Universidade de Aveiro Departamento de Química
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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biotecnologia Molecular, realizada sob a orientação científica da Doutora Maria do Céu Gomes dos Santos, Professora auxiliar convidada do Departamento de Biologia da Universidade de Aveiro e co-orientação da Doutora Xin Lu, Diretora do Ludwig Institute for Cancer Research da Universidade de Oxford.

À memória dos nossos papás

o júri

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palavras-chave tumurigénese, ASPP2, família p53, cancro epitelial

resumo

Na pele surgem dois dos tipos mais comuns de cancro epitelial, o carcinoma basocelular (BCC) e o carcinoma escamoso da pele (SCC). Neste trabalho, investigámos como ASPP2, membro da família de proteínas que interage com a família p53, pode afectar a tumurigénese da pele. Estudou-se a regulação por ASPP2 das vias de sinalização envolvidas na homeostasia normal do tecido epitelial, tais como as vias de p63 e Notch. A activação anormal de ΔNp63 no epitélio é uma causa conhecida para o surgimento do SCC e os nossos resultados indicam que a ASPP2 é importante a limitar a expressão de ΔNp63 no epitélio diferenciado, prevenindo a proliferação das células na pele. Para além disso, observámos que ASPP2 coopera com vias de sinalização pró-diferenciação, tais como as de Notch e p73. Os nossos resultados mostram um possível mecanismo pelo qual a expressão de p63 pode ser regulada na pele e sugerem um novo modelo para a formação espontânea de SCC.

keywords tumourigenesis, ASPP2, p53 family, skin cancer

abstract The skin is 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. We analysed the regulation of ASPP2 in pathways involved in the normal homeostasis of the epithelium, such as the p63 and Notch. Aberrant or misplaced activation of ΔNp63 in the epithelium is a known initiating cause for SCC and our results indicate that ASPP2 is important in limiting ΔNp63 expression in the differentiated epithelium, preventing cell proliferation in the skin. Additionally, we found that ASPP2 can cooperate with skin pro-differentiation pathways, such as Notch and p73. 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.

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List of Abbreviations

Ab – antibody

Acryl/Bis – acrylamide/bis-acrylamide

AK – actinic keratosis

Ank – ankyrin repeats

APS – ammonium persulphate

ASPP – ankyrin repeats, SH3 domain and proline-rich region containing-protein

ASPP – apoptosis stimulating protein of p53

ATCC – American type culture collection

BM – basal membrane

BL – basal layer

BSA – bovine serum albumin

BSC – basal cell carcinoma

ChIP – chromatin immunoprecipitation

cSCC – cutaneous squamous cell carcinoma

TA – acidic transactivation domain

CNS – central nervous system

DAB – diaminobenzidine

DBD – DNA-binding domain

DMEM – Dulbecco's Modified Eagle's Medium

EBSS – Earlie's balanced salts solution

EMEM – Eagle's minimum essential medium

FBS – fetal calf serum

FSG – fish skin gelatin

GL – granular layer

H&E – hematoxylin and eosin

HRP – horse-radish peroxidase

iASPP – inhibitory apoptosis stimulating protein of p53

IF – immunofluorescence

IHC – immunohistochemiistry

IP – immunoprecipitation

IVL – involucrin

K1 – keratin-1

K14 – keratin-14

mAb – monoclonal antibody

NICD – notch intracellular domain

OD – oligomerisation domain

P – post-natal day

pAb – polyclonal antibody

PRO – proline-rich region

SAM – sterile alpha motif

SC – stratum corneum

SCC – squamous cell carcinoma

SDS – sodium dodecyl sulphate

SL – spinous layer

TAE – tris-acetate-EDTA

TEMED – N,N,N',N',-tetramethy-ethylenediamine

TP53 – p53 gene

WB – Western blot

Others abbreviations will be explained along the text.

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Thesis outline

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, and is exclusively to the upper and most differentiated layers. To investigate the observation that the absence of ASPP2 from the epithelial compartment leads 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 Squamous cell carcinoma (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. 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 contrast to p63, ASPP2 and Notch are co-expressed in the differentiated layers of the squamous epithelium. Moreover, ASPP2 can interact with components of Notch nuclear transcriptional machinery, and can regulate communal target genes of both Notch and p73 proteins.

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 the capacity of undergoing uncontrolled cell division and invading the surrounding tissues (Hanahan and Weinberg, 2011). 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 to new locations of the body and give rise to new tumour foci. These secondary tumours are called metastases and the presence of distant metastases is the main cause of mortality in cancer patients (Duffy, McGowan *et al.*, 2008).

The crucial changes in cell behaviour which characterize tumour cells have been classified in six main categories, considered the hallmarks of cancer cells, and include 1) self-sufficiency in growth signals, 2) insensitivity to anti-growth signals, 3) limitless replicative potential, 4) evasion of apoptosis, 5) sustained angiogenesis and 6) 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 (Hanahan and Weinberg, 2011).

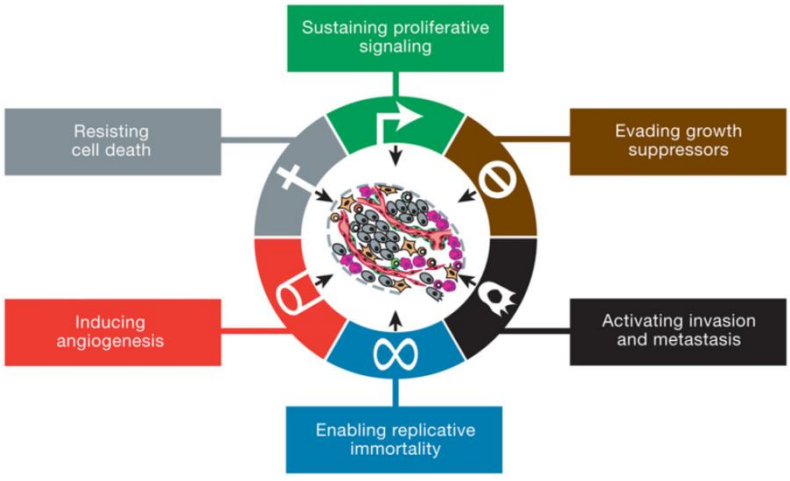


Figure 1 – The hallmarks of cancer - distinctive and complementary capabilities that enable tumor growth and metastatic dissemination (Hanahan and Weinberg, 2011).

The transition from a normal cell to a tumour phenotype is caused by cumulative genetic alterations. These alterations, ascribable to DNA-mutations, can be inherited through parental genes (familiar or hereditary cancer) or can arise *de novo* (non-inherited cancer). Gene mutations in cells result in tumour initiation when these events target genes which are crucially involved in controlling some of 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 crucial growth regulators in normal cell division and in cancer are subjected to activating mutations, becoming the so called "oncogenes". On the contrary, the tumour suppressor genes, which normally act as negative growth regulators, are inactivated by mutations in cancer (Zhao and Epstein, 2011). Oncogenes are characterised by a dominant phenotype, defined as "gain of function". This means that activating mutations in only one allele can result in alterations of cellular behaviour. Whereas, tumour suppressor genes usually require inactivating mutations targeting both alleles in order to produce phenotypic changes in the cell ("loss of function" phenotype) (Croce, 2008).

1.2 The p53 family of proteins

The p53 gene (TP53) is the most well-known and one of the best studied genes in the human genome and its popularity is primarily due its role as a major tumour suppressor (reviewed by 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 plays a pivotal role in controlling cell growth through its ability to induce cell cycle arrest or apoptosis in response to multiple stress signals (Bensaad and Vousden, 2007).

p53 is the most frequently mutated gene in human cancer (Efeyan and Serrano, 2007) and it's now recognised that in about 50% of all human tumours a key step for the development the malignancies is the direct inactivation of p53, via mutations, deletions or interactions with viral proteins. In the remaining half of the malignancies, p53 pathway is deregulated by inactivation of p53 activators and/or downstream targets or activation of its inhibitors. The importance for cancer cells to inactivate p53 comes from its pivotal role in blocking 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 (Lowe, 1999; Crighton, Wilkinson *et al*., 2006). These functions make p53 probably the most important controller of genomic integrity

(Ryan, 2011), referred to as "guardian of the genome" (Lane, 1992). During the past decade numerous studies have further extended p53 functions, showing its involvement in processes such as autophagy, oxidative stress, regulation of metabolism, embryo implantation and angiogenesis (Teodoro, Parker *et al*., 2006; Hu, Feng *et al*., 2007).

Almost twenty years after p53 was first described, two members of the p53 family of proteins, p63 and p73, were discovered (Kaghad, Bonnet *et al*., 1997; Yang, Kaghad *et al*., 1998). These two homologues share high sequence identity with p53, which is reflected in some redundant functions in the gene expression regulation (Bergamaschi, Gasco *et al*., 2003). Despite all these similarities, p63 and p73 can be activated by distinct mechanisms and implicated in several p53-independent pathways, playing an important role in processes such development and differentiation (Levrero, De Laurenzi *et al*., 2000).

1.2.1 Isoforms of the p53 family members

The three family members (p53, p63 and p73) 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 2).

The highest sequence identity among the three family members resides in the DBD 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 97% of all the tumour-associated p53 mutations can be found (Stiewe, 2007). The oligomerisation domain of p53 can be post-translationally modified in different ways, including phosphorylation, methylation, ubiquitynation, 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 (Murray-Zmijewski *et al*., 2006) Moreover, all the three genes can encode two primary transcripts due to the presence of two distinct promoters in their sequence (P1 and P2). P1 promoter gives origin to full proteins, containing the TA, DBD and OD domains (referred to as FLp53, TAp63 and TAp73). Alternatively, when the transcription is initiated from the intronic P2 promoter, N-terminally truncated proteins are produced (referred to as Δ133p53, ΔNp63, ΔNp73).

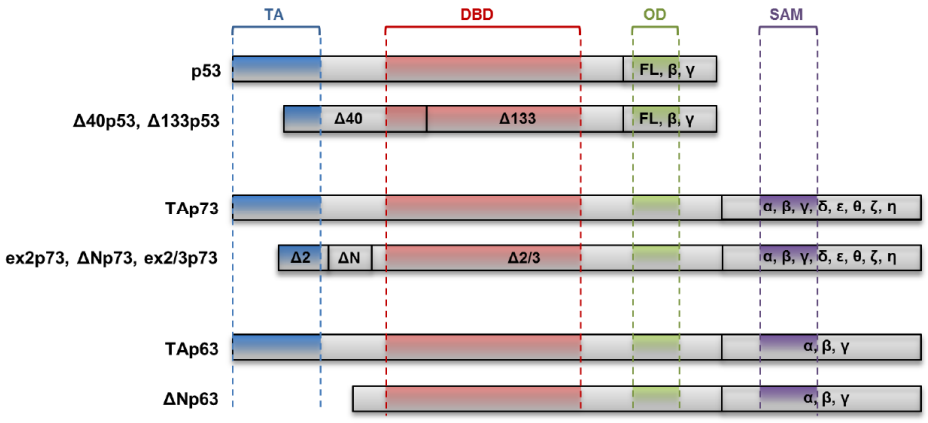


Figure 2 – Structure of the p53 family of proteins (Stiewe, 2007)**.**

In both p53 and p73, additional ΔN variants can be generated by splicing events or alternative initiation of translation (Δ40p53, Δex2p73, Δex2/3p73 and ΔN'p73). Further complexity is added by the fact that the P1 and P2 transcripts can be spliced at the C-terminus. In total, concerning p53 and p63, for each of their TA and ΔN isoforms, three variants can be generated. For p73, a total of eight different isoforms have been identified from the P1 and P2 promoters (Stiewe, 2007).

1.2.2 The p53 family is a key mediator of the apoptotic 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, which can be translated in different patterns of post-translational modifications on p53 protein, with consequent recruitment of characteristic binding partners and induction of a precise combination of target genes (Green and Kroemer, 2009). As a result of this multi-step process, p53 can facilitate the transient adaptation of cells to stressful conditions (summarized in Figure 3).

The critical event during activation of p53 pathway is the stabilization and accumulation of its protein levels. In normal, unstressed conditions, p53 protein level is 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 ubiquitination 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 (Linares, Hengstermann *et al.*, 2003; Lavin and Gueven, 2006).

p53, as a transcription factor, can recognize consensus motifs which are present in the promoters of numerous genes (Green and Kroemer, 2009). Affinity for p53 binding can however vary among all the p53 binding sites. Thus, there are genes whose promoters contain high-affinity p53-binding sites, like p21, MDM2 and PUMA and others with low-affinity sites, as for example Bax (Braithwaite, Del Sal *et al*., 2006).

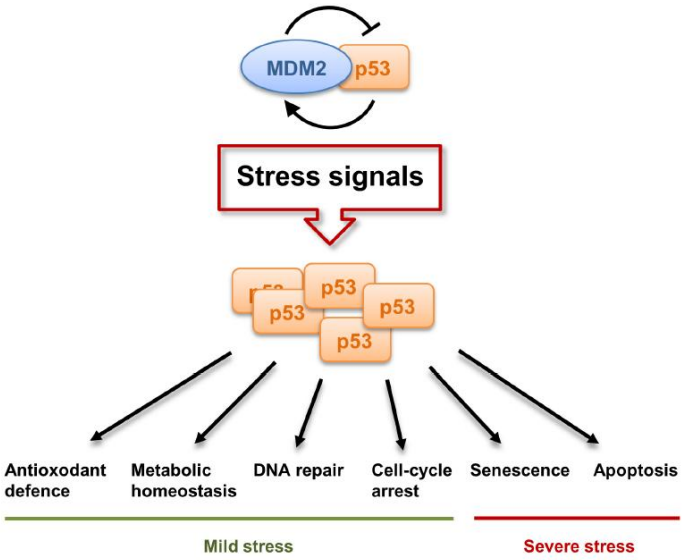
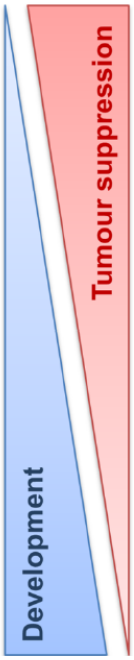


Figure 3 – Simplistic diagram exemplifying the p53 response.
In normal physiological conditions the levels of p53 are maintained low by the p53-MDM2 feedback loop. The release of MDM2 inhibition caused by various stress signals leads to an increase and stabilization of p53 protein levels. The activity 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 The p53 family members in tumourigenesis, development and differentiation

Differentiation is a physiological process which allows cells to switch off a proliferative program and, at the same time, preserving their cellular integrity and function. As the p53 proteins are master regulators in mediating termination of cell proliferation, as well as induction of apoptosis, senescence and cell-cycle arrest, their role in differentiation and development has thoroughly been investigated. Recent evidences have supported the idea that p53/p63/p73 can be activated also by stimuli other than stress signals, regulating 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 appears to have unique roles in mouse and human development (see summary in Table 1).

Table 1 – Summary of the phenotypical characteristics of p53-, p73- and p63-null mice, underlining the differences in tumour predisposition and developmental defects.



Genotype	Phenotype	
	Tumour predisposition	Developmental defects
TP53$^{-/-}$	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
TP73$^{-/-}$	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
TP63$^{-/-}$		Neonatal death due to dehydration caused by lack of epidermis and other squamous epithelia. Absence of limbs, urothelium and secretory epithelia.

1.2.3.1 p53

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 p53 gene can be found in half of all human tumours, with 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 inactivation of the endogenous wild type p53, as well as through oncogenic gain-of-function activities (Brosh and Rotter, 2009). p53 ability to induce apoptosis still represents the key way by which p53 acts as a tumour suppressor (Cicalese, Bonizzi *et al.* 2009).

The observation that p53 negatively regulates the proliferation and survival of both adult and embryonic neural stem cells points to a role for p53 in controlling stem cell function (Armesilla-Diaz *et al.*, 2009). Many studies have demonstrated that expression of p53 in undifferentiated cells can promote differentiation (Lin *et al.*, 2005) suggesting that p53 not only controls cell renewal, but also cell fate.

Studies conducted in p53-deficient mice have shown that p53 is required for normal embryonic development (Armstrong *et al.*, 1995; Meletis *et al.*, 2006). *In utero* exposure of

p53-null embryos to ionizing radiation as also shown that p53 has a role in reducing the rate of birth defects (Norimura, Nomoto *et al*., 1996). p53-mediated apoptosis in response to DNA damage is the mechanism causing the mortality in irradiated wild-type embryos, leading to their early death and preventing birth of individuals with congenital abnormalities. Other studies also shown 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).

1.2.3.2 p63 and p73

Although rarely mutated, p63 and p73 are abnormally expressed in human cancers (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 head and neck squamous cell carcinoma. Similarly, aberrant expression of ΔNp73 was detected in gliomas as well as colon carcinoma (Dominguez, Garcia *et al*., 2006). In contrast, TAp63 and TAp73 tend to be lost or down-regulated in human tumours (Urist, Di Como *et al*., 2002). 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 suppressor functions, as p53.

Genetic experiments on mice have shown that p63 and p73 have a more profound effect on normal development than p53, and p63 deletion has the most striking effect. 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; Candi *et al*., 2006). Functional studies have shown that p63 and its ΔN isoforms in particular are fundamental for the maintenance of the regenerative cell population and therefore the capacity of the epithelium to develop and differentiate (Candi *et al*., 2006).

p73-/- mice have also developmental defects, but they survive postnatally and make it 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 and additional *in vitro* studies showed that p73 expression (TAp73 isoform) in neuroblastoma (undifferentiated neuronal cells) can induce neuronal differentiation

markers. 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). Altogether these experiments confirmed p73 is deeply involved in neuronal differentiation, as p63 is for epithelial differentiation.

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

1.3.1 Overview

The ASPP (Apoptosis Stimulating Protein of p53) proteins have been identified and subsequently characterized as an important regulator of the p53 family members. Interactions with other proteins, as well as post-translational modifications are 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 the acronym ASPP suggests, the ASPP proteins specifically promote p53-dependent apoptosis, but not cell cycle arrest (Takahashi, Kobayashi *et al*. 2004).

The ASPP family consists of three members, ASPP1, ASPP2 and iASPP (inhibitory ASPP). ASPP2 was the first of the family members to be characterized and it was originally identified as an interactor of p53 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. The full-length of ASPP2 however, was only recognised 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, 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), with its size of 828 amino acids (Slee, Gillotin *et al*., 2004). The correspondent protein in *Caenorhabditis elegans* was found to be able 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 Family 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. The family name ASPP also stands for **A**nkyrin repeats, **S**H3 domain and **P**roline-rich region containing-**P**rotein, because of the presence of these three domains at the C-terminal of all the family members (Figure 4). The C-terminal is the most conserved region among the three proteins and the N-terminal is only conserved in ASPP1 and ASPP2 and seems to have a role in determining the cellular localization of the two proteins (Yang, Hori *et al*. 1999). ASPP1 and ASPP2 are, in fact, 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*. ASPP1 and ASPP2, which can be found in mammals, are believed to be derived from iASPP through speciation (Bergamaschi, Samuels *et al*., 2003).

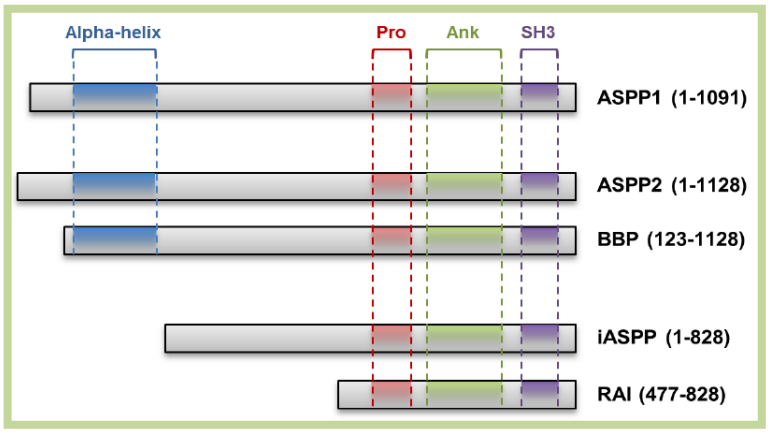


Figure 4 – Structure of the ASPP family members.
The ASPP family of protein consists of three family members: ASPP1, ASPP2 and iASPP. ASPP2 and iASPP are shown with their splice variants, respectively named BBP (Bcl-2 binding protein) and RAI (RelA/p65 inhibitor). The figure shows the length of the proteins and the common structural elements, highlighted with different colours Pro stands for proline-rich region, Ank for ankyrin repeats and SH3 for SH3 domain (Notari, 2011).

1.3.3 Functions of the ASPP family

The best characterised function of the ASPPs is their ability to regulate apoptosis 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).

ASPP1 and ASPP2 mode of action consists in 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 genes, such as CDKN1A or MDM2 (Samuels-Lev, O'Connor *et al*., 2001; Bergamaschi, Samuels *et al*., 2004). ASPP1 and ASPP2 have been found in complex 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 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 (Ahn, Byeon *et al*., 2009).

Additionally to p53/p63/p73, already mentioned, other ASPP binding partner have been identified, such as protein phosphatase 1 (PP1) (Helps, Barker *et al*., 1995), 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), Par3 (Sottocornola, Royer *et al*., 2010) or *Helicobacter pylori* cytotoxin-associated gene A (CagA) (Buti, Spooner *et al*., 2011). 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's natural to think that the ASPPs can have multiple roles in the cell, other than inducing apoptosis, showing stimulating new prospective studies of this family.

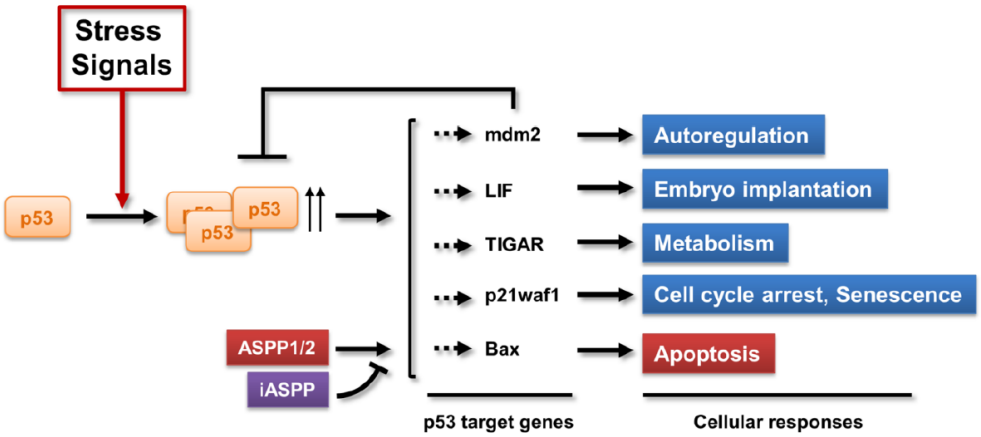


Figure 5 – The ASPP proteins confer selectivity to the p53-stress response.
Stress signals lead to stabilisation and activation of p53 protein, which can now direct different cellular responses by inducing the transcription of several different target genes. The same type of stress signals can also lead 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 trans-repressor of the same genes trans-activated by ASPP1/2, allowing cell proliferation to occur (Tordella, L., 2011).

1.3.4 Role of ASPP family in tumourigenesis

Given the great importance of the ASPP proteins in regulating the apoptotic process, it is logical to expect they can 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 have shown that ASPP1/2 are tumour suppressors and iASPP is a proto-oncogene. One of the major contributions to support a role of ASPP2 in tumour suppression came from our laboratory, with a study on the ASPP2 knock-out mouse model (Vives, Su *et al*., 2006). ASPP2 heterozygous mice were viable but found to have higher incidence in tumour formation, both spontaneous and induced by ionising radiations when compared to wild-type mice. Consistent with the role of ASPP2 in regulating p53-mediated apoptosis, a combination of p53 and ASPP2 heterozygosity accelerated the onset of tumour development (Vives, Su *et al*., 2006).

Another ASPP2 knock-out mouse has been recently generated by another laboratory and the subsequent tumour study showed overlapping results (Kampa, Acoba *et al*., 2009), confirming 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 that ASPP2 behaves like a haploinsufficient tumour suppressor gene. This is supported by the fact

that ASPP2 has been found to be down-regulated in human tumours, but has not been found deleted or mutated (Liu, Lu *et al*., 2005).

Finally, crystal structure analysis of the ASPPs bound with p53, added further insights into the relevance of these proteins in tumourigenesis. One important finding was 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). These analysis suggested that the disruption of ASPP2 binding to p53, with its consequent apoptotic-stimulatory effect, can be one way by which tumours inactivate p53 tumour suppressor function.

1.3.5 Role in normal 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 displayed 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 other identified binding partners, concerning functions that go beyond the regulation of apoptosis (Samuels-Lev, O'Connor *et al*., 2001).

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). 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, as well as number and orientation of the hair follicles (Notari, Hu *et al*., 2011). Our group also showed that in the skin, iASPP cooperates with p63 for the maintenance of the homeostasis of the stratified epithelium (Notari, Hu *et al*., 2011).

1.4 The Notch pathway

1.4.1 Overview

Notch signalling pathway is evolutionary conserved from *Drosophila* until human and it is involved in many fundamental cellular processes during development of the organism, as well as 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 (like Alagille syndrome, Tetralogy of Fallot, syndactyly, spondylcostal dysostosis, familiar aortic valve disease) (Gridley, 2003; Garg, Muth *et al*., 2005), diseases in adulthood (like CADASIL) (Louvi, Arboleda-Velasquez *et al*., 2006) and cancer (Weng, Ferrando *et al*., 2004; Ranganathan, Weaver *et al*., 2011).

1.4.2 Role in development and differentiation

Activation of Notch signalling results from the 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 neighboring cells with different properties.

Notch is a pleiotropic pathway, whose final output is highly context 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 posttranslational 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. Several experimental evidences agreed in defining Notch as a factor important for the maintenance of the stem cell properties. 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 (Rangarajan, Talora *et al*., 2001).

1.4.3 Role in tumourigenesis

Despite the major role played by deregulated Notch pathway activity in solid tumours, few genetic alterations have been reported in Notch genes. This could be explained with 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, cross-talk with other pathways, as the cells here are in touch with each other. Any abnormal variation in these factors can lead to inappropriate activation or inhibition of Notch pathway and cause tumour transformation (Miyamoto, Maitra *et al*., 2003; Santagata, Demichelis *et al*., 2004; Brennan, Momota *et al*., 2009).

In contrast with what was described so far, in tissues where Notch function is associated with induction of differentiation and growth suppression, Notch behaves as a tumour suppressor and inactivation of its activity can lead to tumour formation. This is the case of the skin, where Notch activity is physiologically important to promote terminal differentiation of the keratinocytes in various ways (Radtke and Raj, 2003). *In vitro* experiments conducted with keratinocytes, alongside with Notch defective mouse models, confirmed that the down-regulation of Notch signalling promotes skin tumourigenesis (Koch and Radtke, 2007).

Interestingly, in tumours in which Notch signalling is involved, its deregulation seems to be the consequence of the cross-talk with alerted pathways, which are physiologically important for the normal homeostasis of a given tissue. This suggests that 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. Alterations in such tissue-specific factors can directly affect Notch behaviour, providing the required contribution for tumour transformation (Ridgway, Zhang *et al*., 2006).

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 by two compartments, an inner one called dermis and a more superficial one

called 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 by proteins secreted by cells belonging to both epidermal and dermal compartment. The epidermis, as uppermost compartment of the skin, has the role of providing a physical and permeable barrier for the body, protecting the organism from dehydration, mechanical or bacterial harms. What makes the epidermis an efficient barrier is the nature of its composition: a multi-layered compartment. Keratinocyte cells are the "bricks" which compose such structure (Koster and Roop, 2004; Koster and Roop, 2007)

The keratinocytes in the epidermis are organised as a stratified squamous epithelium. The stratification of the epidermis consists in a proliferative active basal layer, surmounted by progressively more differentiated supra-basal layers, named spinous, granular and cornified (Figure 6). New keratinocytes are originated by mitosis in the basal layer and then they move upwards throughout the layers, undergoing transcriptional and morphological changes during the transit. As the keratinocytes move up, they flatten and lose their proliferative potential, committing themselves to terminal differentiation by the expression of various forms of keratins (in a process known as keratinisation) until they eventually die when they reach the surface and they are sloughed off in process named desquamation. Lost dead cells are constantly replaced by new keratinocytes originated from asymmetric cell divisions of the stem cells resident the basal layer. These undifferentiated cells which have the ability of self-renewal, play a fundamental role in skin homeostasis and repair (Blanpain and Fuchs, 2009). The overall epithelial stratification can be noticed by looking at the morphological diversity of each layer, which is associated to changes in the expression of keratins and others molecular markers (Koster and Roop, 2004).

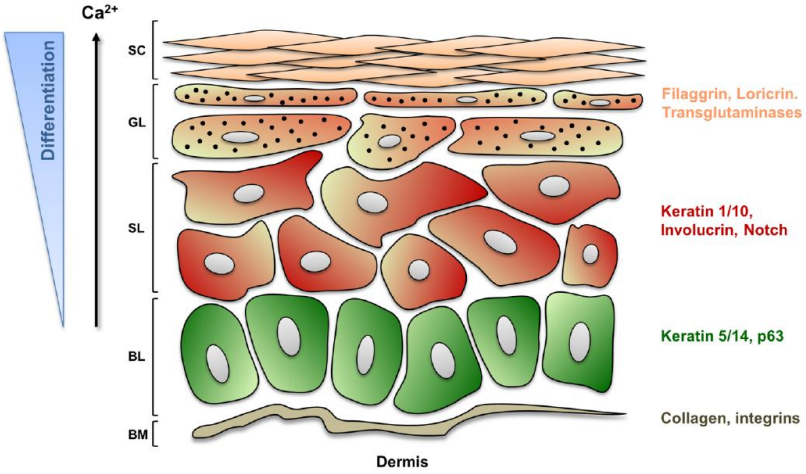


Figure 6 – Model for epidermal stratification.
The layers composing the multi-stratified squamous epithelium of the skin with their characteristic protein markers are shown. The names of the layers are abbreviated as: BM, basal membrane; BL, basal layer; SL, spinous layer; GL, granular layer; SC, stratum corneum. The calcium concentration, which goes increasing throughout the layers of the stratified epithelium, is an index of progression of cell differentiation.

Keratins are intermediate filaments 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 spinuos cell compartment they switch to expression of K10 and K1(Koster and Roop, 2007). As the terminal differentiation of the keratinocytes proceed to the granular layer, protein kinase C (PKC) activity is important to repress the expression of the keratins 1 and 10. Ca^{2+} is a fundamental trigger in promoting epidermal differentiation and its concentration increases (as you go up) in the layers. Ca^{2+} in fact, not only regulates PKC activity, but also promotes cell-cell contacts formation, which is a hallmark of differentiated keratinocytes (Koster and Roop, 2007).

1.5.2 Pathways involved in epithelial differentiation

Whereas the morphological changes that occur during epidermal development have been extensively studied, the molecular mechanisms that govern this process still remain poorly understood. The main known pathways involved in skin differentiation and homeostasis 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 a reciprocal antagonistic activity in the upper versus lower layer, of p63 and Notch proteins (Koster and Roop, 2007) (Figure 7).

1.5.2.1 Role of Notch signaling in epithelial differentiation

Notch complex is one of the main pathways 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 Notch function promotes carcinogenesis (Talora *et al.*, 2002; Nicolas *et al.*, 2003).

Additionally, Notch can directly promote the expression of epithelial differentiation markers, such as keratin-1 and involucrin, and can down-regulate integrins expression (Rangarajan, Talora *et al*., 2001) (Figure 7). Other works showed the existence of a direct negative cross-talk between Notch and p63, which regulates the balance between proliferation and differentiation (Figure 7). A work by Nguyen and colleagues (2006) showed that Notch 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). Indirect evidences showed that mouse epidermis lacking of p63 present defective expression of Notch and, conversely, activation of p53 in human keratinocytes leads to Notch expression (Laurikkala, 2006; DiRenzo, 2003). Despite these findings, convincing evidences for a direct transcriptional regulation on Notch promoter by p53 and p63 are still missing.

1.5.2.2 Role of p63 signaling 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 of p63. Indeed, $p63^{-/-}$ mice have dramatic defects in the epidermis and all the epithelial appendages. The epidermal stratification does not take place in these mice, which are deprived of any skin stratification and die for dehydration early after birth (Mills *et al*., 1999; 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 work by Candi and co-workers

(2006) tried to address this problematic, by re-introducing single p63 isoforms into a $p63^{-/-}$ background and the result was 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 indicates that TAp63 probably just contributes in promoting 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 and it is strongly down-modulated with cell differentiation (Laurikkala, Mikkola *et al*., 2006). In the adult epithelia its general role is to promote proliferation of the keratinocytes, allowing stratification to occur in development and guaranteeing skin renewal and this isoform still represent the most abundant p63 isoform in the epidermis (99% versus 1% of TAp63) (Candi, 2007; Koster and Roop, 2007).

At molecular level, this is achieved in several ways. For example, ΔNp63 can directly promote the expression of the basal keratins K5 and K14 (Candi, 2006; Romano, 2007) or the isoform can affect Notch signaling pathway at different levels, as counteracting Notch effect on the expression of some of its target genes or suppressing Hes1 and $p21^{WAF1}$ expression, whilst promote integrin receptors (Nguyen, Lefort *et al*., 2006; Okuyama, Ogawa *et al*., 2007).

Conversely, ΔNp63 can also synergise with 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 7). Additionally, p63 has been reported to be capable of binding directly the Notch promoter, although little evidence of any transcriptional effect has been reported so far in keratinocytes (Laurikkala, Mikkola *et al*., 2006).

Regarding its own gene expression, despite reports showing activating effects by p53, STAT3, Beta-catenin or ΔNp63 itself (Lanza, Marinari *et al*., 2006; Chu, Dai *et al*., 2008; Ruptier, De Gasperis *et al*., 2011), the transcriptional modulation of ΔNp63 is, to date, largely unknown.

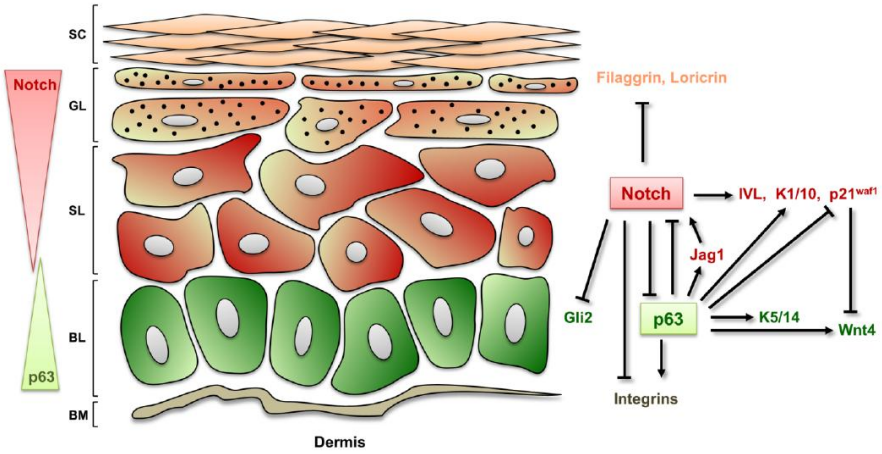


Figure 7 – Interplay between Notch and p63 in regulating epithelial stratification.
Cross-talk between p63 and Notch pathway, whose gradients of expression proceed in opposite direction throughout the stratified epithelium (see left side of the figure), is fundamental for maintaining the correct balance between self-renewing and terminal differentiated keratinocytes populations. BM, basal membrane; BL, basal layer; SL, spinous layer; GL, granular layer; SC, stratum corneum. (Tordella L, 2012).

1.6 Epithelial cancer

1.6.1 Overview

Cancer is a disease mainly of epithelial cells; in fact, around 80% of human tumours derive from epithelial cells. Such tumours are called carcinomas and have the capability of invading 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 (SCC), two common descriptive terms for epithelial tumours, reflect the fact that these cells may have glandular or squamous cell appearances respectively. 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; Proweller, Tu *et al*., 2006).

1.6.2 Squamous cell carcinoma

Neoplasms originating from cutaneous epithelial cells, including adenocarcinoma, Basal Cell Carcinoma (BSC) and Squamous Cell Carcinoma (SCC), are the most common among the carcinomas. Cutaneous squamous cell carcinoma (cSCC) is the second most common human cancer with over 250,000 new cases annually in the US and is second in incidence only to basal cell carcinoma. cSCC typically manifests as a spectrum of progressively advanced

malignancies, ranging from a precursor actinic keratosis (AK) to squamous cell carcinoma (SCC) and finally metastatic SCC (Ratushny, Vladimir *et al*., 2012).

Squamous cell carcinoma 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, and in these organs, it always arise from the keratinising cells of the squamous epithelium (Czarnecki, Staples *et al*., 1994; Bradford, Porcia T. *et al*., 2010).

Unlike most BCCs, SCCs of the skin are associated with a risk of metastasis. In fact, of the three, SCC is the most malignant tumour type with the highest metastatic potential and refractory to treatment. SCCs can arise *de novo* or from precursor lesions (Salehi, Z. *et al*., 2007), and it displays metastatic potential and furthermore, 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 (Wato, Kiyomi *et al*., 2012).

1.6.2.1 Etiology

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. When UV induced mutations occur in the "guardian of the genome" *p53* gene, altering its capacity to induce apoptosis and cell-cycle arrest, there are high chances to develop BCC and SCC. Mutations in *TPp53* are, in fact, detected in about 56% of BCC and 90% of SCC of the skin (van Kranen, Westerman *et al*., 2005; Efeyan and Serrano, 2007).

Experimental observations suggested that p53 inactivation may not be a critical rate-limiting step in SCC formation, but mainly important for the progression of the malignancy and, additionally, for causing susceptibility in the case of the radiation-induced SCCs. Interestingly, what seems 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-regulation of the Notch pathway (Lefort, Mandinova *et al*., 2007).

1.6.2.1.1 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 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, mouse models carrying disruption in Notch signalling pathway led to epidermal abnormalities and spontaneous formations of SCC or BCC (Nicolas, Wolfer *et al*., 2003). A model which has been proposed for Notch pathway downregulation during SCC involves the possibility that p53 could normally act as a positive modulator of Notch expression and, therefore, when its function gets compromised, by cancer-related mutations, then Notch would 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).

1.6.2.1.2 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 expression of p63, which is often due to gene amplification (Hibi, Trink *et al*., 2000; Yamaguchi, Wu et al. 2000). As for the normal squamous epithelium, the major isoform of p63 expressed in SCC is the ΔNp63α isoform, and a correlation between high levels of ΔNp63 and poor prognosis has been also established in SCC of the head and neck carcinomas (Choi, Batsakis *et al*., 2002). Mechanistically, the tumorigenic 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).

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 Notch gene (via binding a p53-responsive element present on Notch promoter), establishing a link with the observation that high p63- expressing tumours have low Notch expression (Yugawa, Narisawa-Saito *et al*., 2010).

Beyond the interaction with the other p53 family members and the effects derived by its own transcriptional properties, ΔNp63 up-regulation has also been shown to contribute to tumorigenesis via the activation of the beta-catenin signaling pathway, through reduction of its phosphorylation and consequent intra-nuclear accumulation (Patturajan, Nomoto *et al*., 2002). Nuclear beta-catenin is another factor which has been found 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 findings, 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 at date, both *in vitro* and *in vivo*.

Aim of the work

The goal of this work was to identify and characterise new functions for the protein ASPP2 in the skin epithelium. The main objectives were:

1. To evaluate whether ASPP2 positively or negatively regulates the expression of p63 in squamous epithelium and which pattern of expression and location these two proteins have in the skin.
2. To characterise the behaviour of both proteins at transcriptional level in the presence of different factors and in different cell lines. We wanted to elucidate whether ASPP2 can regulate the transcription of ΔNp63 promoter on genes involved in epithelial differentiation.
3. To test for a putative relationship between ASPP2 and Notch, another protein important during the differentiation of the epidermis.
4. Lastly, we wanted to study Notch and p73 target genes regulated by ASPP2, as ASPP2 is a common activator of p73 and p63. In central nervous system one of the key factors determining cell fate determining factors is p73 but, in the epidermis, this role is taken p63 that determines cell fate, so it is emerging that many Notch target genes are also p73/p63 targets.

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 was purchased from Promega (WI, USA) and the QIAGEN Plasmid Maxi kit was purchased from Qiagen (UK).

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.

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.

Freezing Medium

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

10% (v/v) DMSO

Made fresh every time.

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.

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.

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.

Protein Molecular weight markers

Pre-stained protein markers were purchased from New England Biolabs (UK) and were used as a size standard for SDS-polyacrylamide gel electrophoresis.

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™ Protein Inhibitor Cocktail

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.

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.

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 SDS-polyacrylamide Gels

Table 2 – List of reagents used to prepare resolving and stacking gels at different concentrations.

	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).

2.1.3 Antibodies

2.1.3.1 Primary antibodies

Table 3 – List of primary antibodies.

antigen	Ab name	host/type	source	applications
Actin	C-2	mouse mAb	Santa Cruz (sc-8432)	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
Envoplakin	M-20	goat pAb	Santa Cruz (sc-16751)	WB (1:1000)
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)
Ku80	Ab-2	mouse mAb	Thermo Fisher	ICC/IF (1:200)
myc-tag	9E10	mouse mAb	ascite	WB (1:1000)
Notch1	C-20	rabbit pAb	Santa Cruz (sc-6014-	IHC (1:400), WB

			R)	(1:1000)
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)
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)

2.1.3.2 Secondary antibodies

Table 4 – List of secondary antibodies.

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

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, 488 Anti-Mouse IgG (H+L)	Donkey	Invitrogen	IF (1:400)
Alexa Fluor® 647, 488 Anti-Rabbit IgG (H+L)	Donkey	Invitrogen	IF (1:400)
Anti-Mouse Immunoglobulins/HRP	rabbit	Dako	IHC (1:250), WB (1:2000)
Anti-Rabbit Immunoglobulins/HRP	swine	Dako	IHC (1:250), WB (1:2000)

2.1.4 Plasmids

Table 5 – List of plasmids used in Luciferase Assay System.

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
Notch intracellular domain (ID)	pcDNA3.1	human Notch ID	Myc	Dr T. Kadesh, Universityof 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	*envoplakin* 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
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
Hey2-luciferase	pGL3	*Hey2* promoter cloned 5' to the firefly luciferase gene	-	Prof M. Gessler, Biozentrum, Germany

2.1.5 Cell lines

Table 6 – List of established cell lines used in this work.

name	tissue/type	source
H1299	human lung carcinoma	ATCC
HSC3	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
CAL-27	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
Saos-2	Human osteosarcoma	ATCC

Abbreviations – American Type Culture Collection (ATCC)

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) 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 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 techniques

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 LB medium containing the appropriate antibiotic (100μg/ml ampicillin) in flasks shaking at 37 °C.

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 ºC for 1 hour before plating on LB-agar plates with an appropriate antibiotic. Plates were then incubated at 37 ºC 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 ºC 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 ºC. The cells were centrifuged at 6,000g for 15 minutes at 4 ºC (Sorvall RC 5C Plus, rotor SLA-3000). The large scale DNA preparation was carried out according to Qiagen Qiafilter Maxi DNA kit protocol.

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 ºC for 10 minutes. 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. 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 electrophoresed 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 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.

2.2.4 Cell-based assays

Cell transfection

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

***In vivo* Transcription assays**

Cells were seeded at about 80% confluence in wells of a 24-well plate and transfected 24h 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.

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.

CHAPTER III: RESULTS

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

To evaluate the role of ASPP2 in skin tumour formation we performed morphological analysis of epithelial tumours. We found that tumour cells from ASPP2 Δexon3 homozygous and heterozygous mice did not express vimentin, according to the immunohistological results. On the contrary, we found high levels of keratin-14 (K14) and keratin-1 (K1). Tumours from the ASPP2 mutant mice (Δexon3) also revealed marked expression of nuclear p63 (Figure 8). Histological analysis of the epithelial tumours spontaneously formed in the ASPP2 mutant mice revealed them to be poorly differentiated, characterized by elevated expression of K14, K1 and nuclear p63.

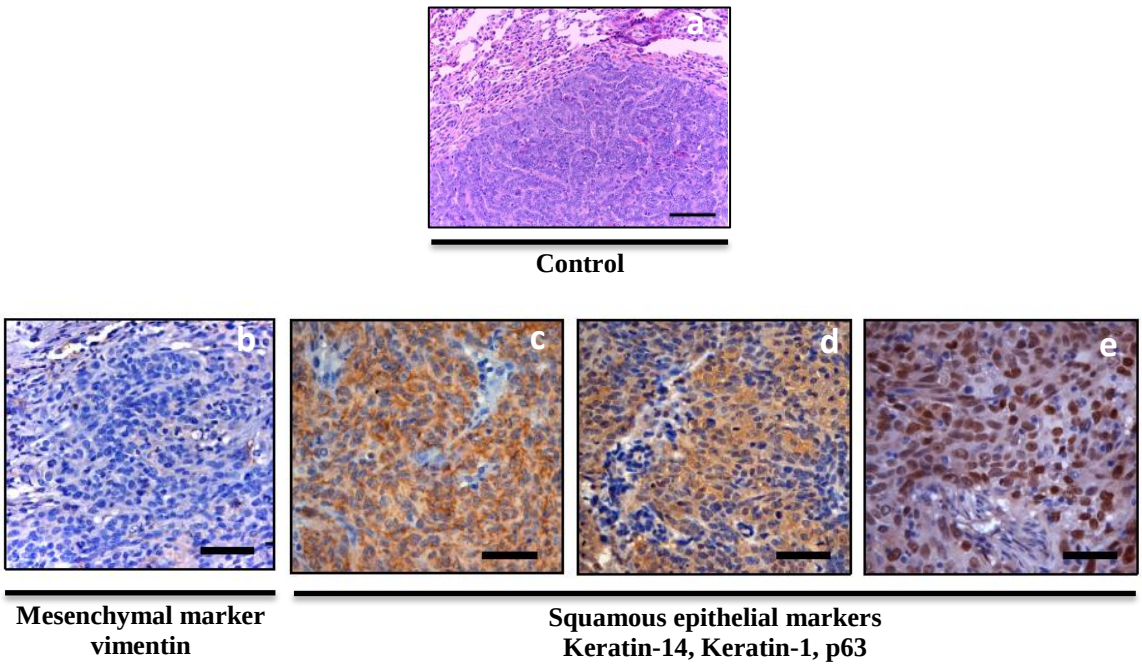


Figure 8 – Immunostaining of the epithelial tumours. a) Control result, hematoxylin and eosin staining of lung metastases. Detection of b) vimentin (negative), c) Keratin-14 (positive), d) Keratin-1 (positive) and e) p63 (positive). The last three are all markers for SCC. Scale bars: 100 μm in a) and 50 μm in b) – e).

3.2 Cooperation between ASPP2 and p53 in tumour suppression

3.2.1 p63 expression is up-regulated in ASPP2-deficient cells and both negatively correlate in squamous epithelium

In order to verify whether ASPP2 could be a significant factor in epithelial homeostasis, we compared ASPP2 expression with that of p63 expression in human stratified epithelium. Immunofluorescence analysis showed high levels of ASPP2 expression in the adult human skin epithelium. Interestingly, the pattern of expression of ASPP2 was mutually exclusive with the one of p63, as no expression of ASPP2 was detected in the basal layer of the skin, where p63 is highly abundant (Figure 9).

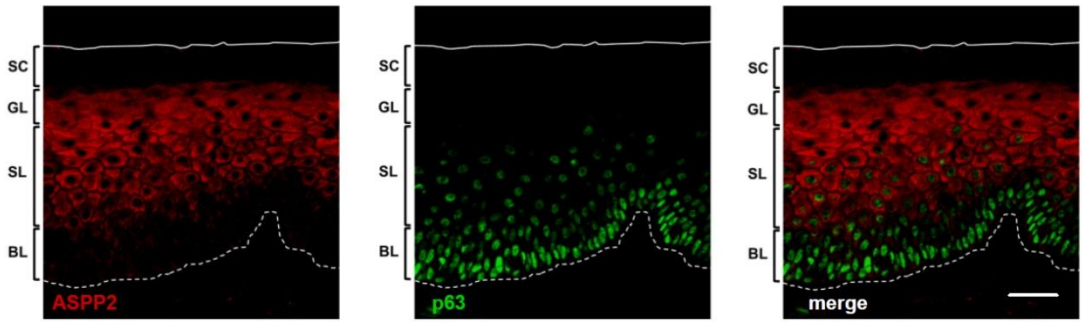


Figure 9 – ASPP2 and p63 mutual exclusive pattern of expression in skin tissue. Double staining of human squamous epithelium of the skin with anti-ASPP2 and anti-p63 antibodies showing that ASPP2 and p63 have an almost mutually exclusive pattern of expression. Scale bar: 50 μm.

To test whether ASPP2 might be a factor involved in the differentiation of the squamous epithelium, we analysed the pattern of ASPP2 expression during cell differentiation in primary mouse keratinocytes by Western Blotting. Pluripotent keratinocytes isolated from 3 days 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 in the cell growth medium, allowing cells to establish cell-cell contacts, to withdraw from the cell-cycle and differentiate, mimicking the physiological process of cell stratification taking place in the squamous epithelium. As a control for the successful outcome of the process of cell-differentiation, the presences of envoplakin, a protein induced with terminal differentiation (Karashima, T. and Watt FM., 2002) as well as the disappearance of p63, marker of cell pluripotency (Davydova, DA *et al.*, 2009) were evaluated. Interestingly, ASPP2 expression was found to be up-regulated during differentiation, alongside with envoplakin and concomitant with p63 down-regulation (Figure 10).

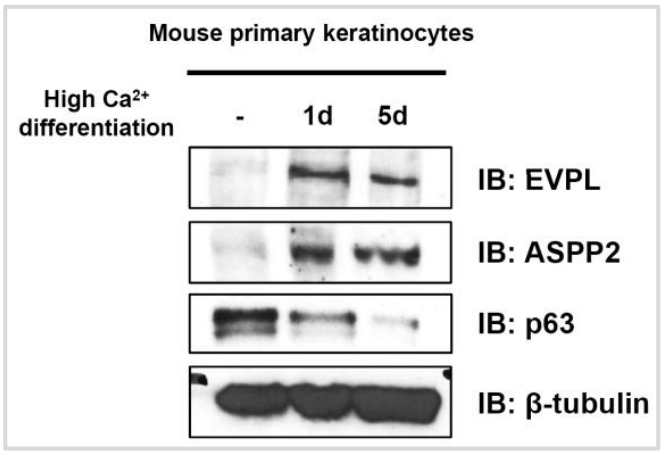


Figure 10 – Evaluation of ASPP2 levels of expression compared to other skin markers during differentiation of mouse primary keratinocytes. Lysates were prepared from cells cultured in absence of calcium (Ca^{2+}) in the medium and then after one and five days upon the addition of Ca^{2+}. Immunoblotting was performed using antibodies anti-envoplakin (EVPL), anti-ASPP2, anti-p63 and anti-βtubulin (loading control).

The following hypothesis could explain why when ASPP2 expression is compromised, as it is in the ASPP2-deficient mice, we observed over-expression of p63 in the skin and spontaneous formation of SCCs (Figure 11). The absence of ASPP2 expression from the upper *strata* would release the inhibition on p63, which could be expressed upwards throughout all the epithelium and lead to SCC formation, as observed in the ASPP2 deficient mice.

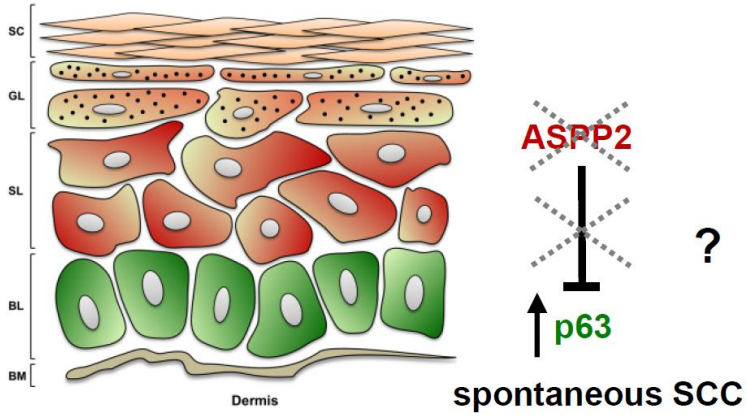


Figure 11 – Model of ASPP2 importance in preventing p63 expression and therefore suppress tumour formation. BM, basal membrane; BL, basal layer; SL, spinous layer; GL, granular layer; SC, *stratum corneum*.

3.2.2 ASPP2 regulates p63 expression at transcriptional level

All previous experiments were performed in adult tissue, and so afterwards we investigated how ASPP2 can regulate p63 at transcriptional level, i.e., understand how ASPP2 controls the regulation of transcription of p63 factor. In the first experiment we tested whether

ASPP2 could repress directly ΔNp63 expression, doing a luciferase transactivation assay in H1299 human cell line. Transcriptional activity of ΔNp63 promoter was measured transfecting ΔNp63 and ASPP2-V5 expression vectors (V5 is an epitope recognized by the mouse monoclonal antibody V5, a specific tag for exogenous ASPP2) into H1299 cells. Luciferase activity was analysed from cell lysates 24h after transfection, and normalised for the signal from renilla and this is expressed as fold induction. Using the same lysates, the transfection was confirmed by Western blotting analysis, using antibodies anti-V5 (ASPP2), anti-p63 and anti-actin (loading control). In this analysis, we observed that ΔNp63α (the main p63 isoform expressed in the skin) is capable of activating the ΔNp63 promoter and ASPP2 shows no effect when co-transfected in combination with ΔNp63α or p53 (Figure 12). Moreover, p53 protein levels seemed to have no effect on ΔNp63 transcription. So, we can conclude that likely ASPP2 did not directly repress ΔNp63 expression in transactivation assay.

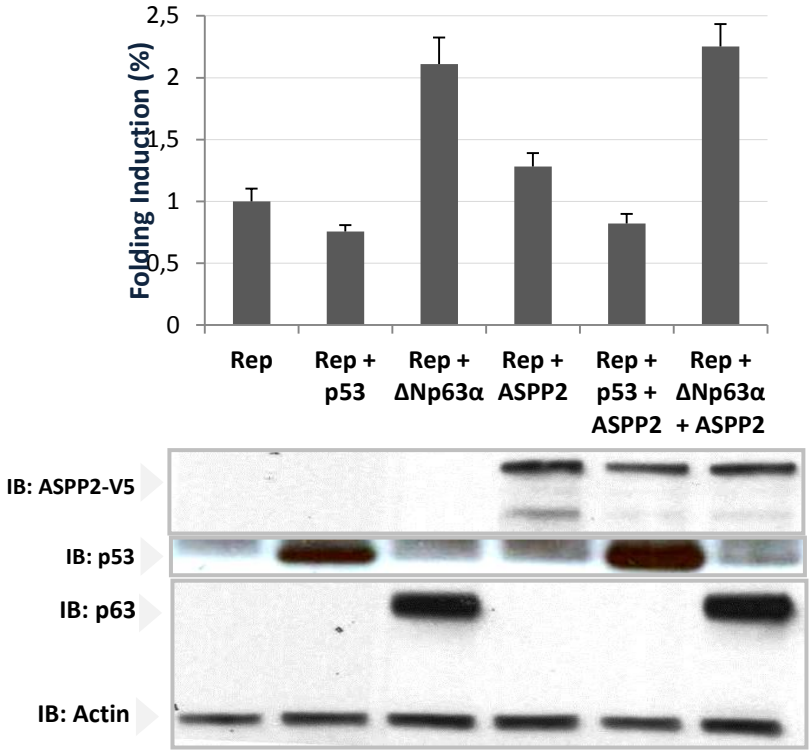


Figure 12 – Transcriptional activity of ΔNp63 promoter measured by Luciferase reporter assay, transfecting into H1299 cells growing in 24-well plate, 1 μg of ΔNp63-luciferase reporter construct in presence or absence of ΔNp63α (400 ng) and ASPP2-V5 (1 μg) expression vectors and together with 6 ng of a renilla expression vector.

In normal basal epithelial cells, ΔNp63α promotes proliferation through regulation of some target genes such as p21. Transcriptional activity of p21 promoter was measured by Luciferase reporter assay, by transfecting into H1299 ΔNp63α in the presence or absence of

ASPP2-V5 expression vectors. Luciferase activity was analysed from cell lysates 24h after transfection and using the same lysates, the result was confirmed by Western blotting analysis, using antibodies anti-V5 (ASPP2), anti-p63 and anti-βtubulin (loading control). When testing the transcriptional activity of p21 promoter by transfecting ΔNp63α in presence of ASPP2, we observed an increased ΔNp63α proportional to the increasing amounts of ASPP2, while neither ΔNp63 nor ASPP2 alone seem to have significant effect (Figure 13).

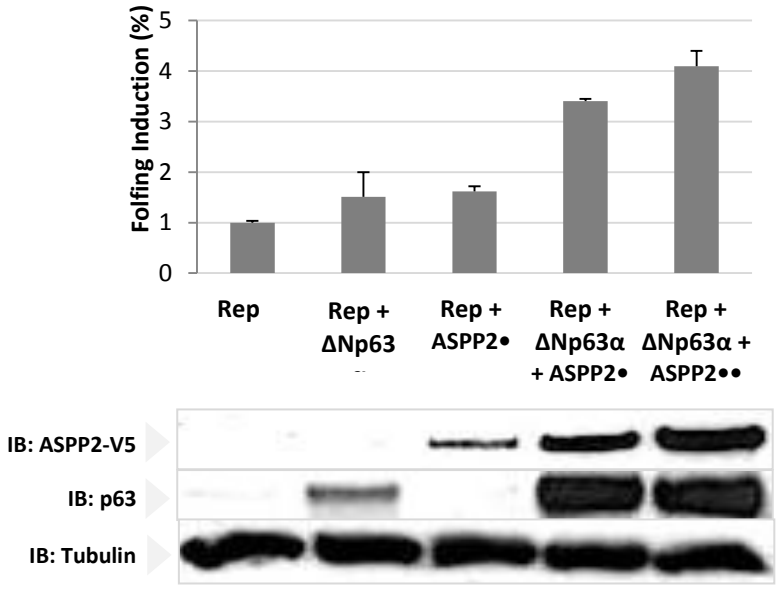


Figure 13 – Transcriptional activity of p21 promoter. In this luciferase reporter assay, was transfected into H1299 cells growing in 24-well plate, 200 ng of p21 promoter in presence or absence of ΔNp63α (100 ng) and ASPP2-V5 (400, 600 ng) expression vectors and together with 6 ng of a renilla expression vector.

We then investigated whether ASPP2 could repress ΔNp63 expression at transcriptional level using p63 promoter mutants, a mutant of a p63 isoform that lacks the N-terminal transactivation domain (a longer reporter) and a mutant that lacks the binding site for β-catenin, a protein studied by our group and that is known to activate p63 expression and also because ASPP2 can inhibit its expression (a shorter reporter).

As we hypothesize that in the -740/+139 promoter there is a group of factors, still unknown, that prevent ASPP2 to repress the expression of ΔNp63, and Notch is a known-repressor of p63, the first approach was to test the expression of Notch alone when transfected with both promoters. Transcriptional activity of -704/+139 and -404/+139 promoters was measured by Luciferase reporter assay, by transfecting into H1299 cells each promoter in presence or absence of Notch expression vectors and together with renilla expression vector (internal control). Luciferase activity was analysed from cell lysates 24h after transfection, and the analysis showed that Notch had no significant effect on the longer promoter but,

interestingly, Notch could repress the transactivation of the -404/+139 shorter promoter (Figure 14).

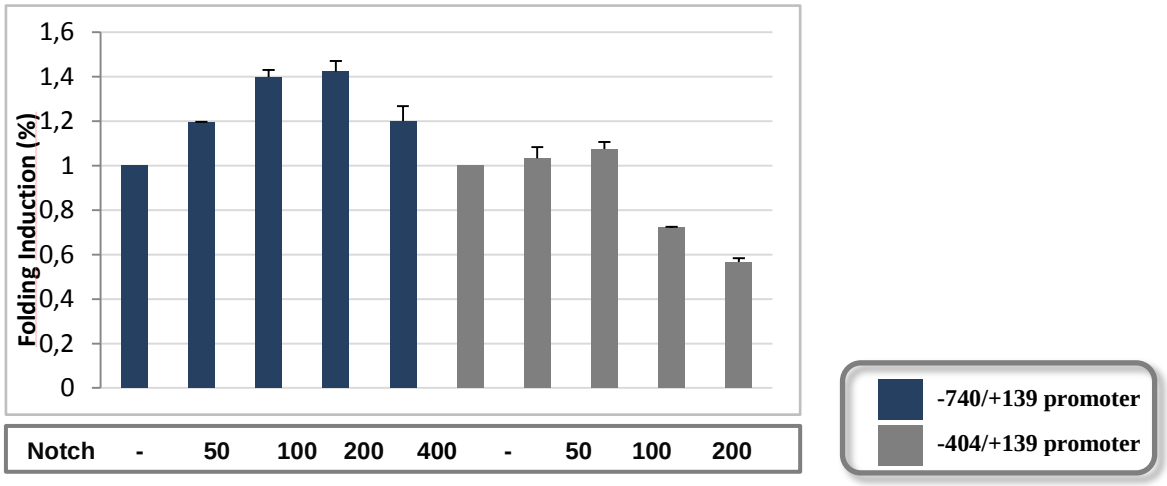


Figure 14 – Effect of Notch on ΔNp63 mutant's transcription determined by luciferase assay. Transcriptional activity of -704/+139 and -404/+139 promoters measured by transfecting into H1299 cells growing in 24-well plate 50 ng of each promoter in presence of Notch expression vectors and together with 6 ng of a renilla expression vector.

Considering the hypothesis that ASPP2 could have a role similar to Notch, we hypothesized that, in the shorter promoter, ASPP2 can inhibit the function of p63 (Figure 30). We tested ASPP2 alone on the same reporter mutants, as seen previously for Notch, and we verified that in the longer promoter ASPP2 had no significant effect (Figure 15, A); however, ASPP2 represses the transactivation of the promoter of the short ΔNp63 promoter only (Figure 15, B).

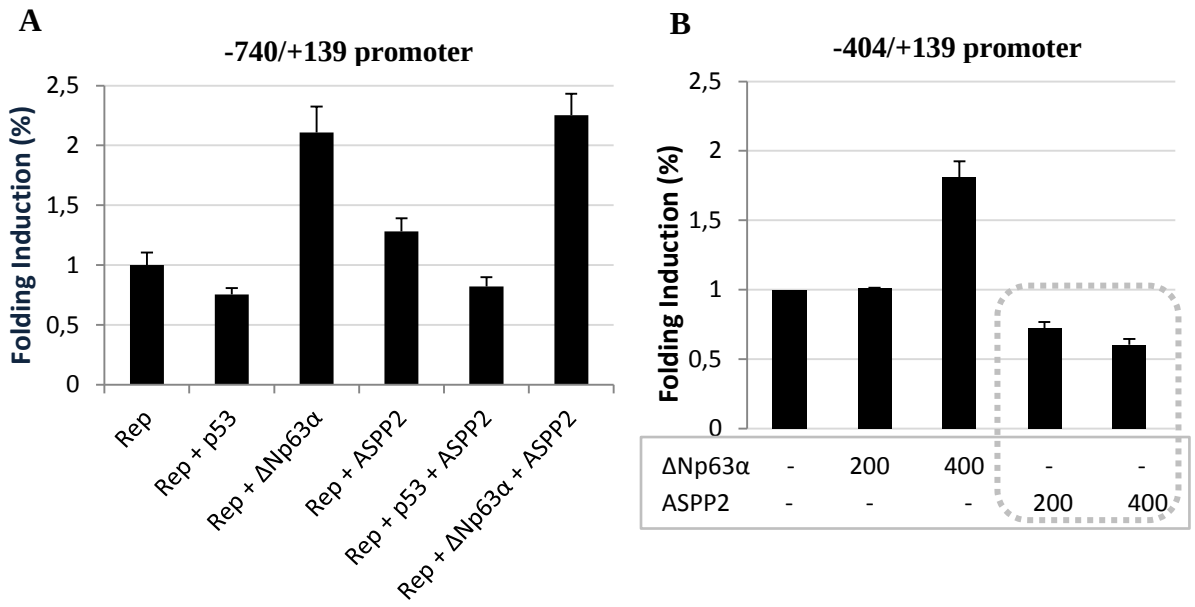


Figure 15 – Effect of ASPP2 on transcription of both ΔNp63 mutants by luciferase assay. Transcriptional activity of -704/+139 (A) and -404/+139 (B) promoters was measured by Luciferase reporter assay, by transfecting into H1299 cells growing in 24-well plate 1 µg (A) or 200 ng (B) of the promoter in presence or absence of ΔNp63α (400 ng, A) and ASPP2-V5 (1 µg, A) expression vectors and together with 6 ng of a renilla expression vector. Luciferase activity was analysed from cell lysates, 24h after transfection, and normalised on the signal from renilla and this is expressed as fold induction.

In order to evaluate whether ASPP2 can repress ΔNp63 expression at transcriptional level in a squamous cell carcinoma cell line HSC3, we transfected cumulative amounts of ASPP2 alone on ΔNp63 promoter and analysed the expression of p63 and ASPP2 transfected by Western Blot (Figure 16). Transcriptional activity of ΔNp63 promoter was measured by Luciferase reporter assay, by transfecting into HSC3 cells growing in 24-well plate 200 ng of promoter in increasing amounts of ASPP2-V5 expression vector. Luciferase activity was analysed from cell lysates, 24h after transfection and the transfection was confirmed by Western blotting analysis, using antibodies anti-p63 and anti-actin (loading control). The results showed that the expression of p63 decreases in accordance with increased values of ASPP2, corroborating that ASPP2 can repress p63 function.

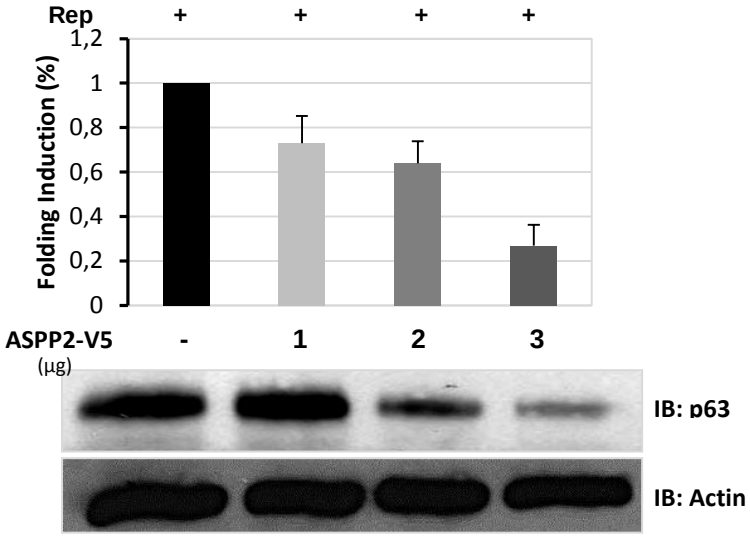


Figure 16 – Effect of ASPP2 on ΔNp63 transcription by luciferase assay. Transcriptional activity of ΔNp63 promoter was measured by Luciferase reporter assay, by transfecting into HSC3 cells growing in 24-well plate 200 ng of promoter in increasing amounts of ASPP2-V5 expression vector and together with 6 ng of a renilla expression vector.

3.3 ASPP2 regulates ΔNp63 transcriptional function

As ASPP2 was shown to directly bind to p63, we decided to test whether ASPP2 in combination with the repression of ΔNp63 expression levels could also regulate ΔNp63 transcriptional activity. We investigated ΔNp63 transcriptional activity, in the absence or presence of ASPP2, on Keratin-14, Keratin-10 and envoplakin target genes. By luciferase transactivation assay, we found that the transcription of all the three genes, K14, K10 and envoplakin, was induced by ΔNp63 expression, whereas ASPP2 had no significant effect. Nevertheless, ASPP2 expression is able to repress ΔNp63α ability to induce K14 (A) and K10 (B) transcription, while it enhances ΔNp63α ability to induce envoplakin transcription (C).

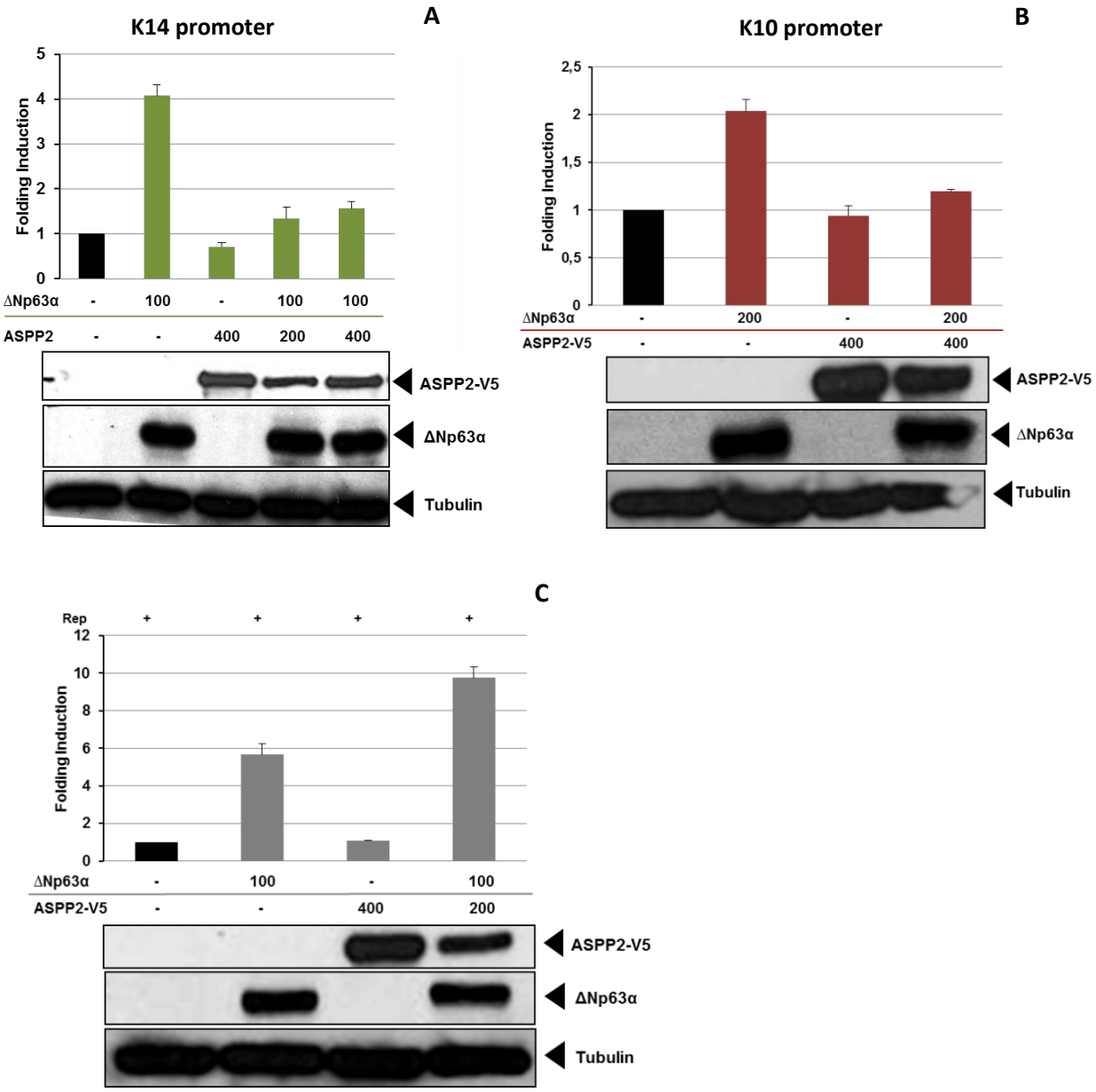


Figure 17 – ASPP2 regulates ΔNp63 transcription on genes involved in epithelial differentiation. (A) K14 luciferase reporter assay in H1299 cells. Transcriptional activity of K14 (A), K10 (B) and envoplakin (C) promoters was individually measured by transfecting into H1299 cells growing in 24-well plate 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 the reading the luciferase activity, using antibodies anti-V5 (ASPP2), anti-p63 and anti-βtubulin (loading control).

3.4 Cross-regulation between ASPP2 and Notch/ΔNp63 pathways

To test if ASPP2 and Notch could interact, we decided to investigate whether their expression levels could regulate Keratin-14 transcriptional activity. We evaluated the K14

transcriptional activity, in absence or presence of Notch/ASPP2 (Figure 18). Luciferase activity was analysed from cell lysates harvested 24h after transfection of the expression vectors and the result was evaluated by Western blotting analysis of the same lysates. We found that Notch expression induced the transcription of K14 and ASPP2 had no significant effect alone. Nevertheless, the transcriptional induction when ASPP2 was transfected with Notch was almost completely eliminated and so ASPP2 was crucial to inhibit the induction of K14 mediated by both p63 and Notch.

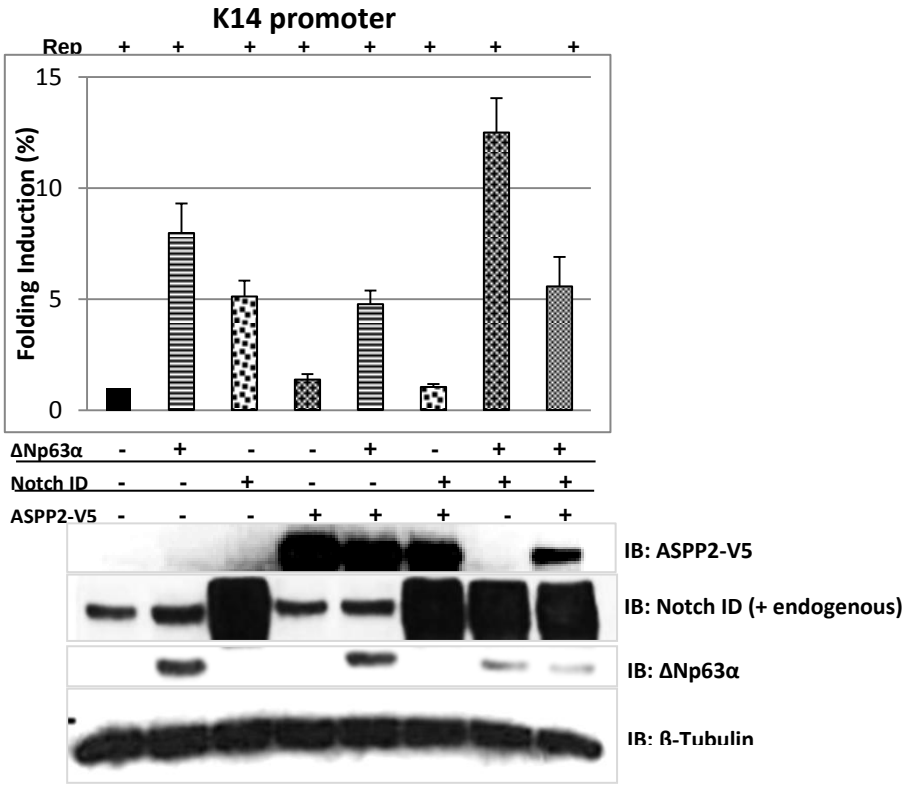


Figure 18 – Effect of Notch and ASPP2 on K14 transcription by luciferase assay. Transcriptional activity of K14 promoter was measured by transfecting into H1299 cells growing in 24-well plate 200 ng of reporter construct in presence or absence of ΔNp63α and ASPP2 (V5-tagged) expression vectors (concentrations 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 was evaluated by Western blotting analysis of the same lysates used for the reading the luciferase activity, using antibodies anti-V5 (ASPP2), anti-p63, anti-Notch and anti-βtubulin (loading control).

3.5 Notch and p73 share communal target genes and they are both regulated by ASPP2

We decided to focus on TAp73 because p73 and Notch have similar roles (Talos, F. *et al.*, 2010). Transcriptional activity of Bax promoter was measured by Luciferase reporter

assay, by transfecting into Saos-2 cells the promoter in presence of TAP73/ΔNp63α and ASPP2 expression vector. Luciferase activity was analysed from cell lysates, 24h after transfection. In this analysis we verified, firstly, that ΔNp73 and Tap73, both isoforms of p73, alone, can stimulate the transcription of Bax promoter; the TA isoform has a better effect on the activation of Bax and ASPP2 is effective only at high levels to promote the transcription of the reporter (Figure 19).

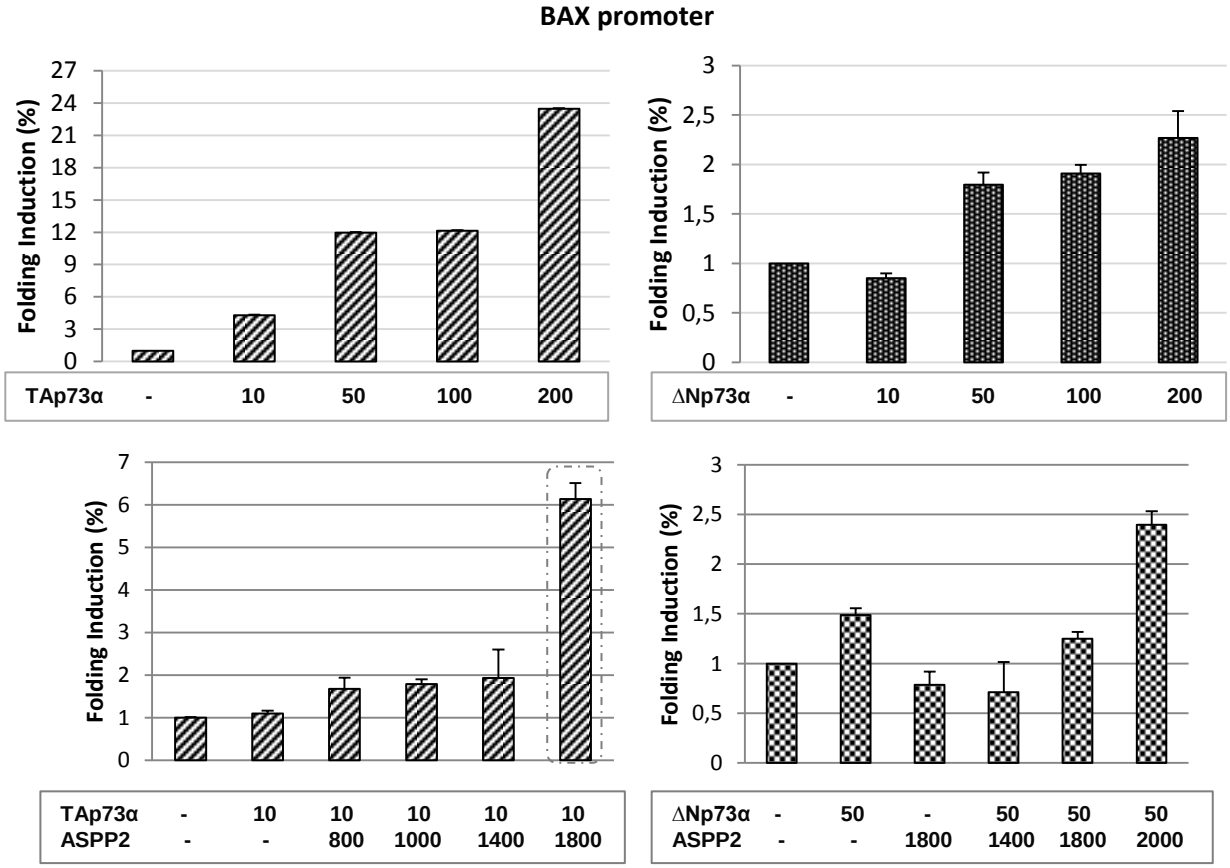


Figure 19 – Effect of Tap73α/ΔNp73α and ASPP2 on Bax transcription by luciferase assay.

After that, we tested the same conditions for Hey2 promoter, a Notch target gene. We analysed the transcriptional activity of Hey2, in presence of Tap73 or ΔNp73 alone and in combination with ASPP2 (Figure 20). We could verify that both isoforms can induce the transcription of Hey2 promoter, and the TA isoform has a better effect on the activation of the reporter, additionally high levels of ASPP2 only induces the transcription of the promoter.

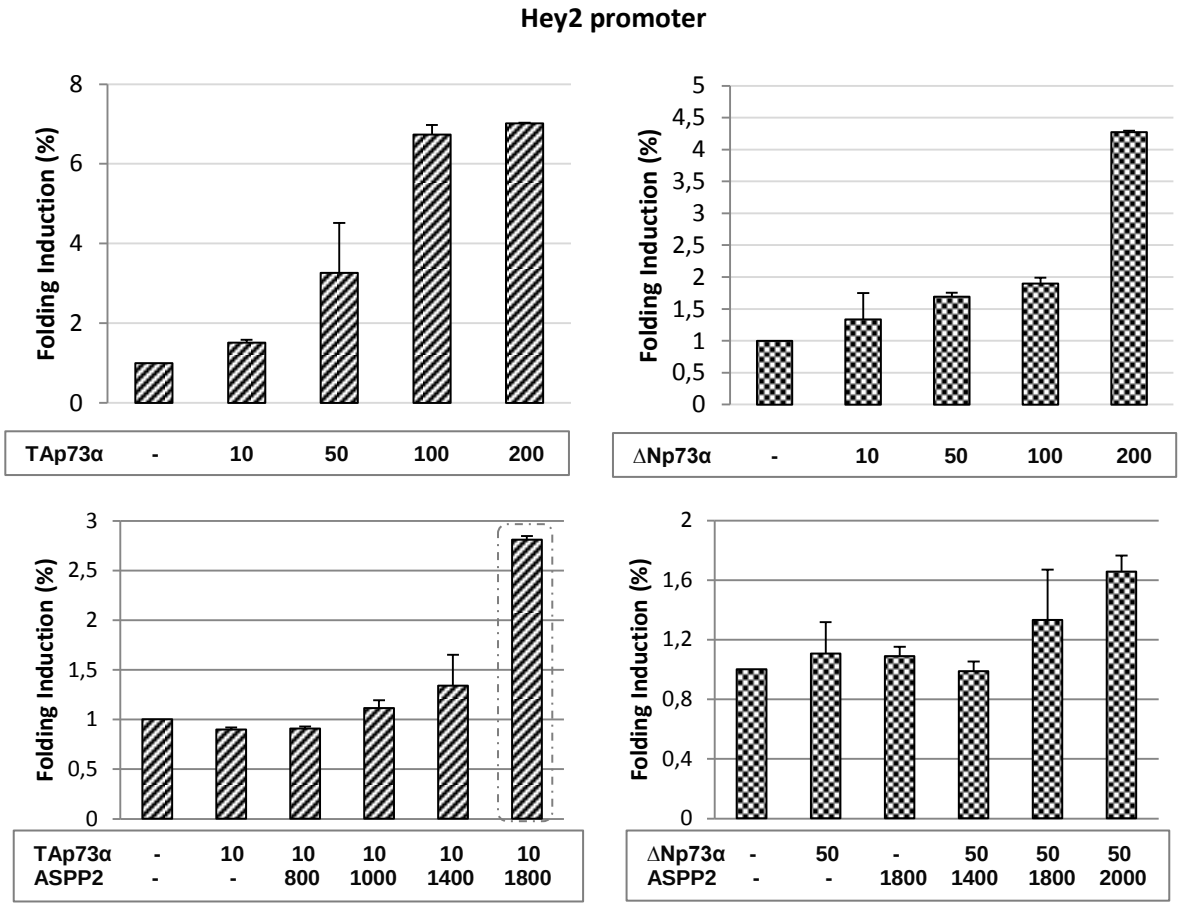


Figure 20 – Effect of Tap73α/ΔNp73α and ASPP2 on Hey2 transcription by luciferase assay. Transcriptional activity of Hey2 promoter was measured by Luciferase reporter assay, by transfecting into Saos-2 cells growing in 24-well plate 200 ng of promoter in presence or absence of TAP73α/ΔNp63α and ASPP2-V5 (concentrations indicated in the graphs) expression vectors and together with 6 ng of a renilla expression vector. Luciferase activity was analysed from cell lysates, 24h after transfection and normalised on the signal from renilla and this is expressed as fold induction. Tap73α and ΔNp73α expression, both isoforms of p73 alone, induce Hey2 transcription.

Since the absence of ASPP2 had a selective impact on the expression of some of the major Notch target genes, as Hey2, we tested if ASPP2 can have a direct effect on its transcription in transactivation assay. We transfected Hey2 promoter with increasing amounts of ASPP2 expression construct, in H1299 cells. Interestingly, ASPP2, which is not a transcriptional factor itself, can regulate Hey2 transcriptional activity when transfected alone.

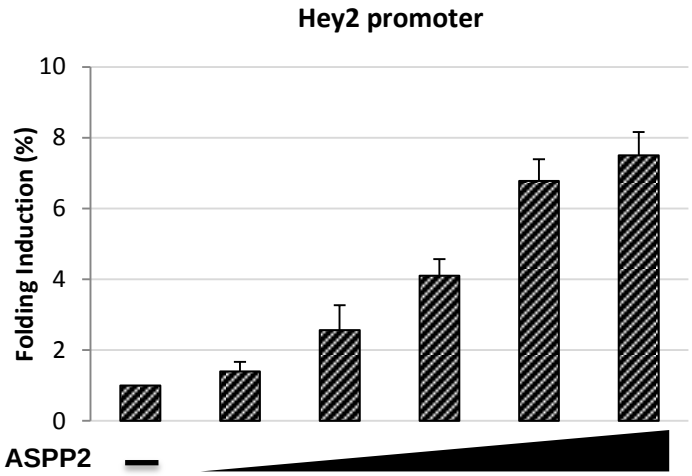


Figure 21 – Effect of ASPP2 on Hey2 transcription by luciferase assay. Transcriptional activity of Hey2 promoter was measured by Luciferase reporter assay, by transfecting into H1299 cells growing in 24-well plate 200 ng of the promoter in increasing amounts of ASPP2 expression vector and together with 6 ng of a renilla expression vector. Luciferase activity was analysed from cell lysates, 24h after transfection and normalised on the signal from renilla and this is expressed as fold induction.

Afterwards, we analysed the expression of Hey2 and Bax, some of the most important p73 target genes, in presence of Tap73 isoform. Notch was capable to induce activation on the promoters, while Tap73 had no effect, contrary to the expression of TAp73 that when transfected with Notch could reduce its expression and decrease the transcription of both target genes (Figure 22).

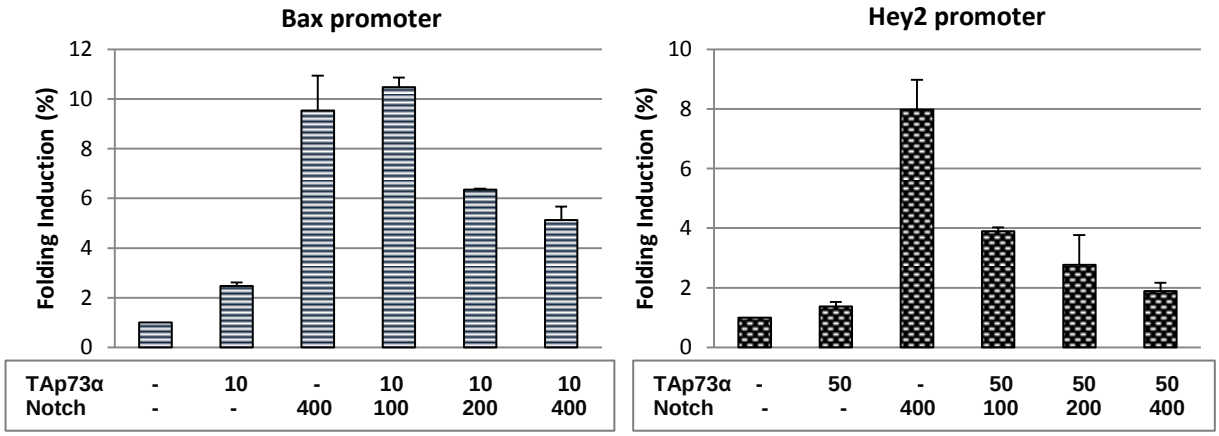


Figure 22 – Effect of Tap73α and Notch on Hey2 and Bax transcription. Transcriptional activity of Hey2 and Bax promoters was measured by Luciferase reporter assay, by transfecting into Saos-2 cells growing in 24-well plate 200 ng of promoter in presence or absence of TAP73α and Notch (concentrations indicated in the graphs) expression vectors and together with 6 ng of a renilla expression vector. Luciferase activity was analysed from cell lysates, 24h after transfection and normalised on the signal from renilla and this is expressed as fold induction.

3.6 Effect of Notch and p73 on reciprocal target genes

Given the apparent similarities between Notch and p73 target genes, we thought it would be interesting to make a brief study about both factors on reciprocal target genes. The next graph summarizes several experiments to check the effect of Notch, p73 and ASPP2 on Hey2, Bax, Hes1 and Hes5 promoters into Saos-2 cells. Interestingly, we could see that Notch induces Bax promoter – which is a known p73 target gene – and ASPP2 alone activates Hey2 promoter.

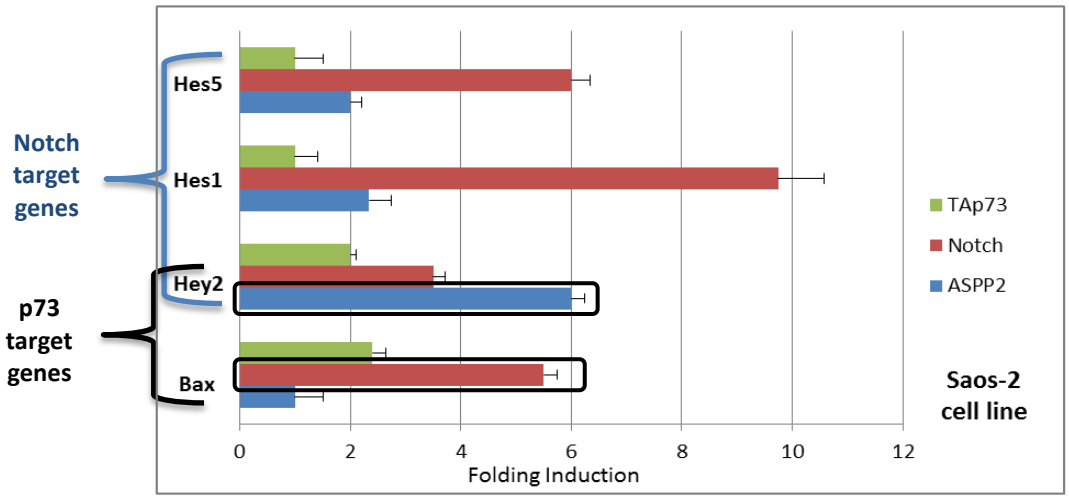


Figure 23 – Effect of Notch and Tap73 on reciprocal target genes. Transcriptional activity of Hes1, Hes5, Hey2 and Bax promoters was measured by Luciferase reporter assay, by transfecting into Saos-2 cells growing in 24-well plate 200 ng of each promoter in presence of ASPP2, Notch or TAP73α expression vectors and together with 6 ng of a renilla expression vector. Luciferase activity was analysed from cell lysates, 24h after transfection and normalised on the signal from renilla and this is expressed as fold induction.

Hereafter, we focused only on Bax and Hey2 promoters and all possible combinations between ASPP2, Notch and Tap73 (Figure 24), to try to understand how ASPP2 can enhance or inhibit Notch transcription on some of its target genes.

We verified that ASPP2, which is not a transcriptional factor itself, can regulate both Bax and Hey2 transcriptional activity when co-transfected with Tap73, more specifically on Bax promoter, or with Notch, on Hey2 promoter. Notch in combination with Tap73, can also induce the expression of Bax promoter, which is interesting, because Bax is known to be a p73 target gene. These results showed that ASPP2 is a factor capable of influencing Notch transcriptional action and both Notch and p73 share communal target genes.

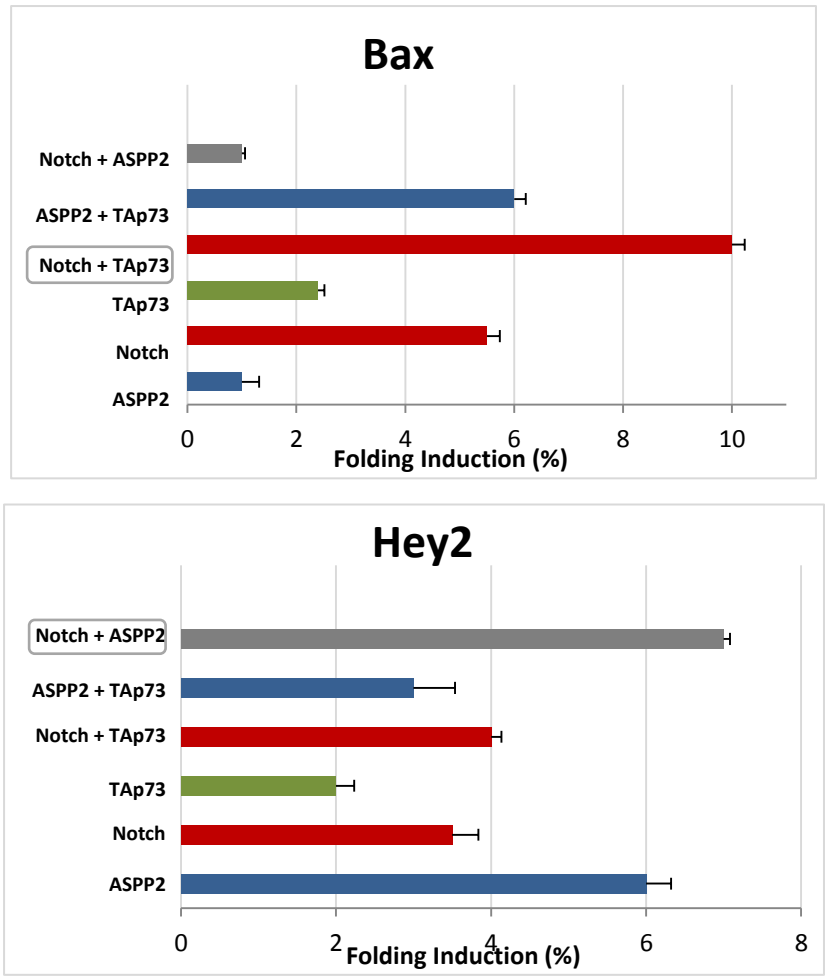


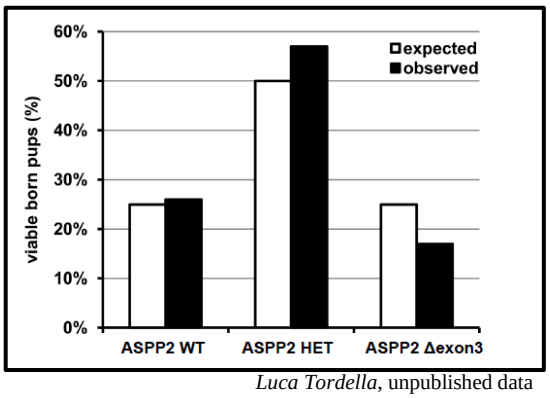
Figure 24 – Effect of ASPP2, Notch and TAp73 on Bax and Hey2 promoters. Transcriptional activity of Bax and Hey2 promoters was measured by Luciferase reporter assay, by transfecting into Saos-2 cells growing in 24-well plate, 200 ng of each promoter in presence of ASPP2, Notch or TAP73α expression vectors and their combinations and together with 6 ng of a renilla expression vector. Luciferase activity was analysed from cell lysates, 24h after transfection and normalised on the signal from renilla and this is expressed as fold induction.

CHAPTER IV: DISCUSSION

4.1 ASPP2 expression promotes formation of spontaneous squamous cell carcinoma

Squamous cell carcinoma (SCC) is among the most common cancer worldwide (Zhang *et al*., 2011) and is a malignancy of epidermal keratinocytes. SCC is a common form of invasive skin cancer (along with basal cell carcinoma (BCC)) (Zhang *et al*., 2005) and ASPP2 is generally expressed in squamous epithelium. In order to provide an *in vivo* model system to study the biological functions of ASPP2, ASPP2 Δexon3 mice were generated by our group. The results which follow have been performed by Luca Tordella, another member of Professor Xin Lu's lab, and are shown here because of their relevance to the present study.

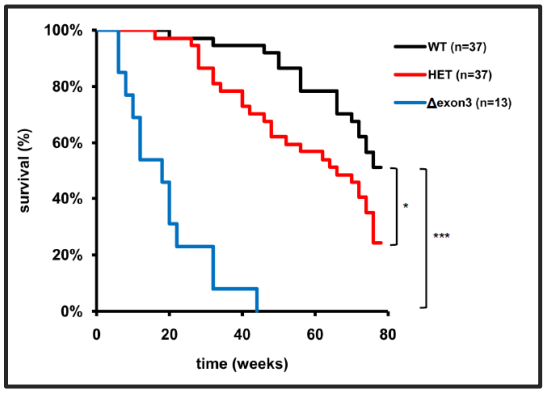
Balb/c is one of the most used mouse experimental strains for preclinical research and because it has a very low spontaneous incidence of tumours, is a suitable tool for long term lab experiments (Jackson Laboratory, last update: June 2012). In an attempt to obtain viable ASPP2 Δexon3 mice and considering the variations between the different genetic backgrounds of inbred mice, ASPP2 mutant mice were inter-crossed to a Balb/c background and, interestingly, the pups were viable and born according to the Mendelian segregation (Figure 25).



Luca Tordella, unpublished data

Figure 25 – Birth rate for ASPP2 wild type, ASPP2 heterozygous and ASPP2 Δexon3. Birth rate for each of the three possible genotypes generated by the intercross between ASPP2 heterozygous mice is shown comparing the Mendelian expected frequency (in white) with the observed frequency (in black). No significant anomalies in the distribution of birth rates were observed.

The majority of the ASPP2 Δexon3 homozygous mice in the Balb/c background were therefore able to survive up to one year after weaning. Since there are abnormalities that affect CNS, the general survival rate after birth was nevertheless significantly reduced when compared with heterozygous and wild type littermates. Moreover, intriguingly increased mortality, this time in the adult age, was also observed for ASPP2 Δexon3 heterozygous (Figure 26) (Tordella L., unpublished data).

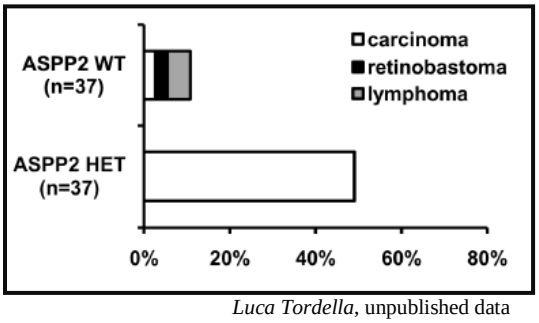


Luca Tordella, unpublished data

Figure 26 – ASPP2 Δexon3 mice in Balb/c genetic background are viable, but their lifespan is reduced. General survival study showing the decreasing percentage of ASPP2 Δexon3, heterozygous and wild type mice. 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.

4.2 Reduction of ASPP2 induces development of spontaneous carcinomas

The susceptibility to develop spontaneous tumours was evaluated in a study comparing mutant ASPP2 heterozygous and wild type mice. An important finding of this study was that all the tumours found in the ASPP2-deficient group were classified as epithelial tumours, also known as carcinomas, while more variation in the tumour spectrum was observed in the wild type cohort (Figure 27).



Luca Tordella, unpublished data

Figure 27 – ASPP2 wild type and mutant mice develop spontaneous epithelial tumours. The graph shows the percentage and the spectrum of tumour types spontaneously developed by wild type and heterozygous mice.

At macroscopic level tumours appeared as solid compact masses, occasionally capsulated, with some degree of vascularisation (Figure 28).

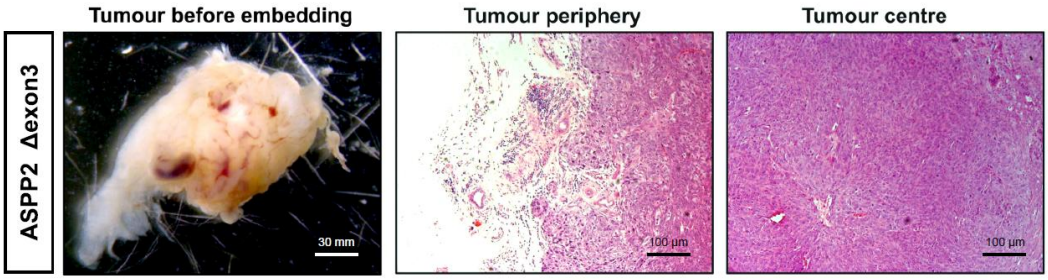


Figure 28 – Whole view and H&E-stained sections from the periphery and center of an epithelial tumour harvested from an ASPP2 heterozygous mouse (scale bars values are indicated) (*Tordella L.*, unpublished data).

The morphological analysis of the tumoural masses was then complemented with immunohistological staining for several cell markers. The results showed that the tumour cells from ASPP2 Δexon3 homozygous and heterozygous mice did not express vimentin, a marker of mesenchymal cells. On the contrary, the expression of several keratins was detected in all of the tumours analysed from the ASPP2 mutant cohort. In particular, high levels of keratin-14 (K14) and keratin-1 (K1) were found (Figure 8), both well-known markers to keratinocytes, whose pattern of expression is a factor for tumour progression in SCC (Choi *et al.*, 2010).

Tumours from the *ASPP2* mutant mice also revealed marked expression of nuclear p63 (Figure 8), another conventional marker for SCC. p63 expression in epithelial cancers was previously found to be elevated in cells with high proliferative capacity (Pietenpol, A. *et al.*, 2004) and it is essential for the development of most tissues in which it is expressed, as p63-*null* mice exhibit profound developmental abnormalities of the skin, limbs and other epithelial tissues (Mills *et al.*, 1999; Pellegrini, D. *et al.*, 2001).

However, it is indeed still not very clear why SCCs express both basal cells markers, such as Keratin-5/14 and p63, and differentiated squamous cell markers, such as Keratin-1/10. It is also unclear why SCC-initiating basal cells need to express differentiation markers, as Keratin-1. A possibility is that keratinocytes fail to switch off basal genes, as Keratin-14 and p63, during differentiation. Alternatively, SCC might develop from partially differentiated K1 positive suprabasal cells, which are still considered partially dynamic, but 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 (Pellegrini, D. *et al.*, 2001).

Poorly differentiated SCCs are distinguishable from well-differentiated carcinomas because they contain more pleomorphic cells, and no keratinous pearls. Relaying on these assumptions, 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. Furthermore, supported a role for ASPP2 in suppressing tumourigenesis and uncovered a possible critical and specific involvement in skin tumour formation.

4.3 Cooperation between ASPP2 and p53 in tumour suppression

ASPP2 stimulates the apoptotic function of the p53 family *in vivo* (Lu *et al*., 2006) and ASPP2 was previously identified as an interacting partner for a number of proteins, including p53 (Iwabuchi *et al*., 1994), binding to its evolutionarily conserved DNA binding domain (Gorina and Pavletich, 1996).

Consistent with the previously characterised role of ASPP2 in regulating p53-mediated apoptosis (Samuels-Lev, O'Connor *et al*., 2001), we decided to test if the ability of ASPP2 to suppress SCC was mediated by the p53 tumour suppressor properties. In order to test this hypothesis, the kinetic of tumourigenesis was measured and we observed that in the absence of p53, earlier tumour formation was detected in the ASPP2 mutant mice, suggesting that the combined loss of ASPP2 and p53 can accelerate tumour onset (data not shown). Loss of just one allele of p53 accelerated the onset of tumour formation in ASPP2 Δexon3 mice, allowing the appearance of a significant number of tumours in these mice before they prematurely die for CNS defects. 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 the homeostasis of the epidermis.

Other works showed 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), indicating p53 loss may not be a critical step in SCC initiation, but mainly important for the progression of malignancy. Altogether, these observations support the existence of a co-operation between ASPP2 and p53 in tumour suppression in a more comprehensive way.

4.3.1 p63 expression is up-regulated in ASPP2-deficient cells

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

To test whether ASPP2 could be an important 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 the adult human skin epithelium with a peculiar localisation, restricted to the spinous and granular layer, which contain exclusively differentiated cells (Figure 9). Interestingly, ASPP2 pattern of expression was mutually exclusive with the one of p63, as no expression of ASPP2 was detected in the basal layer of the skin, containing undifferentiated and proliferative cells, which is where p63 expression resides. These experiments could support that ASPP2 protein is present in the stratified squamous epithelium of the skin and therefore it is conceivable that its disappearance from this site might be a direct cause for SCC formation, as observed in the ASPP2 deficient mice.

Additionally, this specific localised expression of ASPP2 in the differentiated strata of the epidermis, led us to hypothesise that ASPP2 might 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.

Remarkably, ASPP2 expression was found to be up-regulated during differentiation, alongside with envoplakin, a protein induced with terminal differentiation (Karashima, T. and Watt FM., 2002) and in an opposite trend with p63, marker of cell pluripotency (Davydova, DA *et al*., 2009) (Figure 10). In more detail, ASPP2 protein seemed to be absent from the undifferentiated keratinocytes, with abundant p63 expression, marker of cell pluripotency (Davydova, DA *et al*., 2009), for then appearing immediately after one day from the stimulation of differentiation, characterised by the induction of envoplakin expression, a protein induced with terminal differentiation (Karashima, T. and Watt FM., 2002). The appearance of ASPP2 expression corresponded to the decrease in p63 protein level, which became even more substantial after 5 days after the addition of Ca^{2+}-rich culture-medium. This observation – that ASPP2 induction during keratinocytes differentiation was found concomitant with p63 down-regulation – combined with the previously assessed tissue-localisation confined to the differentiated layers of the epithelium, indicates that ASPP2 is a *bona fide* marker of epithelial differentiation in the skin. In other words, these findings suggested that ASPP2 expression might be physiologically important in repressing p63 during skin development and in preventing p63 to be expressed throughout the upper layers of the adult skin. 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 11). Consistent with this, p63 overexpression has been reporter as potential cause of SCC formation in human SCC (Senoo, Tsuchiya *et al*., 2001; Reis-Filho, Torio *et al*., 2002).

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. 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.

4.3.2 Role of ASPP2 in regulating p63 at transcriptional level

Concerning p63 localisation in SCC we already know, from our own results with the ASPP2 mutant mice, as well as results from other groups, that p63 is expressed at very high level in the cells constituting the tumour mass. We have shown that ASPP2 can suppress p63 expression in human SCC cell lines 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. Since previous results were performed in adult tissue, our next important question was to investigate how ASPP2 can regulate p63 at transcriptional level. We started by checking whether ASPP2 could directly repress ΔNp63 expression. In this analysis we measured the transcriptional activity of ΔNp63 promoter by transfecting ΔNp63-luciferase reporter alone and/or in combination with ASPP2. It is important to mention that 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 (both in normal and in SCC cells) (Yang *et al*., 1998), where it drives the proliferation of basal keratinocytes, and it is also the isoform which is over-expressed in SCC (Proweller, Tu *et al*., 2006). In this assay, we could identify that ΔNp63 is capable of activating the expression of ΔNp63 promoter. This analysis was performed by luciferase transactivation assay in H1299, a human carcinoma cell line which expresses very low levels of both ASPP2 and p63. We observed that when ASPP2 was transfected in combination with ΔNp63 or p53, the luciferase activity shows no induction thus, we concluded that ASPP2 seems to have no additive effect on ΔNp63 induction, but just on its own promoter. Our results therefore suggest that ΔNp63 is able to activate the expression of ΔNp63 promoter, and the p53 protein seems to have no effect on ΔNp63 transcription (Figure 12). So, we can conclude that ASPP2 does not directly repress ΔNp63 expression.

In normal basal epithelial cells, ΔNp63 promotes proliferation through regulation of shared p53 gene targets, such as p21, and possibly through other pathways as well (Westfall *et al*., 2003). p63 is similar to p53 with regards to its ability to modulate specific genes that mediate cell cycle arrest and apoptosis – including BAX, p21 and PUMA (Mills, Alea A., 2006). Therefore, we measured the transcriptional activity of p21 promoter by transfecting ΔNp63 in presence of ASPP2 and we observed an increased expression of ΔNp63 proportional to increasing amounts of ASPP2, while neither ΔNp63 nor ASPP2 alone seem to have no additive effect (Figure 13).

Thereafter, we investigated whether ASPP2 could repress ΔNp63 expression at transcriptional level. We did not use the entire ΔNp63 promoter, but two deletion mutants, a longer and a shorter version, -740/+139 and -404/+139 promoters, respectively. Our hypothesis was that in the longer promoter there is a group of factors, still unknown, that prevent ASPP2 to bind and repress the expression of ΔNp63 (Figure 29).

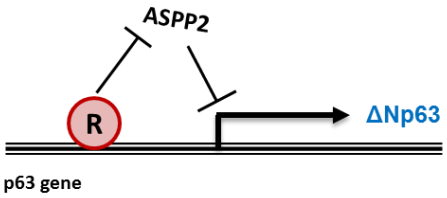


Figure 29 – Hypothetical model of the effect of the longer promoter (-740/+139).

As it is known that Notch is a repressor of p63, our approach was to test the expression of Notch alone when transfected with -740/+139 and -404/+139 promoters. Our results showed that Notch had no significant effect on the longer promoter but, interestingly, could repress the transactivation of the -404/+139 shorter promoter (Figure 14).

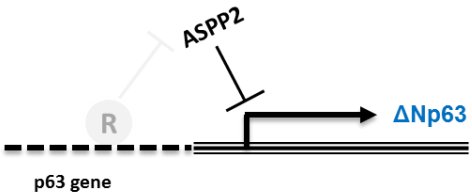


Figure 30 – Hypothetical model of the effect of the shorter promoter (-404/+139).

Considering that ASPP2 could behave similarly to Notch, we hypothesized that, the shorter promoter does not have the factors that function as repressors of ASPP2 expression, and consequently ASPP2 can inhibit the function of p63 (Figure 30). To test this hypothesis we transfected ASPP2 alone with the same reporter mutants, as we did previously for Notch. We observed that with the longer promoter ASPP2 had no significant effect neither alone nor when co-transfected with ΔNp63 (Figure 15, A). Interestingly, and in accordance with Notch activity, ASPP2 represses the transactivation of the shorter promoter (Figure 15, B) and had about 40% inhibitory effect on the short ΔNp63 promoter only.

In order to verify whether ASPP2 can repress ΔNp63 expression at transcriptional level and in a human SCC cell line HSC3, we transfected increasing amounts of ASPP2 alone with ΔNp63 promoter and, using the same lysate, we assessed the expression of endogenous p63 and ASPP2 by Western Blot (Figure 16). We could verify that the expression of p63 decreases while the values of ASPP2 increase, proving further evidence that ASPP2 can repress p63 function. In this experiment there was a problem with the loading control and, although the HSC3 cells might work better, they have lower transfection efficiency. Therefore, in the future, a squamous cell carcinoma cell line may be useful, but one with higher efficiency of transfection.

4.4 ASPP2 regulates ΔNp63 transcriptional function

Keratins, the major structural protein of all epithelia, are a diverse group of cytoskeletal scaffolding proteins that form intermediate filament networks, providing structural support to keratinocytes that maintain the integrity of the skin. Expression of keratin genes is usually regulated by differentiation of the epidermal cells within the stratifying squamous epithelium (Chamcheu, Jean *et al*., 2011).

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 found up-regulated in the tumours developed by the ASPP2-mutant mice (Figure 8). As ASPP2 was shown to directly bind to p53 and its family members, including p63, and to confer them binding selectivity onto specific DNA promoters (Samuels-Lev, O'Connor *et al*., 2001; Bergamaschi, Samuels *et al*., 2004), we 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 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 cell line and 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 Keratin10, the transcriptional induction due to ΔNp63 transfection was almost completely abolished and the transcription was brought back to basal levels (Figure 17 A-B). In an opposite direction, the transcription of envoplakin was further stimulated when ASPP2 was added to ΔNp63 (Figure 17 C). This finding indicates that ASPP2, which is not a transcriptional factor itself, has no intrinsic capacity to transactivate the ΔNp63 target genes but, nevertheless, it can regulate ΔNp63 transcriptional activity on them.

Interestingly, these results correspond to what we had previously observed in the mouse tumours characterised by decreased ASPP2 expression, which showed up-regulation of K14 and K1 (Figure 8). 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 10). 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.

Recent works from Dr Bergamaschi's group (2006) 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, regulating its 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 opposite effects on p63 expression, indicating 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 31).

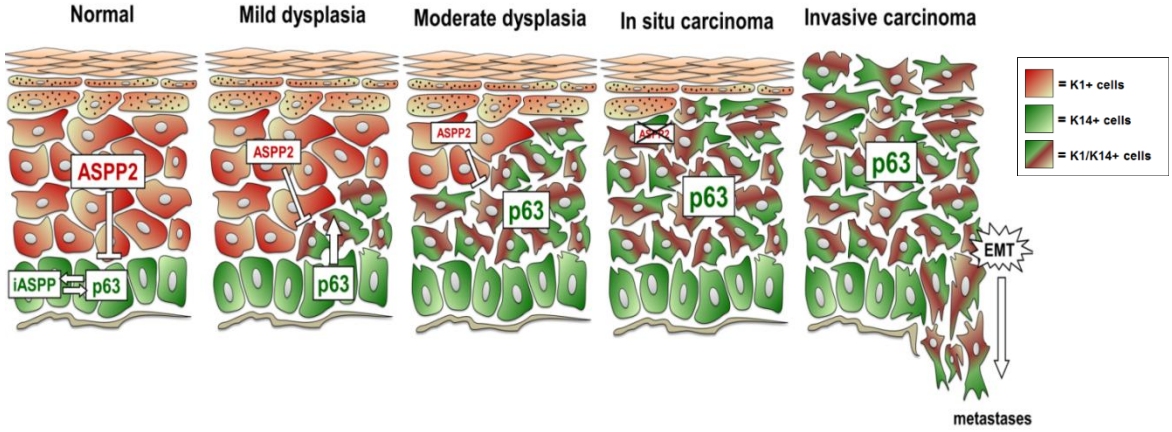


Figure 31 – Proposed model for ASPP2-mediated suppression of SCC. The presence of ASPP2 in the suprabasal layers of the epithelium repress and prevent p63 expression in these areas of the tissue. In case of impairment of ASPP2 function the expression of p63 would not be switched off in the transitional population of partially differentiated (K1 expression) suprabasal keratinocytes, allowing them to keep proliferating and giving origin to uncontrolled cell expansion (dysplasia and then carcinoma). Further decrease in ASPP2 expression levels could eventually favour formation of distant metastases (Notari M., 2011).

4.5 Cross-regulation between ASPP2 and Notch/ΔNp63 pathways

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. Notch signalling participates in the development of multicellular organisms by maintaining the self-

renewal potential of some tissues and inducing the differentiation of others (Raj K., Radtke F., 2003; Proweller, Tu *et al*., 2006).

In the epidermis the lack of Notch activity leads to tumour formation, making Notch a tumour suppressor protein (Proweller, Tu *et al*., 2006). In fact, in skin squamous epithelium, one of the principal ways by which Notch has a pro-differentiation role is by its ability to antagonise the expression and the activity of p63, whose role is to sustain the self-renewal of 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.

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. Since ASPP2 has a biological significance in the squamous epithelium, our group decided to test if ASPP2 and Notch pathways could physically and functionally interact. There are results showing that ASPP2 and Notch are co-expressed in the differentiated layers of the stratified epithelium and in the same cells in tumours. The consequences of ASPP2 and Notch interaction were also analysed, and it was found that the lack of ASPP2 resulted in deregulated Notch activity (Tordella L., unpublished data).

The Notch pathway regulates cell fate and differentiation through cell-cell communication (Kopan R. *et al*., 2009) and Keratins-14 are expressed in undifferentiated and proliferative keratinocytes in the basal cell layer. Nakagawa's group (2010) reconstituted the squamous epithelium in culture and found that Notch inhibition suppressed sharply epithelial stratification as well as early and late differentiation markers without affecting basal cell marker, i.e., K14 expression and proliferation (Nakagawa H. *et al*., 2010). These data suggest there is canonical Notch signalling in the early stages of squamous epithelial differentiation.

To better understand this interaction, we decided investigate whether ASPP2 combining with the Notch expression levels could also regulate Keratin-14 transcriptional activity. Then, we tested K14 transcriptional activity, in absence or presence of Notch/ASPP2 on the target genes (Figure 18). Here, we found that the transcription of K14 was induced by Notch expression, while ASPP2 had no significant effect when transfected alone. Nevertheless, when ASPP2 was transfected with Notch, the transcriptional induction due to Notch transfection was almost completely abolished and the transcription resulted in basal levels. The same effect appeared when ASPP2 was transfected with ΔNp63, as we already discussed in the previous section. We can conclude that the presence of ASPP2 is crucial to inhibit the induction of K14 mediated by both p63 and Notch.

4.6 Notch and p73 share communal target genes and they are both regulated by ASPP2

Cell cycle control is paramount in maintaining normal growth and differentiation of cells and both p53 and p73 are important tumor suppressor genes that regulate the cell cycle via apoptosis and cell cycle arrest. Although the p73 protein does not function as a traditional tumor suppressor gene, its high level of sequence homology with the DNA-binding domains of p53 enables p73 to transactivate p53-response genes, resulting in cell cycle arrest, DNA repair and apoptosis (Li, G. *et al.*, 2012).

The p53 family member p73 is known to be an important mediator of apoptosis in response to DNA damage, chemotherapy, and other stimuli (Urist *et al.,* 2004). Nevertheless, mutation of p73 is not observed at a significant frequency as it is in p63 in human cancers and, additionally, despite their remarkably similar structures, p63 and p73 appear to regulate largely non-overlapping sets of cell-cycle regulatory genes (Melino *et al.,* 2003; Ellisen, L. *et al*., 2006). Furthermore, a significant prevalence of p73 overexpression has been found in numerous different tumour types including tumours of breast, neuroblastoma, lung, colon, stomach, oesophagus, colon carcinoma and head and neck squamous carcinoma. Importantly, patients with high global p73 expression had a worse survival rate than patients with undetectable levels (Slade, N. *et al.*, 2004).

Su's group (2009) identified that TAp73 as a critical factor for neural stem cell maintenance. Indeed, the TAp73 isoform is more highly expressed than the ΔNp73 isoform in neural stem cells (Talos, F. *et al.,* 2010). This phenotype is reminiscent of the identified role of TAp63 in maintaining stem cells in the dermis, which are necessary for wound healing and hair regeneration (Su, X. *et al*., 2009). As the Notch and p73 have similar roles; TAp73 isoform is more highly expressed than the other isoform (Talos, F. *et al*., 2010); and we verified a better effect and a higher activation with TAp73 isoform than with ΔN isoform (Figures 19-20), later on, our group decided to focus on only one isoform, TAp73. Here we tested Bax transcriptional activity, in absence or presence of TAp73/ΔNp73 alone or in combination with ASPP2 on the target genes (Figure 19). We could verify that both isoforms can induce the transcription of Bax promoter, the TA isoform has a better effect on the activation of the reporter and ASPP2 clearly enhanced the ability of p73 to transactivate the Bax promoter at high levels (Figure 19).

After that, we tested the same conditions for Hey2 promoter, a Notch target gene (Fisher A. *et al.*, 2004). We analysed the transcriptional activity of Hey2, in absence or presence of TAp73 or ΔNp73 alone or in combination with ASPP2 (Figure 20). We could verify that both isoforms can induce the transcription of Hey2 promoter, and the TA isoform has a better effect on the activation of the reporter, additionally ASPP2 only induces the transcription of the promoter at high levels.

There are data that define a Tap73-Hey2 transcriptional pathway that, when disrupted, causes depletion of adult neural stem cells (Kaplan, D. *et al.*, 2010; Flores, 2011). Having observed that the absence of ASPP2 had a selective impact on the expression levels of some of the major Notch target genes, as Hey2, we next tested if ASPP2 can have a direct effect on its transcription using transactivation assays. We transfected H1299 cells – chosen because of their high degree of transfectability – with luciferase reporter plasmids for Hey2 promoter in combination with different amounts of ASPP2 expression construct. Interestingly, ASPP2, which is not a transcriptional factor itself, can induce Hey2 transcriptional activity when transfected alone, however these are preliminary data and the mechanism is still unknown.

Notch pathway has a broad fundamental role in cell fate determination and its outcome then acquires specific connotations in different tissues depending on the interactions with different environmental factors. As Notch has an important role in promoting differentiation in the skin, we analysed the expression of some of p73 major target genes, Hey2 and Bax, in absence or presence of TAp73. Notch was found able to induce activation of the promoters in a dose dependent manner, while Tap73 by itself had no impact on them. However, when Tap73 was expressed together with Notch, it was able to modulate Notch capacity to transactivate its gene targets. In particular, Tap73 diminished the ability of Notch to transactivate Hey2 and Bax and, in the specific case of Hey2, the transcription almost returned to basal levels (Figure 22).

4.7 Effect of Notch and p73 on reciprocal target genes

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) (Notari *et al*., 2011). This variety is due to Notch transcriptional selectivity producing differential combinations of genes being expressed for different tissues or cell types, according to the scope required. A study on the effect of Notch and p73 on reciprocal target genes was carried

out with ASPP2, Tap73 and Notch factors. Transcriptional activity of Hey2, Bax, Hes1 and Hes5 promoters was measured by Luciferase reporter assay, by transfecting into Saos-2 cells each promoter in presence of ASPP2, Notch or Tap73 expression vectors. The most interesting results, as explained above, were that Notch activates Bax promoter - which is known to be a p73 target gene - and this is consistent with results from Shen's group in which they observed that when Notch is induced, a significant transcriptional up-regulation of the target gene Bax occurs (Shen J. *et al*, 2004); and the most interestingly is that ASPP2 alone activates Hey2 promoter – as data are still preliminary, the mechanism is still unknown.

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, on the fact that deregulation in Notch pathway activity is observed in the ASPP2 Δexon3 mice and that the two proteins, ASPP2 and Notch were found co-expressed in same cells *in vivo* (Ohashi, Natsuizaka *et al*., 2011), there is a possibility for a direct interaction. Indeed, results from our laboratory 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 (data not shown).

Following the same reasoning, we focused on both promoters Bax and Hey2, and proceeded to all possible combinations between ASPP2, Notch and Tap73 (Figure 23). It was the first time that these results were observed with these promoters (Bax and Hey2) and in the specific Saos-2 cell line, so these results are preliminary and the mechanisms will be investigated later. Currently, the group has been interested in understanding mechanistically how ASPP2 can enhance or inhibit Notch transcription on some of its target genes. Chromatin immunoprecipitation results 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.

For Bax and Hey2 promoters, we verified consistently that ASPP2, which, as already mentioned, is not a transcriptional factor itself and has no intrinsic capacity to transactivate, can regulate both Bax and Hey2 transcriptional activity when co-transfected with TAp73, more specifically on Bax and Hey2 promoters. We also verified that Notch in combination with TAp73, is able to increase its effect and together induce the expression of Bax promoter, which is interesting, because Bax is known to be a p73 target gene. Considering the implications of the ASPP2-Notch heterodimer formation, we verified again that ASPP2 is generally able to enhance Notch transcription of genes. In relation to the latter results, it is worth to mention that they seem to be cell line dependent, as we repeated the same experiments in H1299 cell line

and the results were slightly different. For example, in H1299 cells, ASPP2 did not activate Hey2 promoter, or TAp73, instead, it activated BAX and Notch, both in a lesser extent than in Saos-2 cells.

TAp73α is induced by a wider variety of chemotherapeutic agents in different tumour cell lines and, conversely, blocking TAp73 function leads to enhanced chemoresistance, which is independent of the p53 gene status (Slade, 2004). Notch function, in general, is important to confer cell fate determination and its function is critically context dependent. Importantly, Notch activity seems to be generally regulated by cross-talks with pathways physiologically important for the homeostasis of each given tissue. There are evidences that support the existence of a possible cross-regulation between ASPP2 and Notch pathway, and also that both proteins are expressed in the differentiated layers of the squamous epithelium and the depletion of any of them from this compartment leads to the formation of spontaneous SCC with elevated expression of p63 (Sottocornola R. *et al*., 2010). These results support that ASPP2 is a factor capable of influencing Notch transcriptional programme and both Notch and p73 share communal target genes and they can be both regulated by ASPP2.

Chapter V: Conclusions and Outlook

Having observed that ASPP2 and p63 gene expression are negatively correlated in the squamous epithelium, this would argue that in the adult tissue ASPP2 would be important to prevent p63 to be expressed in differentiated cells. Our data provides evidence that ASPP2 plays an important physiological role in the maintenance of the squamous epithelium. We have unequivocally demonstrated that one of ASPP2 roles in the epithelium is characterised by its inhibition of ΔNp63 (is the main p63 isoform expressed in the skin where it has a) potentially oncogenic expression and, as a consequence, ASPP2 is found down-regulated also in human SCCs. We showed that ASPP2-mediated suppression of SCC is achieved by repressing ΔNp63 expression. p63 transcriptional factor is a master regulator of epithelial homeostasis, fundamental during development as well in the adult tissue to maintain the proliferative and stemness potential of the basal layer of the squamous epithelium.

Interestingly p63, despite been predominantly a basal cell marker, can be still expressed at low level in suprabasal cells and 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. ASPP2 expression is induced upon keratinocytes differentiation and this coincides with a reduction in p63. On the contrary, during tumour progression, ASPP2 is downregulated from normal epithelium to SCC, while p63 is upregulated, defining a mutual exclusive expression pattern between the two proteins. Additionally, ASPP2 mode of action does not seem to be limited to the repression of p63 protein levels, but it also applies to the regulation of p63 transcriptional program, by inhibiting p63-mediated transcription. This indicates that ASPP2 is not only a marker of cell differentiation, but also an active player in the process, mainly acting on p63 regulation. We can conclude that ASPP2 could be a gatekeeper of epidermal differentiation challenging p63 function.

Notch protein is another key regulator of p63 and promotes cell differentiation in the squamous epithelium; it has been shown to be expressed in suprabasal keratinocytes and, once activated, is able to repress p63 expression (Nguyen, Lefort *et al*., 2006). Here we showed that ASPP2 is a factor capable of influencing Notch transcriptional programme, both Notch and p73 share communal target genes and they can be both regulated by ASPP2.

ASPP2 is a factor able to confer transcriptional selectivity and our model shows that ASPP2 is a new important player in the cross-talk with Notch and this interplay could be important for the homeostasis of the squamous epithelial compartment. 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 that p63 and Notch expression and activity can be balanced in the squamous epithelium, providing a correct equilibrium between proliferation and differentiation.

As 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 – in the future, it could be interesting to explore this possibility, which would allow us to establish a fundamental link between our experimental findings and the human clinical data. It could be also interesting to produce expression constructs for the known mutated forms of Notch found in human SCC samples and see if they fail to interact with ASPP2 (physically and functionally). In order to obtain more information about the mechanism, it would also be interesting to investigate the differences between the two cell lines, Saos-2 and H1299, since we found there is an effect Saos-2 dependent on p73 target genes. Consequently, it could be tested more Notch and p73 target genes for the study of communal target genes regulated by ASPP2.

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