Investigation of the role of ASPP2 in tumourigenesis

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Declaration

All the work presented in this thesis is the result of my own work unless otherwise stated and does not constitute part of any other thesis. The work herein described was carried out while I was a graduate student at the Ludwig Institute for Cancer Research, under the supervision of Professor Xin Lu.

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Abstract

The skin is the site where two of the most common types of epithelial cancer, basal cell carcinoma (BCC) and squamous cell carcinoma (SCC), arise. In this work, we have investigated how ASPP2, a member of a family of proteins that interact with the p53 family, can affect skin tumourigenesis. ASPP2 is expressed in the squamous epithelia of various organs, localising exclusively in the upper and most differentiated layers. We show here that Balb/c ASPP2-null and heterozygous mice develop spontaneous SCCs. To investigate how the absence of ASPP2 from the epithelial compartment could lead to tumour formation, we analysed ASPP2's relationship with pathways involved in the normal homeostasis of the epithelium, such as p63 and Notch. ΔNp63 is the main p63 isoform expressed in the adult epidermis, and its function is to drive the proliferation of the basal keratinocytes. Aberrant or misplaced activation of ΔNp63 in the epithelium is a known initiating cause for SCC. Consistent with this, ΔNp63 was found to be highly expressed in tumours derived from *ASPP2*-deficient mice. Our results indicate that ASPP2 is important in limiting ΔNp63 expression in the differentiated epithelium, preventing cell proliferation in the upper layers of the skin. This is achieved by antagonising ΔNp63 transcript and protein expression, resulting in a mutually exclusive expression pattern during differentiation of keratinocytes, as well as in epithelial cancer. ASPP2 expression was found reduced or lost in human SCC cell lines and during head and neck cancer progression, reflecting what was observed in *ASPP2*-deficient mice. Overall, our results indicate a possible mechanism by which p63 expression can be regulated in the skin, and provide a new model for the spontaneous formation of SCC *in vivo*. Additionally, we found that ASPP2 can cooperate with and enhance the activity of skin pro-differentiation pathways, such as Notch. In contrast to p63, ASPP2 and Notch1 are co-expressed in the differentiated layers of the squamous epithelium. Moreover,

ASPP2 can interact with components of Notch nuclear transcriptional machinery, and it is shuttled into the nuclear compartment upon activation of Notch pathway. This recruitment results in modulation of Notch transcriptional activity on specific target genes with a differential pattern of binding sites, providing new insights into the understanding of Notch transcriptional regulation.

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Chapter I: Introduction

1.1 Tumourigenesis in brief

The term tumourigenesis describes the process of transformation of normal cells into tumour cells, leading to a group of heterogeneous pathologies known as cancer. Transformed cells are characterized by their capacity to undergo uncontrolled cell divisions and invade the surrounding tissues (Hanahan and Weinberg 2000). Tumour progression is a multi-step process which begins with the acquisition of behavioural changes by a single cell, resulting in growth advantages compared to the normal cell population. These properties are transmitted to the descendent cells, which can undergo further changes and rapidly expand in size and number into the host tissue. Once the tumour is formed, some of the cancer cells can leave the tumour mass, invade the adjacent host tissue and reach the circulatory or lymphatic system, through which they can be transported in new locations of the body and give rise to new tumour foci. These secondary tumours are called metastases (Nowell 1976; Duffy, McGowan et al. 2008). The presence of distant metastases is the main cause of mortality in cancer patients. The crucial changes in cell behaviour which characterise tumour cells have been classified in six main categories, considered the hallmarks of cancer cells. These hallmarks are: self-sufficiency in growth signals, insensitivity to anti-growth signals, limitless replicative potential, evasion of apoptosis, sustained angiogenesis and tissue invasion and metastases (Hanahan and Weinberg 2000). Recently, two additional hallmarks have been added to the list: the capacity of tumour cells to reprogram their energy metabolism and to avoid immune destruction. To facilitate the acquisition of the hallmarks, the tumour has to possess enabling traits such as intrinsic genomic instability and the ability to promote inflammation (Hanahan and Weinberg 2011).

The transition from a normal cell to a tumour phenotype is caused by genetic alterations. These genetic alterations, ascribable to DNA mutations, can be inherited through parental genes (familiar or

hereditary cancer) or can arise during life (non-inherited cancer). Often, cancer originates by a combination of mutations both inherited and non-inherited.

Gene mutations in cells result in tumour initiation when they target genes that are crucially involved in controlling the cell behaviours described before. In general, these genes are divided in two classes: proto-oncogenes and tumour suppressor genes. The proto-oncogenes act as a crucial growth regulators in normal cell division and in cancer are subjected to activating mutations, becoming so called "oncogenes". Conversely, the tumour suppressor genes, which normally act as negative growth regulators, are inactivated by mutations in cancer (Monier 1990; Bishop 1991). Oncogenes are characterised by a dominant phenotype, defined as "gain of function". This means that activating mutations in only one of the two alleles can result in alterations of cellular behaviour (Croce 2008). Tumour suppressor genes instead usually require inactivating mutations targeting both alleles in order to produce phenotypic changes in the cell ("loss of function" phenotype) (Stanbridge 1990). Some tumour suppressor genes, however, have been shown to require the inactivation of just one allele to confer a selective advantage towards tumour growth. This condition is called haploinsufficiency and can to be attributed to a reduction in the gene dosage of the tumour suppressor (Cook and McCaw 2000).

1.2 The p53 family of proteins

1.2.1 Overview

The *p53* gene (*TP53*) is one of the most well-known and extensively studied genes in the human genome, with more than 60,000 publications currently displayed on Pub Med. The popularity of p53 is primarily due its role as a major tumour suppressor (Royds and Iacopetta 2006). P53 was first discovered in 1979 as an interacting protein of the large T-antigen in SV40-transformed cells (DeLeo, Jay et al. 1979; Lane and Crawford 1979; Linzer and Levine 1979), and was initially thought to be an oncoprotein itself (DeLeo, Jay et al. 1979; Rotter 1983). This notion was consequently proved to be

wrong, as p53 is in fact a tumour suppressor, and the most frequently mutated in human cancer (Eliyahu, Michalovitz et al. 1989; Finlay, Hinds et al. 1989). It is now recognised that in about 50% of all human tumours, a key step for the development of malignancies is the direct inactivation of wild type p53, via mutations, deletions, or interactions with viral proteins. In the remaining half of malignancies, indirect forms of p53 deregulation are likely to occur, such as inactivation of p53 activators and downstream targets or activation of p53 inhibitors. The importance of p53 inactivation for cancer cells arises from its pivotal roles in inducing the blockade of cell proliferation and preventing the generation of genetically altered cells. Fundamentally, p53 can integrate different stress signals, such as DNA damage or oncogenic stress, into a response which ranges from a transient cell-cycle arrest, to allow DNA repair, to a more definitive cell death via apoptosis or senescence (Levine 1997; Lowe 1999). These functions make p53 probably the most important controller of genomic integrity (Lane 1992). During the past decade numerous studies have identified additional p53 functions, showing the involvement of p53 in processes such as autophagy, oxidative stress, regulation of metabolism, embryo implantation and angiogenesis (Crighton, Wilkinson et al. 2006; Teodoro, Parker et al. 2006; Bensaad and Vousden 2007; Hu, Feng et al. 2007).

Almost twenty years after p53 was first described, the other two members of the p53 family of proteins, p63 and p73, were discovered (Kaghad, Bonnet et al. 1997; Schmale and Bamberger 1997; Osada, Ohba et al. 1998; Yang, Kaghad et al. 1998). The two homologues share high sequence identity with p53, which is reflected in some redundant functions in their regulation of gene expression (Levrero, De Laurenzi et al. 2000). Similar to p53, protein levels of p63 and p73 also increase upon treatment with DNA damaging agents (Katoh, Aisaki et al. 2000; Okada, Osada et al. 2002; Bergamaschi, Gasco et al. 2003). Despite all these similarities, p63 and p73 can be activated by distinct mechanisms and are implicated in several p53-independent pathways, playing an important role in processes such development and differentiation (Levrero, De Laurenzi et al. 2000).

1.2.2 Members and structure

The three p53 family members have a high degree of sequence similarity and domain conservation. The hallmark of their structure is the presence of three main domains: an acidic transactivation domain (TA) at the N-terminal, a core DNA-binding domain (DBD) and an oligomerisation domain at the C-terminal (OD) (Figure 1.1). The highest sequence identity among the three family members resides in the DNA-binding domain (around 60%), suggesting p53, p63 and p73 can bind to the same DNA sequences and drive the transcription of the same set of genes. DBD is also the region where more than 95% of all the tumour-associated p53 mutations can be found. The oligomerisation domain of p53 can be post-translationally modified in different ways, including phosphorylation, methylation, and ubiquitylation, resulting in a tight regulation of its function and stability. Unlike p53, both proteins p63 and p73 have an additional C-terminal tail containing a sterile alpha motif (SAM) domain, important for protein-protein interactions and often found in proteins involved in the regulation of development. Moreover, all three genes can encode two primary transcripts because of the presence of two distinct promoters in their sequence (P1 and P2). The P1 promoter gives rise to full proteins, containing the TA, DNA-binding and oligomerisation domain (FLp53, TAp63 and TAp73). Alternatively, when the transcription is initiated from the intronic P2 promoter, N-terminally truncated proteins are produced (Δ133p53, ΔNp63, ΔNp73). In both p53 and p73, further splicing events and alternative initiation of translation can take place generating additional ΔN isoforms (Δ40p53, Δex2p73, Δex2/3p73 and ΔNp73). More complexity is finally derived by alternative splicing of the C-terminal exons. The different variants derived by this event are indicated by the Greek letters α, β, γ, δ, ε, θ, ζ and η. Concerning p53 and p63, for each of their TA and ΔN isoforms three variants can be generated. For p73, eight variants are described (Figure 1.1).

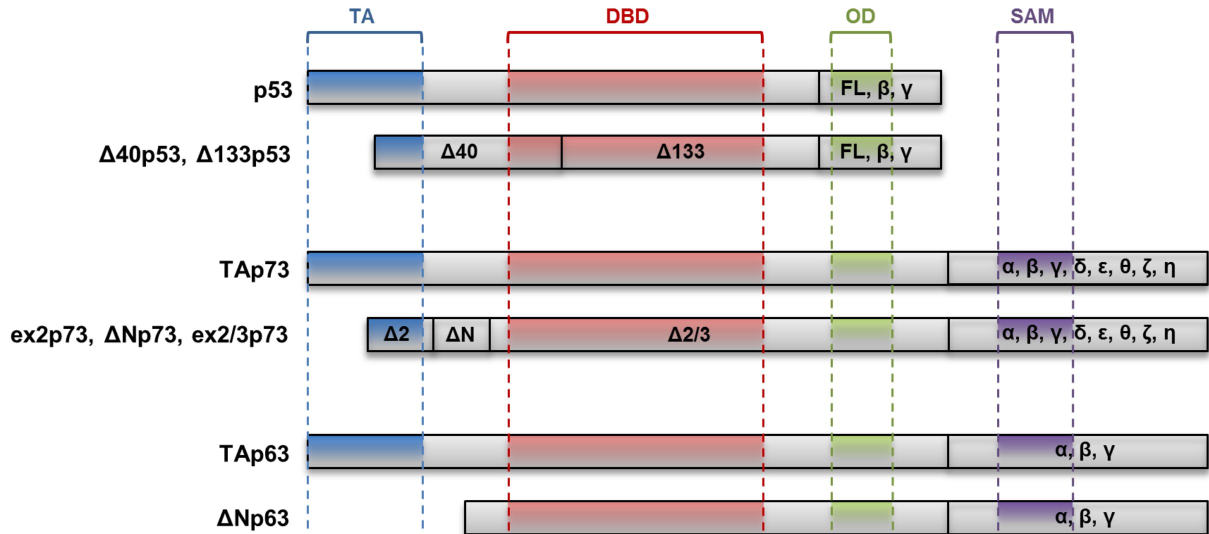


Figure 1.1 Structure of the p53 family of proteins

The p53 family consists of three members, p53, p63 and p73, with a conserved modular structure organised from N-terminal to C-terminal with a transactivation domain (TA), a DNA binding domain (DBD) and oligodimerisation domain (OD). In addition, p73 and p63 possess in their longer C-terminal a sterile alpha motif (SAM), a domain implicated in protein-protein interactions. N-terminal truncated proteins Δ40p53, Δ133p53, ex2p73, ΔNp73, ex2/3p73 and ΔNp63, can be produced as a result of alternative initiation of translation and splicing events. Alternative splicing of the C-terminal adds more complexity generating different isoforms named with Greek letters. There are two for p53 (β, γ), eight for p73 (α, β, γ, δ, ε, θ, ζ, η) and three for p63 (α, β, γ). Because the ΔN isoforms lack of the initial transactivation domain, they are considered functionally different from their full-length counterparts and can behave as dominant-negative. Consistent with this, our current understanding indicates that in cancer TA isoforms are pro-apoptotic and tumour suppressive while ΔN isoforms are anti-apoptotic and therefore oncogenic (Stiewe 2007).

1.2.3 The p53 response

As p53 plays an important role in regulating cell proliferation and death, its activation has to be tightly controlled. The selectivity of p53 response is given by a combination of several factors, starting with the nature of the activating stress. This can be translated into different patterns of post-translational modifications on the p53 protein, with consequent recruitment of characteristic binding partners and induction of a precise combination of target genes. As a result of this multi-step process, p53 can facilitate the transient adaption of cells to stressful conditions (summarised in Figure 1.2). The critical event during activation of p53 pathway is the stabilisation and accumulation of its protein levels. In normal, unstressed conditions, p53 protein levels are kept low mainly by the intervention of two crucial regulators, E3-ubiquitin ligase MDM2 and its related protein MDM4 (also called MDMX). MDM2 alone or in complex with MDM4 promotes ubiquitylation of p53 and targets it to proteasomal degradation Additionally, both MDM2 and MDM4 can inhibit p53 activity by binding it to the amino-terminal region and blocking p53 transactivation domain (Oliner, Pietenpol et al. 1993; Haupt, Maya et al. 1997; Honda, Tanaka et al. 1997; Linares, Hengstermann et al. 2003). Different stress signals, such as DNA damage, induce post-translational modifications (mainly phosphorylation) of p53 and its regulators MDM2/MDM4, with the effect of disrupting their binding and preventing the capacity of MDM2/MDM4 to inhibit and degrade p53. This would ultimately lead to p53 protein accumulation and consequent p53-mediated transcriptional activity (Lavin and Gueven 2006). P53 main effects are mediated by its direct nuclear transcriptional activity, but it can also be involved in transcription-independent cytosolic functions via protein-protein interactions, as for apoptosis induction through binding with Bcl-2 family members (Green and Kroemer 2009). P53, as a transcription factor, can recognize consensus motifs which are present in the promoters of numerous genes, as it was estimated that p53 can bind to at least 542 different loci (Wei, Wu et al. 2006). Affinity for p53 binding can however vary across all the p53 binding sites. Thus, there are genes whose promoters contain high-affinity p53-binding sites, like *p21*^{WAF1}, *MDM2* and *PUMA*, and others with low-affinity sites, as for *Bax* (Chen, Ko et al. 1996). Further selectivity to p53

transcriptional activity is conferred by the nature of its post-translational modifications, as shown for phosphorylation of p53 at Ser46, which confers more effective transactivation of *p53AIP1* gene, than *p21^{WAF1}* (Oda, Arakawa et al. 2000). Finally, interactions with transcriptional coactivators also contribute to direct p53 on specific targets and prevent its binding on others, defining a precise cellular response. Examples of regulators are the E2F and the ASPP family of proteins, which are important in directing p53-decision on inducing cell cycle arrest or apoptosis (Braithwaite, Del Sal et al. 2006). As a consequence of its numerous gene targets, p53 is involved in many cellular responses, among which the best characterized so far are cell cycle arrest and apoptosis.

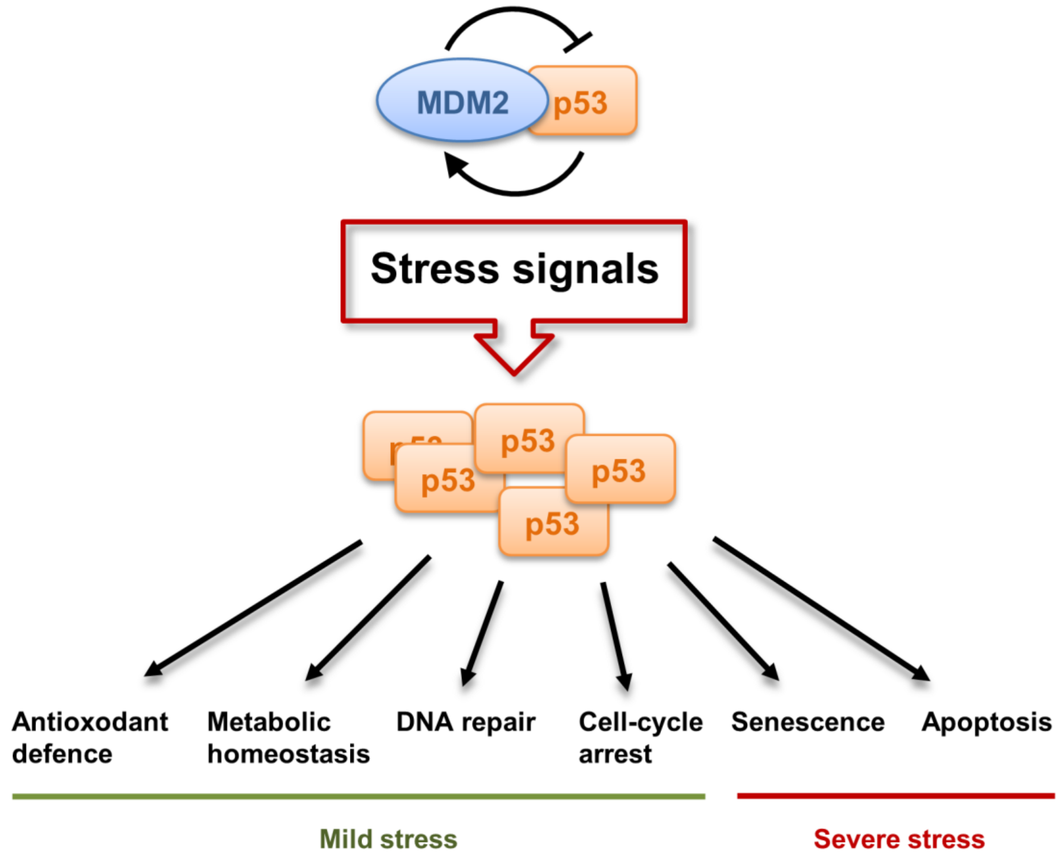


Figure 1.2 Simplistic diagram exemplifying the p53 response
In normal physiological conditions the levels of p53 are maintained low by the p53-MDM2 feedback loop. The release of MDM2 inhibition caused by various stress signals leads to an increase and stabilization of p53 protein levels. The activity of p53 is also increased by post-translational modifications inducing various cellular responses. The nature of the activating stress dictates the severity of the response. Therefore mild stress can produce a temporary growth arrest, functional for dealing with the repair of the stress-inducing damage, while more severe stress can cause more dramatic and irreversible responses, such as senescence and apoptosis.

1.2.3.1 Cell cycle arrest

Upon activation by low level of stress caused by DNA-damaging agents, all the members of p53 family can induce a program of temporary cell cycle arrest (at both G1 and G2 stages) and DNA repair preventing the propagation of oncogenic mutations. The p53 transcriptional targets responsible for consequent cell cycle arrest are $p21^{WAF1}$, crucial inhibitor of cyclin-dependent kinases (CDKs), and others such as *Reprimo*, *14-3-3σ* and *GADD45* (el-Deiry, Tokino et al. 1993; Hermeking, Lengauer et al. 1997; Zhan, Antinore et al. 1999; Ohki, Nemoto et al. 2000). Inhibition of CDKs and their corresponding cyclin proteins is a critical event for blocking cell cycle progression. Similarly to p53, p63 and p73 can be induced by DNA-damaging drugs and activate transcription of cell cycle related genes such as $p21^{WAF1}$ and *GADD45* (Shimada, Kato et al. 1999; Levrero, De Laurenzi et al. 2000).

1.2.3.2 Apoptosis

Depending on the level of the initial stress, p53 activation can lead to the elimination of irreparably damaged cells by apoptosis. Apoptosis, a type of programmed cell death, can be generated by following two main ways: an extrinsic pathway, coming from activation of cell-membrane receptors, and an intrinsic pathway dependent on stress conditions, such as DNA damage. Both processes culminate with the activation of the aspartate-specific cysteine proteases (caspases) that mediate cell death.

Acting as a transcriptional factor, p53 can regulate the expression of genes involved in both pathways (Haupt, Berger et al. 2003). For instance, p53 can activate the extrinsic apoptotic pathway by inducing the expression of genes coding for death receptor Fas, the TRAIL receptor DR5 and another trans-membrane protein called PERP (Wu, Burns et al. 1997; Muller, Wilder et al. 1998; Attardi, Reczek et al. 2000).

In the intrinsic pathway p53 can affect the transcription of many components of the Bcl-2 family, which have the central role of regulating the release of cytochrome c from the mitochondria, allowing the formation of the apoptosome in the final step of the apoptotic process. Bcl-2 proteins can be divided into anti-apoptotic factors, such as Bcl-2 itself and Bcl-X_L, and pro-apoptotic components such as Bax, NOXA and PUMA. P53 activation leads to the transcriptional repression of the anti-apoptotic genes *Bcl-2* and *Bcl-X_L* and promotes the expression of the pro-apoptotic genes *Bax*, *Bak*, *NOXA* and *PUMA* (Oda, Ohki et al. 2000; Ryan, Phillips et al. 2001; Yu, Zhang et al. 2001). The role of p53 in the intrinsic apoptotic pathway is therefore to affect the balance within the Bcl-2 proteins, shifting it towards pro-apoptotic effects. Further down in the apoptotic pathway, transcription of *APAF-1*, a critical component of the apoptosome, can also be directly induced by p53 (Robles, Bemmels et al. 2001). Additionally, cytoplasmic p53 can promote apoptosis in a transcriptional-independent manner by direct interaction with Bcl-2, Bcl-X_L and Bak at the mitochondria, inducing mitochondrial outer-membrane permeabilisation (MOMP) (Green and Kroemer 2009). Given their similarity in the DNA-binding sequence, p53 family members p63 and p73 have been also implicated in transcriptional activation of p53-induced pro-apoptotic genes, although with some differences in selectivity and degree of activation (Jost, Marin et al. 1997; Yang, Kaghad et al. 1998). The two proteins have been also shown to be required for p53 to transactivate its pro-apoptotic target genes, since combined loss of p63 and p73 resulted in the failure of cells containing functional p53 to undergo apoptosis in response to DNA damage (Flores, Tsai et al. 2002). While no transcription-independent mechanism for p63 induced-apoptosis has been identified to date, recent evidence suggests that p73 can induce apoptosis by the mitochondrial pathways (Melino, Bernassola et al. 2004; Sayan, Sayan et al. 2008). A separate discussion must be reserved for the ΔN isoforms of the p53 family, which are believed to inhibit the transcriptional activity of their full-length counterparts by competing for the same DNA binding sites, or physically interacting with and sequestering them, resulting in anti-apoptotic effects (Yang, Kaghad et al. 1998).

1.2.4 The p53 family members in tumourigenesis

Failure of the apoptotic program is one of the hallmarks of tumourigenesis, leading to the uncontrolled proliferation of genetically mutated cells (Hanahan and Weinberg 2011). Because of its pivotal role in inducing apoptosis, p53 is considered a major tumour suppressor gene with the highest frequency of genetic alterations in all human cancers (Hollstein, Sidransky et al. 1991; Hollstein, Shomer et al. 1996). Emerging evidence suggests that p63 and p73 can also exert tumour-suppressive functions by mediating the apoptotic response in various cellular contexts.

1.2.4.1 p53

The role of p53 in tumour suppression is corroborated by several lines of evidence. First, human individuals carrying *TP53* germline mutations are affected by Li-Fraumeni syndrome, characterized by early susceptibility to different types of cancers (Malkin, Li et al. 1990; Srivastava, Zou et al. 1990). Second, *Trp53* (the mouse *p53* gene) knockout mice develop spontaneous tumours with high frequency (Donehower, Harvey et al. 1992). In human tumours, p53 is typically inactivated by gene deletion, mutation, or overexpression of the p53-ubiquitin ligase MDM2 (Vousden and Lu 2002). Mutations on the *p53* gene can be found in half of all human tumours, with more than 95% of mutations occurring in the central region of the gene, which is responsible for sequence-specific DNA binding. Such p53 mutants can promote cancer onset by dominant-negative inactivation of the endogenous wild type p53, as well as through oncogenic gain-of-function activities (Brosh and Rotter 2009). P53's ability to induce apoptosis still represents the fundamental process by which p53 acts as a tumour suppressor (Ryan and Vousden 1998). Recently, however, a new study provided insights into a different mechanism by which p53 can function as a tumour suppressor. In breast cancer, loss of p53 can favour symmetric cell division, leading to an increase of the cancer stem cell population (CSCs) and therefore promoting tumourigenesis (Cicalese, Bonizzi et al. 2009).

1.2.4.2 p63 and p73

P63 and p73 are rarely mutated in human cancers. However, they were found to be abnormally expressed in different types of tumours (Muller, Schleithoff et al. 2006). The dominant-negative isoforms ΔNp63 and ΔNp73 are normally up-regulated in cancer. For instance, ΔNp63 was found frequently over-expressed in squamous cell carcinoma of the head and neck, cervix and lung (Hibi, Trink et al. 2000; Wang, Chen et al. 2001; Massion, Taflan et al. 2003). Similarly, aberrant expression of ΔNp73 was detected in gliomas and colon carcinoma (Dominguez, Garcia et al. 2006; Wager, Guilhot et al. 2006). In contrast, TAp63 and TAp73 tend to be lost or down-regulated in human tumours (Corn, Kuerbitz et al. 1999; Urist, Di Como et al. 2002). Instead of being subjected to inactivating mutations in their coding sequence, as for p53, TAp63 and TAp73 function in tumours can be blocked by sequestration in transcriptionally inactive complexes with mutant p53 or the ΔN isoforms (Gaiddon, Lokshin et al. 2001; Moll and Slade 2004). One example is TAp73 inactivation by binding with ΔNp63 in squamous cell carcinoma (Rocco, Leong et al. 2006). *In vivo* studies in mutant mouse models for p63 and p73 produced contrasting results. Two independent studies showed that $p73^{-/-}$ and $p63^{+/-}$ mutant mice are not tumour prone, and strains doubly heterozygous for both *p53* and *p63* genes develop fewer tumours than single $p53^{+/-}$ mice (Yang, Walker et al. 2000; Keyes, Vogel et al. 2006). However, another study showed that $p73^{-/-}$ and $p63^{+/-}$ mice did develop spontaneous tumours, and that double heterozygous $p53^{+/-}$ $p63^{+/-}$ and $p53^{+/-}$ $p73^{+/-}$ have greater tumour incidence and metastasis relative to the single $p53^{+/-}$ mutants (Flores, Sengupta et al. 2005). Additionally, mutant mice selectively depleted of the TAp73 isoform, but not ΔNp73, displayed a predisposition to tumour formation (Tomasini, Tsuchihara et al. 2008) suggesting TAp73 isoforms have tumour suppressive functions, as for p53. Future clarifications on the exact role of p63 and p73 in cancer-related processes might therefore come from studies aimed at resolving the distinct effect of each of the p63 and p73 isoforms.

1.2.5 The p53 family members in development and differentiation

Differentiation is a physiological process which allows cells to switch off a proliferative program while preserving their cellular integrity and function. As the p53 proteins are master regulators in mediating termination of cell proliferation via induction of apoptosis, senescence and cell-cycle arrest, their role in differentiation and development has been investigated. Recent evidence supports the idea that p53/p63/p73 can be activated by stimuli other than stress signals, and can regulate the expression of genes involved in diverse cellular processes like cell adhesion, motility and angiogenesis, suggesting they might be important to maintain normal cell function (Riley, Sontag et al. 2008). Despite their structural similarity, the p53 family of proteins appear to have unique roles in mouse and human development. Major contributions in this area come from the phenotypical analysis of the *p53*-, *p63*- and *p73*-null mice. Both *p63*- and *p73*-null mice have profound developmental defects, in contrast with *p53*-deficient mice, whose anomalies are almost exclusively due to the spontaneous cancer formation (see summary in Table 1).

1.2.5.1 p53

P53$^{-/-}$ mice are viable and generally develop normally. Around 20-30% of females embryos of certain strains however develop exencephaly, a condition resulting from neural tube closure defects. A similar phenotype has been observed for transgenic embryos lacking of components of mitochondrial death pathways, indicating that the overproduction on neural tissue could be due to the absence of apoptosis in the progenitor cell population (Armstrong, Kaufman et al. 1995; Sah, Attardi et al. 1995). *In utero* exposure of *p53*-null embryos to ionizing radiation also shows that p53 has a role in reducing the rate of birth defects. Upon ionizing irradiation, a higher 70% incidence of developmental abnormalities and a lower 7% incidence of death was observed in *p53*-null embryos, compared to a 20% incidence of abnormalities and 60% death rate in wild-type mice (Norimura, Nomoto et al.

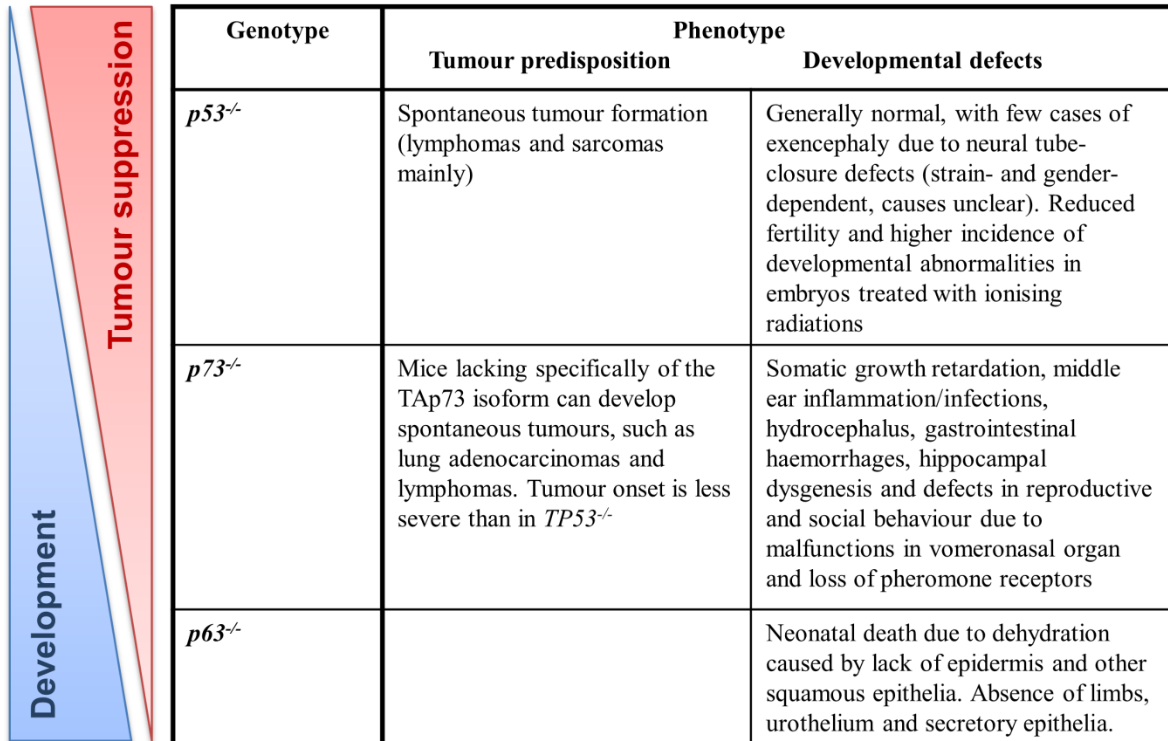
1996). Again, p53-mediated apoptosis in response to DNA-damage is the mechanism causing the mortality in irradiated wild-type embryos, leading their early death and preventing birth of individuals with congenital abnormalities. Other studies also show that *p53*-null animals, both males and females, have reduced fertility. In females this is due to low levels of LIF, a protein important for embryo implantation, whose expression is normally induced by p53, while in males, the abnormality seems to be linked to an higher number of multinucleated giant cells found within the testis, believed to be a result of an inability to complete meiosis (Rotter, Schwartz et al. 1993; Hu, Feng et al. 2007).

1.2.5.2 p63 and p73

P63$^{-/-}$ mice have major developmental abnormalities such as the absence or truncation of limbs, craniofacial malformations and failure to develop skin and other epithelial tissues (Mills, Zheng et al. 1999; Yang, Schweitzer et al. 1999). Born alive, these mice die in few hours from dehydration. Consistent with this severe phenotype in mice, six rare autosomal dominant developmental disorders found in humans seem be caused by germ line point mutations in *p63* gene, including EEC (Ectrodactyly Ectodermal dysplasia–Clefting syndrome) (Celli, Duijf et al. 1999). Similarly to the *p63*$^{-/-}$ mice, patients affected by EEC display limb and craniofacial abnormalities and severe skin defects. Functional studies have shown that p63 and its ΔN isoforms in particular are fundamental for the maintenance of the regenerative cell population, and therefore for the capacity of the epithelium to develop and differentiate (Koster, Kim et al. 2004; Candi, Rufini et al. 2006). Taken together, these data establish a fundamental role for p63 in epithelial development.

P73$^{-/-}$ mice have also developmental defects, but they survive into adulthood. The range of abnormalities observed here clearly differs from those displayed by the *p63*$^{-/-}$ mouse, suggesting p73 has distinct developmental roles. *P73*$^{-/-}$ mice have neurological defects, including hippocampal dysgenesis and hydrocephalus (from excessive secretion of cerebrospinal fluid by the choroid

plexus), abnormalities in the pheromonal sensing pathway and inflammatory defects caused by hypersecretion of mucus by the respiratory epithelium (Yang, Walker et al. 2000). This mouse model demonstrates that p73 plays an important role in neurogenesis, pheromonal sensing and in normal fluid secretion in both choroid plexus and respiratory mucosa. Additional *in vitro* studies showed that p73 expression (TAp73 isoform) in neuroblastoma (undifferentiated neuronal cells) can induce neuronal differentiation markers. Accumulation of TAp73 itself can also be detected upon spontaneous or retinoic acid-induced differentiation of neuroblastoma cells (Kovalev, Marchenko et al. 1998; De Laurenzi, Raschella et al. 2000). The ΔNp73 isoform was also shown to play a role in developing neurons by opposing the apoptotic functions of TAp63 and p53 (Pozniak, Radinovic et al. 2000). These experiments confirmed that p73 is deeply involved in neuronal differentiation, as p63 is for epithelial differentiation. However, unlike *p63*, no germ line mutations on *p73* gene have been associated with any human genetic disease to date.



Genotype	Phenotype: Tumour predisposition	Phenotype: Developmental defects
***p53**$^{-/-}$*	Spontaneous tumour formation (lymphomas and sarcomas mainly)	Generally normal, with few cases of exencephaly due to neural tube-closure defects (strain- and gender-dependent, causes unclear). Reduced fertility and higher incidence of developmental abnormalities in embryos treated with ionising radiations
***p73**$^{-/-}$*	Mice lacking specifically of the TAp73 isoform can develop spontaneous tumours, such as lung adenocarcinomas and lymphomas. Tumour onset is less severe than in *TP53*$^{-/-}$	Somatic growth retardation, middle ear inflammation/infections, hydrocephalus, gastrointestinal haemorrhages, hippocampal dysgenesis and defects in reproductive and social behaviour due to malfunctions in vomeronasal organ and loss of pheromone receptors
***p63**$^{-/-}$*		Neonatal death due to dehydration caused by lack of epidermis and other squamous epithelia. Absence of limbs, urothelium and secretory epithelia.

Table 1 Summary of the phenotypical characteristics of *p53*-, *p73*- and *p63*-null mice

1.3 The ASPP family of proteins: new regulators of p53, p63 and p73

1.3.1 Overview

The ASPP (Apoptosis Stimulating Protein of p53) proteins have been identified and subsequently characterised as important regulators of the p53 family members. Interactions with other proteins, as well as post-translational modifications, are one of the ways by which p53, p63 and p73 can differentiate among the variety of diverse cellular functions they have to accomplish. In particular, one of the common features of the p53-binding proteins appears to be in dictating whether p53 induces a cell cycle arrest or apoptosis. As their name suggests, the ASPP proteins specifically promote p53-dependent apoptosis, but not cell cycle arrest. The ASPP family consists of three members, ASPP1, ASPP2 and iASPP (inhibitory ASPP). ASPP2 was the first of the family members to be characterised, and it was originally identified as an interactor of p53 (mouse residues 73-390 used as bait) in a yeast two-hybrid screen of a transformed B cell cDNA library (Iwabuchi, Bartel et al. 1994). The newly identified p53-interacting protein was called 53BP2, and consisted of only the last 529 amino acids of the full length ASPP2. 53BP2 was also identified as a Bcl-2 interacting protein in another yeast two-hybrid screen, this time of a human Epstein-Barr virus-transformed B cell cDNA library. Subsequently *53BP2* cDNA was cloned and found to encode a protein of 1005 amino acids, which was renamed BBP, as Bcl-2 Binding Protein (Naumovski and Cleary 1996). The full-length of ASPP2 however, was only identified few years later as being 1128 amino acids long (Samuels-Lev, O'Connor et al. 2001). Currently we know that ASPP2 can exit as two splice variants derived from alternative splicing of exon3, called 53BP2S (BBP) or 53BP2L (ASPP2) which are 1005 and 1128 amino acids long respectively (Takahashi, Kobayashi et al. 2004). The second member of the ASPPs, named ASPP1, was initially identified as an N-terminal truncated form of 948 amino acids (KIAA0771), with high C-terminal homology to ASPP2 (Nagase, Ishikawa et al. 1998). That original sequence was further extended to 1091 amino acids, to obtain the complete full length

ASPP1 (Samuels-Lev, O'Connor et al. 2001). Finally, iASPP was first reported as an inhibitor of RelA/p65 (RAI), just 351 amino acids long (Yang, Hori et al. 1999), but was later extended to 828 amino acids, the current known size of iASPP (Slee, Gillotin et al. 2004). The corresponding protein in *C. elegans* was found to inhibit p53-dependent apoptosis, and since this organism does not express RelA/p65, the newly discovered protein was then called iASPP for its inhibitory function (Bergamaschi, Samuels et al. 2003).

1.3.2 Members and structure

ASPP1, ASPP2 and iASPP are encoded by three different genes positioned on three different chromosomes, respectively 14q32.33, 1q42.1 and 19q13.32-3. To date, only ASPP2 and iASPP possess known splicing variants, called BBP (1005 amino acids long) for ASPP2 and RAI (403 amino acids long) for iASPP. The family name ASPP also stands for Ankyrin repeats, SH3 domain and Proline-rich region containing-Protein, because of the presence of these three domains at the C-terminal of all the family members (Figure 1.3). The C-terminal is also the most conserved region among the three proteins and the binding site for several interaction partners, including p53, RelA/p65 and Bcl-2 (Gorina and Pavletich 1996; Naumovski and Cleary 1996; Yang, Hori et al. 1999). The N-terminal is only conserved in ASPP1 and ASPP2 and seems to have a role in determining the cellular localisation of the two proteins. ASPP1 and ASPP2 are mainly cytoplasmic, but when deprived of their N-terminal, the remaining C-terminal, which contains a nuclear localisation signal (Sachdev, Hoffmann et al. 1998), localises to the nucleus (Samuels-Lev et al., 2001). From an evolutionary point of view, iASPP is the most conserved member of the ASPP family, since an orthologue of the human iASPP can be found in *C. elegans* (Bergamaschi, Samuels et al. 2003). ASPP1 and ASPP2, which can be found in mammals, are believed to be derived from iASPP during evolution through the species.

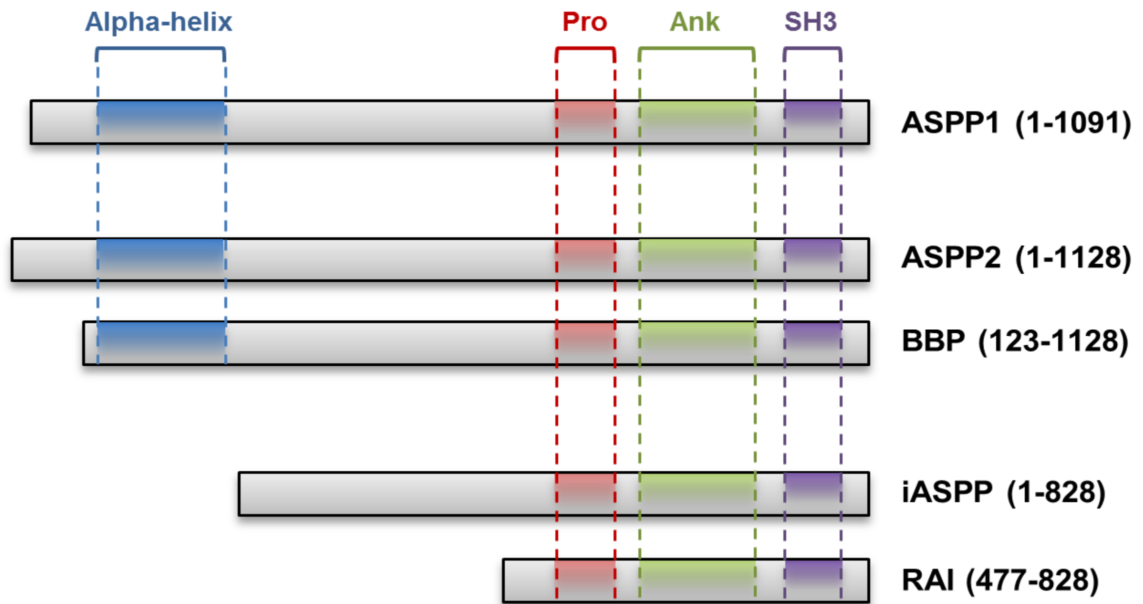


Figure 1.3 Structure of the ASPP family members
The ASPP family of protein consists of three family members: ASPP1, ASPP2 and iASPP. ASPP2 and iASPP are shown with their splice variants, respectively named BBP (Bcl-2 binding protein) and RAI (RelA/p65 inhibitor). The figure shows the length of the proteins and the common structural elements, highlighted with different colours. Pro stands for proline-rich region, Ank for ankyrin repeats and SH3 for SH3 domain.

1.3.3 Functions

The best characterised function of the ASPPs is their ability to regulate apoptosis by acting on the p53 family of proteins. ASPP1 and ASPP2 are able to enhance the capacity of p53, p63 and p73 to induce apoptosis, but not cell cycle arrest (Samuels-Lev, O'Connor et al. 2001; Bergamaschi, Samuels et al. 2004). iASPP, the inhibitory member of the family, has opposite effects, thus allowing cell proliferation to occur (Bergamaschi, Samuels et al. 2003). RAI, the shorter isoform of iASPP, has an even stronger inhibitory effect on p53-mediated apoptosis (Bergamaschi, Samuels et al. 2003) . This might be due to iASPP's predominantly cytoplasmic localisation as opposed to the almost exclusive nuclear localisation of RAI. This also suggests that the additional N-terminal portion of iASPP might represent a regulatory domain involved in its retention in the cytoplasm. ASPP1 and ASPP2's mode of action consists of selectively stimulating p53, p63 and p73 binding to promoters of pro-apoptotic genes, such as *Bax*, *PUMA* and *PIG3* (p53-induced gene 3), resulting in their transactivation, but not to promoters of cell cycle arrest, such as *CDKN1A* or *MDM2* (Samuels-Lev, O'Connor et al. 2001; Bergamaschi, Samuels et al. 2004) (Figure 1.4). The precise mechanism by which this occurs has not been completely clarified. However, ASPP1 and ASPP2 have been found in complexes with p53 on promoters of pro-apoptotic genes by ChIP analysis (Samuels-Lev, O'Connor et al. 2001), and the crystal structure of the C-terminal of ASPP2 (containing the third and fourth ankyrin repeats and the SH3 domain) bound to the DNA core binding domain of p53 has also been solved (Gorina and Pavletich 1996). The physical presence of the ASPPs, in complex with p53, might thus induce conformational changes or recruitment of other factors, resulting in p53 binding to low-affinity sites. Although the C-terminal of the ASPPs seems to be the only region involved in the interaction with p53, the N-terminals of ASPP1 and ASPP2 were also shown to participate in stimulating p53-dependent apoptosis, and were required for maximal transactivation activity (Samuels-Lev, O'Connor et al. 2001). Additionally, a recent study has demonstrated that the ASPP2 N-terminal has an ubiquitin-like structure which can mediate its interaction with other components of the apoptotic network, providing a further mechanism by which ASPP2 could regulate apoptosis

(Tidow, Andreeva et al. 2007). As this domain is the main differentiator between pro-apoptotic (ASPP1 and ASPP2) and anti-apoptotic (iASPP) members of the ASPP family, its contribution is believed to be crucial in promoting apoptosis. Consistent with this, the crystal structure of the C-terminal of iASPP with the DNA binding domain of p53 showed a similar interaction to the one described for ASPP2, suggesting that iASPP could simply function as a competitive inhibitor of ASPP1/2 (Robinson, Lu et al. 2008). A more recent characterisation of the p53-ASPP complex identified a new region of p53 (proline rich domain), outside the DNA binding domain, that interacts with the ASPPs, and in particular iASPP with higher affinity, thus providing additional insights into the way by which iASPP can interfere with the apoptotic function of p53 (Bergamaschi, Samuels et al. 2006; Ahn, Byeon et al. 2009).

In addition to p53/p63/p73 and Bcl-2, already mentioned in the chapter, several other ASPP binding partner have been identified over the past ten years. These include protein phosphatase 1 (PP1) (Helps, Barker et al. 1995), RelA/p65 (Yang, Hori et al. 1999), adenomatous polyposis coli-like (APCL) (Nakagawa, Koyama et al. 2000), YES-associated protein 1 (YAP1) (Espanel and Sudol 2001), amyloid-precursor protein-binding protein 1 (APP-BP1) (Chen, Liu et al. 2003), the hepatitis-C core protein (Cao, Hamada et al. 2004), SAM68 (Thornton, Dalgleish et al. 2006), the DEAD box protein Ddx42p (Uhlmann-Schiffler, Kiermayer et al. 2009), DDA3 (Sun, Hsieh et al. 2008), Par3 (Sottocornola, Royer et al. 2010), *H. Pylori* cytotoxin-associated gene A (CagA) (Buti, Spooner et al. 2011), and dCSK in *Drosophila* (Langton, Colombani et al. 2007). Interestingly, the majority of these interactions are mediated by the ankyrin repeats and SH3 domain, conserved among the three ASPPs. Considering the long list of interacting partners with their diverse cellular functions, it is reasonable to suppose that the ASPPs have multiple roles in the cell, other than inducing apoptosis. Some of the new functions have been already characterised, showing intriguing new perspectives in the study of this family of proteins. For instance, the ASPP2-Par3 interaction was shown to be crucial for the maintenance of apical-basal cell-polarity *in vitro* and *in vivo* (Sottocornola, Royer et al. 2010). This finding, together with the discovery that in *Drosophila* ASPP2 acts as a negative regulator of dSrc

(Langton, Colombani et al. 2007), an important kinase involved in the establishment of cell-cell junctions in epithelial cells, suggests ASPP2 can play a role in the maintenance of epithelial integrity. This is also consistent with the observation that in epithelial cells ASPP2 can normally localise at the cell-cell junctions. However, despite this recent finding, for many of the other interactions, any possible apoptotic-independent function still remains to be elucidated.

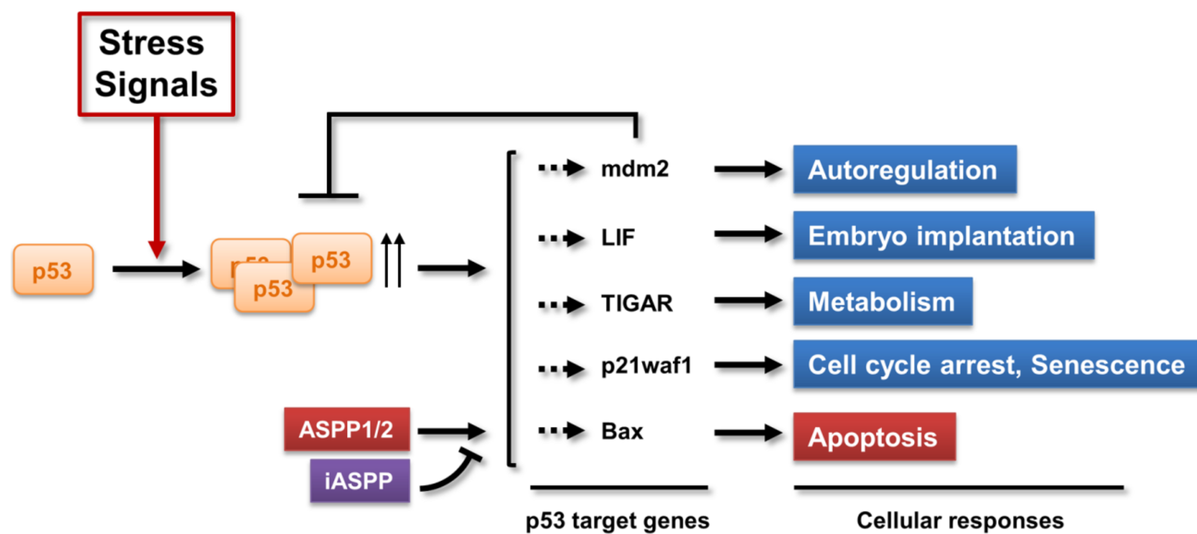


Figure 1.4 The ASPP proteins confer selectivity to the p53-stress response
Stress signals lead to stabilisation and activation of p53 protein, which can now direct different cellular responses by inducing the transcription of several different target genes. The same type of stress signals can also lead to accumulation of ASPP1/2, which can then interact with p53 family members and increase their ability to bind promoters specific for pro-apoptotic genes. iASPP, binding with p53 proteins acts instead as a transrepressor of the same genes transactivated by ASPP1/2, allowing cell proliferation to occur.

1.3.4 Regulation

There are three known ways by which the ASPP proteins can be regulated at the expression level. First, ASPP1 and ASPP2 expression can be induced by the transcriptional factor E2F1, at both mRNA and protein level (Fogal, Kartasheva et al. 2005). E2F1 expression is induced by various DNA damaging agents, and when activated it can mediate apoptosis in both a p53-dependent and independent manner. E2F1-mediated activation of the pro-apoptotic ASPP members is therefore another way by which it can promote apoptosis. Second, epigenetic modifications on the ASPP genes can contribute to the regulation of their expression, as ASPP1 and APP2 expression were found to be silenced by gene methylation in different studies (Liu, Lu et al. 2005; Agirre, Roman-Gomez et al. 2006; Zhao, Wu et al. 2010). Finally, ASPP2 can be targeted for proteasomal degradation upon ubiquitylation on its central region. In fact, proteasome inhibition by treatment with a specific inhibitor (bortezomib) or chemotherapeutic agents results in increase of ASPP2 protein, but not RNA, levels (Zhu, Ramos et al. 2005). Overall, the mechanisms involved in the regulation of the ASPP proteins are still poorly understood, and further clarification in this sense will be very useful in the future, especially given the importance of the cellular processes in which the ASPPs are implicated and their relevance in cancer. An aspect that will be important to elucidate is how the cell localisation of the ASPPs is regulated. The ASPP proteins seem to have distinct functions depending on where they localise in the cells. For instance, ASPP2 can regulate cell-polarity when localised at the cell membrane or promote p53–mediated apoptosis when it is the nucleus. Therefore, it will be of great interest to uncover which external stimuli and post-translational modifications can trigger the shuttling of the ASPPs to different cell compartments.

1.3.5 Role in tumourigenesis

Given the great importance of the ASPP proteins in regulating the apoptotic process, it is logical to expect them to have a central role in tumourigenesis. Consistent with the fact that ASPP1/2 can induce apoptosis, while iASPP is an inhibitor of this process, several lines of evidence accumulated over the past ten years have shown that *ASPP1/2* are tumour suppressor genes and *iASPP* is a proto-oncogene. One of the major contributions to proving ASPP2's role in tumour suppression came from our laboratory, with a study on the *ASPP2* knock-out mouse model (Vives, Su et al. 2006). *ASPP2* knock-out mice in the mixed 129SvJ x C57BL/6 background exhibit postnatal lethality. *ASPP2* heterozygous mice were viable but had a higher incidence of tumour formation, both spontaneous and induced by ionising radiations, when compared to wild-type mice (Vives, Su et al. 2006). Consistent with the role of ASPP2 in regulating p53-mediated apoptosis, a combination of *p53* and *ASPP2* heterozygosity accelerated the onset of tumour development. Interestingly, however, ASPP2/p53 cooperation in tumour suppression seemed to be restricted to certain tumour types, at least as observed in this mixed 129SvJ x C57BL/6 mouse strain, as p53 was required for ASPP2 to suppress the development of lymphomas, but not sarcoma or carcinoma (Vives, Su et al. 2006). This suggests ASPP2 might have some tumour suppressive functions which go beyond p53 regulation.

ASPP2 knock-out mice were recently generated by another laboratory, and the consequent tumour study showed overlapping results (Kampa, Acoba et al. 2009), confirmed that ASPP2 is a tumour suppressor *in vivo*. The work published by our laboratory (Vives, Su et al. 2006), additionally showed that the spontaneous tumour formation in the *ASPP2* heterozygous mice was not accompanied by a loss of heterozygosity, suggesting *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 to be deleted or mutated (Samuels-Lev, O'Connor et al. 2001; Liu, Lu et al. 2005). The main mechanism by which ASPP2 expression is repressed in cancer is by promoter methylation. The expression of the ASPP2 and ASPP1 was found to be frequently down-regulated in a variety of human cancers, as shown for human breast tumours (Samuels-Lev, O'Connor et al. 2001), in hepatitis

B virus (HBV)-positive hepatocellular carcinomas (HCC), and several tumour cell lines (Mori, Okamoto et al. 2000; Liu, Zhang et al. 2004; Mori, Ito et al. 2004; Liu, Lu et al. 2005; Zhao, Wu et al. 2010). Other studies have linked a lowered ASPP2 expression to poor clinical outcome in diffuse large B-cell lymphomas (Lossos, Natkunam et al. 2002) and to poor distant recurrence-free survival in breast cancer patients (Cobleigh, Tabesh et al. 2005). In addition, four single nucleotide polymorphisms within the *ASPP2* gene were linked with significant susceptibility to gastric cancer (Ju, Lee et al. 2005). Consistent with these findings, reduced expression of ASPP2 was observed in metastatic breast cancer samples in a microarray study (Sgroi, Teng et al. 1999). More recent work also found ASPP2 to be targeted for inactivation by *H. pylori* cytotoxin-associated gene (*CagA*), in order to mediate its transforming properties (Buti, Spooner et al. 2011). CagA expression in mice can promote gastrointestinal and intestinal carcinomas as well as myeloid and B-cell lymphomas.

Similar observations were made regarding ASPP1 expression in leukaemia. In one study on leukaemia cell lines, ASPP1 was down-regulated, with a concomitant up-regulation of iASPP (Liu, Zhang et al. 2004). Two further successive studies showed aberrant methylation of the *ASPP1* gene promoter with consequent reduction of its expression in acute lymphoblastic leukaemia (ALL); in this context, ASPP1 down-regulation was linked with poor prognosis (Roman-Gomez, Jimenez-Velasco et al. 2005; Agirre, Roman-Gomez et al. 2006).

In contrast to ASPP1/2, iASPP is believed to have oncogenic properties, as significantly higher levels of iASPP were detected in human tumour samples, such as breast carcinoma (Bergamaschi, Samuels et al. 2003), prostate cancer (Zhang, Xiao et al. 2011), acute leukaemia (AL) and leukaemic cell lines (Zhang, Wang et al. 2005). In addition, *in vitro* experiments showed that iASPP potentiates the transforming activity of the *Ras* oncogene combined with E7 or E1A, by its ability to inactivate p53. In contrast, ASPP1 and ASPP2 reduced the transformation potentials of these oncoproteins (Bergamaschi, Samuels et al. 2003).

Finally, crystal structure analysis of the ASPPs binding with p53 added further insights into the relevance of these proteins in tumourigenesis. One important finding is that four amino acids in p53

DNA-binding domain involved in the interaction with ASPP2 (178His, 181Arg, 243Met and 247Arg) are frequently mutated in human cancer (Gorina and Pavletich 1996). Their mutation frequency is similar to those in contact with DNA and, importantly, the two most frequently mutated p53 residues (248Arg and 273Arg) are in contact with both DNA and ASPP2 (Robinson, Lu et al. 2008). These analysis suggested that the disruption of ASPP2 binding to p53, and abrogation of its consequent apoptotic-stimulatory effect, could be one way by which p53 tumour suppressor function is inactivated in malignancies.

iASPP, unlike ASPP1/2, binds p53 with higher affinity on its proline rich domain, with a selective preference for one common p53 polymorphic variant, called p53Pro72 (p53 containing a proline at codon 72 instead of an arginine) (Bergamaschi, Samuels et al. 2006). As a result, the inhibitory effect of iASPP is more pronounced on p53Pro72 compared to p53Arg72, which turns out to be more active in inducing apoptosis (Sullivan, Syed et al. 2004). Consistent with this, iASPP frequency of over-expression was found significantly higher in a panel of human breast carcinomas with wild-type p53, who are homozygous for the p53Pro72 variant than in those homozygous for the p53Arg72 (Bergamaschi, Samuels et al. 2006), adding further evidence that iASPP is involved in cancer development.

1.3.6 Role in development and differentiation

Studies conducted in genetically modified mice showed that the ASPP proteins play a critical role in normal development. Additionally, all the individual ASPP-deficient mice display developmental defects largely not ascribable to abnormalities in p53/p63/p73 pathways. The importance of the ASPPs in development is therefore due to their interaction with newly identified binding partners, concerning functions that go beyond the regulation of apoptosis. Interestingly, the distinct ASPP mutant mice have a unique phenotype, showing that the three family members have a tissue-specific, non-redundant function during development. Nevertheless, the observation that some tissues in the ASPP deficient mice are unaffected by developmental defects could be instead explained by some degree of functional redundancy between the ASPP proteins in such tissues. ASPP1 and ASPP2 in particular are highly homologous at sequence level, and have been shown to possess overlapping functions, at least in regarding p53 regulation (Samuels-Lev, O'Connor et al. 2001; Bergamaschi, Samuels et al. 2004). A systematic *in vivo* analysis of their temporal and spatial expression would therefore be very informative in order to distinguish between their unique tissue-specific functions and those which are redundant.

iASPP knock-out mice generated in our laboratory suffer from sudden death due to cardiomyopathy and display severe skin abnormalities, affecting the expression of several differentiation markers together with number and orientation of the hair follicles (Notari, Hu et al. 2011). iASPP function in the heart seems to be important for the integrity of the cardiomyocytes, by mediating contacts between cytoskeleton and junctional components (M. Notari, personal communication). In the skin, one of our recent publications showed that iASPP cooperates with p63 for the maintenance of the homeostasis of the stratified epithelium (Notari, Hu et al. 2011).

ASPP1-deficient mice have a lymphatic vascular phenotype, as ASPP1 was shown to be required for the initial assembly and function of the lymphatic vasculature. Analysis of *ASPP1/p53* double knock-out mice demonstrated this phenotype is independent of p53 (Hirashima, Sano et al. 2008). ASPP1 involvement in the developing vasculature has been already suggested by a gene trap expression

screen, which identified *ASPP1* as an endothelial specific gene functioning during mouse embryogenesis (Hirashima, Bernstein et al. 2004).

Characterisation of the *ASPP2* knock-out mouse carried out in our laboratory showed that the tumour suppressor ASPP2 is a key regulator of central nervous system (CNS) development (Sottocornola, Royer et al. 2010). All *ASPP2* knock-out pups in a mixed 129SvxC57BL/6J background died around birth due to severe defects in the CNS, such as hydrocephalus and neural tube closure defect (Vives, Su et al. 2006; Sottocornola, Royer et al. 2010). They also showed extensive loss of cell polarity in the neuroepithelium and abnormal expansion of the pool of neuroprogenitor cells within the brain and retina, forming rosette-like structures similar to those observed in human tumours of neuronal origin (Sottocornola, Royer et al. 2010). Experiments conducted in our lab showed that ASPP2 controls the structural organisation of the neuroepithelium by maintaining the integrity of tight/adherence junctions. This is achieved by controlling the cellular localisation of Par3, an important member of the polarity complex Par3/aPKC/Par6 (Sottocornola, Royer et al. 2010). Impairment of epithelial integrity due to ASPP2 deficiency was found to be responsible for hydrocephalus, because it compromised the capacity of the choroid plexus to regulate the flux of cerebrospinal fluid in the brain cavity. Loss of cell polarity in the neuroepithelium was also responsible for the largely disorganised structure of the *ASPP2*-null brains. Lack of apical/basal polarity may also be partially responsible for the expansion of the progenitor pools, since it has been associated with increased cell proliferation, in particular in cancer (Royer and Lu 2011). Interestingly, this characteristic of the *ASPP2*-null mice is totally independent of p53-mediated apoptosis, since the increase in the number of proliferating cells in the mutant brains was not due to a decrease in apoptosis. Shortening of the cell cycle was instead observed, which could partially explain the increase in the pool neural stem cell (Sottocornola, Royer et al. 2010).

The expansion of the progenitor pools which characterises *ASPP2* mutant mice could also be explained by increased Notch signalling. *In situ* hybridisation with a probe against the Notch signalling target *Hes5* revealed an expansion of cells with active Notch signalling (R. Sottocornola,

personal communication). *Hes5* is a Notch target gene known to promote stemness in the neuroepithelium, as it can be used to identify NSCs (neural stem cells) (Basak and Taylor 2007). Elevated mRNA expression levels of Notch targets *Hes1* and *Hes5* were detected in the mutant brains by quantitative real-time PCR (R. Sottocornola, personal communication). Altogether, these observations suggest that one possible way, as an alternative to p53-mediated apoptosis, by which ASPP2 can inhibit proliferation and promote differentiation is by regulating the Notch pathway.

1.4 The Notch pathway

1.4.1 Overview

The Notch signalling pathway is evolutionary conserved from *Drosophila* to humans, and is implicated in many fundamental cellular processes, during the development of the organism and in self-renewing of adult tissues (Artavanis-Tsakonas, Rand et al. 1999; Schweisguth 2004). Processes that can be mediated by Notch pathways include promotion or suppression of cell proliferation, cell death, acquisition of cell fates and activation of specific programs of differentiation. Accordingly, Notch proteins play a crucial role in differentiation and determination of cell fate. Their function however is critically context dependent, as in some cells/tissues they can promote differentiation (as in the skin) and in other sites Notch proteins are important for the maintenance of the proliferative potential of stem cell populations (as in the brain) (Artavanis-Tsakonas, Rand et al. 1999). Given the physiological importance of Notch signalling pathway, its loss or aberrant gain of function were found to be associated with several human disorders. Such disorders include developmental diseases (e.g. Alagille syndrome, Tetralogy of Fallot, syndactyly, spondylcostal dysostosis, familiar aortic valve disease) (Gridley 2003; Garg, Muth et al. 2005), diseases in adulthood (e.g. CADASIL) (Louvi, Arboleda-Velasquez et al. 2006) and cancer (Weng, Ferrando et al. 2004; Ranganathan, Weaver et al. 2011).

1.4.2 Signal transduction

Transmission along the Notch signalling pathway arises from communication between neighbouring cells, requiring physical cell-cell contact. This is because the Notch family of proteins are trans-membrane receptors which became active by binding with ligands of Delta and Jagged families, trans-membrane proteins themselves expressed on adjacent cells. Upon engagement with their ligands, Notch proteins are subjected to three sequential proteolytic cleavages (De Strooper, Annaert et al. 1999; Mumm, Schroeter et al. 2000). The first cleavage is mediated by metalloproteases of the

ADAM/TACE family, while the second and third cleavages by the γ-secretase complex, composed of presenilin, nicastrin, PEN2 and APH1, resulting in the intracellular release of the Notch intracellular domain (NICD). NICD translocates into the nucleus where it forms a ternary transcription-active complex with a transcription factor of the CSL family, RBP-jκ (also known as CBF1) and the co-activator protein MAML (Mastermind-like). Following the formation of the ternary complex, additional co-activators are recruited, such as histone acetyl transferases (HATs; p300 and/or PCAF/GCN5) and chromatin remodelling complexes (BRM, TRA1/TRRAP and Dom) (Bray 2006). This allows the activation of the complex and the transcription of target genes, including *Hes* (hairy enhancer of split), and *Hey* family genes (hair-enhancer of split-related with YRPW motif) (Borggrefe and Oswald 2009). In absence of NICD, RBP-jκ constitutively binds the DNA on the target gene regions, together with co-repressors, functioning therefore as a transcriptional repressor complex. The repressor complex is also associated with histone deacetylases which keep the local chromatin to a transcriptionally silent form (Lai 2002). Upon activation of Notch pathway, NICD displaces the co-repressor complex bound to RBP-jκ, leading the formation of the ternary complex, which is completed with MAML recruitment. Modifications at NICD C-terminal, by cyclin-dependent kinase-8 (CDK8) and FBXV7/SEL10 E3 ligase, target NICD for proteasome-mediated degradation, terminating its transcriptional activity (Figure 1.5).

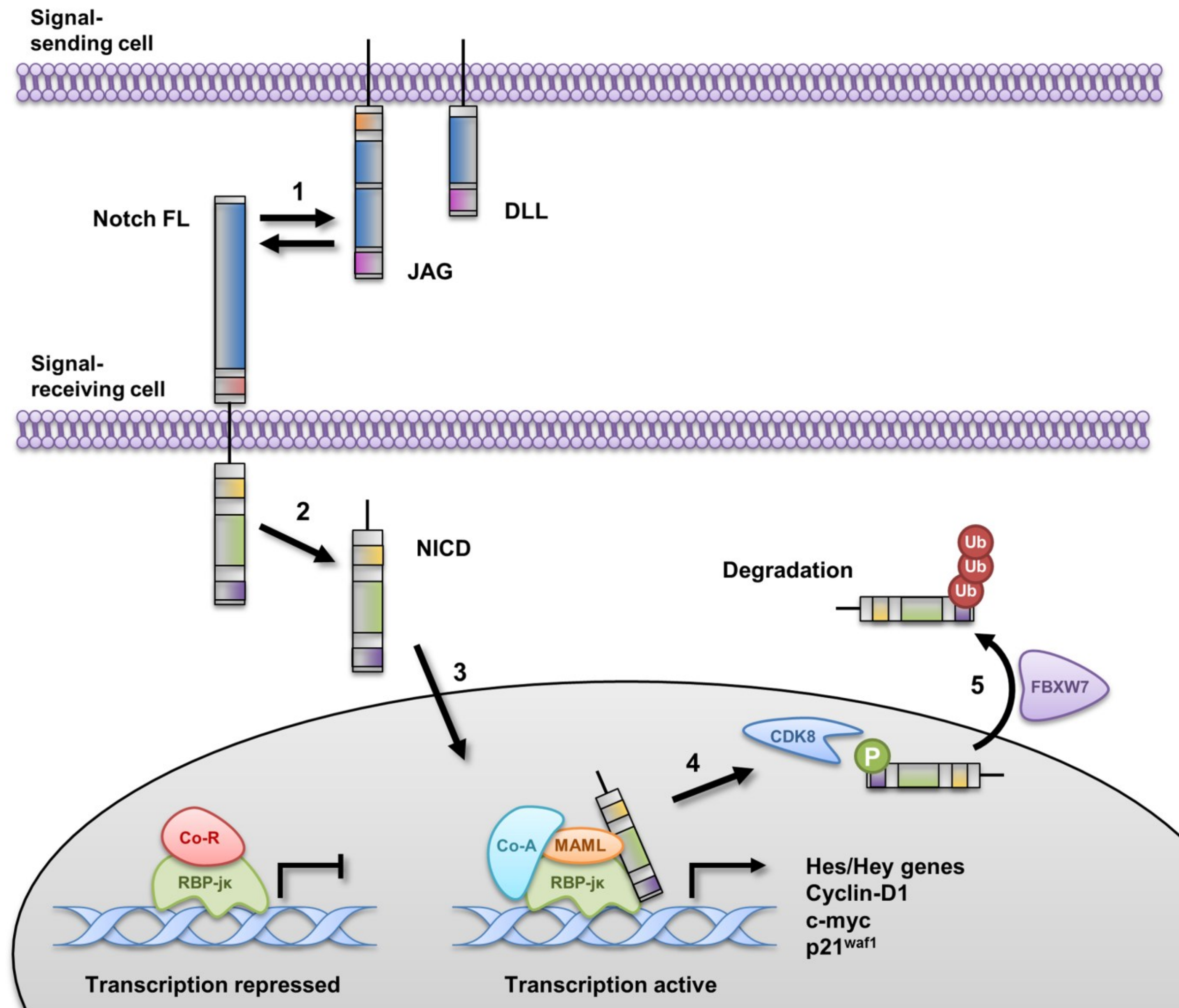


Figure 1.5 The Notch pathway signal transduction
(1) The activation of Notch pathway is initiated by the binding of the Notch receptors with their ligands expressed on the cell-membrane of a neighbouring cell. (2) The activation of the Notch receptor is followed by a series of proteolytic cleavages leading to the intracellular release of the Notch intracellular domain (NICD). (3) NICD enters into the nucleus and recruits the co-activator (Co-A), Mastermind-like (MAML) and other factors necessary to convert the RBP-jκ repressor complex, normally bound to DNA along with co-repressors (Co-R), into a transcriptional activator complex and drives the transcription of target genes. (4) The signal is terminated when by the cyclin-dependent kinase-8 (CDK8) phosphorylate (P) the PEST domain of NICD causing its subsequent ubiquitylation (Ub) by FBXW7 (5) and proteasomal degradation.

1.4.3 Members and structure

Mammals have four Notch paralogues, named Notch1-4. They are all single-pass type I transmembrane receptors, whose expression can vary in different tissues, displaying both redundant and unique functions. All the four Notch proteins have the same structural composition, with two subunits, one entirely extracellular and one predominantly intracellular (NICD), linked together by non-covalent interactions. The extracellular subunit contains a large N-terminal epidermal growth factor (EGF)-like repeats, involved in the interaction with ligands, followed by a unique negative regulatory region composed by three cysteine-rich Lin12-Notch repeats (LNRs), important to prevent ligand independent activation of the receptor. The NICD subunits display conserved domains in their intracellular regions, such as a RAM domain (RBP-jκ associated module), seven ankyrin repeats and a C-terminal PEST sequence (proline/glutamic acid/serine/threonine-rich motifs) (Kopan and Ilagan 2009). The RAM and Ankyrin domains mediate the interaction between NICD, RBP-jκ and MAML, while the PEST domain contains degradation signals which are important in regulating NICD stability (Figure 1.5). The five mammalian Notch-ligands are known as DSL (Delta/Serrate/LAG-2) proteins, belonging to the Jagged (Jagged1-2) and Delta families (Delta-like1, 3 and 4). Similar to the Notch proteins, DSLs are type I transmembrane receptors, with an extracellular EGF-like repeats domain which mediates the binding with Notch receptors expressed on neighbouring cells (Figure 1.6). The Notch target genes from the *Hes* and *Hey* families encode basic helix-loop-helix transcription factors, which function as transcriptional repressors. Hes and Hey proteins are important in regulating differentiation processes by the repression of lineage-specifying genes such as *MASH-1* and *neurogenin* (neurogenesis), and *MyoD* (myogenesis) (Kuroda, Tani et al. 1999; Kageyama, Ohtsuka et al. 2008). Beyond the *Hes* and *Hey* gene families, other Notch transcriptional targets have been identified. Interestingly some of them are genes implicated in cancer, such as *c-myc*, *cyclinD1* and $p21^{WAF1}$ (Rangarajan, Talora et al. 2001; Ronchini and Capobianco 2001; Weng, Millholland et al. 2006)

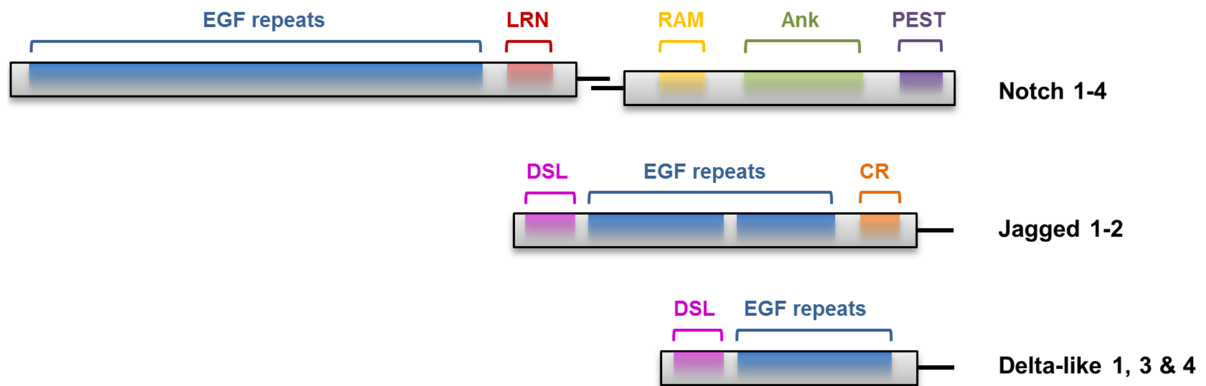


Figure 1.6 Structural organisations of the Notch receptors and their ligands
Vertebrates express four Notch receptors (Notch 1-4) and five ligands (Jagged 1-2 and Delta-like 1,3 and 4). Notch proteins are composed by an extracellular subunit, containing epidermal growth factor (EGF)-like repeats followed by cysteine-rich LIN12 and Notch repeats (LNRs) and an intracellular domain, named NICD, composed of a conserved RBP-jκ associated module (RAM), six ankyrin repeats (Ank) and a C-terminal PEST region. Notch ligands consist of a single unit containing an N-terminal DSL structure (Delta, Serrate and LAG-2) followed by an EGF-like repeats. The ligands are divided in two classes, depending on the presence or absence of a cysteine-rich domain (CR). The CR domain is only present in the members of the Jagged family and not in those of the Delta-like family.

1.4.4 Functions

Activation of Notch signalling results from cell-cell contact, thus is not surprising that many of its biological functions, such as proliferation and differentiation, are mediated by specific cells within a population, affecting neighbouring cells with different properties. Three modalities by which Notch pathways propagate in cell populations have been described to date. They have been named "lateral inhibition", "asymmetric cell fate assignation" and "boundary formation" (Fiuza and Arias 2007). All these mechanisms have been well characterised in *Drosophila* neurogenesis (Bray 1998).

Lateral inhibition is probably the best characterised among the three Notch modes of action and it has been shown to be crucial for neuronal development (Artavanis-Tsakonas, Rand et al. 1999). In this context Notch signalling serves to amplify small differences within a roughly uniform cell population. During neurogenesis, many cells have the potential to adopt a determined cell fate, but only few manage to do it. This is because those few cells which have higher levels of Notch ligands, can exert an inhibitory effect on the surrounding cells, via induction of Notch pathway (thus the name lateral inhibition) (Figure 1.7).

Another way by which the Notch pathway can affect cell fate decisions is during cell division. Neuronal precursors have stem cells properties and therefore they can undergo symmetric cell divisions, generating two identical pluripotent daughter cells, or asymmetric cell division, resulting in two different daughter cells, one identical to the mother and one committed to a determined cell lineage. Asymmetric cell division relies on the unequal inheritance of cell fate determinants between the two daughter cells. Notch regulators, such as Numb and Neuralised, act as cell fate determinants, enabling only one of cells in the progeny to respond to Notch pathway, thus inducing differential transcriptional programmes in the two daughter cells (Frise, Knoblich et al. 1996; Le Borgne and Schweisguth 2003). Additionally, the signal-sending cell can prevent the sister cell from acquiring the same fate, by activating the Notch pathway. After many repetitions the process eventually leads to distinct populations of differentiated cells. The fate induced in the sister cell where the Notch

pathway is active can vary according to the tissue and the stage of development of the organism; it can be a stem-like state, or a terminally differentiated fate (Figure 1.7).

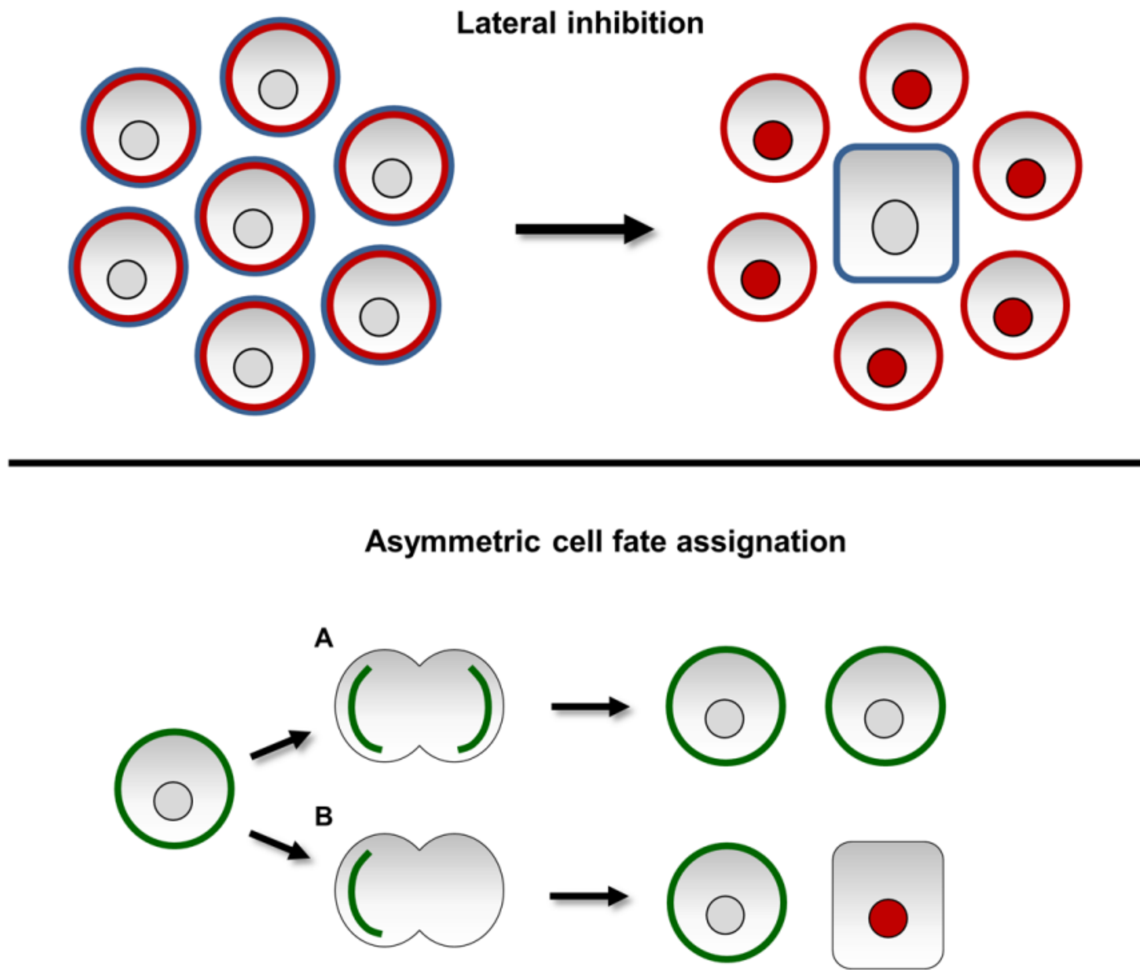


Figure 1.7 Notch modes of action in development and differentiation
The scheme in the upper panel explains Notch-mediated lateral inhibition. From left, a group of cells of the same type expressing equal amounts of Notch receptors (red border) and Notch ligands (blue border). At right, within the group of cells, one cell starts differentiating and increases the expression of the Notch ligands on its surface. This leads to the activation of Notch pathway in the neighbouring cells (red nuclei), thus inhibiting their capacity to differentiate. This process is taking place, for instance, during neuronal development, where the activation of Notch pathway is important for the maintenance of the pool of neuronal stem cells. The lower panel shows the process of asymmetric cell fate assignation. When a cell divides into two daughter cells, certain regulators of the Notch pathway (like Numb, shown in green) can be inherited in a symmetric (A) or asymmetric way (B). If

the inheritance is symmetric, the daughter cells will have the same level of Notch activity (no activity, in this case since Numb is a repressor of Notch pathway) and therefore they will adopt the same fate (A). If instead, the Notch regulator is unequally segregated into the daughter cells, then Notch activity will be present only in one of them leading to a different cell fate (B). This mechanism can be observed in the epidermis, where asymmetric cell-division in the basal stem cells leads to Notch pathway activation and causes terminal differentiation.

1.4.5 Regulation

Notch is a pleiotropic pathway, whose final output is highly context- and time-dependent. The modulation of its activity appears therefore to be crucial. Regulators of Notch signalling are multiple and can intervene at different levels. Events that affect the activation of Notch pathway are post-translational modifications, intracellular trafficking, epigenetic modifications and cross-talk with tissue-specific regulators (Bray 2006). Post-translational modifications and intracellular trafficking in particular are important in regulating ligand and receptor availability, by limiting their expression spatially and temporally. This results in a modulation of ligand-receptor interactions and a tuning of Notch pathway activation.

1.4.5.1 Ubiquitylation and trafficking

Ubiquitylation of Notch ligands by Neuralised and Mind bomb E3 ubiquitin ligases seems to be crucial for Notch ligand activation (Le Borgne, Bardin et al. 2005; Chitnis 2006). Prior to modification by Neuralised and Mind bomb, Notch ligands are inactive, even if expressed at the cell membrane, and can be endocytosed and degraded. Upon Neuralised- or Mind bomb-mediated ubiquitylation, the receptors are endocytosed in clathrin coated vesicles and are recycled at the cell membrane in a functional active form. At present, it is still unclear how the ubiquitylation-mediated endocytosis can activate Notch ligands. A hypothetical model suggests that endocytosis can cause an activating conformational change in the structure of the receptors (Parks, Klueg et al. 2000). Ubiquitylation might also promote clustering of the ligands, and in this arrangement they have been shown to be more effective in activating Notch pathway (Varnum-Finney, Wu et al. 2000). Another

possibility is that the trafficking of the ligands allows them to be re-inserted into specific membrane domains which render them active.

Notch receptors have been also linked with ubiquitylation and endosomal trafficking. The NEDD4 family of HECT domain E3 ligases can modify Notch and target it for degradation, acting as negative regulators of the signalling. The activity of Deltex, another E3 ligase, acting on NICD can instead promote Notch accumulation in endocytic vesicles and positively regulate its signalling, as seen in *Drosophila* (Hori, Fostier et al. 2004). In several mammalian cell types, however, Deltex was shown to antagonise Notch (Sestan, Artavanis-Tsakonas et al. 1999). Further analysis aimed at analysing the balance of all the different E3 ligase activities will clarify the precise role of ubiquitylation-induced endocytosis in regulating Notch localisation and activation.

At the nuclear level, ubiquitylation on NICD domains by FBW7/SEL10 E3 ligase results in turnover of NICD, shutting down transcriptional activity. Earlier phosphorylation by CDK8 is required to render NICD suitable for the binding with FBW7/SEL10 (Fryer, White et al. 2004).

1.4.5.2 Glycosylation

Glycosylation of the EGF-like repeats of Notch is a critical post-translation modification which allows the receptor to be functionally active. The enzyme *O*-fucosyl transferase (*O*-Fut) is responsible for adding the first fucose on Notch EGF-like repeats domain (Shi and Stanley 2003). Furthermore, *O*-Fut can also function as Notch chaperone protein, being responsible for its delivery from the endoplasmic reticulum to the plasma membrane (Okajima, Xu et al. 2005). Consistent with this, depletion of the *O*-Fut enzyme in *Drosophila* and mice resembles the characteristics of mutants lacking of Notch activity (Okajima and Irvine 2002; Shi and Stanley 2003). After the first fucose, extra carbohydrates can be then added by glycosyl transferases of the Fringe family (Haines and Irvine 2003). Given the multiple number of EGF-repeats present of Notch receptors, the pattern of modifications generated by Fringe can be varied. Different glycosylation patterns have been shown to

confer different binding affinities between Notch receptors and their ligands, making glycosylation an important way of modulating Notch pathway activation (Okajima, Xu et al. 2003).

1.4.5.3 Proteolytic cleavage

As mentioned above in the text, membrane-Notch has to undergo three successive proteolytic cleavages on three different sites, S2, S3 and S4, in order to produce the active NICD form. At the present time still not much is known about how the activity of proteins mediating the cleavages, ADAM10/TACE (on S2) and γ-secretase complex (on S3-S4), is regulated. Some studies nevertheless suggest that the length of the Notch extracellular domain might affect the efficiency of cleavage in S3 (Struhl and Adachi 2000), which is also probably taking place after Notch endocytosis, since ubiquitylation on a juxtamembrane lysine was shown to be required for γ-secretase-mediated cleavage in S3 (Gupta-Rossi, Six et al. 2004). Interestingly, drugs regulating presenilin activity (a member of the γ-secretase complex) have been previously developed for treatment of Alzheimer's disease, and are now in clinical trials also for certain types of cancer associated with high Notch activity (Aster 2005). Finally, proteolytic cleavage by Furin-like convertase on site S1 takes place in the Golgi before mature Notch can be exported to the cell membrane.

1.4.5.4 Epigenetics regulation and microRNAs

A recent work by Martinez *et al*. has provided evidence for epigenetic silencing of the Notch locus in *Drosophila* eyes (Martinez, Schuettengruber et al. 2009). Other works showed that Notch-dependent transcription requires chromatin modifications, since chromatin-remodelling factors such as GCN5 and BRM are recruited in order to activate transcription (Kurooka and Honjo 2000; Kadam and Emerson 2003). Notch transcriptional activity can also be modulated by microRNAs (miRNAs), as in Drosophila three different miRNA families were found to regulate Notch target genes (Lai, Tam et al.

2005). A time-regulated and tissue-specific expression of such miRNAs might therefore partially explain variations in Notch pathway activity observed during development and in different organs.

1.4.5.5 Protein partners and cross-talk with other pathways

Currently, the best characterised Notch inhibitor is the protein Numb. Numb is one of the crucial factors which are unequally inherited during asymmetric division of neuronal precursor cells. Its presence in one of the daughter cells generated upon mitosis is sufficient to block Notch activation and direct that cell to a different fate (Figure 1.7). Numb fulfils its function by binding to membrane-associated Notch, promoting Notch endocytosis and consequent degradation by interacting with α-adaptin, a component of the adaptor protein-2 complex (AP2) involved in the clathrin-coated vesicles-mediated endocytosis (Berdnik, Torok et al. 2002). Additionally, Numb can target Notch to degradation by inducing its direct ubiquitylation (McGill and McGlade 2003).

Nuclear transcriptional factor RBP-jκ was shown to bind to at least one partner other than NICD, the bHLH protein p48/PTF1a (Beres, Masui et al. 2006). This, together with the observation that RBP-jκ in cells is found in large excess compared to NICD, suggests RBP-jκ might have other functions or binding partners which are independent by being a repressor- or an activator-complex in Notch pathway. Some of the effects of Notch signalling could therefore be the result of NICD titrating RBP-jκ away from other binding partners. The same could be assumed for Notch co-activator protein MAML, which was shown not to be exclusive for NICD, but to functionally interact with several other proteins, such as β-catenin, Mef2c and p53 (McElhinny, Li et al. 2008).

Evidence of additional nuclear co-factors for NICD have also emerged, showing NICD can interact with SMADs, NFκB and HIF1α, getting involved in multiple known signalling pathways (Kopan and Ilagan 2009).

Recently, works conducted in Stephen C. Blacklow's laboratory showed that two coupled RBP-jκ/NICD/MAML complexes are required for the activation of certain Notch target genes, such as *Hes1* and *Hes5* (Nam, Sliz et al. 2007; Arnett, Hass et al. 2010). Notably, this interaction takes place

between the two ankyrin repeats domain of two adjacent NICD molecules, and since this is a structural motif found in several known proteins, it is possible to speculate that other ankyrin repeats-containing proteins might participate in forming a heterotypic dimeric complex with NICD. Heterodimeric complexes, assembled with the participation of tissue-specific factors, might confer binding selectivity for different Notch-target promoters, helping to understand how Notch pathway signalling can have differential outcomes in different tissues.

Several points of cross-talk between Notch and other signalling pathways are also known, as reviewed by G. Paolo Dotto and P. Ranganathan (Dotto 2009; Ranganathan, Weaver et al. 2011). Notch can induce activation of the PI3K-AKT and receptor tyrosine kinase signalling (RTK) pathways, via down-regulation of PTEN expression and activation of EGF-receptor and PDGF-receptor expression, respectively. Conversely, Notch pathway activation can have an inhibitory effect on Wnt signalling, by $p21^{WAF1}$- and Hes1-mediated down-regulation of Wnt ligands Wnt3/4. An inhibitory effect of the Notch pathway on p63 expression is also observed through a mechanism involving a modulation of interferon-responsive factors and NFκB.

In the opposite direction, TGFβ, Wnt and p63 pathways have been shown to induce the expression of Notch ligand Jagged1, thus synergising with the Notch pathway. P63 can also negatively affect Notch signalling by suppressing the expression of the Notch-target genes *Hes1* and $p21^{WAF1}$. The Wnt pathway can also have an inhibitory effect on Notch, as phosphorylation of NICD by glycogen synthase kinase 3β (GSK3β) leads to inhibition of Notch-mediated transcription.

Despite extensive studies of Notch signalling, only a few tissue-specific and time-regulated binding partners and interacting pathways have been characterised so far. This seems to be the most promising direction to explain the extent of diversity in Notch pathway activity observed in nature.

1.4.6 Role in development and differentiation

Notch1 knock-out mice are embryonic lethal from vascular, neuronal, and somitic defects, suggesting that Notch plays various developmental roles in different tissues (Swiatek, Lindsell et al. 1994; Conlon, Reaume et al. 1995; Huppert, Le et al. 2000; Krebs, Xue et al. 2000). Notch's role in development has been deeply studied in *Drosophila,* where it is crucial in the determination of the neuronal-epidermal fate. Here, Notch acts as a repressor of neuronal differentiation by inhibiting the expression of pro-neural genes (Parks, Huppert et al. 1997). Inhibition of neurogenesis was observed also in vertebrates, as conditional loss of Notch in mice led to premature induction of neurogenesis (Lutolf, Radtke et al. 2002). Mutants for Notch target genes *Hes1* and *Hes5* revealed a similar neuronal phenotype, indicating that Hes1 and Hes5 are key mediators in Notch-induced inhibition of neuronal differentiation (Ishibashi, Ang et al. 1995; Ohtsuka, Ishibashi et al. 1999). Hes1 and Hes5 are in fact repressors of pro-neural genes, such as *MASH-1* and *neurogenins* (Kageyama, Ohtsuka et al. 2008). Several works also showed that other Notch target genes, such as *Hey1*, *Hey2* and *ErbB2*, are expressed in neural progenitor cells and are believed to be important for their maintenance (Patten, Peyrin et al. 2003; Sakamoto, Hirata et al. 2003). Similarly, in the developing pancreas Notch signalling has been reported to be important for the maintenance of undifferentiated precursor cells (Jensen and Robertsen 2002). In melanocytes, selective genetic inactivation of *RBP-jκ* or *Notch1/Notch2* together resulted in loss of melanocyte progenitor cells, due to increased apoptotic rate (Moriyama, Osawa et al. 2006; Schouwey, Delmas et al. 2007). A role for the Notch pathway in somitogenesis was also suggested, because of the defects in somite morphology found in *Notch1* and *RBP-jκ* mutant mice (Conlon et al. 1995, Oka et al. 1995), and was further confirmed by subsequent investigations (Weinmaster and Kintner 2003; Ferjentsik, Hayashi et al. 2009). In the intestine, Notch has a fundamental role in the self-renewal of the tissue, by promoting the maintenance of the stem cells residing in the crypt compartment. Consequently, ectopic expression of NICD resulted in inhibition of differentiation of the intestinal stem cells (Fre, Huyghe et al. 2005).

Overall, various experimental studies agree in defining Notch as a factor important for the maintenance of stem cell properties in the developing CNS, pancreas, post-natal intestine and mammary gland by inhibiting pro-differentiation genes.

Notch signalling, however, can have different effects in different organs and tissues. In the skin for example, Notch induces terminal differentiation. Here, Notch has an active role in promoting expression of differentiation markers, such as Keratin1 and 10, and cell cycle regulator $p21^{WAF1}$ (Rangarajan, Talora et al. 2001). Epidermal Notch signalling will be discussed more in detail in a following section of this chapter ("Epithelial development"). Vascular defects were observed in mice mutated for *Notch* (1, 2 and 1-4 together), *RBP-jκ*, *Hey1*, *Hey2*, *Jagged1* and *Delta-like 4* genes, highlighting an important role of Notch signalling also in vasculogenesis (Rehman and Wang 2006). Additionally, the human pathology CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarction and leukoencephalopathy), which causes stroke and vascular dementia, has been associated with a mutation in the *Notch1* and *Notch3* genes (Joutel, Corpechot et al. 1996; Gridley 2003). An inactivating mutation in the human *Jagged1* gene also leads to various vascular anomalies known as Alagilles syndrome (Li, Krantz et al. 1997). Experimental studies showed that Notch is indeed required for the successful completion of several steps during vascular morphogenesis and differentiation, particularly for the arterial versus venular specification (Alva and Iruela-Arispe 2004). Furthermore, starting from endothelial cells, Notch can promote generation of hematopoietic stem cells during embryonic development, via activation of *GATA-2* (Robert-Moreno, Espinosa et al. 2005). Later in haematopoiesis, Notch can induce progenitor cells to differentiate in T-lymphocytes and inhibit B-cell fate (Pui, Allman et al. 1999).

1.4.7 Role in tumourigenesis

Considering the multifaceted role of Notch in differentiation, where it can be involved in driving opposite processes (promote either stemness or commitment to terminal differentiation), depending on the context or stage of development, it is not surprising that in cancer Notch has been associated with both oncogenic and oncosuppressor roles (Koch and Radtke 2007).

The best studied role of Notch in tumourigenesis is in T-cell acute lymphoblastic leukaemia (T-ALL). Genetic alterations, such as a chromosomal translocation (~1% of T-ALLs) and activating mutations in the *Notch1* locus (more than 50% of T-ALLs), where found in patients affected by T-ALL. These genetic abnormalities often result in constitutive activation of the Notch pathway, leading to formation of lymphoma (Ellisen, Bird et al. 1991; Weng, Ferrando et al. 2004).

Despite the major role played by deregulated Notch pathway activity in solid tumours, few genetic alterations have been reported in Notch pathway genes. This could be explained by the fact that in solid tumours Notch signalling is more dependent on the spatial context, such as the presence or absence of ligands, cellular inhibitors, and cross-talk with other pathways, as the cells here are in contact with each other. Any abnormal variation in these factors can lead to inappropriate activation or inhibition of the Notch pathway and cause tumour transformation (Miyamoto, Maitra et al. 2003; Santagata, Demichelis et al. 2004; Brennan, Momota et al. 2009).

In tissues where Notch activity is important for the maintenance of progenitor cells, such as brain, intestine, pancreas and mammary gland, aberrant activation of its pathway has been associated with the expansion of the stem cell pool and tumour growth. In the brain, Notch activity has been linked with medulloblastoma, a malignant brain tumour of childhood, as increased mRNA expression of *Notch2*, *Hes1* and *Hes5* has been observed (Fan, Mikolaenko et al. 2004; Hallahan, Pritchard et al. 2004). Abnormal activation of Notch in these tumours appears to be a consequence of aberrant sonic hedgehog signalling (Shh), which has been already established as a primary cause of medulloblastoma (Kenney, Cole et al. 2003). In the intestine compartment, the activation of Wnt signalling is instead one of the main promoting agents for colorectal cancer. Consistent with the

observation that Wnt and Notch signalling physiologically cooperate in the crypt cells of the intestine, activation of Notch pathway via Hes1 expression has been observed in colorectal tumour mouse models and in human colorectal tumours (van Es, van Gijn et al. 2005; Fernandez-Majada, Aguilera et al. 2007). Moreover, in human and mouse examples of pancreatic cancers, up-regulation of Notch proteins, ligands and target genes have been reported (Hingorani, Petricoin et al. 2003; Kimura, Satoh et al. 2007). Work by Miyamoto and colleagues suggests that the increase in Notch activity observed in pancreatic tumourigenesis is a consequence of transforming growth factor α (TGFα) activation, whose causative role in pancreatic cancer is well characterised (Hruban, Wilentz et al. 1999). Abnormal expression of Notch receptors and ligands was found in human melanoma specimens. In melanoma cell lines Notch activation has been linked with acquisition of transformation properties, probably associated with enhancement of MAPK, AKT and β-catenin signalling (Balint, Xiao et al. 2005; Liu, Xiao et al. 2006). Finally, consistent with its role in promoting the self-renewal of the normal mammary stem-cells, several studies indicate that Notch pathway activation plays a role in breast cancer (Dontu, Jackson et al. 2004; Liu, Dontu et al. 2005). Over-expression of Notch1, but also down-regulation of the Notch inhibitory protein Numb have been observed in different studies on human breast cancer samples (Parr, Watkins et al. 2004; Pece, Serresi et al. 2004)

In contrast with what has been described so far, Notch behaves as a tumour suppressor in tissues where its function is associated with induction of differentiation and growth suppression, and its inactivation can lead to tumour formation. This is the case in the skin, where Notch activity is physiologically important to promote terminal differentiation of keratinocytes (Radtke and Raj 2003). *In vitro* experiments conducted with keratinocytes, along with Notch defective mouse models and abnormal expression of Notch1, Notch2 and ligands in human skin tumours, confirmed that down-regulation of Notch signalling promotes skin tumourigenesis, (Koch and Radtke 2007). A closer examination of Notch's role in skin tumourigenesis will be provided later in the chapter. A tumour suppressor role for the Notch pathway has been also reported in other tissues, such as in the prostatic

epithelium, small cell lung cancer and hepatocellular carcinoma (Dotto 2008). Finally, several studies showed that Notch receptors and ligands play a key role in neo-angiogenesis (Patel, Li et al. 2005; Zeng, Li et al. 2005). The development of drugs capable of modulating Notch signalling could therefore be an important approach to disrupt tumour associated neovasculature and inhibiting tumour growth, as already successfully demonstrated in two independent studies (Noguera-Troise, Daly et al. 2006; Ridgway, Zhang et al. 2006).

Interestingly, in all the examples of solid tumours in which Notch signalling is involved, its deregulation seems to be the consequence of cross-talk with alerted pathways, which are physiologically important for the normal homeostasis of the given tissue. This suggests that the Notch pathway has a broad fundamental role in differentiation, and its outcome then acquires specific connotations in different tissues according to the interactions with environmental neighbouring factors. Initiating alterations in such tissue-specific factors can directly affect Notch behaviour, providing the required contribution for tumour transformation.

1.5 Epithelial physiology

1.5.1 Overview

Epithelial tissues are located at the interface between the organism and the outside world, as simple monolayers that cover the digestive, respiratory, urinary and reproductive tracts, as glandular acini/alveolae (pancreas, salivary glands, breast gland, prostate, liver and others) or as multilayered tissues (for example, skin, mucosae of upper digestive and upper respiratory tracts, cornea). The largest epithelial tissue in the human body is the skin. The skin is composed of two compartments, an inner layer called the dermis and a more superficial one called the epidermis. Both dermis and epidermis are responsible for the formation of epidermal appendages, such as hair follicles, nails, sweat and mammary glands. The separation between dermis and epidermis is defined by a basement membrane (basal lamina) composed of protein secreted by cells belonging to both the epidermal and dermal compartments. The epidermis, as the uppermost compartment of the skin, has the role of providing a physical and permeability barrier for the body, protecting the organism from dehydration, mechanical or bacterial harm. What makes the epidermis an efficient barrier is the nature of its composition: a multilayered compartment. Keratinocyte cells are the "bricks" which form this structure. Other more specialised cell types that can be found intercalated inside the epidermis are Merkel cells, melanocytes and Langerhans cells.

The keratinocytes in the epidermis are organised as a stratified squamous epithelium. The stratification of the epidermis consists of a proliferative active basal layer, surmounted by progressively more differentiated supra-basal layers, named spinous, granular and cornified layers (Figure 1.8). New keratinocytes are originated by mitosis in the basal layer and move upwards throughout the layers, undergoing transcriptional and morphological changes during their transit. As the keratinocytes move up, they flatten and lose their proliferative potential, committing to terminal differentiation by the expression of various forms of keratin (the process of keratinisation), until they eventually die when they reach the surface, and are sloughed off through desquamation. Dead cells

which are lost are constantly replaced by new keratinocytes originated from asymmetric cell divisions of the stem cells resident in the basal layer. These undifferentiated cells, which have the ability to self-renew, play a fundamental role in skin homeostasis and repair (Blanpain and Fuchs 2009). The overall epithelial stratification can be seen by looking at the morphological diversity of each layer, which is associated with changes in the expression of keratins and others molecular markers (Koster and Roop 2004). Keratins are intermediate filament proteins, which exist in form of heteropolymers assembled with individual keratin proteins of two distinct types. For instance, Keratin-14 (K14) is coupled with Keratin-5 (K5), and Keratin-10 (K10) with Keratin-1 (K1). The proliferative basal layer of the epidermis is characterised by the expression of K14 and K5, while when cells enter the spinous cell compartment they switch to expression of K10 and K1(Koster and Roop 2007). As the terminal differentiation of the keratinocytes proceeds in the granular layer, protein kinase C (PKC) activity is important to repress the expression of the K1 and K10, and promote the expression of granular markers, such as loricrin, filaggrin and transglutaminase (Dlugosz and Yuspa 1993; Dlugosz and Yuspa 1994). Ca^{2+} is a fundamental trigger in promoting epidermal differentiation and its concentration increases moving up the layers. Ca^{2+} not only regulates PKC activity, but also promotes cell-cell contact formation, which is a hallmark of differentiated keratinocytes (Koster and Roop 2007). Conversely, cell adhesion with the extracellular matrix, which is a peculiar characteristic of cells resident in the basal layer, is antagonised by down-regulation of integrins (transmembrane receptors which mediate cell-matrix adhesion), in order to allow differentiated keratinocytes to migrate upwards. Finally, the top cornified envelope, where terminal differentiation is completed, is composed of flattened dead cells expressing structural proteins (keratins, loricrin, filaggrin, involucrin), which are cross-linked by transglutaminases with lipids on the outside, forming a barrier with high physical resistance and waterproof properties.

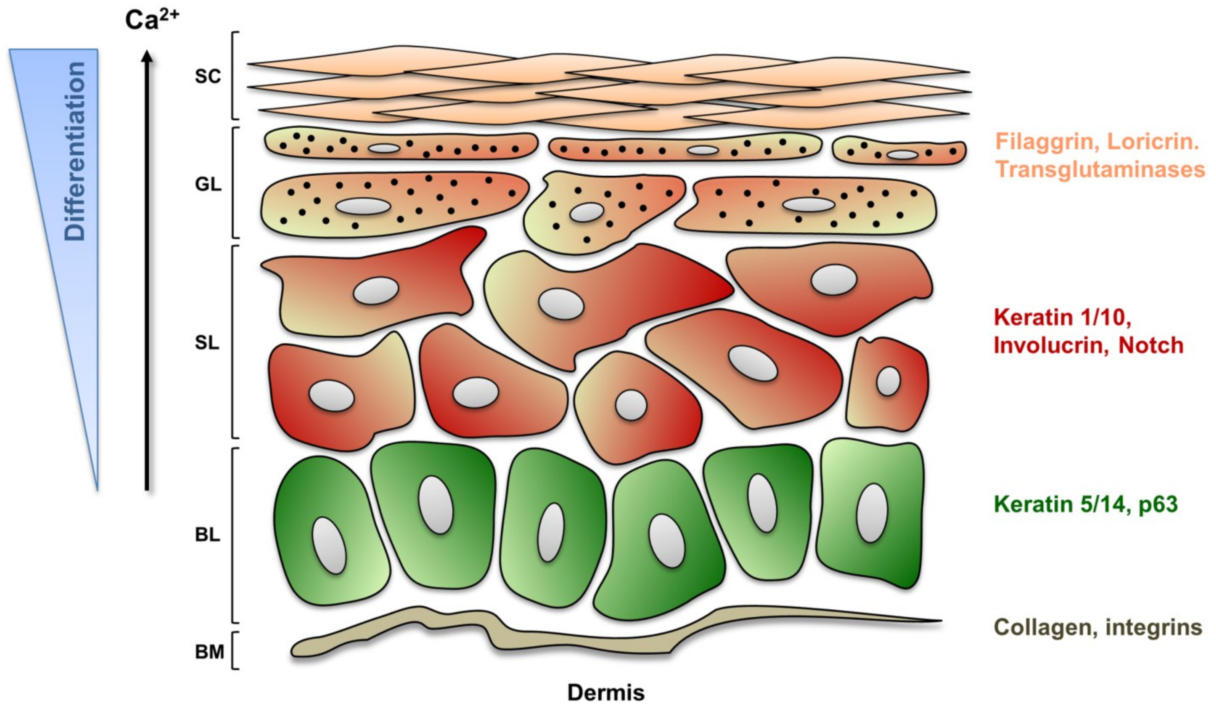


Figure 1.8 Model for epidermal stratification
The layers composing the multistratified squamous epithelium of the skin with their characteristic protein markers are shown. The names of the layers are abbreviated as: BM, basal membrane; BL, basal layer; SL, spinous layer; GL, granular layer; SC, stratum corneum. The calcium concentration, which goes increasing throughout the layers of the stratified epithelium, is an index of progression of cell differentiation.

1.5.2 Pathways involved

Whereas the morphological changes that occur during epidermal development have been extensively studied, the molecular mechanisms that govern this process still remain poorly understood (Koster and Roop 2007). Wnt and Sonic-hedgehog pathways contribute to maintaining the proliferative potential of the basal stem cells, via their signalling components β-catenin and Gli2 (Zhu and Watt 1999; Bigelow, Jen et al. 2005). The main known pathways involved in skin differentiation and homeostasis, however, are Notch and p63 pathways, whose importance has been underscored by the phenotypes of their mutant mice. The dynamic equilibrium existing in the skin between proliferating and terminally differentiated keratinocytes is in fact regulated by the reciprocal antagonistic activity of p63 and Notch proteins, expressed in an inverse gradient in the upper versus lower layer (Figure 1.9).

1.5.2.1 Role of Notch signalling in epithelial differentiation

The Notch pathway is one of the main signals involved in controlling differentiation in the skin. Its expression is restricted to the supra-basal differentiated layers of the squamous epithelium and absent from the proliferative basal layer. In keratinocytes, increased Notch activity causes exit from cell cycle and commitment to differentiation (Lowell et al, 2000; Rangarajan et al 2001; Nickoloff et al 2002), whereas down-modulation or loss of Notch1 function promotes carcinogenesis (Talora et al, 2002; Nicolas et al 2003). Mechanistically, activated Notch1 in keratinocytes induces $p21^{WAF1}$ expression, leading to cell cycle exit and terminal differentiation (Rangarajan et al, 2001). It also directly activates caspase 3, which is required in embryonic keratinocyte terminal differentiation (Okuyama, 2004). Notch-mediated $p21^{WAF1}$ expression is also involved in down-regulation of the Wnt pathway, which is believed to be important for the maintenance of the stemness properties of the basal keratinocytes (Zhu and Watt 1999). $p21^{WAF1}$ has, in fact, a direct transcriptional inhibitory effect on the gene expression of the Wnt ligand Wnt4 (Devgan, Mammucari et al. 2005).

Additionally, Notch1 can directly promote the expression of epithelial differentiation markers, such as K1 and involucrin, and can down-regulate integrins' expression (Rangarajan, Talora et al. 2001) (Figure 1.9).

Other studies proved the existence of a direct negative cross-talk between Notch and p63 (implicated in the promotion of keratinocyte self-renewal), which regulates the balance between proliferation and differentiation (Figure 1.9). Work by Nguyen and colleagues showed that Notch1 activation results in down-regulation of ΔNp63α (the main p63 isoform expressed in the skin) at mRNA and protein levels, both *in vitro* (human and mouse keratinocytes) and *in vivo* (mouse epidermis) (Nguyen, Lefort et al. 2006). The precise mechanism at the origin of this inhibition has not been totally clarified. The model proposed involves a Notch-mediated down-regulation of interferon-responsive-factors (IRFs), which are positive regulators of p63, and activation of the nuclear factor-κB (NF-κB) signalling pathway, which instead is a negative regulator of p63 (Nguyen, Lefort et al. 2006). This regulation however may be just cell-type specific, since the same effect cannot be observed in other cell models, such as fibroblasts (Ross and Kadesch 2004).

Conversely, Notch expression could also be potentially regulated by transcriptional factors of the p53 family, as they have a DNA recognition sequence on the *Notch1* promoter. Indirect evidence showed that mouse epidermis lacking p63 displays defective expression of Notch1, and conversely, activation of p53 in human keratinocytes leads to Notch expression (Laurikkala 2006 and DiRenzo Oncogene 2003). Despite these findings, convincing evidence for direct transcriptional regulation of the *Notch* promoter by p53 and p63 is still missing.

Consistent with its role in restricting growth and promoting keratinocytes differentiation, mice with a deletion of the *Notch1* gene in the epidermis spontaneously develop cutaneous basal cell carcinoma (Nicolas, Wolfer et al. 2003). Moreover, transgenic mice expressing the pan-Notch inhibitor, Dominant Negative Mastermind-Like 1 (DNMAML1), repressing Notch signalling exclusively in the epidermis, spontaneously develop cutaneous squamous cell carcinoma (Proweller, Tu et al. 2006). Similarly, mouse skin affected by conditional ablation of the downstream mediator of the Notch

signalling pathway RBP-Jκ, presented epidermal cyst formation and reduction of the thickness of the spinous layer (Yamamoto, Tanigaki et al. 2003; Blanpain, Lowry et al. 2006). Conversely, induced over-expression of NICD in the mouse skin, driven by *K14*- or *involucrin*-promoters, caused an expansion of the spinous layer (Uyttendaele, Panteleyev et al. 2004; Blanpain, Lowry et al. 2006).

1.5.2.2 Role of p63 signalling in epithelial differentiation

As the generation of Notch mutant mice revealed its importance in epidermis homeostasis, the same can be said for transgenic mice lacking p63. Indeed, *p63*$^{-/-}$ mice have dramatic defects in the epidermis and all the epithelial appendages. Epidermal stratification does not take place in these mice, which are deprived of any skin stratification and die of dehydration early after birth (Mills 1999 and Yang 1999). As previously mentioned, p63 can exist in several forms, mainly distinguished as full-length TA and truncated-ΔN isoforms. The discovery of an additional trans-activation domain at the C-terminal of ΔNp63, showed that the ΔN isoforms do not just behave as dominant negative of the TA isoforms, but are also capable of inducing their own program of gene transcription (Candi, Dinsdale et al. 2007). To date, it is still unclear what specific contribution is given by the single isoforms, TA and ΔN, to the development of epithelial stratification. A recent work by Dr Candi and co-workers tried to address this problematic, by re-introducing single p63 isoforms into a *p63*$^{-/-}$ background (Candi, Rufini et al. 2006). Because only the ΔNp63-expressing mutant led to a rescue, though partial, of the *p63*$^{-/-}$ phenotype, this isoform is believed to be crucial for the process of epithelisation. Nevertheless, the re-introduction of both isoforms together, TA and ΔN, resulted in a higher degree of epithelial rescue, suggesting TAp63 also contributes to epithelial stratification (Candi, Rufini et al. 2006). Its relatively late timing of expression during mouse development (after E13) indicates TAp63 probably contributes in promoting only the last step of differentiation in mature keratinocytes, while ΔNp63 is more active in the early stages of development when it sustains proliferation of basal cells, leading to expansion of the epidermis. ΔNp63 is consistently expressed in the skin throughout mouse development, from around day E8.5 (Laurikkala, Mikkola et al. 2006).

Even after day E13, when TAp63 starts being expressed, ΔNp63 still represent the most abundant p63 isoform in the epidermis (99% versus 1% of TAp63). Other sources however claim that the TA-isoforms are the first to be expressed in epidermal development, adding further complications to the present scenario (Koster and Roop 2007). The field however agrees that the ΔN-isoforms, α and to a lesser extent γ, are the ones which are predominately expressed and have the major role in epithelial morphogenesis (Koster and Roop 2007 and Candi 2007). ΔNp63 is strongly down-modulated with cell differentiation and in the adult epithelia its expression is restricted to transit-amplifying and stem cells residing in the basal layer. Its general role is to promote proliferation of the keratinocytes, allowing stratification to occur in development and guaranteeing skin renewal in the adult. At the molecular level, this is realised by various means. First, ΔNp63 can directly inhibit the expression of $p21^{WAF1}$, 14-3-3σ, which are known to promote terminal differentiation in the epidermis (Westfall, Mays et al. 2003), as well as the expression of known differentiation markers such as filaggrin and loricrin (King, Ponnamperuma et al. 2006). Second, ΔNp63 can directly promote the expression of the basal keratins K5 and K14 (Candi 2006, Romano 2007) (Figure 1.9). Finally, ΔNp63 can affect Notch signalling pathway at different levels. ΔNp63 can counteract Notch's effect on the expression of some of its target genes, and suppress Hes1 and $p21^{WAF1}$ expression, while promoting expression of integrin receptors and Wnt4 (Nguyen, Lefort et al. 2006; Okuyama, Ogawa et al. 2007). Alternatively, ΔNp63 was shown to synergise with the Notch pathway during the early stages of differentiation, via a paracrine mechanism involving the expression of Jagged ligands, which leads to activation of Notch in neighbouring cells (Sasaki, Ishida et al. 2002). During development, ΔNp63 and Notch can also cooperate in promoting the expression of the early differentiation marker K1 (Nguyen, Lefort et al. 2006) (Figure 1.9). Additionally, p63 was reported to be capable of binding the *Notch1* promoter directly in keratinocytes, although little evidence of any transcriptional effect has been reported so far (Laurikkala, Mikkola et al. 2006).

Regarding its own expression, despite reports showing activating effects by p53, STAT3, Beta-catenin or ΔNp63 itself (Harmes, Bresnick et al. 2003; Lanza, Marinari et al. 2006; Chu, Dai et al.

2008; Ruptier, De Gasperis et al. 2011), the transcriptional modulation of ΔNp63 is, at date, largely unknown.

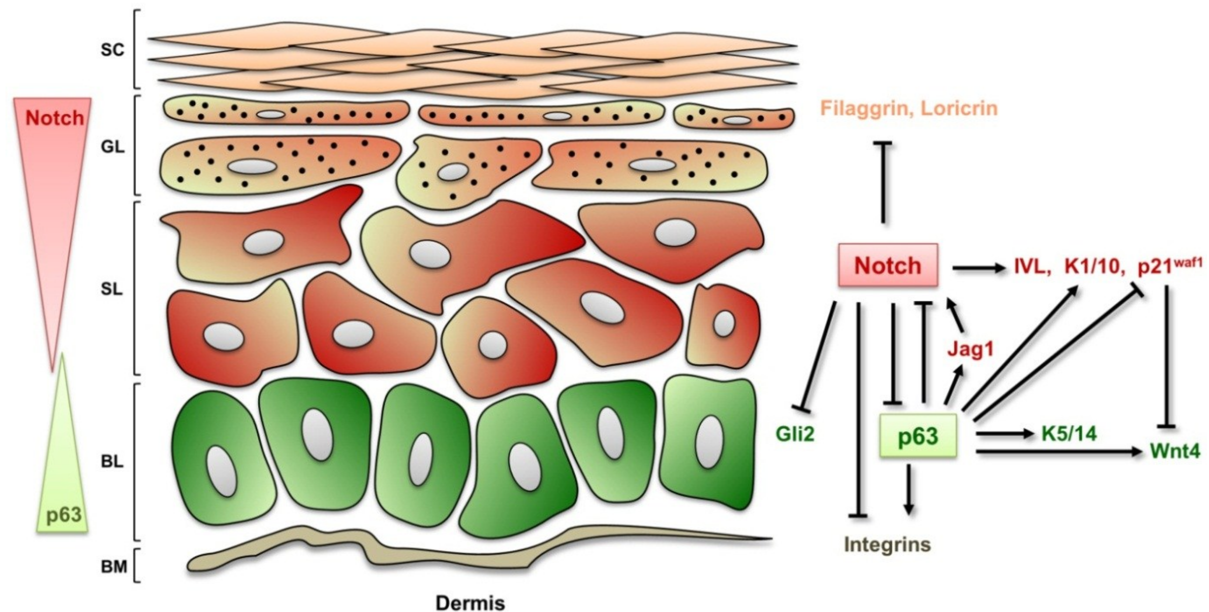


Figure 1.9 Interplay between Notch and p63 in regulating epithelial stratification
Cross-talk between p63 and the Notch pathway, whose gradients of expression proceed in opposite directions throughout the stratified epithelium (see left side of the figure), is fundamental for maintaining the correct balance between self-renewing and terminally differentiated keratinocyte populations. P63, expressed exclusively in the basal cell population, is important for maintaining the proliferative potential of these cells, by blocking the expression of cell-cycle inhibitor $p21^{waf1}$ and promoting the pro-proliferative Wnt pathway via induction of Wnt4 expression. Additionally p63 positively regulates the expression of basal cells keratins, K5 and K15 and promotes cell-adhesion to the basal membrane inducing integrins expression. Conversely Notch, which is active in the upper layers, promotes programs for terminal differentiation, by inducing cell-cycle withdrawal effector $p21^{waf1}$, which in turn can also down-regulate Wnt pathway via inhibition of Wn4 expression. Notch can also inhibit other basal-cell-active pathways, such as Sonic-hedgehog, via transcriptional repression of Gli2. Finally, Notch directly activates the expression of spinous and granular layer markers, such as involucrin (IVL), K1 and prevents the expression of upper markers filaggrin and loricrin. Beyond the induction of differential programs of gene transcription, Notch and p63 also antagonise each other's expression directly. Concomitant with this antagonistic effect, p63 can also induce Notch pathway activation via up-regulation of Jagged 1 expression (Jag1). BM, basal membrane; BL, basal layer; SL, spinous layer; GL, granular layer; SC, stratum corneum.

1.6 Epithelial cancer

1.6.1 Overview

Cancer is a disease mainly of epithelial cells; around 80% of human tumours derive from epithelial cells. Such tumours are called carcinomas, have the capability to invade surrounding tissues and organs, and may metastasize, or spread, to lymph nodes and other sites.

Carcinomas, like all neoplasias, are classified by their histopathological appearance. Adenocarcinoma and squamous cell carcinoma, two common descriptive terms for epithelial tumours, reflect the fact that these cells may have glandular or squamous cell appearances respectively. As the epithelial cells are normally present in different organs and in various form of organization, the aetiology of carcinomas varies depending on the physiological context. In general, as assumed for the other types of tumour transformation, dysfunctions in the major signal transduction pathways, such as Delta/Notch, Wnt/Frizzled, Hedgehog/Patched, TGFβ/BMPs, and RTKs, have been selectively identified in carcinomas. Mutations in the *p53* gene have been detected in 50% of all human cancers and in almost all skin carcinomas (Basset-Seguin, Moles et al. 1994).

1.6.2 SCC

Neoplasms originating from cutaneous epithelial cells, including adenocarcinoma, Basal Cell Carcinoma (BSC) and Squamous Cell Carcinoma (SCC), are the most common among the carcinomas with annual incidences of nearly 1 million and 250,000 cases in the United States alone (Bagheri and Safai 2001).

SCC, which represents approximately 20% of all non-melanoma skin cancers in the US (Bagheri and Safai 2001), is the most common cancer type with metastatic properties in the US and worldwide, although it occurs less frequently than BCC (Czarnecki, Staples et al. 1994; Miller and Weinstock 1994).

SCC is a form of epithelial cancer that may occur in many different organs, including the skin, lips, mouth, oesophagus, urinary bladder, prostate, lungs, vagina, anus and cervix. In these organs, SCC always arises from the keratinising cells of the squamous epithelium. In the skin it can be distinguished from BCC, which begins in a different layer of the epidermis, the deep basal cell layer, and from adenocarcinoma, which originates from the ductal epithelium. Of the three, SCC is the most malignant tumour type with the highest metastatic potential, and the most refractory to treatment. SCC can arise *de novo* or from precursor lesions (in actinic keratosis up to 60% of the time) (Marks, Rennie et al. 1988), and displays metastatic potential with frequencies approaching 12.5%. Variants of SCC include noninvasive (well-differentiated) and invasive (high risk, poorly differentiated). Because it originates from differentiated layers of the epithelium, SCC retains features of the expression pattern characteristic of squamous cell differentiation, such as the presence of keratins 1 and 10.

1.6.2.1 Aetiology

The formation of SCC is based on genetic background, combined with environmental factors. The principal environmental risk factor for the SCC of the skin is the chronic exposure to ultraviolet (UV) light. Thus, individuals with fair or sun-damaged skin are most susceptible. When UV-induced mutations occur in the “guardian of genome” *p53* gene, altering its capacity to induce apoptosis and cell-cycle arrest, there is a high chance of developing BCC and SCC. Mutations in *TP53* are, in fact, detected in about 56% of BCC and 90% of SCC of the skin (Ziegler, Jonason et al. 1994; Soehnge, Ouhtit et al. 1997). In general, alterations in *p53* gene found in all SCC types, which account for about 50% of the cases, seem to be principally important only for the UV-induced tumours (van Kranen, Westerman et al. 2005), as germline mutations in *TP53* in human (Li-Fraumeni syndrome) do not increase the risk of SCC formation. Additionally, *p53* (-/-) mice develop spontaneous lymphoid malignancies and sarcomas, but fail to develop spontaneous carcinomas (Donehower, Harvey et al. 1992). However, upon chemical induction of skin tumours, *p53* (-/-) mice showed an

enhanced progression from papillomas to carcinomas compared to wild type mice (Kemp, Donehower et al. 1993).

Taken together, these observations suggest that p53 inactivation may not be a critical rate-limiting step in SCC initiation, but is instead mainly important for the progression of the malignancy and, additionally, for causing susceptibility in the case of the radiation-induced SCCs. Interestingly, what seems instead to be a primary natural cause for the formation of SCCs is the aberrant over-expression of the p53-family member p63 (Hibi, Trink et al. 2000). Tumours with up-regulated p63 expression also showed frequent down-modulation of the Notch pathway (Lefort, Mandinova et al. 2007).

Other additional genetic alterations that can be found in SCC are the inactivation of $p16^{INK4a}$ and $p14^{ARF}$, two genes generated by the same gene locus *CDKN2A* and functionally responsible for G cell cycle regulation and MDM2-mediated down-regulation of p53 (Kubo, Urano et al. 1997; Soufir, Moles et al. 1999).

1.6.2.2 Role of Notch signalling in SCC

Consistent with its role in promoting terminal differentiation in the epidermis, the activity of Notch was found significantly reduced in in SCC and BCC samples (Thelu, Rossio et al. 2002; Lefort, Mandinova et al. 2007), making it a tumour suppressor protein in the skin. As mentioned earlier on in the introduction, mouse models carrying disruption in Notch signalling pathway led to epidermal abnormalities and spontaneous formations of SCC or BCC. The BCC tumours formed in the $Notch1^{-/-}$ mouse model showed an increase in Shh (high Gli2 expression) and Wnt signalling (presence of nuclear β-catenin) (Nicolas, Wolfer et al. 2003). Similarly, tumours found in the *DNMAML1* knock-in mouse model (downstream inhibition of all Notch proteins) presented up-regulation of active β-catenin, accompanied by increase in nuclear Cyclin-D1 (Proweller, Tu et al. 2006). These observations are consistent with the fact that Wnt and Shh are pathways normally down-regulated by Notch activity in the epidermis (Radtke and Raj 2003). According to the authors of these works, the absence of Notch1 from the skin would allow Shh and Wnt pathways to be active in regions where

they are normally repressed, setting the scene for tumour development. Furthermore, Notch1 deficiency in the skin was shown to accelerate chemical-induced carcinogenesis. Because chemical-induced mutations tend to result in activation of the Ras gene, further experiments were performed to show that Notch1-deficient keratinocytes expressing an activated form of Ras were able to form SCCs *in vivo* upon injection into immunosuppressed mice (Nicolas, Wolfer et al. 2003). Similarly, in primary human keratinocytes with active Ras, the suppression of Notch signalling resulted in aggressive SCCs in a xenograft model (Lefort and Dotto 2004). Consistent with these experimental observations, the expression of Notch1-2 was reduced in a panel of oral and skin SCC cell lines, as well as in SCC human biopsy specimens (Lefort, Mandinova et al. 2007). A proposed model for Notch pathway down-regulation during SCC involves the possibility that p53 could normally act as a positive modulator of Notch1 expression; when p53 function is compromised by cancer-related mutations, Notch would therefore follow the same fate (Lefort, Mandinova et al. 2007). Another possibility is that Notch down-modulation is a consequence of p63 aberrant up-regulation, as p63 has been shown to have antagonistic effects towards Notch in the normal skin, and its expression is frequently up-regulated in SCC. This hypothesis is supported by the observation that Notch expression appears to be down-regulated particularly in those SCCs where p63 is found up-regulated (Lefort, Mandinova et al. 2007).

1.6.2.3 Role of p63 signalling in SCC

A common characteristic of almost all SCCs (about 90% of the cases) (Di Como, Urist et al. 2002) is the high level of expression of p63, which is often due to gene amplification (Hibi, Trink et al. 2000; Yamaguchi, Wu et al. 2000). As in the normal squamous epithelium, the major isoform of p63 expressed in SCC is the ΔNp63α isoform. A correlation between high levels of ΔNp63 and poor prognosis has been also established in SCC of the head and neck (Choi, Batsakis et al. 2002).

Mechanistically, the tumourigenic effect caused by the up-regulation of ΔNp63 expression seems to be linked with its inhibitory effects towards p53 and p73 on their capacity to induce cell death via

cell-cycle arrest, apoptosis and senescence (Levrero, De Laurenzi et al. 2000; Rocco, Leong et al. 2006). However, following the discovery of a second transactivation domain present in the C-terminal region of ΔNp63, other studies have shown that ΔNp63 possesses peculiar transcriptional properties which can promote a stem-like proliferation in SCC tumour cells (Ghioni, Bolognese et al. 2002). For instance, in squamous carcinoma cell lines from the head and neck type, ΔNp63 was shown to regulate the expression levels of CD44 and basal layer K14, known markers of more immature precursors, promoting the maintenance of a non-differentiated phenotype (Boldrup, Coates et al. 2007).

Another well characterised gene target for ΔNp63 is the cell-cycle suppressor $p21^{waf1}$, whose inhibition in cancer allows tumour cells to become unresponsive towards growth-arrest signals. Indeed, inhibiting $p21^{WAF1}$ expression has been hypothesised to be a mechanism by which over-expression of ΔNp63 can drive proliferation in SCC (Chiang, Chu et al. 2009). Another work has proposed that the ability of ΔNp63 to maintain the self-renewing capacity of normal keratinocytes as well as cancer cells is partly due the transcriptional repression of the *Notch1* gene (via binding a p53-responsive element present on Notch1 promoter), establishing a link with the observation that high p63-expressing tumours have low Notch expression (Yugawa, Narisawa-Saito et al. 2010). Recently, more ΔNp63 target genes involved in different cellular functions have been discovered, including a chromatic remodelling gene, *Lsh*, suggesting ΔNp63 may promote tumour progression by acting at several different levels (Keyes, Pecoraro et al. 2011).

Beyond its interaction with the other p53 family members and the effects derived from its own transcriptional properties, ΔNp63 up-regulation has been also shown to contribute to tumourigenesis via the activation of the beta-catenin signalling pathway, through reduction of its phosphorylation and consequent intra-nuclear accumulation (Patturajan, Nomoto et al. 2002). Nuclear beta-catenin is another factor often present in SCC, and a recent report suggests it can also promote the expression of ΔNp63, acting in a positive feedback loop (Ruptier, De Gasperis et al. 2011).

Despite the amount of recent data, the precise mechanism by which p63 mediates cell-survival and proliferation in SCC is still unclear and subject to investigation. Another important open question is what leads to altered expression of p63 in cancer cells, as putative binding sites for several transcriptional factors have been identified, but only modest effects in terms of activation have been reported to date, both *in vitro* and *in vivo*.

Aim of the study

The aim of this work is to investigate the role of ASPP2, a member of the evolutionarily conserved ASPP (ankyrin repeats, SH3 domain and proline-rich region containing protein) family of proteins, in preventing the formation of tumours of epithelial origin. The research originated from the observation that Balb/c mice lacking of ASPP2 expression develop spontaneous squamous cell carcinomas, one of the most common types of epithelial cancer. This remarkable phenotype led us to examine the expression pattern of ASPP2 in the squamous epithelium of the skin and subsequently, its relationship with known pathways involved in maintaining the homeostasis of the tissue, such as p63 and Notch.

Chapter II: Materials and Methods

2.1 Materials

2.1.1 Reagents

All chemicals, unless otherwise stated, were obtained from Sigma (Sigma, MO, USA) or BDH Chemicals (UK). Autoradiography films (Hyperfilm) and ECL (Enhanced Chemi-Luminescence) reagents were purchased from Amersham Pharmacia Biotech (UK). Nitrocellulose membrane was purchased from Whatman, Germany. All tissue culture dishes and flasks were obtained from BD (Becton, Dickinson and Company, NJ USA). 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.

COmplete™ protease inhibitor cocktail

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

DAPI staining solution

Powdered DAPI was dissolved at 1mg/ml and stored at -20°C in aliquots.

DNA Marker

100bp and 1kb DNA ladders were purchased from New England Biolabs (UK) 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.

Freezing Medium

90% (v/v) Fetal Calf Serum (FBS)

10% (v/v) DMSO

Made fresh every time.

GNT buffer

50 mM KCL

1.5 mM $MgCl^{2+}$

10 mM Tris-HCl (ph: 8.5)

0.01% gelatine

0.45% NP-40

0.45% Tween-20 (Fisher)

Kanamycin stock solution

Kanamycin was dissolved at 50mg/ml, filter sterilized and stored at -20°C in aliquots.

LB (Luria Broth)-agar plates

LB-agar powder was dissolved as recommended by the manufacturer's instruction and autoclaved. The solution was then cooled to 55°C before addition of 100µg/ml ampicillin or 50µg/ml kanamycin. 20ml of LB-agar was then poured into 10cm dishes, allowed to set and plates were stored at 4°C.

LB medium

LB powder was dissolved as recommended by the manufacturer's instruction, autoclaved and stored at RT.

Lipotransfection reagents

Lipotransfections were performed with Fugene 6 (Roche) or Lipofectamine®2000 (Invitrogen), following the manufacturer's recommendations or as otherwise stated.

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 Lipofectamine2000 (Invitrogen), following the manufacturer's recommendations or as otherwise stated.

Mowiol Solution

6ml Glycerol

2.4g Mowiol 4-88 (Calbiochem)

12ml Tris-HCL 200 mM (pH 8.5)

The solution was made up to 50ml final volume, heated to 60°C overnight to dissolve all components, filtered through a 0.45µm filter and stored in aliquots at -20°C.

NETN buffer

50mM Tris pH 8.0

150mM NaCl

1mM EDTA

1% (v/v) NP40

Stored at RT. Complete protease inhibitors were added before use.

Paraformaldehyde Solution (4%)

A 20% paraformaldehyde stock solution was made by dissolving 20g of paraformaldehyde powder in 700 ml of PBS. The solution was heated to dissolve the powder and the volume made up to 1L with PBS. The solution was diluted to 4% with PBS and stored at -20°C in aliquots.

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)

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.

Primers used in PCR

All primers were synthesized by Operon, Germany. The lyophilized primers were dissolved in sterile water to a stock concentration of 100μM.

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

Prestained protein markers were purchased from New England Biolabs (UK) 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

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^{TM} 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).

RNeasy Mini Kit

Kit for purification of total RNA from cells and tissues purchased from Qiagen and kept at room temperature.

Purified Notch ligands

Recombinant mouse Dll1 Fc, rat Jagged1 Fc and human Dll4 were purchased by R&D System (UK). Lyophilised proteins were reconstituted at 200µg/mL in sterile PBS, 0.1% BSA.

SDS Solution

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

2X SDS-PAGE loading dye

100mM	Tris-HCl (pH 6.8)
4% (w/v)	SDS
20% (v/v)	Glycerol
0.2% (w/v)	Bromophenol Blue

Before use, 5% (v/v) β-Mercaptoethanol was added fresh to the 2X SDS-PAGE loading dye.

5X SDS-PAGE loading dye

250mM	Tris-HCl
10% (w/v)	SDS
50% (v/v)	Glycerol
12.5% (v/v)	β-Mercaptoethanol
0.5% (w/v)	Bromophenol blue

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

62.5 mM 15.5 ml of 1 M Tris-HCl, pH 6.7

100 mM 1.75 ml of β-mercaptoethanol

2% 5 g SDS

Make up to 250 ml in distilled water.

SuperScript® First Stand Synthesis System

Kit for cDNA production from RNA purchased from Invitrogen and stored at -20°C

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.

TO-PRO

The nuclear dye was purchased as liquid from Invitrogen and stored at -20°C in aliquots.

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)

121g Tris base

36.53g NaCl

250ml Tween-20

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

Triton® X-100

A 20% (v/v) stock solution in PBS was made and stored at room temperature

Water

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

2.1.2 Chromatin immunoprecipitation (ChIP) materials

Formaldehyde

37% Formaldehyde was used at a final concentration of 1%.

Glycine

Final concentration of 0.125M glycine was used by dissolving 0.9383g of glycine in 10ml water.

Sodium Hydrogen Carbonate ($NaHCO_3$)

0.042g of $NaHCO_3$ was used.

Commercial kits

Easy ChIP Kit (EZ ChIP) from Millipore. The kit contains: ChIP dilution buffer, SDS Lysis Buffer, Protease inhibitors cocktail, Low Salt Immune Complex Wash Buffer, High Salt Immune Complex Wash Buffer, LiCl Immune Complex Wash Buffer, TE buffer, 0.5M EDTA, 1M Tris-HCl, 5M NaCl, Proteinase K, and RNase A. The kit was stored at 4°C.

Qiaquick Purification Kit from Qiagen was used according to manufacturer's instructions for DNA extraction. The kit contained spin columns, PBI buffer, PE buffer and collection tubes

2.1.3 SDS-polyacrylamide Gels

Table 2 Gels composition

	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).

2.1.4 Antibodies

2.1.4.1 Primary antibodies

Table 3 List of primary antibodies

antigen	Ab name	host/type	source	applications
1methyl-Histone H3 (K4)	-	rabbit pAb	Cell Signaling	ICC/IF (1:200)
3methyl-Histone H3 (K27)	-	rabbit pAb	Millipore	ICC/IF (1:200)
3methyl-Histone H3 (K4)	C42D8	rabbit pAb	Cell Signaling	ICC/IF (1:200)
3methyl-Histone H3 (K9)	-	rabbit pAb	Millipore	ICC/IF (1:200)
Actin	C-2	mouse mAb	Santa Cruz (sc-8432)	WB (1:1000)
anti-flag	F3165	mouse mAb	Sigma Aldrich	ICC/IF (1:400), WB (1:1000)
anti-flag	F7425	rabbit pAb	Sigma Aldrich	ICC/IF (1:400), WB (1:1000)
anti-His	H-15	mouse mAb	Santa Cruz (sc-803)	WB (1:1000)
ASPP2	S-80	rabbit pAb	serum	IHC (1:400)
ASPP2	LX141.2	mouse mAb	ascite	ICC/IF (1:100), WB (1:1000)
ASPP2	S-32	rabbit pAb	serum	ICC/IF (1:100), WB (1:1000)
ASPP2	DX54.10	mouse mAb	ascite	IHC (1:400), ICC/IF (1:100), WB (1:1000), IP
ASPP2	BP.77	rabbit pAb	serum	WB (1:1000), IP
Cdk8	-	rabbit pAb	Abcam (ab2955)	ICC/IF (1:200)
Cyclin-D1	M-20	rabbit pAb	Santa Cruz (sc-718)	IHC (1:400)
E-cadherin	610182	mouse mAb	BD Pharmigen	ICC/IF (1:200)
Envoplakin	M-20	goat pAb	Santa Cruz (sc-16751)	WB (1:1000)
Gli2	AF3635	mouse mAb	R&D Systems	IHC (1:200)
HDAC1	H-51	rabbit pAb	Santa Cruz (sc-7872)	ICC/IF (1:200)
HP1α	GA-62	mouse mAb	Santa Cruz (130446)	ICC/IF (1:200)
HP1β	H-50	rabbit pAb	Santa Cruz (sc-20699)	ICC/IF (1:200)
HP1γ	-	rabbit pAb	Millipore	ICC/IF (1:200)
iASPP	LX49.3	mouse mAb	ascite	ICC/IF (1:200), WB (1:1000)
Keratin-1	AE1	mouse mAb	Abcam (ab9286)	IHC (1:400)
Keratin-14	PRB-155P-100	rabbit pAb	Covance	IHC (1:400)

Keratin-18	C-04	mouse mAb	Abcam (ab668)	IHC (1:400)
Ki67	-	rabbit pAb	Abcam (ab15580)	IHC (1:400)
Ku80	Ab-2	mouse mAb	Thermo Fisher	ICC/IF (1:200)
MAML1	-	rabbit pAb	Abcam (ab70486)	ICC/IF (1:200)
myc-tag	9E10	mouse mAb	ascite	WB (1:1000)
Notch1	C-20	goat pAb	Santa Cruz (sc-6014)	IHC (1:400)
Notch1	C-20	rabbit pAb	Santa Cruz (sc-6014-R)	IHC (1:400), WB (1:1000)
Notch1	Val1744	rabbit pAb	Abcam (ab52301)	ICC/IF (1:200)
Notch1	ChIP grade	rabbit pAb	Abcam (ab27526)	ChIP (2µg)
p300	C-20	rabbit pAb	Santa Cruz (sc-585)	ICC/IF (1:200)
p53	DO-1	mouse mAb	Santa Cruz (sc-126)	ICC/IF (1:200)
p63	4A4	mouse mAb	Santa Cruz (sc-8431)	IHC and ICC/IF (1:400), WB (1:1000)
p63	-	rabbit pAb	Abcam (ab53039)	IHC and ICC/IF (1:400)
Par3	-	rabbit pAb	Millipore	ICC/IF (1:200)
PCNA	PC-10	mouse mAb	ascite	IHC (1:200)
RBP-Jκ	H-50	rabbit pAb	Santa Cruz (sc-28713)	ICC/IF (1:200), WB (1:1000)
RBP-Jκ	-	mouse pAb	Abcam (ab21930)	ICC/IF (1:200), WB (1:1000)
SC-35	S4045	mouse mAb	Sigma Aldrich	ICC/IF (1:400)
V5-tag	-	chicken pAb	Abcam (ab9113)	ICC/IF (1:400)
V5-tag	SV5-Pk1	mouse mAb	Serotec	ICC/IF (1:400), WB (1:1000)
V5-tag	-	rabbit pAb	Abcam (ab9116)	ICC/IF (1:400), WB (1:1000)
Vimentin	RV202	mouse mAb	Abcam (ab8978)	IHC (1:400)
β-tubulin	TUB 2.1	mouse mAb	Abcam (11308)	WB (1:1000)

2.1.4.1 Secondary antibodies

Ab name	host/type	source	applications
Alexa Fluor® 488 F(ab')2 fragment anti-rabbit IgG	goat	Invitrogen	IF (1:400)
Alexa Fluor® 488 F(ab')2 fragment anti-mouse IgG	goat	Invitrogen	IF (1:400)
Alexa Fluor® 546 F(ab')2 fragment anti-rabbit IgG	goat	Invitrogen	IF (1:400)
Alexa Fluor® 546 F(ab')2 fragment anti-mouse IgG	goat	Invitrogen	IF (1:400)
Alexa Fluor® 647 Anti-Mouse IgG (H+L)	Donkey	Invitrogen	IF (1:400)
Alexa Fluor® 488 Anti-Mouse IgG (H+L)	Donkey	Invitrogen	IF (1:400)
Alexa Fluor® 568 Anti-Goat IgG (H+L)	Donkey	Invitrogen	IF (1:400)
Alexa Fluor® 488 Anti-Rabbit IgG (H+L)	Donkey	Invitrogen	IF (1:400)
Alexa Fluor® 647 Anti-Rabbit IgG (H+L)	Donkey	Invitrogen	IF (1:400)
Alexa Fluor® 546 Anti-Chicken IgG (H+L)	Goat	Invitrogen	IF (1:400)
Anti-Goat Immunoglobulins/HRP	rabbit	Dako	IHC (1:250), WB (1:2000)
Anti-Rabbit Immunoglobulins/HRP	swine	Dako	IHC (1:250), WB (1:2000)
Anti-Mouse Immunoglobulins/HRP	rabbit	Dako	IHC (1:250), WB (1:2000)

Table 4 List of secondary antibodies

Abbreviations – antibody (Ab), monoclonal antibody (mAb), polyclonal antibody (pAb), horse-radish peroxidase (HRP), Immunohistochemistry (ICH), Immunocytochemistry/Immunofluorescence (ICC/IF), Western blot (WB), Immunoprecipitation (IP), Chromatin Immunoprecipitation (ChIP).

2.1.5 Plasmids

Table 5 List of plasmids

name	vector	information	tag	Source
ASPP2	pcDNA3.1	human ASPP2	V5 and His	Dr S. Llanos, UCL, UK
iASPP	pcDNA3.1	human iASPP	V5 and His	Dr S.Llanos, UCL, UK
Control plasmid	pcDNA3.1	empty vector	V5 and His	Invitrogen
GFP	pEGFP-C1	green fluorescent protein (GFP)	-	Clontech
Notch1 intracellular domain (ID)	pcDNA3.1	human Notch1 ID	Myc	Dr T. Kadesh, University of Pennsylvania, USA
Renilla	pRL-TK		-	Promega
EVPL-Luciferase	pGL3	*envoplakin* promoter cloned 5’ to the firefly luciferase gene	-	Prof G. Melino, University of Leicester, UK
K14-Luciferase	pGL3	*K14* promoter cloned 5’ to the firefly luciferase gene	-	Prof G. Melino, University of Leicester, UK
ΔNp63α	pcDNA3	human ΔNp63α	-	Dr I. Shachar, Weizmann Institute of Science, Rehovot, Israel
MAML1	pFLAG-CMV2	human MAML1	flag	Dr L. Wu, Harvard Medical School, Boston, USA
MAML1(1–302)	pFLAG-CMV2	N-terminal fragment (1-302) of human MAML1	flag	Dr L. Wu, Harvard Medical School, Boston, USA
K10-Luciferase	pGL3	*K10* promoter cloned 5’ to the firefly luciferase gene	-	Dr B. Andersen, Laboratory University of California, USA
ΔNp63-Luciferase	pGL3	*ΔNp63* promoter cloned 5’ to the firefly luciferase gene	-	Dr E. Candi, University of Tor Vergata, Italy
ASPP2 mutant 1	pcDNA3.1	fragment (1-360) of human ASPP2	V5 and His	Dr S. Llanos, UCL, UK
ASPP2 mutant 2	pcDNA3.1	fragment (360-925) of human ASPP2	V5 and His	Dr S. Llanos, UCL, UK
ASPP2 mutant 3	pcDNA3.1	fragment (925-1128) of human ASPP2	V5 and His	Dr S. Llanos, UCL, UK
NICD K1946E	pcDNA3	human Notch ID containing K to E mutation at position 1946	-	Dr S.C. Blacklow, Harvard Medical School, Boston, USA
NICD E1950K	pcDNA3	human Notch ID containing E to K mutation at position 1950	-	Dr S.C. Blacklow
NICD K1946E/E1950K	pcDNA3	human Notch ID containing K to E mutation at position 1946 and E to K mutation at position 1950	-	Dr S.C. Blacklow
Hey2-luciferase	pGL3	*Hey2* promoter cloned 5’ to the firefly luciferase gene	-	Prof M. Gessler, Biozentrum, Germany

All plasmids constructed by our laboratory or received from other laboratories were sent to Operon for sequencing to confirm their identity.

2.1.6 Cell lines

2.1.6.1 Established cell lines

Table 6 List of established cell lines

name	tissue/type	source
H1299	human lung carcinoma	ATCC
MCF7	human breast carcinoma	ATCC
MDA-MB-231	human breast carcinoma	ATCC
UPCI-SCC-040	Human squamous cell carcinoma of the head and neck	Dr S. Feller, University of Oxford, UK
HSC3	Human squamous cell carcinoma of the head and neck	Dr S. Feller, University of Oxford, UK
UT-SCC-10	Human squamous cell carcinoma of the head and neck	Dr S. Feller, University of Oxford, UK
UT-SCC-14	Human squamous cell carcinoma of the head and neck	Dr S. Feller, University of Oxford, UK
UT-SCC-16A	Human squamous cell carcinoma of the head and neck	Dr S. Feller, University of Oxford, UK
UT-SCC-21	Human squamous cell carcinoma of the head and neck	Dr S. Feller, University of Oxford, UK
UT-SCC-24A	Human squamous cell carcinoma of the head and neck	Dr S. Feller, University of Oxford, UK
UT-SCC-30	Human squamous cell carcinoma of the head and neck	Dr S. Feller, University of Oxford, UK
UT-SCC-40	Human squamous cell carcinoma of the head and neck	Dr S. Feller, University of Oxford, UK
UT-SCC-67	Human squamous cell carcinoma of the head and neck	Dr S. Feller, University of Oxford, UK
UT-SCC-73	Human squamous cell carcinoma of the head and neck	Dr S. Feller, University of Oxford, UK
UT-SCC-74A	Human squamous cell carcinoma of the head and neck	Dr S. Feller, University of Oxford, UK
UT-SCC-76A	Human squamous cell carcinoma of the head and neck	Dr S. Feller, University of Oxford, UK
UT-SCC-87	Human squamous cell carcinoma of the head and neck	Dr S. Feller, University of Oxford, UK
UPCI-SCC-056	Human squamous cell carcinoma of the head and neck	Dr S. Feller, University of Oxford, UK
UPCI-SCC-075	Human squamous cell carcinoma of the head and neck	Dr S. Feller, University of Oxford, UK

UPCI-SCC-103	Human squamous cell carcinoma of the head and neck	Dr S. Feller, University of Oxford, UK
UPCI-SCC-122	Human squamous cell carcinoma of the head and neck	Dr S. Feller, University of Oxford, UK
UPCI-SCC-154	Human squamous cell carcinoma of the head and neck	Dr S. Feller, University of Oxford, UK
BICR-16	Human squamous cell carcinoma of the head and neck	Dr S. Feller, University of Oxford, UK
BICR-56	Human squamous cell carcinoma of the head and neck	Dr S. Feller, University of Oxford, UK
SCC-4	Human squamous cell carcinoma of the head and neck	Dr S. Feller, University of Oxford, UK
SCC-9	Human squamous cell carcinoma of the head and neck	Dr S. Feller, University of Oxford, UK
SCC-15	Human squamous cell carcinoma of the head and neck	Dr S. Feller, University of Oxford, UK
SCC-25	Human squamous cell carcinoma of the head and neck	Dr S. Feller, University of Oxford, UK
CAL-27	Human squamous cell carcinoma of the head and neck	Dr S. Feller, University of Oxford, UK
CAL-33	Human squamous cell carcinoma of the head and neck	Dr S. Feller, University of Oxford, UK
HSC4	Human squamous cell carcinoma of the head and neck	Dr S. Feller, University of Oxford, UK
OSC-19	Human squamous cell carcinoma of the head and neck	Dr S. Feller, University of Oxford, UK
OSC-20	Human squamous cell carcinoma of the head and neck	Dr S. Feller, University of Oxford, UK
SAS	Human squamous cell carcinoma of the head and neck	Dr S. Feller, University of Oxford, UK
SIHN-005A	Human squamous cell carcinoma of the head and neck	Dr S. Feller, University of Oxford, UK
SIHN-006	Human squamous cell carcinoma of the head and neck	Dr S. Feller, University of Oxford, UK
Saos-2	Human osteosarcoma	ATCC

Abbreviations – American Type Culture Collection (ATCC)

2.1.6.2 Primary cell lines

Table 7 List of primary cell lines

name	origin
Mouse Embryonic Fibroblasts (MEFs)	E13.5 mouse embryos
Mouse keratinocytes	epidermis dissected from mouse pups at P2

Abbreviations – embryonic day (E), post-natal day (P)

2.2 Methods

2.2.1 Tissue culture

Basic Media

Dulbecco's Modified Eagle's Medium (DMEM) was purchased from Gibco-BRL, UK and stored at 4°C. Eagle's Minimum Essential Medium (EMEM) with Earlie's balanced salts solution (EBSS), non-essential amino acids and L-Glutamine without Calcium Chloride was purchased from Lonza, MD USA.

Media supplements

Fetal 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.

Undifferentiated primary keratinocytes were cultured in Eagle's Minimum Essential Medium (EMEM) without calcium chloride, supplemented with 0.05mM $CaCl_2$, L-Glutamine, 1X Antibiotic Antimycotic Solution (Sigma, MO,USA) and 8% $CaCl_2$ chelate fetal calf serum (lab stock). When needed undifferentiated primary keratinocytes were differentiated by the addition of $CaCl_2$ in the medium to a final concentration of 1.2mM (Hennings, Holbrook et al. 1980).

Freezing/thawing of cells

Cells were grown to about 80% confluency and collected by trypsinization (as described above). The cell pellet was resuspended in the appropriate amount of freezing medium and aliquoted in cryovials (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 liquid nitrogen tank for long term storage.

To thaw cells from 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 for recovery.

2.2.2 DNA-RNA techniques

Agarose gel electrophoresis

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

DNA extraction from ear biopsies

Mice were ear-punched for identification and the resulting ear tissue was used for genotyping. Ear tissue was digested overnight at 55ºC in 100µl of extraction buffer GNT and 7.8 mg/ml of proteinase K (Quiagen). The digested tissue was boiled for 10 min at 97 ºC and 1 µl used for polymerase chain reaction (PCR) analysis.

DNA extraction from embryonic yolk sac

Embryos were carefully dissected from the uterus of the pregnant female, and a small part of the yolk sac (<25 mg) was excised from individual embryos and digested overnight at 55ºC in 200µl ATL buffer supplemented with 20µl of proteinase K (7.8 mg/ml, Quiagen). After the overnight incubation, 200µl of AL buffer and 200µl of ethanol (96-100%) were add to the mixture and vortexed from 15 sec. The mixture was then applied to a DNeasy Mini spin column and placed into a 1.5 ml microcentrifuge tube. The column was then centrifuged at 8000 rpm for 1 min. The flow-through was subsequently discarded and the column washed twice with 500µl of AW1 buffer first, then with 500µl of AW2 buffer. The column was placed into a new microcentrifuge tube and 200 µl of AE buffer added to the centre of the column. After 1 min, the column was centrifuged at 8000 rpm for 1 minute to elute the DNA. The obtained DNA was then used for PCR analysis.

Bacterial strains and culture

Chemically competent *Escherichia coli* strain alpha-select™ silver efficiency (Bioline) was used as a host for plasmid DNA. Bacteria were cultured in Luria Broth media containing the appropriate antibiotic (100µg/ml ampicillin or 50µg/ml kanamycin) in flasks shaking at 37°C.

ChIP

ChIP was performed following the protocol provided with the EZ ChIP Kit from Millipore.

Crosslinking and lysis

Cells were fixed in 1% formaldehyde and incubated at 37ºC for 10 minutes. Glycine was added to a final concentration of 125mM and cells were incubated for 5 minutes to quench unreacted formaldehyde. The media was aspirated off and the cells were washed twice with ice cold PBS and scraped into a 15 ml centrifuge tube. Cells were then centrifuged at 700 g, 4ºC for 5 minutes and the supernatant discarded. The SDS lysis buffer was warmed to room temperature. To each 1ml of SDS lysis buffer, 5µl of Protease Inhibitor Cocktail II were added. The cell pellets were resuspended in 250µl of SDS lysis buffer and 2µl of PMSF (1mM) was added.

Sonication to shear DNA

The lysates were sonicated using the Diagenode Bioruptor 200 on high for 40 cycles (30 seconds on/30 seconds off) to shear DNA. 10µl of DNA was taken from each sonicated fraction to analyze sheared chromatin size by gel electrophoresis.

Immunoprecipitation of crosslinked Protein/DNA

The remainder of the supernatants were split into two 1.5ml microfuge tubes and samples were diluted 10 fold with ChIP dilution buffer (0.01% SDS, 1.1% Triton-X100, 1.2mM EDTA, 16.7mM Tris-HCl (pH 8.1), 167mM NaCl. To reduce non-specific background precipitation, 30µl of Dynabeads Protein G (Invitrogen) were added to each eppendorf and incubated at 4ºC for 30 minutes with agitation. The beads were collected using a Magna Rack (Invitrogen), the supernatant was transferred to new microcentrifuge tubes and 50µl of the supernatant was removed for use as the

Input sample (stored at -80°C). To each tube, the antibody for immunoprecipitation was added: 2µg of anti-NICD (ab27526, Abcam) or 2µg of non-specific rabbit IgG as negative control. The samples were incubated for 4 hours at 4ºC with rotation.

To precipitate the chromatin:antibody complex, 40µl of dynabeads Protein G was added and the mixture was incubated for 1 hour at 4ºC with rotation. The complex was collected using Magna Rack and washed for 5 minutes at 4ºC with rotation with a low salt buffer (0.1% SDS, 1% Triton X- 100, 2mM EDTA, 20mM Tris-HCl, 150mM NaCl), high salt buffer (0.1% SDS, 1% Triton X- 100, 2mM EDTA, 20mM Tris-HCl (pH 8.1) and lithium chloride buffer (0.25M LiCl, 1% IGEPAL-CA630, 1% deoxycholic acid, 1mM EDTA, 10mM Tris, pH 8.1). Last 2 washes were performed at room temperature for 5 minutes with TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0).

Elution of Protein/DNA complexes

An elution buffer was made fresh by combining 500µl 10% SDS, 0.042g NaHCO3 and made up to 5ml with dH2O to elute the chromatin:antibody complex. Elution buffer was added to the pellet (100µl) and incubated at room temperature for 15 min with rotation. The eluted sample was left in a magnetic rack for 1 minute and then supernatant was collected and transferred to a new 1.5ml microcentrifuge tube. A further 100µl of elution buffer was added to the pellet and incubated at room temperature for 15 minutes with rotation.

Reversal of formaldehyde crosslinks

The formaldehyde crosslinks were reversed by addition of 8µl of 5M NaCl to the immunoprecipitated samples and to the Input fraction; and were incubated at 65ºC overnight. After overnight incubation, 1µl of RNAse A was added to the samples and incubated at 37ºC for 30 minutes. Then 4µl of 0.5 M EDTA, 8µl of 1M Tris-HCl and 1µl of proteinase K were added to the samples and incubated at 45ºC for 2 hours.

DNA extraction

DNA was extracted using Qiaquick Purification Kit Protocol.

Real-time quantitative PCR (RT-qPCR)

The RT-qPCR reaction was performed as described later in the apposite section. The primes used are reported in Table 8.

Transformation

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

Large scale preparation of plasmid DNA (maxi-prep)

A single bacterial colony was used to inoculate 5ml of LB/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 LB/antibiotic medium and shaken for a further 16 hours at 37°C. The cells were centrifuged at 6,000g 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.

RNA extraction and reverse transcription

RNA was extracted from cells or tissues according to the Qiagen RNeasy mini kit protocol, including a treatment with DNase I (Qiagen) to eliminate possible genomic DNA contamination. Up to 5 µg of total RNA were used to generate cDNA with the SuperScript® First Stand Synthesis System (Invitrogen) following the manufacture's protocol.

Real-time quantitative PCR (RT-qPCR)

Real-time quantitative PCR was performed on the 7500 real time PCR system (Applied Biosystem) using the QuantiTect SYBR Green PCR kit (Qiagen). Each reaction was performed in triplicate using 1 µl of cDNA in a final volume of 25 µl. The following thermal cycle was used for all the samples: 10 min-95°C; 40 cycles of 30s-95°C, 40s-primer specific annealing temperatures, 40s-72°C. For each experiment, the threshold was set to cross a point at which real-time PCR amplification was linear. Previously published primers were used for all the genes analyzed (sequences reported in Table 8). The expression level of each target gene was analysed based on ΔΔCt method, with *GAPDH* as internal control.

Table 8 List of primers

name	Sequence (5'-3')
GAPDH F	TGTCAGCAATGCATCCTGCA
GAPDH R	TGTATGCAGGGATGATGTTC
ΔNp63 F	ATGTTGTACCTGGAAAACAATG
ΔNp63 R	GATGGAGAGAGGGCATCAAA
TAp63 F	AGACAAGCGAGTTCCTCAGC
TAp63 R	TGCGGATACAATCCATGCTA
HEY1 F	CACTGCAGGAGGGAAAGGTTAT
HEY1 R	CCCCAAACTCCGATAGTCCAT
HEY2 F	AAGCGCCCTTGTGAGGAAA
HEY2 R	TCGCTCCCCACGTCGAT
HES1 F	GCTTCAGCGAGTGCATGAAC
HES1 R	CGGTGTTAACGCCCTCACA
HES5 F	GCACCAGCCCAACTCCAA
HES5 R	GGCGAAGGCTTTGCTGTGT
HEYL F	AAGCGCAGAGGGATCATAGAG
HEYL R	CCAATCGTCGCAATTCAGAAAG
HES7 F	GGACCCCGCTTCACCAATC
HES7 R	GCCCCGTCTTGTCTGTAAGG
c-Myc F	AACAGGAACTATGACCTCG
c-Myc R	AGCAGCTCGAATTTCTTC
FJX1 F	CAGGCTGTTTCCTTTCCAAG
FJX1 R	ATGGACTGCATCTCCCAAAG
HES1 promoter F	GCAAAGCCCAGAGGAAAGAGTTAG
HES1 promoter R	AGGAGAGAGGTAGACAGGGGATTC
HEY2 promoter F	CCGCCCCTCCATATTAACCA
HEY2 promoter R	GCTCCTGGAGGTTCTTTCC

Abbreviations – forward (F), reverse (R)

2.2.3 Protein manipulation

Sample preparation

Cells grown in monolayers were washed three times with 1X PBS and lysed in appropriate lysis buffer (100-500µl per 10cm dish). The cells were scraped with a sterile disposable cell scraper (Greiner), transferred to eppendorf tubes and left on ice for 30 minutes, vortexing occasionally. The mixture was cleared by centrifugation at 15,000 g, at 4˚C for 10 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. 1µl 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 6%-12% depending on the size of the protein of interest (see Table 2). 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. 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.

Immunoblotting

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 (Schleicher and Schull, Germany) for 1-3 hours at a constant voltage of 65V, 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 5% fat-free milk (Marvel, UK) in 1X TBS-Tween at room temperature for 40-60 minutes. Primary antibody was added at the recommended concentrations, diluted in TBS-Tween plus 5% milk, for 3h at RT or overnight at 4ºC. After three 15min washes in 1X TBS-Tween, the secondary HRP-labeled antibody was added for 1h at RT (1:2000). Membranes were washed another three times for 15min in TBS-Tween. The results were visualized by enhanced chemoluminescent detection, ECL (Amersham Biosciences) using X-ray films (Fujifilm). If probing with another primary antibody was required, the gels were either immediately reprobed or incubated with stripping buffer for 30 min at 55°C and reblocked in 5% milk, before incubation with the new primary antibody.

Immunoprecipitation (with cell lysates)

Cells grown in monolayers were washed three times with 1X PBS and lysed in the appropriate lysis buffer (0.5-1ml per 10cm dish). The cells were scraped using a sterile disposable cell scraper (Greiner), transferred to an eppendorf tube and left on ice for 30 minutes before centrifugation at 15,000 g, 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 40µl 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 2,000g for 2 minutes and the supernatant was removed into a fresh tube. About 2µg of purified antibody or 2-5µl of polyclonal antiserum/ascite and 30µl of protein G sepharose beads (50% slurry in PBS) was then added to 1mg of pre-cleared lysate. The mixture was left on an eppendorf rotating wheel overnight at 4˚C. Immunocomplexes were collected by centrifugation at 2,000g 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 15,000 g 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

Lipofectamine 2000 (Invitrogen) or FuGene (Promega) were used as DNA-lipid carrier and used according to the manufacturer's protocol.

Immunocytochemistry

Cells were seeded on coverslips (VWR) in 24-well plates at 70-90% density. Once experimental procedures were carried out, cells were fixed with 200 ml of 4% paraformaldehyde in PBS for 20 minutes at RT then permeabilized with 0.1% Triton X-100 in PBS for 1-4 minutes at RT. Primary antibodies were diluted in 0.2% Fish Skin Gelatin (FSG) and incubated with the cells for 60 min at RT. After three washes of 10 minutes each with PBS, the secondary fluorescent labelled antibody (Alexa Fluor®), diluted 1:400 in 0.2% FSG, was added for another 30min at RT. This was followed by another three washes with PBS. Where necessary, TO-PRO® (Molecular Probes) or DAPI (Sigma) was added to the second wash as a nuclear counterstain. The coverslips were then mounted onto poly-lysine microscope slides using a mowiol-glycerol based solution containing 2.5% DABCO anti-fading agent. After drying, the slides were analysed by confocal microscopy (Zeiss), using LSM image viewer software.

***In vivo* Transcription assays**

Cells were seeded at about 80% confluence in wells of a 24-well plate and transfected 24 hrs later using Lipofectamine2000 with the various expression plasmids, including a luciferase reporter plasmid. Twenty-four hours after transfection, cells were washed twice with PBS, lysed in 100 µl 1x Reporter Lysis Buffer, collected with a pipette and put in eppendorf tubes. The lysate was left on ice for 15 minutes before spinning them at 15,000 g for 5 minutes. 20 µl of supernatant was then placed

in a wash tube (Sarstedt, Germany) and its luciferase activity measured in an automated Luminometer (AutoLumat LB 953, EG&G, Berthold) using the the Dual-luciferase® reporter assay system (Promega, USA). The mean values were calculated from at least two independent experiments.

Cell-electroporation

Cell-electroporation was performed in order to deliver higher amount of DNA-plasmids into cell lines resistant to transfection with Lipofectamine2000 or FuGene, such as MEFs, UPCI-SCC-040 and HSC3. The procedure was carried out using the electroporating machine from Lonza (Germany), and the Amaxa® Human Keratinocyte Nucleofector® Kit, following the manufacture's protocol (Lonza, Germany).

Notch pathway activation

A coating with Notch ligands Dll4, Dll1 or Jagged1 (R&D System, UK) was prepared in culture dishes (24-well plates or 6 cm dishes) by diluting the named purified proteins in PBS at the concentration of 3 µg/ml and left at 4°C O/N. The following day, the coating was aspirated under a tissue culture hood and washed once with PBS. MEF cells were then plated into the coated dishes and left growing for indicated times prior processing for immunofluorescence or mRNA extraction.

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**

iASPP (625-828), ASPP2 (905-1128) and AnkyrinR (D34) *in vitro* translated proteins were pre-bound nickel beads and incubated individually with *in vitro* translated C-Notch or C-Notch mutants in NP40 buffer for 2 hours at room temperature. Blank beads were used as control. After 4 washes with NP40 buffer, the bound proteins were released in SDS sample buffer and analysed by 10% SDS–PAGE

2.2.6 Mouse work

Mouse colonies

ASPP2 Δexon3 mutant mice were generated on a mixed C57BL/6Jx129SvJ background (Vives et al, 2006) and backcrossed in a Balb/c background for 9 generations. *ASPP2* Δexon3 mutant mice were genotyped as described in Vives et al., using the following primers: 5'-CTCCACCCCAGGAAATTACA-3' (intron 3), 5'-CGGTTTGGAAGTCAAAGGAA-3' (exon 3) and 5'-GGACCGCTATCAGGACATA-3' (neomycin resistance gene).

P53 heterozygous mice (on a Balb/c background) were obtained from Prof Guillermina (Gigi) Lozano, University of Texas, USA. *P53* and *ASPP2* single heterozygous mice were crossed together to generate double heterozygotes. The latter were intercrossed to generate compound genotypes. *P53*

knockout mice were genotyped using the following primers: 5’-CCCGAGTATCTGGAAGACAG-3’ (exon6), 5’-ATAGGTCGGCGGTTCAT-3’ (exon7), and 5’-GGACCGCTATCAGGACATA-3’ (neomycin resistance gene).

P63 heterozygous mice (on a Balb/c background) were obtained from the Professor Frank McKeon, Harvard Medical School, USA. *P63* and *ASPP2* single heterozygous mice were crossed together to generate double heterozygotes. The latter were intercrossed to generate compound genotypes. *P63* knockout mice were genotyped using the following primers: 5’-TTCTCAGATGGTACCGCTCC-3’ (exon3), 5’- GGTGCTTTGAGGCCCGGATC-3’ (exon4), and 5’- TACCCGCTTCCATTGCTCAG-3’ (neomycin resistance gene).

Tumour analysis

Mice that developed visible tumours or showed signs of ill health were killed and subjected to complete necropsy with careful examination for tumours, tissues with possible neoplasm, and other abnormalities. Tumours and samples from other organs were fixed in 10% buffered formalin, and processed for paraffin histology. Four-micrometer-thick sections were stained with hematoxylin and eosin (H&E). The tumours characterization at morphological level (macroscopic and H&E), prior to immunohistochemistry, was conducted in collaboration with Dr F. Fritzsche, pathologist at University Hospital Zurich, Switzerland.

Hematoxylin and Eosin staining

Tissue sections were dewaxed, rehydrated, immersed in Harris haematoxylin (3 minutes), differentiated with acid alcohol (1 dip), blued in Scott Water (30 seconds) and then immersed in eosin (5 minutes) with washes in water between the steps. Sections were then dehydrated and permanently mounted (Vetcamount – Vector Labs, CA, USA)

Histology and immunohistochemistry

Tissues 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 after blocking with 5% goat (or donkey) serum in PBS for 1h at RT, sections were incubated overnight at 4°C with the primary antibody diluted in blocking solution. Next, sections were incubated with either biotinylated or Alexa Fluor® (1:400, Molecular Probes) labelled secondary antibodies, always in blocking solution, for 30 minutes at room temperature. Bright light **s**taining was visualised using the peroxide substrate solution DAB (diaminobenzidine, Vector). Primary antibodies used and relative concentrations are listed in Table 3.

Generation of murine embryonic fibroblasts (MEFs)

MEFs were prepared from E13.5 mice embryos. A 13.5-days 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.

Generation of murine primary keratinocytes

Newborn mice were sacrificed in a CO_2 chamber. All steps were performed in aseptic conditions in a laminar-flow hood. The entire skin was dissected from the newborn pup and left to float overnight over 0.25% Trypsin-EDTA. The next day, the epidermis was detached from the dermis and place in a

10cm petri dish with 10 ml of Ca^{2+}-depleted complete media. The epidermis was subsequently minced with scissors into fine pieces and resuspended with a 10 ml pipette. Finally, the resulting suspension was transfer to a 50ml falcon tube through a 100-µm cell strainer (BD Falcon n°352360), to get rid of most of the stratum corneum and debris. Cells were centrifuged at 700 rpm for 7 mins, and the resulting pellet resuspended in low calcium media. Primary keratinocytes were plated on different sized petri dishes or glass cover slips which had been coated with rat collagen (BD Falcon) prior to use.

2.2.7 Human tissue samples

Table 9 List of tissue samples

Organ	Sample	Source
Cervix	Full cross-section. 29 samples (tumour and normal)	Prof R. Goldin, St Mary's Hospital, London, UK
Oral/Oropharyngeal	Tissue-array (tumour and normal)	Prof H. Moch, University of Zurich, Switzerland
Skin	Full cross-section. 15 samples (tumour and normal)	Prof R. Goldin, St Mary's Hospital, London, UK

2.2.8 Data analysis

Structure homology modeling

Homology modeling was performed using the ICM software package in collaboration with Dr. Wen Hwa Lee (SGS, University of Oxford).

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. ImageJ® software was used to quantify gel bands from Western blots.

Statistical analysis

Comparisons between genotype frequencies and between the types of tumours developed by mice of different genotypes were performed using the χ^2 test. The log-rank test was used to determine the statistical significance of differences in tumor-free survival of different genotypes. Differences were considered significant at a value of $P \leq 0.05$. The T-test and Fisher's test were used to calculate the statistical significance of other measurements.

<u>Chapter III</u>: Impaired ASPP2 expression promotes *in vivo* formation of spontaneous squamous cell carcinoma

3.1 Introduction

In order to better understand the biological importance of ASPP2 *in vivo*, *ASPP2* Δexon3 mice were generated by our group. The strategy employed for the inactivation of the murine *ASPP2* gene was to target and replace *ASPP2* exon3 in 129Sv IT2 embryonic stem (ES) cells with a vector carrying a neomycin resistance gene cassette (Vives, Su et al. 2006). Neomycin-resistant ES clones, in which the targeting vector was integrated by homologous recombination, were selected and injected into C57BL6/6J blastocysts. The resulting germline transmitting chimeric mice were crossed onto a C57BL6/6J background to produce *ASPP2* Δexon3 heterozygous mice, which were further crossed to generate homozygous *ASPP2* Δexon3 mutants. Successful removal of exon3 in the murine *ASPP2* gene was established at RNA level by RT-PCR and at protein level by using a polyclonal antibody raised against the N-terminal portion of ASPP2 protein (residues 59-383) (Vives, Su et al. 2006). The *ASPP2* Δexon3 homozygous deletion was found to be lethal in C57BL6/6J mice, since no such pups were born. In a mixed 129SvxC57BL/6J background, only around 6.4% of the *ASPP2* mutant pups, out of the expected Mendelian segregation of 25%, survived birth, and all died before weaning. The causes for death were attributed to severe defects in the central nervous system, such as neural tube closure defects and hydrocephalus (Vives, Su et al. 2006). The study which followed identified ASPP2 as a key regulator in controlling neuronal cell differentiation (Sottocornola, Royer et al. 2010).

ASPP2 Δexon3 heterozygous mice were born at the expected Mendelian segregation of 50%, in both pure C57BL6/6J and mixed 129SvxC57BL/6J background (Vives, Su et al. 2006). In an attempt to obtain viable *ASPP2* Δexon3 mice and in light of the variations between the different genetic

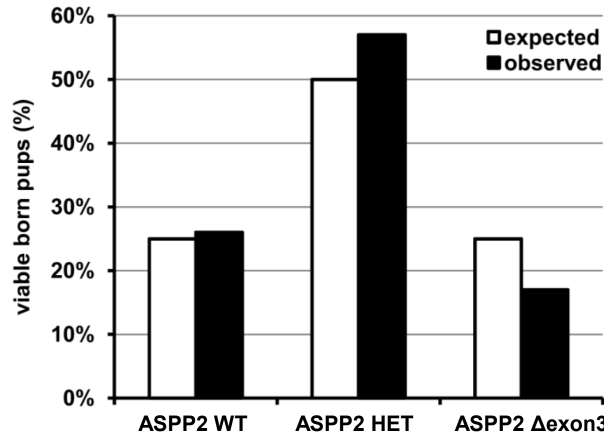
backgrounds of inbred mice, *ASPP2* mutant mice were back-crossed to a Balb/c background to generate a pure strain.

3.2 Results

3.2.1 ASPP2 mutant mice in Balb/c background are viable, but they have a reduced lifespan compared with wild type mice

Balb/c is a mouse strain in which the severity of CNS phenotypes is known to be reduced, and in which there is very low spontaneous incidence of tumours, making it a suitable tool for long term lab experiments (Clapp, Tyndall et al. 1971). Balb/c mice can, however, develop cancers later in life, including mammary tumours, reticular neoplasms, primary lung tumours, renal tumours, and rare spontaneous myoepitheliomas (source: The Jackson Laboratories). Interestingly, *ASPP2* Δexon3 mice in Balb/c background were viable and were born according to Mendelian segregation (Figure 3.1 A). The *ASPP2*-null mice still developed hydrocephalus, similarly to the mixed background, but with some variation in the severity of the phenotype. Despite the presence of hydrocephalus, the mice did not show significant abnormalities in neural tube closure, which was the main cause of prenatal lethality in the mixed background (data not shown). The majority of the *ASPP2* Δexon3 homozygous mice in the Balb/c background were therefore able to survive up to one year after weaning. Their general survival rate after birth was nevertheless significantly reduced when compared with heterozygous and wild type littermates ($p<0.0001$) and only about 20% of the *ASPP2* Δexon3 homozygous mice were able to survive up to 20 weeks of age (Figure 3.1 B). The leading causes for this early post-natal mortality were still the abnormalities affecting the CNS. Intriguingly, increased mortality, this time in the adult age, was also observed for *ASPP2* Δexon3 heterozygotes ($p=0.012$), which are unaffected by CNS defects (Figure 3.1 B).

A



B

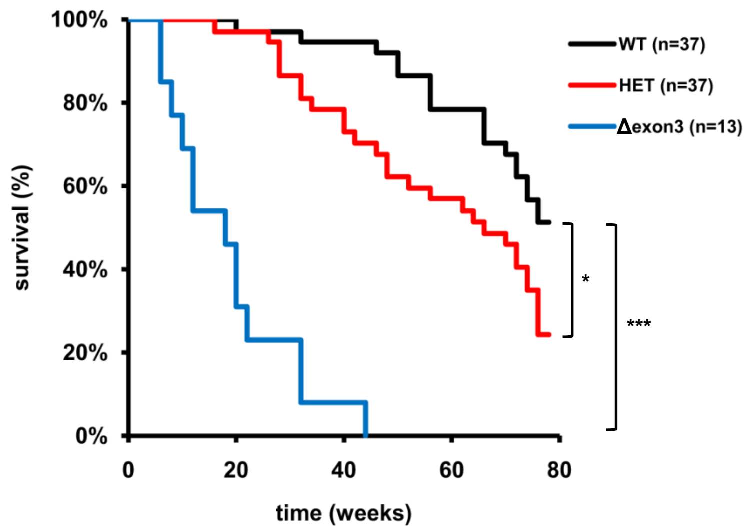


Figure 3.1 ASPP2 Δexon3 mice in Balb/c genetic background are viable, but their lifespan is reduced

(A) Birth rate for each of the three possible genotypes generated by the intercross between *ASPP2* heterozygous mice is shown (236 pups born), comparing the Mendelian expected frequency (in white) with the observed frequency (in black). No significant anomalies in the distribution of birth rates were observed. (B) General survival study, showing the decreasing percentage of *ASPP2* Δexon3, heterozygous and wild type mice alive over a period of 80 weeks. *ASPP2* has a gene dosage effect on mice survival, as mice lacking of both alleles of wild type *ASPP2* die earlier than the *ASPP2* heterozygous, and both have a poorer survival than the *ASPP2* wild type mice. (*) indicates significant at $p=0.012$, while (***) indicates significant at $p<0.0001$ by Log-rank (Mantel-Cox) Test

3.2.2 ASPP2 Δexon3 homozygous and heterozygous Balb/c mice develop spontaneous epithelial tumours

The increased death rate in adult *ASPP2* Δexon3 heterozygous mice was positively affected by the formation of spontaneous solid tumours starting from around 20 weeks of age. Tumours were also found in those few *ASPP2* Δexon3 homozygous mice which survived longer than 20 weeks, but the total number was too small to be part of a statistically significant study. Thus, the susceptibility to develop spontaneous tumours was evaluated in a tumour study by comparing *ASPP2* Δexon3 heterozygous and wild type mice over a period of 2 years. At the conclusion of the study, the final number of tumour-bearing mice was significantly higher in *ASPP2* Δexon3 heterozygous than in wild type mice (p=0.0002) (Figure 3.2 A). No significant differences in the tumour incidence were observed between males and females in the tumour study, indicating that the tumour-onset was not influenced by the gender of the subjects (data not shown).

The second important finding of this study was that all the tumours found in the *ASPP2*-deficient group were classified as epithelial tumours (18 out of 18), also known as carcinomas, while more variation in the tumour spectrum was observed in the wild type cohort, which counted two lymphomas and a single case of retinoblastoma and carcinoma each (Figure 3.2 B). The tumours derived from the *ASPP2* Δexon3 heterozygous mice were all superficial, with occasional ulceration, and restricted to certain areas of the body, such as neck, abdomen (low and high) and flanks, with an average of about 1 cm in size (Figure 3.2 C). At macroscopic level they appear as solid compact masses, occasionally encapsulated, with some degree of vascularisation (Figure 3.2 D). From histological sections, residues of normal skin surrounding the tumour area was still detectable at the edge of the tumour mass, but in fact these were deeply affected and almost undistinguishable from the tumour itself (Figure 3.2 D).

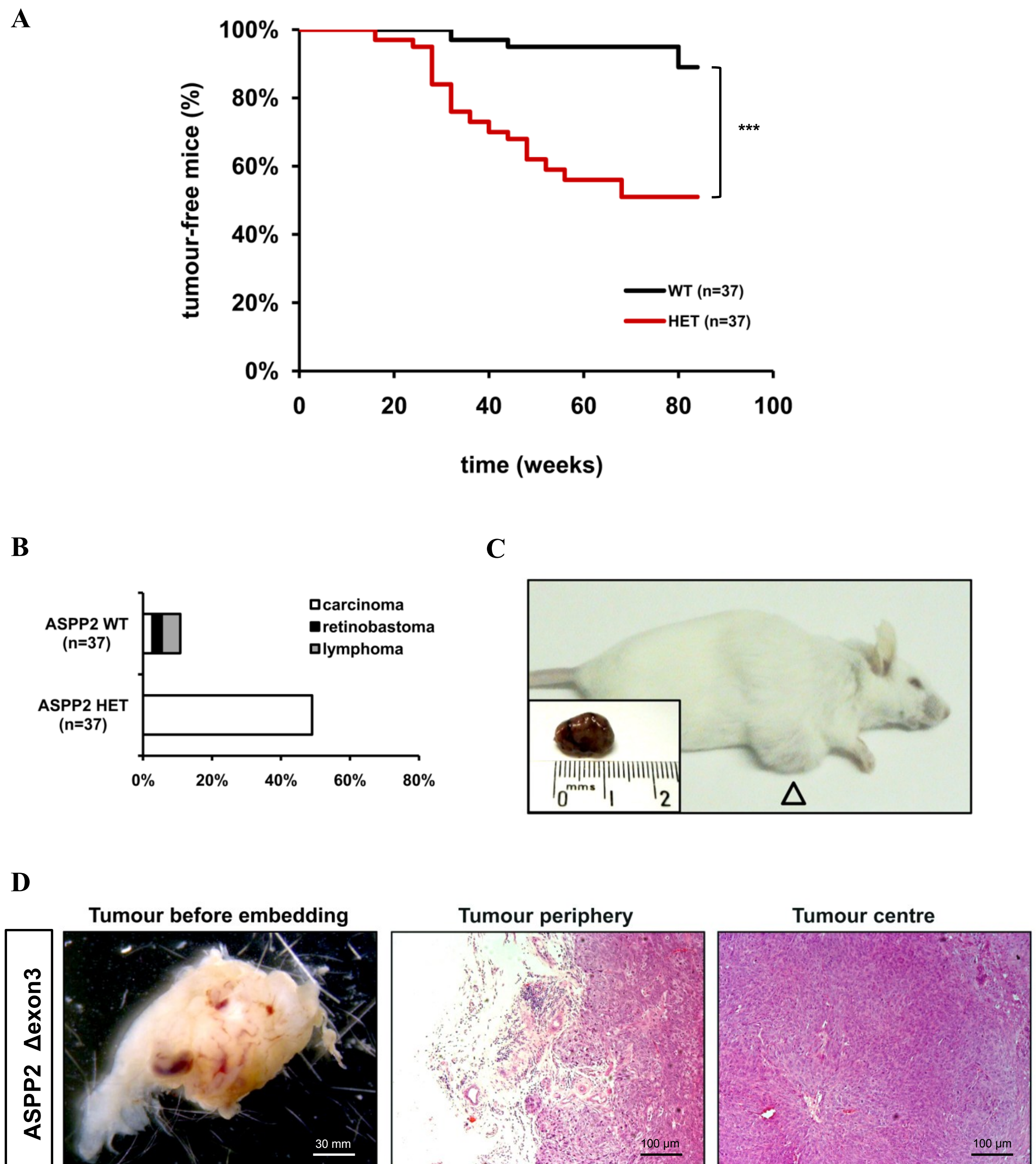


Figure 3.2 ASPP2 Δexon3 mutant mice develop spontaneous epithelial tumours.
(A) Tumour study showing how the onset of spontaneous tumours affects the survival of *ASPP2* heterozygous and *ASPP2* wild type mice over a period of 88 weeks. (***) indicates significant at p=0.0002 by Log-rank (Mantel-Cox) Test. (B) The percentage and the spectrum of tumour types spontaneously developed by wild type and heterozygous mice. (C) Example of an *ASPP2* heterozygous mouse affected by spontaneous tumour. Empty arrowhead indicates site of tumour. Size of tumour harvested is also shown on the left (about 1 cm long). (D) Whole view and H&E-stained sections from the periphery and centre of an epithelial tumour harvested from an ASPP2 heterozygous mouse (scale bars values are indicated).

3.2.3 The tumours developed by the ASPP2 mutant mice are poorly differentiated SCC

Histological analysis of the epithelial tumours spontaneously formed in the *ASPP2* mutant mice revealed them to be poorly differentiated squamous cell carcinomas (SCCs). Their main histological characteristics were the presence of barrel-shaped masses of tumoural cells (Figure 3.3 A, i-i') within reduced surrounding stroma (Figure 3.3 A, s). Frequent areas of necrosis could also be observed within groups of proliferating cells (Figure 3.3 A, n). Poorly differentiated SCCs are distinguishable from well-differentiated carcinomas because they contain more pleomorphic cells, and no keratinous pearls. *ASPP2* Δexon3 homozygous and heterozygous tumours presented high figures of large pleomorphic cells, with vesicular nuclei (Figure 3.3 A, i) and no keratinous pearls. A large number of mitotic nuclei were also observed, indicating a high proliferative index (Figure 3.3 B, arrows). Occasionally, local invasion of tumoural cells into the sub-cutaneous tissue was also detected and in two cases (2 out of 18) distant metastases were found at lymph nodes and lung sites (Figure 3.3 C). Upon lymph node colonisation, the lung is one of the preferential sites for SCC metastases, along with bones (Alam and Ratner 2001). The morphological analysis of the tumoural masses was then complemented with immunohistological staining for several cell markers. Immunohistological analysis showed that the tumour cells from *ASPP2* Δexon3 homozygous and heterozygous mice did not express vimentin, a marker of mesenchymal cells (Figure 3.3 D). Instead, the expression of several keratins was detected in all of the tumours analysed from the *ASPP2* mutant cohort. In particular, we found high levels of Keratin-14 (K14) and Keratin-1 (K1), both well-known markers for SCC (Figure 3.3 D). Tumours from the *ASPP2* mutant mice also revealed marked expression of nuclear p63, another conventional marker for SCC (Figure 3.3 D). P63 expression in epithelial cancers was previously found elevated in cells with high proliferative capacity (Pellegrini, Dellambra et al. 2001). Co-immunofluorescence analysis of tumour sections confirmed that K14, K1 and p63 proteins were coexpressed by the tumour cell population (Figure 3.4 A). Meanwhile, vimentin and K18 (markers of differentiated ductal epithelium) were found to be excluded from the tumour cells

and instead expressed, respectively, in the stromal compartment surrounding the tumour cells and in non-transformed sweat ducts occasionally found scattered within the tumour mass (Figure 3.4 B).

In conclusion, based on our current understanding, it has been established that SCCs of the skin express basal cell markers, namely the two Keratins, 14 and 5 (which form an heterodimer) and nuclear p63, as well as differentiated squamous cell markers as K1 and K10 (also present in form of heterodimer). Our morphological and immunohistochemical analysis indicated that the tumours found in the *ASPP2* mutant mice have all the features of poorly differentiated, highly proliferative SCCs. These data confirm the role of ASPP2 in suppressing tumourigenesis *in vivo* and uncover a possible critical involvement specifically in skin tumour formation, which has not been shown before.

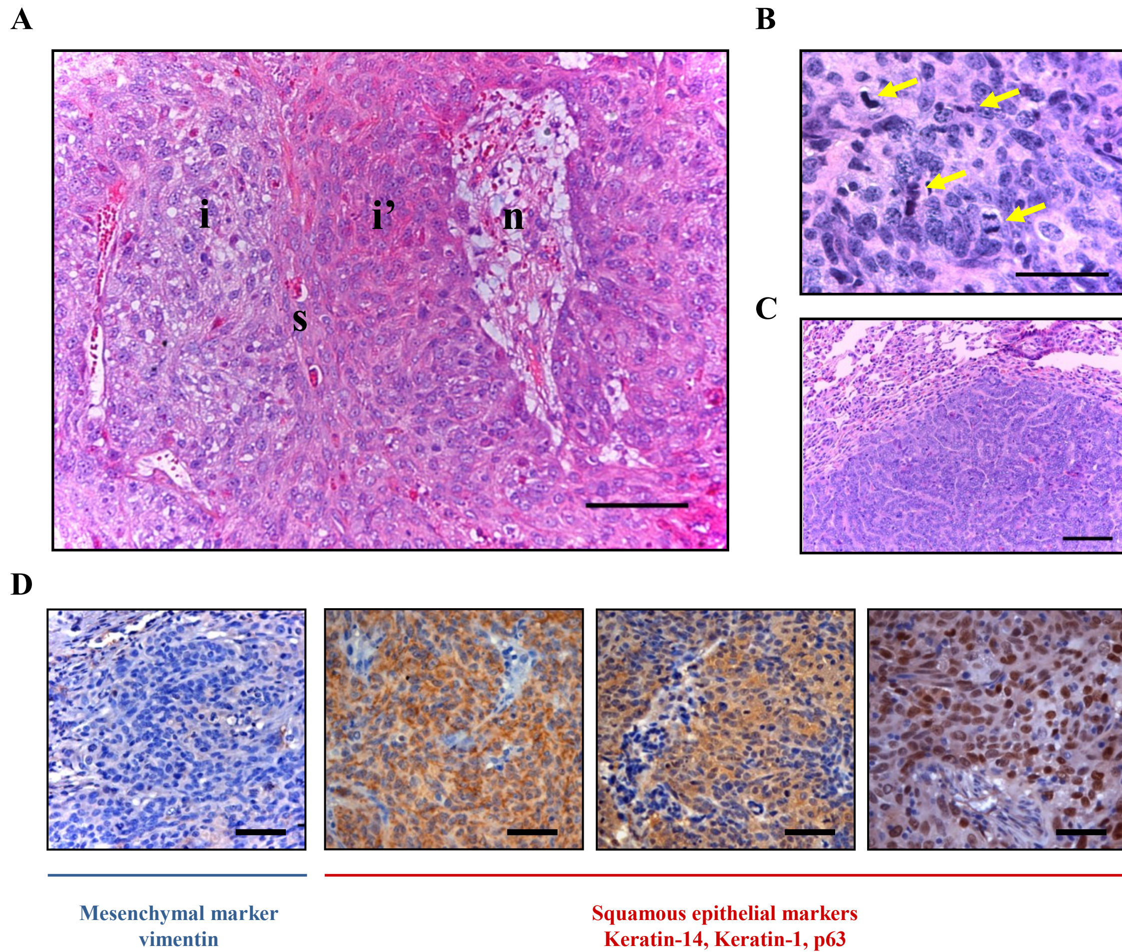
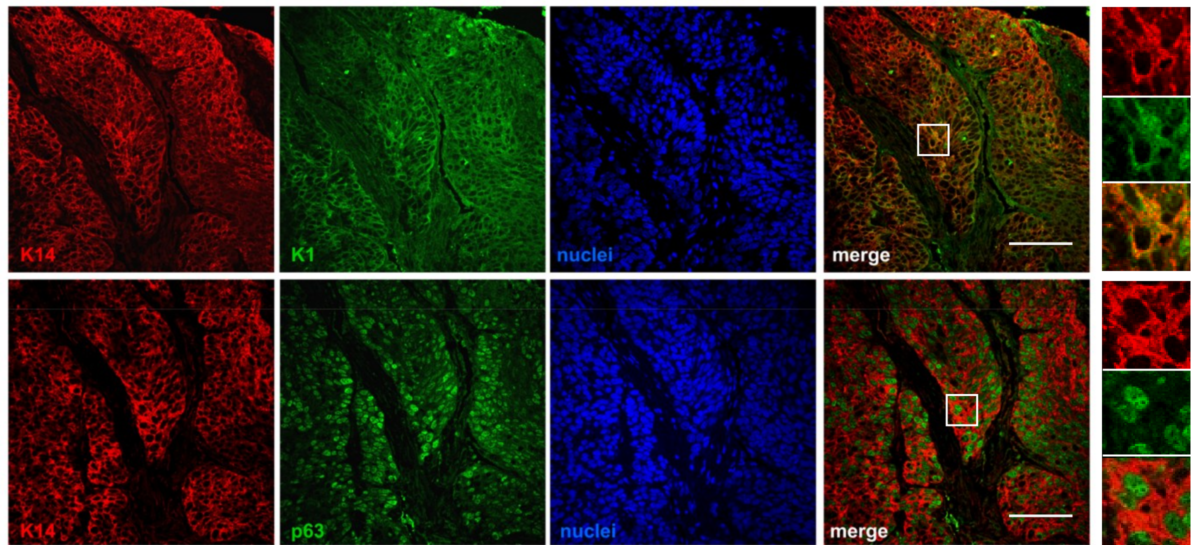


Figure 3.3 Tumours in *ASPP2* Δexon3 heterozygous and homozygous mice are poorly differentiated SCCs

All the images in this panel show tumour tissues harvested from *ASPP2* mutant mice. (A) Example of H&E-stained section showing the morphology characteristics of SCC (i-i', adjacent barrel-shaped masses of tumour cells; n, necrotic region; s, stroma). (B) Tumour cells in mitosis are indicated by yellow arrows in an H&E section. (C) H&E-staining of lung metastases. (D) From left to right, immunostaining of the tumour mass for vimentin (negative), Keratin-14 (positive), Keratin-1 (positive) and p63 (positive). The last three are all markers for SCC. Scale bars: 100 µm in C, 50 µm in A, B and D.

A



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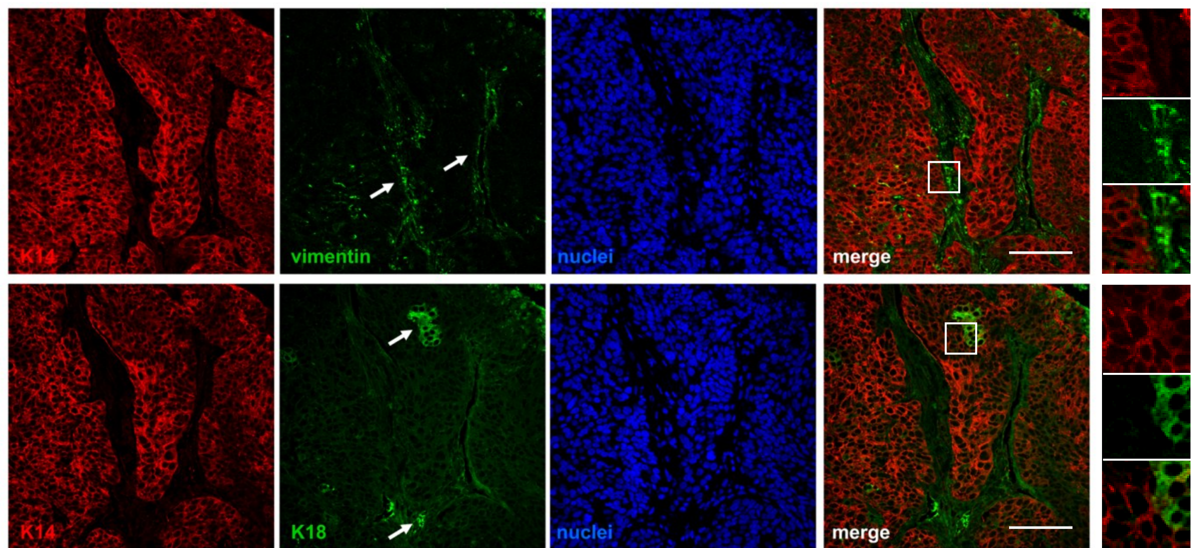


Figure 3.4 Tumour cells co-express markers of SCC, K14, K1 and p63, and do not express either vimentin or K18

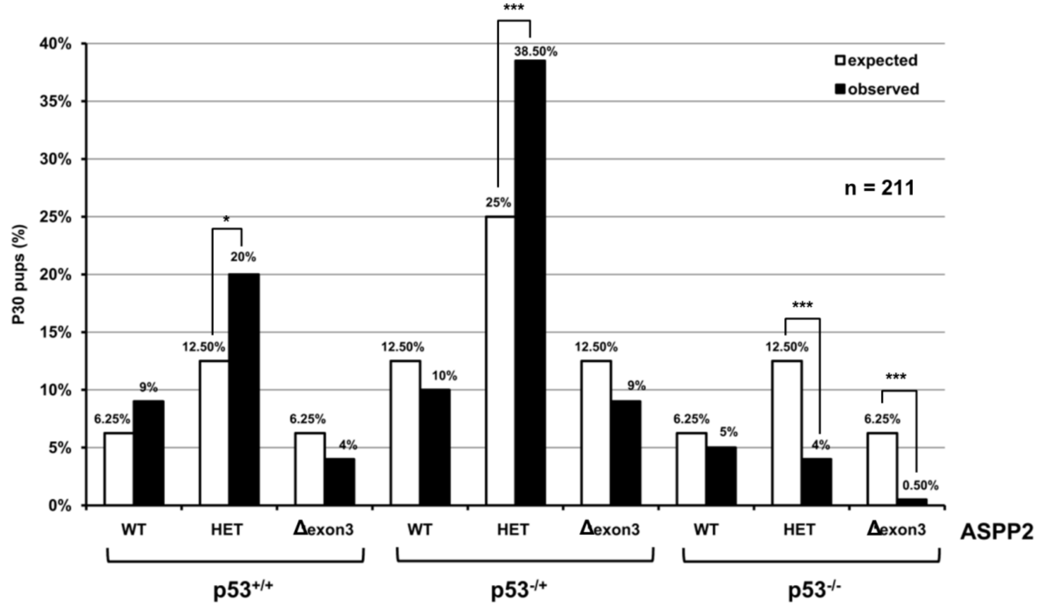
Co-immunostainings for different cell-markers performed in four consecutive sections of the same tumour-area from an *ASPP2* mutant mouse. The white squares define areas within the tumour sections presented at higher magnification on the right side of the panel. (A) Co-expression of K14-K1 and K14-p63 in the tumour cell population. (B) Tumour cells marked by K14 staining are not positive for vimentin (expressed in the stromal compartment) and K18 (expressed in the ductal epithelium). Scale bar: 50 μm

3.2.4 Cooperation between ASPP2 and p53 in tumour suppression

3.2.4.1 Reduced p53 expression accelerates tumour progression in ASPP2 deficient mice

Consistent with the previously characterised role of ASPP2 in regulating p53-mediated apoptosis (Samuels-Lev, O'Connor et al. 2001), we decided to test whether p53 function was required for ASPP2 to suppress SCC. A previous work by our group showed that a combination of *p53* and *ASPP2* heterozygosity accelerated the onset of tumour development in 129SvJ x C57BL/6 mice and that p53 was required for ASPP2 to suppress the development of lymphomas (Vives, Su et al. 2006). We therefore inter-crossed *ASPP2* and *p53* doubly heterozygous mice in a Balb/c background and monitored tumour onset and tumour survival over 100 weeks for each of the genotypes obtained. The rates of birth of pups generated by this inter-cross confirmed previous observations by Vives et al. in 2006, namely that the double *ASPP2*- and *p53*-null mice are not viable (Figure 3.5 A), therefore the tumour study was performed only with the eight genotypes available. We then observed that the *ASPP2* Δexon3 mice start developing tumours as early as 9 weeks of age in the presence of *p53* heterozygosity, compared to after 20 weeks in the single *ASPP2* Δexon3 transgenic mice cohort (Figure 3.5 B-C). Loss of just one allele of *p53* accelerated the onset of tumour formation in *ASPP2* Δexon3 mice, allowing us to get a significant number of tumours in these mice before they prematurely die of CNS defects. Consequently, we were able to show that *ASPP2* has a gene dosage effect on suppression of tumour development, as significant differences were observed in tumour-survival by comparing *ASPP2* wild-type with *ASPP2* heterozygous ($p=0.038$) and *ASPP2* heterozygous with *ASPP2* homozygous Δexon3 mice ($p<0.0001$) in a *p53* heterozygous background (Figure 3.5 B-C). These observations prove the existence of cooperation between ASPP2 and p53 in tumour suppression in a more comprehensive way than Vives et al. 2006, as the present tumour study was carried out on eight different genotypes, compared to the six of the previous work.

A



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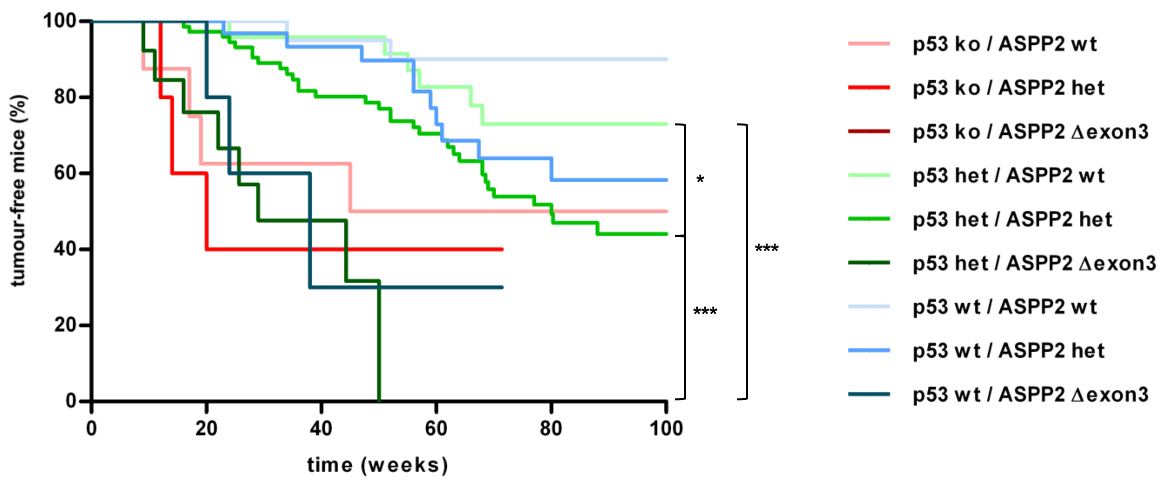

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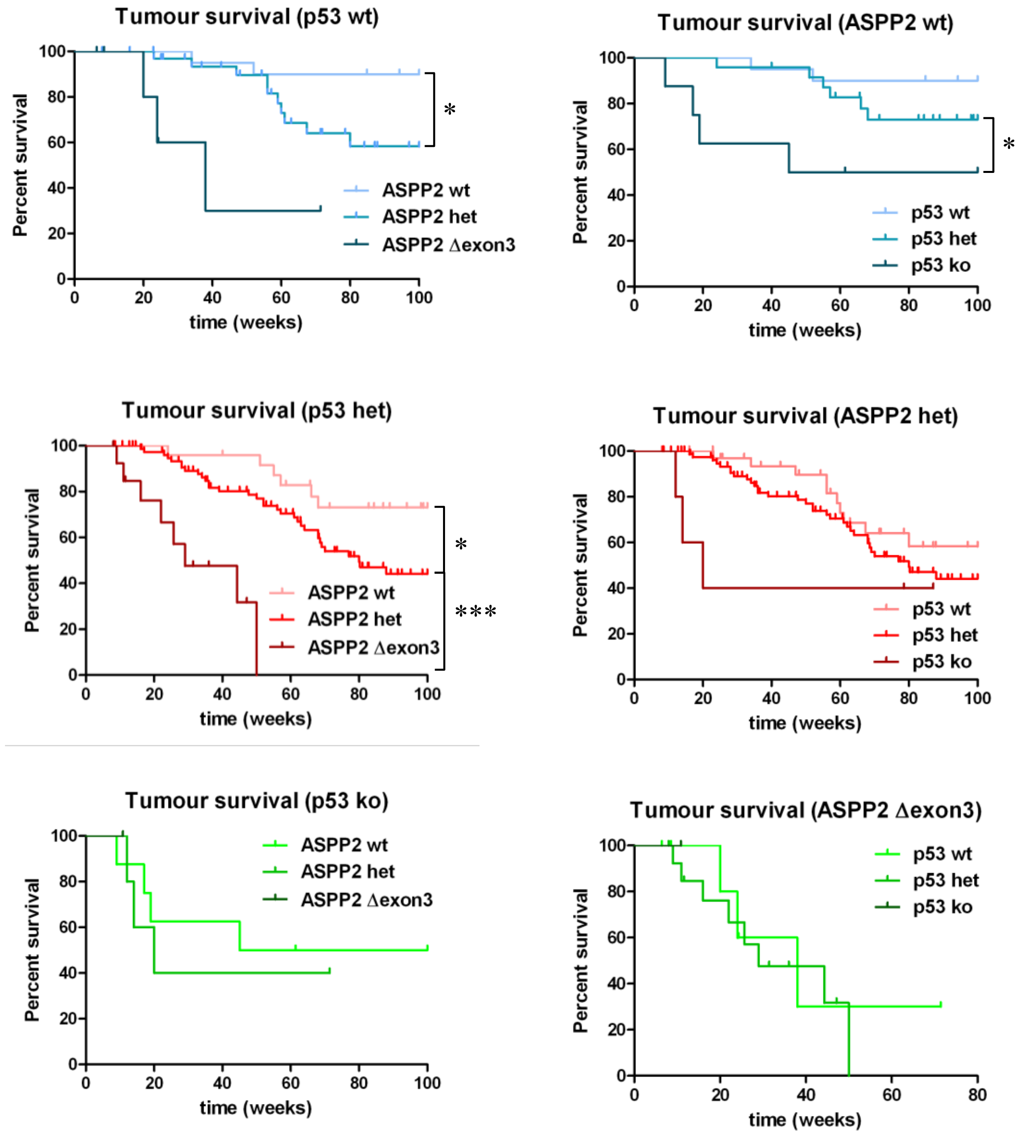


Figure 3.5 Tumour study in *ASPP2/p53* mutant mice: lack of p53 accelerates tumour formation in *ASPP2* Δexon3 mice

(A) Genotype distributions of 211 pups born from intercrosses between *ASPP2* and *p53* single heterozygous mice. The Mendelian segregation frequency (expected) and the observed frequency (observed) are shown. The absence of p53 negatively affects the number of *ASPP2* mutant mice born, while the double heterozygous mice develop normally and compensate for the absence of the other genotypes. (*) indicates significant at p=0.02 and (***) indicates significant at p<0.002 by χ^2 test Test. (B) The graph shows the onset of spontaneous tumours affecting the survival of *ASPP2/p53* mutant mice over a period of 100 weeks. (C) The data from the tumour study shown in B, divided in separate graphs to highlight the gene dosage effect of *ASPP2* and *p53* on tumour onset and tumour survival. (*) indicates significant at p=0.03 and (***) indicates significant at p<0.0001 by Log-rank (Mantel-Cox) Test.

3.2.4.2 ASPP2-suppression of SCC is independent of p53

The frequency of the cancer types obtained for each genotype was analysed. According to morphological and immunohistological analyses, the tumours could be grouped in three main categories: lymphomas, sarcomas of various types, and carcinomas. All carcinomas belonged to the SCC subtype (Figure 3.6 A-B). Here we observed that, despite the fact that the progressive loss of *p53* alleles led to a higher total percentage of tumours in *ASPP2* heterozygous mice, the total percentage of carcinomas only was actually diminished (Figure 3.6 A, compare bars marked by the asterisks). The increase in the total percentage of tumours developed in absence of one or two alleles of *p53* was in fact due to an increment in the incidence of lymphomas and sarcomas, tumours typically related to the loss of p53 (Donehower, Harvey et al. 1992). Indeed, in those genotypes where the absence of p53 was dominant on the absence of ASPP2 (*p53*het/*ASPP2*wt, *p53*ko/*ASPP2*wt, *p53*ko/*ASPP2*het), only sarcomas and lymphomas, and not carcinomas, were observed (Figure 3.6 A, bars marked by black triangles). Conversely, when the absence of ASPP2 was dominant (*p53*wt/*ASPP2*het, *p53*wt/*ASPP2*Δexon3, *p53*het/*ASPP2*Δexon3) the majority of tumours were SCCs (Figure 3.6 A, bars marked by white triangles). As p53-loss did not seem to have a critical impact on the formation of SCC in our tumour study, we concluded that ASPP2-suppression of SCC is independent of p53.

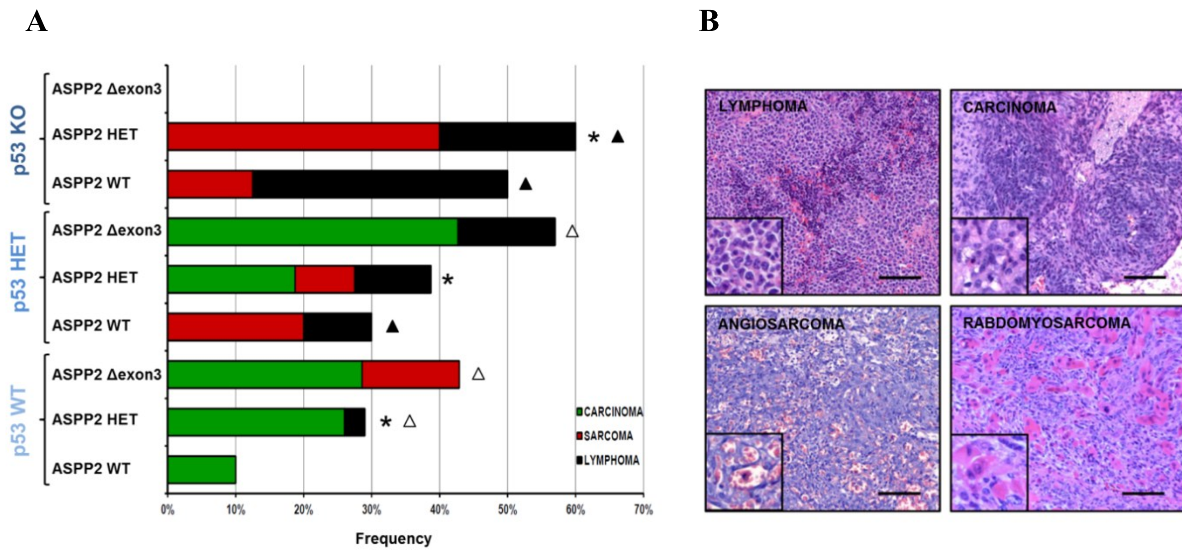


Figure 3.6 Combined loss of ASPP2 and p53 increases the overall spontaneous tumour incidence, but do not significantly increase the number of SCCs

(A) The graph shows the incidence of different tumour-types by genotype. (B) H&E staining of tumour sections showing the variety of tumour-types that can be found in the *ASPP2*-mutant mice. An image at higher magnification is presented on the left-bottom corner of each single picture. Scale bar: 100 µm.

3.3 Summary

The generation of *ASPP2* mutant mice in a pure Balb/c genetic background allowed us to obtain *ASPP2* Δexon3 homozygous mice which are viable, because of an attenuation of the severe neuronal phenotypes observed in C57BL6/6J and mixed 129SvxC57BL/6J mice. Both *ASPP2* Δexon3 homozygous and heterozygous mice suffered from spontaneous tumour formation, starting from about 20 weeks of age. Notably, the only tumour type developed in *ASPP2* mutant mice was poorly differentiated SCC, characterized by elevated expression of K14, K1 and nuclear p63. SCC is caused by dysfunctions in pathways involved in the normal physiology of the multistratified epithelium, such as the Wnt, Sonic-hedgehog, Notch and p63 pathways (see Chapter I for more details). The observation that a single-gene disruption, as in the case of the *ASPP2* Δexon3 mice, can cause such a specific epithelial phenotype suggests that ASPP2 might play an important role in the maintenance of epidermal homeostasis. Since ASPP2 was originally discovered as regulator of p53, we tested whether the ability of ASPP2 to suppress SCC was mediated by p53's tumour suppressor properties. In order to verify this hypothesis, we intercrossed the *ASPP2*-heterozygous with the p53-heterozygous mice (both tumour-prone mouse models) and measured the kinetics of tumourigenesis and the spectrum of tumour-types obtained. Interestingly, in the absence of p53, earlier tumour formation was observed in the *ASPP2* mutant mice, suggesting that the combined loss of ASPP2 and p53 can accelerate tumour onset. Following this observation, we could also show that the presence of *ASPP2* has a gene dosage effect on tumour suppression, since a higher number of tumours was found in the mice missing both alleles of *ASPP2* compared to the mice missing only one allele. Subsequently, the analysis of the tumour-types indicates that p53 does not promote the initiation of new SCCs and therefore it is not influential in the capacity of ASPP2 to suppress SCC. This is consistent with the observation that germline mutations of *TP53* in human (Li-Fraumeni syndrome), which predispose to tumour development, do not lead to formation of SCCs. Similarly to humans, mice deprived of p53 expression are prone to develop spontaneous lymphoid malignancies and

sarcomas, but fail to develop spontaneous SCCs (Donehower, Harvey et al. 1992). Other works show that *p53* mutations in carcinomas can be involved in tumour progression, as opposed to tumour initiation (Kemp, Donehower et al. 1993; Feldser, Kostova et al. 2010). Therefore p53 loss may not be a critical step in SCC initiation, but rather mainly important for the progression of the malignancy. As this seemed also to be the case of the SCCs derived from our ASPP2 mutant mice, we therefore decided to focus our attention on other possible causative factors to explain ASPP2-suppression of SCC.

Chapter IV: ASPP2 expression in the squamous epithelium is important to prevent tumourigenesis by antagonising p63 expression and function

4.1 Introduction

The aberrant over-expression of the p53-family member p63 (ΔN isoforms) was shown to be a primary cause for tumour formation in SCC (Hibi, Trink et al. 2000), and is believed to be important for proliferative potential and resistance to apoptosis in tumour cells (Rocco, Leong et al. 2006; Chiang, Chu et al. 2009). P63 plays an important role in in epidermal homeostasis by promoting the maintenance of the epidermal stem cell population in the basal layer of the skin, while opposing growth arrest and differentiation stimuli mediated by p53-p73 and Notch. In order to maintain the homeostasis of the tissue, p63 activity has to be limited to the basal layer of the skin. Factors specifically expressed in the upper layers of the epithelium, such as Notch, are believed to be important for antagonising p63 expression and activity in these layers, thereby preventing cell-proliferation and protecting their differentiated status. Consistent with this protective role, the depletion of Notch activity or expression from the skin was shown to lead to formation of SCCs (Proweller, Tu et al. 2006). Since p63 was found to be highly and almost ubiquitously expressed in the tumours developed by ASPP2 transgenic mice, we decided to investigate whether ASPP2 could also have a role as physiological repressor of p63 expression and/or activity in the squamous epithelium.

4.2 Results

4.2.1 Mutual exclusive expression of ASPP2 and p63 in adult squamous epithelium in vivo and during differentiation of primary keratinocytes in vitro

To test whether ASPP2 could be a factor important for the maintenance of the epithelial homeostasis, we first examined ASPP2 expression in the normal stratified epithelium, comparing it with p63 expression. By co-immunofluorescence analysis, good levels of ASPP2 expression were detected in in the adult human skin epithelium with a well-defined localisation, restricted to the spinous and granular layer, which contain exclusively differentiated cells (Figure 3.7 A). Interestingly, ASPP2's pattern of expression was mutually exclusive with that of p63, as no expression of ASPP2 was detected in the basal layer of the skin, containing undifferentiated and proliferative cells, where p63 is expressed (Figure 3.7 A). A similar pattern of expression was detected in mouse tissue, where ASPP2 expression was enriched in the upper and most differentiated layers of the epidermis (Figure 3.7 B). In mouse tissue, we observed an increasing gradient of ASPP2 expression towards the apical layers rather than a segregation of ASPP2 expression in a defined area (Figure 3.7 B), due the reduced thickness of the squamous epithelium compared to human skin.

These experiments prove that ASPP2 protein is present in the stratified squamous epithelium of the skin. It is conceivable therefore that its disappearance from this site might be a direct cause for SCC formation, as observed in the *ASPP2* deficient mice. The specific expression pattern of ASPP2 in the differentiated *strata* of the epidermis led us to hypothesise that ASPP2 might also be a factor involved in the differentiation of the squamous epithelium. To address this hypothesis, we monitored how ASPP2 expression can be modulated during cell differentiation in primary mouse keratinocytes. Pluripotent keratinocytes isolated from 3-day-old wild type mice were allowed to grow in culture in a Ca^{2+}-free medium, which permits proliferation while inhibiting terminal differentiation, resembling the properties of the keratinocytes present in the basal layer of the skin. After two days, Ca^{2+} was

added to the cell growth medium, allowing cells to establish cell-cell contacts, to withdraw from the cell-cycle and to differentiate, mimicking the physiological process of cell stratification in the squamous epithelium. Cells were then harvested before and at two different time points after the addition of Ca^{2+} to the medium, and the protein expression levels of ASPP2 were evaluated by Western blot. As a control for the successful outcome of the process of cell-differentiation, the presence of envoplakin, a protein induced with terminal differentiation, as well as the disappearance of p63, a marker of cell pluripotency, were evaluated. Remarkably, ASPP2 expression was found to be up-regulated during differentiation, along with envoplakin and in the opposite manner to p63 (Figure 3.7 C). ASPP2 protein seemed to be absent from the undifferentiated keratinocytes, which are rich in p63 expression, then appeared one day after the stimulation of differentiation, characterised by the induction of envoplakin expression. The appearance of ASPP2 expression corresponded to the decrease in p63 protein levels, which became even more substantial 5 days after the addition of Ca^{2+}-rich culture-medium.

These observations, combined with the previously assessed tissue-localisation within differentiated layers of the epithelium, indicate that ASPP2 is a *bona fide* marker of epithelial differentiation in the skin. Since ASPP2 induction during keratinocytes differentiation was found concomitantly with p63 down-regulation, and in the adult epithelium the two proteins are segregated in two distinct regions, this also indicates that the expression of ASPP2 might exclude that of p63. In other words, these findings also suggest that ASPP2 expression might be physiologically important in repressing p63 expression during skin development, and in preventing p63 expression throughout the upper layers of the adult skin, as described for Notch. This hypothesis could explain why, when ASPP2 expression is impaired as in the *ASPP2*-deficient mice, we observed over-expression of p63 in the skin and spontaneous formation of SCCs (Figure 3.7 D).

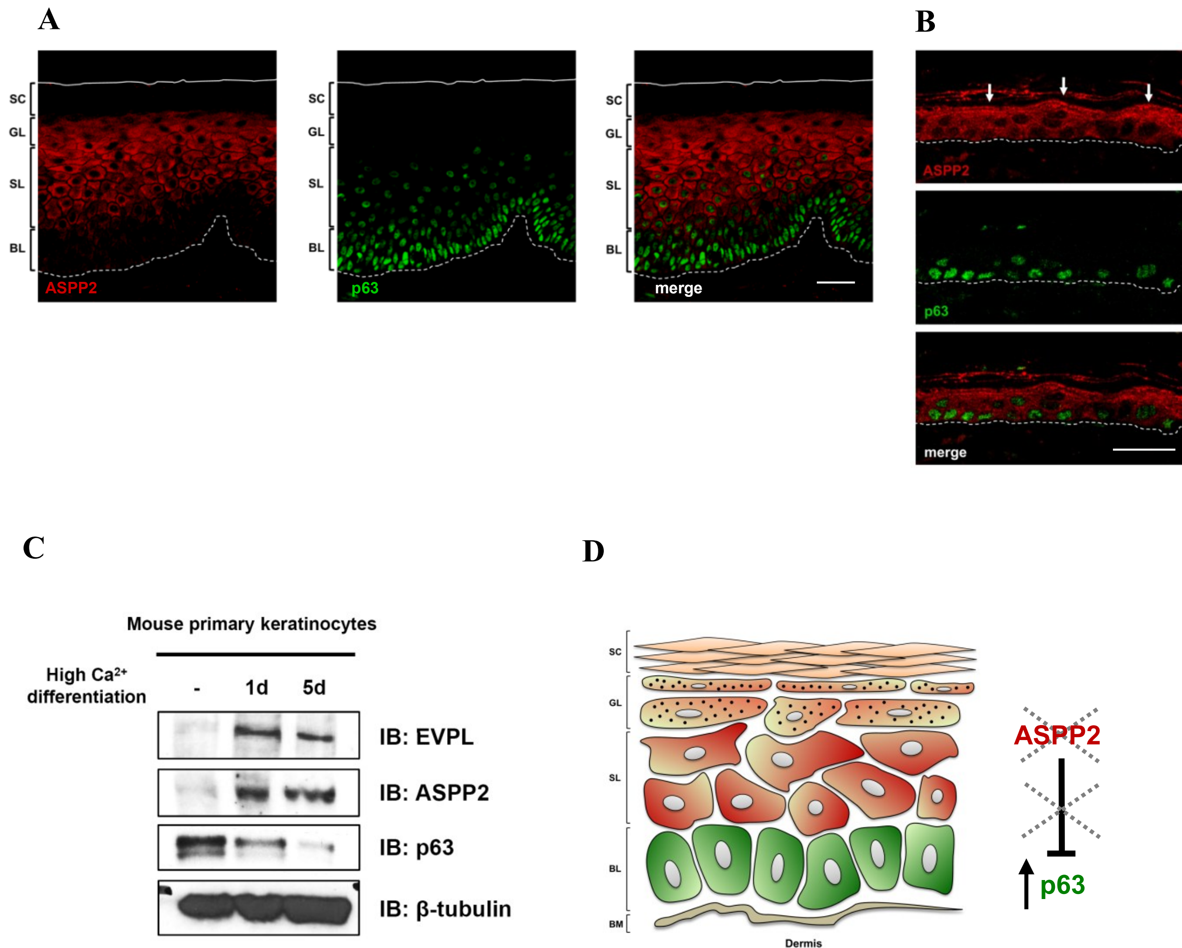


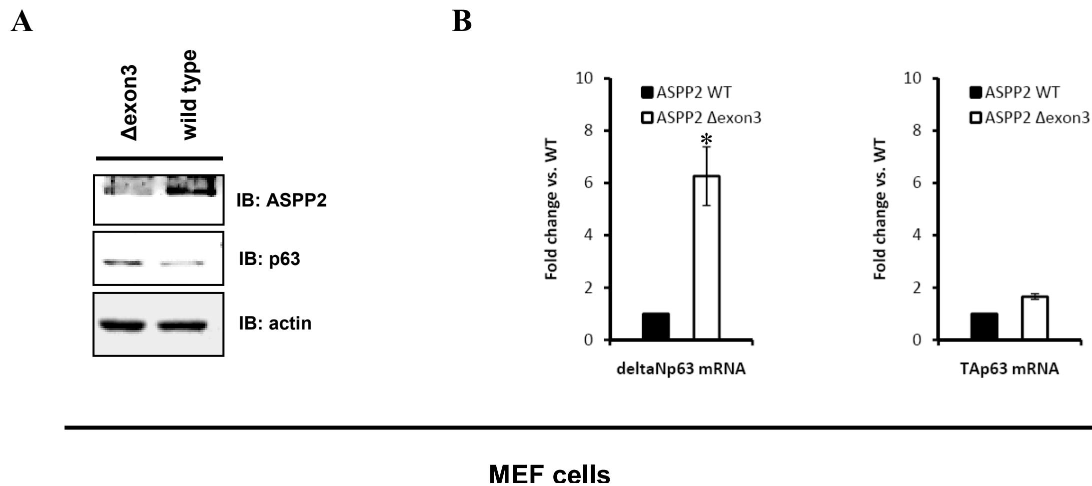
Figure 3.7 ASPP2 and p63 mutual exclusive pattern of expression in skin tissue and in primary keratinocytes

Double staining of human (A) and mouse (B) squamous epithelium of the skin with anti-p63 and anti-ASPP2 antibodies shows that ASPP2 and p63 have an almost mutually exclusive pattern of expression. (C) Evaluation by Western blotting of ASPP2 levels of expression compared to other skin markers during differentiation of mouse primary keratinocytes. Lysates were prepared from cells cultured in absence of calcium (Ca^{2+}) in the medium and then after one and five days upon the addition of Ca^{2+}. Immunoblotting was performed using antibodies anti-envoplakin (EVPL), anti-ASPP2, anti-p63 and anti-βtubulin (loading control). ASPP2 expression is induced upon differentiation, as EVPL, whereas p63 is decreased. (D) Hypothetical model, according to which, ASPP2 localising in the differentiated layers of the stratified epithelium would be important in preventing p63 expression in the upper *strata* and therefore suppress tumour formation. The absence of ASPP2 expression from this site would release the inhibition on p63, which could now be expressed upwards throughout all the epithelium and lead to SCC formation, as observed in the ASPP2 deficient mice. BM, basal membrane; BL, basal layer; SL, spinous layer; GL, granular layer; SC, *stratum corneum*. Scale bars: 20 µm.

4.2.2 p63 expression is upregulated in ASPP2 deficient cells

To acquire further evidence that ASPP2 can physiologically repress p63 expression, we first analysed p63 protein levels in ASPP2 deficient mouse embryonic fibroblasts (MEFs) by Western blotting. Consistent with our hypothesis, we found that p63 expression was higher in the absence of ASPP2 (Figure 3.8 A). Secondly, to verify whether this difference in protein abundance was due to an increase in *p63* transcription, we measured *p63* mRNA levels, distinguishing between *ΔN* and *TA p63* transcripts, in *ASPP2* wild-type and *ASPP2* Δexon3 MEFs by RT-qPCR. Here we found that *ΔNp63* mRNA in *ASPP2* Δexon3 was about 5 times more abundant than in wild-type MEFs. Interestingly, no significant differences in the mRNA levels of *TAp63* were observed (Figure 3.8 B). In MEFs, as well as in keratinocytes, ΔNp63 is the isoform predominantly expressed and the only one normally detectable by Western blot analysis (Figure 3.8 A). The detection of the TA isoform is possible only after a very long exposure of the film.

A selective up-regulation of the *ΔNp63* transcript was also observed in *ASPP2* Δexon3 primary keratinocytes (Figure 3.8 C), suggesting this phenomenon is probably not cell-type specific. The elevated levels of *ΔNp63* transcript in *ASPP2* Δexon3 cells indicates that the higher amount of p63 protein observed in these cells is probably not due to an increase in the protein stability, but instead to an greater transactivation of its promoter. A higher transcriptional activity on the *ΔNp63* promoter was indeed measured by transactivation-assay in *ASPP2* Δexon3, in MEFs upon transfection with a plasmid carrying the *ΔNp63* promoter sequence followed by the luciferase gene, used as a reporter. This result confirms that the increased level of mRNA observed in *ASPP2* deficient cells is due to a higher rate of transcription (Figure 3.8 D). Of note, ΔNp63, which we had found up-regulated in *ASPP2*-deficient cells, is the isoform representing the majority of p63 expressed in the basal layer of the epithelium, where it drives the proliferation of basal keratinocytes, and it is also the isoform which is over-expressed in SCC.



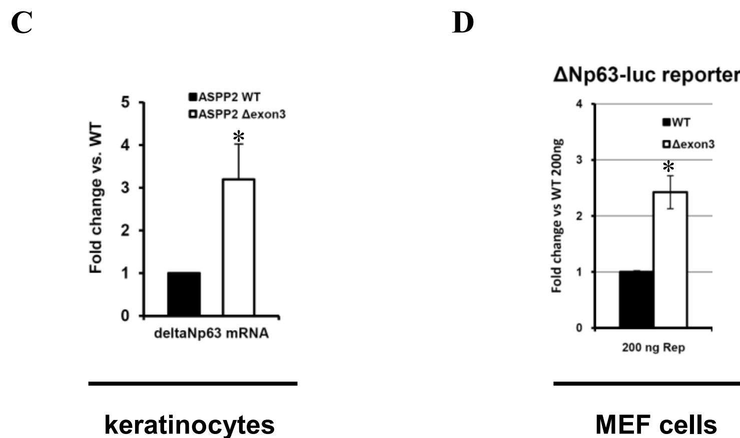


Figure 3.8 ***ASPP2*** **deficient cells express less ΔNp63 than wild type cells**
(A) p63 protein expression is up-regulated in ASPP2 Δexon3 MEFs compared with wild type as measured by Western blotting performed using antibodies anti-ASPP2, anti-p63 and anti-actin (loading control). (B) *ASPP2* Δexon3 MEFs show specific up-regulation (six folds, *p=0.016) of *ΔNp63* but not *TAp63* transcript, as measured by RT-qPCR performed using specific primers distinguishing between the two p63 isoforms. (C) Up-regulation of *ΔNp63* transcript (three folds, *p=0.034) is also verified by RT-qPCR in mouse primary keratinocytes. (D) *ASPP2* Δexon3 MEFs show a higher rate of transcription of *ΔNp63* than wild type cells (*p=0.013). Transcriptional activity of *ΔNp63* promoter was measured by electroporating 200ng of *ΔNp63*-luciferase reporter construct together with 6ng of a renilla expression vector, as internal control, into *ASPP2* wild type and Δexon3 cells growing in 24-well plate. Luciferase activity was analysed from cell lysates harvested 24h after the electroporation and normalised on the signal from renilla. Graphs in B and C were produced based on ΔΔCt method using *GAPDH* as an internal control. The data in B, C and D represent the average of three different paired batches of MEFs or keratinocytes isolated from individual mice. Error bars represent standard deviation of the mean.

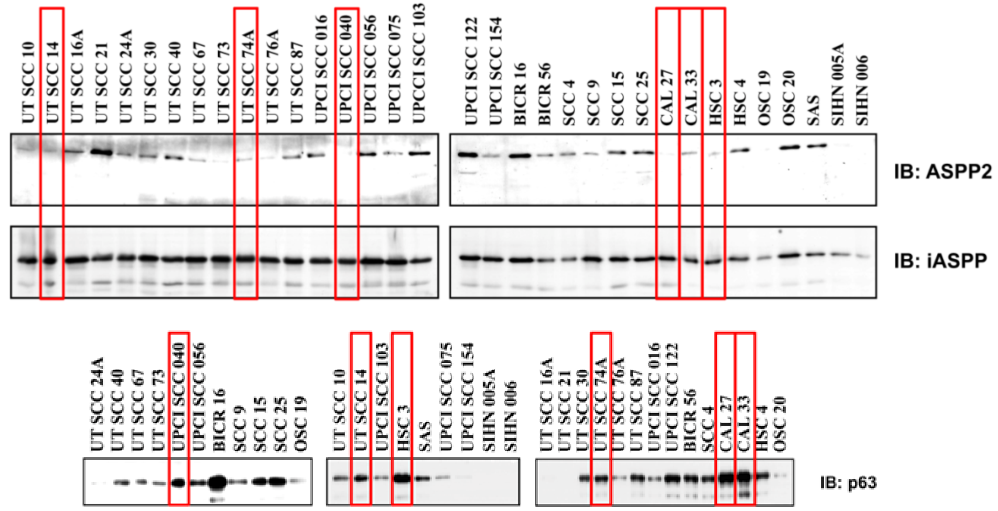
4.2.3 ASPP2 and p63 expression negatively correlate in human SCC cell lines

To establish whether ASPP2 could be an important factor in preventing p63 expression and SCC formation in mice and humans, we analysed a panel of 34 established human SCC cell lines, which had been screened by Western blotting for ASPP2 and p63 protein expression. As expected, we found that in most of the cell lines analysed p63 was present at high protein levels, consistent with its role as a diagnostic marker for SCC (Figure 3.9 A). Similarly, iASPP (the inhibitory member of the ASPP family of protein), was found to be consistently highly expressed throughout the entire panel of the cell lines examined (Figure 3.9 A). This data is consistent with the observation that iASPP is generally up-regulated in cancer (Bergamaschi, Samuels et al. 2003; Zhang, Wang et al. 2005; Zhang, Xiao et al. 2011) and also that iASPP and p63 expression levels positively correlate, as recently seen in mouse keratinocytes (Chikh, Matin et al. 2011; Notari, Hu et al. 2011).

Conversely, ASPP2 was found generally expressed at low levels, or not expressed at all in some of the cell lines (Figure 3.9 A with quantification in Figure 3.9 C). A direct comparison obtained from the quantification of the total p63 and ASPP2 expression levels in all the 34 SCC cell lines analysed showed that p63 is significantly more expressed than ASPP2 ($p=0.0009$) (Figure 4.3 B). In more detail, we noticed that in some of the cell lines in which ASPP2 expression was found particularly low or absent; here p63 was generally expressed at very high levels (Figure 3.9 A, red frames). This inverse correlation could also be visually appreciated in the graph from Figure 3.9 C, where picks of p63 expression correspond to low levels of ASPP2.

This indicates that a decrease in ASPP2 cellular amount might allow cells affected by tumour transformation to reach higher levels of p63 expression.

A



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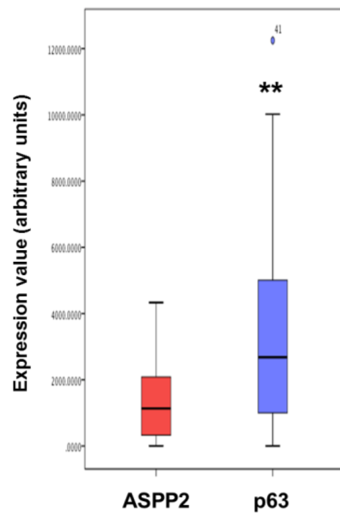

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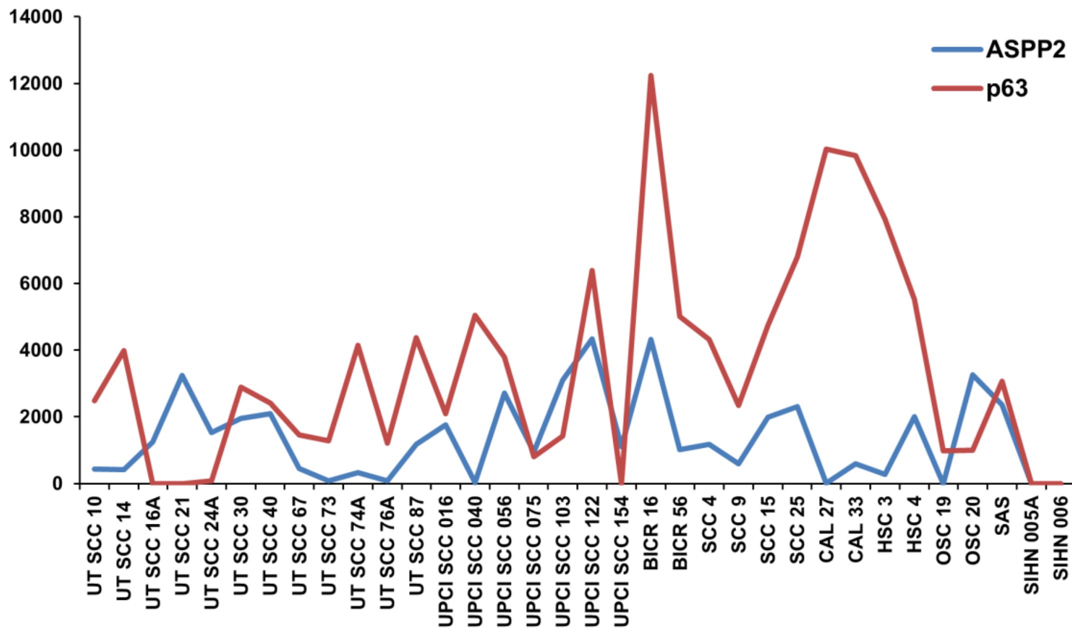


Figure 3.9 ASPP2 expression is generally downregulated in SCC cell lines with high p63 expression
(A) Analysis of lysates from 34 different SCC cell lines aimed by Western blotting using antibodies anti-ASPP2, anti-iASPP and anti-p63. The samples highlighted by red frames indicate conditions of high p63 protein expression corresponding to low ASPP2. (B) Box plot indicating that the mean p63 expression in SCC cell lines is significantly different and higher that the mean expression of ASPP2 in the same cell lines. (**) indicates significant at p=0.0009 by Wilcoxon rank-sum test. (C) Quantification of ASPP2 and p63 protein expression levels based on Western blot shown in A. The expression bands from the gel have been quantified using ImageJ software (“gel analysis” option). ASPP2 and p63 expression values have been normalised on iASPP expression, as it appeared to be fairly constant in all the 34 cell lines. As it can be observed, the trends of ASPP2 and p63 expression across the 34 SCC cell lines largely diverge from each other.

4.2.4 ASPP2 expression is downregulated from normal epithelium to neoplastic tissue in human SCC samples

The results obtained from the analysis of the panel of human SCC cell lines allowed us to define an inverse correlation between ASPP2 and p63 expression in SCC, indicating that in general, p63 is up-regulated when ASPP2 is down-regulated. This however did not tell us where these two proteins are normally expressed in SCC. Concerning p63 localisation in SCC, we already know from several works, including ours in the *ASPP2* mutant mice, that p63 is expressed at very high frequency in the cells constituting the tumour mass (cells of epithelial origin, not in the stromal component). To clarify ASPP2 expression pattern in SCC we immunostained available human specimens of cervical SCC using anti-ASPP2 antibody. Interestingly, as we show in Figure 4.1, we found that ASPP2 was either not present or expressed at a very low level inside the neoplastic mass, whilst it could be clearly detected in portions of relatively normal-looking, non-transformed squamous epithelium present next to the neoplastic lesion (Figure 4.1 e, normal epithelium; n, neoplastic mass).

These data from the tumour-tissue samples add further insights to the previous observations in the SCC cell lines, suggesting that ASPP2 loss of expression from the normal stratified epithelium in humans, as in mice, might lead to an over-expression of p63 and promote tumour formation. This idea implies that ASPP2 would be physiologically important for inhibiting p63 expression in the upper layers of the normal squamous epithelium.

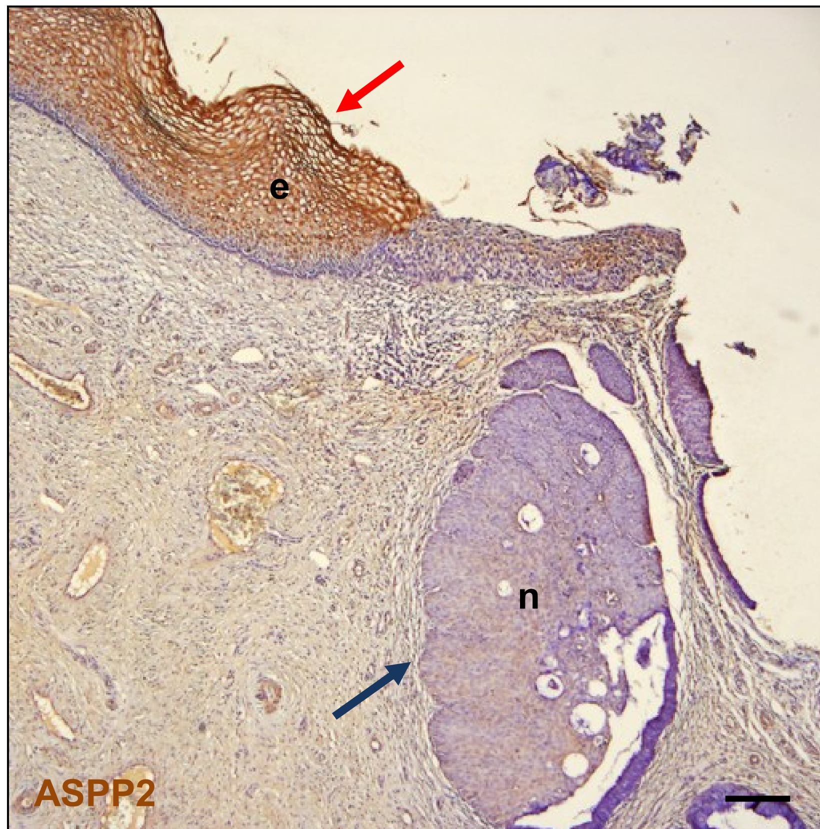


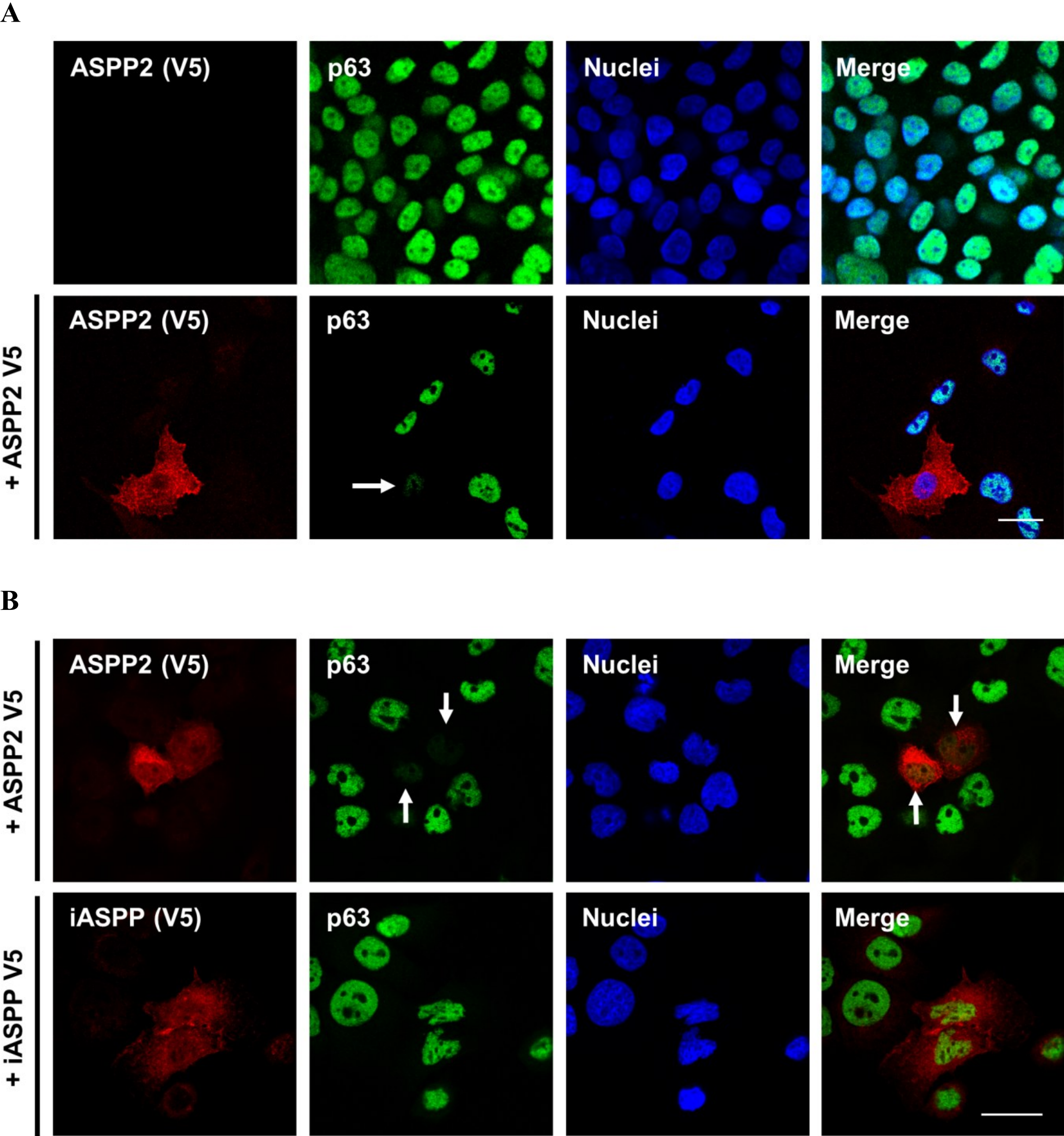
Figure 4.1 ASPP2 is down-regulated in transformed compared with non-transformed squamous epithelium

Immunostaining of cervical squamous cell carcinoma using antibody anti-ASPP2. The red arrow indicates ASPP2 normal expression in the non-transformed squamous epithelium (e), while the blue arrow indicates ASPP2 downregulation within the neoplastic mass (n). Scale bar: 100 µm.

4.2.5 ASPP2 represses p63 expression in vitro and in vivo

To directly prove that ASPP2 can repress p63 expression, we set up an experiment in which we exogenously expressed ASPP2 in some of the human SCC cell lines with very low or undetectable levels of ASPP2, and we then analysed if and how the levels of p63 were going to be affected, using immunofluorescence, RT-qPCR and Western blotting techniques. Remarkably, we observed by co-immunostaining for endogenous p63 and exogenous ASPP2 that in the UPCI SCC 040 cell line (as well as in HSC3, not shown) p63, which is present at high level consistently in all the cells, was selectively down-regulated only in the cells that were transfected with the ASPP2 expression-plasmid (Figure 4.2 A). In contrast, iASPP exogenous expression in the same cell line left p63 expression levels unchanged (Figure 4.2 B). A down-regulation of about 40% in *ΔNp63* transcript was measured by RT-qPCR upon ASPP2 re-expression, and a similar decrease was also observed at protein level by Western blotting (Figure 4.2 C-D), indicating that ASPP2 re-expression can indeed inhibit p63 expression (Figure 4.2 E). Of note, the effect of ASPP2 on p63 inhibition evaluated by RT-qPCR and Western blotting is probably an underestimation of the real effect, as the transfection efficiency of UPCI SCC 040 cells, as well as other SCC cell lines, was rather poor. Nevertheless, these results imply that the presence of ASPP2 in cultured cells tends to exclude that of p63. To test whether this was also true *in vivo*, we went back to analyse the tumours derived from the *ASPP2* heterozygous mice, performing double staining for p63 and ASPP2, measuring the degree of cell co-localisation between the two proteins. Here we found very few ASPP2-positive cells clustered together in areas scattered within the tumour mass. Quantification of the total number of ASPP2-expressing cells in three different tumours told us that ASPP2 was present in less than 5% of all the cells within the tumours, while p63 was expressed in almost 80% of them (Figure 4.3 A) . Remarkably, no co-localisation between the two markers was detected in any of the tumours analysed. This can be appreciated in Figure 4.3 B, which shows one of the rare ASPP2-positive tumour-regions, illustrating the fact that ASPP2 was found expressed in a precise mutually exclusive fashion with p63. This is

quite remarkable if we consider that almost 80% of the total number of the cells in these tumours was positive for p63.



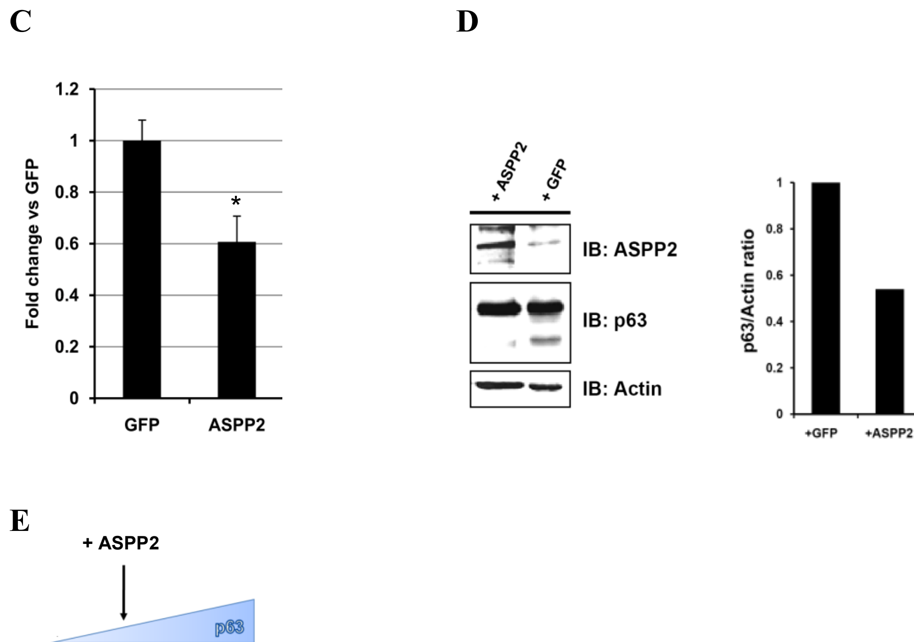
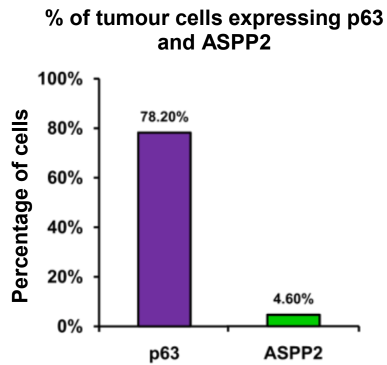


Figure 4.2 Re-expression of ASPP2 in ASPP2-negative SCC lines induce down-regulation of p63

(A) Double staining of UPCI SCC 040 cells transfected with an ASPP2 V5-tagged expression vector, and untransfected, using antibodies anti-V5 and anti-p63. TO-PRO was used to visualise the nuclei. P63 staining indicates that the protein is found uniformly expressed, except in the cells expressing ASPP2, where it is down-regulated (white arrows). Scale bar: 10 µm. (B) Transfection with ASPP2 (staining V5) induces p63 downregulation in UPCI SCC 040 cells (white arrows), while transfection with iASPP (staining V5) does not affect p63 expression. Scale bar: 10 µm. (C) RT-qPCR expression analysis of ΔNp63 mRNA in UPCI SCC 040 cells upon transfection with GFP- or ASPP2-expression construct. Compared with GFP-transfected cells, ASPP2 expression is able to induce a downregulation of ΔNp63 transcript of about 40% (p=0.031). The quantification is based on ΔΔCt method using *GAPDH* as an internal control. Error bars represent standard deviation of the mean. (D) Western blotting protein expression analysis of p63 in UPCI SCC 040 cells upon transfection with GFP- or ASPP2-expression construct, using antibodies anti-ASPP2, anti-p63 and anti-actin, used as a loading control. Gel quantification performed using ImageJ software showed that compared with GFP-transfected cells, ASPP2 expression induces a downregulation of p63 protein of about 40%. P63 expression levels have been normalised by actin protein expression prior comparison for each of the sample. (E) Drawing summarising what observed in the experiments in A, B and C: ASPP2 expression down-regulates p63.

A



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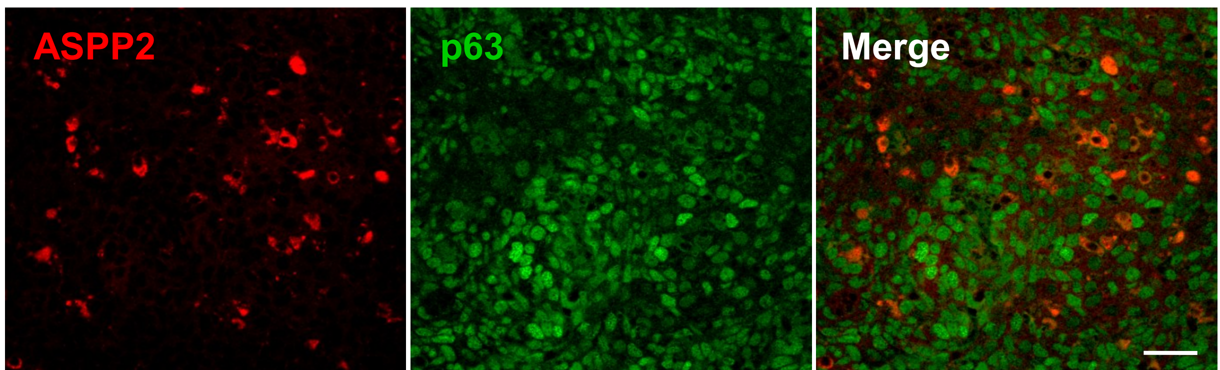


Figure 4.3 ASPP2 and p63 protein expression is mutually exclusive in mouse SCC
(A) Quantification of ASPP2- and p63-expressing cells within the SCCs formed in *ASPP2* heterozygous mice (measurement based on immunostaining of three tumours). (B) Representative area of a tumour section, from *ASPP2* heterozygous mice, immunostained with antibodies anti-ASPP2 and anti-63, showing ASPP2 and p63 reciprocal pattern of expression. The two pattern of expression are mutually exclusive, as ASPP2-positve cells in red, do not show p63 nuclear staining, in green. Scale bar: 20 µm

We then further characterised these two distinct ASPP2- and p63-positive cell populations within the tumours by determining which of them was contributing most strongly to malignant progression. Thus, we performed co-staining with markers of cell-proliferation, such as Ki-67 and nuclear Cyclin-D1. Our results confirmed what already established for human SCC samples, that p63-expressing cells are actively proliferating cells (Figure 4.4 A-B). On the contrary, we found that the ASPP2-expressing cells in the tumours were probably quiescent cells, as no co-localisation with any of the proliferation markers was observed (Figure 4.4 C-D).

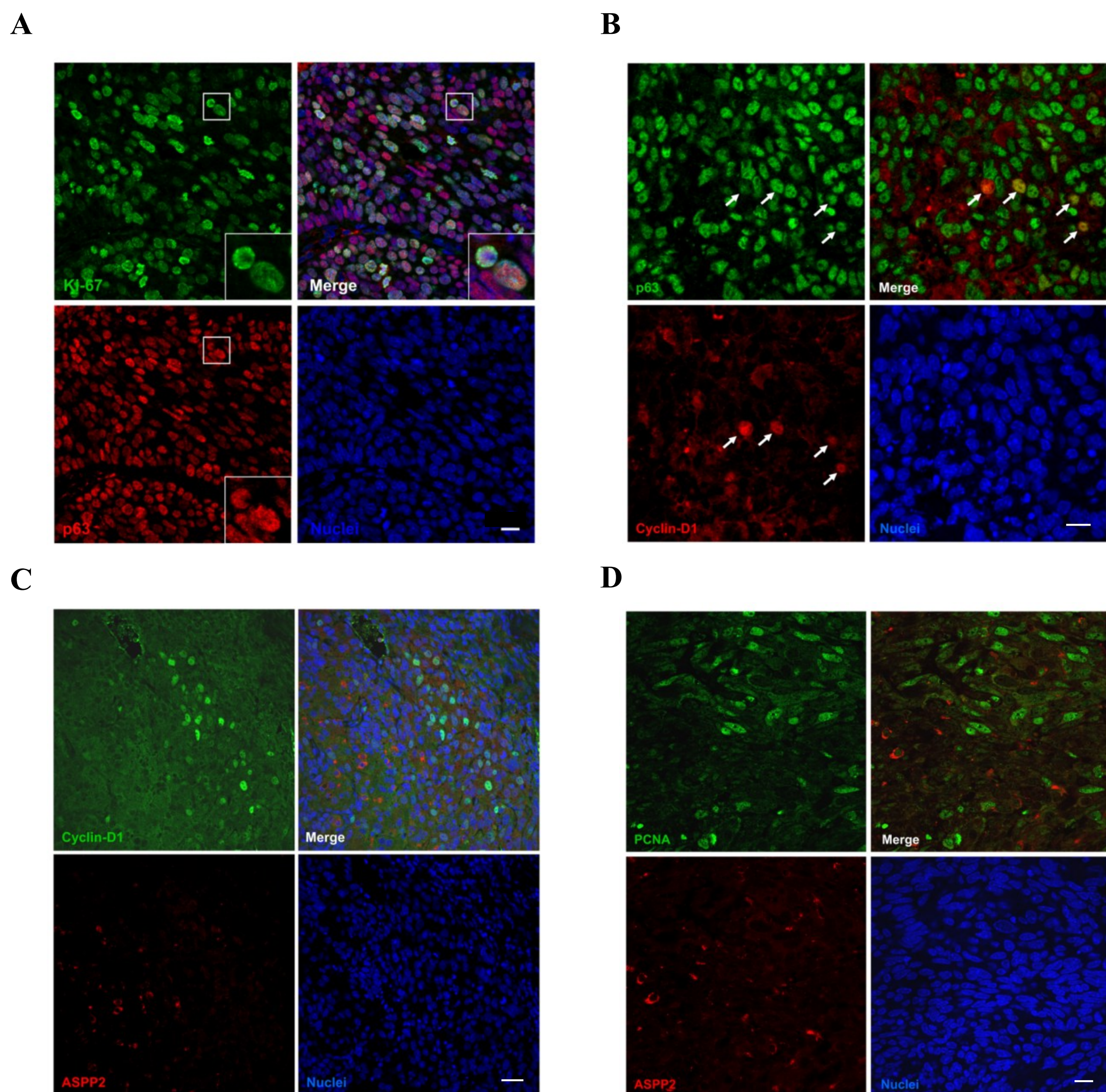


Figure 4.4 p63-positive cells in SCCs express proliferation markers, while ASPP2-positive cells do not
Double staining of p63 with Ki-67 (A) and p63 with Cyclin-D1 (B) showing co-localisation of p63 with both markers (enlargement within white square in A, and white arrows in B). Double staining of ASPP2 with Cyclin-D1 (C) and ASPP2 with PCNA (D) showing expression in distinct cell populations. Ki-67, Cyclin-D1 and PCNA have been used as markers for cell proliferation. Antibodies reacting against the proteins labelled in each figure have been utilised for the immunostainings. It was not possible performing double staining between ASPP2 and Ki-67 because both antibodies were raised in the same animal species (rabbit) and alternative antibodies were not available. PCNA was used instead of Ki-67 in double staining with ASPP2. TO-PRO was used to visualise the nuclei. Scale bars: 10 µm in A, B and D; 20 µm in C

We have shown that ASPP2 can suppress p63 expression in human SCC cell lines leading to a mutually exclusive pattern of expression between the two proteins, not only in cell culture, but also *in vivo* in mouse SCCs. To confirm ASPP2 and p63 negative correlation in humans *in vivo*, we double stained samples of SCC. As seen before, ASPP2 expression was mainly detectable in residual stretches of relatively normal stratified epithelium bordering the tumour region, and again in few clustered cells inside the tumour mass. In all the cases analysed, the cells positive for ASPP2 were negative or poor in p63 expression, matching what observed in the mouse tumours (Figure 4.5).

Collectively, these data show that ASPP2 can repress p63 expression *in vitro* and *in vivo* and its expression is down-regulated in SCC, indicating a possible physiological role for ASPP2 as a “guardian” of the squamous epithelium, whose role is to prevent p63 over-expression and tumour formation.

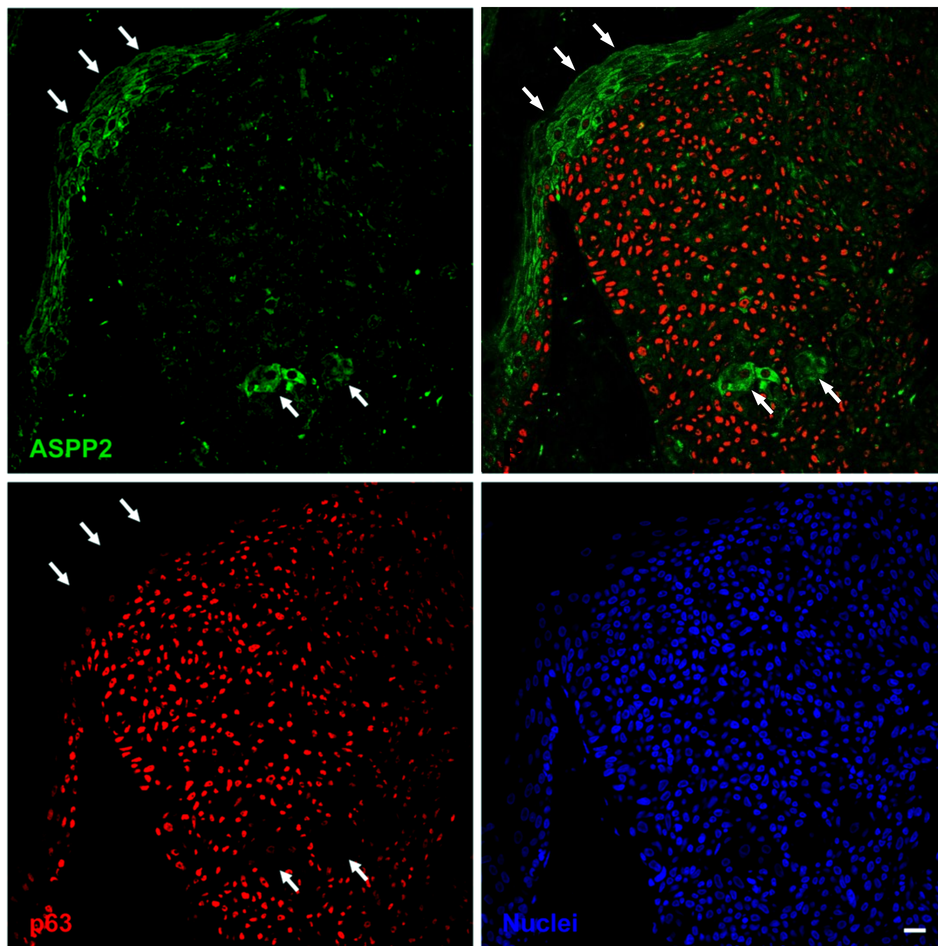


Figure 4.5 ASPP2 expression is down-regulated and mutually exclusive with p63 expression is in human SCC

Double staining of section of human cutaneous squamous cell carcinoma, using antibodies anti-ASPP2 and anti-p63. The majority of tumour cells are p63 positive and ASPP2 negative. Regions expressing ASPP2 are always negative for p63 expression (white arrows). TO-PRO was used to visualise the nuclei. Scale bar: 10 µm.

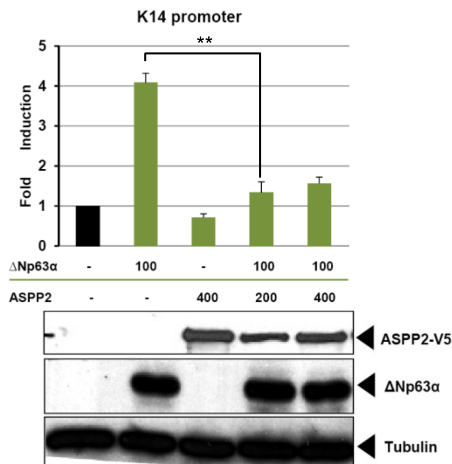
4.2.6 ASPP2 regulates p63 transcriptional function

ΔNp63, as a transcriptional factor, is implicated in the regulation of the expression of several genes involved in epithelial differentiation. Some of these genes, such as *Keratin-14* and *Keratin-1*, were up-regulated in the tumours developed by *ASPP2*-mutant mice (Figure 3.3 D). ASPP2 was previously shown to directly bind to p53 and its family members, including p63, and to confer on them binding selectivity for specific DNA promoters (Samuels-Lev, O'Connor et al. 2001; Bergamaschi, Samuels et al. 2004). We therefore decided to test whether ASPP2, in conjunction with the repression of ΔNp63 expression levels, could also regulate ΔNp63 transcriptional activity. We therefore tested ΔNp63 transcriptional activity, in the absence or presence of ASPP2, on some of its known target genes important for epithelial homeostasis, such as *Keratin-14*, *Keratin-10* and *envoplakin*. This analysis was performed by luciferase transactivation assay in H1299, a carcinoma cell line which expresses very low levels of both ASPP2 and p63. Here we found that the transcription of all the three genes, *K14*, *K10* and *envoplakin*, was induced by ΔNp63 expression, as expected, while ASPP2 had no significant effect when transfected by its own. However, when ASPP2 was expressed in combination with ΔNp63, in the case of *K14* and *K10* the transcriptional induction due to ΔNp63 transfection was almost completely abolished and the transcription was brought back to basal levels (Figure 4.6 A-B). In an opposite direction, the transcription of *envoplakin* was further stimulated when ASPP2 was added to ΔNp63 (Figure 4.6 C). This finding indicates that ASPP2, which is not a transcriptional factor itself, has no intrinsic capacity to transactivate the ΔNp63 target genes, but it can however regulate ΔNp63's transcriptional activity on them, as already shown for p53 on pro-apoptotic genes (Samuels-Lev, O'Connor et al. 2001). In particular, since *K14* and *K10* are normally up-regulated in SCC, while *envoplakin* is down-regulated, these results showed that ASPP2 can orient p63 function towards a pro-differentiation and anti-tumoural program of transcription.

Interestingly, these *in vitro* results correspond to what we had previously observed *in vivo* in the mouse tumours characterised by decreased ASPP2 expression, which showed up-regulation of K14

and K1 (Figure 3.3 D). Moreover, concerning envoplakin expression, we had also previously noticed that during differentiation of primary keratinocytes its appearance was concomitant with ASPP2 expression (day 1 upon addition of Ca^{2+}), confirming that ASPP2 might realistically be involved in its induction (Figure 3.7 C).

A



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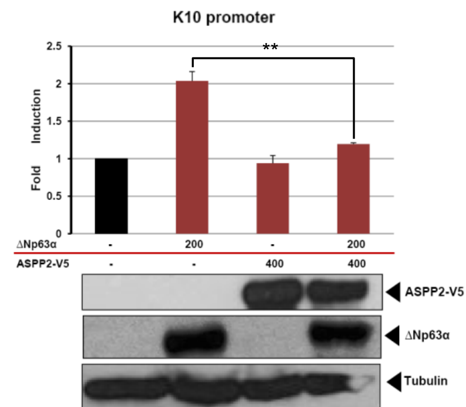

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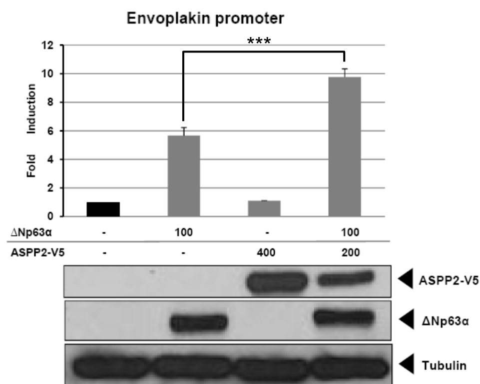

Figure 4.6 ASPP2 regulates ΔNp63 transcription on genes involved in epithelial differentiation
Transcriptional activity of *K14* (A), *K10* (B) and *envoplakin* (C) promoters was individually measured by transfecting into H1299 cells growing in 24-well plate 200 ng of each luciferase reporter construct in presence or absence of ΔNp63α and ASPP2 (V5-tagged) expression vectors (concentrations indicated in the graphs) and together with 6 ng of a renilla expression vector, as internal control. Luciferase activity was analysed from cell lysates harvested 24h after transfection and normalised on the signal from renilla. Effective transfection of ΔNp63α and ASPP2 expression plasmids was evaluated by Western blotting analysis of the same lysates used for reading the luciferase activity, using antibodies anti-V5 (ASPP2), anti p63 and anti-βtubulin (loading control). ASPP2 expression is able to repress ΔNp63α ability to induce *K14* (A, **p=0.0018) and *K10* (B, **p=0.0088) transcription, while it enhances ΔNp63α ability to induce *envoplakin* transcription (C, ***p<0.0001). Each experiment was performed in triplicate and the average values are represented in the graphs. Error bars indicate standard deviation of the mean.

4.2.7 ASPP2 suppress SCC by inhibiting p63

Finally, to demonstrate that ASPP2 suppression of SCC is truly mediated by its inhibitory effects on p63, the genetic proof should come from the inter-cross of *ASPP2*- and *p63*-deficient mice. According to our hypothesis, if p63 expression is required for ASPP2 to suppress SCC, hence mice with combined lack of ASPP2 and p63 expression should not develop SCC. Because of p63's fundamental role in the maintenance of the regenerative cell population of the epithelium, $p63^{-/-}$ mice fail to develop skin and other epithelial tissues and therefore they are not viable (Mills, Zheng et al. 1999; Yang, Schweitzer et al. 1999). As both $p63^{-/-}$ and *ASPP2* Δexon3 mice have poor survival due to developmental defects, the tumour study was performed by comparing the spontaneous tumour development in $ASPP2^{+/-}$ / $p63^{+/+}$ versus $ASPP2^{+/-}$ /$p63^{+/-}$ mice. Here we observed that until the time of one year of age, no *ASPP2/p63* double heterozygous mice had developed any spontaneous tumours, in contrast to about 25% of mice-bearing tumours in the single *ASPP2* heterozygous cohort (Figure 4.7). The tumours found in this group were all carcinomas, as observed in our previous tumour-studies on *ASPP2* deficient mice.

The fact that partial loss of p63 expression prevents *ASPP2*-mutant mice from developing spontaneous skin tumours confirmed our hypothesis, indicating that ASPP2 can supresses SCC via inhibition of p63.

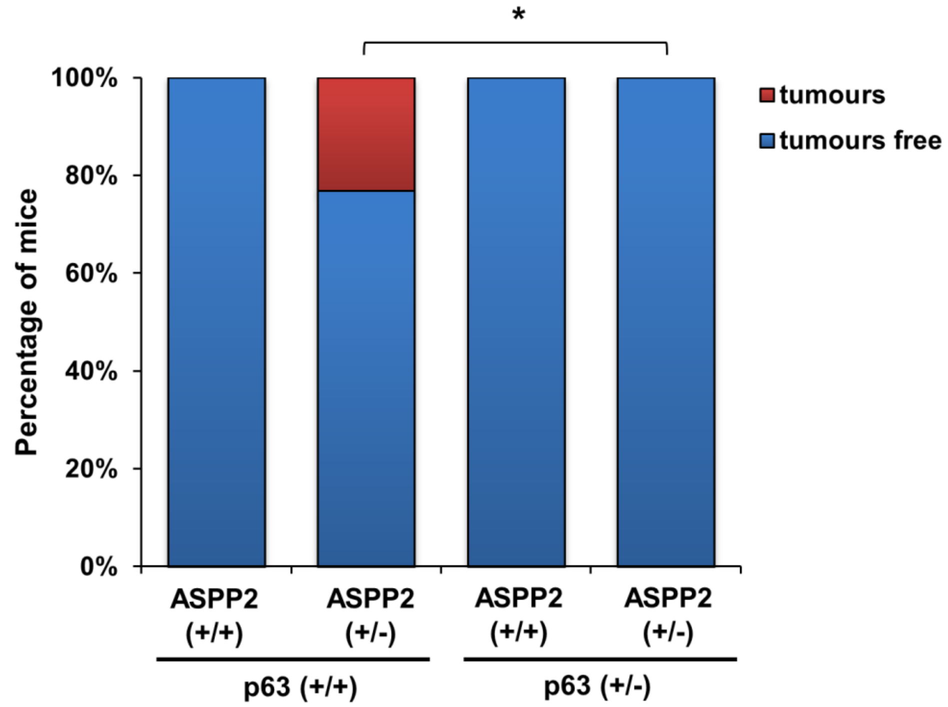

Figure 4.7 ASPP2 heterozygous mice tumour susceptibility is rescued in ASPP2/p63 double heterozygous mice

Presence of spontaneous tumours affecting the indicated mouse cohorts over a period of 1 year. (*) indicates significant at p=0.047. $ASPP2^{+/+}$ / $p63^{+/+}$ n=6, $ASPP2^{+/-}$ /$p63^{+/+}$ n=13, $ASPP2^{+/+}$ / $p63^{+/-}$ n=10, $ASPP2^{+/-}$ / $p63^{+/-}$ n=24.

4.2.8 Downregulation of ASPP2 is associated with tumour progression in human samples of SCC of head and neck

As previously mentioned, only about 20% of the *ASPP2* Δexon3 mice born alive can survive longer than 20 weeks of age, and therefore have a chance to develop tumours (Figure 3.1 B). Among those few *ASPP2* Δexon3 mice which survived longer and developed tumours, we noticed that the frequency of distant metastases was 2-fold higher than in *ASPP2* heterozygous mice bearing tumours (Figure 4.8 A). This suggests that loss of both alleles of *ASPP2* could enhance metastatic spreading of tumour cells. Interestingly, reduced expression of ASPP2 had been already linked with metastatic progression in a microarray study on breast cancer samples (Sgroi, Teng et al. 1999). This prompted us to investigate whether, even within the group of tumours originated from *ASPP2* heterozygous mice, ASPP2 expression could vary according to the stage of the tumour progression. We therefore analysed the expression of ASPP2 by immunohistochemistry, comparing non-metastatic versus metastatic tumours and their respective lung metastases, all derived from *ASPP2* heterozygous mice. Here we found that in one of the two metastatic tumours analysed, ASPP2 already sporadic expression in tumours was further decreased. Moreover, in the other metastatic tumour analysed ASPP2 expression was not detected at all (Figure 4.8 B g-h). In contrast, both the other two non-metastatic tumours used as comparators, expressed limited but higher levels of ASSP2 (Figure 4.8 B e-f). Finally, none of the lung metastases resulted positive for ASPP2 immunostaining, indicating that ASPP2 complete loss of expression in tumours may indeed favour tumour progression and metastases (Figure 4.8 B g'-h').

To verify if this observation was also true in humans, we analysed ASPP2 expression levels in a cohort of 318 human SCCs of the head and neck, including tumours non-metastatic, metastatic and lymph node metastases. Biopsies of non-transformed epithelium were used as a reference for ASPP2 normal levels of expression. Immunostaining and data analysis of the human samples have been performed by Dr Victoria Salter in Prof Xin Lu's lab. As already shown earlier in mice, we found that

ASPP2 expression was also significantly reduced in humans by comparing normal epithelium with the tumour samples. Additionally, further down-regulation of ASPP2 was observed in the tumour samples that were more advanced in the tumour progression. In fact, ASPP2 was found less expressed in metastatic compared to the non-metastatic tumours, and it reached its lowest levels of expression in the samples of metastases (Figure 4.8 C-D).

Taken together, this set of data from human and mouse tumour samples argue that down-regulation of ASPP2 is associated with metastatic progression of SCC.

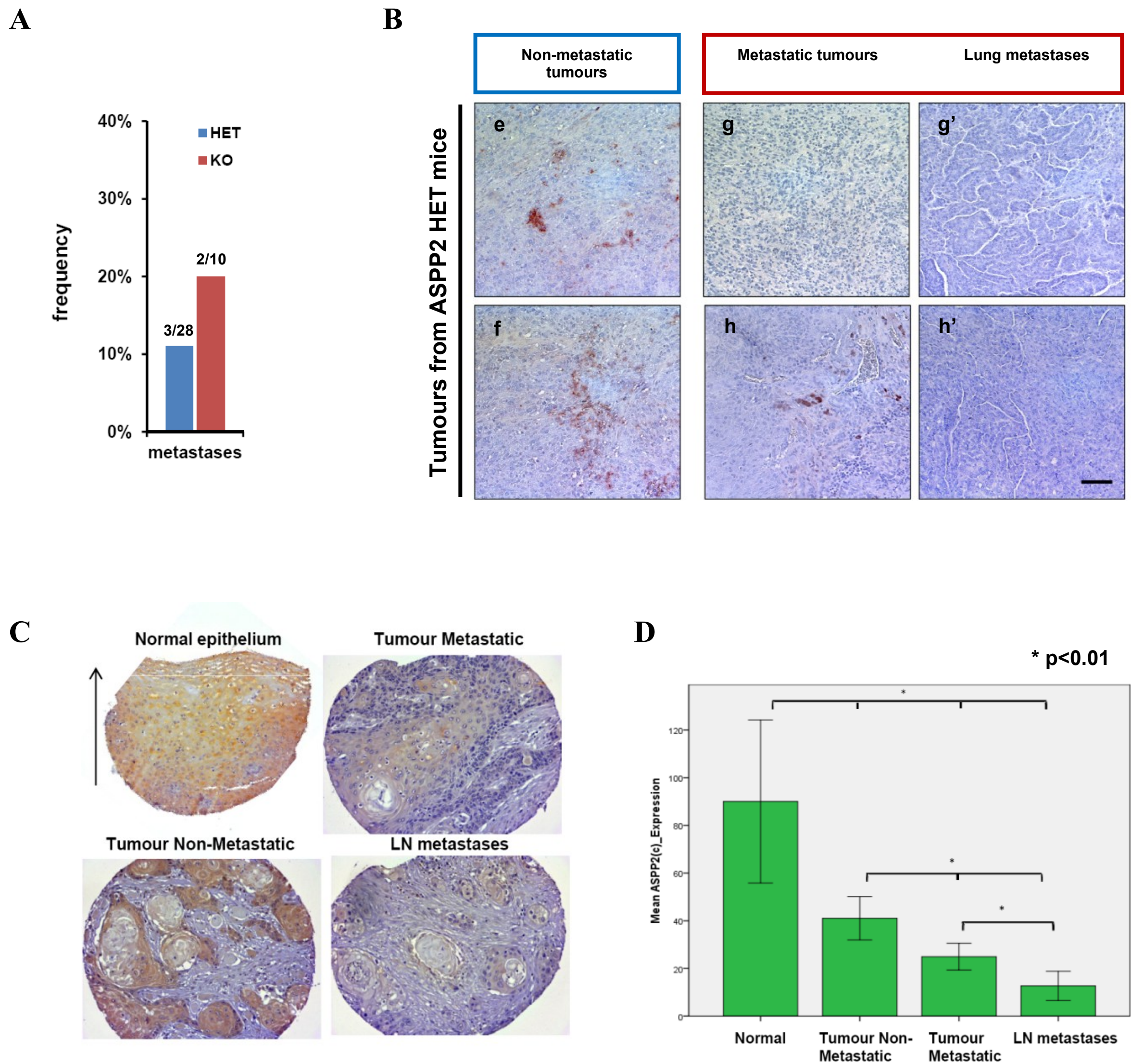

Figure 4.8 ASPP2 expression is decreased during tumour progression in SCCs derived from *ASPP2* mutant mice, as well as in human SCC samples

(A) Frequency of metastatic spreading measured on the total number of tumours observed in *ASPP2* Δexon3 homozygous (2/10 tumours) and heterozygous mice (3/28 tumours), indicating that tumours is *ASPP2* Δexon3 heterozygous mice metastasise more often, although the numbers are not statistically significant (p=0.06). (B) Immunostaining using anti-ASPP2 antibody of sections of two non-metastatic (e-f) compared with two metastatic primary tumours (g-h) and their correspondent lung metastases (g'-h'). ASPP2 expression decreases with tumour progression: scarcely expressed in e-f, further downregulated in g-h and absent in g'-h'. (C) Human squamous cell carcinoma of the head and neck tissue array, comprising various grades of tumour progression for a total of 318 carcinoma cores analysed by immunostaining using an anti-ASPP2 antibody. (D) Quantification of the tissue arrays immunostaining showing that ASPP2 expression is significantly decreased (p<0.01) during tumour progression, as observed in mouse SCC samples. Scale bars: 50 µm.

4.3 Summary

This chapter was dedicated to the investigation of the possible mechanisms which lead *ASPP2* deficient mice to develop spontaneous SCCs. The immunohistological analysis of the tumour masses performed in Chapter III showed an elevated expression of p63, whose aberrant expression have been already linked with SCC in mouse, as well as in human. Since the tumours arose from the superficial squamous epithelium of the mice, to test whether the absence of ASPP2 could be a direct causative factor for p63 over-expression and tumour formation, we first verified ASPP2 expression in the squamous epithelium of the skin. Analysis of human and mouse samples showed that ASPP2 was expressed in the skin and in particular, it localised in the differentiated layers, while it was excluded from the mitotically active basal layer where p63 is expressed. This suggested that ASPP2 could be a factor important for differentiation of the squamous epithelium. Indeed, experiments conducted with mouse primary keratinocytes showed that ASPP2 starts being expressed upon induction of differentiation. Moreover, since we observed that ASPP2 *in vivo* expression in adult skin tissue, as well as during differentiation in cultured keratinocytes, tends to be mutually exclusive with p63 expression, we hypothesised that ASPP2 might also have a role as physiological repressor of p63. This possibility would justify the high p63 expression which leads to tumour formation in the *ASPP2* deficient mice. The hypothesis that the presence of ASPP2 might be important in repressing p63 expression was also corroborated by the observation that *ASPP2* deficient cells, as MEFs as well as primary keratinocytes, were found to express higher levels of specifically ΔN- and not TA-p63 compared to the wild type counterparts. Our results suggested that this up-regulation of ΔNp63, which is also the predominant p63 isoform expressed in both normal and transformed skin, was due to an enhancement of its gene-transcription.

To establish whether the presence or absence of ASPP2 could be linked with the expression of oncogenic ΔNp63 in human tumours, as we have seen in the *ASPP2* deficient mice and mice-derived cells, we analysed human SCC cell lines and tissue samples. In the majority of the 34 cell lines

analysed, ASPP2 was found generally down-regulated, in particular in correspondence of high levels of p63 expression. By immunohistochemistry we were able to visually assess that ASPP2 expression was lost, or strongly diminished, in the tumours, while its expression was still retained in the normal stratified epithelium. This agreed with the concept of ASPP2 as a tumour suppressor in stratified epithelium, as shown before in mice.

As we believe that ASPP2 tumour suppressor function in SCC is due to a repression on p63, we tested this inhibition in human SCC cell lines. Importantly, via re-expression of ASPP2 in ASPP2 low- or not- expressing cells we observed a decrease in p63 level. Moreover, SCCs from both human and mouse specimens showed that the few ASPP2 positive cells present in the tumours were all negative for p63 expression. With these experiments we were able to show that ASPP2 expression could directly induce down-regulation of p63 in SCC and resulted in p63-free cells, which are likely to be non-proliferative cells as shown by the absence of expression of proliferative markers.

Having observed that ASPP2 and p63 expression negatively correlate in the squamous epithelium, this would argue that, in the adult tissue, ASPP2 would be important to prevent p63 from being expressed in differentiated cells. During keratinocyte differentiation however, and localised to a small population of suprabasal cells within the adult epithelium, the expression of the two proteins can coincide, as suggested by our experiments (Figure 3.7 B-C), indicating that a physical interaction between the two would be possible even if only for a short interval of time. As ASPP2 was previously shown to be able to bind and confer gene target selectivity upon p63, favouring the induction of apoptosis, we tested whether this could also happen for genes involved in skin differentiation. Remarkably, we found that ASPP2 could inhibit ΔNp63 activity on *K10* and *K14* promoters, two genes found up-regulated in SCC, and conversely enhance ΔNp63-mediated transactivation of the *envoplakin* promoter, a gene up-regulated during differentiation.

As a final experiment, we tried to rescue the formation of SCCs in the *ASPP2* deficient mice by crossing them with *p63* heterozygous mice to demonstrate that ASPP2 suppresses SCC through inhibition of p63. Although the study was conducted with a relatively small number of animals and for a shorter period of time if compared with the other tumour studies previously mentioned in text, we successfully observed absence of SCC formation in *ASPP2* heterozygous mice defective for *p63* expression.

Furthermore, we analysed ASPP2's role in suppressing tumour metastases. It appeared that in mice, a complete loss of ASPP2 expression (homozygosis for *ASPP2* Δexon3) favoured the formation of distant metastases. Thus, we found that a progressive loss of ASPP2 expression is observed during tumour progression towards a more invasive phenotype until the metastatic stage, in mouse and human samples. Interestingly, other works have shown that ΔNp63 does not seem to be involved in promoting the formation of metastases in SCC (Barbieri, Tang et al. 2006; Graziano and De Laurenzi 2011), suggesting that the total disappearance of ASPP2 from the tumour cells might trigger some other mechanisms, possibly linked with the induction of the epithelial-to-mesenchymal transition (EMT) and increased invasiveness, which will not be investigated in this work.

Altogether these data served to investigate the causes of SCC formation in *ASPP2* mutant mice, indicating that ASPP2 plays an important physiological role in the maintenance of the squamous epithelium. We have demonstrated here that ASPP2 oncosuppressor role in the epithelium is epitomised by its inhibition on ΔNp63 potentially oncogenic expression and as a consequence, ASPP2 is found down-regulated also in human SCCs. In the next chapter, ASPP2 interaction with other fundamental pathways involved in the maintenance of the squamous epithelium, such as the Notch pathway, will be discussed.

Chapter V: ASPP2, a novel player in Notch pathway

5.1 Introduction

Notch is an important regulator of epithelial homeostasis and a repressor of p63. The Notch signalling pathway is one of the most studied because of its importance in both development and disease. In particular, Notch function is important for cell fate determination, perhaps the most critical biological process in tissue homeostasis and development. The choice between different cell fates is strictly context and time dependent, and is the result of the activation of precise transcriptional programs consisting in defined combinations of gene sets. The regulation of gene target selectivity of transcriptional complexes, such as the Notch complex, is thus the event which eventually controls cell fate determination. Because of this differential choice of genes to be transcribed, the Notch pathway can induce diverse and sometimes opposing fate decisions in cells. This diversity of outcomes seems to correlate with the tissue where Notch pathway is active. For instance, in the developing CNS Notch is important for the maintenance of the pool of neural-progenitor cells, while in the keratinocytes of the skin Notch promotes terminal differentiation. Consistent with its pivotal role in cell fate determination, deregulated Notch activity has been linked with cancer. Its role in cancer can however be ambivalent. In tissues where Notch is implicated in promoting stem cell renewal, deregulated Notch activity is considered pro-tumourigenic. In hematopoietic cells for instance, excessive Notch transcriptional activity increases the transcription of *c-myc* oncogene, resulting in uncontrolled expansion of the stem cell pool and development of T-cells lymphoblastic leukaemia (T-ALL) (Sharma, Calvo et al. 2006). On the contrary, in the epidermis it is the lack of Notch activity which leads to tumour formation, making Notch a tumour suppressor protein. In fact, impaired Notch signalling in mouse skin predisposes the animals to develop spontaneous SCCs (Proweller, Tu et al. 2006). Similarly, in humans, in the two major types of non-melanoma skin cancer, SCC and BCC,

Notch activity and gene expression (Notch1 mainly) were found to be reduced (Thelu, Rossio et al. 2002; Lefort, Mandinova et al. 2007). Recently, inactivating mutations in *Notch1* gene have been identified also in SCC of the Head and Neck (HNSCC), indicating that Notch1 may function as a tumour suppressor in all squamous epithelia (Agrawal, Frederick et al. 2011). In skin squamous epithelium, one of the principal ways by which Notch fulfils its pro-differentiation role is through its ability to antagonise the expression and the activity of p63, whose role is to sustain the self-renewal of the basal keratinocytes. Notch and p63 have therefore opposite roles in the epidermis, and the balance between their activities and expression is critical for a correct epithelial homeostasis. In fact, either up-regulation of p63 or down-regulation of Notch can lead to the same outcome, namely skin tumour formation.

In a recent review on the role of Notch in cancer, deregulated Notch activity in solid tumours was ascribed to its interaction with altered pathways that are normally involved in the homeostasis of the tissue where the tumour is occurring, as shown for Notch and the EGFR pathway in astrocytic gliomas and Notch and the RAS-MAPK pathway in pancreatic adenocarcinoma (Ranganathan, Weaver et al. 2011). This indicates that the Notch pathway could simply have a broad fundamental role in cell fate determination, and its outcome then acquires specificity in different tissues depending on the interactions with different cellular factors. Consequently, initiating alterations in such tissue-specific factors can directly affect Notch behaviour, providing the required contribution for tumour transformation. Identification of such cellular factors, which can selectively influence Notch target gene expression, is therefore of vital importance for our understanding of cell fate determination and cancer.

In our study conducted with the *ASPP2* Δexon3 mice, we have previously shown that ASPP2 protein plays a role in epithelial homoeostasis. In particular, like Notch, ASPP2 can inhibit p63 expression and activity (Chapter III-IV). Moreover, reduction of ASPP2 expression in mice predisposes to formation of SCCs, the same types of tumours as are observed upon impairment of Notch activity in

the skin. During the characterisation of the tumours developed by the *ASPP2* deficient mice we also observed over-expression of proteins found in SCCs with impaired Notch activity, such as Gli2 and Cyclin-D1 (Nicolas, Wolfer et al. 2003; Proweller, Tu et al. 2006) (Figure 4.8). Additionally, among the already characterised brain defects of the *ASPP2* Δexon3 mouse, we found an expansion of the pool of neural-progenitor cells accompanied by deregulated expression of some of the Notch target genes, such as *Hes1* and *Hes5* (R. Sottocornola, personal communication).

These findings indicated that altered ASPP2 expression in mouse skin squamous epithelium, as well as in brain, resembles defects caused by impairment of Notch activity, and led us to hypothesis whether an interaction between the two pathways could exist. We have also previously mentioned in the text that Notch differential activity in different tissues relies on its transcriptional selectivity. ASPP2 has been originally characterised as a factor able to confer gene target selectivity, as shown for p53, p63 and p73 (Bergamaschi, Samuels et al. 2004), suggesting that it might also work with Notch in a tissue specific manner in skin and brain.

Collectively, these observations prompted us investigate whether ASPP2 could physically and functionally interact with Notch pathway

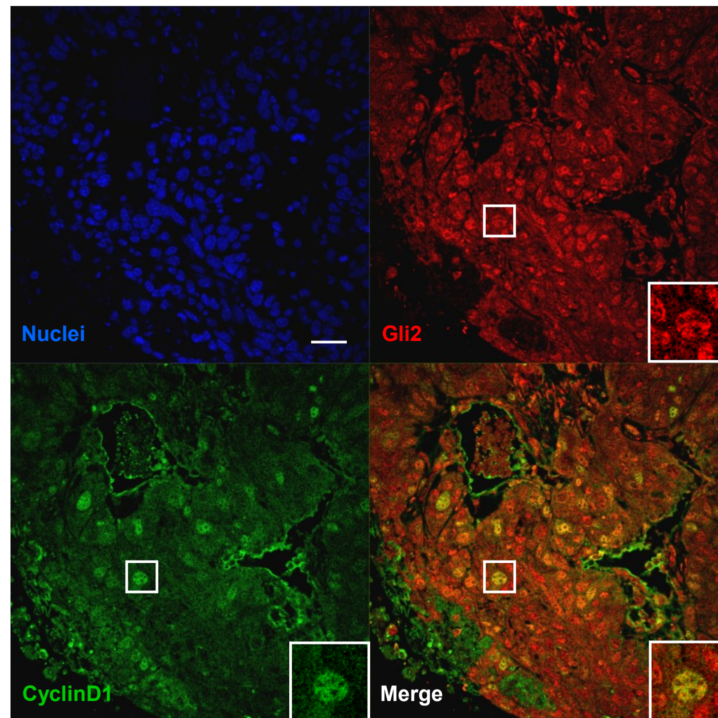

Figure 4.9 Tumours derived from *ASPP2* mutant mice present upregulation of Wnt/β-catenin and Sonic hedgehog pathways, as seen in skin tumours with impaired Notch activity
Double staining of a SCC sample from *ASPP2* deficient mice using antibodies anti-Gli2 and anti-CyclinD1. TO-PRO was used to visualise the nuclei. White frames show an example of double positivity for both markers Gli2 and CyclinD1 at higher magnification. Scale bar: 20 µm.

5.2 Results

5.2.1 Study of ASPP2 and Notch1 expression in adult squamous epithelium in vivo and during differentiation of primary keratinocytes in vitro

As it appears that ASPP2 and Notch1 share overlapping functions in the epithelium, such as the inhibition of p63 and tumour suppressor properties, we decided to investigate their reciprocal physiological expression in human and mouse normal squamous epithelium. The result of co-immunostaining between ASPP2 and Notch1 showed that the two proteins are both predominantly expressed in upper-differentiated layers, and this expression pattern is conserved between human and mouse stratified epithelia (Figure 5.1 A-B). In humans this pattern is more evident than in mice, as the epithelial tissue is visibly more stratified. This observation adds further evidence that ASPP2 and Notch could play similar roles in epithelial homeostasis.

Successively, we checked how the levels of protein expression of ASPP2 and Notch were regulated during differentiation of mouse primary keratinocytes. Western blotting was performed using cell lysates from undifferentiated cells (growth in absence of Ca^{2+}) and from cells harvested upon one and five days upon induction of differentiation (addition of high Ca^{2+}). As seen before (Chapter IV, Figure 3.7) ASPP2 expression was up-regulated immediately upon one day of differentiation (Figure 5.1 C). Notch1 levels of expression were also found regulated upon differentiation and interestingly the two pools of Notch1, the truncated nuclear protein (Notch ID or NICD) and the full-length membrane-associated protein (Notch FL) presented opposite trends of expression (Figure 5.1 C). Notch NICD protein level was found progressively diminished during differentiation, whereas Notch FL enriched, indicating that as we progress towards a more established and definitive differentiated phenotype, Notch nuclear activity is no longer required in large amounts and more transcriptionally inactive Notch membrane receptor is observed. What was however surprising is that, despite the known role of Notch1 in promoting terminal differentiation of keratinocytes, we also detected high amounts of active Notch in undifferentiated cells. This suggests that is probably not the total level of

nuclear Notch but rather its regulated and selective activity on certain target genes which determines a specific outcome, which in this case is the promotion of keratinocyte differentiation. This specificity could be assigned by factors which are not present in undifferentiated cells, but their expression is switched on during differentiation. ASPP2 expression during differentiation of keratinocytes goes from barely detectable in undifferentiated cells to be highly expressed immediately after the addition of Ca^{2+}, indicating that its impact should be relevant in inducing cell differentiation. At this stage (one day after Ca^{2+}) NICD was also present, and therefore ASPP2 could have an impact in directing its transcriptional activity towards a specific pro-differentiation programme.

In conclusion, we first found that ASPP2 and Notch1 co-localised in the same region of the differentiated squamous epithelium, suggesting that the disappearance of one of the two proteins from this site might affect the expression or activity of the other protein, and ultimately the properties of the cells residing there. Secondly, we observed that just after the induction of differentiation of primary keratinocytes the expression of ASPP2 overlaps with that of active Notch, suggesting that they could functionally interact at transcriptional level in the early stages of differentiation.

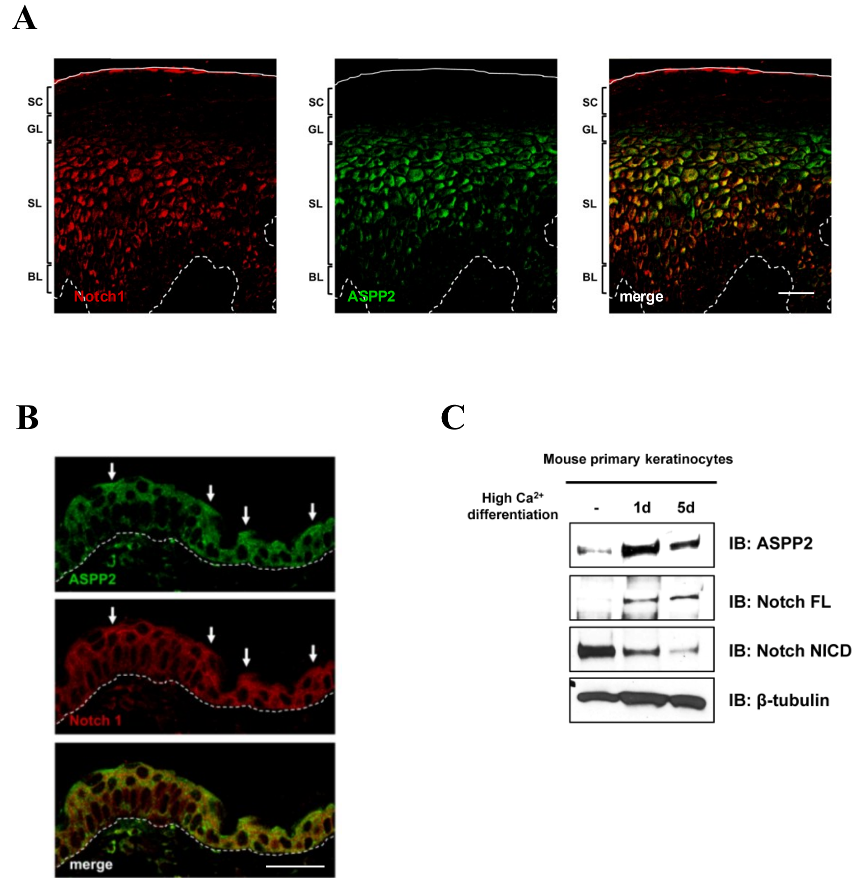

Figure 5.1 ASPP2 and Notch1 are coexpressed in the upper layers of the squamous epithelium and during differentiation in primary keratinocytes

Double staining of human (A) and mouse (B) squamous epithelium of the skin with anti-Notch1 and anti-ASPP2 antibodies shows that ASPP2 and Notch1 have an identical pattern of expression. White arrows in (B) show enrichment of ASPP2 and Notch1 expression in the upper *strata* of the mouse epithelium. (C) Evaluation by Western blotting of ASPP2 levels of expression compared to full-length Notch1 (Notch FL) and cleaved Notch1 (Notch NICD) during differentiation of mouse primary keratinocytes. Lysates were prepared from cells cultured in absence of calcium (Ca^{2+}) in the medium and then after one and five days upon the addition of Ca^{2+}. Immunoblotting was performed using antibodies anti-Notch1 antibody recognising both pools of Notch, FL and NICD, (Santacruz, C-20), anti-ASPP2 and anti-β-tubulin (loading control). ASPP2 expression is induced upon differentiation, as the pool of membrane-associated Notch1 FL, while levels of nuclear Notch1 (NICD) are decreased. BM, basal membrane; BL, basal layer; SL, spinous layer; GL, granular layer; SC, *stratum corneum*. Scale bars: 20 µm.

5.2.2 In SCCs developed by ASPP2 heterozygous mice, Notch expression is down-regulated, mutually exclusive with p63, and associated with ASPP2 expression.

As previously shown, immunostaining of SCCs derived from the *ASPP2* deficient mice revealed the presence of proteins also found up-regulated in cutaneous tumours from mice with impaired Notch activity (Figure 4.9). This suggests that the Notch signalling pathway itself might be affected as a consequence of ASPP2 depletion. We also showed before that ASPP2 and Notch1 are normally co-expressed in the same layers of the normal epithelium, making this hypothesis even more plausible (Figure 5.1 A-B).

To test this hypothesis, we firstly looked at the expression of Notch1 in the ASPP2 deficient tumours. As in the normal epidermis Notch1 and p63 are generally equally expressed, although in different populations of keratinocytes, we decided to compare their reciprocal expression in the *ASPP2* heterozygous tumours. The result showed that p63 expression, as already characterised in Chapters III-IV, was up-regulated (present in about 78% of all the tumour cells) while we found that, consistent with our hypothesis, Notch1 expression was down-regulated in the tumours (present in about 27% of the tumour cells) (Figure 5.2 A-B). Moreover, the two proteins were rarely found co-expressed in the same cells within the tumours, therefore maintaining their original distinctive localisation as in the normal epithelium (Figure 5.2 A and C). The percentage of ASPP2 expressing cells was also quantified in the tumours at around 5%. Reasonably, this low expression of ASPP2 cannot be exclusively attributed to the lack of one of the two alleles, as the tumours originated from *ASPP2* heterozygous mice, but it conceivably indicates that the cells of the epithelium which have been affected by tumourigenic expansion were not the ASPP2 positive cells, which remained more or less the same amount as in the normal tissue. Intriguingly, these few ASPP2-positive cells not only were never found to co-localise with p63 in the tumours, as shown also in Chapter IV, but they were also almost exclusively comprised within the Notch-positive cell population (Figure 5.2 A and C).

These results indicate that Notch expression was found diminished in tumours with partially impaired expression of ASPP2. Nevertheless, the two proteins, Notch and ASPP2, still co-localised in the tumours in a mutually exclusive pattern with p63 as they normally do in the non-transformed epithelium.

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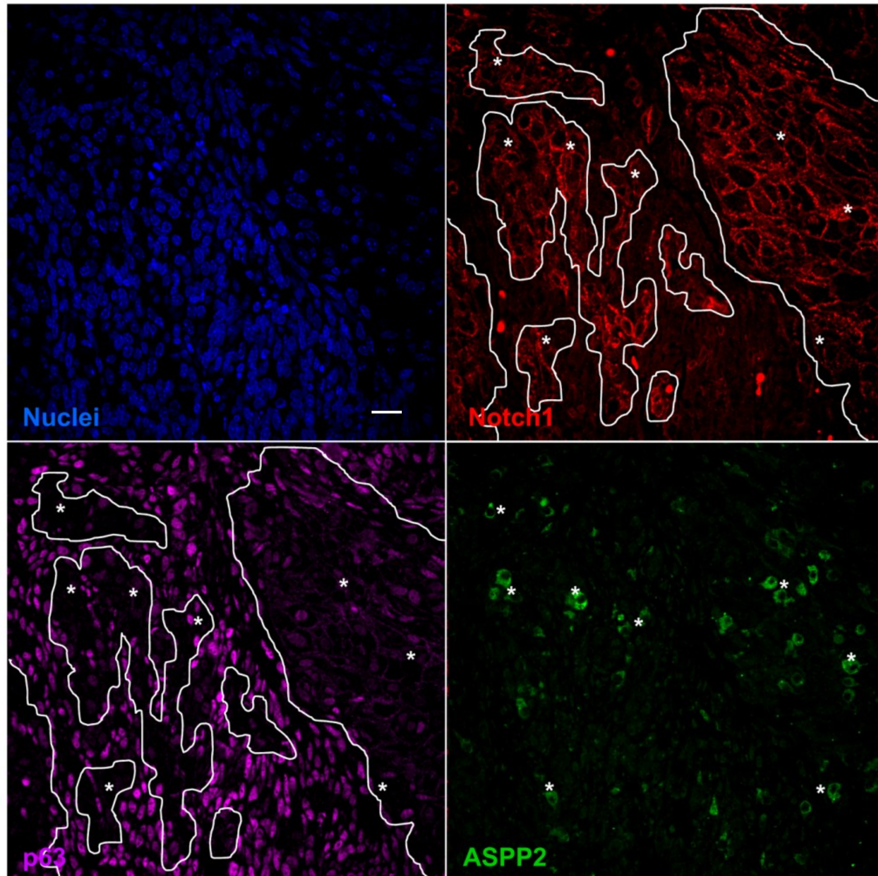

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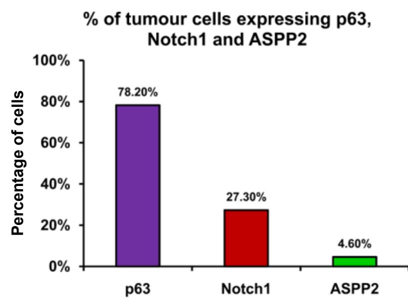

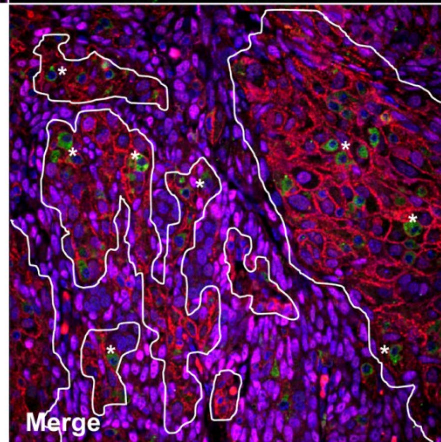

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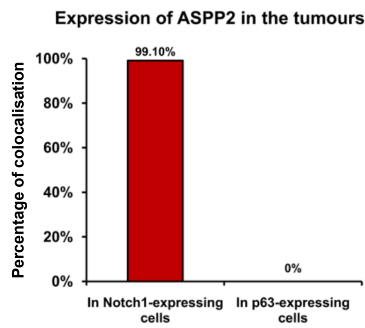

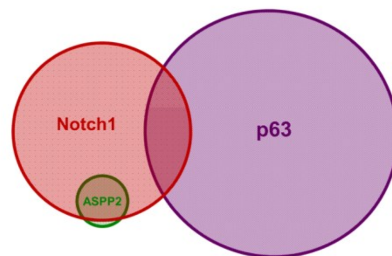

Figure 5.2 In SCCs derived from *ASPP2* Δexon3 heterozygous mice ASPP2 is exclusively expressed in Notch1-positive cells and both markers are mutually exclusive with p63

Triple staining of tumour section from *ASPP2* Δexon3 heterozygous mice using antibodies anti-Notch1 (goat), anti-p63 (mouse) and anti-ASPP2 (rabbit). DAPI was used to visualise the nuclei. Notch1-positive cells have been bordered in white to highlight their mutually exclusive pattern of expression with p63-positive cells. Scattered ASPP2-positive cells have been marked by asterisks to highlight their presence exclusively within groups of Notch-positive cells. Scale bar: 20 μm. (B) Quantification of p63-, Notch1- and ASPP2-expressing cells within the SCCs formed in *ASPP2* heterozygous mice (measurement based on immunostaining of three tumours). (C) Quantification of ASPP2-degree of colocalisation with Notch1- or with p63-positive cells (measurement based on immunostaining of three tumours). Venn diagram next to the bar graph illustrates ASPP2 exclusive localisation in Notch1-expressing cells.

5.2.3 Absence of ASPP2 impairs Notch ability of repressing p63 expression

Having seen that ASPP2 and Notch expression correlate in both normal and transformed epithelium, and that Notch expression was generally down-regulated in tumours derived from *ASPP2* deficient mice, we decided to test whether the absence of ASPP2 could affect also Notch functional activity. In particular, considering the elevated expression of p63 observed in the tumours and the fact that Notch is a known repressor of ΔNp63 in the stratified epithelium, we tested Notch's capacity to inhibit ΔNp63 expression in absence of ASPP2. In order to activate the Notch pathway, *ASPP2* wild type and Δexon3 MEFs were seeded in dishes coated with the Notch ligand Dll4. 24 hours later mRNA extracted from cells grown in the presence and in absence of the Dll4 coating was analysed by RT-qPCR to quantify the levels of *ΔNp63* transcripts. In wild type MEFs, we observed that activation of Notch led to an expected significant decrease of *ΔNp63* mRNA, estimated at about 90%. Interestingly, in *ASPP2* Δexon3 MEFs this decrease was only 40% (Figure 5.3). This suggests that Notch function is defective in *ASPP2* Δexon3 cells. It also argues that Notch can possibly cooperate with ASPP2 to suppress ΔNp63 expression.

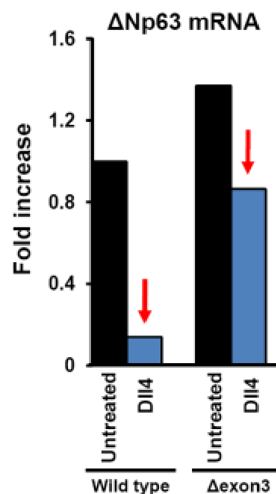

Figure 5.3 Notch-mediated inhibition of p63 is less effective in *ASPP2* Δexon3 cells
ASPP2 wild type and Δexon3 MEFs have been plated in presence or absence of Dll4 coating (3 μg/ml). Dll4 was used to induce activation of Notch signalling. 24 hours later cells were harvested and mRNA levels of *ΔNp63* was analysed by RT-qPCR. *ΔNp63* transcript was found downregulated by about 90% in wild type MEFs upon Notch activation, while only 40% downregulation was

observed in *ASPP2* Δexon3 MEFs. Analysis based on ΔΔCt method using *GAPDH* as an internal control.

To summarise the evidence supporting the existence of a possible cross-talk between ASPP2 and Notch pathway, we have found that: both proteins are expressed in the differentiated layers of the squamous epithelium; the depletion of either of them from this compartment leads to the formation of spontaneous SCC with elevated expression of p63, as well as Gli2 (up-regulation of Shh pathway) and Cyclin-D1; both ASPP2 and Notch can suppress p63 expression; and Notch expression and functional activity is affected by loss of ASPP2 expression (Figure 5.4). This set of data realistically indicates the possible existence of an ASPP2-Notch interaction which will be further investigated in the following part of this chapter.

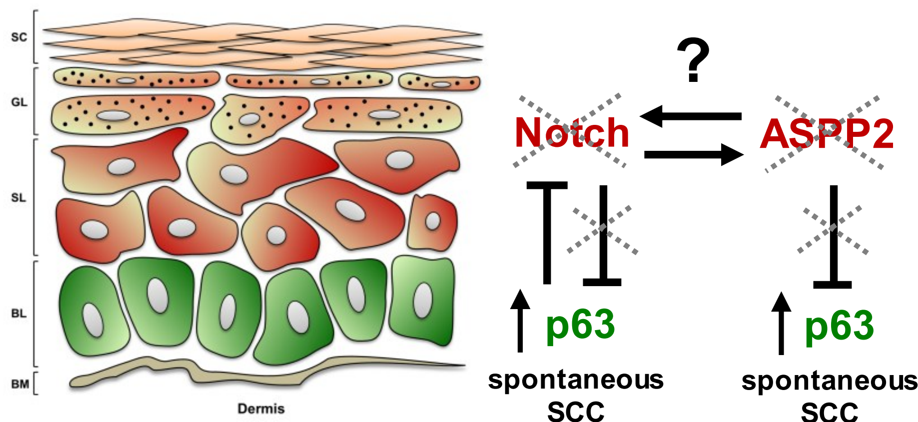

Figure 5.4 Diagram illustrating functional similarity between ASPP2 and Notch in the squamous epithelium
ASPP2 and Notch share the same localisation in differentiated layers, they have the same inhibitory effect on p63 expression, depletion of their expression from the squamous epithelium of the skin leads to the spontaneous formation of the same tumour type (SCC): altogether these data suggest the possibility of an existing crosstalk between the two pathways.
BM, basal membrane; BL, basal layer; SL, spinous layer; GL, granular layer; SC, *stratum corneum*.

5.2.4 Notch and ASPP2 co-localise in the nucleus in vivo

The activity of the Notch signalling pathway is transcriptional, therefore it can be detected by the presence of NICD in the cell nucleus. It appears that in the adult, fully differentiated, stratified epithelium Notch can be rarely detected in the nuclear compartment (Figure 5.1), suggesting that the pathway is generally less active here than during skin development. It was in fact at the early stages of keratinocyte differentiation that we measured good expression levels of NICD (Figure 5.1 C). This indicates that the Notch pathway is not constantly active, but rather is temporally regulated and induced when required. Similarly, ASPP2 is very rarely detected in the nucleus in normal conditions, in both tissues and cell lines. It has been demonstrated however that ASPP2 can be translocated into the nucleus in certain particular conditions, such as upon DNA damage, and affect the transcriptional program of proteins such as p53, p63 and p73 (Samuels-Lev, O'Connor et al. 2001; Bergamaschi, Samuels et al. 2004). Both ASPP2 and Notch are therefore in normal conditions expressed at the cell membrane, and can then become transcriptionally influential upon events which determine their nuclear recruitment. As tumour development can be consider a relevant perturbation of the normal homeostasis of the tissue, we examined the presence of ASPP2 and Notch1 in the nuclear compartment by immunostaining sections of SCCs derived from the *ASPP2* heterozygous mice. Quantification conducted on three tumours showed that nuclear ASPP2 was observed in 7% of all ASPP2 positive cells present in the tumours, while nuclear Notch1 was observed in only about 1% of all Notch1-positive cells (Figure 5.5 A). As the population of Notch1-positive cells in the tumours was larger than the ASPP2-positive population (27% and 5%, respectively), the total fraction of tumour cells with nuclear Notch1 and nuclear ASPP2 turned out to be very similar: 0.35% of total tumour cells with nuclear ASPP2 and 0.27% with nuclear Notch1. Even more interestingly, about 43% of the cells with nuclear ASPP2 also had nuclear Notch1 (Figure 5.5 B-C). This shows that Notch1 and ASPP2 co-localise in the nucleus *in vivo* and therefore they may be activated by similar stimuli.

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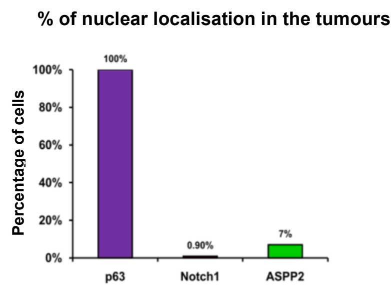

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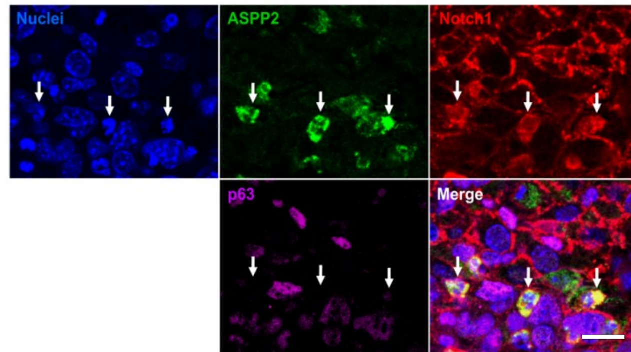

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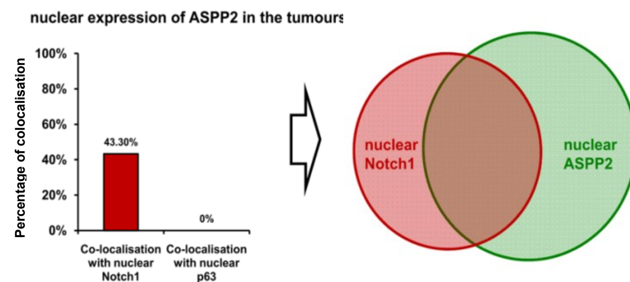

Figure 5.5 In SCCs derived from *ASPP2* Δexon3 heterozygous mice ASPP2 and Notch1 colocalise in the nucleus

(A) Quantification of p63, Notch1 and ASPP2 percentage of nuclear localisation in cells within the SCCs formed in *ASPP2* heterozygous mice (measurement based on immunostaining of three tumours). (B) Triple staining of tumour section from *ASPP2* Δexon3 heterozygous mice using antibodies anti-Notch1 (goat), anti-p63 (mouse) and anti-ASPP2 (rabbit). DAPI was used to visualise the nuclei. Arrows indicate nuclear colocalisation between ASPP2 and Notch1 and contemporaneous absence of p63 nuclear expression. Scale bar: 10 μm. (C) Quantification of ASPP2-degree of colocalisation with Notch1 or with p63 in the nucleus (measurement based on immunostaining of three tumours). Venn diagram next to the bar graph illustrates that nuclear ASPP2 colocalises with nuclear Notch in about 43% of the cases.

5.2.5 Notch pathway activation induces nuclear ASPP2

To test whether the nuclear recruitment of ASPP2 and Notch can be induced by the same stimuli we analysed ASPP2 cellular localisation after activation of Notch pathway in MCF7 cells. In epithelial cells such as MCF7, endogenous ASPP2 is predominantly expressed as a junctional protein localised at the cell-membrane. In order to activate Notch pathway, MCF7 cells were plated in presence, or absence, of a coating with Notch ligand Dll4. Cells were then processed for immunofluorescence aimed at the detection of Notch1 and ASPP2. As expected, Dll4/Notch engagement led to an increase of NICD in the nuclei. Interestingly, Dll4 coating produced an identical effect on ASPP2 localisation by inducing its nuclear recruitment (Figure 5.6 A). Notch pathway activation by Dll4 coating was further verified by immunostaining the cells for the two main components of the Notch nuclear transcriptional machinery, MAML and RBP-jκ. As for Notch, nuclear accumulation of both proteins was observed in presence of Dll4 (figure 5.6 B). This result indicates that ASPP2 cellular localisation is influenced by Notch pathway activation.

To confirm this observation using a different experimental approach, and at the same time to verify whether NICD was physically needed to shuttle ASPP2 into the nucleus, we exogenously expressed NICD in MCF7 cells. Cells were fixed and immunostained for the detection of exogenously expressed NICD and endogenous ASPP2, 24 hours after cell transfection with a construct carrying a myc-tagged DNA coding sequence of NICD. Co-stainings between NICD and RBP-jκ and NICD and iASPP were used as controls. RBP-jκ nuclear staining was enriched in NICD transfected cells, confirming the activation of the Notch transcriptional complex, while iASPP localisation remained unchanged (Figure 5.7). Consistent with the previous experiment conducted using Dll4 ligand, ASPP2 accumulated in the nucleus upon activation of the Notch pathway, this time mediated by the expression of NICD (Figure 5.7).

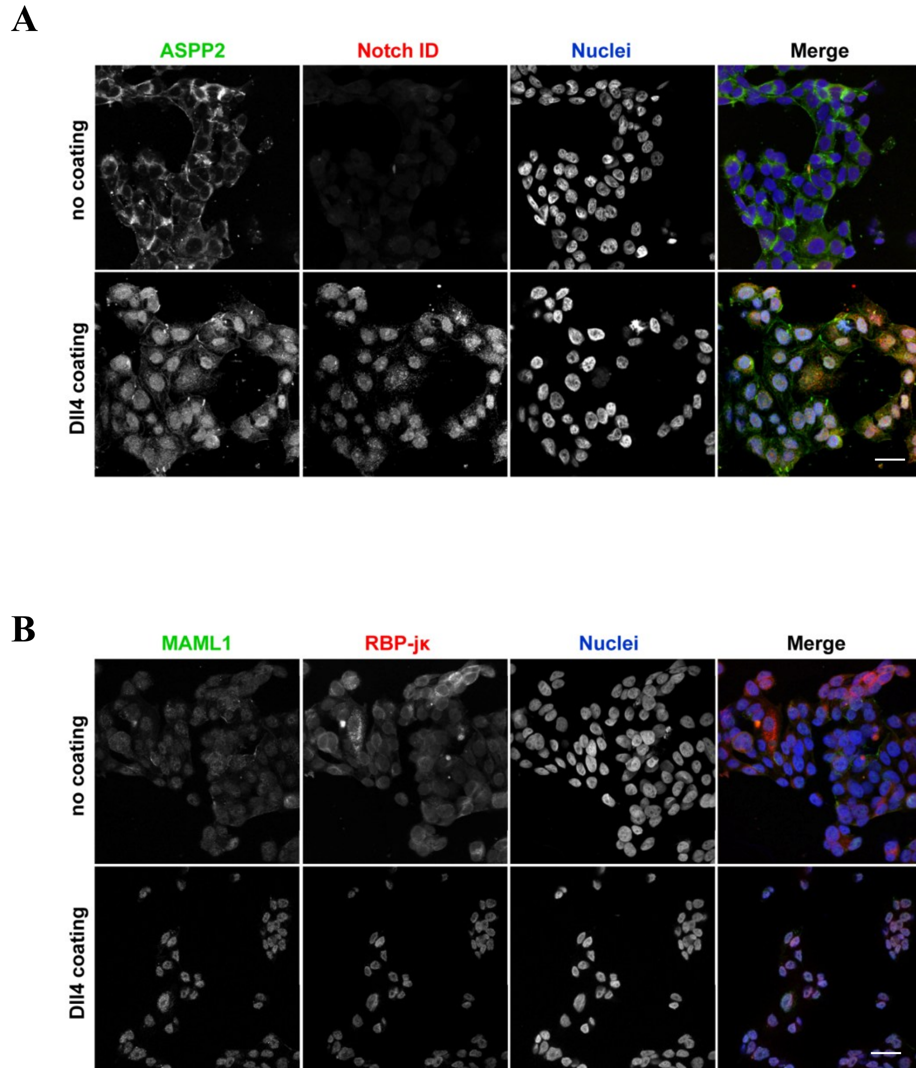

Figure 5.6 Dll4 induces ASPP2 nuclear recruitment
Notch, ASPP2, MAML1 and RBP-jκ cellular localisation was analyzed after activation of Notch pathway via Dll4 ligand. (A) MCF7 cells were plated on glass coverslips on a 24-well plate in presence or absence of a coating with Notch ligand Dll4. Cells were then fixed after 5 hours and processed for immunofluorescence aimed at the detection of endogenous Notch ID, using an antibody recognizing the active form of Notch, and ASPP2, using an anti-ASPP2 antibody. Dll4/Notch engagement led to an increase of Notch ID into the nuclei and produced an identical effect on ASPP2 by inducing its nuclear localisation. (B) Following the same experimental procedure for Notch pathway activation, cells were then immunostained for MAML1 and RBP-jκ. As for Notch ID, nuclear accumulation of both proteins was observed in the presence of Dll4. TO-PRO was used to visualise the nuclei. Scale bar: 20 μm.

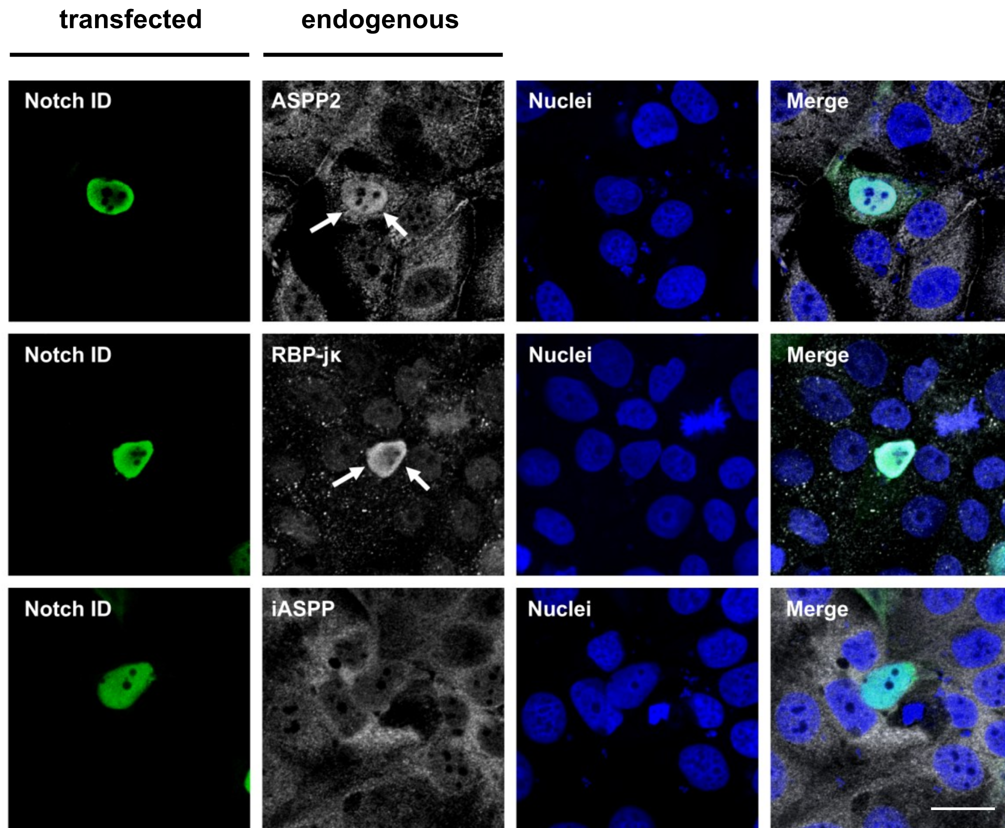

Figure 5.7 Notch ID induces ASPP2 nuclear recruitment
ASPP2, RBP-jκ and iASPP cellular localisation was analyzed after exogenous expression of Notch ID (active form of Notch). MCF7 cells growing on coverslips in a 24-well plate have been transfected with a plasmid expressing a myc-tagged form of Notch ID. 24 hours later cells were fixed and processed for immunofluorescence. Co-immunostaining was performed using an anti-myc antibody in combination with, respectively, anti-ASPP2, anti-RBP-jκ and anti-iASPP antibody. Nuclear ASPP2 was greatly increased in Notch ID transfected cells, similarly to nuclear RBP-jκ, used here as a positive control, while iASPP expression pattern was not affected by Notch ID exogenous expression. TO-PRO was used to visualise the nuclei. Scale bar: 10 μm.

5.2.6 Expression of MAML1 induces ASPP2 in nuclear foci where it localises with components of the Notch transcriptional complex

Once activated, Notch is translocated into the nucleus where it forms a transcriptionally active ternary complex with the co-activator protein MAML and the transcriptional factor RBP-jκ. As RBP-jκ can already be found in the nucleus even in the absence of the other two factors, the formation of the active complex is induced by the nuclear entrance of NICD and MAML. In previous experiments, we showed that expression of NICD in cells can promote nuclear localisation of ASPP2. We next investigated whether expression of MAML can have the same effect on ASPP2 localisation. As in the experiment with NICD, a construct carrying a flag-tagged version of the DNA-coding sequence of MAML1 was transfected into MCF7 cells. Its effect on endogenous ASPP2 was subsequently analysed. Immunofluorescence analysis of transfected MAML1 revealed a defined punctuate nuclear localisation (Figure 5.8 A), as shown in previous works (Wu, Aster et al. 2000; Saint Just Ribeiro, Hansson et al. 2009). Interestingly, upon MAML1 exogenous expression, ASPP2 was also found often present in distinct nuclear regions (Figure 5.8 A i-i'). We therefore performed co-immunostaining between transfected MAML1 and endogenous ASPP2 and found a clear co-localisation of the two proteins to the same defined spots in the nucleus (Figure 5.8 B). Quantification of the cell staining showed that about 85% of the endogenous ASPP2 was localised in nuclear foci upon MAML1 transfection (Figure 5.8 C). The same effect on ASPP2 localisation was observed in MDA-MB-231, indicating that the phenomenon observed is general and not cell line specific (data not shown). Notch1 is one of the major interacting proteins of MAML1 and was previously observed to colocalise with it in nuclear foci, thus we investigated whether Notch1 could also be found in the ASPP2/MAML1 foci. Individual co-localisation experiments between ASPP2-MAML1, ASPP2-RBP-jκ and ASPP2-Notch1 carried out upon MAML1 transfection confirmed that the nuclear regions where ASPP2 is recruited are Notch-complex foci, as both endogenous RBP-jκ and Notch1 were found to co-localise there with ASPP2-MAML1 (Figure 5.8 D). To further confirm the simultaneous

presence of ASPP2 with the two other members of the Notch complex, MAML1 and RBP-jκ, in the nuclear foci, triple co-localisation studies between ASPP2-MAML1-RBP-jκ and Notch1-MAML1-RBP-jκ as a positive control, were also performed in MCF7 cells. As for Notch1, ASPP2 was also detected together with MAML1 and RBP-jκ in the same nuclear foci (Figure 5.8 E). Additionally, we performed costainings between MAML1 and other factors which are known to be temporarily recruited at the level of the Notch transcriptional complex, for different reasons, such as CDK8 (induces proteosomal degradation), p300 (contributes to activation) and HDAC1 (recruited by co-repressors). Their successful detection in the MAML-induced foci again confirmed the specificity of the phenomenon observed (Notch-complex foci) (Figure 5.8 F).

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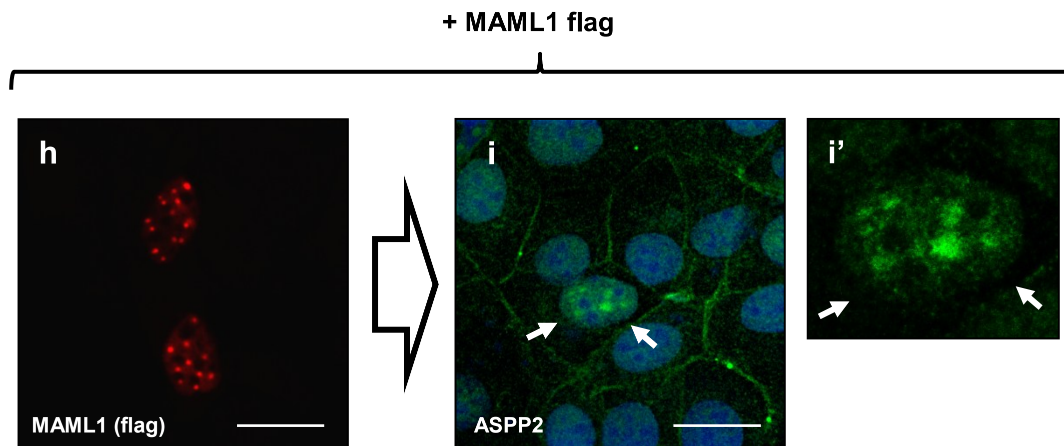

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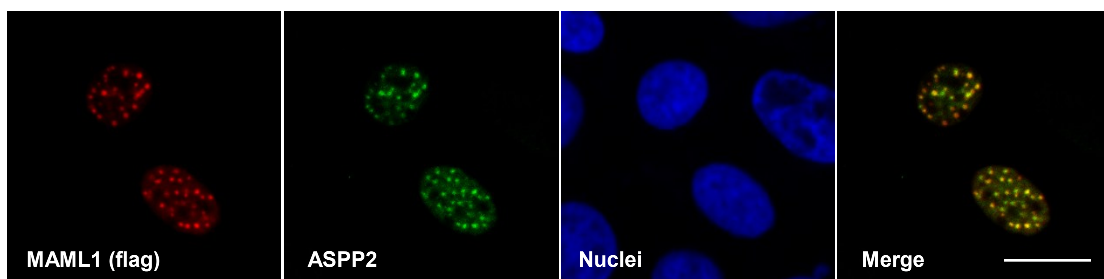

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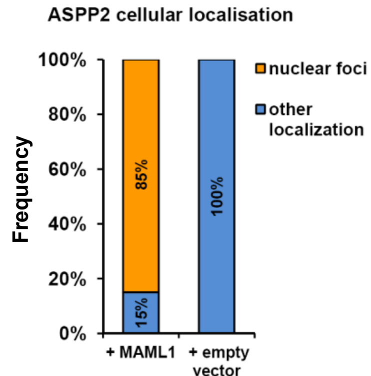

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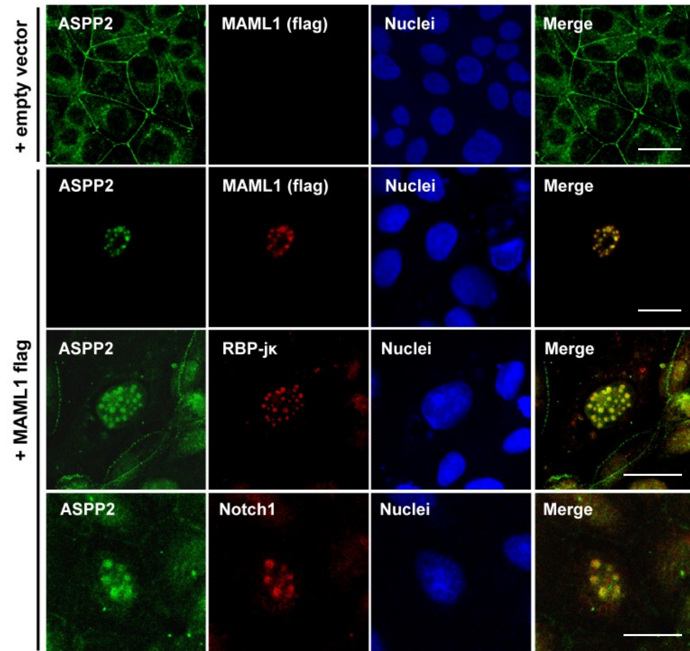

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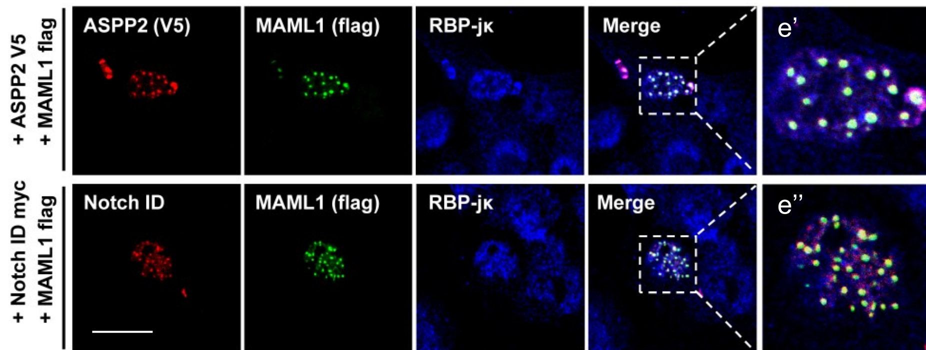

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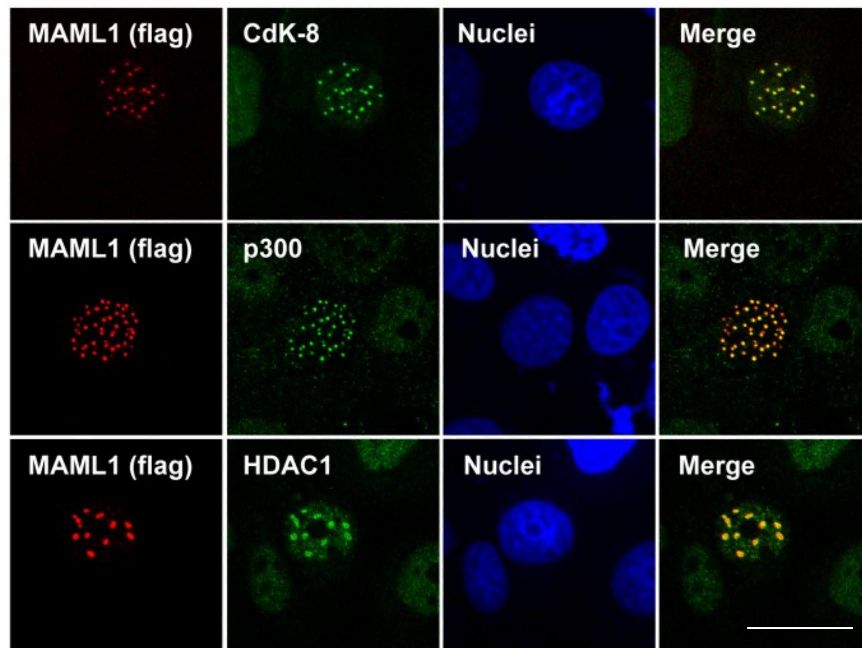

Figure 5.8 MAML1 exogenous expression induces ASPP2 movement into Notch-complex foci
In all the experiments shown here, MCF7 cells growing on coverslips in a 24-well plate were transfected with flag-tagged MAML1 plasmid unless otherwise indicated. Cells were fixed and processed for immunofluorescence 24 hours after transfection. (A) Immunostaining using an anti-flag antibody (h) and using an anti-ASPP2 antibody (i, and higher magnification i') showing similar localisation in nuclear foci. The white arrows indicate nuclear localisation of ASPP2. (B) Double staining using anti-ASPP2 and anti-flag antibodies showing that ASPP2 and MAML colocalise in the nuclear foci. (C) Quantification of the percentage of cells having nuclear ASPP2 upon transfection with MAML1 or with an empty vector (pcDNA3.1). Calculation based on three independent experiments. (D) Double staining of ASPP2 with Notch1 and with the two main components of Notch transcriptional complex, MAML1 and RBP-jκ. Upon MAML1 transfection ASPP2 immunostaining colocalises with MAML1, RBP-jκ and Notch1 immunostaining in nuclear foci. (E) Triple staining between ASPP2 (chicken anti-V5), MAML1 (mouse anti-flag) and RBP-jκ (rabbit anti-RBP-jκ) and between Notch (goat anti-Notch1), MAML1 (mouse anti-flag) and RBP-jk (rabbit anti-RBP-jκ) showing that ASPP2 has the same nuclear pattern as active Notch. (e'-e'') are higher magnification images of the nuclear foci. (F) Double staining using anti-flag antibody in combination with either anti-CdK8 or anti-p300 or and or anti-HDAC1 antibody, showing that MAML1-foci are positive for proteins involved in Notch-transcription. TO-PRO was used to visualise the nuclei in A, B, D and F, while in E was used DAPI. For ASPP2 detection different antibodies were used: mouse monoclonal DX54.10 in A, mouse monoclonal 141.2 in B and rabbit polyclonal S-32 in D. Scale bars: 10 μm.

Regarding ASPP2 localisation in the nuclear foci, several controls were performed to exclude any artefacts due to cross-reactivity between the antibodies used for the immunofluorescence experiments. Firstly, ASPP2 accumulation in the nuclei upon overexpression of MAML1 was detectable even in the absence of MAML1 antibody staining (Figure 5.8 A i-i'). Secondly, co-immunostainings were performed using three different anti-ASPP2 antibodies: DX54.10 and 141.2, both raised in a mouse host, and S-32, raised in a rabbit host. When used in combination with rabbit or mouse anti-flag antibodies for MAML1 detection, the same pattern of co-localisation was obtained (Figure 5.8 A-B-C). Finally, co-staining between MAML1 and other proteins was conducted to confirm the specificity of the ASPP2 recruitment. For instance, we showed that the localisation of other membrane-associated proteins, such as E-cadherin and also Par3 which is a binding partner of ASPP2, was not affected by MAML transfection (Figure 5.9 A). Moreover, several factors characterised for their specific nuclear localisations and spanning different functions from apoptosis to RNA-splicing , such as Ku-80, HP1α, SC-35, H3 K9 3-methyl , H3 K27 3-methyl, HP1γ, HP1β, H3 K4 3-methyl, H3 K4 1-methyl and p53 have been analysed upon MAML1 exogenous expression. None of them showed an overlapping nuclear pattern with MAML1 (Figure 5.9 A and B). Altogether these results indicate that ASPP2 nuclear recruitment can be induced by different stimuli related to the nuclear translocation and activation of Notch, such as the cell-interaction with Dll4 ligand and the expression of nuclear MAML1 or of NICD.

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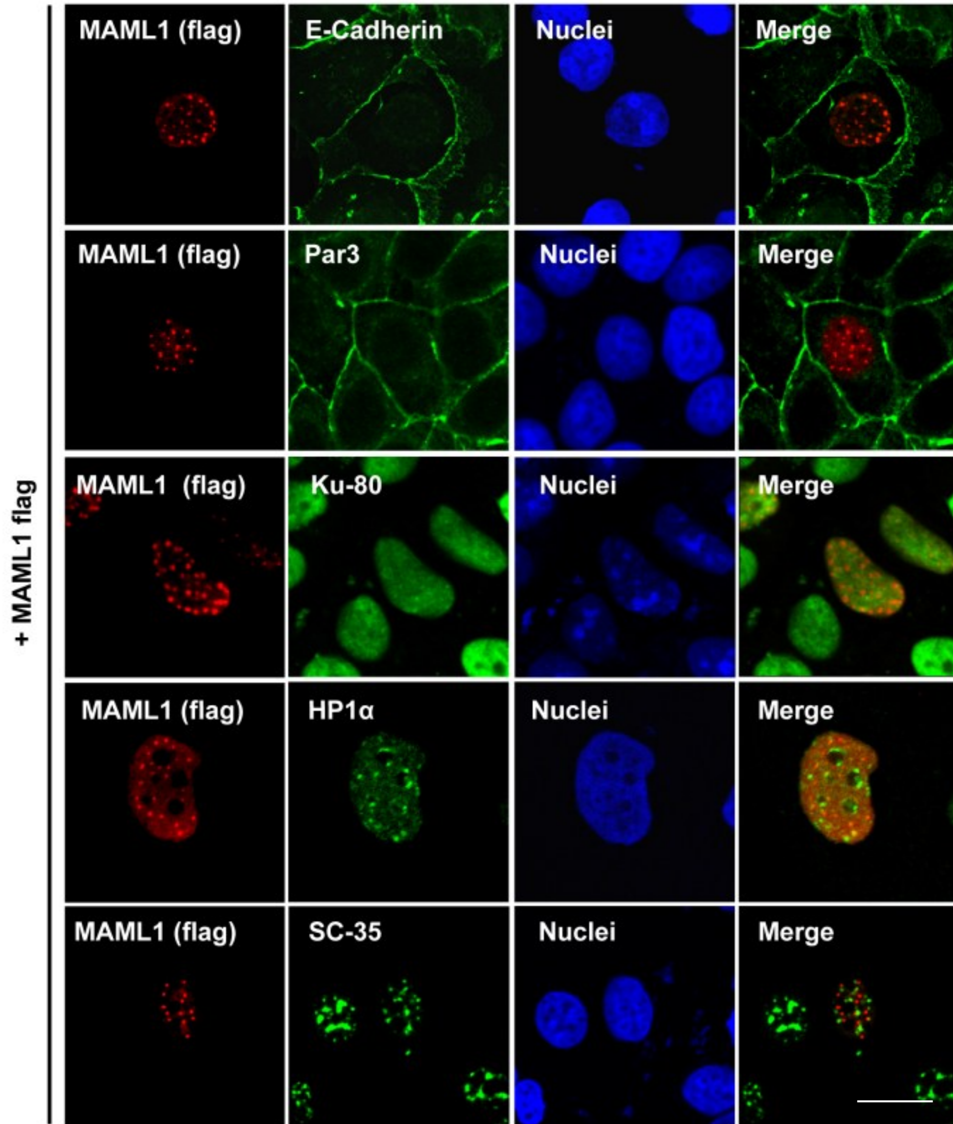

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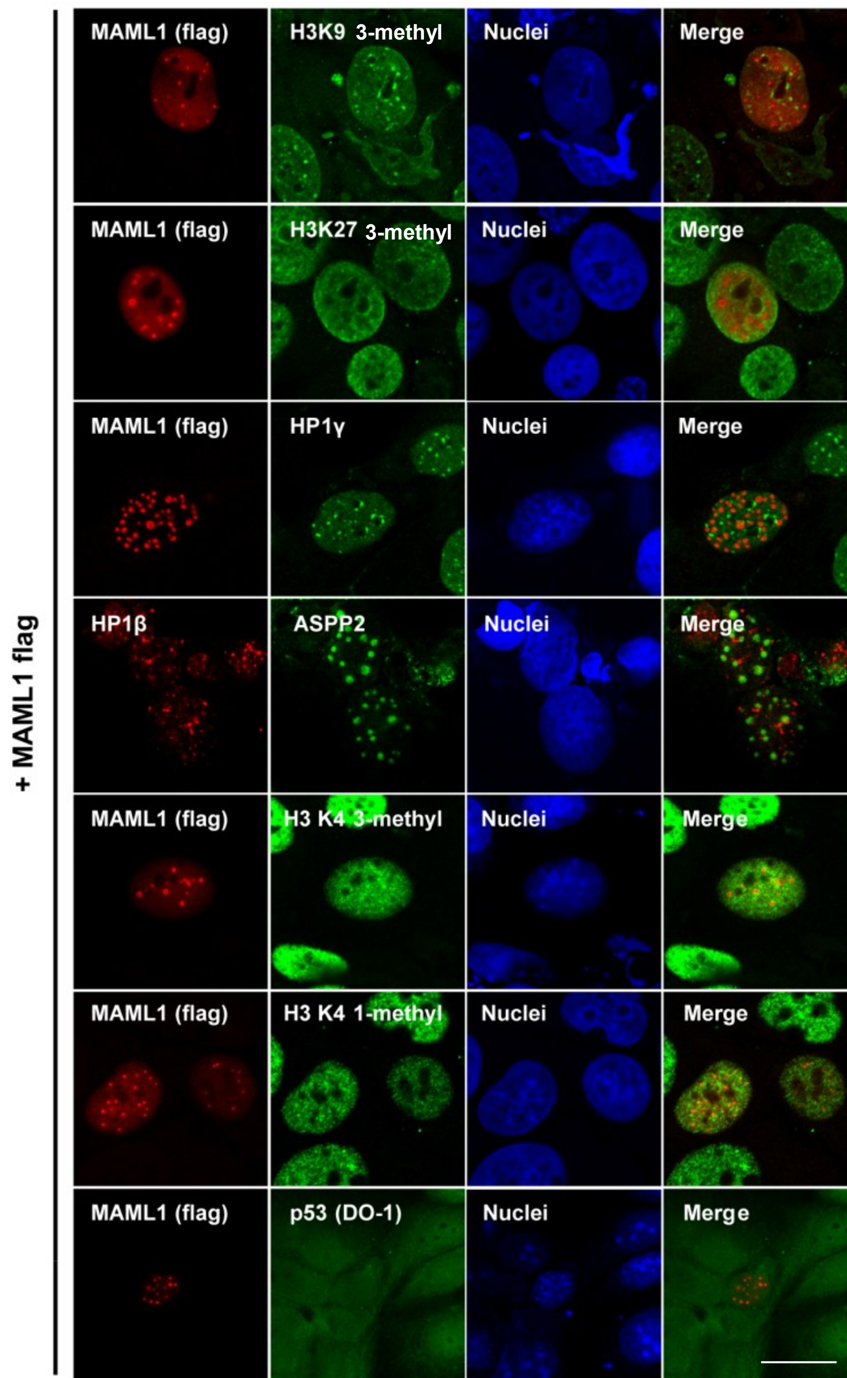


Figure 5.9 MAML1-induced nuclear recruitment is specific for ASPP2 and Notch
In all the experiments shown here, MCF7 cells growing on coverslips in a 24-well plate have been transfected with flag-tagged MAML1 plasmid. Cells were fixed and processed for

immunofluorescence 24 hours after transfection. (A) Double staining between MAML1 (flag staining) and E-cadherin or Par3 showing that other membrane associated proteins, as ASPP2 and Notch, cannot be recruited in nuclear foci. Similarly, double staining between MAML1 (flag staining) and nuclear proteins Ku-80, HP1α or SC-35, shows that MAML-foci are not involved in nuclear functions, such as DNA-repair, heterochromatisation or RNA-splicing. A more complete panel of nuclear functions is analysed in (B), where MAML-foci (flag or ASPP2 staining) have been double stained with several other nuclear markers covering additional nuclear functions, including apoptosis (p53 staining). As shown in A, no colocalisation with any of these nuclear markers has been observed. TO-PRO was used to visualise the nuclei. Scale bars: 10 μm.

5.2.7 Ankyrin repeats containing C-terminal of ASPP2 is required and sufficient to co-localise with MAML in nuclear foci

We next decided to map the interaction between MAML1 and ASPP2. To find which part of ASPP2 was responsible for contacting MAML, resulting in the recruitment into nuclear foci, we tested the ability of MAML to interact with different truncated mutants of ASPP2. We utilised an ASPP2 full-length (FL) expressing construct as a positive control, and three others constructs expressing truncated mutants covering different portions of ASPP2: the N-terminal portion (mutant 1: 1-360 aa), the central part containing the Proline-rich region (mutant 2: 360-925 aa) and the C-terminal, which includes ankyrin repeats and SH3 domain (mutant 3: 925-1128 aa) (Figure 6.1 A). The MAML-expressing plasmid was transfected into MCF7 cells in the presence or absence of each of the four different ASPP2 expression vectors. Twenty-four hours after transfection, cells were fixed and immunofluorescence was carried out to test how efficiently each of the ASPP2 truncated mutants could form nuclear foci with MAML, compared to the ASPP2 FL protein. The pattern of cellular localisation of the transfected ASPP2 FL and the three mutants was then analyzed in the absence and presence of transfected MAML. Representative pictures of the most frequently observed events for the localisation of each of the ASPP2 constructs are shown in Figure 6.1 B, while the exact frequency was quantified and is presented in the graph of Figure 6.1 C. The result showed that full-length ASPP2 expressed alone in cells was localised in the cytoplasm and at the cell-membrane, resembling

the localisation of the endogenous protein, and it could be efficiently recruited in the nuclear foci when co-expressed with MAML (in about 80% of the cases). Mutants 1 and 2, transfected alone, had similar cellular localisation to full-length protein, but both failed to successfully associate with MAML in the nucleus (only in about 11% and 28% of the cases). Mutant 3, whose normal cell localisation was predominantly nuclear, was instead found in nuclear foci in the majority of the cases upon transfection with MAML (about 62%) (Figure 6.1 B and C). Thus, despite being the shortest of the three ASPP2 truncated mutants, the ankyrin-containing mutant 3 was the only one able to mimic the behavior of ASPP2 FL upon MAML transfection. Interestingly, the Notch nuclear interaction with MAML is known to be mediated by its C-terminal ankyrin repeats domain, which is the same type of structural motif also present in the ASPP2 mutant 3. This finding indicates that the ankyrin repeats domain of ASPP2 is required and sufficient to colocalise with MAML in nuclear foci and therefore this is probably the region responsible for the interaction with MAML, as seen for Notch (Figure 6.1 A).

Subsequently, we examined which part of MAML1 interacts with ASPP2. A C-terminal truncated mutant of MAML1 was exogenously expressed in MCF7 and its ability to induce nuclear ASPP2 was analysed by immunostaining (Figure 6.1 D). This MAML1 mutant, which lacks the transactivation domain, was previously shown to be unable to form nuclear foci and unable to transactivate Notch target genes (Wu, Aster et al. 2000). In our experiment, when transfected into MCF7 cells the mutant did not localise in nuclear foci, as expected, and it also failed to recruit endogenous ASPP2 into the nucleus (Figure 6.1 E). This indicates that MAML C-terminal is required for the interaction with ASPP2 (Figure 6.1 D), and it also suggests that MAML has to be present specifically in nuclear foci in order to affect ASPP2 localisation. Additionally, as the MAML mutant is impaired in its transactivation properties, our result argues that ASPP2/MAML is probably a functional interaction with effects at the transcriptional level as for Notch/MAML.

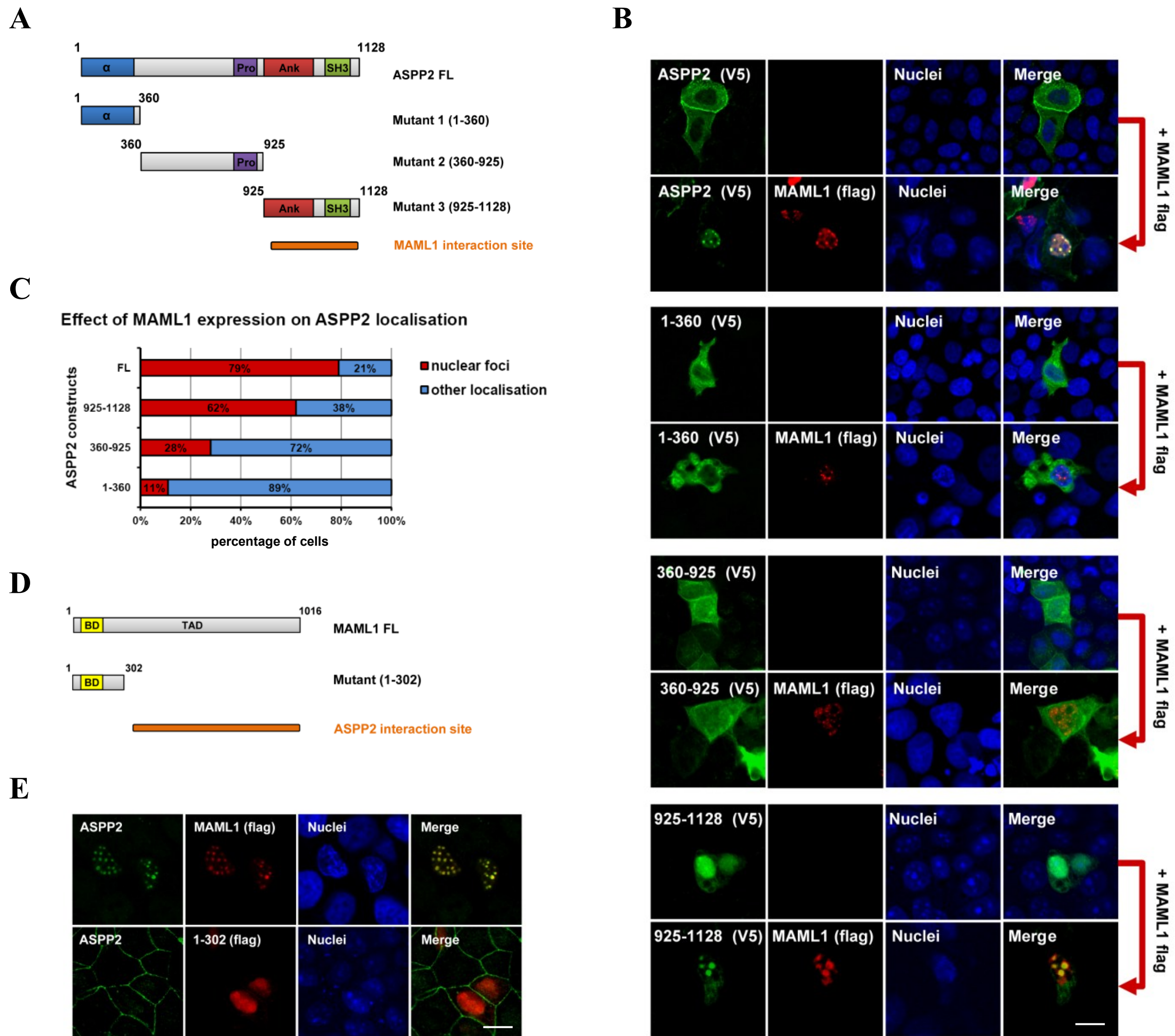


Figure 6.1 Mapping of ASPP2-MAML1 interaction

(A) Graphic representation of the ASPP2 constructs (all V5-tagged) used to map the interaction with MAML1 (FL, full-length). The region of possible interaction with MAML1 at the ASPP2 C-terminal in also shown. (B) The aim of the experiment is to see which of the ASPP2 mutants can be recruited by MAML1 in the nuclear foci, indicating which part of ASPP2 is involved in the interaction with MAML1. MCF7 cells growing on coverslips in a 24-well plate have been transfected with each of the ASPP2 constructs described before in A, alone or in combination with a MAML1-expressing plasmid (flag-tagged) and then 24h later processed for immunofluorescence using antibodies anti-flag and anti-V5. ASPP2 FL, used here as a positive control, and Mutant 3 (925-1128) were the only two which managed to get efficiently recruited by MAML1 in the nuclear foci, indicating that ASPP2 C-terminal is necessary and sufficient for the interaction. (C) Quantification of the frequency of events observed in the previous experiment in B for each of the ASPP2 constructs (presence in nuclear foci or any other cell localisation, upon transfection with MAML1). (D) Graphic representation of the MAML1 constructs (all flag-tagged) used to map the interaction with ASPP2 (FL, full-length; BD,

basic domain; TAD, transcription activation domain). (E) Here, the aim of the experiment is to see if MAML1 mutant (1-302) can recruit endogenous ASPP2 in the nuclear foci as wild type MAML1 does. MCF7 cells growing on coverslips in a 24-well plate have been transfected with each of the MAML1 constructs described in D and then 24h later processed for immunofluorescence using antibodies anti-flag and anti-ASPP2. The result shows that MAML mutant (1-302) does not affect ASPP2 cell localisation, indicating that MAML1 TAD region is requited for the interaction with ASPP2. TO-PRO was used to visualise the nuclei. Scale bars: 10 μm.

5.2.8 ASPP2 binds RBP-jκ and MAML in vivo

As previously established, Notch's interaction with the two major components of its transcriptional complex, RBP-jκ and MAML, is mediated by the ankyrin repeats domain present at its C-terminus. Similarly, ASPP2 is able to translocate into the nucleus upon various Notch activating signals and co-localise with members of Notch complex via its ankyrin containing C-terminal region. Alignment of the Notch and ASPP2 ankyrin coding regions showed high homology, and further bioinformatics analysis, based on the crystal structures of ASPP2 and Notch ankyrin domains (4 and 7 repeats respectively), also revealed a notable overlap between the two ternary structures (Figure 6.2 A). This observation led us to test the possibility that ASPP2 may be able to bind directly to RBP-jk and MAML, similarly to Notch.

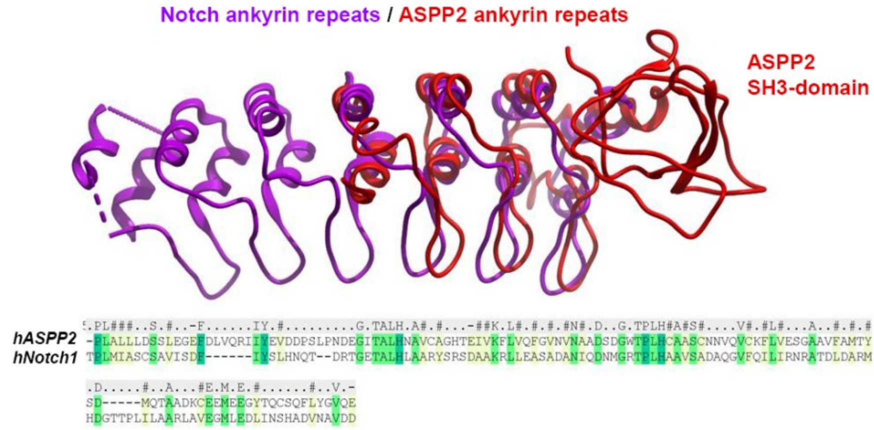
The *in vivo* association between endogenous ASPP2 and RBP-jk was verified by immunoprecipitation assay in MDA-MB-231 cells, a carcinoma cell line with high levels of Notch proteins. Total protein lysates were immunoprecipitated with a monoclonal anti-ASPP2 antibody or with an unrelated antibody, as a negative control. By Western blotting analysis endogenous RBP-jκ was specifically detected in the lysates immunoprecipitated with the ASPP2 antibody and not in the control, indicating that the two proteins interact *in vivo* (Figure 6.2 B, left). The specificity of this binding was proved by using alternative antibodies to immunoprecipitate ASPP2 and to detect RBP-jκ in Western blotting. Again, RBP-jκ was detected in ASPP2 immunoprecipitation only and not in the control (Figure 6.2 B, right).

Subsequently we tested the binding between ASPP2 and MAML1. Because of the low level of MAML1 normally expressed in cell lines, the binding between ASPP2 and MAML was verified by exogenously expressing MAML1 via a flag-tagged expression vector. H1299 cells were chosen because of their high degree of transfectability. The endogenous levels of ASPP2 in H1299 were however rather low, and therefore ASPP2 had to be co-transfected with MAML1 (V5-tagged expression vector). Exogenously expressed ASPP2 was immunoprecipitated with an anti-V5 antibody or a control antibody. Immunodetection performed by Western blotting revealed binding between ASPP2, and both members of the Notch complex, MAML1 and RBP-jκ (Figure 6.2 C).

Finally, we also observed that ASPP2 exogenous expression can increase the endogenous protein abundance of RBP-jk. This phenomenon was observed when we introduce by transfection ASPP2 in UPCI SCC 040 cells, a carcinoma cell line with undetectable endogenous ASPP2. By Western blotting analysis we observed that transfection with MAML1, can generate a modest increase in RBP-jk protein level, while a more substantial up-regulation was observed upon ASPP2 transfection (Figure 6.2 D). A similar effect was observed in H1299 cells, which also express low levels of ASPP2 (M. Genin, personal communication).

In summary, our findings allow us to speculate that by *in vivo* binding to MAML and RBP-jκ, ASPP2 could form a ternary complex via its ankyrin repeats domain in a similar manner to the Notch complex (Figure 6.2 E).

A



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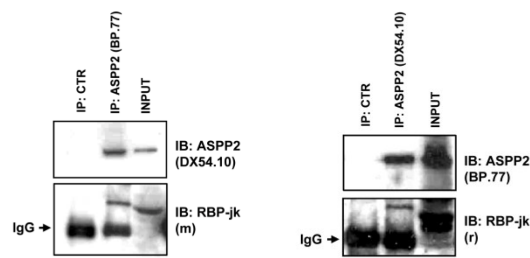

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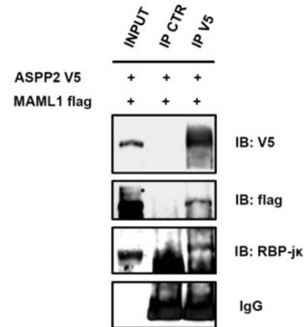

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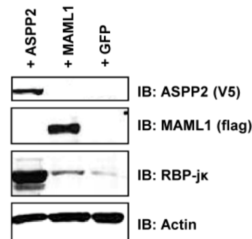

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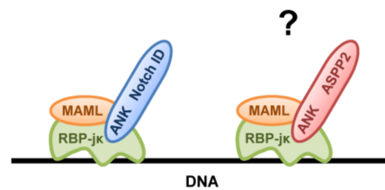


Figure 6.2 ASPP2 can bind the two main component of Notch transcriptional machinery, MAML1 and RBP-jκ

(A) Comparison of sequence and structure of ASPP2 and Notch ankyrin domains, showing high degree of similarity. Overlap of the three dimensional crystal structures of the Notch seven ankyrin repeats (violet) and the four ASPP2 ankyrin repeats (red). SH3 domain of ASPP2 is also present in ASPP2 structure representation. Below, amino acid sequence alignment of the ankyrin domains of Notch and ASPP2. (B, left) ASPP2 binds to RBP-jk. Association between endogenous ASPP2 and RBP-jk was performed by immunoprecipitation assay in MDA-MB-231 cells. Total protein lysates were immunoprecipitated with polyclonal BP.77 anti-ASPP2 antibody (IP ASPP2) or rabbit IgGs (IP CTR) and then were analyzed by blotting with monoclonal DX54.10 anti-ASPP2 and monoclonal anti-RBP-jκ antibody. RBP-jκ protein was detected specifically only in the ASPP2 IP, but not in the CTR IP. (B, right) The same experiment was conducted using the monoclonal DX54.10 anti-ASPP2 antibody to immunoprecipitate ASPP2 and mouse IgGs as a control. RBP-jκ protein was then detected in Western blotting using a polyclonal anti-RBP-jκ antibody. Its presence was again observed only in the ASPP2 immunoprecipitates. (C) ASPP2 binds to MAML1. Association between exogenously expressed ASPP2 (V5-tagged) and MAML1 (flag-tagged) was performed by immunoprecipitation assay in H1299 cells. Total protein lysates were immunoprecipitated with monoclonal anti-V5 antibody (IP V5) or mouse IgGs (IP CTR) and then were analyzed by blotting with monoclonal anti-V5, monoclonal anti-flag and monoclonal anti-RBP-jκ antibody (positive control). MAML1 protein, as well as RBP-jκ were detected specifically only in the ASPP2 IP, but not in the CTR IP. (D) ASPP2 exogenous expression enhances RBP-jκ endogenous protein levels. UPCI SCC 040 cells were transfected with GFP, MAML1 or ASPP2 expressing plasmids, and then lysates were analysed by Western blotting using antibodies anti-RBP-jκ, anti-V5 to detect ASPP2, anti-flag to detect MAML1 and anti-actin (loading control). (D) Graphic model illustrating the possibility that ASPP2 may form a nuclear complex, similar to the Notch one, binding MAML1 and RBP-jκ via its ankyrin repeats domain.

5.2.9 ASPP2 and Notch ankyrin repeats heterodimerises in vitro and colocalise in the nucleus in vivo

A number of recent works have shown that two identical Notch/RBP-jκ/MAML complexes can dimerise at DNA level in a head to head conformation (Nam, Sliz et al. 2007; Arnett, Hass et al. 2010). The interaction appears to be mediated by the ankyrin domain of each Notch molecule present in the two contacting complexes and evolutionary conserved residues involved in the binding have been identified (Nam, Sliz et al. 2007). Among the known Notch target genes, a subset of them contains "sequence paired" binding sites (SPS). The Dimerised Notch transcriptional complex is more active than the monomeric complex on this set of genes. Therefore, Notch target genes can be divided in two groups: the ones containing SPS whose transactivation is dimer dependent and others not containing SPS that are transcribed in a dimer independent manner. These new findings suggest that cellular factors able to dimerise with Notch via their ankyrin repeats might selectively affect Notch target gene expression. . According to the dimerisation model established by Dr. Blacklow's group (Nam, Sliz et al. 2007), the interaction between the ankyrin domains of two Notch proteins is mediated by their positive-charged lysines at position 1946 contacting negative-charged glutamic acid residues in position 1950 of each ankyrin domain. This allows the formation of salt bridge contacts based on hydrogen bonding and electrostatic interactions.

A detailed comparative analysis of the ASPP2 and Notch1 ankyrin repeats sequences identified the presence in ASPP2 of one of the Notch key residues involved in the dimerization: the lysine 1946, found at position 976 on ASPP2 sequence (Figure 6.3 A). Interestingly, this residue is not present in the sequence of iASPP, the inhibitory member of the ASPP family, which as we showed before is unable to colocalise with NICD. As we also showed, ASPP2 can be recruited into the nucleus by NICD and can also co-localise and bind with RBP-jκ and MAML. Therefore, we next tested whether ASPP2 could dimerise with Notch via its ankyrin domain. The experiments which follow were performed by Min Lu, another member of Professor Xin Lu's lab, and are shown here because of the

relevance with the present study. iASPP (625-828) or ASPP2 (925-1128) C-terminals (His-tagged) were pre-bound to nickel beads and incubated with *in vitro* translated NICD (myc-tagged). Blank beads were used as control. The interaction between them was then determined by co-immunoprecipitation using anti-His and anti-myc antibodies. NICD was found able to bind specifically to the ankyrin containing C-terminal of ASPP2 and not to the ankyrin containing C-terminal of iASPP (Figure 6.3 B).

Next, to directly test if the lysine 1946 and glutamic acid 1950 of Notch were involved in the hetero-dimerisation of Notch-ASPP2, we tested the binding between the ankyrin domains of ASPP2 and three isoforms of NICD mutated in such amino acids, named respectively E1946K, with a glutamic acid substituting the Lys-1946, K1950E, with a lysine substituting the Glu-1950 and K1946E E1950K, including both substitutions. The ankyrin domain-containing C-terminal of ASPP2 (925-1128), His-tagged, was pre-bound to nickel beads and then incubated with lysates containing *in vitro* translated NICD or NICD mutants (all myc-tagged). The iASPP C-terminal (625-828) and ankyrin repeat domain from AnkyrinR (D34 region), both His-tagged, were also pre-bound to nickel beads and used as controls. Interactions were then determined by Western blotting after co-immunoprecipitation of the bound proteins and immunoblotting with anti-His and anti-myc antibodies. Here we found that only the non-mutated NICD was able to bind C-terminal ASPP2, while none of the NICD mutants could do the same (Figure 6.3 C). These data suggest that residues K1946 and E1950 of Notch are critical not only for the Notch-Notch dimerization, but also for a Notch-ASPP2 heterodimer formation.

To verify this result *in vivo*, we analyzed by immunofluorescence how the expression of NICD and NICD mutants (E1946K, K1950E and K1946E E1950K) could affect the localisation of endogenous ASPP2. Myc-tagged expression plasmids for NICD and NICD mutants were then transfected into MCF-7 cells. After 24 hours, immunostaining using antibodies anti-ASPP2 and anti-myc was performed. Endogenous ASPP2 was recruited into the nucleus upon transfection with NICD, as

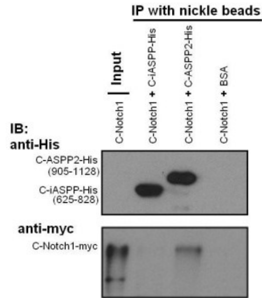
shown before, while none of the NICD mutants was able to affect ASPP2 cellular localisation (Figure 6.3 D). This is consistent with *in vitro* results in Figure 6.3 C, where we showed that none of the NICD mutants were able to bind ASPP2. Conversely, we then tried to transfect the C-terminal ASPP2 (925-1128) into MCF7 cells, whose expression pattern is exclusively nuclear, to see if it could reciprocally induce endogenous Notch in the nucleus. Interestingly, we found that Notch endogenous localisation was unchanged upon transfection with C-terminal ASPP2, suggesting that endogenous ASPP2 can be recruited into the nucleus by the presence of NICD, whereas the localisation of endogenous Notch1 was not affected by expression of the nuclear C-terminal of ASPP2 (Figure 6.3 D).

Altogether, these findings indicate that ASPP2 can interact with Notch1 in the nucleus, forming a heterodimer via its ankyrin repeats domain (Figure 6.3 E).

A

h Notch1 -DAAKRLLEASAD-
h ASPP2 -TEIVKFLVQFGVN-
h iASPP -YSIVDFLITAGAN-

B



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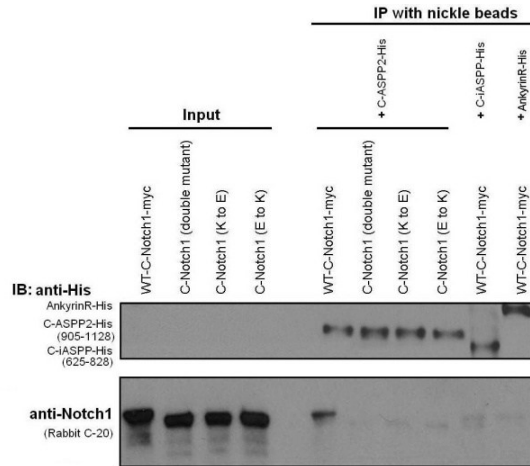

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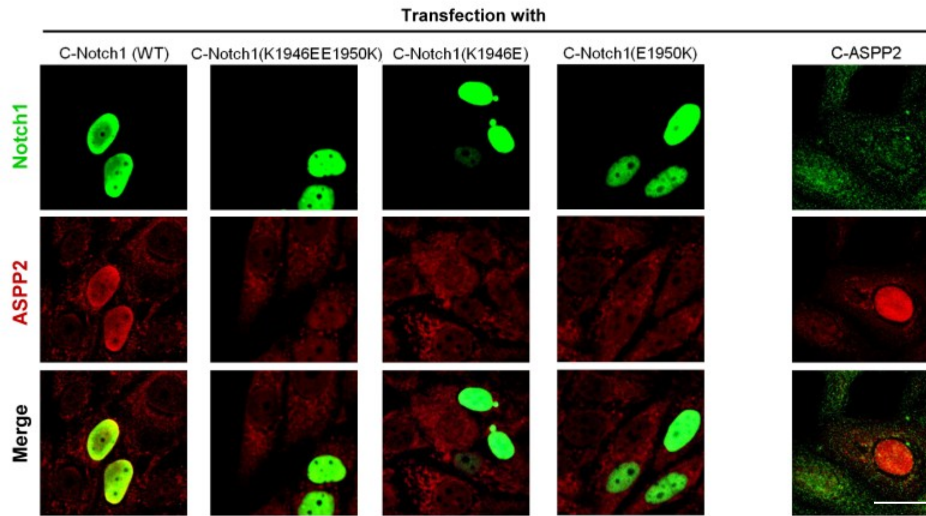

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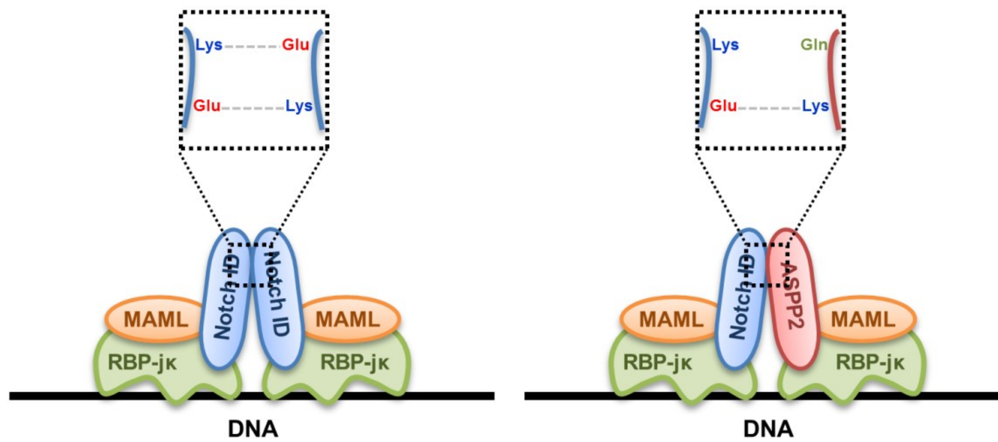


Figure 6.3 ASPP2 ankyrin domain dimerises in the nucleus with the ankyrin domain of Notch1
(A) Multiple alignment of the human sequences of Notch1, ASPP2 and iASPP, corresponding to a part of their ankyrin domain region. Highlighted in red is one of the residues critical for Notch dimerization, the lysine 1946 (K1946), found also in ASPP2 (K976) but not in iASPP sequence (D instead of K). (B) His-tagged ankyrin containing C-terminals of iASPP (625-828) or ASPP2 (925-1128) pre-bound to nickel beads were incubated with *in vitro* translated Notch ID (myc-tagged) lysates in NP40 buffer. Blank beads were used as control. Upon immunoprecipitation using anti-His and anti-myc antibodies, we found by Western blotting analysis that Notch ID (C-Notch1) can bind specifically to ASPP2 C-terminal (C-ASPP2-His) and not to iASPP2 C-terminal (C-iASPP-His). (C) His-tagged ankyrin containing C- terminal of ASPP2 (925-1128) pre-bound to nickel beads was incubated with *in vitro* translated Notch ID lysates or Notch ID mutants (double mutant, K to E and E to K) lysates in NP40 buffer. His-tagged ankyrin containing C-terminal of iASPP (625-828) and ankyrin repeat domain from the protein AnkyrinR (D34) pre-bound to nickel beads were also incubated with C-Notch containing lysates and used as controls (negative and positive respectively). Upon immunoprecipitation using anti-His and anti-myc antibodies we found by Western blotting analysis that only the non-mutated C-Notch1 was able to bind C-ASPP2, while none of the C-Notch1 mutants did bind C-ASPP2. (D) ASPP2-Notch interaction was verified by immunofluorescence in MCF7 cells, analysing how the expression of Notch ID or Notch ID mutants (double mutant, K to E and E to K) affect the localisation of endogenous ASPP2. Expression plasmids for Notch ID or Notch ID mutants were transfected into MCF-7, then after 24 hours cells were fixed and immunostained using antibodies anti-ASPP2 and anti-Notch1. Transfection with Notch ID was able to recruit endogenous ASPP2 into the nucleus, while none of the Notch ID mutants could do so. We then transfected C-ASPP2 (V5 tagged) into MCF7 and tested if it could reciprocally induce endogenous Notch into the nucleus. Immunostaining using antibodies anti-V5 and anti-Notch1 showed that Notch1 endogenous localisation was unchanged upon transfection with C-terminal ASPP2, indicating that Notch ID recruits ASPP2 into the nucleus and not vice versa. Scale bar: 10 µm. (E) Graphic model of the Notch/Notch homodimer and the hypothetic Notch/ASPP2 heterodimer. The region of contact between the ankyrin domains has been enlarged to show the residues mediating the interaction. In Notch/Notch two salt bridges are established between the Lys-1946 of one subunit and Glu-1950 of the other, while in Notch/ASPP2 only one salt bridge is present between Lys-976 of ASPP2 and Glu-1950 of Notch.

5.2.10 ASPP2 alters Notch transcriptional selectivity in vitro and in vivo

In this chapter we have established that ASPP2 can be recruited into the nuclear compartment upon activation of the Notch pathway, and that it can physically interact with NICD and other components of its transcriptional machinery. We then tested whether this interaction could have a functional impact on Notch signalling pathway activity by analysing the expression of Notch target genes in presence or absence of ASPP2 *in vivo*. As Notch has an important role in promoting differentiation in the skin, we analysed the expression of some its major target genes, *Hey1*, *Hey2* and *Hes1*, in primary keratinocytes generated from three-day-old mice. To assess the impact ASPP2 could have on their transcription, we compared mRNA expression levels from *ASPP2* wild type and Δexon3 mouse primary keratinocytes. Interestingly, *Hey2* expression was decreased by 37% in *ASPP2* Δexon3 keratinocytes, while *Hes1* expression increased by about 1.5-fold (Figure 6.4 A). Elevated mRNA expression levels of *Hes1* were also detected by RT-qPCR in the *ASPP2* Δexon3 mouse brain, which is another site phenotypically affected by the loss of ASPP2 (R. Sottocornola, personal communication). As the basal transcription of these genes in absence of Notch activation is low in cells, we decided to analyse their expression after having induced Notch activation. For this experiment we used MEF cells instead of mouse primary keratinocytes, because MEFs allow us to get a better activation of the Notch pathway, as they do not require special culture conditions which can interfere with the Dll4 coating. Primary keratinocytes additionally require for their growth a collagen coating and calcium-free medium. Thus, we seeded *ASPP2* wild type and Δexon3 MEFs in presence or absence of Dll4 coating to activate Notch signalling. Cells were harvested after 24 hours and mRNA levels of *Hey1*, *Hey2* and *Hes1* were analysed by RT-qPCR. Consistent with what we observed in primary keratinocytes, we found that *Hes1* expression was up-regulated by 1.5-fold in *ASPP2* Δexon3 MEFs compared to wild type MEFs upon Dll1-induced Notch activation. Even more interestingly, *Hey2* was very poorly induced by Dll4 in absence of ASPP2 (figure 6.4 B). To add more significance to our results, the experiment was repeated using other Notch ligands as

stimulators, such as Dll1 and Jagged1. Similar results concerning *Hes1* and *Hey2* activation were obtained by comparing the results from all three Notch ligands utilised (Figure 6.4 C).

Having observed that the absence of ASPP2 had a selective impact on the expression levels of some of the major Notch target genes, we next tested whether ASPP2 could have a direct effect on their transcription in transactivation assay. In particular, having seen that Notch activation fails to induce *Hey2* gene in absence of ASPP2, we tested whether ASPP2 could be directly involved in its transcription. Transactivation assays performed in Saos-2 cells showed that this was the case, as ASPP2 induced *Hey2* transcription in a dose dependent manner (Figure 6.4 D). Moreover, chromatin immunoprecipitation (ChIP) experiments performed by Dr Ewa Dudziec in our lab showed that a higher amount of NICD can be found bound to the *Hey2* promoter in the presence of ASPP2 than when ASPP2 is absent (Figure 6.4 E), suggesting that ASPP2 could promote *Hey2* transcription by increasing NICD binding affinity on its promoter. Interestingly, no increase in NICD binding on the *Hes1* promoter was observed in ASPP2-null cells, indicating the higher levels of *Hes1* observed in *ASPP2*-null cells is not due to the presence of more NICD on its promoter, but it has most likely something due with NICD functionality and stability (Figure 6.4 E).

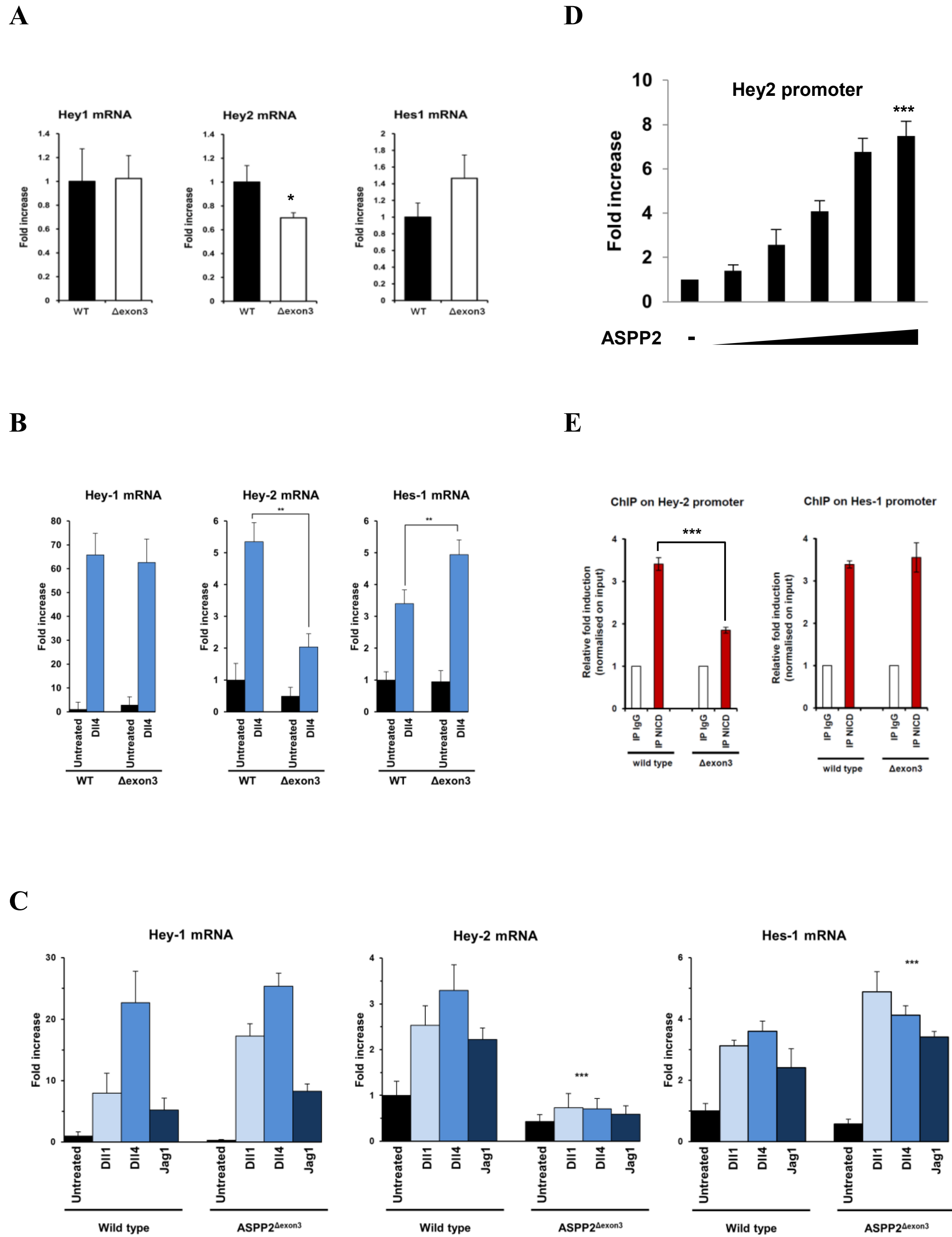


Figure 6.4 ASPP2 affects Notch transcription on its target genes
(A) Total m-RNA has been extracted from primary keratinocytes obtained from three-day-old *ASPP2* wild type and Δexon3 mouse pups. Relative amounts of mRNA of Notch target genes *Hey1*, *Hey2* and *Hes1* were quantified by RT-qPCR. *ASPP2* Δexon3 keratinocytes, compared to wild type,

express lower levels of *Hey2* (significant at *p=0.03) and higher levels of *Hes1* (although not significant at p=0.06). Quantification based on three different pairs (wild type and Δexon3) of mice. (B) *ASPP2* wild type and Δexon3 MEFs were plated in presence or absence of Dll4 coating (3 μg/ml). Dll4 was used to induce activation of Notch signalling. 24 hours later mRNA levels of Notch target genes *Hey1*, *Hey2* and *Hes1* were analysed by RT-qPCR. In *ASPP2* Δexon3 MEFs, compared to wild type, *Hes1* mRNA was found more upregulated upon Dll4 induction (about 1.5 folds, **p=0.008), while *Hey2* mRNA much less upregulated (about 2.5 folds, **p=0.0014). The experiment was repeated using three different pairs of MEFs. (C) Same experiment as in B, with the addition of two more coatings, other than Dll4 (Dll1 and Jag1), to induce activation of Notch pathway in wild type and Δexon3 cells. The results obtained with Dll1 and Jag1 stimulation were comparable to the ones obtained with Dll4 (***p<0.001). For the RT-qPCR experiments in A, B and C, analysis based on ΔΔCt method using *GAPDH* as an internal control. (D) Transactivation assay was performed in Saos-2 cells. Cells plated in 24-well plates were transfected with 200 ng of *Hey2* reporter construct together with 6 ng of renilla and growing amounts of ASPP2 expression plasmid (0.8, 1, 1.4, 1.8 and 2μg). The result shows an ASPP2 dose dependent increase in the activation of *Hey2* promoter (***p<0.001 at 2μg of ASPP2). Data were normalized relatively to the renilla activity and is based on three independent experiments performed in duplicate. (E) ChIP experiment was performed in *ASPP2* wild type and *ASPP2* Δexon3 cells plated for 24h on a Dll4 coating, using an antibody recognizing the transcriptionally active form of Notch (IP NICD) and normal rabbit IgG as control (IP IgG). Both immunoprecipitations were normalized on the input. No differences were observed in the amount of NICD bound on the *Hes1* promoter while a diminished amount of NICD was observed in ASPP2 Δexon3 cells on *Hey2* promoter (***p<0.001). Error bars in all graphs represent standard deviation of the mean.

Of note, the opposing effect exerted by ASPP2 on *Hey2* and *Hes1* genes could be explained by looking at the pattern of RBP-jκ binding sites present on their promoters. The *Hes1* promoter region in fact possesses a pair of recently characterised SPS sites, requiring the presence of a Notch complex dimer in order to get full activation. In contrast, the *Hey2* promoter has regular RBP-jκ binding sites oriented in the same direction. When we extended our analysis on the induction of other Notch-target genes in absence or presence of ASPP2, we found that those with mono-oriented RBP-jκ binding sites generally required ASPP2 to be activated (*Hey2*, *HeyL* and *Hes7*), while those containing SPS sites do not and actually get more activated in absence of ASPP2 (*Hes1* and *Hes5*) (Figure 6.5 A-B). Interestingly, *Hes5* did not respond to Dll4-mediated Notch activation in wild-type MEFs, but it was activated (2-fold increase) in ASPP2 Δexon3 MEFs. This may indicate that *Hes5*, despite not being an important gene in MEFs physiology and thereby not induced on Dll4 coating, its Notch-mediated activation can be restored by removing ASPP2 expression.

This suggests that ASPP2 could differentially affect Notch target genes, which contain different patterns of RBP-jκ binding sites, at a functional level. In particular, our data suggest that ASPP2 can enhance or sustain the activation of genes with mono-directionally oriented RBP-jκ binding sites, but instead shows an inhibitory effect on head-to-head oriented RBP-jκ binding sites, possibly competing with other NICD molecules for dimerization (Figure 6.5 C-D).

As very few Notch target genes with SPS sites have been currently identified, and some of them do not seem to respond to Notch activation in our assay, like *c-myc* or *FJX1* (data not shown), we could not expand further our data on SPS-containing genes.

In conclusion, with these experiments we demonstrated that ASPP2 expression has a major impact on Notch transcriptional activity *in vivo,* and this modulation seems to be selective for genes with different patterns of RBP-jκ binding sites.

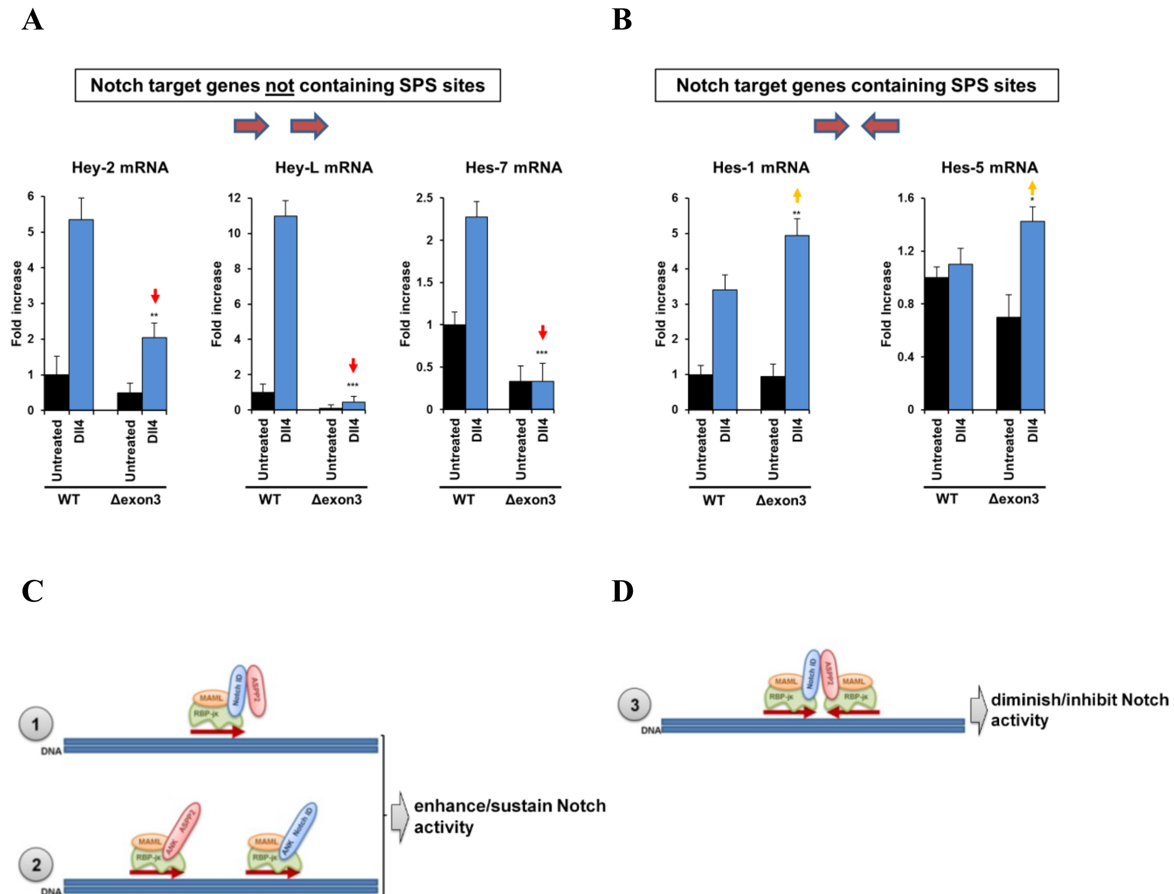


Figure 6.5 ASPP2 has an effect on Notch target selectivity based on the orientation of the RBP-jκ DNA binding sites

(A) Analysis by RT-qPCR of Notch target genes not containing SPS sites (*Hey2*, *HeyL* and *Hes7*), upon Notch induction by Dll4 in *ASPP2* wild type and Δexon3 MEFs. The result shows that they are all poorly induced or not induced at all in *ASPP2* Δexon3 cells compared to wild type (**p=0.0014; ***p<0.001). (B) Vice versa genes containing SPS sites (*Hes1* and *Hes5*), are all more induced upon Notch activation by Dll4 in *ASPP2* Δexon3 MEFs (**p=0.008; *p=0.012). (C) Diagram describing the mode of action of ASPP2 in combination with Notch. On mono-directionally oriented RBP-jκ binding sites ASPP2 could dimerise with Notch on a single site, without binding MAML1 and RBP-jκ, or forming its own transcriptional complex on a different site, with the result of promoting Notch transcriptional activity. (D) In presence of head-to-head oriented SPS sites, ASPP2 could instead dimerise with Notch forming a second transcriptional complex sitting on the adjacent site. This heterodimer however would be less functional (possibly because less stable) than the Notch/Notch homodimer, negatively affecting the transcriptional activity. Error bars in all graphs represent standard deviation of the mean.

5.3 Summary

In the previous chapters we have described ASPP2 as having a new role as a suppressor of SCC, by regulating p63 expression and function. Notch is another key regulator of p63 and promotes cell differentiation in the squamous epithelium. Because of this, mice with impaired activity of Notch in the skin develop spontaneous SCCs, as do *ASPP2* deficient mice. Notch function in general is important to confer cell fate determination, and its function is critically context dependent. Importantly, either in health or in cancer, Notch activity seems to be generally regulated by cross-talks with pathways physiologically important for the homeostasis of each given tissue. ASPP2 is a factor able to confer transcriptional selectivity, as shown in relation to p53 and family members, and given its biological significance in the squamous epithelium, we decided to test whether the ASPP2 and Notch pathways could physically and functionally interact.

We first analysed ASPP2 and Notch1 reciprocal expression patterns in normal and transformed squamous epithelium. We found that, in contrast with p63 expression, ASPP2 and Notch1 are co-expressed in the differentiated layers of the stratified epithelium and in the same cells in tumours. In particular, the observation that they can co-localise in the nuclear compartment *in vivo* prompted us to test if they can be activated by the same *stimuli*. We utilised different methods to induce Notch nuclear localisation in cells, such as employing a Dll4 coating, transfecting MAML1 or directly expressing NICD. With all the conditions tested, we were able to induce ASPP2 nuclear recruitment indicating that ASPP2 localisation can be regulated by Notch activating signals. Moreover, we found that ASPP2 in the nucleus can localise in Notch-complex foci. Binding experiments confirmed that ASPP2 can also bind MAML1 and RBP-jκ, the two main components of the Notch transcriptional complex. The region of ASPP2 which is likely to form the nuclear complex with MAML1 and RBP-jκ is the ankyrin repeats containing C-terminal. This was found to be required and sufficient for ASPP2 nuclear localisation in Notch-foci, and shares high homology with the ankyrin domain of Notch1, the structural motif that allows Notch itself to bind MAML1 and RBP-jκ. Recent works have

shown that the ankyrin domain of Notch is also involved in the formation of a Notch-Notch dimer at particular SPS sites present in the promoter of certain Notch target genes. Since ASPP2 possess an ankyrin domain with high sequence and structural homology with the one of Notch and it has been shown before to be able to bind transcriptional factors as p53, p63 and p73, and confer promoter selectivity, we next tested if ASPP2-ankyrin could form a heterodimer with Notch-ankyrin. Indeed we found that ASPP2 and Notch ankyrin domains can heterodimerise, and we discovered that the Notch residues K1946 and E1950, which are important to mediate the Notch-Notch interaction, are also fundamental for the ASPP2-Notch connection.

In the final step, we examined the functional consequences of the ASPP2-Notch pathways' interaction. Here we found that the absence of ASPP2 *in vivo* resulted in deregulated Notch activity in inducing some of its target genes, as well as in inhibiting p63 expression as already shown at the beginning of this chapter. Considering the implications of the ASPP2-Notch heterodimer formation in more details, we found that ASPP2 is generally able to enhance Notch transcription of genes with mono-directionally oriented RBP-jκ binding sites. Conversely, despite limited data, our findings on Notch target genes containing SPS sites show that ASPP2 here interferes with Notch transcription.

In conclusion our model shows that ASPP2 is a new important player in the cross-talk with Notch1 and that this interplay could be important for the homeostasis of the squamous epithelial compartment, as well as the central nervous system.

Chapter VI: Discussion

The aim of the work presented in this thesis is to characterise novel and important functions of the ASPP2 protein, based on *in vivo* observations derived from the study of the *ASPP2* Δexon3 mouse. Together with defects associated with the central nervous system, these mice showed a predisposition to developing spontaneous squamous cell carcinomas (SCCs). The investigation of the mechanisms involved in these phenotypical manifestations led us to discover a new role for ASPP2 in antagonising p63 in the squamous epithelium, and possibly a broader role as a regulatory factor for Notch-mediated transcription.

6.1 The Balb/c ASPP2 Δexon3 mouse: a novel one-gene-deletion mouse model for SCC

The homozygous deletion of *ASPP2* in mice in C57BL6/6J pure and 129SvxC57BL6/6J mixed backgrounds has been previously proven to be lethal (Vives, Su et al. 2006). Here we were able to generate viable *ASPP2* Δexon3 homozygous mice in a pure Balb/c background, which allowed us to perform a more comprehensive study on the effects of *ASPP2* gene deletion *in vivo*. The *ASPP2* Δexon3 homozygous mice were able to survive after weaning, although the majority died before 20 weeks of age due to CNS defects. We also found that, as already observed in the other genetic backgrounds, the *ASPP2*-deficient mice are prone to develop spontaneous tumours. Interestingly, in Balb/c all the tumours found belong to the SCC subtype, expressing the characteristic pattern of cellular markers of SCC, such as K1, K14, p63 and no expression of vimentin.

Around 80% of all human cancers develop in the epithelium, and of all the different epithelial cancer types SCC is the most malignant and has the highest metastatic potential. To date, mouse genetics has

played an important role in our understanding of the molecular pathways involved in SCC formation. The most effective existing mouse models of SCC require multistage genetic alterations, such as the chemically induced multistage carcinogenesis approach for mouse skin, in which one treatment with a low dose of the mutagen 9, 10-dimethyl-1, 2-benzathracene (DMBA) followed by repeated treatment with tumour promoting agent 12-O-tetradecnoyl phorbol-13-acetate (TPA) induces formation of papillomas containing mutations in the *Ras* gene (DiGiovanni 1992). Successively, the use of an inducible mutant *Ras* knock-in mouse model confirmed that *Ras* mutations are important for SCC initiation, and that additional mutations such as loss or gain of p53 function are required to convert papilloma to SCC (Boyle, Hakim et al. 1993; Shahnavaz, Regezi et al. 2000; Vitale-Cross, Amornphimoltham et al. 2004; Caulin, Nguyen et al. 2007). These additional mutations can be caused by external stimuli, including irradiation or injury. Collectively, these experimental data are in agreement with the high frequency of mutations affecting *Ras* and *p53* genes found in human SCC samples. These results however also indicate that *Ras* and *p53* mutations alone are not sufficient to induce SCC. Moreover, the nature of the multistage models also creates complexity that may contribute to the difficulties in identifying the precise origin of SCC. Thus, the establishment of a novel "one gene deletion-caused SCC model", as in the *ASPP2* Δexon3 mouse generated in our laboratory, could be a very powerful tool for future studies of SCC. In our work we also showed that *ASPP2* has a gene-dosage effect on tumour suppression, as the loss of both alleles accelerates the onset of the tumours. Additionally, we found that this effect is p53-independent and, as observed in other mouse models, loss of p53 contributes to SCC progression, but it is not required for tumour initiation. Consistent with this newly discovered role of ASPP2 in suppressing SCC, mutations in the *ASPP2* gene have been found in samples of human cutaneous SCC (source : http://www.sanger.ac.uk/genetics/CGP/cosmic/). The identification of molecular signatures for SCC-initiation, as loss of ASPP2 function appears to be, is considered critical for any therapeutic approaches aimed at the early diagnosis of SCC and ultimately at the development of more effective and targeted anti-SCC therapies.

Our mouse model could also be employed to shed some light into the precise cellular origin of SCC, which remains to date still largely unknown. It is not clear why SCCs express both basal cell markers, such as Keratin-5/14 and p63, and differentiated squamous cell markers, such as Keratin-1/10. Early findings on mutant Ras indicate that, when overexpressed in basal cells, it induces SCC formation (Brown, Strathdee et al. 1998). It is however unclear why SCC-initiating basal cells need to express differentiation markers, such as Keratin-1/10. Another possibility is that keratinocytes fail to switch off basal genes, such as *Keratin-5/14* and *p63*, during differentiation. However, the question which still remains is: why do these basal cells not develop into a basal cell carcinoma (BCC), but instead become an SCC? Alternatively, SCC might develop from partially differentiated K1 positive suprabasal cells, which are still considered partially dynamic, but are committed to enter terminal differentiation within a short time frame. The distribution and intensity of ASPP2 expression in normal stratified squamous epithelium indicates that ASPP2 is predominantly expressed in differentiated suprabasal keratinocytes rather than the proliferative keratinocytes of the basal layer. This suggests that perturbation of ASPP2 expression is more likely to have an impact on suprabasal K1-expressing keratinocytes rather than on K14-expressing basal keratinocytes. Testing the hypothesis that SCC might originate from differentiated suprabasal keratinocytes could help to identify the precise cellular source of SCC, and could be experimentally realised with our mouse model by inducing *ASPP2* deletion selectively in K1 positive cells. This can be achieved taking advantage of newly generated transgenic mice capable of switching off ASPP2 expression only in presence of Cre recombinase, which will be crossed with transgenic mice that express the Cre enzyme exclusively in K1 positive cells.

6.2 ASPP2 a gatekeeper of epidermal differentiation by regulating p63 function

Mechanistically, we found that ASPP2-mediated suppression of SCC is probably achieved by its repression of ΔNp63 (main p63 isoform expressed in the skin) expression and pro-proliferative function. The transcriptional factor p63 is a master regulator of epithelial homeostasis, fundamental both during development and in adult tissue to maintain the proliferative potential and stemness of the basal layer of the squamous epithelium. Consistent with this, its overexpression has been reported as potential cause of SCC formation in humans (Senoo, Tsuchiya et al. 2001; Reis-Filho, Torio et al. 2002), as well as in our *ASPP2*-null mouse model.

Interestingly p63, despite been predominantly a basal cell marker, can be still expressed at low level in suprabasal cells. Knowing that an increase in its expression will result in cell proliferation, the maintenance of low levels of p63 expression in this site would be crucial to maintain the stability of the squamous epithelium and prevent any cancerous transformation. Notch protein has been proposed as a candidate for this maintenance role, as it is expressed in suprabasal keratinocytes and, once activated, is able to repress p63 expression (Nguyen, Lefort et al. 2006). Similarly, we found that ASPP2 is also highly expressed in suprabasal layers of differentiated squamous epithelia, such as in cervix, oesophagus and skin, and its expression pattern is conserved between mouse and human. Furthermore, we showed that ASPP2 expression is induced upon keratinocyte differentiation, and that this coincides with a reduction in p63. Conversely, during tumour progression, ASPP2 is downregulated from normal epithelium to SCC, while p63 is upregulated. This defines a mutually exclusive expression pattern between the two proteins, with ASPP2 acting as oncosuppressor and p63 as oncogene. To explain this pattern we showed that the ASPP2 expression has a direct impact on p63 expression levels, as forced re-expression of ASPP2 in ASPP2-negative SCC cell lines led to a decrease in p63 at both mRNA and protein level. Additionally, ASPP2's mode of action does not seem to be limited to the repression of p63 protein levels, but also applies to the regulation of the p63

transcriptional program by inhibiting p63-mediated transcription of basal genes, and conversely, promoting the transcription of suprabasal genes. This indicates that ASPP2 is not only a marker of cell differentiation, but also an active player in the process, mainly through acting on p63 regulation. Finally, to provide genetic evidence for the hypothesis that ASPP2 suppresses SCC by inhibiting p63, we crossed *ASPP2* Δexon3 heterozygous mice with *p63* heterozygous mice and showed that if p63 expression is reduced, *ASPP2*-deficient mice no longer develop SCC.

At present, despite the known importance of p63 in skin homeostasis and cancer, the molecular mechanisms controlling its expression and transcriptional regulation are not understood. Our results indicate a possible new mechanism by which p63 expression and activity can be balanced in the squamous epithelium, providing a correct equilibrium between proliferation and differentiation.

Recent works from our laboratory and Dr Bergamaschi's group have shown that the ASPP family inhibitory member iASPP is also important for epithelial homeostasis, being expressed exclusively in the basal layer of the squamous epithelium together with p63, and regulating p63 transcriptional activity and promoting its expression (Chikh, Matin et al. 2011; Notari, Hu et al. 2011). Thus, ASPP2 and iASPP have different expression patterns in the stratified epithelium and generally opposing effects on p63 expression. This indicates that, similarly to what has been described for p53-mediated apoptosis, even in epithelial differentiation the presence of different members of the ASPP family of proteins in combination with p63 could lead to different outcomes, which are in this specific case a basal- (iASPP) or differentiated-cell fate (ASPP2) (Figure 6.6).

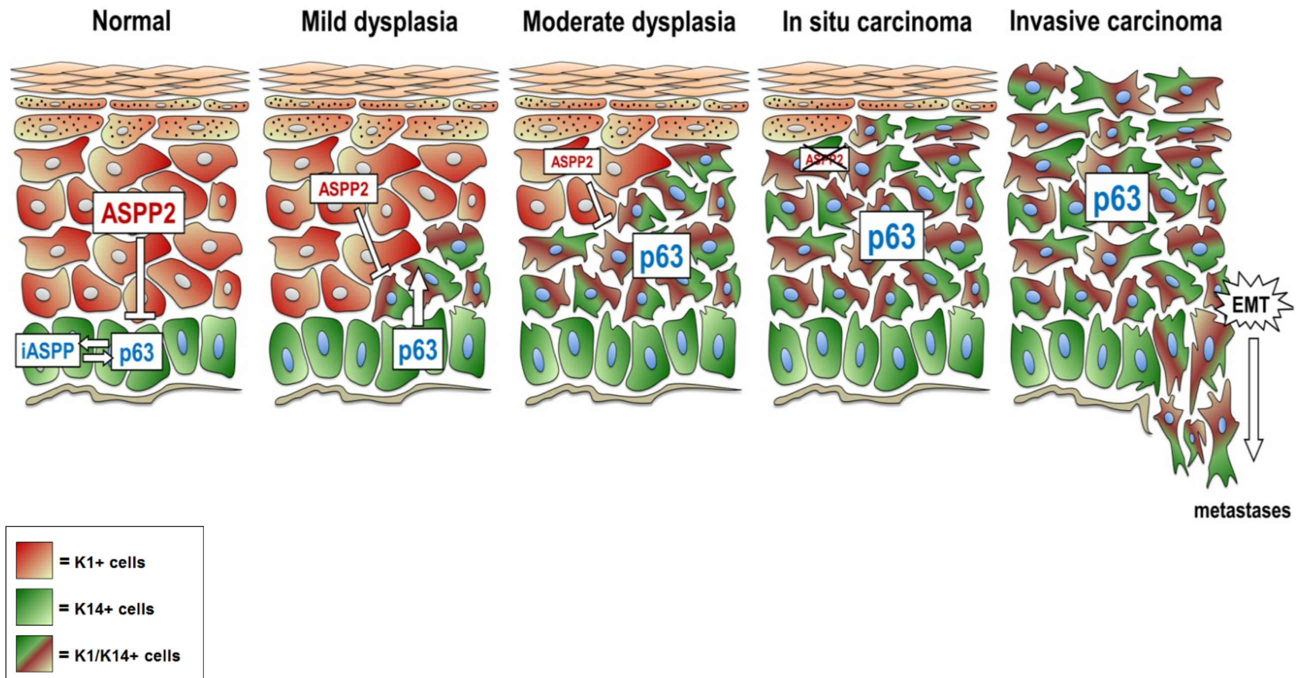


Figure 6.6 Proposed model for ASPP2-mediated suppression of SCC
The presence of ASPP2 in the suprabasal layers of the epithelium can serve to repress and prevent p63 expression in these areas of the tissue. In case of impairment of ASPP2 function or expression, p63 would not be switched off in the transitional population of partially differentiated (K1 expression) suprabasal keratinocytes, allowing them to keep proliferating and give origin to uncontrolled cell expansion (dysplasia and then carcinoma). Further decrease in ASPP2 expression levels could eventually favor EMT and formation of distant metastases.

6.3 ASPP2, helping Notch to take the right decision

A recent microarray experiment performed in our lab using mRNA extracted from the epidermis of 3-day-old *ASPP2* wild type and Δexon3 mice, revealed that the absence of ASPP2 causes a marked downregulation of differentiation markers, such as *envoplakin*, *filaggrin* and *loricrin*, while upregulation of genes belonging to pathways critical for the maintenance of basal layer of the epithelium and activated in cancer, such as Wnt/βcatenin and Shh pathways (data not shown). This result confirmed that ASPP2 is generally important in promoting differentiation in the squamous epithelium, and its absence allows keratinocytes to maintain basal signalling pathways in a more active state. This in turn confers a higher degree of plasticity to these cells, and therefore a higher predisposition to proliferate and develop carcinomas. Importantly, the microarray data also showed alterations in Notch pathway target genes: *involucrin*, *p21*, and *Hey2* were all downregulated in *ASPP2* Δexon3, whereas *Gli-2*, *CyclinD1* and *Hes1* were all upregulated in *ASPP2* Δexon3. The overexpression of Gli-2 and CyclinD1 has been already previously noted in SCCs from *ASPP2*-deficient mice (Chapter V), and the same tumour type (SCC) and cellular markers have been also observed in mice with impaired Notch expression or activity (Nicolas, Wolfer et al. 2003; Proweller, Tu et al. 2006). More generally, considering the overexpression of the Notch target genes *Hes1* and *Hes5* observed in the brain of the *ASPP2* Δexon3 mouse, we found that both the major phenotypes of the *ASPP2* Δexon3 mouse, such as the expansion of neuroprogenitor cells inside the brain and the formation of SCCs, resemble defects in the Notch pathway. The Notch pathway is notoriously important for being able to affect cell fate determination across a wide range of different tissues, with outcomes that can be very different, such as committing cells to differentiation (in skin) or promote cell pluripotency (in brain). This variety is due to Notch transcriptional selectivity, producing differential combinations of genes being expressed in different tissues or cell types, according to the scope required. Obviously in normal cell physiology as well as in disease, and in particular in cancer, it is very important to understand how Notch is regulated in light of its dual behaviour as an

oncogene/oncosuppressor. ASPP2 belongs to a family of adaptor proteins which are able to confer gene target selectivity, as shown for the p53 family of transcriptional factors, eventually affecting cell decisions and behaviour. Based on this assumption, and in light of the facts that deregulation in Notch pathway activity is observed in the *ASPP2* Δexon3 mice, and that ASPP2 and Notch were found co-expressed in the same cells *in vivo*, we examined the possibility of a direct interaction. In the present work we showed that ASPP2 and Notch not only co-localise in the nucleus, the site where transcriptional activity takes place, but they can also be shuttled there by the same stimuli. We then determined that ASPP2 can bind to transcriptional components of the Notch complex, and in particular to Notch itself via an ankyrin-ankyrin interaction. Our data support ASPP2 as being an enhancer of Notch transcriptional activity on certain promoters, such as those of *Hey2*, *Heyl* and *Hes7*, rather than those of *Hes1* and *Hes5*. This was established by the observed failure of active Notch to induce the transcription of this set of genes in the absence of ASPP2.

We next tested whether this transcriptional selectivity attributed by ASPP2 to Notch could be extended into a general pattern of genes regulating similar functions *in vivo*. We broadened our approach and performed a microarray experiment with mRNA extracted from our established experimental system of *ASPP2* wild type and Δexon3 MEFs stimulated (vs. unstimulated) by Dll4. Interestingly, we found more than 50 genes whose expression is induced exclusively in wild type MEFs upon Notch activation, and not in Δexon3, suggesting that ASPP2 is required for Notch to transcribe them (data not shown). Among those genes we were able to confirm the previously identified *Hey2* and *Heyl* and to discover some new ones such as *Notch3* and *involucrin*. Consistent with this, *Hey2*, *Notch3* and *involucrin* have been already found to be downregulated in the absence of ASPP2 in the previously mentioned microarray on mouse epidermis, while *Heyl* was also previously found downregulated in another microarray experiment performed in the past in our laboratory on brains of *ASPP2* Δexon3 mice. Additionally, various data available in the literature show that these genes take part in determining cell fate in brain or squamous epithelium, linking our experimental data with the phenotypical defects observed in the *ASPP2* Δexon3 mice. *Heyl,* for

instance, has been reported to promote neuronal differentiation (Jalali, Bassuk et al. 2011), while *Hey2*, *Notch3* and *involucrin* are involved in differentiation of the squamous epithelium (Nguyen, Lefort et al. 2006; Ohashi, Natsuizaka et al. 2010; Ohashi, Natsuizaka et al. 2011). Conversely, the *Hes1* gene, towards which ASPP2 seems to have an inhibitory effect and which was found to be highly expressed in both brain and epidermis of the *ASPP2* Δexon3 mice, has been reported to have a pro-stemness role in both neuronal cells and keratinocytes.

Our results indicate that Notch transcriptional target selectivity, which leads to different and sometimes opposite outcomes in different tissues, might be simply explained by the concurrent presence or absence of ASPP2. This theory can be applied to at least two tissues, the skin-epithelium and CNS. When ASPP2 is not temporally and spatially coexpressed with Notch, Notch transcriptional activity would preferentially be based on the formation of Notch/Notch homodimers, promoting the expression of pro-stemness genes such as *Hes1* and *Hes5*. In certain areas of the tissue, and/or during certain stages of development, Notch expression meets ASPP2 expression and Notch/Notch homodimer formation is diminished, because of the possibility of forming the heterodimer Notch/ASPP2. The heterodimer then inhibits the transcription of the pro-stemness genes *Hes1* and *Hes5*, while favouring the expression of pro-differentiation genes such as *involucrin*, *Hey2*, *Heyl*, *Hes7* and *Notch3*. Altogether, ASPP2 appears to drive Notch transcriptional activity towards a crucial pro-differentiation program, that when missing, causes the onset of undifferentiated phenotypes in both squamous epithelium and CNS, as observed in the *ASPP2* Δexon3 mice (model summarised in Figure 6.7 A-B).

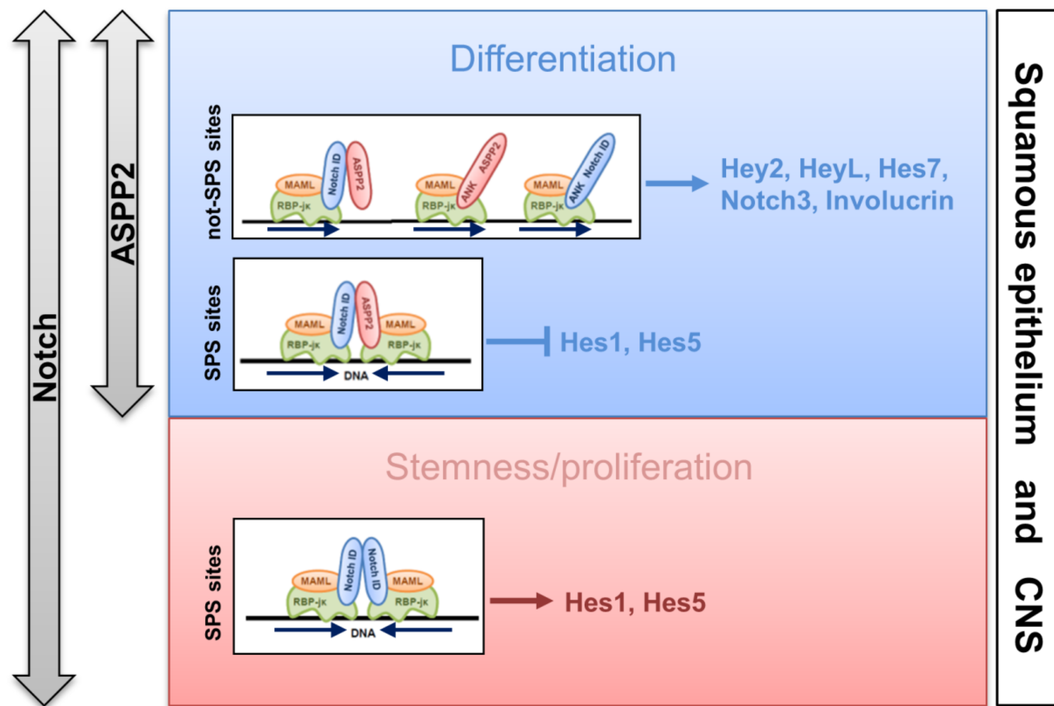
In the future, other known Notch-related genes which emerged as being altered by the absence of ASPP2 in our microarray experiment, such as *Jag1*, *GATA3* and *Flt1* (a gene coding for the VEGF receptor), will be subjected to further investigation in order to acquire a more comprehensive view of the functional implications of the ASPP2/Notch interaction.

In parallel to this, we are also trying to understand mechanistically how ASPP2 can enhance or inhibit Notch transcription of some of its target genes. Our chromatin immunoprecipitation result showed that less active Notch can be found bound to the *Hey2* promoter in absence of ASPP2, suggesting that ASPP2 could act by increasing the binding affinity or the stability of Notch on certain promoters. A broader genome-wide experiment, currently on-going, of ChIP-sequencing conducted by immunoprecipitating NICD-bound DNA in *ASPP2* wild type and Δexon3 cells will help us to understand whether this is a general mechanism applicable also to other genes transcriptionally regulated by ASPP2/Notch.

Moreover, three recent works have uncovered the great impact of *Notch* mutations in human SCC (cutaneous, lung and head and neck), which appear to target some of the regions important for Notch functional activity, such as the EGF-like and ankyrin repeats domains (Agrawal, Frederick et al. 2011; Stransky, Egloff et al. 2011; Wang, Sanborn et al. 2011). This is in contrast with the mutations previously identified in haematopoietic tumours, which selectively target Notch regulatory regions, such as the heterodimerisation and PEST domain, and are believed to produce an over activation of Notch signalling. Among these other mutations found in SCC, and in particular among those present in the ankyrin repeats domain of Notch, some result in a truncated protein clearly leading to an ablation of Notch signalling (nonsense mutations), while others involve single amino acid changes (missense mutations). These more subtle changes might be responsible for producing alterations in the functionality of the protein and, more intriguingly, could alter its capacity to interact with ASPP2. Since the independent loss of ASPP2 or Notch protein from the squamous epithelium can lead to the formation of SCC in mouse models, hence the main mechanism lying behind this phenomenon might be the same: the loss of the Notch pro-differentiation transcriptional program.
In the future we aim to explore this possibility, which would allow us to establish a fundamental link between our experimental findings and the human clinical data. We will therefore produce expression constructs for the known mutated forms of Notch found in human SCC samples and establish

whether they are defective in interacting with ASPP2 (physically and functionally). Similarly, as ASPP2 was also found mutated in human SCC samples, mutant forms of ASPP2 derived from clinical data will be tested for their ability to interact with wild type Notch.

In conclusion, with our study we believe that we have revealed in ASPP2 a time-regulated, tissue specific factor capable of influencing the Notch transcriptional programme. This improves our understanding of the complex pattern of different and sometimes opposing effects mediated by the Notch signalling pathway in different tissues.



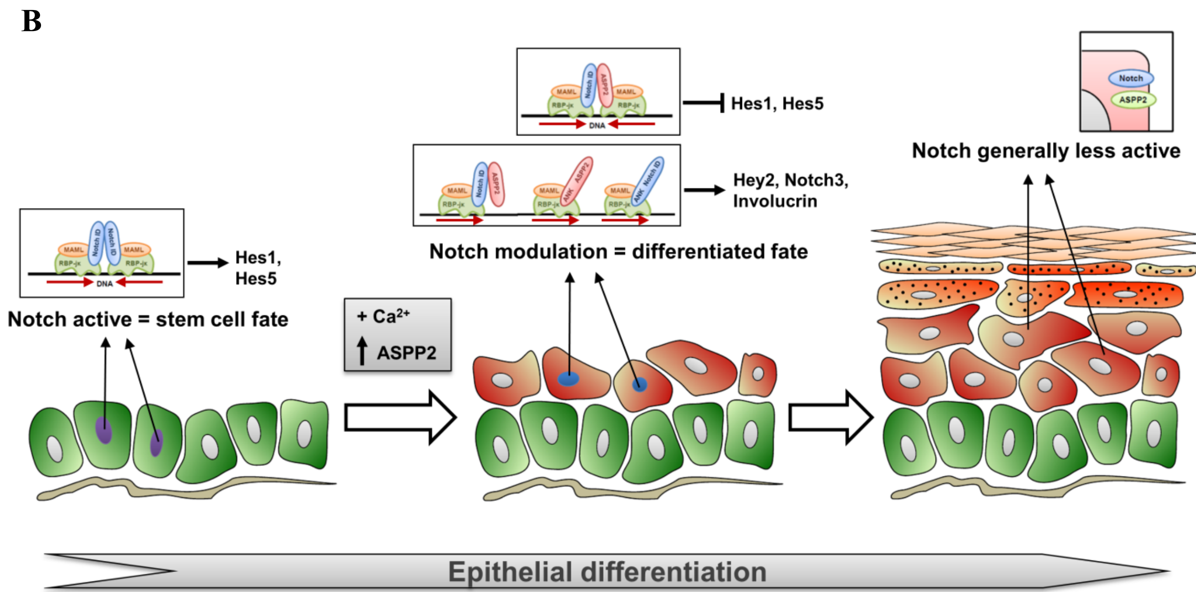


Figure 6.7 Proposed model for ASPP2-Notch involvement in cell fate determination
(A) The simultaneous expression of ASPP2 together with Notch can alter Notch transcriptional selectivity, favouring a program for cell-differentiation. (B) Model applied to epithelial differentiation. The expression of ASPP2 is temporally regulated during epithelial differentiation (induced by Ca^{2+}), while Notch is already present in undifferentiated keratinocytes. The presence of ASPP2 can affect Notch transcriptional program, promoting the expression of pro-differentiation genes and inhibiting others pro-stemness, with the overall effect of orienting Notch action towards the specification of a differentiated cell fate. A similar mechanism could take place also in the neural epithelium where the spatial absence of ASPP2 would allow Notch to transcribe mainly pro-stemness genes (*Hes1*-*Hes5*), while in regions where ASPP2 is expressed genes pro-differentiation would be induced (*Heyl*).

Bibliography

Agirre, X., J. Roman-Gomez, et al. (2006). "ASPP1, a common activator of TP53, is inactivated by aberrant methylation of its promoter in acute lymphoblastic leukemia." Oncogene **25**(13): 1862-1870.

Agrawal, N., M. J. Frederick, et al. (2011). "Exome sequencing of head and neck squamous cell carcinoma reveals inactivating mutations in NOTCH1." Science **333**(6046): 1154-1157.

Ahn, J., I. J. Byeon, et al. (2009). "Insight into the structural basis of pro- and antiapoptotic p53 modulation by ASPP proteins." J Biol Chem **284**(20): 13812-13822.

Alam, M. and D. Ratner (2001). "Cutaneous squamous-cell carcinoma." N Engl J Med **344**(13): 975-983.

Alva, J. A. and M. L. Iruela-Arispe (2004). "Notch signaling in vascular morphogenesis." Curr Opin Hematol **11**(4): 278-283.

Armstrong, J. F., M. H. Kaufman, et al. (1995). "High-frequency developmental abnormalities in p53-deficient mice." Curr Biol **5**(8): 931-936.

Arnett, K. L., M. Hass, et al. (2010). "Structural and mechanistic insights into cooperative assembly of dimeric Notch transcription complexes." Nat Struct Mol Biol **17**(11): 1312-1317.

Artavanis-Tsakonas, S., M. D. Rand, et al. (1999). "Notch signaling: cell fate control and signal integration in development." Science **284**(5415): 770-776.

Aster, J. C. (2005). "Deregulated NOTCH signaling in acute T-cell lymphoblastic leukemia/lymphoma: new insights, questions, and opportunities." Int J Hematol **82**(4): 295-301.

Attardi, L. D., E. E. Reczek, et al. (2000). "PERP, an apoptosis-associated target of p53, is a novel member of the PMP-22/gas3 family." Genes Dev **14**(6): 704-718.

Bagheri, M. M. and B. Safai (2001). "Cutaneous malignancies of keratinocytic origin." Clin Dermatol **19**(3): 244-252.

Balint, K., M. Xiao, et al. (2005). "Activation of Notch1 signaling is required for beta-catenin-mediated human primary melanoma progression." J Clin Invest **115**(11): 3166-3176.

Barbieri, C. E., L. J. Tang, et al. (2006). "Loss of p63 leads to increased cell migration and up-regulation of genes involved in invasion and metastasis." Cancer Res **66**(15): 7589-7597.

Basak, O. and V. Taylor (2007). "Identification of self-replicating multipotent progenitors in the embryonic nervous system by high Notch activity and Hes5 expression." Eur J Neurosci **25**(4): 1006-1022.

Basset-Seguin, N., J. P. Moles, et al. (1994). "TP53 tumor suppressor gene and skin carcinogenesis." J Invest Dermatol **103**(5 Suppl): 102S-106S.

Bensaad, K. and K. H. Vousden (2007). "p53: new roles in metabolism." Trends Cell Biol **17**(6): 286-291.

Berdnik, D., T. Torok, et al. (2002). "The endocytic protein alpha-Adaptin is required for numb-mediated asymmetric cell division in Drosophila." Dev Cell **3**(2): 221-231.

Beres, T. M., T. Masui, et al. (2006). "PTF1 is an organ-specific and Notch-independent basic helix-loop-helix complex containing the mammalian Suppressor of Hairless (RBP-J) or its paralogue, RBP-L." Mol Cell Biol **26**(1): 117-130.

Bergamaschi, D., M. Gasco, et al. (2003). "p53 polymorphism influences response in cancer chemotherapy via modulation of p73-dependent apoptosis." Cancer Cell **3**(4): 387-402.

Bergamaschi, D., Y. Samuels, et al. (2004). "ASPP1 and ASPP2: common activators of p53 family members." Mol Cell Biol **24**(3): 1341-1350.

Bergamaschi, D., Y. Samuels, et al. (2003). "iASPP oncoprotein is a key inhibitor of p53 conserved from worm to human." Nat Genet **33**(2): 162-167.

Bergamaschi, D., Y. Samuels, et al. (2006). "iASPP preferentially binds p53 proline-rich region and modulates apoptotic function of codon 72-polymorphic p53." Nat Genet **38**(10): 1133-1141.
Bigelow, R. L., E. Y. Jen, et al. (2005). "Sonic hedgehog induces epidermal growth factor dependent matrix infiltration in HaCaT keratinocytes." J Invest Dermatol **124**(2): 457-465.
Bishop, J. M. (1991). "Molecular themes in oncogenesis." Cell **64**(2): 235-248.
Blanpain, C. and E. Fuchs (2009). "Epidermal homeostasis: a balancing act of stem cells in the skin." Nat Rev Mol Cell Biol **10**(3): 207-217.
Blanpain, C., W. E. Lowry, et al. (2006). "Canonical notch signaling functions as a commitment switch in the epidermal lineage." Genes Dev **20**(21): 3022-3035.
Boldrup, L., P. J. Coates, et al. (2007). "DeltaNp63 isoforms regulate CD44 and keratins 4, 6, 14 and 19 in squamous cell carcinoma of head and neck." J Pathol **213**(4): 384-391.
Borggrefe, T. and F. Oswald (2009). "The Notch signaling pathway: transcriptional regulation at Notch target genes." Cell Mol Life Sci **66**(10): 1631-1646.
Boyle, J. O., J. Hakim, et al. (1993). "The incidence of p53 mutations increases with progression of head and neck cancer." Cancer Res **53**(19): 4477-4480.
Braithwaite, A. W., G. Del Sal, et al. (2006). "Some p53-binding proteins that can function as arbiters of life and death." Cell Death Differ **13**(6): 984-993.
Bray, S. (1998). "Notch signalling in Drosophila: three ways to use a pathway." Semin Cell Dev Biol **9**(6): 591-597.
Bray, S. J. (2006). "Notch signalling: a simple pathway becomes complex." Nat Rev Mol Cell Biol **7**(9): 678-689.
Brennan, C., H. Momota, et al. (2009). "Glioblastoma subclasses can be defined by activity among signal transduction pathways and associated genomic alterations." PLoS One **4**(11): e7752.
Brosh, R. and V. Rotter (2009). "When mutants gain new powers: news from the mutant p53 field." Nat Rev Cancer **9**(10): 701-713.
Brown, K., D. Strathdee, et al. (1998). "The malignant capacity of skin tumours induced by expression of a mutant H-ras transgene depends on the cell type targeted." Curr Biol **8**(9): 516-524.
Buti, L., E. Spooner, et al. (2011). "Helicobacter pylori cytotoxin-associated gene A (CagA) subverts the apoptosis-stimulating protein of p53 (ASPP2) tumor suppressor pathway of the host." Proc Natl Acad Sci U S A **108**(22): 9238-9243.
Candi, E., D. Dinsdale, et al. (2007). "TAp63 and DeltaNp63 in cancer and epidermal development." Cell Cycle **6**(3): 274-285.
Candi, E., A. Rufini, et al. (2006). "Differential roles of p63 isoforms in epidermal development: selective genetic complementation in p63 null mice." Cell Death Differ **13**(6): 1037-1047.
Cao, Y., T. Hamada, et al. (2004). "Hepatitis C virus core protein interacts with p53-binding protein, 53BP2/Bbp/ASPP2, and inhibits p53-mediated apoptosis." Biochem Biophys Res Commun **315**(4): 788-795.
Caulin, C., T. Nguyen, et al. (2007). "An inducible mouse model for skin cancer reveals distinct roles for gain- and loss-of-function p53 mutations." J Clin Invest **117**(7): 1893-1901.
Celli, J., P. Duijf, et al. (1999). "Heterozygous germline mutations in the p53 homolog p63 are the cause of EEC syndrome." Cell **99**(2): 143-153.
Chen, X., L. J. Ko, et al. (1996). "p53 levels, functional domains, and DNA damage determine the extent of the apoptotic response of tumor cells." Genes Dev **10**(19): 2438-2451.
Chen, Y., W. Liu, et al. (2003). "ASPP2 inhibits APP-BP1-mediated NEDD8 conjugation to cullin-1 and decreases APP-BP1-induced cell proliferation and neuronal apoptosis." J Neurochem **85**(3): 801-809.
Chiang, C. T., W. K. Chu, et al. (2009). "Overexpression of delta Np63 in a human nasopharyngeal carcinoma cell line downregulates CKIs and enhances cell proliferation." J Cell Physiol **219**(1): 117-122.

Chikh, A., R. N. Matin, et al. (2011). "iASPP/p63 autoregulatory feedback loop is required for the homeostasis of stratified epithelia." EMBO J **30**(20): 4261-4273.
Chitnis, A. (2006). "Why is delta endocytosis required for effective activation of notch?" Dev Dyn **235**(4): 886-894.
Choi, H. R., J. G. Batsakis, et al. (2002). "Differential expression of p53 gene family members p63 and p73 in head and neck squamous tumorigenesis." Hum Pathol **33**(2): 158-164.
Chu, W. K., P. M. Dai, et al. (2008). "Transcriptional activity of the DeltaNp63 promoter is regulated by STAT3." J Biol Chem **283**(12): 7328-7337.
Cicalese, A., G. Bonizzi, et al. (2009). "The tumor suppressor p53 regulates polarity of self-renewing divisions in mammary stem cells." Cell **138**(6): 1083-1095.
Clapp, N. K., R. L. Tyndall, et al. (1971). "Differences in tumor types and organ susceptibility in BALB-c and RF mice following dimethylnitrosamine and diethylnitrosamine." Cancer Res **31**(2): 196-198.
Cobleigh, M. A., B. Tabesh, et al. (2005). "Tumor gene expression and prognosis in breast cancer patients with 10 or more positive lymph nodes." Clin Cancer Res **11**(24 Pt 1): 8623-8631.
Conlon, R. A., A. G. Reaume, et al. (1995). "Notch1 is required for the coordinate segmentation of somites." Development **121**(5): 1533-1545.
Cook, W. D. and B. J. McCaw (2000). "Accommodating haploinsufficient tumor suppressor genes in Knudson's model." Oncogene **19**(30): 3434-3438.
Corn, P. G., S. J. Kuerbitz, et al. (1999). "Transcriptional silencing of the p73 gene in acute lymphoblastic leukemia and Burkitt's lymphoma is associated with 5' CpG island methylation." Cancer Res **59**(14): 3352-3356.
Crighton, D., S. Wilkinson, et al. (2006). "DRAM, a p53-induced modulator of autophagy, is critical for apoptosis." Cell **126**(1): 121-134.
Croce, C. M. (2008). "Oncogenes and cancer." N Engl J Med **358**(5): 502-511.
Czarnecki, D., M. Staples, et al. (1994). "Metastases from squamous cell carcinoma of the skin in southern Australia." Dermatology **189**(1): 52-54.
De Laurenzi, V., G. Raschella, et al. (2000). "Induction of neuronal differentiation by p73 in a neuroblastoma cell line." J Biol Chem **275**(20): 15226-15231.
De Strooper, B., W. Annaert, et al. (1999). "A presenilin-1-dependent gamma-secretase-like protease mediates release of Notch intracellular domain." Nature **398**(6727): 518-522.
DeLeo, A. B., G. Jay, et al. (1979). "Detection of a transformation-related antigen in chemically induced sarcomas and other transformed cells of the mouse." Proc Natl Acad Sci U S A **76**(5): 2420-2424.
Devgan, V., C. Mammucari, et al. (2005). "p21WAF1/Cip1 is a negative transcriptional regulator of Wnt4 expression downstream of Notch1 activation." Genes Dev **19**(12): 1485-1495.
Di Como, C. J., M. J. Urist, et al. (2002). "p63 expression profiles in human normal and tumor tissues." Clin Cancer Res **8**(2): 494-501.
DiGiovanni, J. (1992). "Multistage carcinogenesis in mouse skin." Pharmacol Ther **54**(1): 63-128.
Dlugosz, A. A. and S. H. Yuspa (1993). "Coordinate changes in gene expression which mark the spinous to granular cell transition in epidermis are regulated by protein kinase C." J Cell Biol **120**(1): 217-225.
Dlugosz, A. A. and S. H. Yuspa (1994). "Protein kinase C regulates keratinocyte transglutaminase (TGK) gene expression in cultured primary mouse epidermal keratinocytes induced to terminally differentiate by calcium." J Invest Dermatol **102**(4): 409-414.
Dominguez, G., J. M. Garcia, et al. (2006). "DeltaTAp73 upregulation correlates with poor prognosis in human tumors: putative in vivo network involving p73 isoforms, p53, and E2F-1." J Clin Oncol **24**(5): 805-815.
Donehower, L. A., M. Harvey, et al. (1992). "Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours." Nature **356**(6366): 215-221.

Dontu, G., K. W. Jackson, et al. (2004). "Role of Notch signaling in cell-fate determination of human mammary stem/progenitor cells." Breast Cancer Res **6**(6): R605-615.
Dotto, G. P. (2008). "Notch tumor suppressor function." Oncogene **27**(38): 5115-5123.
Dotto, G. P. (2009). "Crosstalk of Notch with p53 and p63 in cancer growth control." Nat Rev Cancer **9**(8): 587-595.
Duffy, M. J., P. M. McGowan, et al. (2008). "Cancer invasion and metastasis: changing views." J Pathol **214**(3): 283-293.
el-Deiry, W. S., T. Tokino, et al. (1993). "WAF1, a potential mediator of p53 tumor suppression." Cell **75**(4): 817-825.
Eliyahu, D., D. Michalovitz, et al. (1989). "Wild-type p53 can inhibit oncogene-mediated focus formation." Proc Natl Acad Sci U S A **86**(22): 8763-8767.
Ellisen, L. W., J. Bird, et al. (1991). "TAN-1, the human homolog of the Drosophila notch gene, is broken by chromosomal translocations in T lymphoblastic neoplasms." Cell **66**(4): 649-661.
Espanel, X. and M. Sudol (2001). "Yes-associated protein and p53-binding protein-2 interact through their WW and SH3 domains." J Biol Chem **276**(17): 14514-14523.
Fan, X., I. Mikolaenko, et al. (2004). "Notch1 and notch2 have opposite effects on embryonal brain tumor growth." Cancer Res **64**(21): 7787-7793.
Feldser, D. M., K. K. Kostova, et al. (2010). "Stage-specific sensitivity to p53 restoration during lung cancer progression." Nature **468**(7323): 572-575.
Ferjentsik, Z., S. Hayashi, et al. (2009). "Notch is a critical component of the mouse somitogenesis oscillator and is essential for the formation of the somites." PLoS Genet **5**(9): e1000662.
Fernandez-Majada, V., C. Aguilera, et al. (2007). "Nuclear IKK activity leads to dysregulated notch-dependent gene expression in colorectal cancer." Proc Natl Acad Sci U S A **104**(1): 276-281.
Finlay, C. A., P. W. Hinds, et al. (1989). "The p53 proto-oncogene can act as a suppressor of transformation." Cell **57**(7): 1083-1093.
Fiuza, U. M. and A. M. Arias (2007). "Cell and molecular biology of Notch." J Endocrinol **194**(3): 459-474.
Flores, E. R., S. Sengupta, et al. (2005). "Tumor predisposition in mice mutant for p63 and p73: evidence for broader tumor suppressor functions for the p53 family." Cancer Cell **7**(4): 363-373.
Flores, E. R., K. Y. Tsai, et al. (2002). "p63 and p73 are required for p53-dependent apoptosis in response to DNA damage." Nature **416**(6880): 560-564.
Fogal, V., N. N. Kartasheva, et al. (2005). "ASPP1 and ASPP2 are new transcriptional targets of E2F." Cell Death Differ **12**(4): 369-376.
Fre, S., M. Huyghe, et al. (2005). "Notch signals control the fate of immature progenitor cells in the intestine." Nature **435**(7044): 964-968.
Frise, E., J. A. Knoblich, et al. (1996). "The Drosophila Numb protein inhibits signaling of the Notch receptor during cell-cell interaction in sensory organ lineage." Proc Natl Acad Sci U S A **93**(21): 11925-11932.
Fryer, C. J., J. B. White, et al. (2004). "Mastermind recruits CycC:CDK8 to phosphorylate the Notch ICD and coordinate activation with turnover." Mol Cell **16**(4): 509-520.
Gaiddon, C., M. Lokshin, et al. (2001). "A subset of tumor-derived mutant forms of p53 down-regulate p63 and p73 through a direct interaction with the p53 core domain." Mol Cell Biol **21**(5): 1874-1887.
Garg, V., A. N. Muth, et al. (2005). "Mutations in NOTCH1 cause aortic valve disease." Nature **437**(7056): 270-274.
Ghioni, P., F. Bolognese, et al. (2002). "Complex transcriptional effects of p63 isoforms: identification of novel activation and repression domains." Mol Cell Biol **22**(24): 8659-8668.
Gorina, S. and N. P. Pavletich (1996). "Structure of the p53 tumor suppressor bound to the ankyrin and SH3 domains of 53BP2." Science **274**(5289): 1001-1005.

Graziano, V. and V. De Laurenzi (2011). "Role of p63 in cancer development." Biochim Biophys Acta **1816**(1): 57-66.
Green, D. R. and G. Kroemer (2009). "Cytoplasmic functions of the tumour suppressor p53." Nature **458**(7242): 1127-1130.
Gridley, T. (2003). "Notch signaling and inherited disease syndromes." Hum Mol Genet **12 Spec No 1**: R9-13.
Gupta-Rossi, N., E. Six, et al. (2004). "Monoubiquitination and endocytosis direct gamma-secretase cleavage of activated Notch receptor." J Cell Biol **166**(1): 73-83.
Haines, N. and K. D. Irvine (2003). "Glycosylation regulates Notch signalling." Nat Rev Mol Cell Biol **4**(10): 786-797.
Hallahan, A. R., J. I. Pritchard, et al. (2004). "The SmoA1 mouse model reveals that notch signaling is critical for the growth and survival of sonic hedgehog-induced medulloblastomas." Cancer Res **64**(21): 7794-7800.
Hanahan, D. and R. A. Weinberg (2000). "The hallmarks of cancer." Cell **100**(1): 57-70.
Hanahan, D. and R. A. Weinberg (2011). "Hallmarks of cancer: the next generation." Cell **144**(5): 646-674.
Harmes, D. C., E. Bresnick, et al. (2003). "Positive and negative regulation of deltaN-p63 promoter activity by p53 and deltaN-p63-alpha contributes to differential regulation of p53 target genes." Oncogene **22**(48): 7607-7616.
Haupt, S., M. Berger, et al. (2003). "Apoptosis - the p53 network." J Cell Sci **116**(Pt 20): 4077-4085.
Haupt, Y., R. Maya, et al. (1997). "Mdm2 promotes the rapid degradation of p53." Nature **387**(6630): 296-299.
Helps, N. R., H. M. Barker, et al. (1995). "Protein phosphatase 1 interacts with p53BP2, a protein which binds to the tumour suppressor p53." FEBS Lett **377**(3): 295-300.
Hennings, H., K. Holbrook, et al. (1980). "Growth and differentiation of mouse epidermal cells in culture: effects of extracellular calcium." Curr Probl Dermatol **10**: 3-25.
Hermeking, H., C. Lengauer, et al. (1997). "14-3-3 sigma is a p53-regulated inhibitor of G2/M progression." Mol Cell **1**(1): 3-11.
Hibi, K., B. Trink, et al. (2000). "AIS is an oncogene amplified in squamous cell carcinoma." Proc Natl Acad Sci U S A **97**(10): 5462-5467.
Hingorani, S. R., E. F. Petricoin, et al. (2003). "Preinvasive and invasive ductal pancreatic cancer and its early detection in the mouse." Cancer Cell **4**(6): 437-450.
Hirashima, M., A. Bernstein, et al. (2004). "Gene-trap expression screening to identify endothelial-specific genes." Blood **104**(3): 711-718.
Hirashima, M., K. Sano, et al. (2008). "Lymphatic vessel assembly is impaired in Aspp1-deficient mouse embryos." Dev Biol **316**(1): 149-159.
Hollstein, M., B. Shomer, et al. (1996). "Somatic point mutations in the p53 gene of human tumors and cell lines: updated compilation." Nucleic Acids Res **24**(1): 141-146.
Hollstein, M., D. Sidransky, et al. (1991). "p53 mutations in human cancers." Science **253**(5015): 49-53.
Honda, R., H. Tanaka, et al. (1997). "Oncoprotein MDM2 is a ubiquitin ligase E3 for tumor suppressor p53." FEBS Lett **420**(1): 25-27.
Hori, K., M. Fostier, et al. (2004). "Drosophila deltex mediates suppressor of Hairless-independent and late-endosomal activation of Notch signaling." Development **131**(22): 5527-5537.
Hruban, R. H., R. E. Wilentz, et al. (1999). "Pathology of incipient pancreatic cancer." Ann Oncol **10 Suppl 4**: 9-11.
Hu, W., Z. Feng, et al. (2007). "p53 regulates maternal reproduction through LIF." Nature **450**(7170): 721-724.
Huppert, S. S., A. Le, et al. (2000). "Embryonic lethality in mice homozygous for a processing-deficient allele of Notch1." Nature **405**(6789): 966-970.

Ishibashi, M., S. L. Ang, et al. (1995). "Targeted disruption of mammalian hairy and Enhancer of split homolog-1 (HES-1) leads to up-regulation of neural helix-loop-helix factors, premature neurogenesis, and severe neural tube defects." Genes Dev **9**(24): 3136-3148.
Iwabuchi, K., P. L. Bartel, et al. (1994). "Two cellular proteins that bind to wild-type but not mutant p53." Proc Natl Acad Sci U S A **91**(13): 6098-6102.
Jalali, A., A. G. Bassuk, et al. (2011). "HeyL promotes neuronal differentiation of neural progenitor cells." J Neurosci Res **89**(3): 299-309.
Jensen, I. and B. Robertsen (2002). "Effect of double-stranded RNA and interferon on the antiviral activity of Atlantic salmon cells against infectious salmon anemia virus and infectious pancreatic necrosis virus." Fish Shellfish Immunol **13**(3): 221-241.
Jost, C. A., M. C. Marin, et al. (1997). "p73 is a simian [correction of human] p53-related protein that can induce apoptosis." Nature **389**(6647): 191-194.
Joutel, A., C. Corpechot, et al. (1996). "Notch3 mutations in CADASIL, a hereditary adult-onset condition causing stroke and dementia." Nature **383**(6602): 707-710.
Ju, H., K. A. Lee, et al. (2005). "TP53BP2 locus is associated with gastric cancer susceptibility." Int J Cancer **117**(6): 957-960.
Kadam, S. and B. M. Emerson (2003). "Transcriptional specificity of human SWI/SNF BRG1 and BRM chromatin remodeling complexes." Mol Cell **11**(2): 377-389.
Kageyama, R., T. Ohtsuka, et al. (2008). "Roles of Hes genes in neural development." Dev Growth Differ **50 Suppl 1**: S97-103.
Kaghad, M., H. Bonnet, et al. (1997). "Monoallelically expressed gene related to p53 at 1p36, a region frequently deleted in neuroblastoma and other human cancers." Cell **90**(4): 809-819.
Kampa, K. M., J. D. Acoba, et al. (2009). "Apoptosis-stimulating protein of p53 (ASPP2) heterozygous mice are tumor-prone and have attenuated cellular damage-response thresholds." Proc Natl Acad Sci U S A **106**(11): 4390-4395.
Katoh, I., K. I. Aisaki, et al. (2000). "p51A (TAp63gamma), a p53 homolog, accumulates in response to DNA damage for cell regulation." Oncogene **19**(27): 3126-3130.
Kemp, C. J., L. A. Donehower, et al. (1993). "Reduction of p53 gene dosage does not increase initiation or promotion but enhances malignant progression of chemically induced skin tumors." Cell **74**(5): 813-822.
Kenney, A. M., M. D. Cole, et al. (2003). "Nmyc upregulation by sonic hedgehog signaling promotes proliferation in developing cerebellar granule neuron precursors." Development **130**(1): 15-28.
Keyes, W. M., M. Pecoraro, et al. (2011). "DeltaNp63alpha is an oncogene that targets chromatin remodeler Lsh to drive skin stem cell proliferation and tumorigenesis." Cell Stem Cell **8**(2): 164-176.
Keyes, W. M., H. Vogel, et al. (2006). "p63 heterozygous mutant mice are not prone to spontaneous or chemically induced tumors." Proc Natl Acad Sci U S A **103**(22): 8435-8440.
Kimura, K., K. Satoh, et al. (2007). "Activation of Notch signaling in tumorigenesis of experimental pancreatic cancer induced by dimethylbenzanthracene in mice." Cancer Sci **98**(2): 155-162.
King, K. E., R. M. Ponnamperuma, et al. (2006). "Unique domain functions of p63 isotypes that differentially regulate distinct aspects of epidermal homeostasis." Carcinogenesis **27**(1): 53-63.
Koch, U. and F. Radtke (2007). "Notch and cancer: a double-edged sword." Cell Mol Life Sci **64**(21): 2746-2762.
Kopan, R. and M. X. Ilagan (2009). "The canonical Notch signaling pathway: unfolding the activation mechanism." Cell **137**(2): 216-233.
Koster, M. I., S. Kim, et al. (2004). "p63 is the molecular switch for initiation of an epithelial stratification program." Genes Dev **18**(2): 126-131.
Koster, M. I. and D. R. Roop (2004). "Genetic pathways required for epidermal morphogenesis." Eur J Cell Biol **83**(11-12): 625-629.

Koster, M. I. and D. R. Roop (2007). "Mechanisms regulating epithelial stratification." Annu Rev Cell Dev Biol **23**: 93-113.
Kovalev, S., N. Marchenko, et al. (1998). "Expression level, allelic origin, and mutation analysis of the p73 gene in neuroblastoma tumors and cell lines." Cell Growth Differ **9**(11): 897-903.
Krebs, L. T., Y. Xue, et al. (2000). "Notch signaling is essential for vascular morphogenesis in mice." Genes Dev **14**(11): 1343-1352.
Kubo, Y., Y. Urano, et al. (1997). "Mutations of the INK4a locus in squamous cell carcinomas of human skin." Biochem Biophys Res Commun **232**(1): 38-41.
Kuroda, K., S. Tani, et al. (1999). "Delta-induced Notch signaling mediated by RBP-J inhibits MyoD expression and myogenesis." J Biol Chem **274**(11): 7238-7244.
Kurooka, H. and T. Honjo (2000). "Functional interaction between the mouse notch1 intracellular region and histone acetyltransferases PCAF and GCN5." J Biol Chem **275**(22): 17211-17220.
Lai, E. C. (2002). "Keeping a good pathway down: transcriptional repression of Notch pathway target genes by CSL proteins." EMBO Rep **3**(9): 840-845.
Lai, E. C., B. Tam, et al. (2005). "Pervasive regulation of Drosophila Notch target genes by GY-box-, Brd-box-, and K-box-class microRNAs." Genes Dev **19**(9): 1067-1080.
Lane, D. P. (1992). "Cancer. p53, guardian of the genome." Nature **358**(6381): 15-16.
Lane, D. P. and L. V. Crawford (1979). "T antigen is bound to a host protein in SV40-transformed cells." Nature **278**(5701): 261-263.
Langton, P. F., J. Colombani, et al. (2007). "Drosophila ASPP regulates C-terminal Src kinase activity." Dev Cell **13**(6): 773-782.
Lanza, M., B. Marinari, et al. (2006). "Cross-talks in the p53 family: deltaNp63 is an anti-apoptotic target for deltaNp73alpha and p53 gain-of-function mutants." Cell Cycle **5**(17): 1996-2004.
Laurikkala, J., M. L. Mikkola, et al. (2006). "p63 regulates multiple signalling pathways required for ectodermal organogenesis and differentiation." Development **133**(8): 1553-1563.
Lavin, M. F. and N. Gueven (2006). "The complexity of p53 stabilization and activation." Cell Death Differ **13**(6): 941-950.
Le Borgne, R., A. Bardin, et al. (2005). "The roles of receptor and ligand endocytosis in regulating Notch signaling." Development **132**(8): 1751-1762.
Le Borgne, R. and F. Schweisguth (2003). "Unequal segregation of Neuralized biases Notch activation during asymmetric cell division." Dev Cell **5**(1): 139-148.
Lefort, K. and G. P. Dotto (2004). "Notch signaling in the integrated control of keratinocyte growth/differentiation and tumor suppression." Semin Cancer Biol **14**(5): 374-386.
Lefort, K., A. Mandinova, et al. (2007). "Notch1 is a p53 target gene involved in human keratinocyte tumor suppression through negative regulation of ROCK1/2 and MRCKalpha kinases." Genes Dev **21**(5): 562-577.
Levine, A. J. (1997). "p53, the cellular gatekeeper for growth and division." Cell **88**(3): 323-331.
Levrero, M., V. De Laurenzi, et al. (2000). "The p53/p63/p73 family of transcription factors: overlapping and distinct functions." J Cell Sci **113 (Pt 10)**: 1661-1670.
Li, L., I. D. Krantz, et al. (1997). "Alagille syndrome is caused by mutations in human Jagged1, which encodes a ligand for Notch1." Nat Genet **16**(3): 243-251.
Linares, L. K., A. Hengstermann, et al. (2003). "HdmX stimulates Hdm2-mediated ubiquitination and degradation of p53." Proc Natl Acad Sci U S A **100**(21): 12009-12014.
Linzer, D. I. and A. J. Levine (1979). "Characterization of a 54K dalton cellular SV40 tumor antigen present in SV40-transformed cells and uninfected embryonal carcinoma cells." Cell **17**(1): 43-52.
Liu, S., G. Dontu, et al. (2005). "Mammary stem cells, self-renewal pathways, and carcinogenesis." Breast Cancer Res **7**(3): 86-95.
Liu, Z. J., X. Lu, et al. (2005). "Downregulated mRNA expression of ASPP and the hypermethylation of the 5'-untranslated region in cancer cell lines retaining wild-type p53." FEBS Lett **579**(7): 1587-1590.

Liu, Z. J., M. Xiao, et al. (2006). "Notch1 signaling promotes primary melanoma progression by activating mitogen-activated protein kinase/phosphatidylinositol 3-kinase-Akt pathways and up-regulating N-cadherin expression." Cancer Res **66**(8): 4182-4190.
Liu, Z. J., Y. Zhang, et al. (2004). "Abnormal mRNA expression of ASPP members in leukemia cell lines." Leukemia **18**(4): 880.
Lossos, I. S., Y. Natkunam, et al. (2002). "Apoptosis stimulating protein of p53 (ASPP2) expression differs in diffuse large B-cell and follicular center lymphoma: correlation with clinical outcome." Leuk Lymphoma **43**(12): 2309-2317.
Louvi, A., J. F. Arboleda-Velasquez, et al. (2006). "CADASIL: a critical look at a Notch disease." Dev Neurosci **28**(1-2): 5-12.
Lowe, S. W. (1999). "Activation of p53 by oncogenes." Endocr Relat Cancer **6**(1): 45-48.
Lutolf, S., F. Radtke, et al. (2002). "Notch1 is required for neuronal and glial differentiation in the cerebellum." Development **129**(2): 373-385.
Malkin, D., F. P. Li, et al. (1990). "Germ line p53 mutations in a familial syndrome of breast cancer, sarcomas, and other neoplasms." Science **250**(4985): 1233-1238.
Marks, R., G. Rennie, et al. (1988). "Malignant transformation of solar keratoses to squamous cell carcinoma." Lancet **1**(8589): 795-797.
Martinez, A. M., B. Schuettengruber, et al. (2009). "Polyhomeotic has a tumor suppressor activity mediated by repression of Notch signaling." Nat Genet **41**(10): 1076-1082.
Massion, P. P., P. M. Taflan, et al. (2003). "Significance of p63 amplification and overexpression in lung cancer development and prognosis." Cancer Res **63**(21): 7113-7121.
McElhinny, A. S., J. L. Li, et al. (2008). "Mastermind-like transcriptional co-activators: emerging roles in regulating cross talk among multiple signaling pathways." Oncogene **27**(38): 5138-5147.
McGill, M. A. and C. J. McGlade (2003). "Mammalian numb proteins promote Notch1 receptor ubiquitination and degradation of the Notch1 intracellular domain." J Biol Chem **278**(25): 23196-23203.
Melino, G., F. Bernassola, et al. (2004). "p73 Induces apoptosis via PUMA transactivation and Bax mitochondrial translocation." J Biol Chem **279**(9): 8076-8083.
Miller, D. L. and M. A. Weinstock (1994). "Nonmelanoma skin cancer in the United States: incidence." J Am Acad Dermatol **30**(5 Pt 1): 774-778.
Mills, A. A., B. Zheng, et al. (1999). "p63 is a p53 homologue required for limb and epidermal morphogenesis." Nature **398**(6729): 708-713.
Miyamoto, Y., A. Maitra, et al. (2003). "Notch mediates TGF alpha-induced changes in epithelial differentiation during pancreatic tumorigenesis." Cancer Cell **3**(6): 565-576.
Moll, U. M. and N. Slade (2004). "p63 and p73: roles in development and tumor formation." Mol Cancer Res **2**(7): 371-386.
Monier, R. (1990). "Oncogenes and anti-oncogenes in tumorigenesis." Reprod Nutr Dev **30**(3): 445-454.
Mori, S., G. Ito, et al. (2004). "p53 apoptotic pathway molecules are frequently and simultaneously altered in nonsmall cell lung carcinoma." Cancer **100**(8): 1673-1682.
Mori, T., H. Okamoto, et al. (2000). "Aberrant overexpression of 53BP2 mRNA in lung cancer cell lines." FEBS Lett **465**(2-3): 124-128.
Moriyama, M., M. Osawa, et al. (2006). "Notch signaling via Hes1 transcription factor maintains survival of melanoblasts and melanocyte stem cells." J Cell Biol **173**(3): 333-339.
Muller, M., E. S. Schleithoff, et al. (2006). "One, two, three--p53, p63, p73 and chemosensitivity." Drug Resist Updat **9**(6): 288-306.
Muller, M., S. Wilder, et al. (1998). "p53 activates the CD95 (APO-1/Fas) gene in response to DNA damage by anticancer drugs." J Exp Med **188**(11): 2033-2045.
Mumm, J. S., E. H. Schroeter, et al. (2000). "A ligand-induced extracellular cleavage regulates gamma-secretase-like proteolytic activation of Notch1." Mol Cell **5**(2): 197-206.

Nagase, T., K. Ishikawa, et al. (1998). "Prediction of the coding sequences of unidentified human genes. XII. The complete sequences of 100 new cDNA clones from brain which code for large proteins in vitro." DNA Res **5**(6): 355-364.
Nakagawa, H., K. Koyama, et al. (2000). "APCL, a central nervous system-specific homologue of adenomatous polyposis coli tumor suppressor, binds to p53-binding protein 2 and translocates it to the perinucleus." Cancer Res **60**(1): 101-105.
Nam, Y., P. Sliz, et al. (2007). "Cooperative assembly of higher-order Notch complexes functions as a switch to induce transcription." Proc Natl Acad Sci U S A **104**(7): 2103-2108.
Naumovski, L. and M. L. Cleary (1996). "The p53-binding protein 53BP2 also interacts with Bc12 and impedes cell cycle progression at G2/M." Mol Cell Biol **16**(7): 3884-3892.
Nguyen, B. C., K. Lefort, et al. (2006). "Cross-regulation between Notch and p63 in keratinocyte commitment to differentiation." Genes Dev **20**(8): 1028-1042.
Nicolas, M., A. Wolfer, et al. (2003). "Notch1 functions as a tumor suppressor in mouse skin." Nat Genet **33**(3): 416-421.
Noguera-Troise, I., C. Daly, et al. (2006). "Blockade of Dll4 inhibits tumour growth by promoting non-productive angiogenesis." Nature **444**(7122): 1032-1037.
Norimura, T., S. Nomoto, et al. (1996). "p53-dependent apoptosis suppresses radiation-induced teratogenesis." Nat Med **2**(5): 577-580.
Notari, M., Y. Hu, et al. (2011). "Inhibitor of apoptosis-stimulating protein of p53 (iASPP) prevents senescence and is required for epithelial stratification." Proc Natl Acad Sci U S A **108**(40): 16645-16650.
Nowell, P. C. (1976). "The clonal evolution of tumor cell populations." Science **194**(4260): 23-28.
Oda, E., R. Ohki, et al. (2000). "Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis." Science **288**(5468): 1053-1058.
Oda, K., H. Arakawa, et al. (2000). "p53AIP1, a potential mediator of p53-dependent apoptosis, and its regulation by Ser-46-phosphorylated p53." Cell **102**(6): 849-862.
Ohashi, S., M. Natsuizaka, et al. (2011). "A NOTCH3-mediated squamous cell differentiation program limits expansion of EMT-competent cells that express the ZEB transcription factors." Cancer Res **71**(21): 6836-6847.
Ohashi, S., M. Natsuizaka, et al. (2010). "NOTCH1 and NOTCH3 coordinate esophageal squamous differentiation through a CSL-dependent transcriptional network." Gastroenterology **139**(6): 2113-2123.
Ohki, R., J. Nemoto, et al. (2000). "Reprimo, a new candidate mediator of the p53-mediated cell cycle arrest at the G2 phase." J Biol Chem **275**(30): 22627-22630.
Ohtsuka, T., M. Ishibashi, et al. (1999). "Hes1 and Hes5 as notch effectors in mammalian neuronal differentiation." EMBO J **18**(8): 2196-2207.
Okada, Y., M. Osada, et al. (2002). "p53 gene family p51(p63)-encoded, secondary transactivator p51B(TAp63alpha) occurs without forming an immunoprecipitable complex with MDM2, but responds to genotoxic stress by accumulation." Exp Cell Res **276**(2): 194-200.
Okajima, T. and K. D. Irvine (2002). "Regulation of notch signaling by o-linked fucose." Cell **111**(6): 893-904.
Okajima, T., A. Xu, et al. (2003). "Modulation of notch-ligand binding by protein O-fucosyltransferase 1 and fringe." J Biol Chem **278**(43): 42340-42345.
Okajima, T., A. Xu, et al. (2005). "Chaperone activity of protein O-fucosyltransferase 1 promotes notch receptor folding." Science **307**(5715): 1599-1603.
Okuyama, R., E. Ogawa, et al. (2007). "p53 homologue, p51/p63, maintains the immaturity of keratinocyte stem cells by inhibiting Notch1 activity." Oncogene **26**(31): 4478-4488.
Oliner, J. D., J. A. Pietenpol, et al. (1993). "Oncoprotein MDM2 conceals the activation domain of tumour suppressor p53." Nature **362**(6423): 857-860.
Osada, M., M. Ohba, et al. (1998). "Cloning and functional analysis of human p51, which structurally and functionally resembles p53." Nat Med **4**(7): 839-843.

Parks, A. L., S. S. Huppert, et al. (1997). "The dynamics of neurogenic signalling underlying bristle development in Drosophila melanogaster." Mech Dev **63**(1): 61-74.
Parks, A. L., K. M. Klueg, et al. (2000). "Ligand endocytosis drives receptor dissociation and activation in the Notch pathway." Development **127**(7): 1373-1385.
Parr, C., G. Watkins, et al. (2004). "The possible correlation of Notch-1 and Notch-2 with clinical outcome and tumour clinicopathological parameters in human breast cancer." Int J Mol Med **14**(5): 779-786.
Patel, N. S., J. L. Li, et al. (2005). "Up-regulation of delta-like 4 ligand in human tumor vasculature and the role of basal expression in endothelial cell function." Cancer Res **65**(19): 8690-8697.
Patten, B. A., J. M. Peyrin, et al. (2003). "Sequential signaling through Notch1 and erbB receptors mediates radial glia differentiation." J Neurosci **23**(14): 6132-6140.
Patturajan, M., S. Nomoto, et al. (2002). "DeltaNp63 induces beta-catenin nuclear accumulation and signaling." Cancer Cell **1**(4): 369-379.
Pece, S., M. Serresi, et al. (2004). "Loss of negative regulation by Numb over Notch is relevant to human breast carcinogenesis." J Cell Biol **167**(2): 215-221.
Pellegrini, G., E. Dellambra, et al. (2001). "p63 identifies keratinocyte stem cells." Proc Natl Acad Sci U S A **98**(6): 3156-3161.
Pozniak, C. D., S. Radinovic, et al. (2000). "An anti-apoptotic role for the p53 family member, p73, during developmental neuron death." Science **289**(5477): 304-306.
Proweller, A., L. Tu, et al. (2006). "Impaired notch signaling promotes de novo squamous cell carcinoma formation." Cancer Res **66**(15): 7438-7444.
Pui, J. C., D. Allman, et al. (1999). "Notch1 expression in early lymphopoiesis influences B versus T lineage determination." Immunity **11**(3): 299-308.
Radtke, F. and K. Raj (2003). "The role of Notch in tumorigenesis: oncogene or tumour suppressor?" Nat Rev Cancer **3**(10): 756-767.
Ranganathan, P., K. L. Weaver, et al. (2011). "Notch signalling in solid tumours: a little bit of everything but not all the time." Nat Rev Cancer **11**(5): 338-351.
Rangarajan, A., C. Talora, et al. (2001). "Notch signaling is a direct determinant of keratinocyte growth arrest and entry into differentiation." EMBO J **20**(13): 3427-3436.
Rehman, A. O. and C. Y. Wang (2006). "Notch signaling in the regulation of tumor angiogenesis." Trends Cell Biol **16**(6): 293-300.
Reis-Filho, J. S., B. Torio, et al. (2002). "p63 expression in normal skin and usual cutaneous carcinomas." J Cutan Pathol **29**(9): 517-523.
Ridgway, J., G. Zhang, et al. (2006). "Inhibition of Dll4 signalling inhibits tumour growth by deregulating angiogenesis." Nature **444**(7122): 1083-1087.
Riley, T., E. Sontag, et al. (2008). "Transcriptional control of human p53-regulated genes." Nat Rev Mol Cell Biol **9**(5): 402-412.
Robert-Moreno, A., L. Espinosa, et al. (2005). "RBPjkappa-dependent Notch function regulates Gata2 and is essential for the formation of intra-embryonic hematopoietic cells." Development **132**(5): 1117-1126.
Robinson, R. A., X. Lu, et al. (2008). "Biochemical and structural studies of ASPP proteins reveal differential binding to p53, p63, and p73." Structure **16**(2): 259-268.
Robles, A. I., N. A. Bemmels, et al. (2001). "APAF-1 is a transcriptional target of p53 in DNA damage-induced apoptosis." Cancer Res **61**(18): 6660-6664.
Rocco, J. W., C. O. Leong, et al. (2006). "p63 mediates survival in squamous cell carcinoma by suppression of p73-dependent apoptosis." Cancer Cell **9**(1): 45-56.
Roman-Gomez, J., A. Jimenez-Velasco, et al. (2005). "Lack of CpG island methylator phenotype defines a clinical subtype of T-cell acute lymphoblastic leukemia associated with good prognosis." J Clin Oncol **23**(28): 7043-7049.

Ronchini, C. and A. J. Capobianco (2001). "Induction of cyclin D1 transcription and CDK2 activity by Notch(ic): implication for cell cycle disruption in transformation by Notch(ic)." Mol Cell Biol **21**(17): 5925-5934.
Ross, D. A. and T. Kadesch (2004). "Consequences of Notch-mediated induction of Jagged1." Exp Cell Res **296**(2): 173-182.
Rotter, V. (1983). "p53, a transformation-related cellular-encoded protein, can be used as a biochemical marker for the detection of primary mouse tumor cells." Proc Natl Acad Sci U S A **80**(9): 2613-2617.
Rotter, V., D. Schwartz, et al. (1993). "Mice with reduced levels of p53 protein exhibit the testicular giant-cell degenerative syndrome." Proc Natl Acad Sci U S A **90**(19): 9075-9079.
Royds, J. A. and B. Iacopetta (2006). "p53 and disease: when the guardian angel fails." Cell Death Differ **13**(6): 1017-1026.
Royer, C. and X. Lu (2011). "Epithelial cell polarity: a major gatekeeper against cancer?" Cell Death Differ.
Ruptier, C., A. De Gasperis, et al. (2011). "TP63 P2 promoter functional analysis identifies beta-catenin as a key regulator of DeltaNp63 expression." Oncogene **30**(46): 4656-4665.
Ryan, K. M., A. C. Phillips, et al. (2001). "Regulation and function of the p53 tumor suppressor protein." Curr Opin Cell Biol **13**(3): 332-337.
Ryan, K. M. and K. H. Vousden (1998). "Characterization of structural p53 mutants which show selective defects in apoptosis but not cell cycle arrest." Mol Cell Biol **18**(7): 3692-3698.
Sachdev, S., A. Hoffmann, et al. (1998). "Nuclear localization of IkappaB alpha is mediated by the second ankyrin repeat: the IkappaB alpha ankyrin repeats define a novel class of cis-acting nuclear import sequences." Mol Cell Biol **18**(5): 2524-2534.
Sah, V. P., L. D. Attardi, et al. (1995). "A subset of p53-deficient embryos exhibit exencephaly." Nat Genet **10**(2): 175-180.
Saint Just Ribeiro, M., M. L. Hansson, et al. (2009). "GSK3beta is a negative regulator of the transcriptional coactivator MAML1." Nucleic Acids Res **37**(20): 6691-6700.
Sakamoto, M., H. Hirata, et al. (2003). "The basic helix-loop-helix genes Hesr1/Hey1 and Hesr2/Hey2 regulate maintenance of neural precursor cells in the brain." J Biol Chem **278**(45): 44808-44815.
Samuels-Lev, Y., D. J. O'Connor, et al. (2001). "ASPP proteins specifically stimulate the apoptotic function of p53." Mol Cell **8**(4): 781-794.
Santagata, S., F. Demichelis, et al. (2004). "JAGGED1 expression is associated with prostate cancer metastasis and recurrence." Cancer Res **64**(19): 6854-6857.
Sasaki, Y., S. Ishida, et al. (2002). "The p53 family member genes are involved in the Notch signal pathway." J Biol Chem **277**(1): 719-724.
Sayan, A. E., B. S. Sayan, et al. (2008). "P73 and caspase-cleaved p73 fragments localize to mitochondria and augment TRAIL-induced apoptosis." Oncogene **27**(31): 4363-4372.
Schmale, H. and C. Bamberger (1997). "A novel protein with strong homology to the tumor suppressor p53." Oncogene **15**(11): 1363-1367.
Schouwey, K., V. Delmas, et al. (2007). "Notch1 and Notch2 receptors influence progressive hair graying in a dose-dependent manner." Dev Dyn **236**(1): 282-289.
Schweisguth, F. (2004). "Regulation of notch signaling activity." Curr Biol **14**(3): R129-138.
Senoo, M., I. Tsuchiya, et al. (2001). "Transcriptional dysregulation of the p73L / p63 / p51 / p40 / KET gene in human squamous cell carcinomas: expression of Delta Np73L, a novel dominant-negative isoform, and loss of expression of the potential tumour suppressor p51." Br J Cancer **84**(9): 1235-1241.
Sestan, N., S. Artavanis-Tsakonas, et al. (1999). "Contact-dependent inhibition of cortical neurite growth mediated by notch signaling." Science **286**(5440): 741-746.
Sgroi, D. C., S. Teng, et al. (1999). "In vivo gene expression profile analysis of human breast cancer progression." Cancer Res **59**(22): 5656-5661.

Shahnavaz, S. A., J. A. Regezi, et al. (2000). "p53 gene mutations in sequential oral epithelial dysplasias and squamous cell carcinomas." J Pathol **190**(4): 417-422.
Sharma, V. M., J. A. Calvo, et al. (2006). "Notch1 contributes to mouse T-cell leukemia by directly inducing the expression of c-myc." Mol Cell Biol **26**(21): 8022-8031.
Shi, S. and P. Stanley (2003). "Protein O-fucosyltransferase 1 is an essential component of Notch signaling pathways." Proc Natl Acad Sci U S A **100**(9): 5234-5239.
Shimada, A., S. Kato, et al. (1999). "The transcriptional activities of p53 and its homologue p51/p63: similarities and differences." Cancer Res **59**(12): 2781-2786.
Slee, E. A., S. Gillotin, et al. (2004). "The N-terminus of a novel isoform of human iASPP is required for its cytoplasmic localization." Oncogene **23**(56): 9007-9016.
Soehnge, H., A. Ouhtit, et al. (1997). "Mechanisms of induction of skin cancer by UV radiation." Front Biosci **2**: d538-551.
Sottocornola, R., C. Royer, et al. (2010). "ASPP2 binds Par-3 and controls the polarity and proliferation of neural progenitors during CNS development." Dev Cell **19**(1): 126-137.
Soufir, N., J. P. Moles, et al. (1999). "P16 UV mutations in human skin epithelial tumors." Oncogene **18**(39): 5477-5481.
Srivastava, S., Z. Q. Zou, et al. (1990). "Germ-line transmission of a mutated p53 gene in a cancer-prone family with Li-Fraumeni syndrome." Nature **348**(6303): 747-749.
Stanbridge, E. J. (1990). "Human tumor suppressor genes." Annu Rev Genet **24**: 615-657.
Stiewe, T. (2007). "The p53 family in differentiation and tumorigenesis." Nat Rev Cancer **7**(3): 165-168.
Stransky, N., A. M. Egloff, et al. (2011). "The mutational landscape of head and neck squamous cell carcinoma." Science **333**(6046): 1157-1160.
Struhl, G. and A. Adachi (2000). "Requirements for presenilin-dependent cleavage of notch and other transmembrane proteins." Mol Cell **6**(3): 625-636.
Sullivan, A., N. Syed, et al. (2004). "Polymorphism in wild-type p53 modulates response to chemotherapy in vitro and in vivo." Oncogene **23**(19): 3328-3337.
Sun, W. T., P. C. Hsieh, et al. (2008). "p53 target DDA3 binds ASPP2 and inhibits its stimulation on p53-mediated BAX activation." Biochem Biophys Res Commun **376**(2): 395-398.
Swiatek, P. J., C. E. Lindsell, et al. (1994). "Notch1 is essential for postimplantation development in mice." Genes Dev **8**(6): 707-719.
Takahashi, N., S. Kobayashi, et al. (2004). "Expression of 53BP2 and ASPP2 proteins from TP53BP2 gene by alternative splicing." Biochem Biophys Res Commun **315**(2): 434-438.
Teodoro, J. G., A. E. Parker, et al. (2006). "p53-mediated inhibition of angiogenesis through up-regulation of a collagen prolyl hydroxylase." Science **313**(5789): 968-971.
Thelu, J., P. Rossio, et al. (2002). "Notch signalling is linked to epidermal cell differentiation level in basal cell carcinoma, psoriasis and wound healing." BMC Dermatol **2**: 7.
Thornton, J. K., C. Dalgleish, et al. (2006). "The tumour-suppressor protein ASPP1 is nuclear in human germ cells and can modulate ratios of CD44 exon V5 spliced isoforms in vivo." Oncogene **25**(22): 3104-3112.
Tidow, H., A. Andreeva, et al. (2007). "Solution structure of ASPP2 N-terminal domain (N-ASPP2) reveals a ubiquitin-like fold." J Mol Biol **371**(4): 948-958.
Tomasini, R., K. Tsuchihara, et al. (2008). "TAp73 knockout shows genomic instability with infertility and tumor suppressor functions." Genes Dev **22**(19): 2677-2691.
Uhlmann-Schiffler, H., S. Kiermayer, et al. (2009). "The DEAD box protein Ddx42p modulates the function of ASPP2, a stimulator of apoptosis." Oncogene **28**(20): 2065-2073.
Urist, M. J., C. J. Di Como, et al. (2002). "Loss of p63 expression is associated with tumor progression in bladder cancer." Am J Pathol **161**(4): 1199-1206.
Uyttendaele, H., A. A. Panteleyev, et al. (2004). "Activation of Notch1 in the hair follicle leads to cell-fate switch and Mohawk alopecia." Differentiation **72**(8): 396-409.

van Es, J. H., M. E. van Gijn, et al. (2005). "Notch/gamma-secretase inhibition turns proliferative cells in intestinal crypts and adenomas into goblet cells." Nature **435**(7044): 959-963.
van Kranen, H. J., A. Westerman, et al. (2005). "Dose-dependent effects of UVB-induced skin carcinogenesis in hairless p53 knockout mice." Mutat Res **571**(1-2): 81-90.
Varnum-Finney, B., L. Wu, et al. (2000). "Immobilization of Notch ligand, Delta-1, is required for induction of notch signaling." J Cell Sci **113 Pt 23**: 4313-4318.
Vitale-Cross, L., P. Amornphimoltham, et al. (2004). "Conditional expression of K-ras in an epithelial compartment that includes the stem cells is sufficient to promote squamous cell carcinogenesis." Cancer Res **64**(24): 8804-8807.
Vives, V., J. Su, et al. (2006). "ASPP2 is a haploinsufficient tumor suppressor that cooperates with p53 to suppress tumor growth." Genes Dev **20**(10): 1262-1267.
Vousden, K. H. and X. Lu (2002). "Live or let die: the cell's response to p53." Nat Rev Cancer **2**(8): 594-604.
Wager, M., J. Guilhot, et al. (2006). "Prognostic value of increase in transcript levels of Tp73 DeltaEx2-3 isoforms in low-grade glioma patients." Br J Cancer **95**(8): 1062-1069.
Wang, N. J., Z. Sanborn, et al. (2011). "Loss-of-function mutations in Notch receptors in cutaneous and lung squamous cell carcinoma." Proc Natl Acad Sci U S A **108**(43): 17761-17766.
Wang, T. Y., B. F. Chen, et al. (2001). "Histologic and immunophenotypic classification of cervical carcinomas by expression of the p53 homologue p63: a study of 250 cases." Hum Pathol **32**(5): 479-486.
Wei, C. L., Q. Wu, et al. (2006). "A global map of p53 transcription-factor binding sites in the human genome." Cell **124**(1): 207-219.
Weinmaster, G. and C. Kintner (2003). "Modulation of notch signaling during somitogenesis." Annu Rev Cell Dev Biol **19**: 367-395.
Weng, A. P., A. A. Ferrando, et al. (2004). "Activating mutations of NOTCH1 in human T cell acute lymphoblastic leukemia." Science **306**(5694): 269-271.
Weng, A. P., J. M. Millholland, et al. (2006). "c-Myc is an important direct target of Notch1 in T-cell acute lymphoblastic leukemia/lymphoma." Genes Dev **20**(15): 2096-2109.
Westfall, M. D., D. J. Mays, et al. (2003). "The Delta Np63 alpha phosphoprotein binds the p21 and 14-3-3 sigma promoters in vivo and has transcriptional repressor activity that is reduced by Hay-Wells syndrome-derived mutations." Mol Cell Biol **23**(7): 2264-2276.
Wu, G. S., T. F. Burns, et al. (1997). "KILLER/DR5 is a DNA damage-inducible p53-regulated death receptor gene." Nat Genet **17**(2): 141-143.
Wu, L., J. C. Aster, et al. (2000). "MAML1, a human homologue of Drosophila mastermind, is a transcriptional co-activator for NOTCH receptors." Nat Genet **26**(4): 484-489.
Yamaguchi, K., L. Wu, et al. (2000). "Frequent gain of the p40/p51/p63 gene locus in primary head and neck squamous cell carcinoma." Int J Cancer **86**(5): 684-689.
Yamamoto, N., K. Tanigaki, et al. (2003). "Notch/RBP-J signaling regulates epidermis/hair fate determination of hair follicular stem cells." Curr Biol **13**(4): 333-338.
Yang, A., M. Kaghad, et al. (1998). "p63, a p53 homolog at 3q27-29, encodes multiple products with transactivating, death-inducing, and dominant-negative activities." Mol Cell **2**(3): 305-316.
Yang, A., R. Schweitzer, et al. (1999). "p63 is essential for regenerative proliferation in limb, craniofacial and epithelial development." Nature **398**(6729): 714-718.
Yang, A., N. Walker, et al. (2000). "p73-deficient mice have neurological, pheromonal and inflammatory defects but lack spontaneous tumours." Nature **404**(6773): 99-103.
Yang, J. P., M. Hori, et al. (1999). "Identification of a novel inhibitor of nuclear factor-kappaB, RelA-associated inhibitor." J Biol Chem **274**(22): 15662-15670.
Yu, J., L. Zhang, et al. (2001). "PUMA induces the rapid apoptosis of colorectal cancer cells." Mol Cell **7**(3): 673-682.

Yugawa, T., M. Narisawa-Saito, et al. (2010). "DeltaNp63alpha repression of the Notch1 gene supports the proliferative capacity of normal human keratinocytes and cervical cancer cells." Cancer Res **70**(10): 4034-4044.

Zeng, Q., S. Li, et al. (2005). "Crosstalk between tumor and endothelial cells promotes tumor angiogenesis by MAPK activation of Notch signaling." Cancer Cell **8**(1): 13-23.

Zhan, Q., M. J. Antinore, et al. (1999). "Association with Cdc2 and inhibition of Cdc2/Cyclin B1 kinase activity by the p53-regulated protein Gadd45." Oncogene **18**(18): 2892-2900.

Zhang, B., H. J. Xiao, et al. (2011). "Inhibitory member of the apoptosis-stimulating protein of p53 (ASPP) family promotes growth and tumorigenesis in human p53-deficient prostate cancer cells." Prostate Cancer Prostatic Dis.

Zhang, X., M. Wang, et al. (2005). "The expression of iASPP in acute leukemias." Leuk Res **29**(2): 179-183.

Zhao, J., G. Wu, et al. (2010). "Epigenetic silence of ankyrin-repeat-containing, SH3-domain-containing, and proline-rich-region- containing protein 1 (ASPP1) and ASPP2 genes promotes tumor growth in hepatitis B virus-positive hepatocellular carcinoma." Hepatology **51**(1): 142-153.

Zhu, A. J. and F. M. Watt (1999). "beta-catenin signalling modulates proliferative potential of human epidermal keratinocytes independently of intercellular adhesion." Development **126**(10): 2285-2298.

Zhu, Z., J. Ramos, et al. (2005). "Control of ASPP2/(53BP2L) protein levels by proteasomal degradation modulates p53 apoptotic function." J Biol Chem **280**(41): 34473-34480.

Ziegler, A., A. S. Jonason, et al. (1994). "Sunburn and p53 in the onset of skin cancer." Nature **372**(6508): 773-776.