle's Medium (DMEM) were purchased from Gibco-BRL, UK and stored at 4°C.

Media supplements

Foetal calf serum (FCS) was purchased from PAA Laboratories and tested for its ability to support growth of various cell lines. It was heat inactivated for 30 minutes at 55°C and stored at -20°C in 50 ml aliquots.

L-Glutamine was purchased from Gibco-BRL at a 200mM concentration stored at -20°C and used at a final concentration of 2 mM.

Penicillin / Streptomycin was purchased from Gibco-BRL at 10,000 units/ml stored at -20°C and used at a final concentration of 200 units/ml.

Maintaining cell lines

All cell lines were cultured in Complete Medium (DMEM or RPMI 1640) supplemented with L-Glutamine, penicillin / streptomycin and 10% (v/v) foetal calf serum in flasks or dishes (Falcon) maintained in a Heraeus incubator at 37°C in the presence of 10% CO_2. Medium was changed every 3-5 days depending on the cell lines. On reaching confluence, the cells were washed once with 1X PBS and incubated with 2-4ml pre-warmed Trypsin-EDTA (Gibco-BRL) at 37°C until the cells detached from the flasks or dishes. Trypsin was inhibited by addition of an appropriate volume of fresh growth medium and this culture was then seeded on to fresh flasks or dishes at the desired density.

Freezing / Thawing of cells

Cells were seeded the day prior to freezing at a density such that they would be 70% confluent on the day of freezing. Cells from the growing culture were detached by trypsinising with 0.5ml Trypsin-EDTA then resuspended in 2ml of freezing medium (10% v/v DMSO , 90% v/v FCS) at a concentration of around 1-5 $x10^7$ cells per ml. 1ml aliquots of the cell suspensions were transferred into 2ml freezing ampoules (Corning). The vials were then labelled and cooled at the rate of 1°C per minute in a Nalgen Cryo 1°C freezing container or in a tissue-insulated polystyrene box when placed in a -80°C freezer (New Brunswick Scientific) for at least 24 hr before being transferred to a liquid nitrogen tank for long term storage.

To thaw cells from the liquid nitrogen stock, vials were placed in the 37°C water bath for 2 minutes and then transferred to a 6cm or 10cm dish with the appropriate pre-warmed fresh growth medium and kept in the 37°C incubator overnight to recover.

2.2.2 DNA techniques

Bacterial strains and culture

The *Escherichia coli* strains DH5α or BL21 were used as host strains for plasmid DNA. Bacteria were cultured at 37°C in 2X TY broth with the appropriate antibiotic (for example 100μg/ml Ampicillin) according to the resistance gene carried by the plasmid DNA for selection of transformed bacterium.

Preparing competent bacterial cells

A single colony of the required *E.coli* strain was picked from a 2x TY plate, inoculated in 3ml 2x TY without any antibiotics and cultured at 37°C with shaking overnight. 1ml of this culture was used to inoculate a 500ml 2x TY flask without antibiotics, which was incubated at 37°C with shaking for about 2-3 hr;

the optical density at 600nm was measured at appropriate intervals until it reached 0.95. The cells were then pelleted by centrifugation for 5 minutes at 490g (IEC PR-7000) at 4°C and washed with 10ml of sterile ice-cold solution containing 80mM $CaCl_2$ and 50mM $MgCl_2$. The bacteria were pelleted again and then resuspended in 10ml sterile cold 0.1M $CaCl_2$ (to a final concentration of 5 x 10^9 cells /ml) and incubated on ice for 20 minutes. After the addition of sterile-filtered 50% glycerol, the bacteria cells were aliquoted into sterile Eppendorf tubes and snap frozen on dry ice/ethanol. The tubes were then stored at -70°C. Re-freezing and thawing the same aliquot was avoided.

Transformation

Competent cells were thawed on ice, followed by addition of the desired plasmid DNA (100ng) to a vial of competent cells. The mixture was incubated on ice for 30 minutes. The bacteria were subjected to heat shock for 20 seconds at 42°C in a water bath followed by incubation on ice for another 2 minutes. 500µl of 2x TY without antibiotics was added to the Eppendorf tube and the sample left to shake at 37°C for 30 minutes to 1 hour before plating on 2x TY plates with an appropriate antibiotic. Plates were then incubated at 37°C overnight.

Small scale preparation of plasmid DNA (mini-prep)

A single bacterial colony was used to inoculate 3ml of 2X TY/antibiotic medium in a sterile test tube. The medium was shaken at 37°C for 16 hours followed by the removal of 2ml, which was then centrifuged at 3,000rpm, at 4°C for 2 minutes (eppendorf 5417R centrifuge/F-45-24-11 rotor). The supernatant was discarded into bleach and the pellet resuspended in 300µl of Qiagen Solution 1. Subsequently, 300µl of Solution 2 was gently mixed in by inverting a few times and left for 5 minutes at room temperature. 300µl of Qiagen Solution 3 was added on ice, mixed and left for 5 minutes. The mixture was then centrifuged (eppendorf 5417R centrifuge/F-45-24-11 rotor) at 14,000rpm at 4°C for 10 minutes and the supernatant removed to a fresh eppendorf followed by mixing with 650µl of iso-propanol. The solution was inverted a few times to mix and was then centrifuged at 14,000rpm for 10 minutes at 4°C. The supernatant was discarded carefully so as not to disrupt the pellet. 500µl of 70% ethanol was used to wash the pellet twice. The DNA was dried in a DNA speed vacuum (Savant DNA 10) at high speed and high temperature for 10 minutes. 50µl of TE buffer was added to resuspend the DNA pellet and the concentration measured by OD260 using a Nanodrop spectrophotometer.

Large scale preparation of plasmid DNA (maxi-prep)

A single bacterial colony was used to inoculate 5ml of 2X TY/antibiotic medium in a sterile test tube, and shaken at 37°C for 4 hours. The resulting bacterial suspension was used to inoculate a 250ml flask of 2X TY/antibiotic medium and shaken for a further 16 hours at 37°C. The cells were centrifuged at 5,000g for 15 minutes at 4°C (Sorvall RC 5C Plus, rotor SLA-3000). The large scale DNA preparation was carried out according to Qiagen Qiafilter Maxi DNA kit protocol.

Agarose gel electrophoresis

DNA samples were mixed with 5xDNA loading buffer to a final concentration of 1x before being run on an agarose gel of appropriate percentage according to the size of the DNA bands to be visualised. The gels were made with TAE buffer containing ethidium bromide and run at 60-80 V with DNA makers. The ethidium bromide stained DNA bands were then visualised under UV-irradiation.

2.2.3 Protein manipulation

Sample preparation

Cells grown in monolayers were washed three times with 1X PBS and lysed in appropriate lysis buffer (150-250µl per 10cm dish). The cells were scraped with a sterile disposable cell scraper (Greiner), transferred to an eppendorf tube and centrifuged at 14,000rpm, at 4°C or at room temperature depending on the lysis buffer for 30 minutes (eppendorf 5417R). The resulting lysate was removed to a fresh eppendorf tube and the cell debris discarded.

Protein concentration determination

The protein concentrations of cell extracts were determined using the BioRad protein assay reagent system. 0.5µl of cell lysate was mixed with 200µl of 1x BioRad assay reagent and then measured at 595nm in the spectrophotometer (Anthos Labtech instrument). All samples were measured in duplicate and the absorbance was compared against a standard curve made at the same time from known concentrations of bovine serum albumin (BSA; Sigma-Aldrich) in the same solutions, using the same method.

Preparation of SDS-polyacrylamide gels

All plates were washed with water and detergent, dried and assembled in the casting trays (Pharmacia BioTech, UK). The acrylamide content of the gels varied between 8%-12% depending on the size of the protein of interest. The acrylamide gels were overlaid with 70% isopropanol solution and left to polymerise. After polymerisation, the isopropanol was removed and a 4% stacking gel was set with the appropriate number and size wells.

SDS-polyacrylamide gel electrophoresis (PAGE)

Known concentrations of protein were mixed with appropriate volumes of 5x SDS-PAGE Sample Loading Buffer and boiled for 5 min. Equal amounts of proteins were loaded in respective wells cast in the stacking gel. The resolving gels were made with 6-12% of polyacrylamide, depending on the size of the proteins to be resolved. The gels were run at 135V for one hour in 1x SDS-PAGE running buffer using a Mighty Small II system (Hoefer) with a protein molecular weight marker.

Immunoblotting

Cell lysates and protein molecular weight marker in sample buffer were loaded onto SDS-polyacrylamide gels in a 1x SDS-PAGE running buffer and the proteins electrophorised at a constant voltage of 100-250V. Equal amounts of protein were loaded in each lane as determined by the BioRad assay system, unless otherwise stated. After the samples were separated through the gel, the gel was transferred to a wet transfer unit containing 1x SDS-PAGE transfer buffer. The proteins were then electrophoretically transferred onto nitrocellulose membrane for 1-3 hours at a constant voltage of 55V, or 20V overnight, in a Hoefer Transphor Electrophoresis unit. The membrane was then stained with Ponceau S solution to determine the success of the transfer of proteins and equal loading of the lanes. The membranes were then washed in water and incubated in 10% non-fat milk at room temperature for 40-60 minutes. The membranes were then ready to be probed with primary antibody at the recommended concentrations for 1-3 hour at room temperature or overnight at 4°C. Membranes were washed with large amounts of water before addition of the secondary antibody at the recommended concentration (generally 1:2000) at room temperature for 1 hour. After incubation with the secondary antibody the membrane was washed with 1X TBS-T extensively with repeated changes of TBS-T. The ECL was then performed according the manufacturer's instructions (Amersham Life Science) or the membrane was scanned with the Licor-Odyssey. If reprobing with another primary antibody was required, stripping of the membranes was performed as follows. Membranes were incubated with a commercial stripping buffer

(Chemicon International) for 15 minutes at room temperature. The membranes were extensively washed with 1X TBS-T and then blocked in 10% milk for 1 hour at room temperature. The blot was then reprobed with primary antibody as before.

Immunoprecipitation (with cell lysates)

Cells grown in monolayers were washed three times with 1X PBS and lysed in the appropriate lysis buffer (150-250µl per 10cm dish). The cells were scraped using a sterile disposable cell scraper (Greiner) and transferred to an eppendorf tube, before centrifugation at 14,000rpm, at 4°C for 30 minutes (eppendorf 5417R). The resulting lysate was removed to a fresh eppendorf tube and the cell debris discarded. The protein concentration was determined using the BioRad assay system. 500-2000µg of cell lysate was precleared with 30µl of protein G sepharose beads (50% slurry in PBS) for 30-60 minutes at 4°C on an eppendorf rotating wheel. The lysate was centrifuged at 2,500rpm for 2 minutes and the supernatant was removed into a fresh tube. 1-4µl of antibody and 30µl protein G sepharose beads (50% slurry in PBS) was then added to the pre-cleared lysate. The mixture was left on an eppendorf rotating wheel overnight at 4°C. Immunocomplexes were collected by centrifugation at 2,500rpm for 3 minutes and the supernatant discarded. The beads were washed with three successive changes of lysis buffer. After removing as much residual supernatant as possible, the IP beads were mixed with 20-60µl of 5X sample buffer and heated at 95°C for

5 minutes. The beads were centrifuged at 14000 rpm for 15 seconds and all or part of the sample loaded onto a SDS-polyacrylamide gel. The separated proteins were processed for immunoblotting.

2.2.4 Cell-based assays

Cell transfection

Adherent cells were split to a confluence of between 70-80% in fresh medium. Typically, a 60mm dish was seeded with 8 x 10^5 Saos-2 cells, plated 24 hours prior to the transfection procedure. 15-30 minutes before the transfection, the medium on the dishes was replaced with 3 ml of fresh medium. 2X HBS buffer was diluted in sterile water to a concentration of 1X and a volume of 300µl per 60mm dish placed in sterile eppendorf tubes. The required amount of DNA was added using sterile Gilson tips and mixed thoroughly. To form the precipitate, 30µl of 2.5 M $CaCl_2$ was added to the transfection mix and left at room temperature for 15 minutes. After this time the mixture was added drop-wise in the tissue culture hood to the cells and the dishes replaced in the incubator for 6 hours. The medium was removed and a wash of DMEM medium without foetal calf serum was applied to the cells. Finally, 3ml growth tissue culture medium was added to each dish and the cells were left for 24 hours, for a transient assay. If

a lipotransfection method was required, the manufacturer's procedures were followed (Fugene 6, Roche; Effectene, Qiagen; Lipofectamine 2000, Invitrogen).

Immunocytochemistry

Cells were seeded on coverslips in 24-well plates at 70-90% density. Once experimental procedures were carried out, cells were fixed with 200ml of 4% paraformaldehyde in PBS for 10 minutes then permeabilized with 0.1% Triton X-100 in PBS for 4 minutes. Anti-β-catenin E-5 antibody (1:100), DX54.10 (1:50) and anti-V5 monoclonal antibody (1:100) were diluted in PBS containing 2% of BSA and applied to the cells for 40 minutes. Protein expression was detected by using Alexa Fluor® (1:400, Molecular Probes) for 20 minutes. TO-PRO (Invitrogen) was used to stain nucleic acids (1:2000).

Transactivation in MEFs

MEFs were plated 24 hours before transfection ($7x10^5$ cells per dish). For each dish, 6μl of FuGene 6 (Roche) was used to transfect 2μg of Lef/Tcf luciferase reporter (TOPFLASH, Upstate biotechnology) and 0.1μg of renilla luciferase. Twenty-four hours after transfection, the medium was changed and cells were treated with LiCl over-night. Finally, the transcriptional assay was carried out

using the Dual-luciferase® reporter assay system (Promega) following the manufacturer's protocol.

Transfection of ASPP2 siRNA in MCF7 cells

The siRNA directed against ASPP2 was obtained from QIAGEN and was designed to target the following sequence: AACGTGAATGCTGCTGATAGT. Cells were transfected in 15cm dishes when they reached 80-90% confluency. For each transfection, 90μg of DNA (pcDNA3.1) were used together with 135μl of siRNA (20mM solution) and 90μl of Lipofectamine™ 2000 (Invitrogen). Transfected cells were selected by using G418 (Invitrogen) (final concentration: 0.5 mg/ml) for 48 hours.

Cytosol and nuclear fractionation

Cells were rinsed and harvested with cold PBS. One-third of the collected cells was pelleted and lysed in NP-40 lysis buffer containing 20mM HEPES (pH 8.4), 150mM NaCl, 0.5% NP-40, 1mM EDTA, 1mM dithiothreitol (DTT), 10% glycerol and a cocktail of protease and phosphatase inhibitors. Lysates were incubated for 15 min on ice and centrifuged for 15 min at 20000g. The supernatants were used as unfractionated lysates (L). The remaining two-thirds of the collected cells were pelleted at 1000g for 5min at 41°C, resuspended in four

volumes of buffer A (20mM HEPES (pH 7.6), 20% glycerol, 10mM NaCl, 1.5mM $MgCl_2$, 0.1% NP-40, 5mM DTT and a cocktail of protease and phosphatase inhibitors) and incubated on ice for 20 min with gentle vortexing. After centrifugation for 5 min at 1000 g at 41°C, the supernatant was collected and referred to as cytoplasmic extract (C). The nuclear pellet was resuspended in two volumes of buffer B (20mM HEPES, pH 7.6), 20% glycerol, 10mM NaCl, 1.5mM MgCl2, 0.1% NP-40, 5mM DTT, 500mM NaCl and a cocktail of protease and phosphatase inhibitors) and incubated for 30 min on ice before centrifugation for 15 min at 20000 g at 41°C. The supernatant was collected and referred to as nuclear extract (N). Equal amounts of proteins were analysed by SDS–PAGE.

2.2.5 *In vitro* assays

In vitro translation of plasmids

In vitro transcription and translation of plasmids was performed using the Promega TNT® T7 Quick coupled Transcription /Translation system. A typical reaction was carried out using 40µl of Reaction mix containing rabbit reticulocyte lysate, reaction buffer, all amino-acids (except methionine), RNase inhibitors and T7 RNA polymerase, together with 1 µg of plasmid containing the T7 promoter. Either 2µl of ^{35}S-Methionine (for radio-labelled proteins) or 1mM Methionine (for

non-labelled proteins) was added to the reaction mix and made up to 50µl with nuclease-free water. This was incubated at 30 °C for 60-90 minutes.

In vitro translation and *in vitro* immunoprecipitation

ASPP2-V5 and β-catenin were translated in vitro with cold methionine using the TNT T7 Quick coupled Transcription/Translation System (Promega). The reticulocyte lysates containing each protein were combined as indicated and incubated together for 1 h at 30°C. The samples were precleared in 1 ml of PBS by rotating for 1 h at 4°C with protein G sepharose beads (Amersham Biosciences). Following removal of the beads antibody immobilized on protein G sepharose beads was added to the binding reactions and rotated at 4°C for 16 h. The beads were then washed with PBS. The bound proteins were released in SDS sample buffer and analysed by 10% SDS–PAGE and immunoblotting.

Preparation of Protein G sepharose

Protein G Sepharose^{TM} 4 Fast Flow beads (Amersham Pharmacia) were washed three times with an excess volume of PBS then resuspended in PBS at a ratio of 1:1. For immunoprecipitations, the protein G beads were added to the required antibody by mixing 30µl of the 50% slurry protein G sepharose with 1-4µl ascites or the required amount of commercially-available solution in a 1.5 ml tube.

2.2.6 Mouse work

Mouse colonies

ASPP2(+/-) mice have been generated on a mixed C57BL/6Jx129SvJ background (Vives et al, 2006). *ASPP2* knockout mice were genotyped using the following primers: 5'-CTCCACCCCAGGAAATTACA-3' (intron 3), 5'-CGGTTTGGAAGTCAAAGGAA-3' (exon 3) and 5'-GGACCGCTATCAGGACATA-3' (neomycin resistance gene).

Histology and immunohistochemistry

Embryos of various stages were fixed in 10% buffered formalin overnight and then dehydrated in an ethanol series, cleared in histoclear and embedded in paraffin wax. Sections were cut at 4μm thickness and either stained with hematoxylin and eosin or processed for immunostaining. Rehydrated paraffin-embedded sections were microwaved in 10mM sodium citrate buffer, pH 6, incubated in 3% hydrogen peroxide in methanol, washed in PBS and incubated overnight with the primary antibody at 4°C, followed by either biotinylated or Alexa Fluor® (1:400, Molecular Probes) secondary antibodies for 30 minutes at room temperature. Bright light staining was visualised using the peroxide

substrate solution DAB (diaminobenzidine, Vector). Primary antibodies used were mouse anti-Ki67 (1:500, Novocastra), rabbit anti-phospho-histone H3 (1:500, Upstate), anti-cleaved caspase 3 (1:500, Cell signalling technology), mouse anti-nestin (rat 401, 1:1000, DSHB), mouse anti-βIII tubulin (TUJ1, 1:1000, Covance), mouse anti-β-catenin (1:50, Transduction laboratories), rabbit anti-N cadherin (1:100, abcam) and mouse anti-cyclin D1 (1:1000, Cell Signalling).

Neurosphere culture and differentiation

The methodology of Reynolds and Weiss was followed. Briefly, the striata were removed from E13.5 embryos and mechanically dissociated with a fire polished Pasteur pipette. The cells were plated in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and F-12 nutrient (DMEM/F-12, Gibco) supplemented with hepes buffer (5 m*M*), glucose (0.6%), sodium bicarbonate (3 m*M*), glutamine (2 m*M*), insulin (25 mg/ml), apo-transferrin (100 mg/ml), progesterone (20 n*M*), putrescine (60 m*M*) and sodium selenite (30 n*M*). Epidermal growth factor (EGF) (20 ng/ml) and fibroblast growth factor 2 (FGF-2) (20 ng/ml). Seven days later, the resulting neurospheres were dissociated and resuspended in 96-well plates coated with poly-L-ornithine (15μg/ml). In the medium used to induce differentiation, EGF and FGF-2 were replaced by 1% foetal bovine serum.

Generation of murine embryonic fibroblasts (MEFs)

MEFs were prepared from E13.5 mice embryos. A 13.5-day-old pregnant mouse was sacrificed by cervical dislocation and the uterus was removed and placed in PBS. The embryos were then isolated from the uterus under sterile conditions and their heads and internal organs were removed. The remaining tissues were disaggregated by fine mincing followed by treatment with 4 ml of trypsine-EDTA (Invitrogen) for 10 minutes at 37°C. The cell suspensions were subsequently transferred to 15 ml Falcon tubes to which 10 ml of DMEM supplemented with 10% FCS was added. After 5 minutes, the cell supernatants were plated into 15 cm dishes.

2.2.7 Data analysis

Computer images

All autoradiographs were scanned using the Epson perfection 1660 Photo scanner and the Adobe Photoshop 7.0 software. Images were manipulated only as a whole for size, brightness and contrast. No signal was modified in relation to the whole image.

Chapter III: APCL and APC positively regulate the function of ASPP1 and ASPP2 in p53 mediated apoptosis

3.1 Introduction

APCL was first described as a brain specific homologue of the adenomatous polyposis coli tumour suppressor (Nakagawa et al. 1998; van Es et al. 1999). Nakagawa et al. showed that, in adult tissues, APCL is exclusively expressed in the brain. In contrast, Van Es et al. found that it was expressed at high levels in the central nervous system, including the whole brain and the spinal cord, although APCL mRNA was also found at lower levels in other organs, such as in the heart, lung, and small intestine. Interestingly, the highest levels of APCL mRNA, like APC, were found in the foetal brain, which suggests that APCL could play a role in the development of the CNS (van Es et al. 1999). The precise expression pattern of APCL during mouse development has also been studied and its expression was found in post-mitotic cells during the development of the cortex, retina and cerebellum (Yamanaka et al. 2002).

APCL was found to be down-regulated in glioma specimens and glioma cell lines, suggesting that APCL may also be associated with progression of brain tumours (Nakagawa et al. 1999). However, APCL was not found to be mutated in a panel of brain tumours, although this does not exclude the possibility of epigenetic inactivation of APCL, for example, by methylation of its promoter. This is in stark contrast to APCL's homologue APC, which is commonly mutated in colorectal cancer.

ACPL, like its homologue APC, has been shown to be able to down-regulate β-catenin; however, the molecular basis of APCL function in the regulation of β-catenin has yet to be clarified (Nakagawa et al. 1998; van Es et al. 1999). As previously mentioned, APCL and APC are mainly conserved in their N-terminal half. Hence, the heptad repeat domain of APC, the armadillo domain, five copies of the 20 amino acid repeats and two of the SAMP repeats are conserved in APCL (Figure 3.1). This suggests that APCL can still bind to β-catenin and Axin through its conserved motifs, and that it might form a similar β-catenin destruction complex as the one containing APC.

However, APC and APCL retain little homology in their C-terminal half, suggesting that this region might provide a relative degree of specificity to their functions. The C-terminus of APCL has been shown to be involved in the binding to 53BP2, a smaller version of ASPP2 (Nakagawa et al. 2000a) (Figure 3.1).

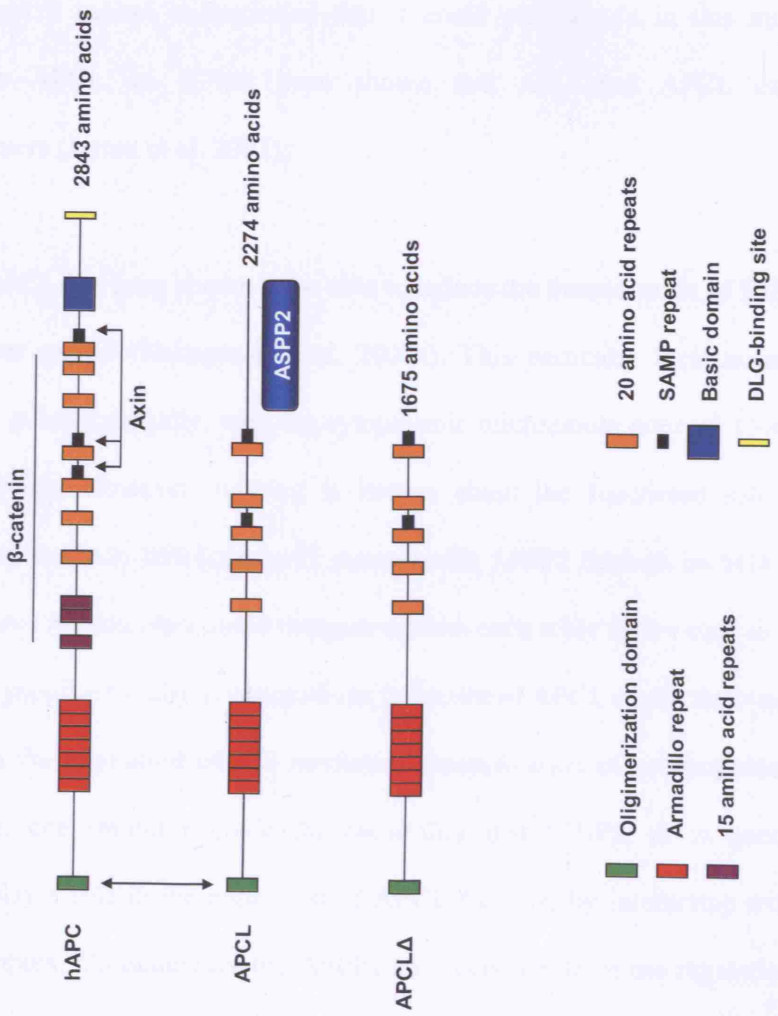


Figure 3.1: Structure of APC, APCL and APCLΔ. The functional domains of APC and APCL are shown, together with APCLΔ, a deletion construct of APCL containing amino acids 1 to 1675, thus lacking the region identified as being required for binding to ASPP2.The key domains are indicated as shown, as well as the known binding regions of β-catenin and ASPP2.

53BP2 interacts with APCL through its SH3 domain and Ankyrin repeats, suggesting that the full-length protein ASPP2, as well as the other members of the ASPP family, could bind to APCL. Current evidence suggests that APC would not be able to bind directly to the ASPPs, as it lacks the APCL C-terminal binding region, but it cannot be excluded that it could play a role in this interaction alongside APCL, as it has been shown that APC and APCL can form heterodimers (Jarrett et al. 2001).

APCL has been shown to be able to induce the translocation of 53BP2 to a perinuclear region (Nakagawa et al. 2000a). This particular localization could coincide, at least partially, with the cytoplasmic microtubule network (Nakagawa et al. 2000b). However, nothing is known about the functional role of this interaction. As both APCL and p53 interact with 53BP2 through its SH3 domain and Ankyrin repeats, they could compete against each other for binding to ASPP2. Thus, we propose to fully investigate the influence of APCL on the function of the ASPPs in the regulation of p53 mediated transactivation of proapoptotic genes. Moreover, one cannot exclude the possibility that 53BP2, or in general the ASPPs, play a role in the regulation of APCL function by interacting within the same complex. Consequently the ASPPs may play a role in the regulation of β-catenin levels and activity.

3.2 Results

3.2.1 APCL induces the translocation of ASPP1 and ASPP2 to a perinuclear region

APCL has been described as being able to influence the localization of the C-terminus of ASPP2 (53BP2) and to drag it to a perinuclear region where they co-localize (Nakagawa et al. 2000a). Therefore, to fully investigate the effect of APCL on the subcellular localization of all the members of the ASPP family, we decided to transfect Saos-2 cells, which are p53 null, and express low levels of APC and ACPL (data not shown), with V5-tagged constructs expressing ASPP1, ASPP2 or iASPP with or without myc-tagged APCL (Figure 3.2). Exogenous expression levels of ASPP1 and ASPP2 were similar to their endogenous levels (data not shown). Using p53 null cells for these experiments allowed us to examine the localization of the ASPPs independently of this of p53.

As previously described, APCL was mainly localized in large aggregates around the nucleus whereas ASPP1, ASPP2 and iASPP were mainly found in the cytoplasm (Figure 3.2A), although expression was also detected at the membrane and in the nucleus of a certain number of cells.

As expected, when APCL was co-expressed with ASPP2, it profoundly affected its localization, to the extent that the majority of ASPP2 was found to co-

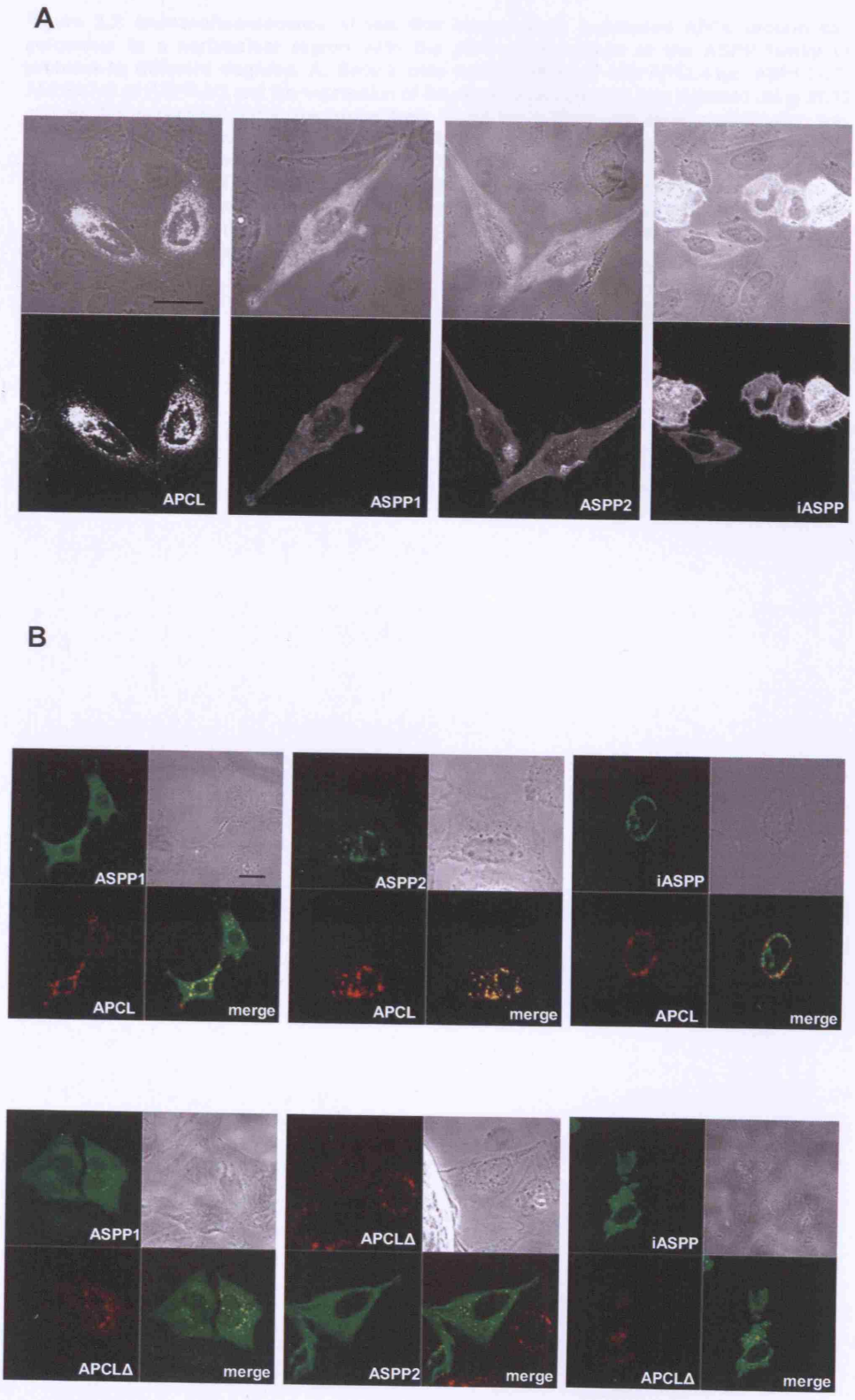

A
APCL
ASPP1
ASPP2
iASPP
B
ASPP1
APCL
merge
ASPP2
APCL
merge
iASPP
APCL
merge
ASPP1
APCLΔ
merge
APCLΔ
ASPP2
merge
iASPP
APCLΔ
merge

Figure 3.2

Figure 3.2: immunofluorescence shows that exogenously expressed APCL protein can colocalize in a perinuclear region with the different members of the ASPP family of proteins to different degrees. A. Saos-2 cells were transfected with APCL-myc, ASPP1-V5, ASPP2-V5 or iASPP-V5 and the expression of the recombinant proteins was detected using 9E10 and anti-V5 monoclonal antibodies respectively. Scale bar = 20μm (the same magnification was used for all the pictures in this figure). **B**. APCL-myc or its truncated form were transfected in SAOS2 cells together with ASPP1-V5, ASPP2-V5 or iASPP-V5. A Goat Anti-Mouse IgG1 specific antibody conjugated to TRITC (red) and a Goat Anti-Mouse IgG2a specific antibody conjugated to FITC (green) were used to detect 9E10 or anti-V5 antibodies respectively. Colocalization can be visualized in yellow on the pictures. Scale bar = 20μm (the same magnification was used for all the pictures in this figure).

localize with APCL in the perinuclear region where ectopically APCL is normally expressed (Figure 3.2B, upper panel). A similar observation was made when APCL was co-transfected with ASPP1, although the relocalization of ASPP1 was not as dramatic, since a portion of ASPP1 remained diffused in the cytoplasm. In contrast, the localization of iASPP did not appear to be affected by the over-expression of APCL. In conclusion, it would appear that the localization of the three members of the ASPP family is regulated to different degrees by APCL, with ASPP2 being the most affected, followed by ASPP1 and finally iASPP.

To determine whether the C-terminus of APCL, which was described as the binding region for ASPP2, was required for the ability of APCL to regulate ASPP1 and ASPP2 localization, we performed a similar experiment using an APCL construct, lacking this region, APCLΔ (Figure 3.1). ASPP1 and ASPP2 localization remained essentially unaffected by the truncated form of APCL, but surprisingly some of the proteins co-localized in small circular structures around the nucleus, suggesting that they could still partially interact (Figure 3.2B, lower panel).

3.2.2 APCL enhances the ability of ASPP1 and ASPP2 to increase p53 transactivation function on the PIG3 promoter

We have demonstrated that APCL and ASPP2 colocalize and Nakagawa et al. have shown that this binding requires domains of 53BP2 which are also involved in its interaction with p53 (namely the ankyrin repeats and the SH3 domain) (Nakagawa et al. 2000a). We, therefore, hypothesised that APCL might me able to interfere with the ability of ASPP2 to enhance the transactivation function of p53 on the promoters of proapoptotic genes. To address this issue, we used a PIG3 reporter construct and transiently transfected p53 and ASPP2 into Saos-2 cells either with or without APCL (Contente et al. 2002). As expected, ASPP2 was able to increase p53 ability to induce the PIG3 promoter (Figure 3.3B). Interestingly, although by itself it had few or no effect on p53 transactivation function on PIG3, APCL was able to significantly enhance the synergy between p53 and ASPP2. The levels of exogenous ASPP2 and p53 were not significantly altered by the expression of APCL, suggesting that APCL did not enhance the activity of p53 and ASPP2 through an increase of the expression levels of these two proteins.

Because the ASPPs are conserved in the region involved in the binding to APCL, we also addressed whether APCL could modify the regulation of p53 by ASPP1 and iASPP. Using a similar experimental design (Figure 3.3C), APCL was able to increase the synergy between ASPP1 and p53. Again, the exogenous

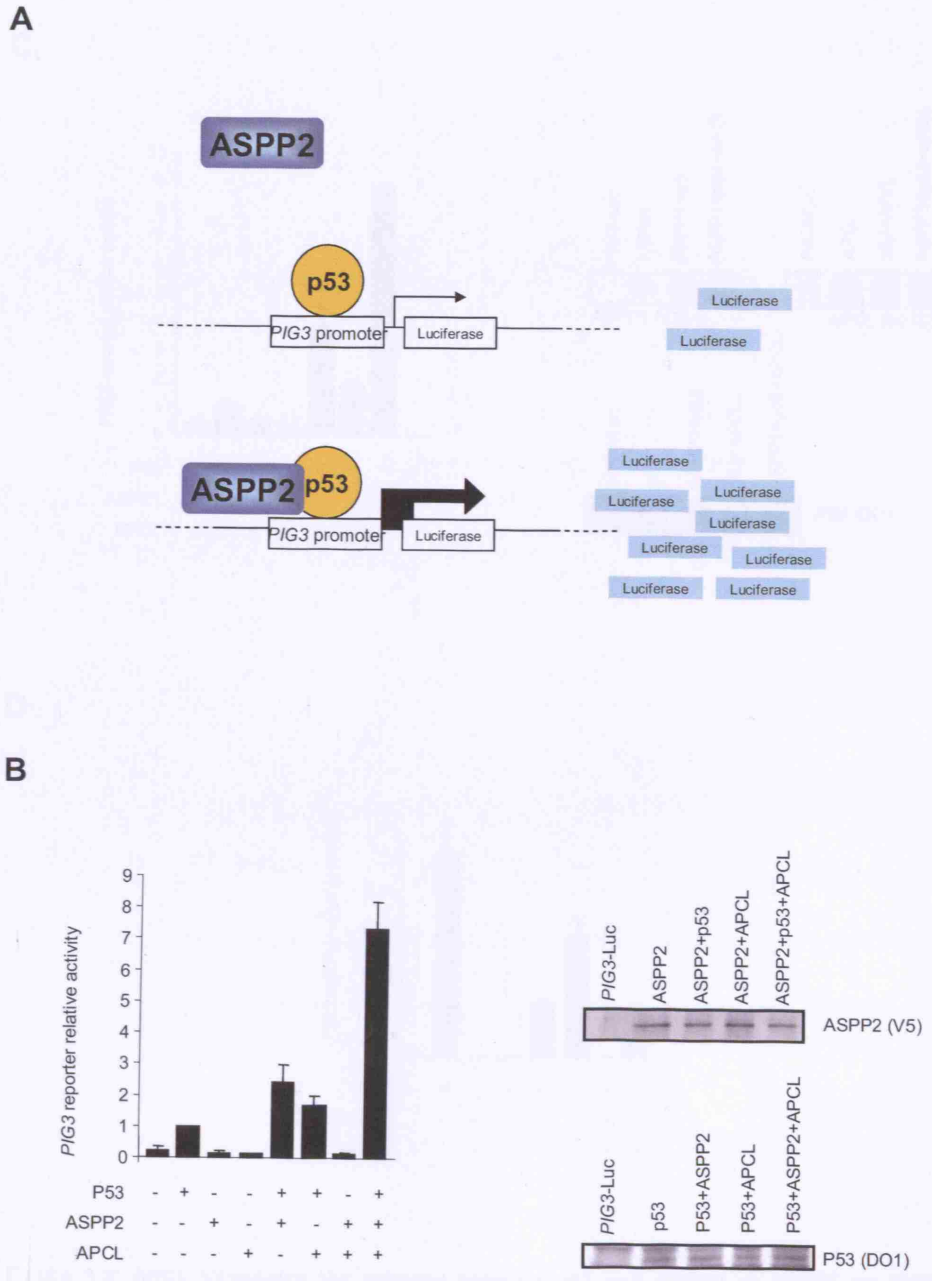
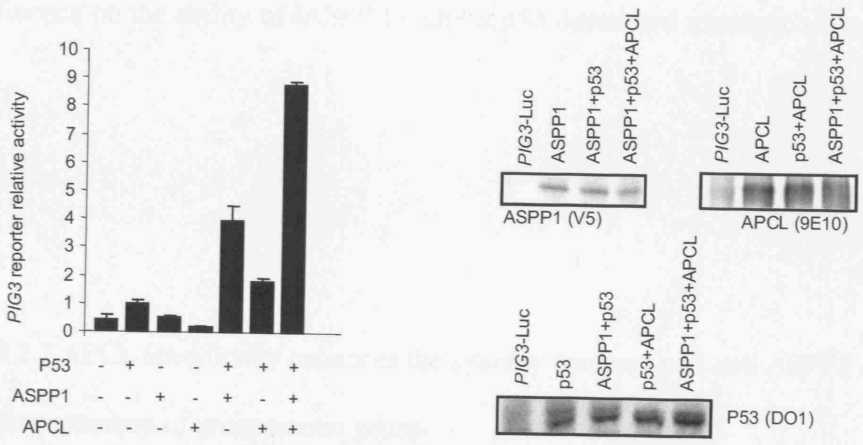

A
ASPP2
p53
PIG3 promoter
Luciferase
Luciferase
Luciferase
ASPP2
p53
PIG3 promoter
Luciferase
Luciferase
Luciferase
Luciferase
Luciferase
Luciferase
Luciferase
Luciferase
Luciferase
B
PIG3 reporter relative activity
9
8
7
6
5
4
3
2
1
0
P53 - + - - + + - +
ASPP2 - - + - + - + +
APCL - - - + - + + +
PIG3-Luc
ASPP2
ASPP2+p53
ASPP2+APCL
ASPP2+p53+APCL
ASPP2 (V5)
PIG3-Luc
p53
P53+ASPP2
P53+APCL
P53+ASPP2+APCL
P53 (DO1)

Figure 3.3

C



D

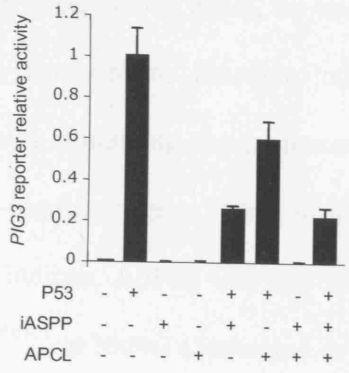


Figure 3.3: APCL stimulates the synergy between p53 and ASPP2 or ASPP1 on PIG3 promoter. **A**. Diagram illustrating the principle of a transactivation assay. The promoter of interest (*PIG3*) is cloned upstream the luciferase gene. In the presence of p53, the promoter is activated which drives the expression of luciferase. This is enhanced in the presence of ASPP2, which results in an increase of the luciferase activity. **B-D**. Transactivation assays were performed in Saos-2 cells. The cells were transfected with a *PIG3* reporter construct (1µg) together with renilla luciferase (0,1µg) and various amounts of the indicated expression plasmids in order to obtain similar levels of protein expression. The total amount of DNA was normalized by addition of the appropriate empty vector. The data were normalized relative to the renilla-luciferase activity. Error bars represent the standard deviation of 3 independent experiments performed in duplicate. Cell lysates were then normalized for total protein content and analyzed by SDS-PAGE/immunoblotting, using DO1, 9E10 and anti-V5 monoclonal antibodies for detecting p53, Myc-tagged APCL and V5-tagged ASPP1 and ASPP2 respectively.

protein levels of ASPP1 and p53 remained consistent upon APCL overexpression. In contrast to ASPP1 and ASPP2, the results in figure 3.3D show that APCL has little influence on the ability of iASPP to inhibit p53 dependent transactivation of PIG3.

3.2.3 APCL specifically enhances the synergy between p53 and ASPP2 on the promoters of proapoptotic genes

As it is known that ASPP1 and ASPP2 both specifically enhance p53 dependent transactivation of proapoptotic genes, we wanted to determine whether the effect of APCL was extended to functions other than apoptosis. Two additional reporter constructs containing the promoters of the cell cycle arrest gene $p21^{WAF-1}$ and the auto-regulatory gene Mdm2 were employed (Figure 3.4). In agreement with previous findings, ASPP2 was unable to substantially enhance p53 transactivation of $p21^{WAF-1}$ or Mdm2 (Samuels-Lev et al. 2001). APCL on its own did not increase p53 transactivation on either $p21^{WAF-1}$ or Mdm2 and, furthermore, this activity was not substantially increased in the presence of ASPP2. The increase of p53-mediated transactivation of Mdm2 observed in the presence of both ASPP2 and APCL was not statistically significant ($p=0.068$).

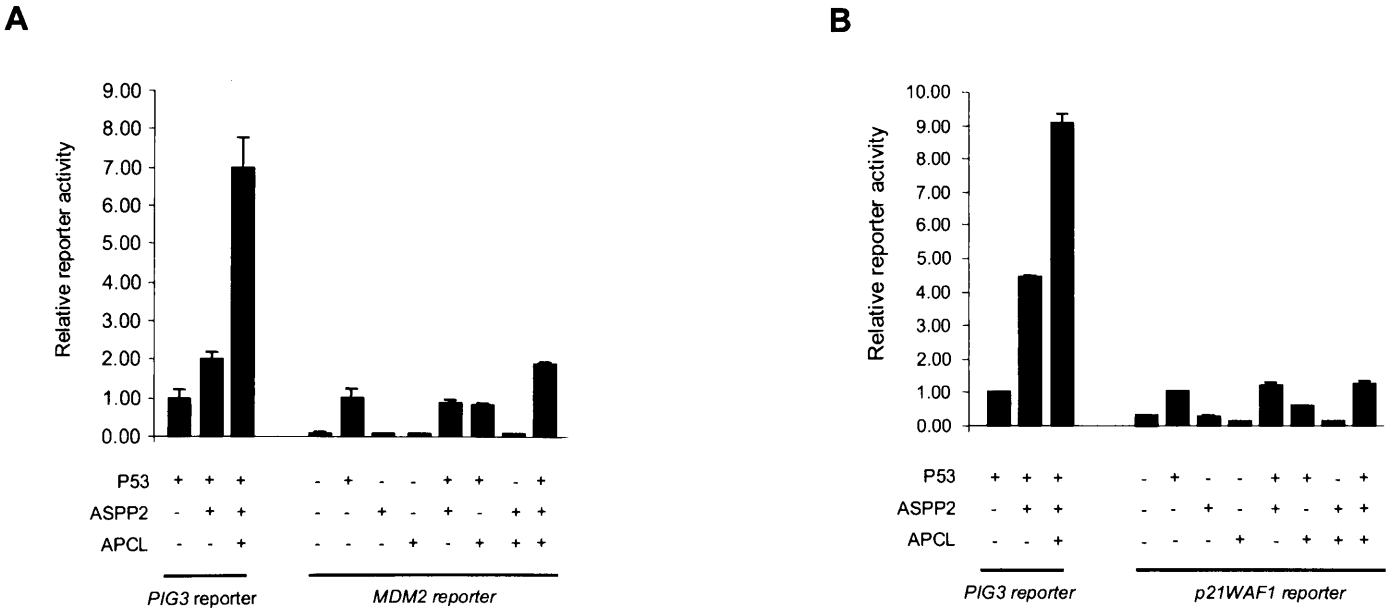


Figure 3.4: APCL specifically enhances the synergy between p53 and ASPP2 on the promoter of the proapoptotic gene *PIG3*. Comparison of the effect of APCL on ASPP2 and p53 using *PIG3* and *MDM2* reporter constructs (**A**) or *PIG3* and *p21WAF1* reporter constructs (**B**). SAOS2 cells were transfected with PIG3 (1µg), Mdm2 (1µg), or p21 (1µg) reporter constructs, together with renilla luciferase (0,1µg) and the indicated expression plasmids. The total amount of DNA was normalized by addition of the appropriate empty vector. The data were normalized relative to the renilla-luciferase activity. Error bars represent the standard deviation of 2 independent experiments. To allow comparison, luciferase activity in the presence of p53 was arbitrarily set to 1.

This suggests that APCL enhances p53 activity in an ASPP dependent mechanism and as a result it has an effect exclusively on p53 regulated apoptotic genes.

3.2.4 The C-terminal ASPP2 binding region of APCL is partially required for the effect of APCL on the synergy between ASPP1&2 and p53

As we showed that APCL could regulate ASPP1 and ASPP2 subcellular localization and function, we wanted to determine whether the interaction between APCL and ASPP1 or ASPP2 was the key element to these observations. Thus, we used the C-terminal truncated form of APCL (APCLΔ) which is not able to bind to the C-terminus of ASPP2, in place of APCL in the transactivation assay. We found that APCLΔ was clearly less potent than APCL in enhancing the synergy between ASPP1 and p53 (Figure 3.5A); an effect that was demonstrated more acutely between ASPP2 and p53 (Figure 3.5B). However, APCLΔ still had some effect in enhancing promoter activity, especially in the presence of ASPP1 and p53. In conclusion, we find that the ASPP2 C-terminal binding region of APCL is required for its full effect on the synergy between ASPP1 or ASPP2 and p53, but not essential, as this can still be partially enhanced by APCLΔ. Moreover, we show that the ASPP2 C-terminal binding region of APCL is more important in its effect on the synergy between ASPP2 and APCL than between ASPP1 and APCL

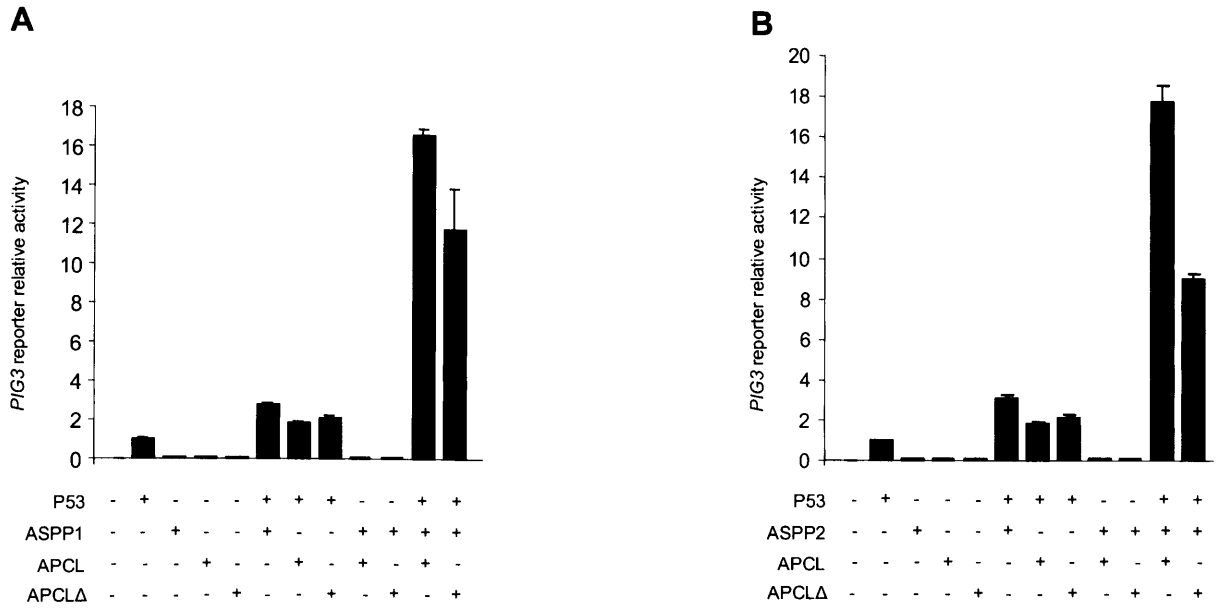


Figure 3.5: the C-terminal truncated form of APCL, APCLΔ, is less potent in its ability to stimulate the synergy between p53 and ASPP1 (A) or ASPP2 (B) on PIG3 promoter. SAOS2 cells were transfected witha PIG3 reporter construct (1µg) together with renilla luciferase (0,1µg) and various amounts of the indicated expression plasmids. The total amount of DNA was normalized by addition of the appropriate empty vector. The data were normalized relative to the renilla-luciferase activity. Luciferase activity in the presence of p53 alone was arbitrarily set as 1. Error bars represent the standard deviation of 3 independent experiments performed in duplicate.

suggesting that APCL does not modulate the activity of ASPP1 and ASPP2 in exactly the same way.

3.2.5 APC colocalizes with and induces the accumulation of ASPP1 and ASPP2 at the cell extremities

APC and its homologous APCL have been described to form heterodimers (Jarrett et al. 2001), therefore, we hypothesised that, although APC does not have the C-terminal region found in APCL which binds to ASPP2, it might still be able to play a role in the regulation of the ASPPs subcellular distribution through interaction with APCL. The subcellular location of APC itself is of a very complex nature and its study has proved particularly difficult mainly due to the absence of an appropriate anti-APC antibody which allows the detection of the protein at endogenous levels (Brocardo et al. 2005). Hence, we decided to examine the effect of APC overexpression on the localization of the ASPPs (Figure 3.6). In agreement with previous findings, the bulk of exogenously expressed APC is located in the cytoplasm (Figure 3.6 top left panel). APC was also detected at the cell extremities, presumably at the microtubules ends were it is known to localize. It has also been described in the literature that APC can shuttle between the cytoplasm and the nucleus; however, no nuclear localization was observed under the conditions used. Surprisingly, when APC was

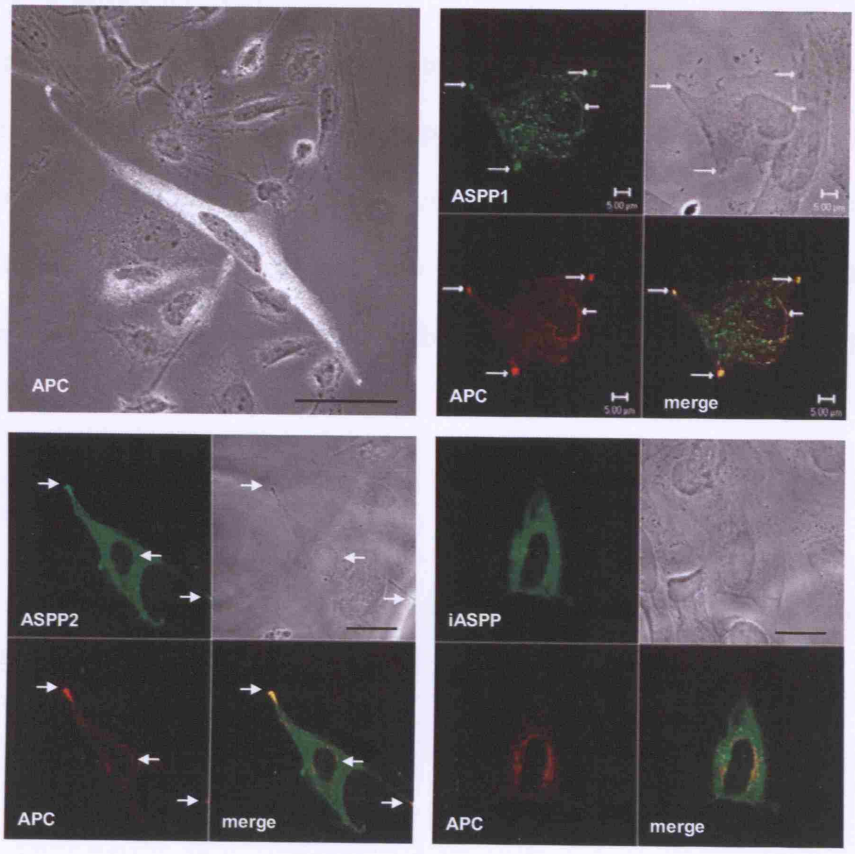


Figure 3.6: Exogenously expressed APC protein can colocalize with the ASPP family of proteins. APC was transfected in SAOS2 cells either alone (top left panel) or together with ASPP1-V5, ASPP2-V5 or iASPP-V5. A Goat Anti-Mouse IgG1 specific antibody conjugated to TRITC (red) and a Goat Anti-Mouse IgG2a specific antibody conjugated to FITC (green) were used to detect anti-APC (Santa Cruz) or anti-V5 antibodies respectively. White arrows indicate colocalization which appear in yellow on the pictures. Scale bar = 20μm unless otherwise stated (the same magnification was used for all the pictures in this figure).

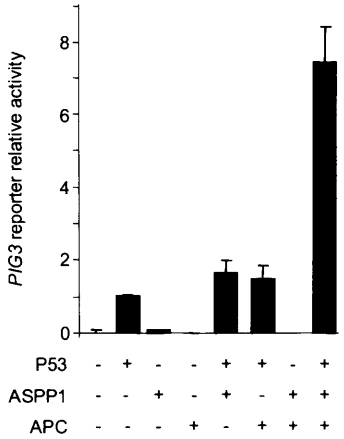
coexpressed with ASPP1 or ASPP2, they were found to colocalize predominantly at the cell extremities. To a lesser extent they also colocalized around the nucleus. No colocalization between iASPP and APC was observed, in agreement with the results obtained with APCL.

Taken together, these data demonstrates that APC potentially interacts with ASPP1 and ASPP2 but not iASPP. Also, these overexpression experiments tend to show that APC can influence the localization of the ASPPs independently of APCL: so that APCL colocalizes with ASPP1 and ASPP2 mainly around the nucleus, whereas APC colocalizes with them principally at the cell extremities.

3.2.6 APC positively modulates the synergy between ASPP1&2 and p53 on the PIG3 promoter

Having established that APCL could have a role in the regulation of the activity of p53 in a mechanism implicating the ASPPs, we raised the question whether the APC tumour suppressor could also behave in a similar way. Supporting this possibility, our data show that APC is able to colocalize with ASPP1 and ASPP2, suggesting that they can interact within the same complex and therefore impinge on their respective functions. It has also been shown that APC and APCL can form heterodimers, allowing APC to interfere with the ASPP2/APCL complex, regardless of whether it can bind or not to ASPP1 or

A


PIG3 reporter relative activity
0
2
4
6
8
P53 - + - - + + - +
ASPP1 - - + - + - + +
APC - - - + - + + +

B

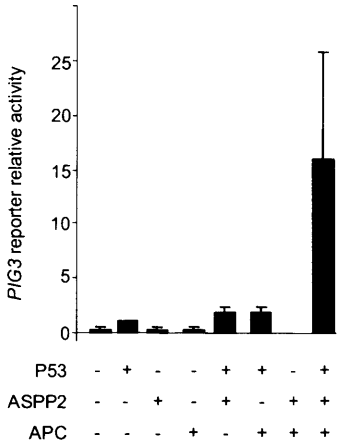

PIG3 reporter relative activity
0
5
10
15
20
25
P53 - + - - + + - +
ASPP2 - - + - + - + +
APC - - - + - + + +

Figure 3.7

C

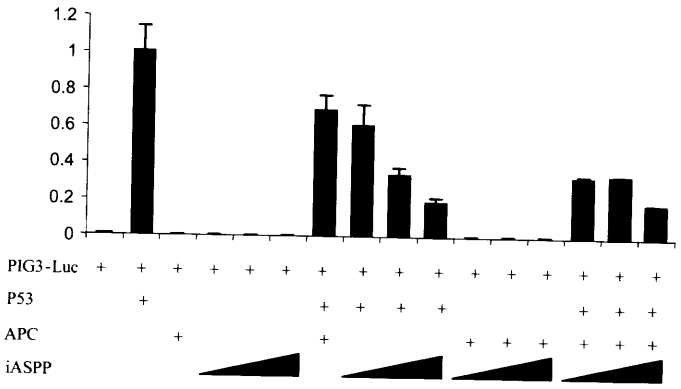


Figure 3.7: APC stimulates the synergy between p53 and ASPP2 or ASPP1 on PIG3 promoter. Transactivation assays were performed in Saos-2 cells, and the ability of APC to modulate the function of ASPP1 (A), ASPP2 (B) and iASPP (C) was tested. The cells were transfected with PIG3 reporter construct (1μg) together with renilla luciferase (0.1μg) and the indicated expression plasmids (ASPP1, ASPP2 and APC: 4μg; iASPP: 2μg; p53: 0.05μg). The total amount of DNA was normalized by addition of the appropriate empty vector. The data were normalized relative to the renilla luciferase activity. Error bars represent the standard deviation of 2 independent experiments performed in duplicate.

ASPP2. Hence, we performed a transactivation assay using a *PIG3* reporter construct to determine the effect APC could have on p53 activity alone or on its synergy with ASPP1 and ASPP2 (Figure 3.7). Under the conditions used, ASPP2 or APC had little effect on p53, whereas together they had a noticeable impact on p53 transactivation activity (Figure 3.7A). A similar result was obtained with ASPP1 (Figure 3.7B). This strongly suggests that APC and ASPP1&2 can work together to enhance p53 transactivation activity on the promoters of proapoptotic genes. Although the C-terminal region of APCL, which was shown to interact with 53BP2, bears little similarity to that of APC, we cannot rule out an interaction between APC and the full-length ASPP proteins. Furthermore, it has been shown that APC and APCL can interact together and this could provide a mechanism by which APC can stimulate the synergy between p53 and ASPP2 without forming a direct interaction with ASPP2.

Although we showed that APCL has little impact on the ability of iASPP to inhibit p53 transactivation function on the PIG3 promoter, we wanted to verify whether this was also the case for APC (Figure 3.7C). As expected, increasing quantities of iASPP gradually inhibited p53 activity on the *PIG3* promoter. However, p53 activity was not dramatically altered by APC. At low concentrations of iASPP, APC was able to enhance the inhibitory effect of iASPP on p53, but this effect was not observed once the inhibition of p53 by iASPP reached more than 60% of its basal activity. This result makes it difficult to determine whether APC plays a biologically significant role in enhancing the function of iASPP to inhibit p53 activity. As we showed that the localization

between APC and iASPP correlates poorly, it is more likely that any effect using this experimental design is indirect rather than the consequence of their organization in the same structural complex.

3.2.7 Inhibition of GSK3β modulates the function of the ASPPs in the regulation of p53

The only presumed function for APCL to date, like APC, is the ability to bind to β-catenin and induce its down-regulation, thus impinging on its transcriptional activity (Nakagawa et al. 1998). However, our results suggest a possible new role for the APC family in the regulation of ASPP1 and 2 mediated enhancement of p53 proapoptotic function. It has also been shown that β-catenin is involved in cell survival and proliferation and that it can regulate p53 (Miyagishi et al. 2000). As a result, we investigated whether β-catenin could influence the relationship between the ASPPs and p53. To see the effects of increased levels of β-catenin, we treated Saos-2 cells with lithium chloride (LiCl), which has been shown to be able to inhibit GSK3β and not other kinases at physiological doses (Figure 3.8) (Stambolic et al. 1996). LiCl as a result inactivates the β-catenin degradation complex and induces the stabilization of β-catenin. Treatments with the proteasome inhibitor MG132 were also used as a control in order to prevent the degradation of β-catenin and stabilize its expression

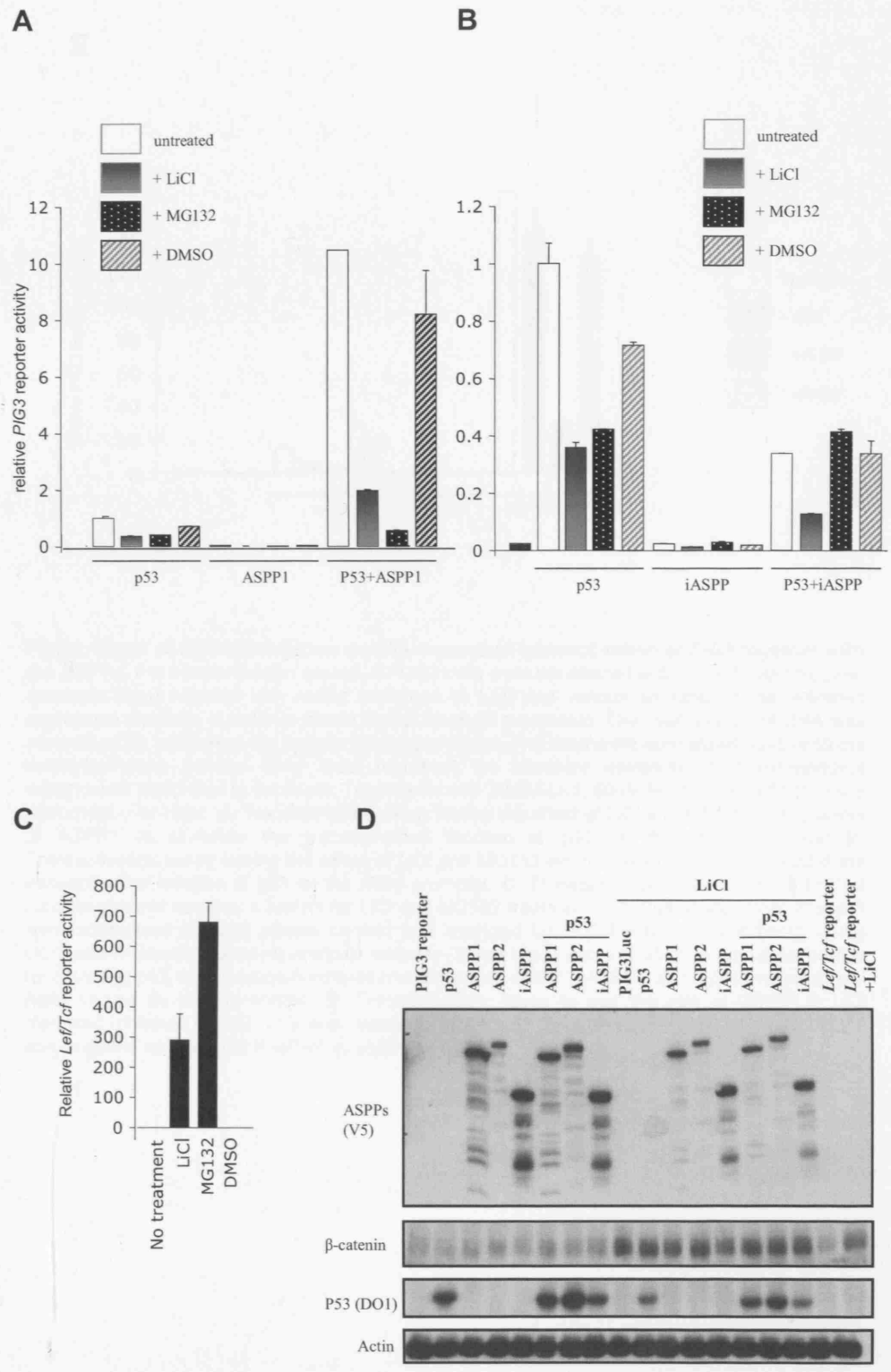

A
B
untreated
+ LiCl
+ MG132
+ DMSO
relative PIG3 reporter activity
p53
ASPP1
P53+ASPP1
iASPP
P53+iASPP
C
D
Relative Lef/Tcf reporter activity
No treatment
LiCl
MG132
DMSO
PIG3 reporter
PIG3Luc
Lef/Tcf reporter
Lef/Tcf reporter +LiCl
ASPP1
ASPP2
iASPP
ASPPs (V5)
β-catenin
P53 (DO1)
Actin

Figure 3.8

E

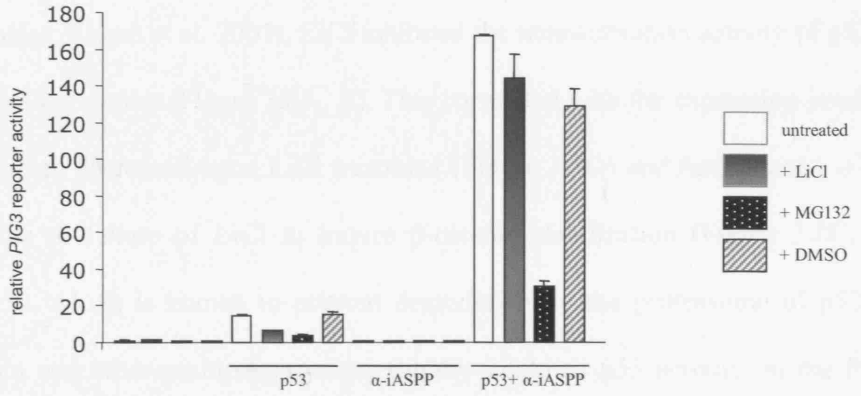


Fig3.8: Effect of GSK3β inhibition on p53 dependant transactivation of *PIG3* together with the ASPPs. For transactivation assays, SAOS2 cells were transfected with the indicated reporter construct (1μg) together with renilla luciferase (0,1μg) and various amounts of the indicated expression plasmids in order to obtain similar levels of expression. The total amount of DNA was normalized by addition of the appropriate empty vector. The data were normalized relative to the renilla-luciferase activity. Error bars represent the standard deviation of 2 independent experiments performed in duplicate. Treatments with 30mM LiCl, 50μM MG132 and DMSO were performed over-night. **A**. Transactivation assay testing the effect of LiCl and MG132 on the ability of ASPP1 to stimulate the transactivation function of p53 on the *PIG3* promoter **B**. Transactivation assay testing the effect of LiCl and MG132 on the ability of iASPP to inhibit the transactivation function of p53 on the *PIG3* promoter. **C**. Transactivation assay with the Lef/Tcf reporter plasmid used as a control for LiCl and MG132 treatments. **D**. Cell lysates from A and B were normalized for total protein content and analyzed by SDS-PAGE/immunoblotting, using DO1, anti-β-catenin mouse monoclonal antibody (Santa Cruz) and anti-V5 monoclonal antibodies for detecting p53, endogenous β-catenin and V5-tagged ASPP1, ASPP2 and iASPP respectively. Actin served as loading control. **E**. Transactivation assay to test the role of iASPP in LiCl mediated inhibition of p53. LiCl was unable to inhibit p53 transactivation of PIG3 when iASPP was depleted with an iASPP siRNA (α-iASPP).

levels (it has to be noted that MG132 induces the stabilization of all the proteins which are regulated by proteasomal degradation). Although it had been shown that LiCl treatments could lead to an increase of p53 levels in wild-type MEFs (Damalas, Kahan et al. 2001), LiCl inhibited the transactivation activity of p53 on the PIG3 promoter (Figure 3.8A, B). This correlated with the expression levels of p53 which decreased upon LiCl treatment (Figure 3.8D) and furthermore, it was not due to failure of LiCl to induce β-catenin stabilization (Figure 3.8C, D). MG132, which is known to prevent degradation by the proteasome of p53, β-catenin and other multiple proteins, slightly inhibited p53 activity on the PIG3 promoter compared to the DMSO control (Figure 3.8A, B).

When ASPP1 was overexpressed with p53, p53 transactivation on PIG3 was induced by 10 fold (Figure 3.8). LiCl prevented ASPP1 from fully enhancing p53 dependent transactivation; however, we cannot definitively conclude whether this is due to β-catenin stabilization alone, since ASPP1 and p53 levels were decreased in these conditions (Figure 3.8A, D). MG132 was a more potent inhibitor of the synergy between ASPP1 and p53 than LiCl (Figure 3.8A) and also increased activation of β-catenin transactivation function (Figure 3.8C). Similar results were obtained with ASPP2 (data not shown).

LiCl but not MG132 was able to enhance the inhibition of p53 transactivation mediated by iASPP (Figure 3.8B). To confirm further that LiCl and iASPP can co-operate to inhibit p53 transactivation we used an siRNA to remove endogenous iASPP from the cells. Under these conditions, LiCl but not MG132 was no longer able to inhibit p53 transactivation on PIG3, suggesting that the inhibition of p53 by LiCl requires the presence of iASPP (Figure 3.8F).

These results suggest that the inhibition of GSK-3β, which results in the accumulation of β-catenin, may play a role in the regulation of p53 transactivation function in an iASPP-dependent manner.

3.3 Discussion

The recently identified ASPP family of proteins have been shown to selectively regulate the apoptotic function of p53 by increasing its transcriptional activity on the promoters of proapoptotic genes (Samuels-Lev et al. 2001; Bergamaschi et al. 2003). Furthermore, numerous ASPP binding partners have been identified over the past decade, including proteins involved in numerous pathways (Trigiante and Lu 2006). However, the roles that these interactions play remain largely uncharacterized. As a result, it is equally possible that the ASPPs are involved in the regulation of the function of these proteins or vice versa. This chapter has focused on the interplay between the ASPPs and two related proteins, APCL and APC to try to have a better understanding of the functional relevance that this interaction might have.

3.3.1 APCL and APC differentially affect the subcellular localization of ASPP1 and ASPP2.

Originally, APCL was shown to bind to 53BP2 and induce its translocation to a perinuclear region where they colocalize (Nakagawa et al. 2000a). Here we show that APCL regulates the full-length ASPP2 protein in a similar fashion (Figure 3.2B). Moreover, as expected, the originally identified C-terminal ASPP2 binding region of APCL appears to play a direct role in the translocation of exogenous ASPP2 to the perinucleus, as an APCL construct lacking this region was less efficient in doing so (Figure 3.2B). This shows that the binding of APCL to ASPP2 is important and plays a role in modulating the subcellular localization of ASPP2. APCL has been shown to interact with the Ankyrin repeats and SH3 domain of 53BP2 which are conserved among the three members of the family. Therefore, it was expected that APCL could also interact with both ASPP1 and iASPP. However, our results indicate that APCL regulates the subcellular localization of the ASPPs to different extents. Thus, APCL-induced perinuclear translocation of ASPP2 was more pronounced than that of ASPP1 and iASPP which remained unaffected. Interestingly, similarly to APCL, APC was also able to influence the subcellular localization of ASPP1 and ASPP2 (Figure 3.6). However, APC induced the accumulation of ASPP1&2 at the tip of cellular extensions rather than at the perinucleus.

It was not possible to investigate whether the complex between APCL and ASPP1&2 could be formed endogenously, as good reagents were not available. It will be essential to develop antibodies against APCL able to detect endogenous levels of the protein in order to confirm the relevance of these experiments.

3.3.2 APC and APCL positively regulate the action of ASPP1 and ASPP2 on p53 transactivation of proapoptotic genes.

Interestingly, both APC and APCL were able to stimulate the function of ASPP1 and ASPP2, whereas they had little effect on iASPP (Figure 3.3-5 and 3.7). The molecular mechanism behind this is not yet understood, as ASPP1 and ASPP2 are mainly cytoplasmic and neither APC nor APCL seem to affect their nuclear levels (Figure 3.2), suggesting that some other mechanisms may be involved in the regulation of the function of ASPP1 and ASPP2 by APC and APCL. It will be interesting to see whether APC or APCL can induce post-translational modifications in ASPP1 and ASPP2 and therefore modulate their ability to stimulate p53 mediated transactivation of proapoptotic genes.

Several lines of evidence indicate that the effect of APC and APCL on p53 is mediated via ASPP1 and ASPP2. Indeed, both APC and APCL had little effect on p53 mediated transactivation of PIG3 on their own, whereas they were found to be able to collaborate together with ASPP1 or ASPP2 (Figure 3.3B-C and

3.7A-B). Moreover, the fact that APCL cooperates with ASPP2 only on the promoter of proapoptotic genes such as PIG3 and not Mdm2 and $p21^{WAF1}$ reinforces the idea that APCL regulates p53 activity in an ASPP dependent manner (Figure 3.4).

Therefore, at least in the case of APCL, these observations seem to rely on its interaction with ASPP1 and ASPP2, as a construct deficient of the ASPP binding domains, APCLΔ was less efficient than full length APCL in enhancing the synergy between ASPP1&2 and p53 (Figure 3.5). However, removing the C-terminus of APCL did not completely abolish its activity towards ASPP1 and ASPP2, suggesting that some activity is contained within the rest of the protein. Interestingly, the APCLΔ construct retains all of the domains involved in the binding and regulation of β-catenin, since the 20 amino acid repeats and the SAMP repeats, which allow the binding to axin, are still present (van Es et al. 1999). Therefore, it is possible that APCLΔ conserves its ability to down-regulate β-catenin levels and activity. This raises the question whether this activity contributes to the effect that APCL has on the synergy between ASPP1&2 and APCL. Moreover, the fact that APCLΔ partially colocalizes with ASPP1 and ASPP2 (Figure 3.2B) suggests that, to some extent, they are still able to be part of the same complex. As the shorter form of ASPP2, 53BP2, was used in the binding study with APCL, it might be possible that the N-terminal part of ASPP2 contains additional binding sites for APCL, which may explain why they can still partially colocalize and why APCLΔ retains some activity. Additionally, APCLΔ might still be able to bind indirectly to ASPP1 and ASPP2 in a cellular context, which would explain why they can still partially colocalize. This could be achieved via,

for example, the formation of a ternary complex with endogenous proteins, such as APCL itself, β-catenin or even APC. Indeed such a ternary complex could take place as both APC and APCL contain an oligomerization domain in their N-terminus which would allow them to homo- or heterodimerize (Jarrett et al. 2001).

In order to address all these questions, it will be essential to study carefully the composition of the complexes involving ASPP1, ASPP2, APC and APCL. It will also be critical to refine the limits of the ASPP2 binding domain in APCL, as the region deleted in the APCLΔ construct is vast and may lead to the misfolding of the resulting exogenous protein. In previous studies, the binding between 53BP2 and APCL has been investigated *in vitro* because of the relative insolubility of APCL (Nakagawa et al. 2000a); however, their interaction remains to be shown in a cellular context, which will be critical to fully understand the biological role of this complex. Moreover, in order to confirm our results obtained with the PIG3 reporter construct, it will be important to determine endogenously whether APC or APCL can collaborate with ASPP1 and ASPP2 to regulate the expression of p53 transcriptional targets. It will be possible to test that using siRNA targeted against these proteins in cell lines expressing wild-type p53. Consequently, the magnitude of the p53 response to various stresses, such as chemotherapy drugs or UV radiations, will be assessed by determining the endogenous expression levels of proteins such as PIG3, BAX and PUMA.

3.3.3 The function of APC and APCL as down-regulators of β-catenin may play a role in the regulation of ASPP1 and ASPP2

APCL, like APC, can target β-catenin for degradation by the proteasome (Nakagawa et al. 1998; van Es et al. 1999). β-catenin has also been shown to regulate and be regulated by p53, and inhibit apoptosis (Damalas et al. 1999; Orford et al. 1999; Damalas et al. 2001; Sadot et al. 2001). Aberrant accumulation of β-catenin can trigger the expression of p53 and, conversely, p53 is able to down-regulate β-catenin. Further complexity in the relationship between these two proteins is demonstrated by the fact that p53 and β-catenin have been shown to compete for CBP/p300, which could be a way for β-catenin to inhibit the activity of p53 (Miyagishi et al. 2000). Our results indicate that an increase in the levels of β-catenin by inhibition of GSK3β could lead to a decrease in p53 dependent transactivation of proapoptotic genes (Figure 3.8A). Additionally, it prevented ASPP1 from being able to induce p53 transactivation of PIG3, although this could possibly be as a result of a reduction in p53 and ASPP1 levels. Interestingly, iASPP was not only able to accentuate the inhibition of p53 by LiCl but it was also required in order for LiCl to be able to down-regulate the activity of p53 (Figure 3.8B,E). However, it will be interesting to further confirm these data using Wnt ligands or siRNA targeting GSK3β in order to avoid any off-target effects that may be caused by the use of LiCl.

Collectively these data suggest a mechanism in which ASPP1&2 collaborate with APCL and APC to promote p53 mediated apoptosis, to the detriment of β-catenin

signalling and its effects on cell survival and proliferation. β-catenin could potentially revert this by inhibiting p53 in an iASPP-dependent manner (Figure 3.9).

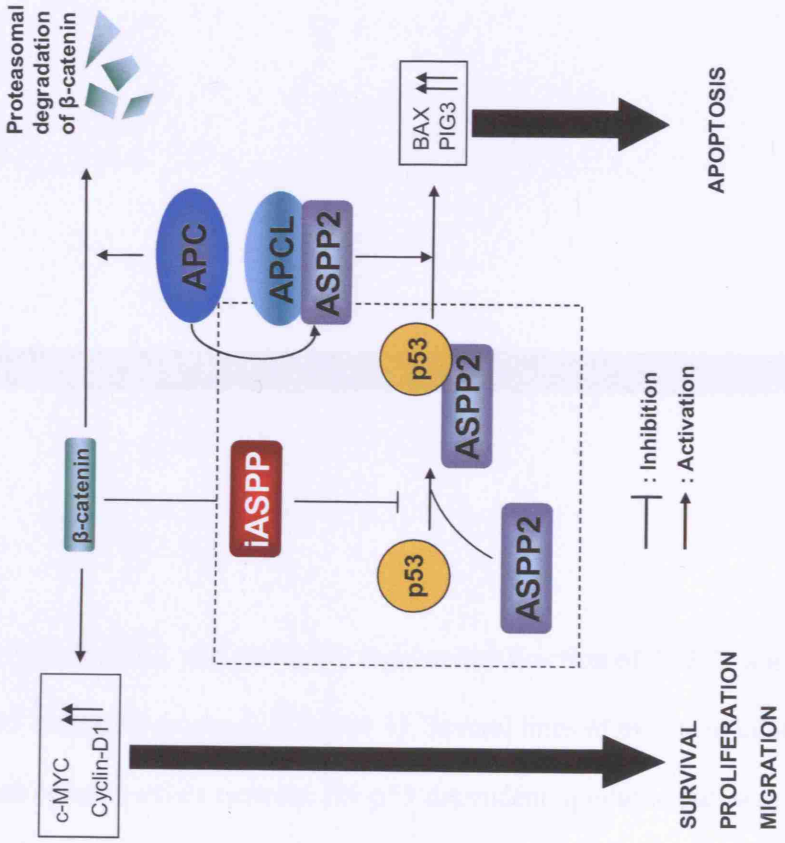


Fig3.9: Model of the regulation of p53 mediated apoptosis by the ASPP family of proteins and by the ASPP2-interacting protein APCL. The members of the ASPP family of proteins specifically regulate the apoptotic function of p53 (dashed square). Hence, ASPP2 (and ASPP1) can bind to p53, thus promoting the binding of p53 on the promoters of proapoptotic genes such as *BAX* and *PIG3*. As a result, they induce apoptosis. iASPP, to the contrary, inhibits p53 mediated apoptosis. Our results indicate that both APCL and APC can positively regulate the function of ASPP2 (and ASPP1) in p53 mediated transactivation of proapoptotic genes. Moreover, we find that β-catenin may down-regulate p53 dependent transcriptional activity on the *PIG3* promoter in a mechanism requiring the presence of iASPP. In this model, one group of protein, involving ASPP1, ASPP2, APC and APCL works against the function of β-catenin, by promoting its destruction or by inducing apoptosis. The second group of proteins, involving iASPP and β-catenin ensures cell survival by either inhibiting p53 mediated apoptosis or by inducing the transcription of genes involved in cell survival such as *c-MYC* and *Cyclin-D1*.

Chapter IV: ASPP2: a new negative regulator of β-catenin during the embryonic development of the CNS

4.1 Introduction

APC and APCL can positively regulate the function of ASPP1 and ASPP2 during p53 mediated apoptosis (Chapter 3). Several lines of evidence indicate that an intricate balance exists between the p53 dependent apoptotic pathway and the Wnt/β-catenin signalling pathway. For example, our data indicate that ASPP1 and ASPP2 can be found in complex with APC or APCL, suggesting that they may be involved in the regulation of the β-catenin cytoplasmic destruction complex and, as a result, in the regulation of β-catenin transcriptional activity. This possibility is corroborated by the fact that ASPP1 is able to interact with the E3 ubiquitin-ligase Siah-1, which is itself involved, in collaboration with APC, in targeting β-catenin for degradation by the proteasome (personal communication from Dr D Elliott). Furthermore, *Siah-1* has been shown to be a transcriptional target of p53 and its transcription is induced upon DNA damage (Liu et al. 2001; Matsuzawa and Reed

2001; Fiucci et al. 2004). P53 itself has been shown to be able to down-regulate β-catenin levels and activity, in a mechanism involving the mobilization of axin to a particular cellular fraction (Sadot et al. 2001; Levina et al. 2004). Altogether, these observations show that there are multiple links between the Wnt/β-catenin signalling pathway and p53, and furthermore, they suggest that the ASPPs, by interacting with p53, Siah-1 or APCL, could play a role in the regulation of β-catenin.

Several lines of evidence, inherent to the fact that they positively regulate the apoptotic function of p53, indicate that ASPP1 and ASPP2 are tumour suppressors. Firstly, their expression has been found to be down-regulated in a panel of human breast cancer tumours (Samuels-Lev et al. 2001). Additionally, in ASPP2 heterozygous mice, the occurrence of tumours was significantly increased and the difference with the wild-type was further increased when the mice were γ-irradiated (Vives et al. 2006b). Interestingly, it is not uncommon for tumour suppressors to play a role during embryonic development. Thus ASPP1 and ASPP2, in addition to their tumour suppressor function, might also be implicated in embryonic development and play a role involving their various binding partners. APCL expression, like APC, is particularly high in the foetal brain, suggesting that it might play an important role in the development of the CNS (van Es et al. 1999). It is tempting to speculate that ASPP1 and ASPP2, through their interaction with APCL, may regulate the function of APCL during this process and, as a result, interfere with its ability to down-regulate β-catenin activity.

The Wnt/β-catenin signalling pathway is important during embryonic development of the CNS, where Wnt1 has been shown to be critical for the development of the brain (Brault et al. 2001). In addition, over-expression of constitutively active β-catenin in the forebrain was reported to increase the cortex size by enhancing progenitor growth and inhibiting neuronal differentiation. Another study demonstrated the pivotal role of β-catenin in the maintenance of the proliferation of neuronal progenitors and in controlling the size of the progenitor pool, thus affecting the decision of neuronal progenitors to proliferate or to differentiate (Zechner et al. 2003). Thus, β-catenin can increase cycling and expansion of neural progenitors, and loss of β-catenin activity causes a reduction in the progenitor compartment (Chenn and Walsh 2002; Zechner et al. 2003). The role of β-catenin in the regulation of the neural stem cell compartment is believed to be mainly achieved via its function as a transcription factor. However, β-catenin, as a component of the *adherens* junctions, could also play a role in the development of the CNS, as it has been shown for instance, that N-cadherin is essential for the cortical organization of the mouse brain (Kadowaki et al. 2007).

This chapter aims to investigate the role of the ASPPs in the regulation of β-catenin. Furthermore, the role of ASPP2 in the regulation of β-catenin will be assessed during mouse embryonic development, with particular focus on its role in the CNS.

4.2 Results

4.2.1 ASPP2 is required to inhibit the WNT/β-catenin pathway in MEFs

To test the hypothesis that ASPP2 is implicated in the negative regulation of the WNT/β-catenin signalling pathway, murine embryonic fibroblasts (MEFs) generated from either *ASPP2* wild-type, heterozygous or null E13.5 embryos were transfected with a Lef-Tcf reporter construct, allowing us to detect differences in the β-catenin relative transcriptional activity (Figure 4.1A). Under normal growth conditions, β-catenin transcriptional activity was around 5-fold higher in *ASPP2*-null MEFs compared to the wild-type, although this difference was not quite statistically significative ($p=0.074$). β-catenin transcriptional activity in heterozygous MEFs was not statistically different compared to the wild-type ($p=0.116$). This observation suggests that ASPP2 may play a role in keeping the β-catenin transcriptional activity low. As GSK3β has been shown to be a major component of the β-catenin degradation pathway, we decided to inhibit its activity using LiCl to further investigate the inhibitory role of ASPP2 on β-catenin transcriptional activity. Upon inhibition of GSK3β with increased concentrations of LiCl, the transcriptional activity of β-catenin was significantly enhanced to a greater extent in the *ASPP2* deficient MEFs compared to the wild-type when 30mM of LiCl were used ($p=0.033$). This shows that ASPP2 provides a second

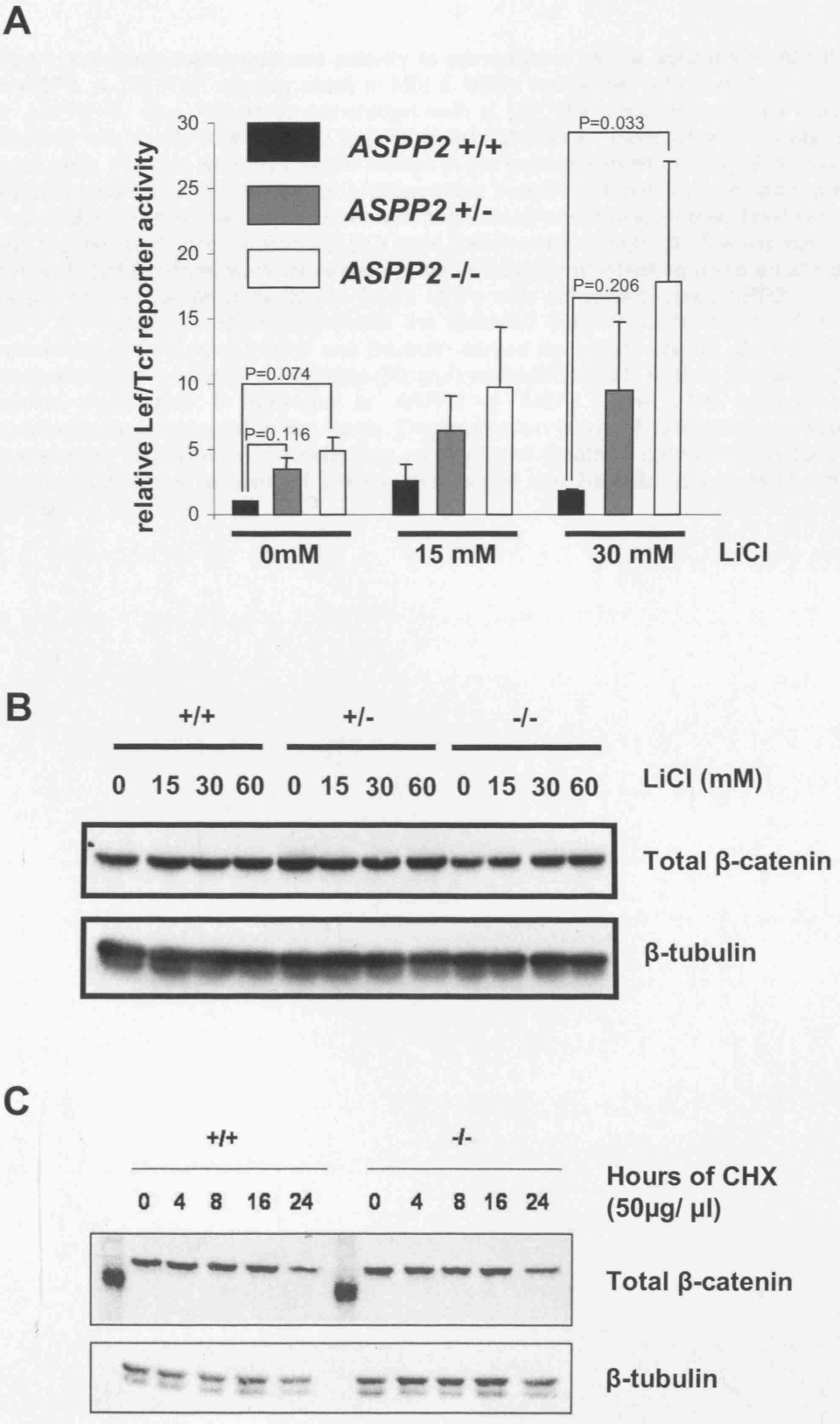

A
relative Lef/Tcf reporter activity
30
25
20
15
10
5
0
ASPP2 +/+
ASPP2 +/-
ASPP2 -/-
P=0.033
P=0.206
P=0.074
P=0.116
0mM
15 mM
30 mM
LiCl
B
+/+
+/-
-/-
0 15 30 60 0 15 30 60 0 15 30 60
LiCl (mM)
Total β-catenin
β-tubulin
C
+/+
-/-
0 4 8 16 24 0 4 8 16 24
Hours of CHX
(50μg/ μl)
Total β-catenin
β-tubulin

Figure 4.1

Fig4.1: β-catenin transcriptional activity is upregulated in the absence of ASPP2 in MEFs. A. LEF/TCF reporter assay in MEFs. MEFs cells either wild-type, *ASPP2* +/- or *ASPP2* -/- were transiently transfected with a LEF/TCF reporter construct (2μg) together with renilla luciferase (0,1μg) to assess β-catenin transcriptional activity in these cells. The data were normalized relative to the renilla-luciferase activity. Error bars represent the standard deviation of 3 independent experiments performed in duplicate. The p values represented in the figure were obtained by performing a t-test. Treatments with the indicated concentrations of LiCl were performed overnight. **B**. The expression levels of total β-catenin were assessed by SDS-PAGE/Immunoblotting using an anti-β-catenin monoclonal antibody (Santa Cruz). MEFs cells either wild-type, *ASPP2* +/- or *ASPP2* -/- were treated overnight with the indicated concentrations of LiCl. Equal amounts of protein were loaded and β-tubulin served as loading control. **C**. A pulse-chase experiment using cycloheximide (50μg/μl) was performed to assess the rate of β-catenin degradation in wild-type or *ASPP2* -/- MEFs. Time after addition of cycloheximide is indicated in the figure. The expression levels of total β-catenin were assessed by SDS-PAGE/Immunoblotting using an anti-β-catenin monoclonal antibody (Santa Cruz). Equal amounts of protein were loaded and β-tubulin served as loading control.

level of regulation of β-catenin, as we can see that it is still able to play an inhibitory role on the β-catenin transcriptional activity even when the canonical degradation pathway of β-catenin is blocked. Interestingly, *ASPP2* heterozygous MEFs showed about half the increase in β-catenin transcriptional activity observed in ASPP2 null MEFs; however, this difference was not statistically different suggesting that ASPP2 is not haploinsufficient regarding its role in the regulation of β-catenin transcriptional activity ($p=0.206$).

The expression levels of β-catenin did not considerably differ between the *ASPP2* wild-type, heterozygous and null MEFs (Figure 4.1B). However, as no drastic changes in the total levels of β-catenin could be seen upon increased concentrations of LiCl, it may be that only a small increase in the nuclear levels of β-catenin caused the changes in transcriptional activity seen in figure 4.1A.

The cytoplasmic levels of β-catenin are regulated by the cytoplasmic destruction complex, which as a result prevents β-catenin from accumulating and going into the nucleus to carry out its function as a transcription factor. In order to verify whether the higher β-catenin transcriptional activity seen in the ASPP2 deficient MEFs was as a result of reduced β-catenin degradation, a pulse-chase experiment using cycloheximide was performed (Figure 4.1C). Over a period of 24 hours, there did not appear to be any noticeable differences in the stability of the total levels of β-catenin depending on whether ASPP2 was expressed or not. Therefore, this set of data shows that ASPP2 is a negative regulator of β-catenin transcriptional activity, and that this is more likely to be as a result of changes in β-catenin location, for example, rather than alterations of the levels and the stability of β-catenin.

4.2.2 ASPP2 delays the accumulation of β-catenin in the nucleus

The absence of changes in β-catenin levels between ASPP2-null MEFs and wild-type MEFs, despite the differences in Wnt/β-catenin signalling activity between the cells, prompted us to investigate whether β-catenin subcellular repartition could be affected by the loss of ASPP2. Analysis of β-catenin localization by immunofluorescence in MEFs showed that ASPP2-null and ASPP2 heterozygous cells contained slightly more β-catenin in their nucleus in comparison to the wild-type cells (Figure 4.2A). To verify this, the accumulation of β-catenin in the nucleus of wild-type and ASPP2-null MEFs was assessed upon WNT3a or LiCl treatment over a 15 hour period of time (Figure 4.2B). In agreement with the results obtained from analysis of β-catenin transcriptional activity, in this particular experiment (n=1), more cells with nuclear β-catenin were counted in ASPP2-null MEFs compared to the wild-type after both Wnt3a and LiCl treatments. Notably, it seemed that the nuclear β-catenin staining in the ASPP2-null MEFs was more intense, suggesting that more β-catenin could accumulate in these cells compared to the wild-type when β-catenin degradation was prevented by either WNT3a or LiCl.

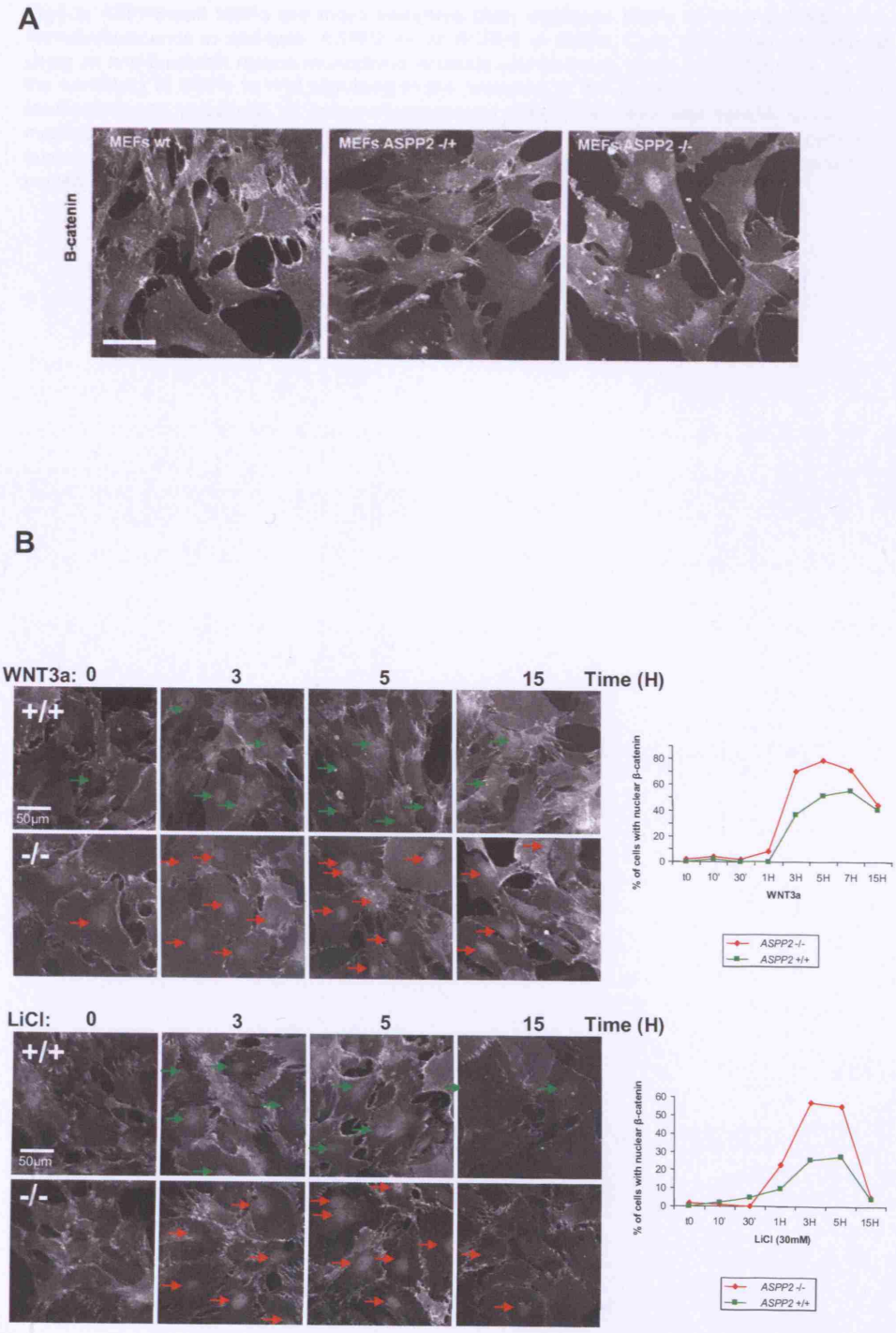

A
MEFs wt
MEFs ASPP2 -/+
MEFs ASPP2 -/-
B-catenin
B
WNT3a: 0
3
5
15
Time (H)
+/+
50µm
-/-
% of cells with nuclear β-catenin
80
60
40
20
0
t0
10'
30'
1H
3H
5H
7H
15H
WNT3a
ASPP2 -/-
ASPP2 +/+
LiCl: 0
3
5
15
Time (H)
+/+
50µm
-/-
% of cells with nuclear β-catenin
60
50
40
30
20
10
0
t0
10'
30'
1H
3H
5H
15H
LiCl (30mM)
ASPP2 -/-
ASPP2 +/+

Figure 4.2

Fig4.2: ASPP2-null MEFs are more sensitive than wild-type MEFs to Wnt signalling. A. Immunofluescence in wild-type, *ASPP2* +/- or *ASPP2* -/- MEFs. Cells were fixed and stained using an anti-β-catenin mouse monoclonal antibody (Santa-Cruz). Scale bar = 50μm **B.** To test the sensitivity of MEFs to Wnt signalling in the presence or the absence of ASPP2, β-catenin localization was visualized by immunofluorescence upon treatments with Wnt3A conditioned medium or with LiCl. Time after treatment is indicated in hours. The percentage of cells with nuclear β-catenin is represented by a graph. Arrows indicate the nucleus of cells containing nuclear β-catenin (green for the wild-type and red for the knockout). Scale bar = 50μm

4.2.3 ASPP2 favours phosphorylation of β-catenin at serine 45 in MEFs

Although the total levels of β-catenin appeared to be relatively similar in wild-type and ASPP2-null MEFs, the possibility remained that the greater tendency of the ASPP2 deficient MEFs to accumulate nuclear β-catenin was due to the impaired function of the β-catenin cytoplasmic destruction complex. When Wnt receptors are not engaged, the excess of newly synthesised cytoplasmic β-catenin is targeted for degradation by the proteasome, as a result of the phosphorylation of four key residues in the N-terminal portion of β-catenin. It is commonly accepted that Casein kinase 1α (CK1 α) phosphorylates β-catenin at Ser-45, following which residues at Ser-33, Ser-37 and Thr-41 are phosphorylated by GSK3β, allowing recognition of β-catenin by the F-box/WD repeat protein β-TrCP, a component of a dedicated E3 ubiquitin ligase complex.

Therefore, we investigated whether the phosphorylation pattern of these four residues was altered in ASPP2 deficient MEFs (Figure 4.3). Phosphorylation of Ser-45 was lower in the knock-out compared to the wild-type whereas, surprisingly, phosphorylation levels of Ser-33, Ser-37 and Thr-41 remained unchanged. As phosphorylation at Ser-45 primes the phosphorylation of Ser-33, Ser-37 and Thr-41, we would have expected a decrease in their phosphorylation levels. However, it has been shown that phosphorylation of β-catenin at Ser-33, Ser-37, and Thr-41 can occur in the absence of phosphorylation at Ser-45 in colon cancer cells (Wang et al. 2003). The decrease in phosphorylation levels at Ser-45 in the absence of ASPP2 might partially disable the β-catenin degradation

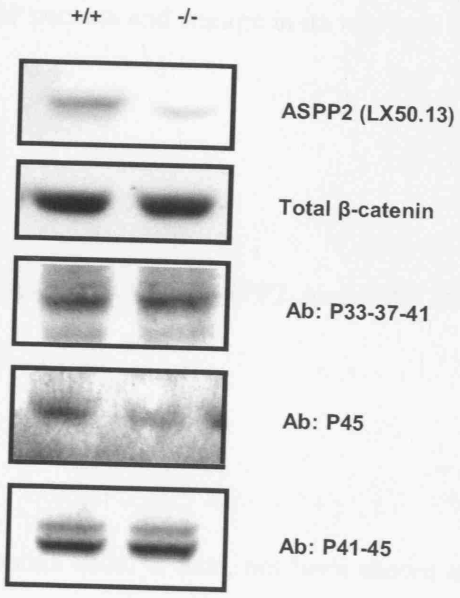


Fig4.3: β-catenin phosphorylation status in wild-type and *ASPP2* -/- MEFs. The levels of phosphorylated β-catenin in wild-type or *ASPP2* -/- MEFs at residues S33, S37, T41 and S45 were assessed by SDS-PAGE/immunoblotting. LX50.13 mouse monoclonal antibody was used to detect ASPP2. β-catenin phospho-antibodies recognizing β-catenin phosphorylated at the indicated residues were obtained from Cell Signaling. The total levels of β-catenin were detected with a mouse monoclonal antibody from Santa Cruz Biotechnology. Equal emounts of proteins were loaded and total levels of β-catenin were used as loading control, as they were shown to be similar in wild-type and *ASPP2* deficient MEFs (Figure 4.1B).

machinery leading to a leakage of newly synthesised β-catenin, which may subsequently enter the nucleus and engage in its role as a transcription factor.

4.2.4 The binding between ASPP2 and PP1 may play a role in the regulation of β-catenin

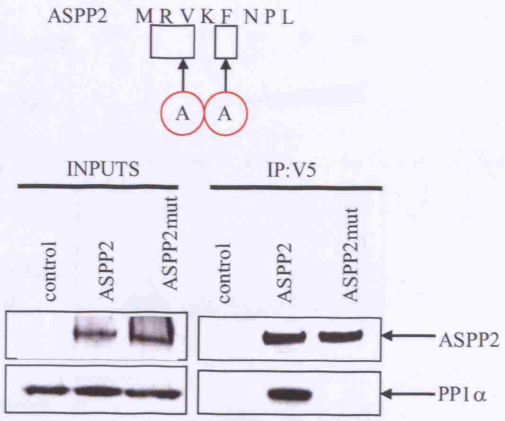
The ASPP proteins have, to date, not been shown to possess any intrinsic enzymatic activity and their effects are most probably mediated through protein/protein interactions. 53BP2, corresponding to the C-terminal region of ASPP2, has been shown to be able to bind to protein phosphatase 1 (PP1) and to inhibit its activity on certain substrates (Helps et al. 1995). Further studies from S Llanos confirmed that ASPP2 contains an RVxF motif, which is required for the full-length ASPP2 protein to bind to protein phosphatase 1 (Figure 4.4A, unpublished data). When the RVxF motif in ASPP2 was mutated, the binding between ASPP2 and PP1α was completely abolished (Figure 4.4). Furthermore, a recent study showed that PP1 is a positive regulator of the Wnt signalling pathway and that inhibition of PP1 increases axin phosphorylation by CK1 (Luo et al. 2007), suggesting that PP1 promotes the disassembly of the β-catenin destruction complex by dephosphorylating Axin. As we saw a decrease in CK1 dependent phosphorylation of β-catenin at Ser-45 in the *ASPP2*-null MEFs, we hypothesised that ASPP2 might negatively regulate PP1 activity and its function in the β-

catenin degradation complex assembly. Therefore we would expect that the PP1 binding mutant of ASPP2 would not be able to regulate β-catenin.

We first examined the effect of wild-type ASPP2 overexpression on the levels of β-catenin in two different fractions of total cell lysate: a Ripa soluble and a Ripa insoluble fraction (Figure 4.4B). As previously described, in the presence of high levels of p53, β-catenin levels were reduced in both the soluble and insoluble fractions (Sadot et al. 2001); Figure 4.4B). ASPP2 did not dramatically affect the levels of β-catenin in the Ripa soluble fraction whereas, surprisingly, it increased them in the Ripa insoluble fraction. When p53 was coexpressed together with ASPP2 and β-catenin, the effect of ASPP2 on the β-catenin levels in the Ripa insoluble fraction was abolished.

The role of the binding between ASPP2 and PP1 in the regulation of β-catenin by ASPP2 was tested using the ASPP2 construct which does not bind to PP1 (Figure 4.4A). Increasing quantities of either wild-type ASPP2 or its PP1 binding mutant counterpart were expressed with constant quantities of β-catenin (Figure 4.4C). As the expression levels of wild type ASPP2 increased, β-catenin levels accumulated in the Ripa insoluble fraction. Interestingly, a ladder above the full-length β-catenin appeared at high concentrations of ASPP2, suggesting that β-catenin is being modified upon overexpression of ASPP2. In contrast, the ASPP2 PP1 binding mutant was unable to increase the levels of β-catenin in the Ripa insoluble fraction, even at high concentrations. This suggests that the ability of ASPP2 to bind to PP1 may be of importance in the regulation of β-catenin activity.

A


ASPP2 M R V K F N P L
A
A
INPUTS
IP:V5
control
ASPP2
ASPP2mut
control
ASPP2
ASPP2mut
ASPP2
PP1α

B

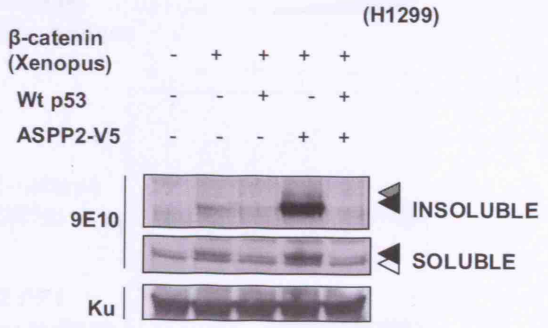
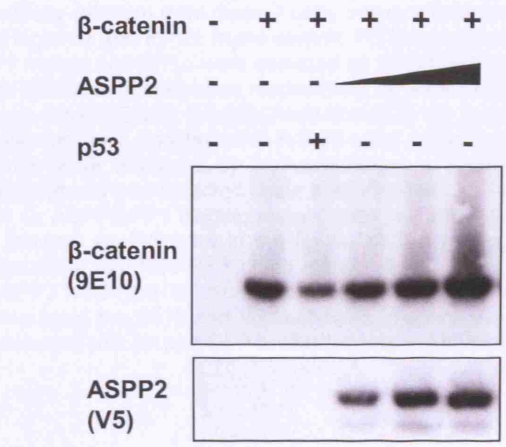

(H1299)
β-catenin (Xenopus) - + + + +
Wt p53 - - + - +
ASPP2-V5 - - - + +
9E10
INSOLUBLE
SOLUBLE
Ku

Figure 4.4

C


β-catenin - + + + + +
ASPP2 - - -
p53 - - + - - -
β-catenin (9E10)
ASPP2 (V5)

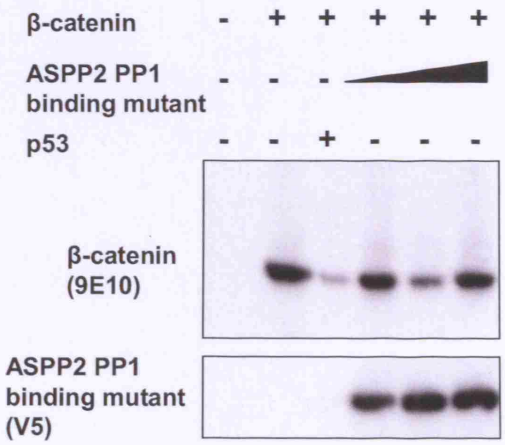

β-catenin - + + + + +
ASPP2 PP1 binding mutant - - -
p53 - - + - - -
β-catenin (9E10)
ASPP2 PP1 binding mutant (V5)

Figure 4.4: The binding of PP1 to ASPP2 is important in ASPP2 mediated regulation of β-catenin. A. Binding study between ASPP2 and PP1α. Diagram illustrates amino acids 920 to 927 of ASPP2. Boxed amino acids indicate the RVxF motif. Two residues of the RVxF motif were mutated to alanines in the ASPP2-PP1 binding mutant construct (circled alanines). Lower panel indicates an immunoprecipitation of wild-type ASPP2 or ASPP2-PP1binding mutant with anti-V5 rabbit polyclonal antibody (abcam) from Saos-2 cells overexpressing wild-type ASPP2 or ASPP2-PP1 binding mutant together with PP1α. In the control, PP1α was transfected on its own. Wild-type ASPP2, ASPP2-PP1 mutant and PP1α were detected by SDS-PAGE/immunoblotting using anti-V5 and anti-PP1 mouse monoclonal antibodies respectively. **B**. ASPP2 promotes the accumulation of modified β-catenin in a Ripa insoluble fraction. As indicated in the figure, ASPP2-V5, p53 and myc-tagged *Xenopus* β-catenin were transfected in H1299 cells, using the calcium phosphate method. Two different fractions were analysed by immunoblotting, a Ripa soluble and Ripa insoluble fraction. Exogenous β-catenin was detected using a 9E10 antibody. An anti-Ku antibody was used as a loading control. **C**. ASPP2-PP1 binding mutant does not affect the pool of β-catenin found in the Ripa insoluble fraction. As indicated in the figure, p53, myc-tagged *Xenopus* β-catenin and ASPP2-V5 (upper panel) or ASPP2-PP1 binding mutant (lower panel) were transfected in H1299 cells. β-catenin, ASPP2 wild-type or mutant expression levels were then detected by SDS-PAGE/immunoblotting using the 9E10 and V5 antibodies, respectively. GFP was transfected in all the conditions and detected with an anti-GFP antibody (data not shown).

4.2.5 ASPP1 and ASPP2 can colocalize with β-catenin where cells form contacts

In order to obtain further insight into how ASPP2, and more generally the ASPPs, can regulate the activity of β-catenin, we investigated more thoroughly their localization with respect to the localization of β-catenin.

In Saos-2 cells, the ASPPs proteins could be found at diverse subcellular locations (see Figure 3.2A). When ASPP1 and ASPP2 were ectopically expressed in Saos2-cells, they were located mainly in the cytoplasm. However, expression was also detected within the nucleus and at the membrane, where cell-cell contacts are made (Figure 4.5A). Some of the cytoplasmic ASPP1 and ASPP2 was able to localize in a perinuclear region, where they may interact with endogenous APCL, since the cellular staining looks similar to what was observed in figure 3.2B when ASPP1 and ASPP2 where coexpressed together with APCL. Interestingly, the membrane pool of ASPP1 and ASPP2 was able to colocalize with endogenous β-catenin, suggesting that they may interact in the same complex. The localization of overexpressed iASPP was mainly cytoplasmic and sometimes nuclear, but it was only rarely found at the membrane. Furthermore, colocalization of iASPP with β-catenin was poor. Hence, ASPP1 and ASPP2 may interact with β-catenin where cell-cell contact are established; however it seems that, at least under the conditions used, iASPP is excluded from such a complex.

In order to further investigate these findings, we analysed the subcellular location of ASPP2 in a panel of epithelial cell lines (Figure 4.5B-E). In U-2 OS

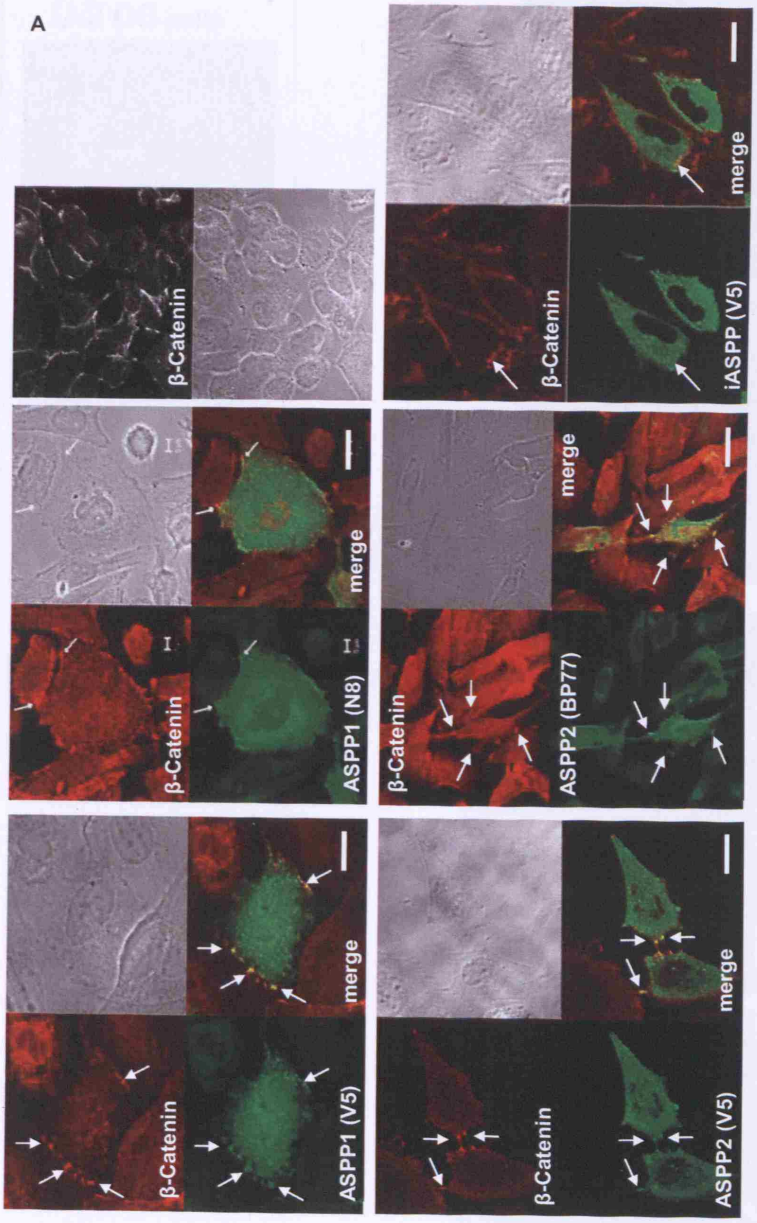

A
β-Catenin
ASPP1 (V5)
merge
β-Catenin
ASPP1 (N8)
merge
β-Catenin
β-Catenin
ASPP2 (V5)
merge
β-Catenin
ASPP2 (BP77)
merge
β-Catenin
iASPP (V5)
merge

Figure 4.5

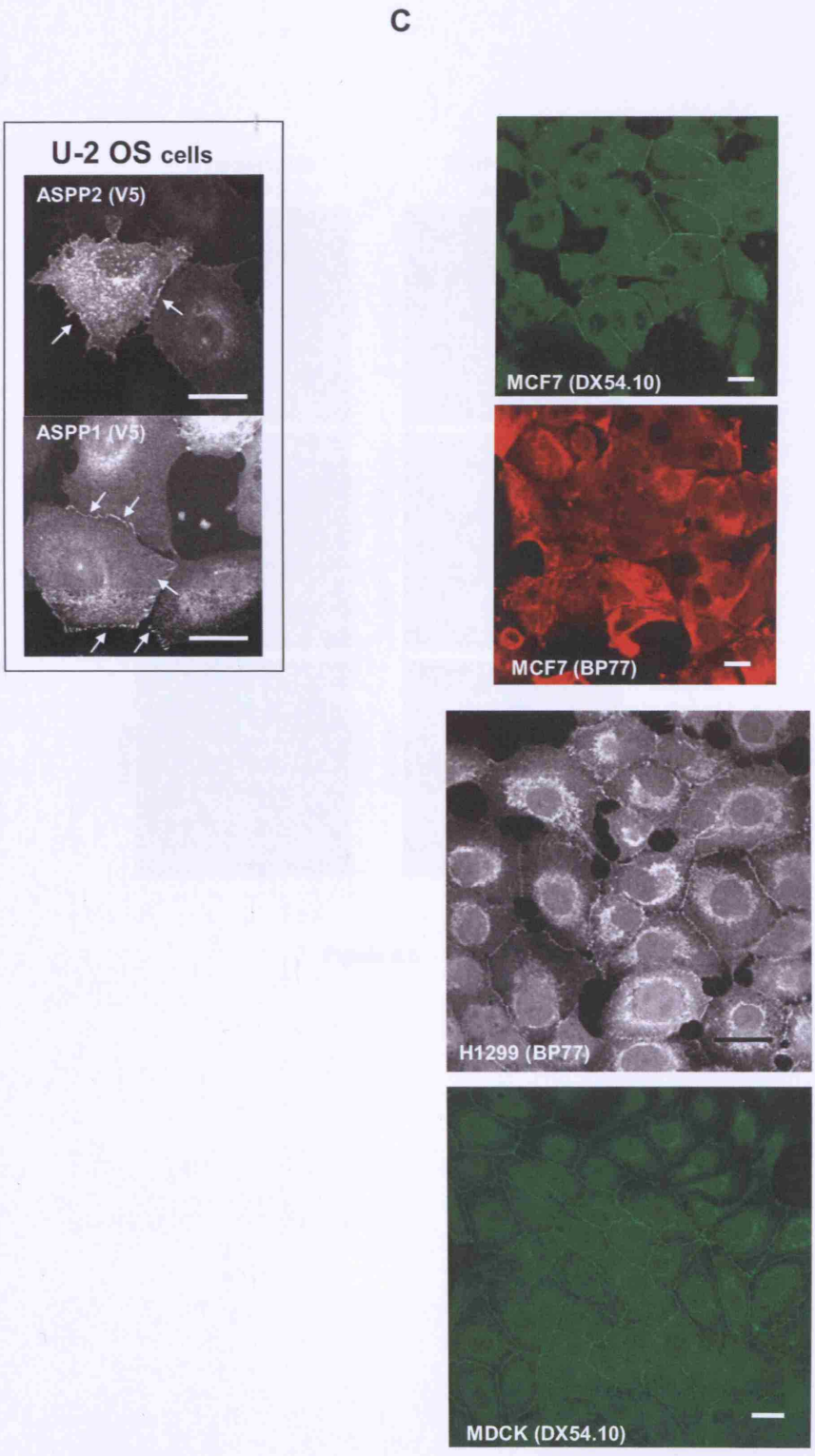

B
C
U-2 OS cells
ASPP2 (V5)
ASPP1 (V5)
MCF7 (DX54.10)
MCF7 (BP77)
H1299 (BP77)
MDCK (DX54.10)

Figure 4.5

D

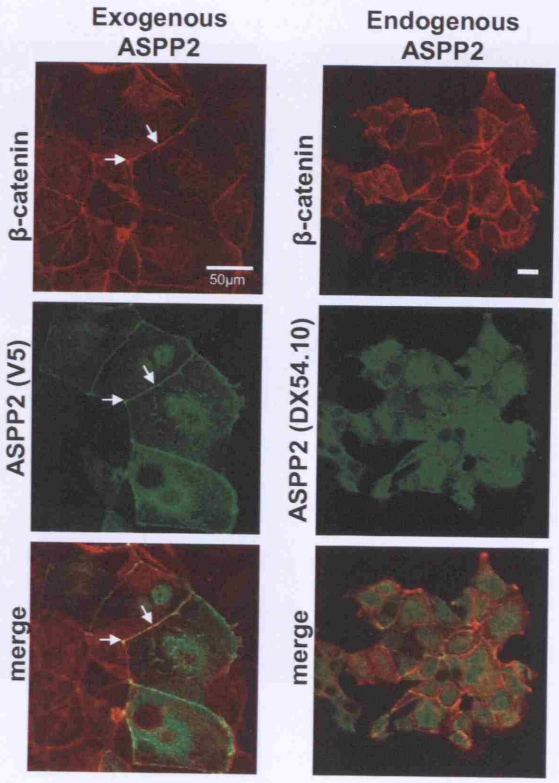

Exogenous ASPP2
Endogenous ASPP2
β-catenin
β-catenin
50μm
ASPP2 (V5)
ASPP2 (DX54.10)
merge
merge

Figure 4.5

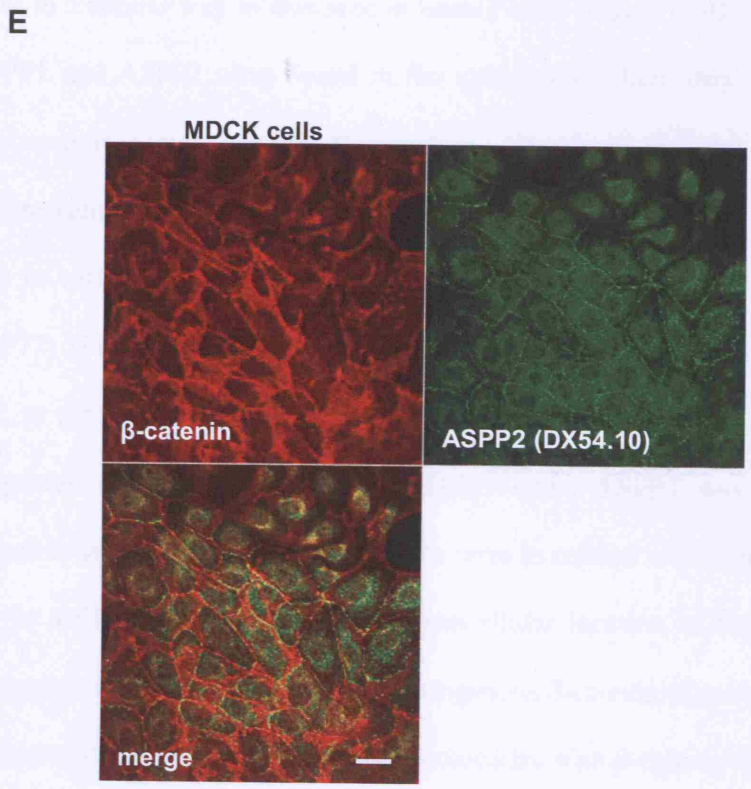


Figure 4.5: The ASPP family of proteins can colocalize with β-catenin in close relation with cell-cell contacts. A. Immunofluorescence of the ASPPs and endogenous β-catenin in Saos-2 cells. Saos-2 cells were transfected with ASPP1-V5, ASPP2-V5 or iASPP-V5 and the expression of the recombinant proteins was detected using an anti-V5 monoclonal antibody. ASPP1 and ASPP2 were also detected using N8 and BP77 rabbit polyclonal antibodies respectively and endogenous β-catenin was detected using an anti-β-Catenin mouse monoclonal antibody (Santa-Cruz). Arrows indicate zones of colocalization between the ASPPs and β-catenin. **B**. Immunofluorescence of exogenously expressed ASPP1-V5 and ASPP2-V5 in U-2 OS cells using a mouse monoclonal anti-V5 antibody. Arrows indicate expression of ASPP1 or ASPP2 where cells interact. **C**. Endogenous localization of ASPP2 in MCF7, MDCK and H1299 cells using two different antibodies (rabbit polyclonal antibody, BP77 and mouse monoclonal antibody, DX54.10). **D**. Comparison of exogenous and endogenous ASPP2 expression in MCF7 cells. ASPP2-V5 was transfected in MCF7 cells and detected with an anti-V5 antibody (left panel). Endogenous ASPP2 was detected with DX54.10 (right panel). Endogenous β-Catenin was detected using an anti-β-Catenin mouse monoclonal antibody (Santa Cruz). Arrows indicate zones of colocalization between exogenous ASPP2 and endogenous β-catenin **E**. Immunofluorescence of endogenous ASPP2 and endogenous β-Catenin in MDCK cells. Endogenous ASPP2 was detected with DX54.10 and endogenous β-Catenin was detected using an anti-β-Catenin mouse monoclonal antibody (Santa Cruz biotechnology). In all the figures, scale bar = 20μm unless otherwise stated.

cells, exogenous ASPP1 and ASPP2 were expressed at the level of cell-cell contacts in a similar way to that seen in Saos-2 cells (Figure 4.5B). The majority of ASPP1 and ASPP2 were found in the cytoplasm, where they were slightly concentrated in regions around the nucleus. To ensure that this was not an overexpression artefact, we checked the subcellular location of endogenous ASPP2 in various epithelial cell lines using two different antibodies (DX54.10 and BP77) (Figure 4.5C). In MCF7 cells, both antibodies were able to detect ASPP2 at the contacts between cells, although some non-specific cytoplasmic staining was evident. In MDCK and H1299 cells, ASPP2 was also clearly expressed at the precise location where cells were in contact with each other.

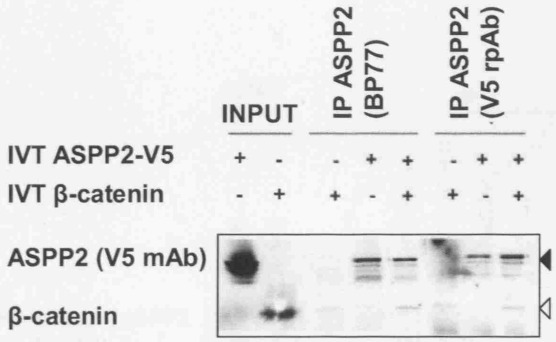
In MCF7 cells we examined the subcellular location of both exogenous and endogenous ASPP2 with respect to endogenous β-catenin (Figure 4.5D). Both exogenous and endogenous ASPP2 could colocalize with β-catenin at the cell-cell junctions. Collectively, as β-catenin is known to be a major component of the *adherens* junctions, these data suggest that ASPP2 is able to interact with β-catenin within these complexes, which could provide evidence as to how ASPP2 can regulate the activity of β-catenin.

4.2.6 ASPP2 can bind to β-catenin

To confirm whether ASPP2 and β-catenin can be part of the same complex, we investigated whether they could bind to each other. When *in vitro* translated ASPP2 was immunoprecipitated with either an anti-ASPP2 antibody or an antibody directed against the V5 tag of ASPP2, *in vitro* translated β-catenin was co-immunoprecipitated, showing that they can bind (Figure 4.6A). To show that the binding can take place *in vivo*, endogenous ASPP2 was immunoprecipitated from MCF7 cell lysates (Figure 4.6B). Similar to what was observed *in vitro*, β-catenin co-immunoprecipitated with ASPP2; however, α-catenin did not, indicating that the interaction is specific. Furthermore, in MDCK cells, more β-catenin co-immunoprecipitated compared to DU145 cells, suggesting that the interaction is stronger in those cells (Figure 4.6C, last two lanes). In this particular experiment small amounts of ASPP2 seemed to be immunoprecipitated in MDCK cells, although this could be due to the fact that the antibody BP77 did not efficiently recognize dog ASPP2. This difference seen in MDCK and DU145 cells agrees with our previous results which indicated that the colocalization between ASPP2 and β-catenin is more extensive in MDCK cells compared to DU145 cells (Figures 4.5E and 4.7A).

Although we could not detect endogenous ASPP1 and β-catenin co-localization, overexpressed ASPP1 could localize at the cell-cell contacts in U-2 OS cells in the same way as ASPP2 (Figure 4.5B). Therefore, we compared the ability of ASPP1 and ASPP2 to bind to β-catenin (Figure 4.6C). To allow a

A


INPUT
IP ASPP2 (BP77)
IP ASPP2 (V5 rpAb)
IVT ASPP2-V5
IVT β-catenin
ASPP2 (V5 mAb)
β-catenin

B

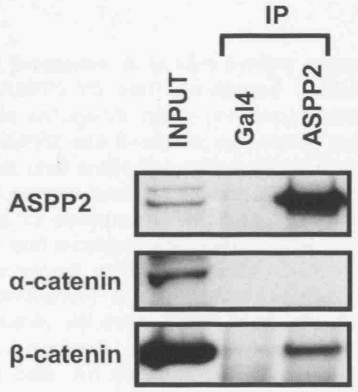

IP
INPUT
Gal4
ASPP2
ASPP2
α-catenin
β-catenin

Figure 4.6

C

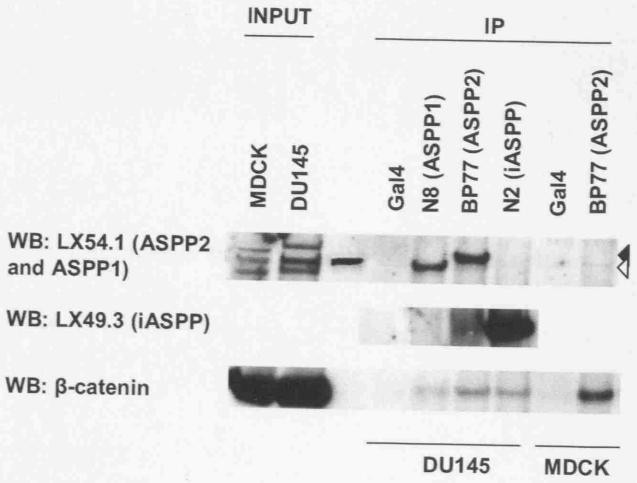


Figure 4.6: ASPP2 can bind β-catenin. **A**. In vitro binding experiment between ASPP2 and β-catenin. *In vitro* translated ASPP2-V5 and Myc-tagged β-catenin were used. ASPP2 was immunoprecipitated either with an anti-V5 rabbit polyclonal antibody or an anti-ASPP2 rabbit polyclonal antibody (BP77). ASPP2 and β-catenin expression was detected by immunoblotting using anti-V5 and 9E10 monoclonal antibodies respectively. Black arrow head: ASPP2. White arrow head: β-catenin. **B**. Endogenous binding between ASPP2 and β-catenin. Anti-ASPP2 rabbit polyclonal antibody was used to co-immunoprecipitate endogenous ASPP2 and β-catenin in MCF7 cells. ASPP2, β-catenin and α-catenin were detected by immunoblotting using respectively DX54.10, anti β-catenin monoclonal antiblody (Santa Cruz) and anti-α-catenin monoclonal antibody (BD transduction laboratories). **C**. Comparison of the binding between ASPP2 and β-catenin and ASPP1 and β-catenin. All three ASPP family members were immuno-precipitated with the indicated rabbit polyclonal antibodies in DU145 cells. ASPP2 was also immunoprecipitated in MDCK cells. An antibody cross-reacting with ASPP1 and ASPP2 was used to detect their expression (LX54.1). LX49.3 monoclonal antibody and β-catenin monoclonal antiblody (Santa Cruz) were used to detect iASPP and β-catenin expression respectively. Black and white arrow heads indicate ASPP2 and ASPP1 respectively. In B and C, a Gal4 rabbit polyclonal antibody (Santa Cruz) was used as a negative control.

comparison, equal amounts of ASPP1 and ASPP2 were immunoprecipitated from a DU145 cell lysate. More β-catenin coimmunoprecipitated with ASPP2 than ASPP1, indicating that ASPP2 is more likely to play an important role in the regulation of β-catenin than ASPP1.

4.2.7 The interaction between ASPP2 and β-catenin is cell-cell contact dependent

The hepatocyte growth factor (HGF) is the natural agonist of the c-Met receptor, a member of the receptor tyrosine kinase family. *In vitro*, upon binding to HGF, c-Met induces the scattering of epithelial cells such as DU145 (Wells et al. 2005). In order to try to further understand whether the interaction between ASPP2 and β-catenin at the membrane is cell contact dependent, we decided to study the effect of HGF induced scattering of DU145 cells. In DU145 cells, as seen in other epithelial cell types, ASPP2 and β-catenin were able to colocalize at the membrane; however, β-catenin was highly expressed at the membranes and ASPP2 was found to colocalize with only a fraction of the β-catenin found at the membrane (Figure 4.7A). Under the experimental conditions used, HGF only induced a partial cell scattering as small colonies of cells remained after treatment (Figure 4.7B). Nonetheless, we could observe a decrease in β-catenin and ASPP2 staining at the membrane in the cells detaching from colonies after HGF treatment

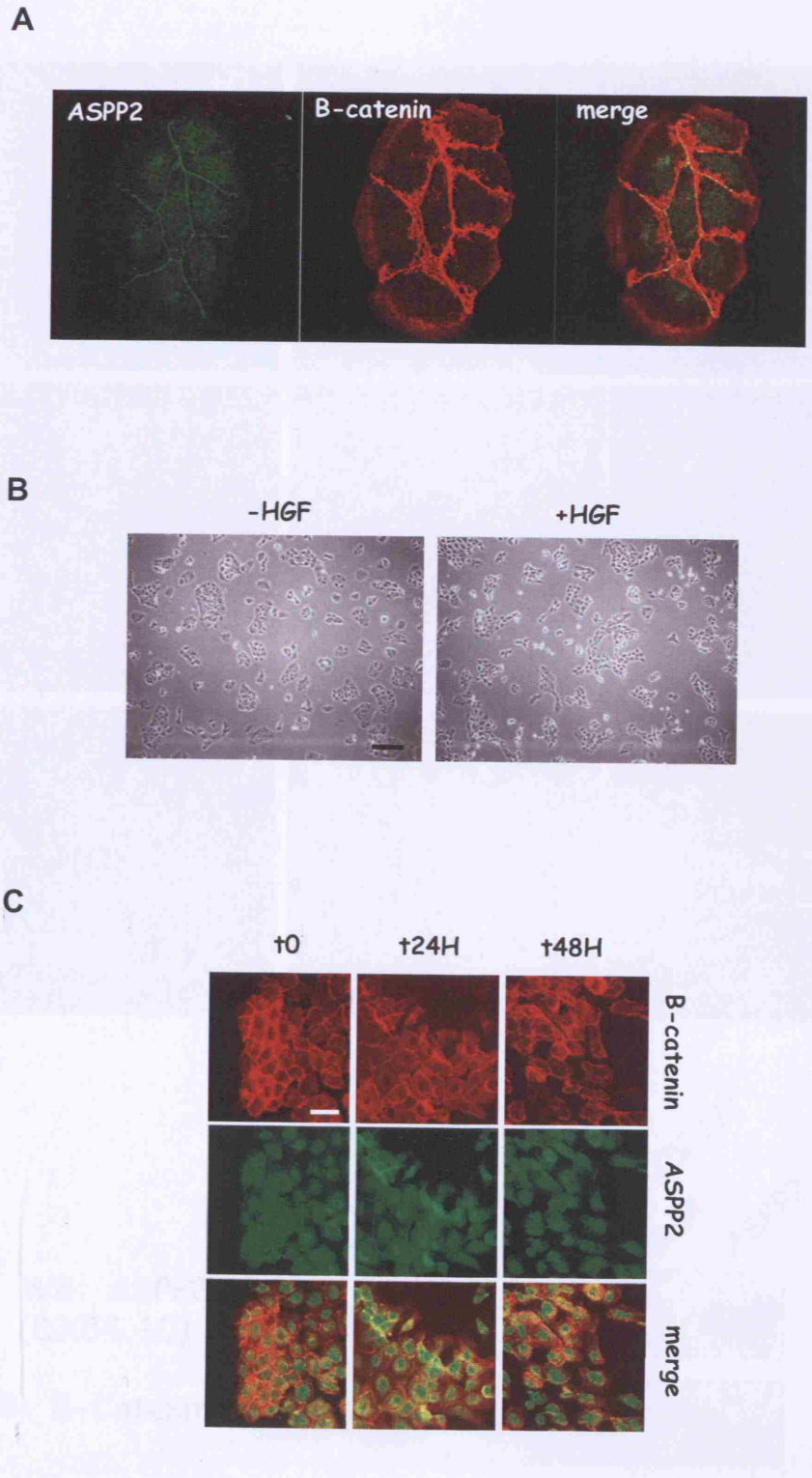
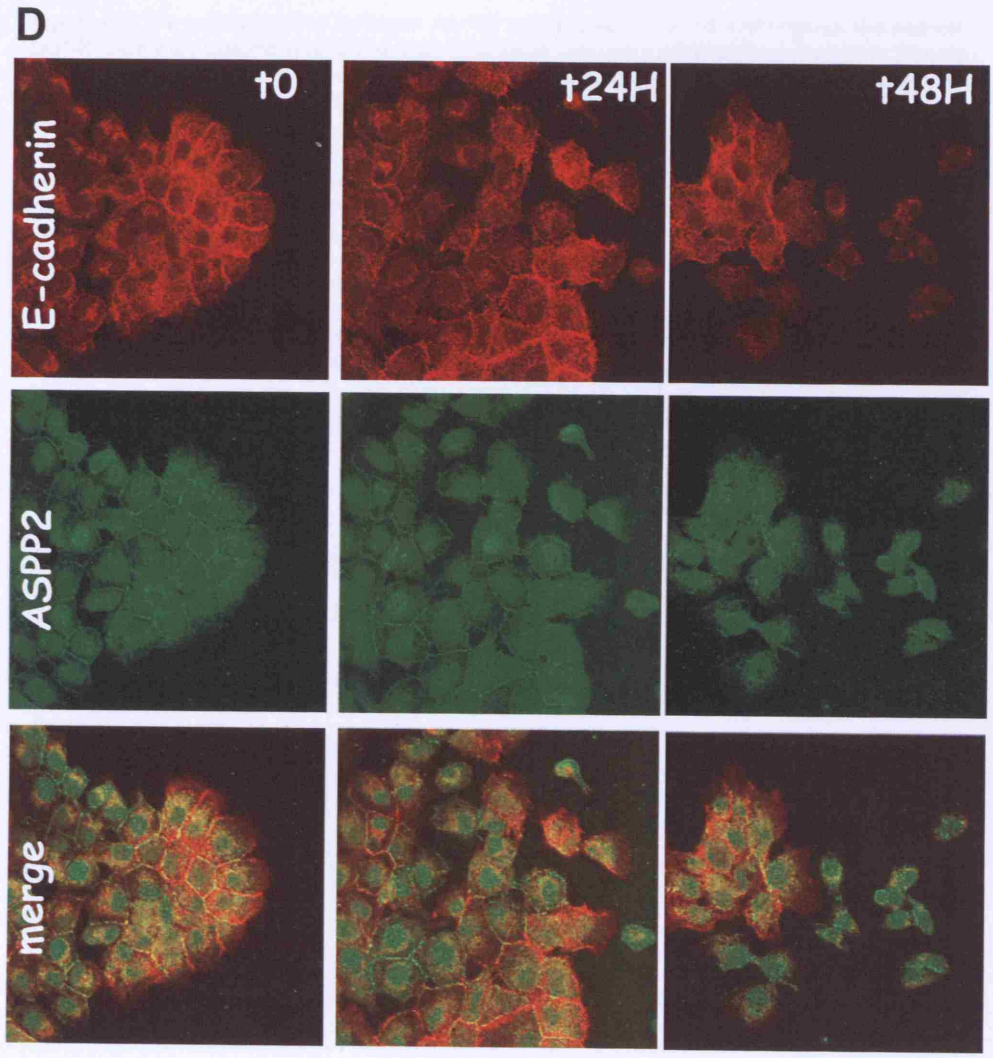

A
ASPP2
B-catenin
merge
B
-HGF
+HGF
C
t0
t24H
t48H
B-catenin
ASPP2
merge

Figure 4.7


D
t0
t24H
t48H
E-cadherin
ASPP2
merge

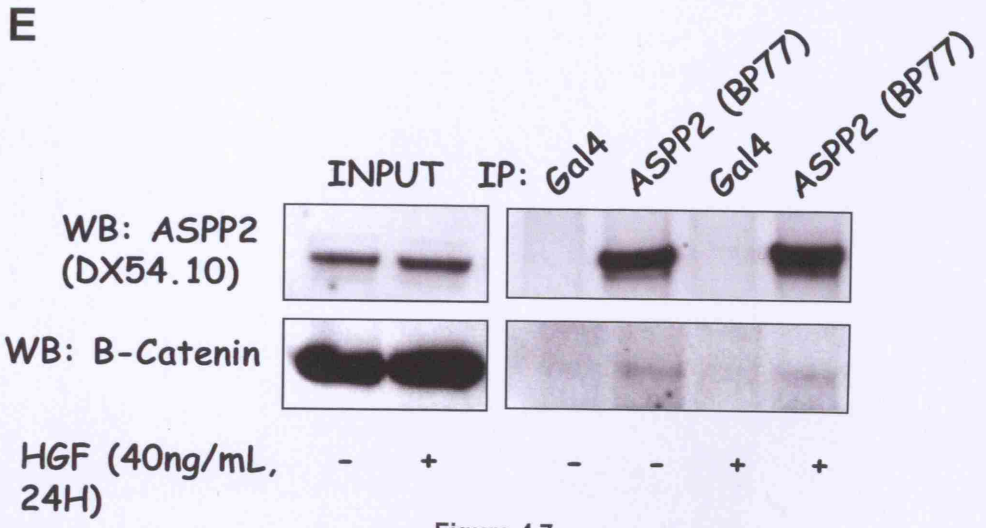

E
INPUT
IP: Gal4
ASPP2 (BP77)
Gal4
ASPP2 (BP77)
WB: ASPP2 (DX54.10)
WB: B-Catenin
HGF (40ng/mL, 24H)
-
+
-
-
+
+

Figure 4.7

Figure 4.7: the colocalization between ASPP2 and β-catenin is cell-cell contact dependant. DU145 cells were seeded sparsely so they form small colonies containing between 10 to 20 cells **A.** Immunofluorescence of endogenous ASPP2 and β-catenin. DX54.10 and an anti-β-catenin polyclonal antibody (Santa Cruz) were used to identify ASPP2 and β-catenin respectively. Scale bar = 20μm **B.** Phase contrast pictures of DU145 cells either untreated or treated with 40ng/ml of HGF for 24 hours. Scale bar = 200μm **C.** Immunofluorescence of ASPP2 and β-catenin in DU145 cells upon HGF treatment. Cells were treated for either 24 hours or 48 hours with 40ng/ml of HGF. Endogenous ASPP2 and β-catenin expression was detected with DX54.10 and a polyclonal β-catenin antibody (Santa Cruz) respectively. Scale bar = 50μm **D.** Immunofluorescence of ASPP2 and E-cadherin in DU145 cells upon HGF treatment. Cells were treated for either 24 hours or 48 hours with 40ng/ml of HGF. Endogenous ASPP2 and E-cadherin expression was detected with DX54.10 and a polyclonal E-cadherin antibody (Cell Signaling) respectively. Scale bar = 50μm **E.** β-catenin binding to ASPP2 in the presence or in the absence of HGF treatment. ASPP2 was immunoprecipitated with BP77 and the amount of ASPP2 and β-catenin was assessed by immunobloting using DX54.10 and an anti-β-catenin monoclonal antibody (Santa Cruz) respectively. A Gal4 rabbit polyclonal antibody (Santa Cruz) was used as a negative control.

Figure 4.7: the colocalization between ASPP2 and β-catenin is cell-cell contact dependant. DU145 cells were seeded sparsely so they form small colonies containing between 10 to 20 cells **A.** Immunofluorescence of endogenous ASPP2 and β-catenin. DX54.10 and an anti-β-catenin polyclonal antibody (Santa Cruz) were used to identify ASPP2 and β-catenin respectively. Scale bar = 20μm **B.** Phase contrast pictures of DU145 cells either untreated or treated with 40ng/ml of HGF for 24 hours. Scale bar = 200μm **C.** Immunofluorescence of ASPP2 and β-catenin in DU145 cells upon HGF treatment. Cells were treated for either 24 hours or 48 hours with 40ng/ml of HGF. Endogenous ASPP2 and β-catenin expression was detected with DX54.10 and a polyclonal β-catenin antibody (Santa Cruz) respectively. Scale bar = 50μm **D.** Immunofluorescence of ASPP2 and E-cadherin in DU145 cells upon HGF treatment. Cells were treated for either 24 hours or 48 hours with 40ng/ml of HGF. Endogenous ASPP2 and E-cadherin expression was detected with DX54.10 and a polyclonal E-cadherin antibody (Cell Signaling) respectively. Scale bar = 50μm **E.** β-catenin binding to ASPP2 in the presence or in the absence of HGF treatment. ASPP2 was immunoprecipitated with BP77 and the amount of ASPP2 and β-catenin was assessed by immunobloting using DX54.10 and an anti-β-catenin monoclonal antibody (Santa Cruz) respectively. A Gal4 rabbit polyclonal antibody (Santa Cruz) was used as a negative control.

(Figure 4.7C). In a similar way, following HGF treatments, a correlation in the disappearance of ASPP2 and E-cadherin staining was evident at the membrane of scattered cells (Figure 4.7D). In an attempt to prove that the loss of ASPP2 and β-catenin staining at the membrane of scattered cells is accompanied by the loss of their binding, we co-immunoprecipitated ASPP2 and β-catenin with or without HGF treatment (Figure 4.7E). HGF treatment did not change the total levels of both proteins, but a subtle reduction in their binding was observed. The incomplete loss of the β-catenin/ASPP2 interaction may be due to the partial effect of HGF on the scattering of DU145 cells. Altogether, the data confirm the idea that the interaction between ASPP2 and β-catenin principally takes place at the membrane level, and that it is cell-cell contact dependent.

4.2.8 ASPP2 interacts with β-catenin in the *adherens* junction complex

As previously mentioned, β-catenin constitutes one of the major components of the *adherens* junction by forming a high affinity, one-to-one complex with the cadherin cytoplasmic domain. As we have evidence that β-catenin and ASPP2 can interact with β-catenin at the level of the cellular membrane in a cell-cell contact dependent mechanism, we hypothesised that this interaction could take place within the *adherens* junction. The formation of *adherens* junctions is dependent on calcium and we, therefore, employed a

A

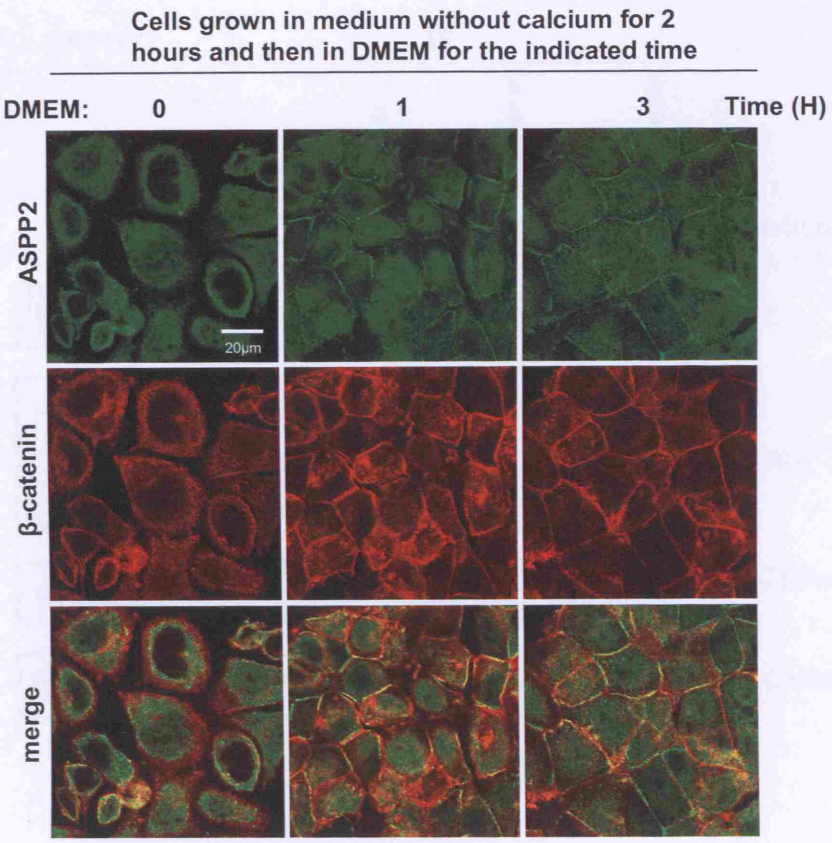

Cells grown in medium without calcium for 2 hours and then in DMEM for the indicated time
DMEM:
0
1
3
Time (H)
ASPP2
β-catenin
merge
20μm

Figure 4.8

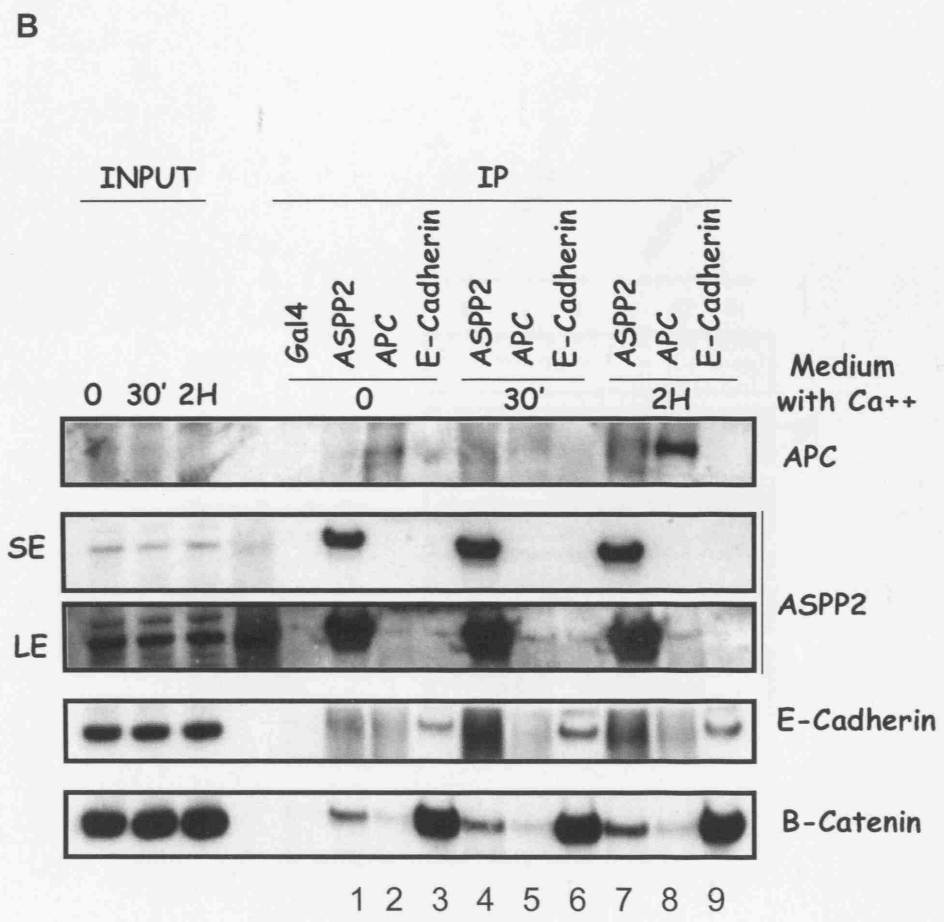

B
INPUT
IP
Gal4
ASPP2
APC
E-Cadherin
ASPP2
APC
E-Cadherin
ASPP2
APC
E-Cadherin
0 30' 2H
0
30'
2H
Medium with Ca++
APC
SE
LE
ASPP2
E-Cadherin
B-Catenin
1 2 3 4 5 6 7 8 9

Figure 4.8

C

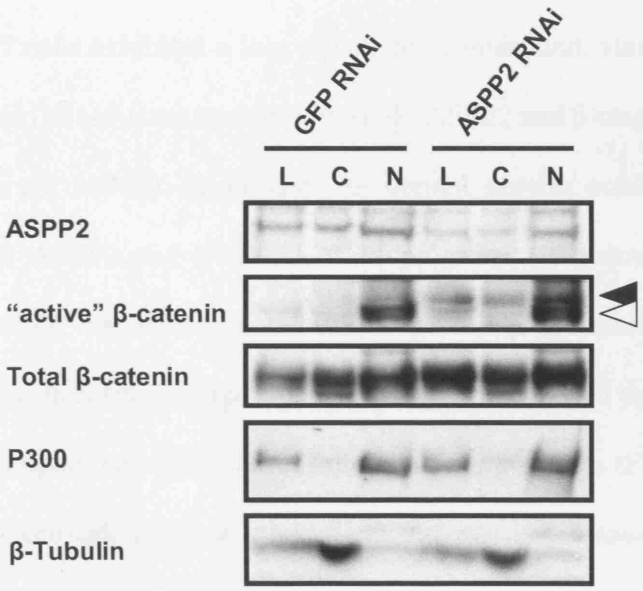


Figure 4.8: ASPP2 interacts with β-catenin within the *adherens* junctions. **A**. ASPP2 and β-catenin colocalize in MCF7 cells, as *adherens* junctions reform. MCF7 cells were grown to confluency and then deprived of calcium to break the *adherens* junctions. At time 0, normal growth conditions were restored. ASPP2 and β-catenin were detected by immunofluorescence as the adherens junctions were reforming. DX54.10 and an anti-β-catenin polyclonal antibody (Santa Cruz) were used. **B**. In a similar experimental set up, ASPP2, APC or E-cadherin were immunoprecipitated at various time points once the normal growth conditions were restored. By immunoblotting, we checked whether the reformation of the adherens junctions could impact the composition of the complexes containing β-catenin. ASPP2, APC and E-cadherin were respectively immunoprecipitated with BP77, APC rabbit polyclonal antibody (hybridoma) and E-cadherin polyclonal antibody (Cell Signaling). DX54.10, APC monoclonal antibody (Abcam), E-cadherin monoclonal antibody (BD transduction laboratories) and β-catenin monoclonal antibody (Santa Cruz) were used to detect the corresponding proteins by immunoblot. SE: short exposure; LE: long exposure. **C**. ASPP2 knock-down and nuclear fractionation in MCF7 cells. An siRNA directed against ASPP2 was used to knock-down ASPP2 expression levels, followed by nuclear fractionation. ASPP2, active β-catenin (non phoshorylated β-catenin on S37 and T41) and levels of total β-catenin were detected by SDS-PAGE/immunoblotting using DX54.10, anti-active-β-catenin polyclonal antibody (Upstate), an anti-β-catenin monoclonal antibody (Santa Cruz) respectively. P300 and β-tubulin levels were also assessed in the different fractions as an indicator of their purity. L = total lysate, C = cytoplasmic fraction, N = nuclear fraction. Black arrow head: non-phosphorylated β-catenin. White arrow head: non-specific band

calcium switch approach to disrupt the *adherens* junctions and subsequently follow the subcellular location of ASPP2 and β-catenin as the junctions reformed (Figure 4.8A). After around two hours of growth in a calcium-deprived medium (at 0 hour), MCF7 cells exhibited a loss of cell-cell contact and, similar to what was observed when DU145 were treated with HGF, ASPP2 and β-catenin staining disappeared from the cellular membrane. As normal growth conditions were reintroduced, both ASPP2 and β-catenin were progressively expressed at the membrane, suggesting that they were added to the newly assembled *adherens* junction complexes. In a similar experimental set-up, we followed the ASPP2/β-catenin interaction upon the reformation of the *adherens* junctions (Figure 4.8B). In the absence of cell-cell contacts, some β-catenin was still bound to ASPP2, suggesting that they can either still interact, at least partially, in the cytoplasm or that the total disassociation of cell-cell contacts was not completely achieved. In agreement with our hypothesis, reintroduction of calcium-containing medium increased the amount of β-catenin being co-immunoprecipitated with ASPP2 (lanes 1, 4 and 7). Similarly, increasing amounts of E-cadherin were co-immunoprecipitated with ASPP2 (lanes 1, 4 and 7). When E-cadherin was directly immunoprecipitated, very little ASPP2 was co-immunoprecipitated (lanes 3, 6 and 9). This is probably because the proportion of ASPP2 within the *adherens* junctions is small compared to the total pool of ASPP2. Additionally, the amount of β-catenin co-immunoprecipitated with E-cadherin did not vary under the different growth conditions, suggesting that cytoplasmic β-catenin and E-cadherin may still form dimers following calcium removal. In conclusion, ASPP2 can be found in complex with E-cadherin and β-catenin and colocalize with both proteins

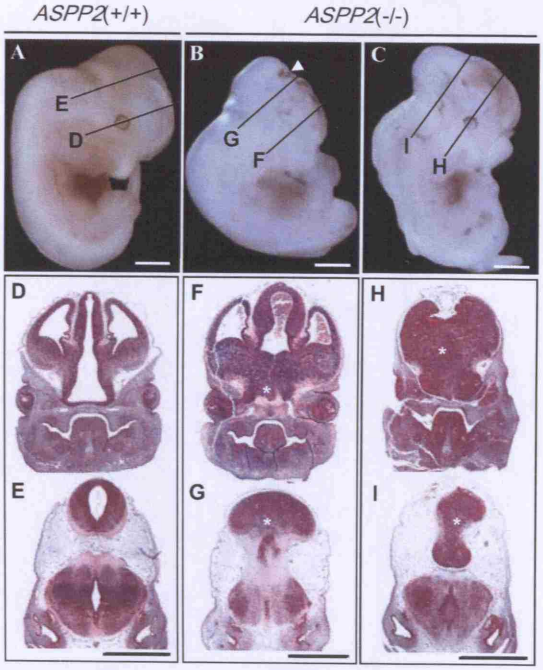
at the membrane of epithelial cells in a calcium dependent manner, thus, strongly suggesting that ASPP2 can interact within the *adherens* junction complexes.

We previously showed that ASPP2 can inhibit β-catenin transcriptional activity (Figure 4.1A); therefore, we wanted to know whether ASPP2 could also be a negative regulator of β-catenin transcriptional activity in epithelial cells. ASPP2 was knocked-down in MCF7 cells using siRNA and total and non-phosphorylated β-catenin levels were monitored in different cellular fractions (Figure 4.8C). In the control cells, ASPP2 could be found in all three fractions (total lysate, cytoplasmic and nuclear fractions), reflecting the immunofluorescence data in various cell types. β-catenin could also be found in all of the fractions. However, the transcriptionally active, non-phosphorylated form of β-catenin was expressed at residual levels. When ASPP2 expression was reduced by siRNA knock-down, β-catenin total levels were increased slightly in all the fractions, but more importantly, non-phosphorylated β-catenin expression increased greatly in the nuclear fraction, demonstrating that ASPP2 expression also inhibits the transcriptional activity of β-catenin in epithelial cells.

4.2.9 ASPP2 is essential for central nervous system development

A recent study from Dr Vives in our laboratory provided information on a new vital role of ASPP2 in CNS development (unpublished data). *ASPP2*

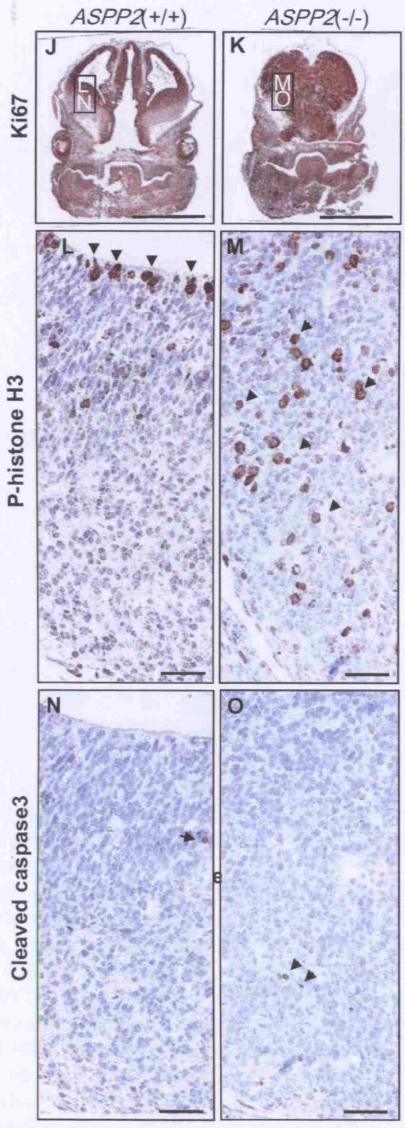
A



V. Vives

Figure 4.9

B



V. Vives

Figure 4.9

C

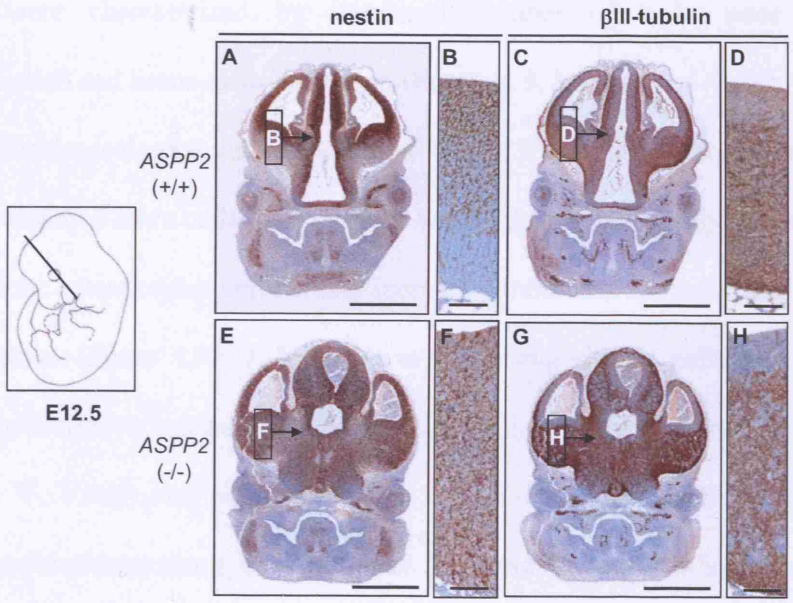


Figure 4.9: Overgrowth in the brain and retina of ASPP2-null embryos is due to overproliferation. A. (A-C) Side views of E12.5 wild-type (A) or ASPP2-null (B-C) embryos with straight lines indicating the location of the coronal sections (D-I). Note the visible haemorrhage in the mutant brain (B, arrowhead).
(D-I) Hematoxylin- and eosin-stained coronal head sections of the embryos shown in (A-C) show excessive cell numbers in the forebrain (asterisks in F,H) and the hindbrain (asterisks in G,I) of E12.5 ASPP2(-/-) embryos, compared with wild-type (+/+) littermates (D,E). **B**. Coronal sections of E12.5 wild-type (J) and ASPP2-null (K). (L,N) and (M,O) respectively correspond to the boxed areas in (J) and (K). Ki-67 (J, K), phospho-histone H3 (L,M) and cleaved caspase 3 (N,O) stainings of wild-type (J,L,N) and ASPP2-null embryos (K,M,O). Note that mitotic cells expressing ki-67 and phospho-histone H3 are confined to the luminal surface of the ventricular zone in wild-type (J, arrowheads in L) whereas ki-67 and H3-positive cells are present at all depths in the ASPP2-null brain (K, arrowheads in M). Only a few apoptotic cells are labelled with the antibody to activated caspase 3 (arrowheads in N,O), with similar staining of wild-type and ASPP2-null brains. **C**. Perturbation of differentiation in the brain of ASPP2-null embryos. A scheme shows the location of the coronal section made in the head of E12.5 wild-type (A-D) and ASPP2-null (E-H) embryos. (A-B, E-F) nestin and betaIII-tubulin (C-D, G-H) immunostainings of wild-type (A-D) and ASPP2-null (E-H) embryos. The boxed areas in (A), (C), (E) and (F) are shown at a higher magnification in (B), (D), (F) and (G) respectively. Note the restriction of the nestin staining in the ventricular area of the wild-type forebrain (A-B) whereas positive cells are scattered in all the tube in the knock-out (E-F). Note the absence of betaIII-tubulin staining in small clusters of nestin-positive cells in the knock-out (F, arrows in G) compared to the wild-type forebrain (C-D).

deficiency in mice led to lethality around birth and to severe abnormalities, especially in the formation of the eyes and the brain during embryogenesis. These organs were characterized by increased number of cells, poor cellular differentiation and tissue disorganization (Figure 4.9, V. Vives).

Histological examination showed that *ASPP2*-null embryo brains and retinas contained more cells than those of wild-type and heterozygous littermates, although the extent of this cell number increase varied between individual *ASPP2*-null embryos (figure 4.9A A-I, V. Vives). The majority of cells in the E12.5 *ASPP2*-null embryo brain expressed the cell proliferation markers Ki67 (figure 4.9B K, V. Vives) and phospho-histone H3 (figure 4.9B M, V. Vives), with positive cells present throughout the brain. In contrast, wild-type brains contained Ki67 (figure 4.9B J, V. Vives) only in the ventricular zone, while mitotic cells expressing phospho-histone H3 were visible only at the surface of the ventricular zone bordering the brain lumen (figure 4.9 L, V. Vives). Similar results were obtained with *ASPP2*-null embryo retinas (data not shown). Hence, at E12.5, most of the cells of the *ASPP2*-null embryo brain and retina retained their proliferation potential and had not embarked on the normal course of post-mitotic differentiation.

The excessive cell number observed in *ASPP2*-null embryo brains did not appear to result from a complete lack of apoptosis. Activated caspase 3, a marker of apoptosis, was observed in a similar low proportion of cells in sections of *ASPP2*-null and wild-type embryo brains suggesting that apoptosis played a minor role in the regulation of the number of cells (Figure 4.9B N-O, V. Vives). Thus, the increased number of cells observed in embryo brains and retinas of the

knockout mice was most probably the consequence of uncontrolled cell proliferation rather than apoptosis. Therefore, ASPP2 must control cell proliferation by a mechanism which does not involve the apoptotic function of p53.

Immunostaining with anti-nestin and anti-βIII-tubulin antibodies, commonly used as neural progenitors and neuronal cell markers respectively, show expansion of the progenitor cell population (figure 4.9C A-B,E-F, V. Vives) and reduction of differentiated neuronal cell population (figure 4.9C C-D,F-G, arrows in G, V. Vives) in the knockout neural tube. The neural progenitor cells in the wild-type embryos localize around the ventricles (figure 4.9C A-B, V. Vives). In contrast, not only the areas around the ventricles but also the overgrowing cells including rosette-like structures are positive for nestin expression in the *ASPP2*-null mutants (figure 4.9C E-F, V.Vives). These data show the perturbation of differentiation in *ASPP2*-null neural tube.

4.2.10 The role of ASPP2 in CNS development involves its function as a regulator of β-catenin

The results from analysis of the CNS in mice embryos suggested that ASPP2 plays a key role in regulating CNS development and that this not predominantly due to its effect in enhancing the apoptotic function of p53. We,

therefore, wanted to investigate how ASPP2 regulates CNS development and begun by analysing the Wnt/β-catenin signalling pathway for several reasons. This pathway has been shown, not only to play an important role in regulating virtually all of the defined human adult stem cell systems including brain (Reya and Clevers 2005), but also to be critical for CNS embryonic development (Brault et al. 2001). In addition, over-expression of constitutively active β-catenin in the forebrain was reported to increase the cortex size by enhancing progenitor growth and inhibiting neuronal differentiation. Another study demonstrated the pivotal role of β-catenin in the maintenance of proliferation of neuronal progenitors, thereby controlling the size of the progenitor pool, and affecting the decision of neuronal progenitors to proliferate or to differentiate (Zechner et al. 2003). Therefore, given that ASPP2 inhibits β-catenin activity *in vitro*, we hypothesised that there might be a link between the over-proliferation and lack of differentiation phenotype observed in the neural tube of *ASPP2*-null embryos and the ability of ASPP2 to regulate β-catenin.

To answer this question we examined the expression patterns of β-catenin and N-cadherin in the brains of wild-type and *ASPP2*-null embryos. As shown in Figure 4.10A, wild-type E12.5 ventral midbrain is well organized, with a localized region with nuclear β-catenin. In contrast, the knock-out counterpart showed a complete structural disorganization of the neuroepithelium with nuclear β-catenin disseminated through the overgrown regions. As expected, N-cadherin co-localized with β-catenin at the cell-cell junctions but the shape of the cells in the knockout were more irregular than in the wild-type, suggesting anomalies in the formation of the *adherens* junctions.

In order to assess the transcriptional activity of β-catenin, we examined the expression pattern of one of β-catenin's transcriptional targets, cyclin D1, which is known to play a role in promoting cellular proliferation (Figure 4.10B). Cyclin D1 was present in the dorsal layers of the wild-type retina (Figure 4.10B B-C) and ventricular region of the midbrain (Figure 4.10B F); however, its expression was scattered through the overgrown embryonic *ASPP2*-null retinal (Figure 4.10B D-E) and midbrain (Figure 4.10B G).

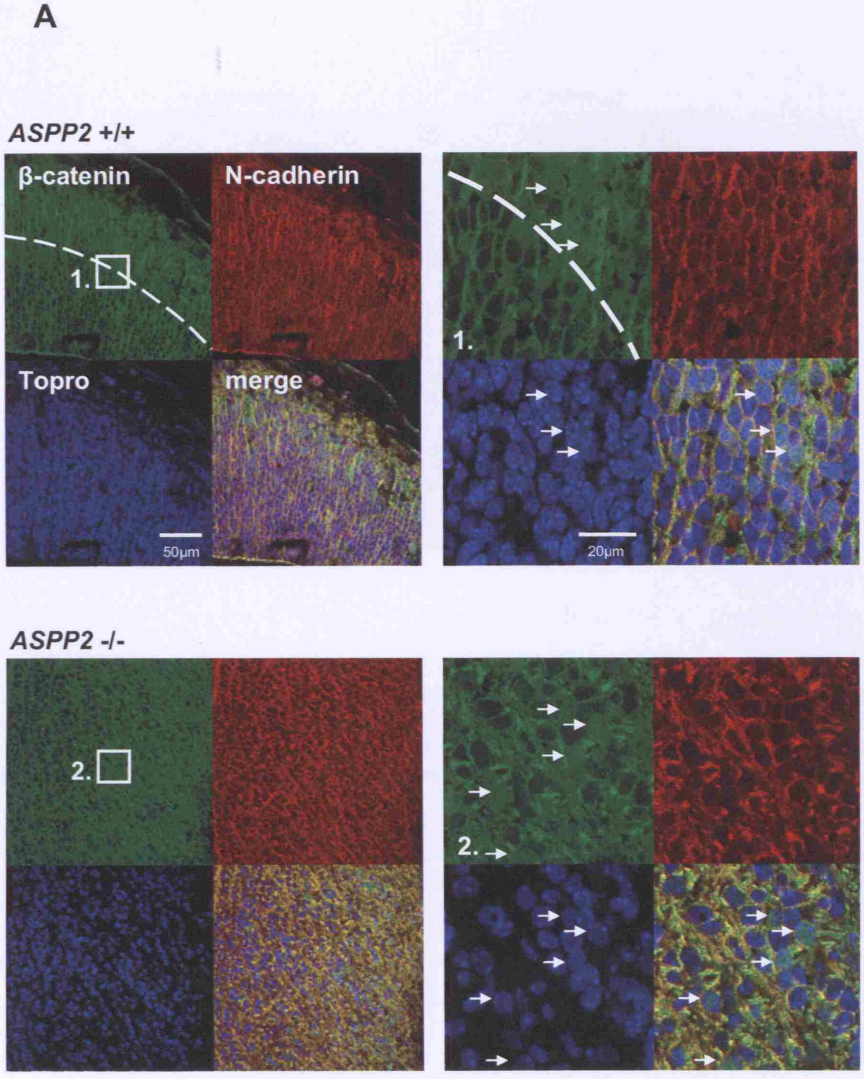

A
ASPP2 +/+
β-catenin
N-cadherin
1.
Topro
merge
50μm
1.
20μm
ASPP2 -/-
2.
2.

Figure 4.10

B

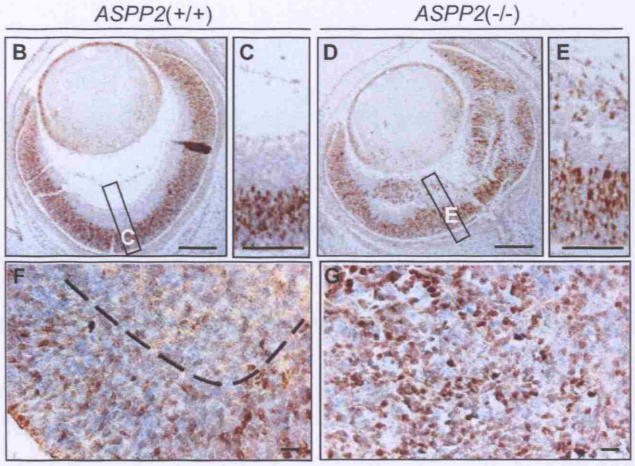


Figure 4.10: β-catenin and cyclin D1 expression in the overgrown regions of the ASPP2 knock-out neural tube. A. β-catenin (BD Transduction Laboratories) (green), N-cadherin (Abcam) (red) and TO-PRO (blue) staining of E12.5 wild-type (top panel) and ASPP2 knock-out (bottom panel) ventral midbrain, using . A magnification of the boxed area labelled 1 (top panel) or 2 (bottom panel) is shown on the right side panel. The arrows show nuclear β–catenin. The dotted line on the top panel delimits nuclear and junctional β–catenin areas. Note that this boundary is not conserved in the knock-out neuroepithelium. **B**. (B-E) Cyclin D1 immunostaining of E15.5 wild-type (B-C) and ASPP2-null (D-E) embryos retinas. Boxed areas in (B) and (D) are enlarged in (C) and (E) respectively. (F-G) Cyclin D1 immunostaining of E12.5 wild-type (F) and ASPP2-null (G) embryos striata.
Scale bars: B-E, 50 µm; F-G, 20 µm

4.3 Discussion

The interest in β-catenin, and more generally Wnt signalling, has been growing over the last two decades. β-catenin is a unique protein with versatile functions as it forms part of the *adherens* junctions and plays a role in cell-cell adhesion at the level of the plasma membrane, while in the nucleus, it binds to the LEF/TCF family of transcription factors to reprogram the gene expression pattern of the cell (Clevers 2006; Hartsock and Nelson 2007). The control of the dual function of β-catenin in cellular adhesion and transcriptional regulation is crucial in maintaining normal cellular function. Often in cancer the equilibrium between these two functions is de-regulated, and the balance leans towards the transcriptional function of β-catenin to the detriment of its role in cellular adhesion. Moreover, during development both functions are required in order to control the balance between proliferation and differentiation, as well as the formation of tissues. As a result, it is critical to understand how the switch between these two functions is controlled. Herein, we explored the role of the ASPPs, and in particular ASPP2, in the regulation of β-catenin in a cellular context and *in vivo* during development in ASPP2 deficient mice.

4.3.1 ASPP2 negatively regulates β-catenin signalling

ASPP2 and β-catenin drive opposite biological effects in the nucleus, as the former is involved in p53-mediated apoptosis and the latter promotes proliferation by inducing the expression of genes involved in cell-cycle regulation such as, for example, cyclin D1. In order to understand the connection between these two proteins, we investigated the role of ASPP2 in regulating β-catenin.

Several lines of evidence, using different cellular systems, suggest that ASPP2 is a negative regulator of the Wnt/β-catenin signalling pathway (Figure 4.1-2, 4.8). However, ASPP2 seems to finely tune β-catenin activity rather then completely block it. For example, ASPP2 deficiency in MEFs leads to a relatively small increase in β-catenin transcriptional activity, which can be further enhanced by blocking the activity of the β-catenin degradation pathway (Figure 4.1A). This observation correlates with immunofluorescence data, showing that ASPP2 deficiency does not lead to an increase in the overall levels of β-catenin, but rather to a leakage of β-catenin to the nucleus (Figure 4.2). Again, this was further enhanced by inducing Wnt signalling and blocking the β-catenin degradation complex, although this will need to be reproduced in order to determine whether this result is significant (Figure 4.2B). Reducing the levels of ASPP2 with siRNA in MCF7 cells led to a similar observation, as the total levels of β-catenin were not dramatically changed, whereas transcriptionally active β-catenin accumulated in the nucleus (Figure 4.8).

As ASPP2 can interact with APCL and as APCL downregulates β-catenin transcriptional activity, we hypothesised that ASPP2 could positively regulate the function of APCL and target β-catenin for degradation (Nakagawa et al. 1998; Nakagawa et al. 2000a). However, our data obtained from analysis of β-catenin phosphorylation status did not allow us to make a definitive conclusion, as the priming phosphorylation for GSK3β on Ser-45 by CK1 was reduced in ASPP2 deficient MEFs, without affecting phosphorylations at Ser-33, Ser-37 and Thr-41 by GSK3β. This, however, does suggest that β-catenin would be targeted for proteasomal degradation as efficiently by the cytoplasmic degradation complex, as phosphorylation of the residues involved in β-TRCP binding remains unaffected (Hart et al. 1999). However, further analysis of the role of ASPP2 in the phosphorylation status of β-catenin needs to be done to fully understand this result. Using kinase assays, it will be essential to investigate the effect of ASPP2 on the phosphorylation of β-catenin by CK1 and GSK3β. Interestingly, ASPP2 has been shown to be able to bind to PP1 (Helps et al. 1995) and the binding motif in ASPP2, and the other members of the family, has been identified by Dr S. Llanos (Figure 4.4A; data for ASPP1 and iASPP are not shown). Moreover, PP1 has recently been shown to positively regulate the Wnt/β-catenin pathway by dephosphorylating Axin and inhibiting its interaction with GSK3β and, as a result, regulating the assembly/disassembly of the cytoplasmic destruction complex (Luo et al. 2007). Our data indicate that overexpression of ASPP2 can promote the formation of modified β-catenin, which may represent ubiquitinated β-catenin (Figure 4.4B), although this remains to be determined. Interestingly, the binding of ASPP2 to PP1 seems to play a role in the modification of β-catenin by ASPP2,

as the ASPP2 construct mutated in the PP1 binding site could not induce the formation of higher molecular weight forms of β-catenin (Figure 4.4C). This raises the question whether ASPP2 can inhibit the activity of PP1 and the subsequent regulation of the assembly of the cytoplasmic destruction complex. As a result, it will be determinant to investigate whether ASPP2 can influence the phosphorylation status of the proteins contained in the β-catenin destruction complex such as Axin, as PP1 can regulate its phosphorylation.

4.3.2 ASPP2, a new component of the *adherens* junction complex

The ASPPs subcellular localization is complex. ASPP2 has been identified as being able to bind to p53 on the promoter of proapoptotic genes in chromatin immunoprecipitation experiments, although it seems predominantly cytoplasmic in immunofluorescence experiments (Samuels-Lev et al. 2001) (Figure 3.1A). Here we report that ASPP2, and to a different extent ASPP1, is able to localize within the *adherens* junctions.

Several lines of evidence indicate that ASPP2 can form part of the adherens junctions. In all epithelial cell types that were examined, both endogenous and exogenous ASPP2 could be found at the membrane, where cell-cell contacts take place (Figure 4.5). Moreover, breaking cell-cell junctions via the activation of the c-Met receptor displaced ASPP2 from the cytoplasmic membrane

(Figure 4.7). Finally, ASPP2 could relocalize to the plasma membrane in a Ca^{2+} dependent manner (Figure 4.8). Biochemical evidence shows that ASPP2 can physically interact within the same complex as β-catenin *in vitro* and *in vivo* (Figure 4.6). The formation of this complex, which also contains E-cadherin, is, at least partially, dependent on the formation of cell-cell contacts (Figure 4.7-8). In contrast, α-catenin could not be found within the complex. The fact that α-catenin could not be detected within the complex, may be due to the highly dynamic interaction between α-catenin and β-catenin, or simply because ASPP2 and α-catenin compete against each other for the binding to the *adherens* junction complex (Drees et al. 2005; Yamada et al. 2005). We currently do not know whether p120 catenin can also form part of the complex, although some preliminary data showed it can efficiently bind to iASPP (data not shown). Therefore, the composition of this complex needs to be further investigated in order to have a deeper understanding of how these complexes form and the role of each component in its formation.

4.3.3 ASPP2 is essential to ensure the proper function of β-catenin during the formation of the neuroepithelium and the eye

In this study we have identified a new vital role of ASPP2 in CNS development. *ASPP2* deficiency in mice led to lethality around birth and to severe

abnormalities, especially in the formation of the eyes and the brain during embryogenesis (Figure 4.9). These organs were characterized by increased number of cells, poor cellular differentiation and tissue disorganization. The over-growth phenotype was the result of over-proliferation as demonstrated by cleaved caspases 3 and phosphor-histone H3 staining experiments (Figure 4.9B). Apoptosis did not seem to be an important factor in the development of the brain, therefore suggesting that the function of ASPP2 in regulating p53 mediated apoptosis was not involved (Figure 4.9B). Poor differentiation was underlined by the presence of dysplasic rosettes and confirmed by the absence of differentiation markers in these rosettes and by the expansion of the progenitor cell population to the over-proliferative regions of the brain (Figure 4.9C). Finally, aberrant tissue organization was obvious in the surviving pups which exhibited defects such as bilateral retinal dysplasia.

We wanted to address whether ASPP2 regulates the formation of the CNS in a β-catenin dependent manner. Our data suggests that *ASPP2* deficiency sensitizes neural stem cells to WNT3A (data not shown), suggesting that the expansion of the progenitor cell population and the reduction of the differentiated neuronal cell population in the overgrown regions of the brain could be due to an increase in the WNT/β-catenin signalling pathway in these cells. Furthermore, in agreement with this observation, cells containing nuclear β-catenin were found in a very limited and organized apical region of the developing cortex in the wild-type, whereas in the *ASPP2*-deficient embryos, they were disseminated throughout the overgrown regions of the brain (Figure 4.10A). Finally, cyclin D1 expression pattern in the brain was consistent with this result, suggesting that

nuclear β-catenin in those cells is transcriptionally active and able to induce cellular proliferation (Figure 4.10B). However, as cyclin D1 expression can be controlled by multiple other pathways, it will be necessary to determine whether the expression of other targets known to be specifically regulated by β-catenin, such as Axin 2, are deregulated in the *ASPP2*-null brains in order to confirm these results.

4.3.4 Summary

In this chapter, we have shown the biological importance of the ASPPs, and in particular ASPP2, in the regulation of the Wnt/β-catenin signalling pathway *in vitro* and *in vivo*. Our data indicate that ASPP2 is able to control and inhibit the transcriptional activity of β-catenin, in a mechanism yet to define. However, our data provide some insight into how this regulation could take place. For instance, we show that the binding of ASPP2 to PP1 is required in order to induce modifications of β-catenin. Additionally, we show that ASPP2 can form a newly identified complex with β-catenin within the adherens junctions. Finally, we believe that the role of ASPP2 in the regulation of β-catenin activity and its interaction with β-catenin at the level of the adherens junctions may be determinant for the organization of the neuroepithelium and the eye and for the control of cell proliferation during embryonic development of the CNS.

Chapter V: APP-BP1 and ubiquitin-like modifications play a role in the regulation of the ASPP family

5.1 Introduction

The ASPP2 interacting protein APP-BP1 was first identified as a protein that interacts with the C-terminus of the amyloid precursor protein (APP) and is a major component of the neddylation pathway (Chow et al. 1996). Amyloid protein precursors are precursors of β-amyloid which play a role in the Alzheimer's disease brain. APP-BP1 is one-half of a bipartite activating enzyme for the ubiquitin-like protein NEDD8. APP-BP1 is homologous to the N-terminal of the ubiquitin activating enzyme E1, but it lacks the conserved cysteine required for E1 ubiquitin activation activity. As a result, APP-BP1 has to bind to hUba3, which is homologous to the C-terminus of E1, in order to form a functional E1-like enzyme for NEDD8 activation. Activated NEDD8 is then loaded onto hUbc12, which is analogous to E2 in the ubiquitination pathway. Finally, NEDD8 is covalently targeted to proteins at a lysine residue.

There are only a few substrates, like cullin family members, which are known to be neddylated in mammalian cells (Hori et al. 1999). Interestingly, the neddylation of Cullin-1 within the SCF complex has been shown to catalyse its ubiquitination function on several substrates, including β-catenin. As a result, ablation of the neddylation pathway in Uba3 deficient mice results, among other effects, in the accumulation of β-catenin (Tateishi et al. 2001).

APP-BP1 binds to ASPP2 in two different portions, one in the C-terminus encompassing the signature domains of the ASPPs (amino acids 693-1128), and one close to the N-terminus (amino acids 332-483) (Chen et al. 2003). Surprisingly, early evidence suggests that ASPP2 can inhibit APP-BP1 mediated apoptosis in primary neurons and that ASPP2 is a negative regulator of the neddylation pathway through its interaction with APP-BP1 (Chen et al. 2003).

In this chapter we will attempt to answer several questions. Firstly, as ASPP2 uses its C-terminus to bind to both p53 and APP-BP1, we will investigate whether APP-BP1 competes with p53 to bind to ASPP2 and, therefore, interferes with the ability of ASPP2 to regulate the proapoptotic function of p53. Moreover, the neddylation of p53 has been shown to play a role in the regulation of its activity (Xirodimas et al. 2004), suggesting that ASPP2, by interacting with APP-BP1 could influence the neddylation status of p53 and as a consequence impact on its activity. As a result one of the aims of this chapter will be to investigate the influence of the neddylation pathway and the interaction between APP-BP1 and ASPP2 on its function in the regulation of p53.

Secondly, we will need to investigate whether ASPP1 and iASPP also functionally interact with APP-BP1. This needs to be clarified, as the interaction with APP-BP1 might be specific to ASPP2, and not the other ASPP family members, as it involves a region close to the N-terminus of ASPP2 which is not conserved in ASPP1 and iASPP. However, the signature domains of the ASPP family are also involved in the interaction, therefore we cannot exclude the possibility that ASPP1 and iASPP also interact with APP-BP1.

Moreover, ASPP2 has been shown to be ubiquitinated and targeted for degradation by the proteasome (Zhu et al. 2005). Additionally, ASPP2 is a binding partner of different proteins, such as APCL, Siah-1 or APP-BP1, which are ultimately involved in the ubiquitination or the neddylation of various substrates, suggesting that ASPP2 and the ASPPs might be subjected to other types of ubiquitin-like modifications. Finally, we will therefore investigate whether the ASPPs are potentially modified by ubiquitin-like molecules as this may play an important role in their regulation, by affecting their protein levels or their localization for example.

5.2 Results

5.2.1 APP-BP1 positively regulates the function of ASPP1 and ASPP2

The effect of APP-BP1 on the ability of the ASPP proteins to modulate p53 apoptotic function was tested, by using a *PIG3* reporter plasmid in a transactivation assay (Figure 5.1). When APP-BP1 was transfected alongside p53 it did not affect the activity of p53 on the promoter. However, APP-BP1 was able to increase the effect that ASPP1 or ASPP2 have on p53, suggesting that APP-BP1 can affect the activity of p53 in an ASPP1 or ASPP2 dependent manner. In contrast, APP-BP1 appeared to have little or no effect on the ability of iASPP to inhibit p53 activity. The protein expression levels indicate that there was more ASPP1 in the presence of APP-BP1, suggesting that it might play a role in the stabilization of ASPP1 (Figure 5.1). This observation was confirmed, as upon expression of increasing amounts of APP-BP1, exogenous ASPP1 was stabilized (Figure 5.2A). This stabilization required less APP-BP1 in the presence of p53.

To verify these overexpression data, we used the thermosensitive hamster cell line ts41 (Figure 5.2B). At the permissive temperature (34°C) the APP-BP1 protein is functional but when the temperature is switched to 39°C (the non permissive temperature), the protein becomes unstable and is degraded, thus down-regulating the neddylation pathway. We analyzed the levels of ASPP1, ASPP2 and iASPP at

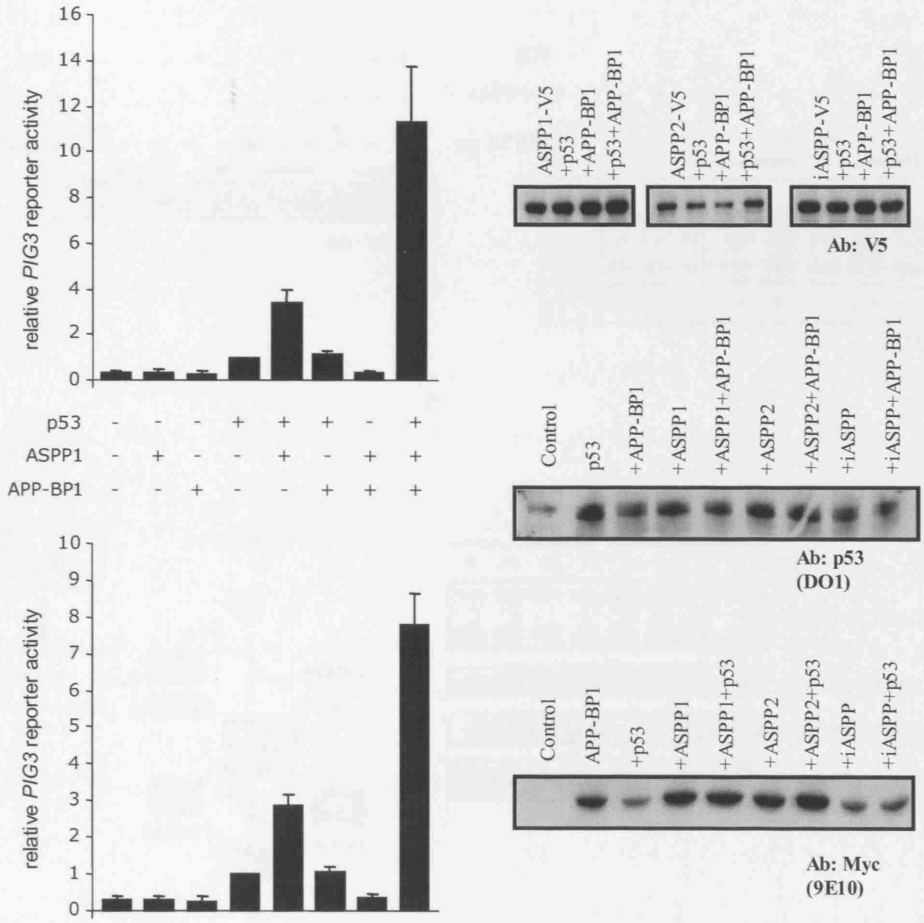


Fig5.1: APP-BP1enhances the synergy between p53 and ASPP2 or ASPP1 on *PIG3* promoter. Left panel: Transactivation assays were performed in Saos-2 cells in order to investigate the influence of APP-BP1 on the regulation of p53 by ASPP1 (top bar graph), ASPP2 (middle bar graph) and iASPP (bottom bar graph) respectively. The cells were transfected with a *PIG3* reporter construct (1µg) and various amounts of the indicated expression plasmids in order to obtain similar levels of protein expression. The total amounts of DNA were normalized by addition of the appropriate empty vector. Error bars represent the standard deviation of 3 independent experiments performed in duplicate. Right panel: cell lysates were then normalized for total protein content and analyzed by SDS-PAGE/immunoblotting, using DO1, 9E10 and anti-V5 monoclonal antibodies for detecting p53, Myc-tagged APP-BP1 and V5-tagged ASPP1, ASPP2 and iASPP, respectively.

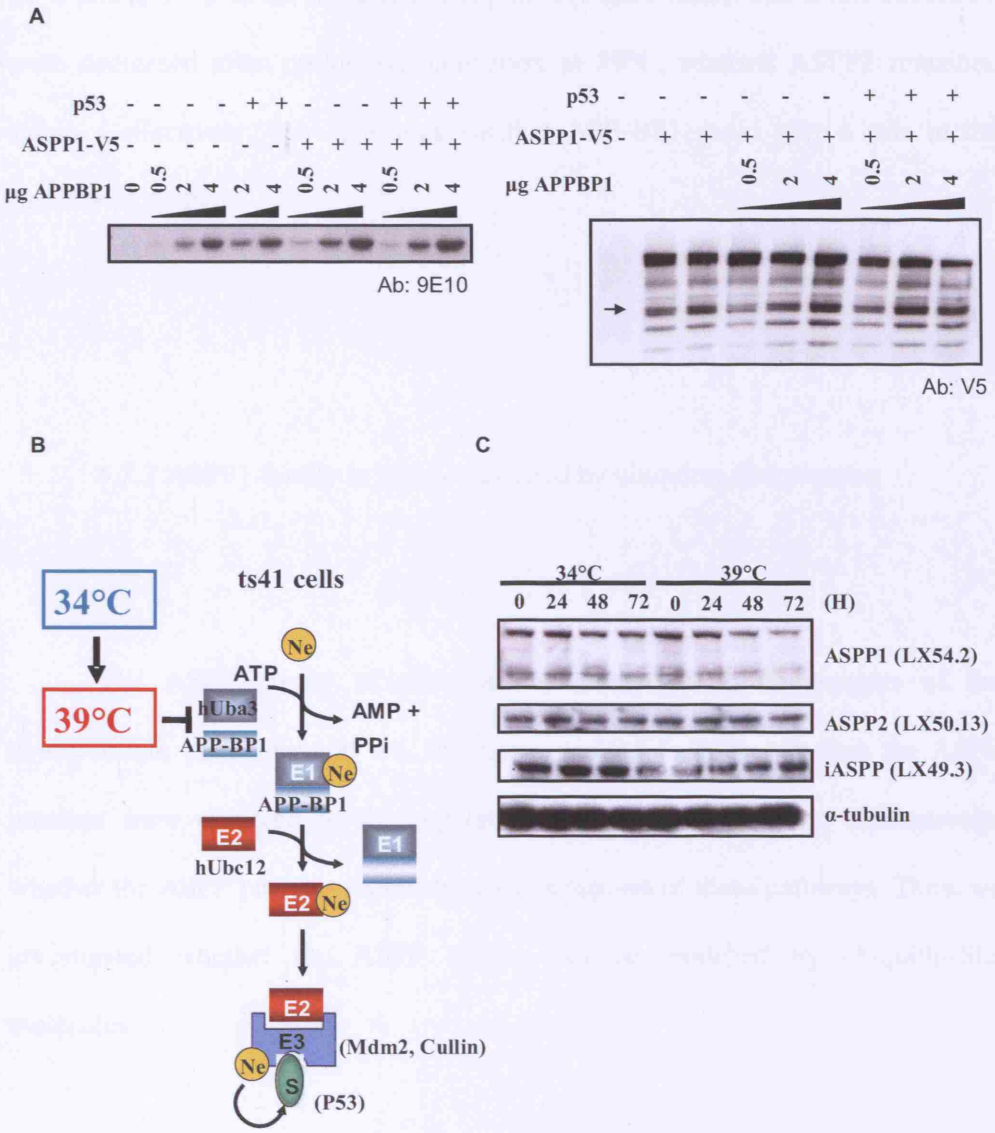


Figure 5.2: APP-BP1 is involved in ASPP1 stabilization. **A.** Titration of APP-BP1 in SAOS2 cells; different plasmids were transfected as indicated in the figure. The total amounts of DNA were normalized by addition of the appropriate empty vector. The expression of APP-BP1 and ASPP1 were analysed by SDS-PAGE/immunoblotting using 9E10 and V5 antibodies, respectively. Black arrow indicates ASPP1. **B.** Scheme of the APP-BP1 thermosensitive mutation in ts41 cells. Upon temperature switch to 39ºC, APP-BP1 becomes unstable and is degraded. As a consequence, the E1-like NEDD8 activating enzyme constituted by hUba3 and APP-BP1 is not functional anymore and the neddylation pathway is disrupted. Ne: NEDD8; S: substrate **C.** Immunoblot analysis of the ASPP family of proteins in the ts41 cells at either 34 ºC (permissive temperature) or 39 ºC (non permissive temperature) at different time points (0, 24, 48 and 72 hours). ASPP1, ASPP2 and iASPP protein levels were detected with LX54.2, LX50.13 and LX49.3 monoclonal antibodies respectively. α-tubulin was used as loading control.

34°C and at 39°C at the indicated time points (Figure 5.2C). The levels of ASPP1 were decreased after prolonged incubation at 39°C, whereas ASPP2 remained stable. Collectively, this data suggests that APP-BP1 could play a role in the stability of ASPP1.

5.2.2 ASPP1 family is highly modified by ubiquitin-like proteins

The ASPP family of proteins can interact with components of the ubiquitin-like pathways and we, therefore, wanted to know whether the ASPP proteins were involved in the regulation of these pathways or, alternatively, whether the ASPP proteins themselves are substrates of these pathways. Thus, we investigated whether the ASPP family can be modified by ubiquitin-like molecules.

First indication that the ASPPs are modified

The first evidence we had that suggested that the ASPPs could be modified was obtained by adding iodoacetamide to whole cell lysates (Figure 5.3). Iodoacetamide is a cysteine-proteases inhibitor and is thus able to inhibit the activity of ubiquitin and ubiquitin-like proteases such as HAUSP (Ubiquitin-

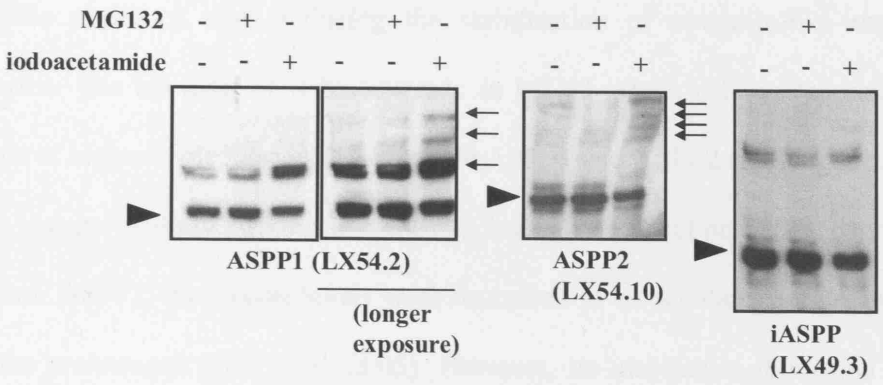


Figure 5.3: The ASPPs are potentially modified by ubiquitin-like proteins. MG63 cells were treated with or without 50μM MG132 for 2 hours and then lysed in RIPA (1% NP40) buffer with or without 5mM iodoacetamide. ASPP1, ASPP2 and iASPP were detected by immunoblotting using LX54.2, LX54.10 and LX49.3 monoclonal antibodies respectively. Full-length proteins are indicated with black arrowheads in the figure and potential modifications with arrows.

specific protease), NEDP1 (NEDD8-specific protease), SENP1 (Sentrin or SUMO specific protease), thus inducing the stabilization of ubiquitin-like modified proteins. The addition of iodoacetamide in MG63 whole cell lysates stabilized bands of higher molecular weight than the ASPP1 and ASPP2 full-length proteins (this was particularly striking for ASPP1), but had no effect on iASPP expression pattern. ASPP2 expression levels were described to be regulated by degradation by the proteasome (Zhu et al. 2005). However, no increase in the levels of the full-length protein was observed upon MG132 treatment under the conditions we used.

ASPP1 is a better candidate than ASPP2 for ubiquitin-like modifications

In order to clarify this first observation, we investigated whether ASPP1 was modified by ubiquitin-like molecules. Endogenous ASPP1 was immunoprecipitated and several antibodies against different members of the ubiquitin-like family were used to detect ASPP1 modified species (Figure 5.4A). From the results, we have evidence to suggest that ASPP1 could be modified in vivo by ubiquitination, sumoylation and neddylation, the latter being the most strongly detected.

In a similar experiment, we immunoprecipitated ASPP1 and ASPP2 to compare their respective potential to be modified by ubiquitin or ubiquitin-like proteins (Figure 5.4B). Endogenous ASPP2 was clearly less subject to this type of

modifications in comparison to ASPP1, which again, appeared to be strongly neddylated.

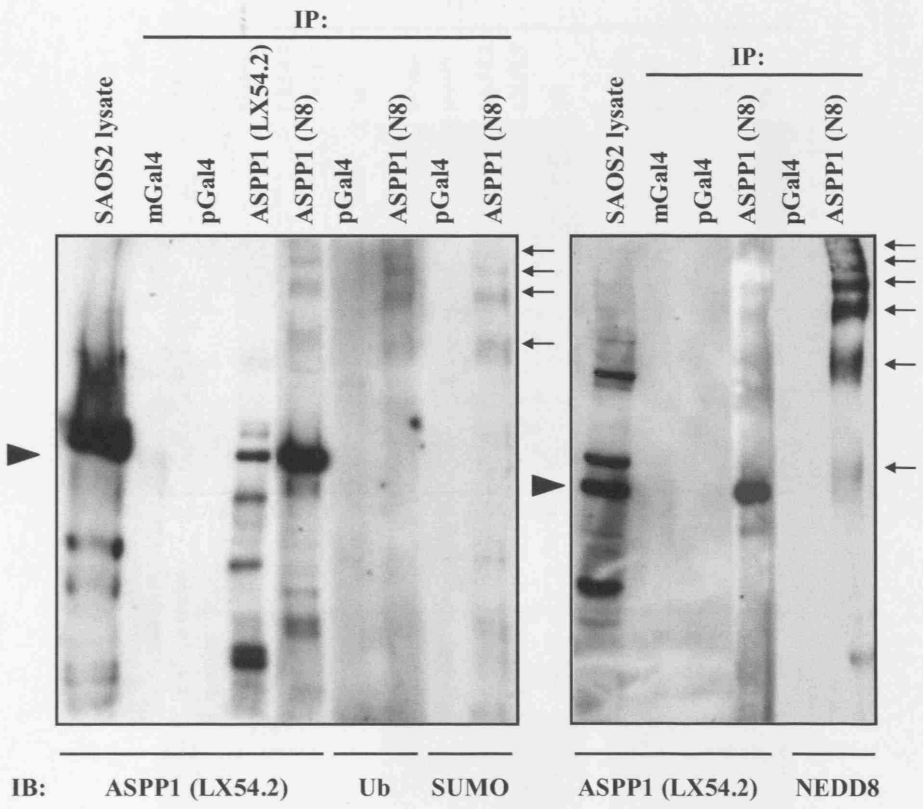
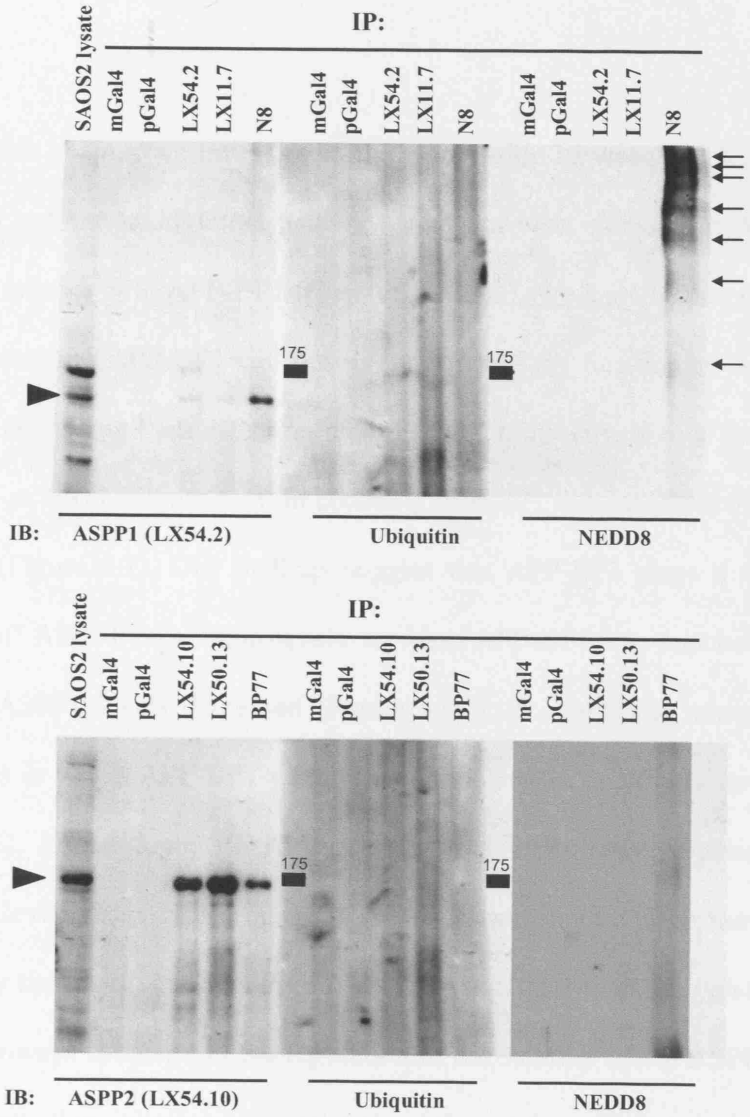


Figure 5.4: ASPP1 is strongly modified *in vivo* compared to ASPP2. A. ASPP1 was immunoprecipitated in Saos-2 cells using N8 or LX54.2 antibodies. Anti-Gal4 Mouse monoclonal or rabbit polyconal antibodies were used as negative controls. ASPP1 expression and ubiquitin-like modifications of ASPP1 were detected by SDS-PAGE/immunoblotting using antibodies against ubiquitin, NEDD8 or SUMO modifications. Black arrow head: full length ASPP1. Arrows: ubiquitin-like modifications. **B.** Immunoprecipitations of endogenous ASPP1 (top panel) or ASPP2 (lower panel) were performed in Saos-2 cells with the indicated antibodies. The lysates were resolved by immunoblotting with antibodies against ASPP1 or ASPP2 and against ubiquitin-like proteins as indicated in the figure. Full-length proteins are shown with black arrowheads and potentially modified proteins with arrows. The 175kDa protein marker is indicated on the figure.

B


IP:
SAOS2 lysate
mGal4
pGal4
LX54.2
LX11.7
N8
mGal4
pGal4
LX54.2
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N8
mGal4
pGal4
LX54.2
LX11.7
N8
175
175
IB:
ASPP1 (LX54.2)
Ubiquitin
NEDD8
IP:
SAOS2 lysate
mGal4
pGal4
LX54.10
LX50.13
BP77
mGal4
pGal4
LX54.10
LX50.13
BP77
mGal4
pGal4
LX54.10
LX50.13
BP77
175
175
IB:
ASPP2 (LX54.10)
Ubiquitin
NEDD8

Figure 5.4

5.3 Discussion

In this chapter we investigated the relationship between the ASPP family of proteins and the neddylation pathway, as it has been shown that ASPP2 can physically interact with APP-BP1 (Chen et al. 2003). We showed that the E1-like activating enzyme APP-BP1 can positively regulate the function of ASPP1 and ASPP2 by enhancing their effect on p53 mediated transactivation of proapoptotic genes such as *PIG3* (Figure 5.1). In contrast, APP-BP1 did not affect the function of iASPP (Figure 5.1). Our findings suggest that APP-BP1 plays a role in the regulation of ASPP1 expression levels, as when APP-BP1 was knocked-down in ts41 cells ASPP1 levels decreased (Figure 5.2C). In contrast to overexpression experiments in which APP-BP1 was able to modulate the activity of both ASPP1 and ASPP2, knock-down of APP-BP1 in ts41 cells only affected ASPP1 expression levels (Figures 5.1 and 5.2C). The discrepancy between these two set of data may be due to artefacts from overexpression. However, it is also possible that the pathways involved in the regulation of the stability of the ASPP proteins are redundant; thus, altering one pathway might not be sufficient to affect their stability. This is corroborated by the fact that ASPP1 levels slowly decrease upon temperature switch in ts41 cells (Figure 5.2C). Finally, at least in the case of ASPP1, the positive regulation exerted by APP-BP1, may be explained by its role in the regulation of ASPP1 expression levels.

We then investigated whether endogenous ASPP1 and ASPP2 could be modified by ubiquitin-like proteins. Our results indicate that ASPP1 is more heavily modified by ubiquitin-like modifications in comparison to ASPP2 (Figure 5.4B). We also found that endogenous ASPP1 was strongly neddylated (Figure 5.4A). Collectively, these data support our previous findings that APP-BP1 is more important for the regulation of ASPP1 expression levels in comparison to ASPP2 (Figure 5.2C). However, we need to further investigate whether the neddylation of ASPP1 is a determinant factor in APP-BP1 mediated regulation of ASPP1 expression levels, as this has not formally been proved. Identifying which residues of ASPP1 are modified by NEDD8 will be essential in order to design ASPP1 mutants which cannot be neddylated and help us understand the role of ASPP1 neddylation in its stability. Importantly, Mdm2 mediated neddylation of p53 has been shown to inhibit its transcriptional activity (Xirodimas et al. 2004). It would be interesting to investigate whether ASPP1 can compete with p53 as a substrate of Mdm2 mediated neddylation, as we would speculate, according to our data, that this may result in the stabilization of ASPP1 and in p53 transcriptional activity being increased and specifically targeted to the promoter of proapoptotic genes.

Furthermore, we found that, although endogenous ASPP1 was strongly neddylated, it was also modified by other ubiquitin-like proteins, such as SUMO and ubiquitin itself (Figure 5.4A). In contrast, under the conditions used, we could not clearly identify endogenous ASPP2 as being modified by ubiquitin-like proteins, although the degradation of ASPP2 has been shown to be regulated by its ubiquitination (Zhu et al. 2005). However, we found that exogenous ASPP2

could be strongly stabilized upon MG132 treatments (data not shown), suggesting that ASPP2 levels are indeed regulated by ubiquitination and proteasomal degradation. Finally, it will be of major interest to understand the role of these various modifications, as they are often involved in the control of the localization or the stability of proteins, and also in the regulation of their functions.

Chapter VI: Final discussion and perspectives

6.1 Overview

In this thesis, we have sought to gain further understanding of the function of the ASPP family of proteins, by exploring their effect on two major pathways: the p53 apoptotic and the Wnt/β-catenin signalling pathways. To date, the ASPPs have essentially been known for their ability to specifically regulate p53 mediated apoptosis (Samuels-Lev et al. 2001; Bergamaschi et al. 2003). However, the ASPPs, and in particular ASPP2, have also been shown to bind to numerous proteins involved in different pathways, largely as a result of their signature domains. The various different ASPP binding partners indicate that this family may be involved in multiple other pathways, in addition to its role in the regulation of p53 mediated apoptosis. Interestingly, some of these proteins, at least in theory, provide a link to the Wnt/β-catenin signalling pathway. ASPP2 binds to APCL, which has been shown to down-regulate β-catenin as efficiently

as its homologue, the adenomatous polyposis coli tumour suppressor (Nakagawa et al. 1998; van Es et al. 1999; Nakagawa et al. 2000a).

Initially, we focused on the interaction between the ASPPs and the APC family of proteins and the consequences that their association have on the function of the ASPPs (Chapter III). We found that both APC and APCL could influence the localization of ASPP1 and ASPP2 and positively regulate their function as regulators of p53 mediated apoptosis. Additionally, several other interacting ASPPs proteins, such as Siah-1, PP1 or even APP-BP1, potentially regulate the activity of β-catenin, mainly by promoting or inhibiting its degradation (Helps et al. 1995; Chen et al. 2003; Chen 2004; Thornton et al. 2006). Therefore, the interaction of the ASPPs with these proteins may affect the stability and the function of β-catenin as a transcription factor. We, therefore, subsequently elected to focus on the effect that the ASPPs, and ASPP2 in particular, have on the function of β-catenin using both cell lines and *in vivo* studies, during embryonic development (Chapter IV). Finally, we also wanted to assess the relationship between the ASPPs and the neddylation pathway (Chapter V). Early results are presented which give some insights into a potential role in the interaction between the ASPPs and APP-BP1.

6.2 ASPP2, a connection between distinct pools of β-catenin

Strong parallels can be made between the ASPPs, in particular ASPP2, and β-catenin, with respect to their function, their localization and their binding partners (Figure 6). For example, both molecules can localize at the plasma membrane, where they interact within the *adherens* junctions. Moreover, they can both interact with APC and APCL, and finally, they execute their function in the nucleus where they play a role in modifying the transcriptional program of cells. These similarities are unusual, for two molecules that ultimately play opposite roles in cellular fate: ASPP2 promotes apoptosis and β-catenin is involved in cellular proliferation and cell survival.

Firstly, we have shown that the function of ASPP2 and ASPP1 during p53 mediated apoptosis can potentially be enhanced by APC and APCL and, additionally, that both APC and APCL can be found in complex with ASPP1 and ASPP2 within cells, thus affecting their localization (Figures 3.2-3; 3.6-7; 6). ASPP1 and ASPP2 colocalize with APC at the extremity of cellular extensions, whereas they colocalize with APCL in a perinuclear region (Figures 3.2; 3.6). It will be interesting to identify precisely how APC and APCL achieve the same effect in regulating ASPP1 and ASPP2 on p53, since the complexes that they form with these proteins are found in different subcellular locations. Neither APC nor APCL seem to induce the accumulation of ASPP1 or ASPP2 in the nucleus

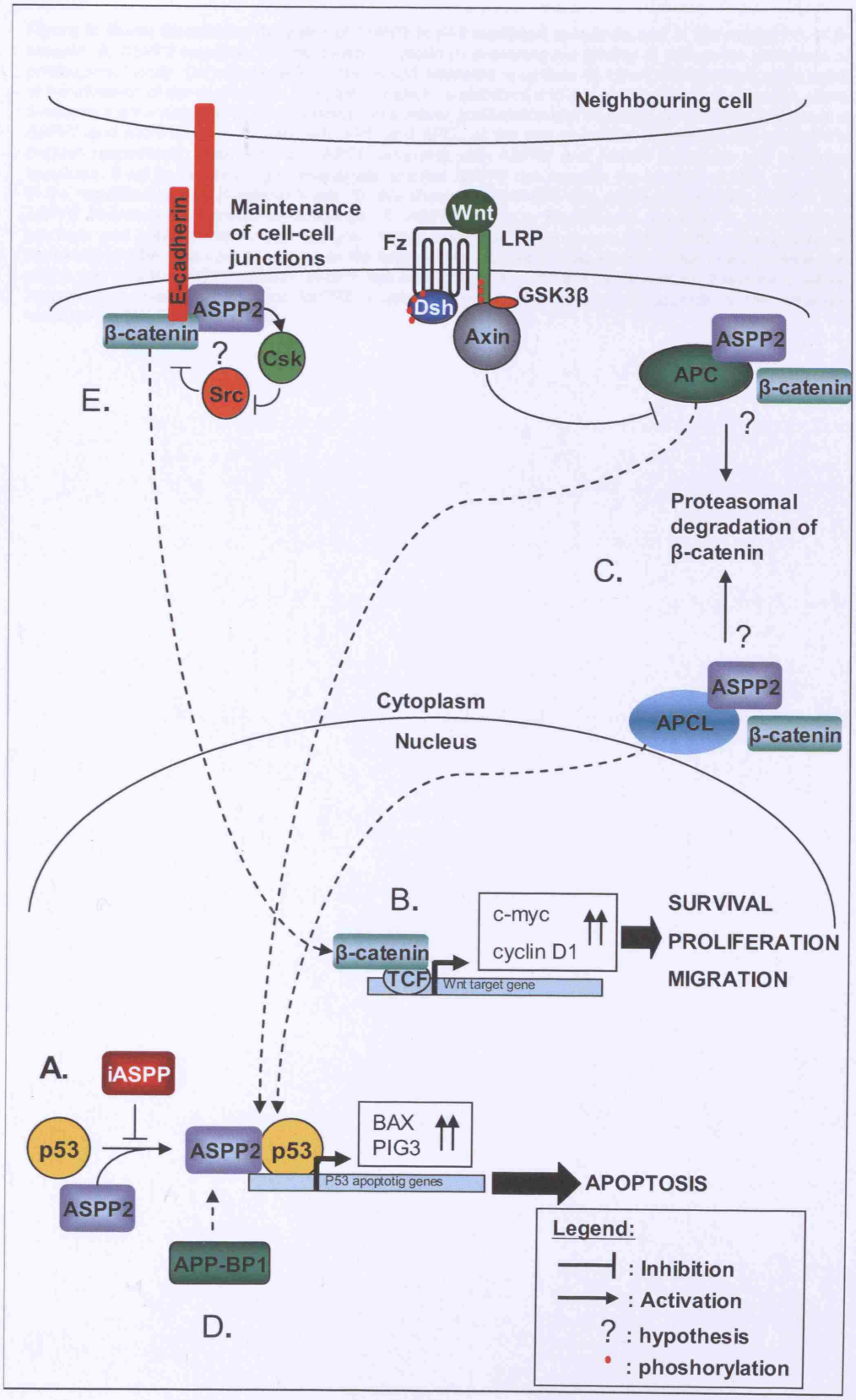
Neighbouring cell
E-cadherin
Maintenance of cell-cell junctions
Wnt
Fz
LRP
GSK3β
Dsh
Axin
ASPP2
β-catenin
Csk
Src
?
E.
ASPP2
APC
β-catenin
?
Proteasomal degradation of β-catenin
C.
?
ASPP2
APCL
β-catenin
Cytoplasm
Nucleus
B.
β-catenin
TCF
Wnt target gene
c-myc
cyclin D1
SURVIVAL
PROLIFERATION
MIGRATION
A.
iASPP
p53
ASPP2
ASPP2
p53
BAX
PIG3
P53 apoptotig genes
APOPTOSIS
APP-BP1
D.
Legend:
: Inhibition
: Activation
? : hypothesis
: phoshorylation

Figure 6

Figure 6: Model illustrating the roles of ASPP2 in p53 mediated apoptosis and in the regulation of β-catenin. **A**. ASPP2 regulates p53 mediated apoptosis by promoting the binding of p53 on the promoters of proapoptotic genes. Conversely iASPP inhibits p53 mediated apoptosis. **B**. Upon Wnt signalling, as a result of the inhibition of the degradation complex, β-catenin is stabilized,and can accumulate in the nucleus where it induces the transcription of genes involved in survival, proliferation and migration. **C**. Our data indicate that ASPP1 and ASPP2 can colocalize with APC and APCL at the end of cellular extensions and around the nucleus respectively. Both APC and APCL synergise with ASPP2 and ASPP1 to induce p53 mediated apoptosis. It will be determining to investigate whether ASPP2 can regulate the function of APC and APCL in the regulation of free β-catenin levels. **D**. We show that APP-BP1 can also synergise with ASPP1 and ASPP2 to induce p53 mediated apoptosis. **E**. ASPP2 binds to β-catenin at the level of the *adherens* junctions and plays a role in their integrity during embryonic development of the CNS. It remains to be answered whether has consequences on the levels of free β-catenin able to translocate to the nucleus and play a role as a transcription factor. dASPP has been shown to positively regulate dCsk, therefore, it will be interesting to investigate whether ASPP2 is able to prevent Src mediated disassembly of the adherens junctions via the activation of Csk.

(Figures 3.2; 3.6). As a result, some other molecular mechanisms must be involved. For instance, It will be interesting, using chromatine immunoprecipitation technics, to test whether APC or APCL induce post-translational modifications of ASPP1 and ASPP2, thereby promoting their binding with p53 on the promoters of proapoptotic genes. It will be essential to understand whether the binding between ASPP2 and APCL or p53 is mutually exclusive or not. The perinuclear complex between ASPP1&2 and APCL needs to be further characterized in order to identify the proteins and subcellular structures that it is associated with. It is not clear yet whether APCL is associated with the actin filaments, the microtubules or the Golgi apparatus and how this regulates its function (Nakagawa et al. 2000b; Jarrett et al. 2001). It will be possible to test whether these structures of the cytoskeleton are required for the function of this complex by inducing their destabilization. Moreover, the complex that APCL forms in order to target β-catenin for degradation by the proteasome has yet to be identified. It probably involves the scaffold protein Axin, which plays an important role in the β-catenin cytoplasmic degradation complex containing APC, as two of the SAMP domains involved in the binding between APC and Axin are retained in APCL (van Es et al. 1999). In contrast, the complex between APC and ASPP1&2 probably forms at the microtubules plus ends, although this remains to be formally shown (Figure 3.6). We currently do not know the specific role of these two different complexes; however, their localization may allow the regulation of specific pools of β-catenin (Figure 6).

Secondly, ASPP2 can bind to β-catenin within the *adherens* junction complex (Figures 4.8A-B). We have yet to demonstrate the role of this interaction, but the disorganization of the neuroepithelium observed in the brains of mouse embryos suggests that ASPP2 could play a role in the maintenance and the integrity of cell-cell junctions (Figures 4.10A; 6). In order to fully understand the role of ASPP2 within the adherens junctions, it will be of particular importance to identify with which molecules ASPP2 interacts directly within the complex, and which domains are required for these interactions. ASPP2 contains several domains involved in protein-protein interactions, such as the Ankyrin repeats and SH3 domains. However, it cannot be excluded that another part of ASPP2 plays a role in the formation of this complex. Interestingly, an Ankyrin containing protein, Ankyrin-G has been shown to bind to the C-terminus of E-cadherin, and to be required, together with β-2-spectrin, for the accumulation of E-cadherin at the lateral membrane in both epithelial cells and early embryo (Kizhatil et al. 2007). This suggests that ASPP2 could bind to this region of E-cadherin through its Ankyrin repeats, thereby promoting the assembly of the *adherens* junctions.

The *Drosophila* homologue, dASPP has recently been identified as a positive regulator of dCsk, resulting in the inhibition of dSrc and loss of epithelial integrity in *Drosophila* wings (Langton et al. 2007). This suggests that the role of ASPP2 in the regulation of epithelial integrity, as shown by the phenotype observed in the eye and the brain of *ASPP2*-null mice, is conserved in its *Drosophila* homologue. Interestingly, Src has been shown to induce the phosphorylation of β-catenin and other components of the adherence junctions

such as N-cadherin and consequently promote the disassembly of the *adherens* junctions (Behrens et al. 1993; Qi et al. 2006). Therefore it will be of major interest to investigate whether ASPP2 can regulate, through Csk and Src, the phosphorylation of these *adherens* junction molecules, thereby regulating the assembly of these complexes (Figure 6). If ASPP2 indeed plays a role in the assembly of, or is important to strengthen the *adherens* junctions, it will be of major interest to understand which underlying mechanisms are involved and whether ASPP2 plays a role in the switch between distinct pools of β-catenin: one contained within the *adherens* junctions and one associated with the β-catenin cytoplasmic destruction complex.

During embryonic development of the CNS, ASPP2 is a major regulator of cell proliferation, cell differentiation and of the organisation of the neuroepithelium (Figures 4.9-10). The role of ASPP2 in the regulation of β-catenin transcriptional activity may explain why the absence of ASPP2 favours proliferation over differentiation. Indeed, some of our data indicates that ASPP2 deficiency leads to the accumulation of active β-catenin in the nucleus (Figures 4.1A, 4.2 and 4.8C); moreover, the role of β-catenin as a transcription factor has already been suggested to be essential for the decision between proliferation and differentiation during CNS development (Chenn and Walsh 2002; Zechner et al. 2003; Woodhead et al. 2006). However, in the overgrown ventricular zones of the brain of ASPP2-null embryos, there was no striking change in the proportion of cells containing nuclear β-catenin in relation to the total number of cells, but rather a dissemination of these cells across the epithelium (Figure 4.10A). As a result, it is hard to conclude whether nuclear β-catenin was the determinant factor

driving cellular proliferation. However, cyclin-D1, one of the transcriptional targets of β-catenin, was more abundant in the neuroepithelium of ASPP2-null mice, suggesting that a rise in cyclin-D1 could be responsible for the over-proliferation phenotype.

However, we have not clearly established which aspect of β-catenin between its function as a transcription factor or its role as a major component of the *adherens* junctions is responsible for the phenotype observed in the CNS of ASPP2-null embryos. The disorganization observed in the neuroepithelium and the eye of ASPP2-null embryos may be as a result of a defect in the *adherens* junctions, as, for instance, N-cadherin has been shown to be essential for cortical organization in the brain and lamination in retinal development (Fu et al. 2006; Kadowaki et al. 2007).

Therefore, we would like to know the answers by further analysing the integrity of the *adherens* junctions in the neuroepithelium of *ASPP2*-null mice. Also, the culture of neuroepithelial cells grown from wild-type and *ASPP2*-null mice will allow a better understanding of the relationship between the role of ASPP2 at the level of the *adherens* junctions and its role in the regulation of β-catenin transcriptional activity.

6.3 More binding partners, a role in ASPP mediated regulation of β-catenin?

We have shown that ASPP1 and ASPP2 can colocalize with APC and APCL and that ASPP2 can regulate β-catenin transcriptional activity (Figure 3.2B; 3.6; 4.1A). It will therefore be interesting to investigate whether ASPP2 or ASPP1 can directly influence the ability of APCL and APC to regulate β-catenin (Figure 6). Several lines of evidence suggest that this could be the case as the ASPPs, in addition to their link to APCL, can interact with a number of proteins involved in the regulation of β-catenin at the level of the cytoplasmic destruction complex. Hence, ASPP1 can interact with the E3 ubiquitin ligase Siah-1, which has been shown to interact with APC and promote the degradation of β-catenin (Liu et al. 2001). Moreover, the binding of ASPP2 to APP-BP1 could affect the neddylation of cullins, which are part of the SCF complex involved in the ubiquitination of β-catenin and one of the substrates of the neddylation pathway (Weissman 2001). As it has been shown that the neddylation of Cullin-1 within the SCF complex catalyses its ubiquitination function, including on β-catenin, it would be interesting to investigate whether ASPP2 could influence this process. Currently our results indicate that APP-BP1 and the neddylation pathway play a role in the stability of ASPP1 (Figure 5.2). Additionally, ASPP1 is heavily neddylated (Figure 5.4A), suggesting that the neddylation of ASPP1 could induce its stabilization and enhance its function. As a result, it will be interesting to

investigate whether this influences the function of ASPP1 in p53 mediated apoptosis. Finally, our result indicating that the binding between ASPP2 and PP1 is important for the regulation of β-catenin modifications, together with the fact that PP1 plays a role in the dephosphorylation of Axin may suggest that ASPP2 plays a role in the regulation of the assembly of the β-catenin cytoplasmic degradation complex (Figure 4.4C; (Luo et al. 2007).

6.4 Concluding remarks and perspectives

This work has contributed to a better understanding of the function of the ASPP family of proteins by characterising their roles in various major cell signalling pathways. The ASPPs appear to be a multifunctional class of proteins that can play various roles depending on where they are located and which proteins they are in complex with. This study has focused on the relationship between the ASPPs and their binding proteins, and how these interactions affect cellular pathways, such as the Wnt signalling pathway and the neddylation pathway. The role of ASPP2 has been studied for the first time during mouse embryonic development, establishing the importance of ASPP2 in the control of cellular proliferation in the CNS and in the architecture of the neuroepithelium. Therefore, it will be critical to understand whether the mechanisms involving ASPP2 in the regulation of cellular proliferation and the maintenance of epithelial integrity are restricted to development of the CNS. Indeed, these aspects of ASPP2 could contribute to its function as a tumour suppressor in normal adult tissues and in cancer. Therefore it will be interesting to investigate whether the tumour suppressor role of ASPP2 is only due to its ability to regulate p53 mediated apoptosis or whether its ability to control the transcriptional activity of β-catenin also play a significant role. Importantly, the discovery that ASPP2 may play a role in epithelial integrity and maintenance of the adherens junctions suggests that it could be determinant in preventing cellular invasion and

metastasis. Indeed, loss of E-cadherin function and loss of adhesion are tightly linked to tumour-cell invasion and metastasis (Christofori and Semb 1999; Hanahan and Weinberg 2000).

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