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Identification of novel and direct target genes of p73

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ABSTRACT

The p53 paralogues p73, p63 and their respective truncated isoforms have been shown to be critical regulators of developmental and differentiation processes. Indeed, both p73 and p63 deficient mice exhibit severe developmental defects. Here, we show that the S100A2 gene, whose transcript and protein are induced during keratinocyte differentiation of HaCaT cells, is a direct transcriptional target of p73β and ΔNp63α and is required for proper keratinocyte differentiation. Transactivation assays reveal that p73β and ΔNp63α exert opposite transcriptional effects on the S100A2 gene. While ΔNp63α is found *in vivo* onto S100A2 regulatory regions predominantly in proliferating cells, p73β is recruited in differentiating cells. Silencing of p73 impairs the induction of S100A2 during the differentiation of HaCaT cells. Moreover, silencing of p73 or S100A2 impairs the proper expression of keratinocyte differentiation markers. Of note, p53 family members do not trigger S100A2 gene expression in response to apoptotic doses of cisplatin and doxorubicin.

The p53 family is also known to be involved in the transcriptional control of growth arrest and apoptosis. Despite the recent identification of specific p73-target genes by genome-wide expression profile techniques, p73-mediated apoptosis occurs mostly through the activation of a set of genes that were originally found to be activated by p53. This suggests that promoter selectivity by both p53 and p73 might be the result of biochemical events such as post-translational modifications and specific protein-protein interactions.

The transcriptional coactivator Yes-associated protein (YAP) has been demonstrated to interact with and to enhance p73-dependent apoptosis in response to DNA damage. Here we show the existence of specific target genes whose transcriptional activation during the apoptotic response requires both p73 and YAP. In particular p73 and YAP are concomitantly recruited onto the regulatory regions of the promyelocytic leukemia gene (PML); an essential event for PML induction after cisplatin treatment. Moreover, sequestring YAP into the cytoplasm by a constitutively active mutant of AKT leads to a reduction of p300 recruitment onto the PML regulatory regions which correlates with a reduction in histone acetylation and a reduction in PML expression. Finally, we show that PML binds to YAP and plays a role in the regulation of YAP half-life, preventing its ubiquitinylation and subsequent degradation.

To my family
and to the love of my life

CONTENTS

INTRODUCTION

p53 family

p53 was first observed to co-immunoprecipitate with the large and small T antigens in Simian virus 40-transformed cells (Kress *et al.*, 1979; Lane and Crawford, 1979; Linzer and Levine, 1979). Since that time, p53 has evolved from a potential oncogene to the principal tumour suppressor in mammals: its inactivation is a precondition to most human cancer. Further confirmation of the role of p53 in tumour suppression has come from animal models that show increased tumorigenesis in p53-null mice (Donehower *et al.*, 1992). p53 has been recognized as a guardian against cellular stressors, particularly those that inflict DNA damage. It is a transcription factor which exerts its protective effects by inducing cell-cycle arrest to allow repair processes or, falling that, by promoting cellular senescence or apoptosis (Levine, 1997).

Although p53 was long considered to be unique, two novel family members were identified and termed p73 and p63 (Kaghad *et al.*, 1997; Yang *et al.*, 1998). Both genes give rise to proteins that have both entirely novel functions and p53-related functions. The gene structure of p53, p63 and p73 is highly conserved from mollusk to human. The three most conserved domains in all three genes are the N-terminal transactivation domain, the central DNA binding domain and the C-terminal oligomerization domain. Both p63 and p73 share >60% amino acid identity with the DNA binding region of p53 (and even higher identity among themselves), including conservation of all DNA contact and structural residues that are hotspots for p53 mutations in human tumours. In addition, p73 shows 38% identity with the p53 tetramerization domain and 29% identity with the p53 transactivation domain. In vertebrates, the p73 and p63 genes are ancestral to p53 and possibly evolved from a common p63/p73 archetype (Kaghad *et al.*, 1997; Yang *et al.*, 1998).

In addition to their similar protein structure, the three members are prone to alternative splicing and their transcription is driven by alternative promoters, giving rise to a complex expression of proteins. So far, three p53 proteins were identified: the full length, the Δ40p53 (also known as ΔNp53 or p47) lacks the first 40 amino acids resulting from either an alternative splicing of the intron 2 or an alternative initiation of translation and p53i9 which results from an alternative splicing of intron 9 and lack the last 60 amino acids. Δ40p53 partially possesses the transactivation domain and it is therefore able to transactivate p53 target genes but mainly acts as a dominant negative inhibitor of the full length protein (Courtois *et al.*, 2002; Ghosh *et al.*, 2004). p53i9 is defective in transcriptional activity and lacks DNA

binding ability (Flaman *et al.*, 1996). However, recent investigations using the RACE technique showed that p53 gene structure is similar to its counterpart members (Bourdon *et al.*, 2005). p53 also possesses a second promoter located within intron 4 from which the mRNA transcript gives rise to a protein lacking the first 133 amino acids and to two C-terminal splice variant proteins lacking the tetramerization domain. The first promoter generates six proteins including the full length, two proteins lacking the tetramerization domain (p53β and γ) and three Δ40p53 proteins (Δ40p53, Δ40p53β and Δ40p53γ).

p63 and *p73* have two promoters: P1 in the 5' untranslated region upstream of the noncoding exon 1 and P2 within the 23 kb spanning intron 3. P1 and P2 promoters produce two diametrically opposing classes of proteins: those containing the TA (TAp63 and TAp73) and those lacking it (ΔNp63 and ΔNp73). ΔNp63 and ΔNp73 occur in human and mouse. In addition, alternative exon splicing of the P1 transcripts of *p63* and *p73* give rise to other isoforms lacking the transactivation domain (e.g., ΔN'p73, Ex2Delp73, and Ex2/3Delp73; Kaghad *et al.*, 1997; Stiewe *et al.*, 2002; Fillippovich *et al.*, 2001; Ishimoto *et al.*, 2002). Of importance, the ΔNp73 and ΔN'p73 transcripts encode the *same* protein due to the use of a second translational start site because of an upstream premature stop in ΔN'p73 (Ishimoto *et al.*, 2002). TA proteins mimic p53 function in cell culture including transactivating many p53 target genes and inducing apoptosis, whereas (the collectively called) ΔTA proteins act as dominant-negative inhibitors of themselves and of other family members in vivo in the mouse and in transfected human cells (Yang *et al.*, 1998; Yang *et al.*, 2000; Pozniak *et al.*, 2000). Strikingly, the *p63* locus is contained within a frequently amplified region in squamous cell carcinoma (Hibi *et al.*, 2000), and squamous epithelium of the skin and squamous carcinoma produce high levels of ΔNp63α (also called $p68^{AIS}$). Furthermore, ΔNp73 is the predominant *p73* product in the developing mouse nervous system and is required to counteract the proapoptotic action of p53 (Yang *et al.*, 2000; Pozniak *et al.*, 2000).

Additional complexity is generated at the COOH terminus: *p73* and *p63* undergo multiple COOH-terminal splicings of exons 10 to 14, skipping one or several exons. Thus far, nine transcripts were found for *p73*: α, β, γ, δ, ε, τ, η, η_1, and φ (α being full-length; Ishimoto *et al.*, 2002; Kaghad *et al.*, 1997; Stiewe *et al.*, 2002), and three were found for p63: α, β, and γ (Yang *et al.*, 1998). The p73 isoforms φ, η, and η_1 lack the second COOH-terminal TA and the tetramerization domain encoded by exon 10

(Stiewe *et al.*, 2002; Ishimoto *et al.*, 2002). In some COOH-terminal isoforms, exon splicing also leads to unique sequences due to frameshifts. For *p63*, three isotypes (α, β and γ) are made. Splicing of different "tails" further modulates the p53-like function of TA proteins, although they do not appear to vary much in their role in tumorigenesis. Structurally, the γ forms of *p73* and *p63* most closely resemble p53 itself, harboring just a small COOH-terminal extension beyond the last 30-amino acid stretch of p53. Surprisingly, whereas TAp63γ (also called p51A) is as powerful as p53 in transactivation and apoptosis assays (Yang *et al.*, 1998), TAp73γ is rather weak. The αforms of *p73* and *p63* contain an additional highly conserved sterile αmotif (SAM). SAMs are protein-protein interaction modules found in a wide variety of proteins implicated in development. In addition, the p73 SAM domain can bind to anionic and zwitterionic lipid membranes (Barrera *et al.*, 2003). The crystal and solution structures of p73 SAM agree with each other and feature a five-helix bundle fold that is characteristic of all SAM domain structures (Chi *et al.*, 1999; Wang *et al.*, 2001). Other SAM-containing proteins are the ETS transcription factor TEL that plays a role in leukemia, the polycomb group of homeotic transcription factors, and the ephrin receptors. Despite predictions of homo- and hetero-oligomerization of SAM-containing proteins, p73 SAM appears monomeric by experimental analysis, casting doubt whether this domain mediates interaction of p73 with heterologous proteins (Wang *et al.*, 2001). There are also functional differences between TAp73α and TAp63α. Whereas TAp73α is comparable with p53 in potency in transactivation and apoptosis assays, TAp63α (also called p51B) is very weak (Yang *et al.*, 1998). One reason for this difference could be that p63α isoforms contain a 27-kDa COOH-terminal region that drastically reduces its transcriptional activity (Serber *et al.*, 2002). This domain is necessary and sufficient for transcriptional inhibition and acts by binding to a region in the NH_2-terminal TA of p63, which is homologous to the MDM2 binding site in p53. Of note, this transactivation inhibitory domain is biologically important, because patients with deletions in this p63 domain have phenotypes very similar to patients with mutations in the DBD (Serber *et al.*, 2002).

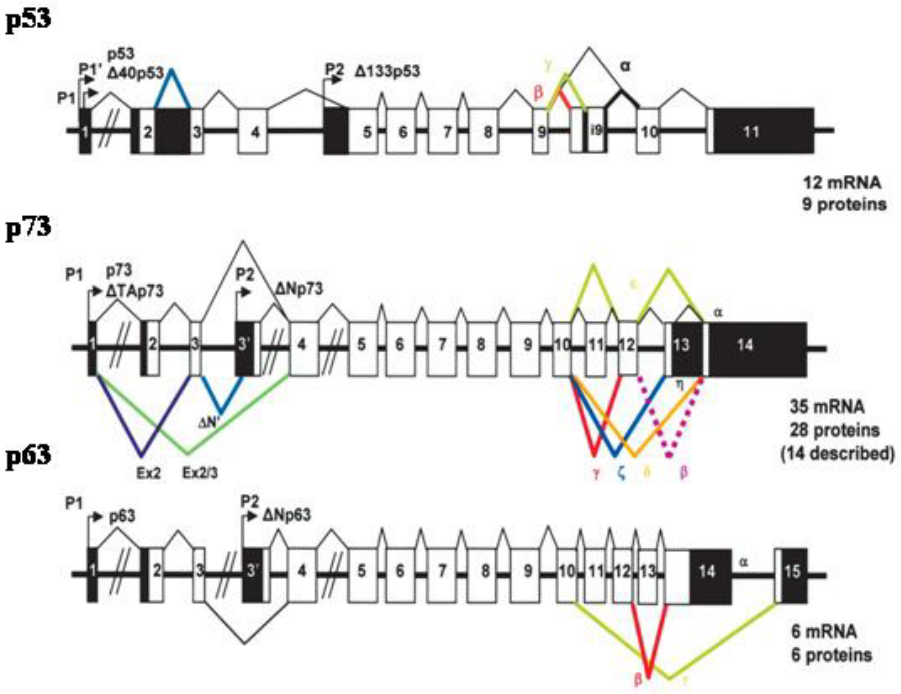


Fig.1. Structure of the human p53, p63, and p73 genes.

The p53 family includes the three genes p53, p63 and p73. They have a modular structure consisting of the transactivation domain (TA), the DNA binding domain (DBD) and the oligomerization domain. All genes are expressed as two major types: full-length proteins containing the TA domain and ΔN proteins missing the TA domain. In addition, extensive COOH-terminal splicing further modulate the p53-like functions of the TA proteins.

p63 and p73 play important roles in development and differentiation

Both genes play important and, despite their structural similarity, surprisingly unique roles in mouse and human development. This is powerfully revealed by the striking developmental phenotypes of p63- and p73-deficient mice (Yang *et al.*, 2000; Yang *et al.*, 1999; Mills *et al.*, 1999) and is in contrast to p53-null mice, which are highly tumor prone but lack a developmental phenotype.

p63

p63 expression is absolutely essential for limb formation and epidermal morphogenesis (integument and tongue) including the formation of adnexa (teeth, hair, mammary and prostate glands, and sweat and lacrimal glands). p63-null animals show severe limb truncations or absence of limbs and absence of skin and craniofacial malformations. They also fail to develop skin and most epithelial tissues (e.g., prostate and mammary glands). The animals do not survive beyond a few days postnatally. Reminiscent of the knockout phenotype in mice, heterozygous germ line point mutations of p63 in humans cause six rare autosomal dominant developmental disorders

Importantly, basal cells of normal human epithelium including the epidermis strongly express p63 proteins, predominantly the ΔNp63 isotype (ratio is ~100:1 of ΔNp63 to TAp63; Yang *et al.*, 1998), but lose them as soon as these cells withdraw from the stem cell compartment (Pellegrini *et al.*, 2001). Consistent with this notion, keratinocyte differentiation is associated with the disappearance of ΔNp63α (Parsa *et al.*, 1999; Nylander *et al.*, 2000; Westfall *et al.*, 2003), whereas the expression of p53 target genes p21 and 14-3-3σ, mediating cell cycle arrest, increase. p63 binds p21 and 14-3-3σ promoters and represses them. p63 is also indispensable for the differentiation of a transitional urothelium and is expressed in normal bladder urothelium. p63 is lost in most invasive bladder cancers (Urist *et al.*, 2002).

Together, these data clearly establish a fundamental role of p63 in epithelial stem cell biology and in the apical ectodermal ridge of the limb bud, where p63-expressing cells create a signaling center (Pellegrini *et al.*, 2001). Whether this role is one in stem cell self-renewal or in stem cell differentiation into stratified epithelium remains a matter of controversy (Yang *et al.*, 1999; Mills *et al.*, 1999). In one model, p63 is required for the ectoderm to commit to epidermal lineages (Yang *et al.*, 1999; Mills *et al.*, 1999), whereas, in the other model, p63 is not required to commit but to

maintain the stem cell pool and prevent it from differentiation (Brunner *et al.*, 2002).

p73

p73 also has distinct developmental roles. *p73* expression is required for neurogenesis of specific neural structures, for pheromonal signaling, and for normal fluid dynamics of cerebrospinal fluid (Yang *et al.*, 2000). The hippocampus is central to learning and memory and continues to develop throughout adulthood. p73-null animals exhibit hippocampal dysgenesis due to the selective loss of large bipolar neurons called Cajal-Retzius in the marginal zone of the cortex and the molecular layers of the hippocampus. These Cajal-Retzius neurons are responsible for cortex organization and coexpress ΔNp73 and the secretory glycoprotein reelin. In addition, p73-null mice have severe malformations of the limbic telencephalon. They also suffer from hydrocephalus (~20%) probably due to hypersecretion of cerebrospinal fluid by the choroid plexus and from a hyperinflammatory response (purulent but sterile excudates) of the respiratory mucosa likely due to mucus hypersecretion. Moreover, the animals are runted and show abnormal reproductive and social behavior due to defects in pheromone detection. The latter abnormality is due to a dysfunction of the vomeronasal organ, which normally expresses high levels of p73.

Role of ΔNp73 in Mouse Development

ΔNp73 is the predominant form in the developing mouse brain and might act as a repressor (Yang *et al.*, 1998; Pozniak *et al.*, 2000). In situ hybridization reveals strong ΔNp73 expression in E12.5 fetal mouse brain in the preplate layer, bed nucleus of stria terminalis, choroid plexus, vomeronasal area, and preoptic area (Yang *et al.*, 2000). Moreover, ΔNp73 is the only form of p73 found in mouse brain and the sympathetic superior cervical ganglia in P10 neonatal mice (Pozniak *et al.*, 2000). Functional studies and knockout mice showed that ΔNp73 plays an essential antiapoptotic role in vivo. ΔNp73 is required to counteract p53-mediated neuronal death during the normal "sculpting" of the developing mouse neuronal system (Pozniak *et al.*, 2000). Withdrawal of nerve growth factor, an obligate survival factor for mouse sympathetic neurons, leads to p53 induction and p53-dependent cell death. Conversely, nerve growth factor withdrawal leads to a decrease of ΔNp73. Importantly, sympathetic neurons are rescued from cell death after nerve growth factor withdrawal when ΔNp73 levels are maintained by viral delivery. Likewise, sympathetic

neurons are rescued from Adp53-mediated neuronal death by coinfected AdΔNp73. In pull-down assays, mixed protein complexes of p53/ΔNp73 were demonstrated, suggesting one biochemical basis for transdominance in addition to possible promoter competition. Together, these data firmly put ΔNp73 downstream of nerve growth factor in the nerve growth factor survival pathway. It also explains why $p73^{-/-}$ mice, missing all forms of p73 including protective ΔNπ73, undergo accelerated neuronal death in postnatal superior cervical ganglia (Pozniak *et al.*, 2000).

In tissue culture models, p73 also plays a role in differentiation of several cell lineages. *p73* expression increases during retinoic acid–induced and spontaneous differentiation of neuroblastoma cells (De Laurenzi *et al.*, 2000; Kovalev *et al.*, 1998). In addition, ectopic TAp73β but not p53 induce morphologic and biochemical markers of neuroblastoma differentiation (De Laurenzi *et al.*, 2000). Moreover, expression of specific COOH-terminal isoforms correlates with normal myeloid differentiation. p73α and p73β are associated with normal myeloid differentiation, whereas p73γ, p73δ, p73ε, and p73θ are associated with leukemic blasts. In fact, p73ε is specific for leukemic blast cells (Tschan *et al.*, 2000). Similarly, TAp73γ and TAp73δ may play a role in the terminal differentiation of human skin keratinocytes (De Laurenzi *et al.*, 2000). This suggests a p73-specific differentiation role that is not shared by p53 and, for the most part, not shared by p63 either. p53 has an important developmental role in early mouse embryogenesis (E7-8d) as revealed when the autoregulatory feedback loop with MDM2 is removed and p53 levels remain uncontrolled (Montes *et al.*, 1995; Jones *et al.*, 1995). Nevertheless, in stark contrast to *p63*- and *p73*-null mice, *p53*-null mice make it through development with essentially no problems (with the exception of rare exencephaly in females; Donehower *et al.*, 1992; Jacks *et al.*, 1994).

p63 and p73 expression in normal human tissues

p73 gene expression occurs at very low levels in all normal human tissues studied (Kovalev *et al.*, 1998; Ikawa *et al.*, 1999), making detection difficult. p63, mainly its ΔN form, occurs at higher levels and is readily detectable at the protein level. In embryonic epidermis, p63 is the molecular switch for initiation of an epithelial stratification program (Koster *et al.*, 2004). In postnatal epidermis, p63 expression is restricted to the nuclei of basal cells of normal epithelia (skin, esophagus, tonsil, prostate, urothelium, ectocervix,

and vagina) and to certain populations of basal cells in glandular structures of prostate, breast, and bronchi (Yang *et al.*, 1999, Di Como *et al.*, 2002). Specifically, p63 is expressed in myoepithelial cells of the breast and considered to be the specific marker for those cells in normal breast tissue (Ribeiro-Silva *et al.*, 2003; Reis-Filho *et al.*, 2003). p63 expression in prostate is restricted to basal cells, making it an excellent diagnostic marker in prostate cancer. The vast majority of prostate cancers and preinvasive prostate intraepithelial neoplasia lesions have lost p63 expression. Basal cells play important roles in differentiation and carcinogenesis of the prostate (Davis *et al.*, 2002; Garraway *et al.*, 2003).

Transcriptional and apoptotic activity of p63 and p73

In general, many functional parallels are found among p53, TAp73, and TAp63 on the one hand and among ΔNp73 and ΔNp63 on the other hand. When ectopically overexpressed in cell culture, p73α and p73β closely mimic the transcriptional activity and biological function of p53. p73β and, to a lesser extent, p73α bind to canonical p53 DNA binding sites and transactivate many p53-responsive promoters (Jost *et al.*, 1997; Di Como *et al.*, 1999; Zhu *et al.*, 1998; Lee *et al.*, 1999), although relative efficiencies on a given p53 target promoter may differ from p53 and also differ among various COOH-terminal isoforms of TAp73 and TAp63 (Zhu *et al.*, 1998; Lee *et al.*, 1999). In reporter assays, p73-responsive promoters include well-known p53 target genes involved in antiproliferative and proapoptotic cellular stress responses such as $p21^{WAF1}$, 14-3-3σ, GADD45, BTG2, PIG3 (Zhu *et al.*, 1998), ribonucleotide reductase p53R2 (Nakano *et al.*, 2000), and IGFBP3 (Steegenga *et al.*, 1999). Bax transactivation is controversial (Zhu *et al.*, 1998, Steegenga *et al.*, 1999). TAp73α and TAp73β also induce MDM2. Conversely, ectopic p73 overexpression leads to transcriptional repression of vascular endothelial growth factor, analogous to the ability of p53 to transcriptionally suppress vascular endothelial growth factor (Salimath *et al.*, 2000). Although there are probably still dozens of common targets that have not yet been described or discovered, it will be important to identify p63/p73-preferred or p63/p73-specific targets. For example, Fontemaggi *et al.* have shown through microarray analysis that inducible expression of p53

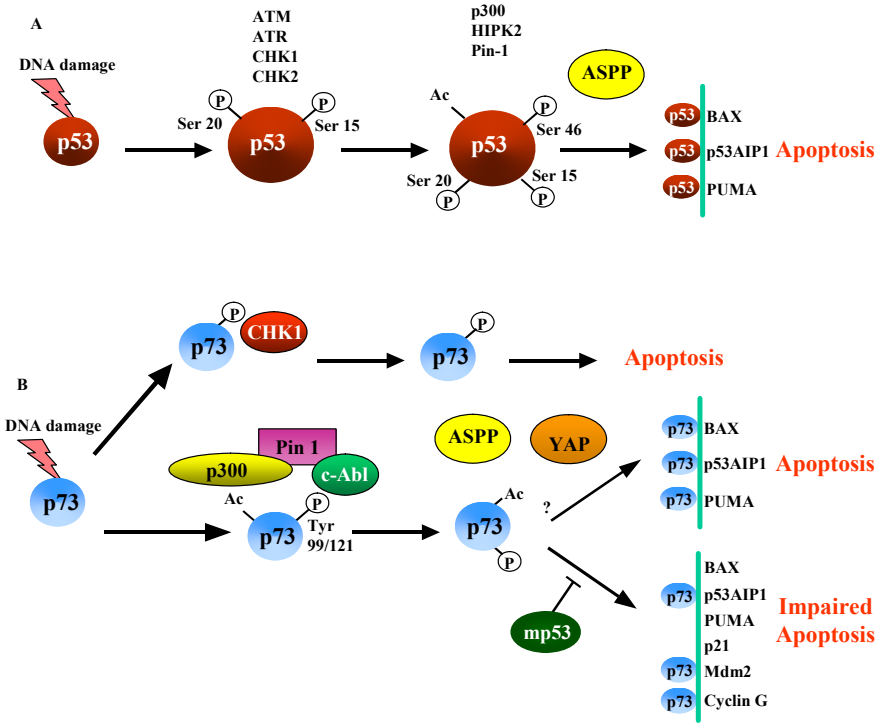


Fig.2. p53 family members and DNA damage-induced apoptosis.

(A) In response to DNA damage, p53 is stabilized, phosphorylated at specific serine and threonine residues and acetylated. These post-traslational modifications result in the potentiation of p53-mediated apoptosis.
(B) In response to DNA damage, p73 is stabilized, phosphorylated in specific tyrosine, serine and threonine residues and acetylated. Both c-Abl-mediated tyrosine phosphorylation of p73 and the recruitment of selective coactivators could define the selectivity of p73-mediated apoptosis. Mutant p53 can act as an inhibitor of p73 by the formation of protein complexes comprising mutant p53 and p73.

or p73α promotes the transcriptional activation or repression of common as well as quite distinct patterns of direct target genes (Fontemaggi *et al.*, 2002). Moreover, 14 novel target genes that are differentially regulated by various p53 family members were recently identified (Chen *et al.*, 2003).
A surprising "essential cooperativity" among family members for transcriptional function was recently found. In response to DNA damage, induction of $p21^{WAF1}$ (mediating cell cycle arrest) occurred normally in $p63^{-/-}$ and $p73^{-/-}$ single null mouse embryo fibroblasts (MEFs) and $p63/p73^{-/-}$ double null MEFs. However, in double null MEFs, the induction of Bax, Noxa, and PERP genes (thought to mediate apoptosis) was suppressed. Chromatin immunoprecipitation assays confirmed that there is no binding of p53 to the Bax, PERP, and NOXA promoters in the absence of p63 or p73, whereas, conversely, p63 still binds to them in $p53^{-/-}$ single null MEFs. These data demonstrate that either p63 or p73 are essential for p53-induced apoptosis (Flores *et al.*, 2002). Ectopic p73 promotes apoptosis in human tumor cell lines independent of their p53 status (Kaghad *et al.*, 1997; Jost *et al.*, 1997). In fact, in a subset of cancer cell lines, p73β is more efficient in inducing apoptosis than p53 itself (Ishida *et al.*, 2000). Potency differences exist among the COOH-terminal isoforms. Overexpression of p73α, p73β, and p73δ suppresses focus formation of p53-deficient Saos-2 cells, whereas p73γ fails or suppresses only very poorly (Jost *et al.*, 1997; Ishida *et al.*, 2000; De Laurenzi *et al.*, 1998). Similarly, TAp63α lacks significant transcriptional and apoptotic ability, whereas TAp63γ is very potent in both (Yang *et al.*, 1999).

Regulation of p73 protein stability and transcriptional activity

Proteasomes are mediating the turnover of p73 proteins because proteasome inhibitors stabilize p73 isoforms (Balint *et al.*, 1999). In sharp contrast to p53, however, p73 degradation is not mediated by MDM2. The molecular basis for the MDM2 resistance of p73 was found by systematic motif swapping. Region 92-112 of p53, which is absent in p73, was identified to confer MDM2 degradability to p53 (Gu *et al.*, 2000). p73 protein is also resistant to human papillomavirus (HPV) E6, which together with E6-AP mediates hyperactive degradation of p53 in HPV-infected cells (Balint *et al.*, 1999; Marin *et al.*, 1998). Just as MDM2 does not mediate p73 degradation, $p19^{ARF}$, which stabilizes p53 levels by antagonizing the degrading action of MDM2, has not been shown to stabilize p73 protein. One

potential consequence of the differential MDM2 sensitivity between p53 and p73 was seen in tissue culture: ectopic coexpression of p73 leads to a selective decrease of ectopic p53 and endogenous induced p53 because p53 is susceptible to MDM2, whereas p73 is not (Wang *et al.*, 2001). This suggests a potential down-modulation of p53 by high levels of TAp73 (because MDM2 is also a p73 target), an interesting family twist to keep in mind with respect to tumor formation. On a transcriptional level, however, the negative feedback regulation between the two genes is preserved. MDM2 is transcriptionally activated by p73 and in turn negatively regulates the transcriptional ability of p73, just as it functions toward p53 (Balint *et al.*, 1999; Dobbelstein *et al.*, 1999; Zeng *et al.*, 1999). However, the mechanism is again distinct from p53. The binding to MDM2 causes the disruption of physical and functional interaction with p300/cAMP-responsive element binding protein by competing with p73 for binding to the NH_2 terminus of p300/cAMP-responsive element binding protein (Zeng *et al.*, 1999). Recently, Rossi *et al.* found that a HECT-type E3 ubiquitin protein ligase Itch interacts with p73 through the WW protein–protein interaction domains of Itch and the p73 region containing the PY motif, and p53 which does not contain the PY motif fails to interact with Itch (Rossi *et al.*, 2005). According to these results, Itch had an ability to ubiquitinate and degrade p73. Upon DNA damage induced by chemotherapeutic drugs including cisplatin, doxorubicin or etoposide, the endogenous expression levels of Itch were significantly down-regulated through an unknown mechanism, thereby increasing the stability and activity of p73. On the other hand, it has been demonstrated that a novel HECT-type E3 ubiquitin protein ligase NEDL2 directly binds to p73, and this interaction is mediated by the WW domains of NEDL2 and the COOH-terminal region of p73 containing the PY motif (Miyazaki *et al.*, 2003). Unexpectedly, NEDL2 promoted the ubiquitination of p73 in cells, however, NEDL2-mediated ubiquitination increased the stability of p73 and enhanced the p73-dependent transcriptional activation, indicating that there exists a non-proteolytic regulatory role of ubiquitination. Other studies demonstrated that the NH_2-terminally truncated form of p73 (ΔNp73) is much more stable than TAp73, suggesting that p73-mediated transcriptional activation is required for the rapid turnover of p73, and that, like p53, one or more transcriptional targets of p73 might promote its proteolytic degradation (Wu *et al.*, 2004). Additionally, Toh *et al.* reported that c-Jun increases the stability of p73 without direct interaction, and c-Jun-mediated stabilization of p73 is regulated in its transactivation function-dependent manner (Toh *et al.*, 2004). Alternatively, several lines of

evidence suggest that the proteolytic degradation of p73 is regulated in a ubiquitination-independent manner. For example, Ohtsuka *et al.* found that cyclin G, one of the direct transcriptional targets of p53 and p73, interacts with p73 and induces the latter's rapid degradation (Ohtsuka *et al.*, 2003). According to these results, cyclin G-mediated degradation of p73 was not associated with an increase in its ubiquitination levels. Recently, it has been demonstrated that a U-box-type E3/E4 ubiquitin protein ligase UFD2a interacts with p73 through its COOH-terminal SAM domain, and induces the proteasomal turnover of p73 (Hosoda *et al.*, 2006) Intrinsic E3/E4 ubiquitin protein ligase activity was not necessary for the UFD2a-mediated proteolytic degradation of p73, and UFD2a failed to increase the ubiquitination levels of p73. Similar to Itch, the intracellular expression levels of UFD2a were significantly down-regulated at protein levels in response to cisplatin, thereby leading to a dissociation of free active p73 from the p73/UFD2a complex. Although the precise molecular mechanisms underlying the proteasome-dependent degradation of p73 mediated by UFD2a are not yet known, it is likely that p73 might be recruited to the proteasome through its interaction with UFD2a.

The NAD(P)H:quinone oxidoreductase-1 stabilizes p73α (as well as p53) but not p73β by binding of its SAM domain to NQO1, which protects p73α from 20S proteasomal degradation that is independent of MDM2. This NQO1-mediated stabilization of p73α and p53 provides one explanation why NQO1 knockout mice have a cancer phenotype and humans with inactive NQO1 polymorphisms are susceptible to cancer (Asher *et al.*, 2002).

In addition to post-translational modifications including phosphorylation and acetylation, the activity of p73 is regulated by physical interaction with several viral and cellular proteins. Like p53, p73 was associated with the adenovirus E1A and the T-cell lymphotropic virus I-derived Tax, and these interactions inhibited the activity of p73 (Irwin *et al.*, 2001). On the other hand, the viral proteins which can bind to and inactivate p53, including the adenovirus E1B, papilomavirus E6 and simian virus 40 T antigen, failed to interact with p73 (Marin *et al.*, 1998; Roth *et al.*, 1998; Steegenga *et al.*, 1999). For cellular proteins, MDM2 interacted with both p53 and p73, and inactivated their activities (Zeng *et al.*, 1999; Dobbelstein *et al.*, 1999).

Recently, several experimental approaches have been employed to identify the specific binding partners of p73. Few laboratories focussed attention on the PY motif of p73 not found in p53, and identified the Yes-associated protein (YAP), NEDL2 and Itch (Rossi *et al.*, 2005; Miyazaki *et al.*, 2003; Strano *et al.*, 2001). As mentioned above, NEDL2 ubiquitinated

p73 but extended its half-life, thereby enhancing its transcriptional activation. Itch promoted the ubiquitination-mediated proteasomal turnover of p73. YAP interacted with the PY motif of p73 through its WW domain, and stimulated p73-mediated transcriptional activation. Nakagawara' lab performed a conventional yeast-based two-hybrid screening using the extreme COOH-terminal tail of p73 not found in p53. Finally, they identified the c-Myc-binding protein (MM1), RACK1 and RanBPM as p73-binding proteins (Kramer *et al.*, 2005; Watanabe *et al.*, 2002; Ozaki *et al.*, 2003). Based on these results, MM1 attenuated the c-Myc-mediated inhibition of transcriptional activity of p73, whereas RACK1 significantly inhibited the function of p73 and its inhibitory effect was counteracted by pRB. RanBPM increased the stability of p73 by reducing its ubiquitination levels. The proteins identified had no detectable effects on p53. By using a new CytoTrap yeast two-hybrid screening, they identified the protein kinase A catalytic subunit β (PKA-Cβ) as a novel binding partner of p73 (Hanamoto *et al.*, 2005). PKA-Cβ bound to both the NH_2- and COOH-terminal regions of p73, and inhibited its transcriptional activity. PKA-Cβ efficiently phosphorylated p73, and PKA-Cβ-mediated inhibition of p73 was dependent on the kinase activity of PKA-Cβ. These observations strongly suggest that the regulatory mechanisms of p73 are distinct from those of p53.

The ankyrin-rich, Src holomogy 3 domain, proline-rich proteins ASPP1 and ASPP2 stimulate the apoptotic function of p53, p63, and p73 (Bergamaschi *et al.*, 2004; Samuels-Lev *et al.*, 2001). By binding to the DBD of p53, p63, and p73, ASPP1 and ASPP2 stimulate the transactivation function of all three proteins on the promoters of Bax, PIG3, and PUMA but not MDM2 or $p21^{WAF-1/CIP1}$. Hence, ASPP1 and ASPP2 are the first two identified common activators of all p53 family members.

Post-translational modifications during activation

p53 stabilization and activation by genotoxic stress is associated with multiple post-translational modifications at the NH_2 and COOH termini of p53 in vivo. In close temporal relationship to stress, the NH_2 terminus undergoes heavy phosphorylation (Ser^{15}, Ser^{20}, Ser^{33}, Ser^{37}, Ser^{46}, Thr^{18}, and Thr^{81}), which is thought to stabilize the protein by interfering with MDM2 binding, thereby disrupting the constitutively targeted degradation. The COOH terminus also undergoes site-specific phosphorylation (Ser^{315} and Ser^{392}), acetylation (Lys^{320}, Lys^{373}, and Lys^{382}), and sumoylation (Lys^{386}).

The COOH-terminal modifications are thought to activate the transcriptional activity of p53 (Appella *et al.*, 2001). So-called stress kinases (e.g., ATM, ATR, and Chk2), which detect genotoxic stress and initiate signal transduction, are in vivo kinases for specific p53 serine residues, whereas the histone acetyltransferases p300/cAMP-responsive element binding protein and PCAF (which at the same time are transcriptional coactivators) acetylate p53.

Recent studies revealed that p73 is induced to be accumulated in response to a subset of DNA-damaging agents, including cisplatin, adriamycin, camptothecin and etoposide (Irwin *et al.*, 2003). p73 is predominantly regulated at the post-translational level. Accumulating evidence strongly suggests that chemical modifications of p73, such as phosphorylation and acetylation, prolong its half-life, which, in turn, enhance its transcriptional and pro-apoptotic activity. During the cisplatin-mediated apoptotic process, p73 is phosphorylated at Tyr-99 and stabilized in a pathway dependent on nuclear non-receptor tyrosine kinase c-Abl (Gong *et al.*, 1999; Agami *et al.*, 1999; Yuan *et al.*, 1999). In addition to c-Abl, exposure to cisplatin promoted a complex formation between p73 and a protein kinase Cδ catalytic fragment, which phosphorylated p73 at Ser-289 and increased its stability and transcriptional activity (Ren *et al.*, 2002). Recently, it has been shown that cisplatin-induced apoptosis is associated with p73 phosphorylation at Ser-47 mediated by Chk1 (Gonzalez *et al.*, 2003). Chk1-dependent phosphorylation resulted in an increase in the transcriptional activity of p73. In contrast, CDK-mediated phosphorylation of p73 led to significant inhibition of its transcriptional activity (Gaiddon *et al.*, 2003) indicating that the phosphorylation of p73 might not always convert a latent form of p73 to an active one. Alternatively, p73 is regulated by acetylation. p73 was previously found to be associated with p300 histone acetyltransferase through its NH_2-terminal transactivation domain, and this interaction resulted in a significant enhancement of p73-mediated transcriptional activation as well as apoptosis (Zeng *et al.*, 2000). Costanzo *et al.* reported that p300 acetylates p73 at Lys-321, Lys-327 and Lys-331 in response to doxorubicin in a c-Abl-dependent manner, and the acetylated forms of p73 have pro-apoptotic activity (Costanzo *et al.*, 2002). Intriguingly, the p300-mediated acetylation of p73 was stimulated by prolyl isomerase Pin1, thereby stabilizing p73 (Mantovani *et al.*, 2004). It is likely that p73 acetylation catalyzed by p300 reduces its ubiquitination levels by competition between acetylation and ubiquitination.

Sumoylation of COOH-terminal Lys^{627} occurs specifically in p73α but not in p73ß in vitro. However, in contrast to sumoylation of p53, which activates its transcriptional activity, sumoylation of p73 promotes its degradation (Minty *et al.*, 2000).

Alteration of p73 expression in human cancer

p73 maps to chromosome 1p36.33, which frequently undergoes loss of heterozygosity in breast and colon cancer, neuroblastoma, oligodendroglioma, and melanoma. This fact, in conjunction with the functional similarity to p53, originally led to the proposal that p73 is a tumor suppressor gene (Kaghad *et al.*, 1997). Genetic data on most cancer types (with the notable exception of leukemias and lymphomas), however, exclude p73 as a classic Knudson-type tumor suppressor, which by definition is targeted to undergo loss of expression or function during tumorigenesis. To date, in a total of >1,100 primary tumors, loss of function mutations in p73 are vanishingly rare (0.6%). Surprisingly, the most common identifiable cancer-specific alteration is overexpression of various isoforms of the wild-type *p73* rather than a loss of expression (Kaghad *et al.*, 1997). This suggests that *p73* plays an oncogenic role in tumorigenesis. The single exceptions to this picture might be lymphoid malignancies and, possibly, bladder cancer. Although overexpression of p73 gene was found in B-cell chronic lymphocytic leukemia (Novak *et al.*, 2001) and during differentiation of myeloid leukemic cells (Tschan *et al.*, 2000), *p73* has been found to be transcriptionally silenced in some lymphoblastic leukemias and lymphomas due to hypermethylation (Corn *et al.*, 1999; Kawano *et al.*, 1999). Likewise, based on one immunocytochemical study with prognostic analysis, invasive high-grade bladder cancers, which had lost p73 (and p63) staining, had a poorer clinical outcome (Puig *et al.*, 2003).

To date, significant prevalence of p73 overexpression has been found in 12 different tumor types including tumors of breast (Zaika *et al.*, 1999), neuroblastoma (Kovalev *et al.*, 1998), lung (Mai *et al.*, 1998; Tokuchi *et al.*, 1999), esophagus (Cai *et al.*, 2000), stomach (Kang *et al.*, 2000), colon (Sunahara *et al.*, 1998), bladder (Chi *et al.*, 1999; Yokomizo *et al.*, 1999), ovarian cancer (70% of cases in one cohort; Ng *et al.*, 2000; Chen *et al.*, 2000; Zwahlen *et al.*, 2000), ependymoma (Zwahlen *et al.*, 2000), liver cancer (Tannapfel *et al.*, 1999a), cholangiocellular carcinoma (Tannapfel *et al.*, 1999b), chronic myelogenous leukemia blast crisis and acute

myelogenous leukemia (Tschan *et al.*, 2000; Peters *et al.*, 1999), colon carcinoma (Guan *et al.*, 2003; Sun *et al.*, 2002), and head and neck squamous carcinoma (associated with distant metastasis; Choi *et al.*, 2002; Weber *et al.*, 2002a; Weber *et al.*, 2002b). Most studies measure overexpression of full-length p73 mRNA (TAp73) by reverse transcription-PCR, but a few studies also measure overexpression of TAp73 protein(s) by either immunoblot or immunocytochemistry. For example, there is overexpression of TAp73 transcripts (5- to 25-fold) in 38% of 77 invasive breast cancers relative to normal breast tissue and in five of seven breast cancer cell lines (13- to 73-fold; Zaika *et al.*, 1999). Likewise, there is overexpression of TAp73 transcripts in a subset of neuroblastoma (8- to 80-fold) and in 12 of 14 neuroblastoma cell lines (8- to 90-fold; Kovalev *et al.*, 1998). A close correlation between p73 mRNA levels and protein levels was shown in ovarian carcinoma cell lines (Ng *et al.*, 2000). In a series of 193 patients with hepatocellular carcinoma, 32% of tumors showed detectable (high) p73 by immunocytochemistry and in situ hybridization, whereas all normal tissue had undetectable levels (low; Tannapfel *et al.*, 1999a). Of note, primary tumors and tumor cell lines with p73 overexpression tend to simultaneously overexpress a complex profile of shorter COOH-terminal splice variants (p73γ, p73δ, p73ε, and p73ϕ), whereas the normal tissue of origin is limited to the expression of p73α and p73β (Zaika *et al.*, 1999). Importantly, patients with high global p73 protein expression had a worse survival than patients with undetectable levels (Tannapfel *et al.*, 1999a; Sun *et al.*, 2002).

There is an emerging sense that the dominant-negative ΔTAp73 isoforms rather than TAp73 might be the physiologically relevant components of tumor-associated p73 overexpression, functionally overriding an often concomitant increase in TAp73 expression. This might have escaped notice because many of the early p73 overexpression studies in human cancers determined total p73 levels (all isoforms). Therefore, up-regulation of ΔTAp73 forms likely contributed to the elevated total p73 levels found previously in human cancers. Although, to date, only a few limited studies of tumors (breast cancer, gynecologic cancers, hepatocellular carcinoma, and neuroblastoma) focused on ΔTAp73, highly prevalent, tumor-specific up-regulation of ΔNp73 or ΔN'p73 (producing the same protein) has already been found in all of them (Zaika *et al.*, 2002; Concin *et al.*, 2004; Stiewe *et al.*, 2002; Putzer *et al.*, 2003; Sayan *et al.*, 2001; Casciano *et al.*, 2002; Douc-Rasy *et al.*, 2002). Moreover, 31% of 52 breast cancers overexpressed ΔNp73 compared with normal breast tissue (Zaika *et al.*, 2002). Of note,

ΔNp73 overexpression appears to have a clinical impact at least in some cancer types. ΔNp73 was found to be an independent prognostic marker for reduced progression-free and overall survival in neuroblastoma patients (Casciano *et al.*, 2002).

Alteration of p63 expression in human cancer

p63 is not a tumor suppressor. The analysis of p63 in cancers of patients with germ line mutations or somatic mutations indicates similar lack of mutations but up-regulation of dominant-negative forms. For example, no p63 mutations were found in 47 bladder cancers (Park *et al.*, 2000) or 68 squamous cell carcinoma of the head and neck (Weber *et al.*, 2002). Only 1 missense mutation ($Ala^{148}Pro$) of 66 various human tumors and 2 missense mutations in 35 tumor cell lines were found.

The human p63 gene is on chromosome 3q27-28 within a region that is frequently amplified in squamous cell, cervical, and prostate carcinomas. Some lung cancers and squamous cell carcinomas of the head and neck show p63 overexpression associated with a modest increase in *p63* copy numbers (Hibi *et al.*, 2000). In 25 primary nasopharyngeal carcinomas, all tumor cells overexpressed predominantly ΔNp63, which in normal nasopharyngeal epithelium is limited to proliferating basal and suprabasal cells (Crook *et al.*, 2000). In esophageal squamous cell carcinoma, ΔNp63 is the major isotype expressed throughout. In contrast, in normal esophagus, p63 staining is restricted to the basal and suprabasal cell layers (Choi *et al.*, 2002, Hu *et al.*, 2002). Thus, the maintenance of the ΔNp63 isoforms in squamous cancers may contribute to keeping the cells in a stem cell–like phenotype, thereby promoting tumor growth. Up-regulation of ΔNp63 was also found in 30 of 47 bladder cancers (Park *et al.*, 2000). Interestingly, TAp63 was concomitantly down-regulated in 25 of those 47 tumors.

Transcriptional regulation of the main promoter of p73

It has been recently established that the cellular and viral oncogenes E2F1, c-Myc, and E1A can induce and activate the endogenous TAp73α and TAp73β proteins for target gene transactivation, apoptosis, and growth suppression in p53-deficient human tumor cells (Zaika *et al.*, 2001; Stiewe *et al.*, 2000; Lissy *et al.*, 2000; Irwin *et al.*, 2000).

E2F1 transcription factor plays an important role in the regulation of cell cycle progression by inducing the transcription of genes whose products are directly or indirectly required for entry into the S phase (Johnson *et al.*, 1993). In addition to the proliferative effect of deregulated E2F1 activity, unscheduled E2F1 activation leads to apoptosis to protect cells from cellular transformation (Shan *et al.*, 1994). Consistent with this notion, E2F1-deficient mice exhibited a high incidence of unusual tumors (Yamasaki *et al.*, 1996; Field *et al.*, 1996). E2F1-induced apoptosis is regulated in a p53-dependent or p53-independent manner. It is interesting that the *p73* promoter region contains a TATA-like box and at least three E2F1-binding sites, and indeed the enforced expression of E2F1 strongly stimulates the transcription of *p73* through the direct binding to the E2F1-responsive elements in the *p73* promoter (Irwin *et al.*, 2000; Stiewe *et al.*, 2000). The E2F1-mediated up-regulation of p73 results in a significant induction of apoptosis. Alternatively, E2F1 might also contribute to the up-regulation of *p73* mRNA levels during muscle and neuronal differentiation of murine C2C12 myoblasts and P19 cells, respectively (Fontemaggi *et al.*, 2001). In addition to E2F1, cellular and viral oncogene products such as c-Myc and E1A indirectly activated the transcription of p73 (Zaika *et al.*, 2001).

Because oncogene deregulation of E2F1 and c-Myc are one of the most common genetic alterations in human tumors, these findings might provide a physiologic mechanism for TAp73 overexpression in tumors. Taken together, these data establish another important link between p73 and human cancer.

p73 is required for antigen-induced death of circulating peripheral T cells after T-cell receptor activation and for tumor necrosis factor-α-induced death of thymocytes (immature T cells). This death pathway is mediated via the E2F1-p73 (Lissy *et al.*, 2000; Wan *et al.*, 2003). Conversely, the survival of antigen-stimulated T cells requires nuclear factor kB–mediated inhibition of p73 expression (Wan *et al.*, 2003). Consistent with this notion, E2F1-null mice exhibit a marked disruption of lymphatic homeostasis with increased numbers of T cells and splenomegaly, suggesting that p73 plays a role in tumor surveillance pathways of lymphoid cells (Yamasaki *et al.*, 1996; Field *et al.*, 1996). Moreover, the p73 gene is transcriptionally silenced in acute lymphoblastic leukemia and Burkitt's lymphoma due to hypermethylation (Corn *et al.*, 1999; Kawano *et al.*, 1999; Liu *et al.*, 2001; Scaruffi *et al.*, 2000). This appears to be restricted to lymphoid tumors because neither other hematopoietic malignancies nor solid tumors show p73 hypermethylation (Corn *et al.*, 1999; Liu *et al.*, 2001). Interestingly, in radiation-induced T-cell

lymphomas of the mouse, the p73 locus undergoes loss of heterozygosity in 33% of the cases (Herranz *et al.*, 1999). Thus, in lymphoid tumors, p73 shows some genetic features of a classic tumor suppressor gene. Early growth response factor-1, an immediate early gene that is activated by mitogens in quiescent postmitotic neurons, induces apoptosis in neuroblastoma cells. This apoptosis seems to be mediated by p73, which is elevated in cells overexpressing early growth response factor-1 (Pignatelli *et al.*, 2003).

Recently, Fontemaggi *et al.* identified a 1 kb negative regulatory fragment within the first intron of *p73* gene (Fontemaggi *et al.*, 2001). This intronic fragment significantly reduced the activity of the *p73* promoter upon E2F1 overexpression. Of note, the *p73* intronic fragment contained six consensus binding sites for transcriptional repressor ZEB. Ectopic expression of ZEB in C2C12 myoblasts attenuated myotube formation, and repressed the transcription of *p73*. In accordance with these results, the dominant negative form of ZEB had an ability to restore the expression levels of p73 in proliferating cells.

Because DNA hypermethylation contributes to the alteration of the entry of transcription factors into the regulatory region, the epigenetic modification of the *p73* promoter region through aberrant hypermethylation could be an alternative molecular mechanism for silencing the *p73* gene. Corn *et al.* described the aberrant promoter methylation of *p73* as occurring frequently in primary acute lymphoblastic leukemias and Burkitt's lymphomas, whereas the *p73* promoter methylation was not detected in normal lymphocytes or bone marrow (Corn *et al.*, 1999). Similar results were also reported by Kawano *et al* (Kawano *et al.*, 1999). In contrast, hypermethylation of the *p73* promoter region was not observed in solid tumors including breast, renal, colon cancers or neuroblastomas (Banelli *et al.*, 2000), suggesting that the methylation-dependent silencing of *p73* transcription might be specific to hematological malignancies.

Mutual regulation between p73 and p53

Previously, it has been shown that tumor-derived p53 mutants but not wild-type p53 interact with p73, and abrogate its function (Di Como *et al.*, 1999). Subsequent studies demonstrated that the ability of p53 mutants to interact with p73 depends on the nature of the p53 mutations as well as the polymorphism at codon 72 (Pro-72 or Arg-72) of p53 mutants (Marin *et al.*,

2000), in particular, p53 mutants carrying Arg-72 bound to p73 better than p53 mutants with Pro-72. Consistent with this notion, p53 mutants carrying Arg-72 act as more potent inhibitors of chemotherapy-induced apoptosis than the p53 mutants with Pro-72 (Bergamaschi *et al.*, 2003). Functionally, formation of such stable complexes leads to a loss of p73- and p63-mediated transactivation and proapoptotic abilities. Moreover, E2F1-induced p73 transactivation, apoptosis, and colony suppression was inhibited by coexpressed $p53His^{175}$ (Stiewe *et al.*, 2000). It suggests that in tumors that express both TAp73 and mutant p53 (typically at very high levels due to deficient MDM2-mediated degradation), the function of TAp73 and TAp63 might be inactivated. This gain-of-function results in increased tumorigenicity compared with p53-null parental cells, increased resistance to cancer agents, and increased genomic instability due to abrogation of the mitotic spindle checkpoint (Dittmer *et al.*, 1993; Shaulsky *et al.*, 1991; Halevy *et al.*, 1990). Other studies focused on the functional interaction between wild-type p53 and p73. Miro-Mur *et al.* reported that p73 induces both accumulation and activation of wild-type p53 by preventing MDM2-mediated degradation through MDM2 titration (Miro-Mur *et al.*, 2003). In addition, Goldschneider *et al.* found that p73 promotes the nuclear localization of wild-type p53 in neuroblastoma cells in which p53 is predominantly expressed in cytoplasm (Goldschneider *et al.*, 2003). These results suggest that p73 has an ability to enhance the activity of wild-type p53. In contrast, Vikhanskaya *et al.* described that p73 reduces the p53-mediated transcriptional activation through the competition of the same DNA-binding site (Vikhanskaya *et al.*, 2000). These controversial results regarding the effects of p73 on wild-type p53 might be at least in part due to the different cell systems used in those studies.

Recently, it has been shown that p53-dependent apoptosis requires the indirect contribution of at least one other p53 family member, p73 or p63 (Flores *et al.*, 2002). Thus, it is likely that p73 cooperates with p53 to promote apoptotic cell death. These findings emphasize the functional importance of p73 in the regulation of the DNA damage-induced apoptotic response.

An autoregulatory feedback loop exists among p53, Tap73 and ΔNp63

p53 and TAp73 regulate ΔNp73 but not ΔNp63 levels by binding to the p73 P2 promoter and inducing its transcription. A p73-specific responsive element was mapped within the P2 region (Nakagawa *et al.*, 2002). This generates a negative feedback loop analogous to the p53-MDM2 loop that in turn negatively regulates the activity of p53 and p73 (Nakagawa *et al.*, 2002; Kartasheva *et al.*, 2002; Vossio *et al.*, 2002; Grob *et al.*, 2001). ΔNp73 blocks p53 and TAp73 activity through heterocomplex formation (Stiewe *et al.*, 2002; Zaika *et al.*, 2002; Nakagawa *et al.*, 2002) or through promoter competition (Stiewe *et al.*, 2002; Kartasheva *et al.*, 2002;) and thus contributes to the termination of the p53/p73 response in cells that do not undergo apoptosis. In contrast to ΔNp73, ΔNp63 expression is transcriptionally repressed by p53 (Waltermann *et al.*, 2003).

p73 and chemosensitivity

Endogenous p73 protein levels increase in response to cisplatin and Adriamycin (Agami *et al.*, 1999; Costanzo *et al.*, 2002; Gong *et al.*, 1999). Although originally thought to respond only to a limited spectrum, it is now clear that TAp73 (α more than β) is induced by a wider variety of chemotherapeutic agents (Adriamycin, cisplatin, taxol, and etoposide) in different tumor cell lines (Irwin *et al.*, 2003; Bergamaschi *et al.*, 2003). p73 accumulation is due to increased transcription and increased protein stabilization and leads to induction of apoptotic target genes such as apoptosis-induced protein-1. Conversely, blocking TAp73 function (either by the inhibitory p73DD fragment or by p73 small interfering RNA) leads to enhanced chemoresistance, which is independent of the p53 gene status. Of note, whereas the presence of p73 is essential for p53 to induce apoptosis in fibroblasts (Flores *et al.*, 2002), p73 on the other hand can induce apoptosis in cells that lack functional p53 (Irwin *et al.*, 2003). This confirms the importance of p73 in the response to chemotherapeutic agents (Bergamaschi *et al.*, 2003).

In cell culture, overexpression of antiapoptotic p73 isoforms can also block chemotherapy-induced apoptosis in wild-type p53 tumor cells (Zaika *et al.*, 2002; Vossio *et al.*, 2002). Moreover, overproduction of certain p53 mutants can block p73 function and chemotherapy-induced apoptosis (Di

Como *et al.*, 1999; Gaiddon *et al.*, 2001; Blandino *et al.*, 1999). This effect is most strongly linked to the Arg^{72} polymorphism of the p53 gene (Irwin *et al.*, 2003; Marin *et al.*, 2000; Bergamaschi *et al.*, 2003) and is mediated by stable hetero-oligomers involving the DBDs. Bergamaschi *et al.* have used different cell lines forced to express a series of p53 mutants as either Arg (72R) or Pro (72P) versions at codon 72. Only Arg mutants correlated with chemoresistance. These data were mirrored in a series of polymorphic head and neck cancer patients with the same p53 mutants: 72R patients showed poor response to chemotherapy and shorter survival (Bergamaschi *et al.*, 2003). Conversely, down-modulation of endogenous p53 mutants enhances chemosensitivity in p53-defective mutant cells (Irwin *et al.*, 2003). Consequently, a promising therapeutic approach includes the use of small interfering RNA specifically directed against particular p53 mutants, which might restore chemosensitivity of tumor.

YAP

Large protein complexes that include transcription factors and specific coactivators frequently govern activation of specific sets of genes (Naar *et al.*, 2001). As in the case of p53, p73 is controlled by interaction partners and these interactions might determine the extent to that p73 contributes to apoptosis. The existence of the protein complex YAP/p73 was the first evidence linking WW domain containing proteins to the p53 family members (Strano *et al.*, 2001). YAP, the first protein in which a WW domain was identified, is a phosphoprotein of 65 kDa that interacts with the SH3 domain of the *c-yes* protooncogene product, a nonreceptor tyrosine kinase of the Src family (Sudol, 1994). WW domains are protein-protein interaction modules that recognize short proline-rich motifs of diverse proteins involved in various signaling pathways (Sudol and Hunter, 2000). The name refers to two signature tryptophan (WW) residues that are spaced 20–22 amino acids apart and play an important role in the domain structure and function (Sudol and Hunter, 2000). In addition to a type I WW domain, YAP also contains a PDZ interaction motif, an SH3 binding motif, and a coiled-coil domain. A recent work has reported the identification of a 14-3-3 binding molecule, named TAZ (transcriptional coactivator with PD*Z* binding motif), that shares a remarkable homology with YAP (Kanai *et al.*, 2000). It has recently been shown that YAP is a potent transcriptional coactivator. YAP binds to and coactivates the Runx and the four TEAD/TEF transcription factors (Yagi *et al.*, 1999 and Vassilev *et al.*, 2001).

A close link between YAP and the transcription factors of the p53 family has recently emerged (Strano *et al.*, 2001 and Basu *et al.*, 2003). We had originally reported that YAP engages in a physical association with p73 and p63 (Strano *et al.*, 2001). In terms of binding to p53 family members, YAP possesses two levels of specificity, binding to long, but not to short, forms of p73 and p63, and it does not bind to p53 at all (Strano *et al.*, 2001). The binding of YAP to p73 or p63 results in a strong transcriptional coactivation (Strano *et al.*, 2001 and Basu *et al.*, 2003; S.S. and G.B., unpublished data). It has been reported that YAP is phosphorylated by AKT, and such modification impairs YAP-nuclear translocation and attenuates p73-mediated apoptosis (Basu *et al.*, 2003).

In a recent work we demonstrated that p73 is required for the nuclear translocation of endogenous YAP in cells exposed to cisplatin and that YAP is recruited by PML into the nuclear bodies (NBs) to promote p73

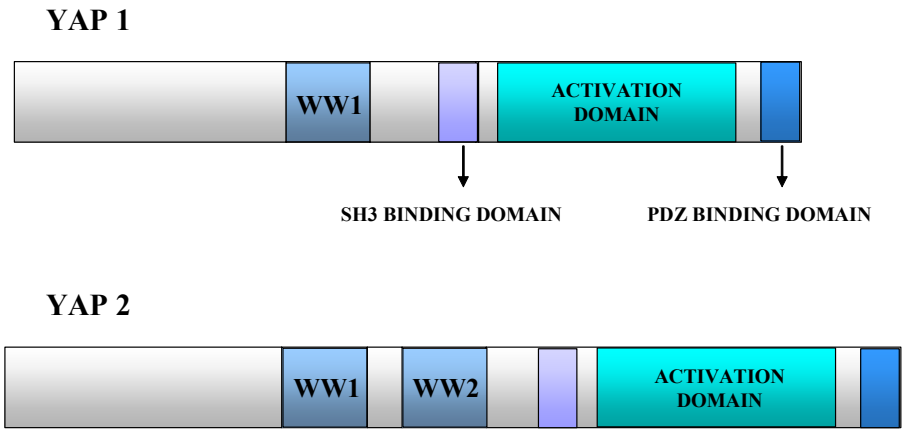


Fig.3. Structure of YAP proteins.

Two YAP isoforms have been identified, characterized by the presence of one (YAP1) or two (YAP2) WW domains. WW domains are protein-protein interaction modules that recognize short proline-rich motifs of diverse proteins involved in various signaling pathways. The name refers to two signature tryptophan (WW) residues that are spaced 20–22 amino acids apart and play an important role in the domain structure and function. In addition to a type I WW domain, YAP also contains a PDZ interaction motif, an SH3 binding motif, and a coiled-coil domain.

transcriptional activity (Strano *et al.*, 2005). We found that YAP contributes to p73 stabilization in response to DNA damage and promotes p73-dependent apoptosis through the specific and selective coactivation of apoptotic p73 target genes and potentiation of p300-mediated acetylation of p73. Indeed, endogenous p73, YAP, and p300 proteins are concomitantly recruited to the regulatory regions of the apoptotic target gene *p53AIP1* only when cells are exposed to apoptotic conditions. Silencing of YAP by specific siRNAs impairs p300 recruitment onto the p73 apoptotic target gene *p53AIP1* upon DNA damage, and this correlates with the reduction of histone acetylation at the same promoter site and delayed or reduced apoptosis (Strano *et al.*, 2005). Altogether, these results identify YAP as an important determinant for p73 target gene specificity through p300 recruitment and p73 acetylation.

Recently, Rossi *et al.* have shown that Itch, a human ubiquitin-protein ligase, which belongs to the Nedd4-like E3 family containing a WW domain (Perry *et al.*, 1998), binds and ubiquitinates p73 via its PPPY motif and determines its rapid proteosome-dependent degradation in a ubiquitin-dependent manner (Rossi *et al.*, 2005). More recently Levy *et al.* have shown that YAP competes with Itch for binding to p73 at the PPPY motif and this prevents Itch ubiquitination, and subsequent degradation, of p73 (Levy *et al.*, 2006). Treatment of cells with cisplatin leads to an increase in p73 accumulation and induction of apoptosis, but both were dramatically reduced in the presence of YAP siRNA.

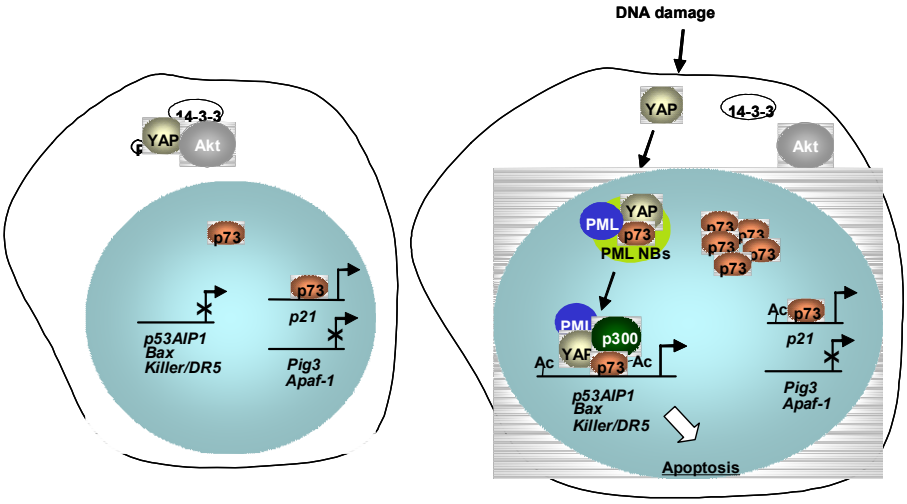


Fig.4. Model for p73 gene target specificity modulation by YAP in response to DNA damage.

DNA damage causes p73 accumulation, release of YAP from cytoplasmic multiprotein complexes containing 14-3-3 and AKT, and YAP relocalization into the nucleus. PML is required to localize YAP into the NBs to coactivate p73. The interaction with YAP promotes p73 stabilization, binding to p300 and its acetylation. Under apoptotic conditions, the transcriptionally active complex that contains acetylated p73, YAP and p300 assembles onto the regulatory regions of the p53-p73 complex proapoptotic target genes p53AIP1 and BAX.

S100 proteins

The S100 proteins are small acidic proteins (10-12 kDa) that are found exclusively in vertebrates (Schafer *et al.*, 1996). With at least 25 members found to date in humans, the S100 proteins constitute the largest subfamily of the EF-hand proteins. First identified by Moore in 1965 (Moore *et al.*, 1965), the S100 proteins have 25-65% identity at the amino acid level characterized by the presence of two Ca2+ binding sites of the EF-hand type (i.e., helixloop-helix), one of which, located in the S100 N-terminal half, is unconventional, while the other one, located in the S100 C-terminal half, is canonical. As a consequence, Ca2+ binding to individual EF hands occurs with different affinities, a lower affinity in the case of the N-terminal site and a ~ 100-times higher affinity in the case of the C-terminal site. The two EF hands are interconnected by an intermediate region, referred to as the hinge region, and the C-terminal EF hand is followed by a C-terminal extension. S100 members differ from one another mostly for the length and sequence of the hinge region and the C-terminal extension, which are thus suggested to specify the biological activity of individual proteins. Three members of the family, i.e., profilaggrin, trychohyalin, and repetin, are large proteins that exhibit an S100 motif along their primary sequence. With the exception of calbindin D9k, which is monomeric, all other small S100 proteins exist within cells as homodimers in which monomers are related by a twofold axis of rotation and are held together by noncovalent bonds.

Upon Ca2+-binding, helix III becomes perpendicular to helix IV, the hinge region swings out, and a cleft forms in each monomer, which is defined by residues in the hinge region, helices III and IV and the C-terminal extension, and is buried in apo S100 monomer. Residues defining this cleft are believed to be important for the Ca2+-dependent recognition of S100 target proteins. The hinge region and the C-terminal extension play a critical role in the interaction of S100A1, S100B, S100A10, and S100A11 with several target proteins (Donato, 2001; McClintock *et al.*, 2002). Thus, upon Ca2+ binding, each S100 monomer opens up to accommodate a target protein (with the exception of S100A10 that is normally in an open-up state), and the S100 dimer can bind target proteins on opposite sides. By this mechanism, an S100 dimer functionally crosslinks two homologous or heterologous target proteins. Given the positions of the helices of one monomer relative to those of the other monomer (particularly helices I and IV and I' and IV'), helix I' might participate in the formation of each of the two binding surfaces on a

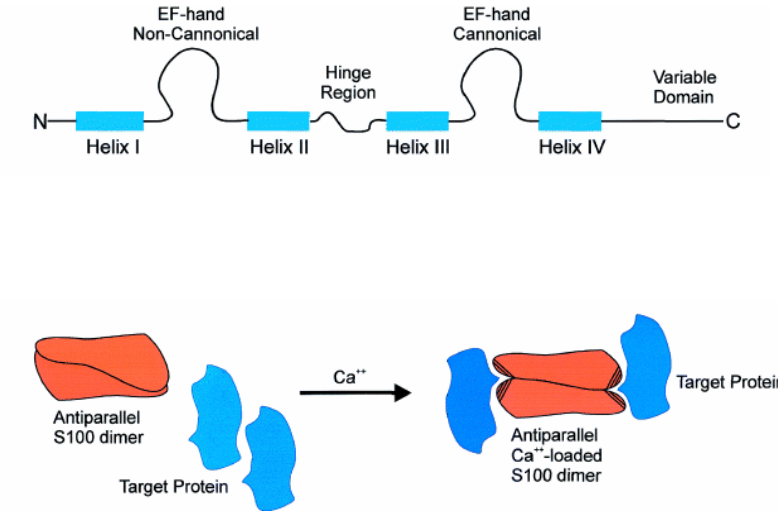


Fig.5. S100 protein structure and model of S100 protein/target protein interaction.

The overall structure of each S100 protein family member includes four alpha-helical segments, two calcium-binding EF-hands (one non-canonical site binds calcium with low affinity, and one canonical), a central hinge region of variable length, and the C- and N-terminal variable domains.
S100 proteins exist as anti-parallel dimers. An increase in calcium concentration results in a conformation change in the dimer that results in exposure of a cleft, which forms the target protein binding site. Once in the calcium-loaded state, each S100 protein dimer can interact with a target protein via its C-terminal domain. Thus, a single S100 protein dimer can ligate two target proteins.

given S100 dimer (Donato, 2001). This would explain why most S100 members form dimers; one S100 monomer is not enough for binding a target protein or, alternatively, target protein binding to S100 occurs with reduced strength. Probably, dimeric S100 proteins use different mechanisms for interacting with their target proteins, as in some cases Ca2+ is not required for S100 to bind to an effector protein, indicating that residues other than those that become exposed to the solvent upon Ca2+ binding might recognize definite target proteins.

Intracellular roles of S100 proteins

S100 proteins have been implicated in the regulation of protein phosphorylation, the dynamics of cytoskeleton constituents, Ca2+ homeostasis, enzyme activities, transcription factors, cell growth and differentiation, and the inflammatory response (Donato, 1999, 2001; Schaf̈er and Heizmann, 1996; Zimmer *et al.*, 1995).

Inhibition of protein phosphorylation

Inhibition of protein phosphorylation by S100 proteins depends on blockade of access of kinases to the pertinent protein substrate. This might represent a means to finely tune the activity of a given effector protein since in most cases the inhibitory effect of S100 protein on protein phosphorylation is Ca2+-dependent. Also, this represents an example of cross-talk between (cytosolic) Ca2+-based activities and protein phosphorylation. There are a few examples of S100-dependent inhibition of protein phosphorylation that are potentially relevant: (1) inhibition of caldesmon phosphorylation by S100B results in the reversal of caldesmon-dependent inhibition of actomyosin ATPase activity (Fujii *et al.*, 1990; Pritchard and Martson, 1991; Skripnikowa and Gusev, 1989); (2) inhibition of microtubule (MT)-associated τ protein phosphorylation by S100B has been suggested to be an important mechanism of neuronal protection from τ hyperphosphorylation in Alzheimer's disease (Yu and Fraser, 2001), in neurons in which S100B is expressed; (3) inhibition of p53 phosphorylation by S100B might result in inhibition of p53-dependent transcription activation via disruption of the p53 tetramer and, hence, of tumor suppressor

activity of p53 (Rustandi *et al.*, 2000; Lin *et al.*, 2001); (4) inhibition of an unknown substrate of protein kinase Cβ might result in inhibition of the hypertrophic response following myocardial infarction (Tsoporis *et al.*, 1997, 1998); (5) inhibition of myosin heavy chain phosphorylation by S100A4 might be linked to modulation of the cytoskeleton dynamics in metastatic cells (Davies *et al.*, 1996; Kriajevska *et al.*, 1998); (6) inhibition of p53 phosphorylation by S100A4 might result in inhibition of p53-dependent transcription activation and, hence, of tumor suppressor activity of p53 (Grigorian *et al.*, 2001); and (7) inhibition of ANXA2 phosphorylation by S100A10 might result the sequestration of ANXA2 in the cytoplasm (Eberhard *et al.*, 2001) and consequent modulation of the activities of ANXA2, a Ca2+-dependent phospholipid-, membrane-, and cytoskeleton-binding protein (Gerke and Moss, 2002).

Regulation of enzyme activity

S100A1 stimulates the sarcomeric, myosin-associated giant kinase twitchin in a Ca2+- and Zn2+-dependent manner in vitro (Heierhorst *et al.*, 1996). Twitchin is a member of a family of giant protein kinases involved in the regulation of muscle contraction and the mechanoelastic properties of the sarcomere in invertebrates. The corresponding vertebrate protein is titin, which was recently shown to interact with S100A1 (Yamasaki *et al.*, 2001). S100B and, to a smaller extent, S100A1 stimulate Ndr, a nuclear serine/threonine protein kinase important in the regulation of cell division and cell morphology, in a Ca2+-dependent manner (Millward *et al.*, 1998). S100B and S100A1 stimulate a membrane-bound guanylate cyclase (GC) activity in photoreceptor outer segments in vitro (Duda *et al.*, 1996; Pozdnyakoz *et al.*, 1997). The S100A8/S100A9 heterodimer modulates the activity of casein kinase I and II, two enzymes that phosphorylate topoisomerase I and RNA polymerases I and II, pointing to a potential role of S100A8 and S100A9 and/or the S100A8/S100A9 heterodimer in the regulation of myeloid cell maturation and function (Lagasse *et al.*, 1988; Murao *et al.*, 1989; Zwadlo *et al.*, 1988). S100A10 inhibits the activity of cytosolic (85-kDa) phospholipase (PL) A2 (Wu *et al.*, 1997).

Although the majority of S100 protein interactions are calcium-dependent, several calcium-independent interactions have been reported. The most common binding partners for the apo-S100 proteins are enzymes. For example, S100B and S100A1 bind with glycogen phosphorylase (Zimmer *et*

al., 1993), whereas S100A10 and S100A11 show interactions with transglutaminase (Ruse *et al.*, 2001).

Regulation of cell growth and differentiation

S100A1 and S100B bind to the basic helix-loop-helix (bHLH) sequence found in the transcription factors MyoD and E12 (Baudier *et al.*, 1995; Onions *et al.*, 1997) and at least S100A1 inhibits MyoD phosphorylation (Baudier *et al.*, 1995). S100B, which interacts with the tumor suppressor protein p53 (Baudier *et al.*, 1992), has been suggested to cooperate with p53 to cause cell growth arrest and apoptosis (Scotto *et al.*, 1998). However, this interpretation has been questioned based on structural and functional analyses of the S100B-p53 interactions (Lin *et al.*, 2001; Rustandi *et al.*, 2000). According to these authors, S100B would actually block p53, a conclusion in accordance with the notion that S100B is up-regulated in cancer cells (Donato, 1999; Ilg *et al.*, 1996) and that inhibition of its synthesis results in a decreased proliferation rate (Selinfreund *et al.*, 1990). Inhibition of expression of S100A1 in PC12 cells also results in a decrease in cell proliferation rate (Zimmer *et al.*, 1998), a finding that was interpreted as suggestive of a role of this protein in the modulation of the activity of an unknown transcription factor. S100A2 is markedly down-regulated in breast tumor biopsies and can be re-expressed in mammary carcinoma cells by azadeoxycytidine treatment (Lee *et al.*, 1992). Thus, S100A2 might have a tumor suppressor function (Wicki *et al.*, 1997), although its mechanism of action is not known. S100A11 has been shown to be phosphorylated in confluent, normal fibroblasts, but not immortalized cells, and to translocate into the nucleus (Sakaguchi *et al.*, 2000). Nuclear translocation of S100A11 resulted in a diminished cell proliferation, suggesting the possibility that the protein might be involved in the contact inhibition of cell growth. The molecular mechanism whereby S100A11 putatively inhibit cell proliferation is not known.

S100 proteins and the cytoskeleton

S100 proteins regulate MTs, intermediate filaments (IFs), microfilaments (MFs), tropomyosin, and myosin. As no additional information has been presented in this field during the last year, if one excludes the interaction of

S100A1 with the giant sarcomeric kinase, titin (Yamasaki *et al.*, 2001), as mentioned above.

Extracellular roles of S100 proteins

In addition to their intracellular functions, several S100 proteins, such as S100B, S100A4, S100A8, S100A9, S100A12, and S100A13, are secreted and act in a cytokine-like manner. For example, the S100A8/A9 heterodimer acts as a chemotactic molecule in inflammation (Newton *et al.*, 1998), S100B exhibits neurotrophic activity (Huttunen *et al.*, 2000), S100A4 has angiogenic effects (Ambartsumian *et al.*, 2001), S100A12 is involved in the host–parasite response, and S100A13 is a component of a secreted complex containing FGF1 and synaptotagmin. A multiligand receptor mediating these extracellular activities of S100 proteins was recently identified. S100 proteins bind to the extracellular domain of the receptor for advanced glycation end products (RAGE) and activate different intracellular signaling pathways, including MAP-kinase or NFκB (Hsieh *et al.*, 2003; Hofmann *et al.*, 1999; Sorci *et al.*, 2003; Arumugam *et al.*, 2004).

S100 proteins and cancer

Different forms of cancer exhibit dramatic changes in the expression of S100 proteins such as S100B, S100A2, S100A4, S100A6, and S100P. Such changes might be caused by rearrangements and deletions in chromosomal region 1q21, which are frequently observed in tumor cells. The mechanisms of how the S100 proteins act as tumor promoter or suppressor are widely different. For example, elevated levels of S100A4 are associated with poor survival rates in breast cancer patients and induce metastasis in mouse models. Recently it was shown that S100A4 protein added to the extracellular space triggers pro-metastatic cascades in tumor cells, emphasizing the important role of S100 proteins in the extracellular space (Schmidt-Hansen *et al.*, 2004). Other S100 proteins act intracellularly, e.g., S100A2, which is primarily found in the cell nucleus (Mandinova *et al.*, 1998). S100A2 was originally described as a tumor suppressor because reduced levels of S100A2 were detected in squamous lung cell carcinoma or breast cancer (Wicki *et al.*, 1997). However, recent studies have shown that S100A2 is highly expressed in tumors such as non-small lung cancer, gastric

cancer, and lymphoma (Hsieh *et al.*, 2003; Nagy *et al.*, 2002; Heighway *et al.*, 2002; El-Rifai *et al.*, 2002), creating a more complex picture of the role of S100A2 in carcinogenesis. Elevated expression levels of other S100 proteins such as S100A6 or S100P were found in breast cancer, progressive lung cancer, and pancreatic tumours (Hsieh *et al.*, 2003; Guerreiro Da Silva *et al.*, 2000). Although in most cases the function of S100 proteins in cancer cells is still unknown, the specific expression patterns of these proteins can be used as a valuable prognostic tool.

S100 proteins in the epidermis

Many members of this family of gene are encoded in the epidermal differentiation complex (EDC) located on chromosome 1q21 (Volz *et al*, 1993; Hardas *et al*, 1996; Wicki *et al*, 1996). This region is of particular interest, since it encodes many genes (involucrin, filaggrin, trichoyalin, repetin, etc.) that are expressed in epidermal keratinocytes (Mischke *et al*, 1996; South *et al*, 1999). Thus, the finding that many S100 genes are clustered within the EDC has heightened interest in their role in the epidermis. Among the 25 S100 proteins that have been cloned to date, 11, including S100A2, S100A3, S100A4, S100A6, S100A7, S100A8, S100A9, S100A10, S100A11, S100A12, and S100A15, are expressed in the human epidermis or in cultured keratinocytes (Boni *et al*, 1997; Xia *et al*, 1997; Broome *et al*, 2003; Wolf *et al*, 2003). The main focus of many studies in keratinocytes has been localizing these proteins in normal and diseased epidermis. This information is summarized in Table I. Compared with other cell types (Heizmann and Cox, 1998; Donato, 1999), information regarding S100 protein function in keratinocytes is limited. Recent studies, however, suggest potential roles for S100 proteins in epidermal wound repair, cancer, differentiation, and response to stress.

In particular, S100A2 (S100L) is localized to the basal layer of normal human epidermis and hair follicles (Boni *et al*, 1997; Shrestha *et al*, 1998). The most common form of S100A2 is overexpressed in psoriasis, although some polymorphic S100A2 forms are not (Stoll *et al*, 2001). S100A2 has a strong tendency to form homodimers as measured by interaction in a yeast two-hybrid screen and by immunoprecipitation (Deshpande *et al*, 2000). Moreover, when exposed to oxidizing conditions, the homodimers become linked via disulfide bonds (Deshpande *et al*, 2000). In cultured normal human keratinocytes, S100A2 is found mainly in the nucleus (Zhang *et al*,

2002). H_2O_2 treatment of normal keratinocytes causes a relocation of S100A2 from the nucleus to the cytoplasm. This translocation is also observed when cells are exposed to an ionophore-dependent increase in intracellular calcium, and both the H_2O_2- and ionophore-dependent translocation is inhibited by treatment with reducing agent. S100A2 translocation occurs within 1 h after treatment with H_2O_2 and cell death follows within 24 h. Thus, S100A2 translocation is an early marker of oxidative stress-related keratinocyte cell death. The inhibition of translocation from nucleus to cytoplasm by reducing agent treatment suggests that disulfide-linked dimer formation is required for movement (Deshpande *et al*, 2000). S100A2 expression is markedly increased in ErbB-driven epidermal hyperplasia, and decreased in the absence of functional p53 in carcinoma cell lines and tumors (Xia *et al*, 1997); however, the biological importance of this S100 protein concentration change is not known.

PML

The *PML* tumor suppressor gene, also known as *myl*, was originally identified cloning the (15;17) chromosomal translocation specific of acute promyelocytic leukemia (APL), a distinct subtype of acute myeloid leukemia (AML). As a consequence of this translocation, *PML* fuses to the retinoic acid (RA) receptor alpha (*RARα*) gene. Two fusion genes are generated encoding PML-RARα and RARα-PML fusion proteins, which coexist in the leukemic cells (Melnick and Licht, 1999). The PML-RARα oncoprotein was found to inhibit RARα transcriptional function, and also to associate physically with PML, thus potentially interfering with its function (Piazza *et al.*, 2001). PML has therefore become the object of intense research on the basis of this premise. This hypothesis was further corroborated by the discovery that while PML is typically concentrated in subnuclear structures variably named PML-nuclear bodies (PML-NBs), Kremer bodies, nuclear domain 10 (ND10) or PML oncogenic domains (POD) (Jensen *et al.*, 2001), in the APL blasts, PML-RAR*a* physically associates with PML and causes its delocalization into microspeckled nuclear structures with consequent disruption of the PML-NB (Melnich and Licht, 1999). Furthermore, *in vivo*, PML-RARα causes leukemia with APL features when expressed in the promyelocytic/myeloid compartment of transgenic mice, while dominant-negative RARα mutants, that do not interfere with PML function, fail to do so, underscoring the importance in leukemogenesis of the functional disruption of PML and the PML-NB (Pandolfi, 2001).

PML is one of the founding members of a large family of proteins harboring a distinctive zinc finger domain termed RING (really interesting gene), some of which have also been implicated in tumor suppression and the control of genomic stability (e.g. BRCA1; Zhong *et al.*, 2000b; Jensen *et al.*, 2001;). The PML RING finger is located N-terminally and is followed by two additional zinc fingers (B-boxes) and an α-helical coiled-coil motif (collectively referred to as the RBCC domain). The RBCC domain mediates protein–protein interactions and is responsible for PML multimerization, localization in the PML-NB and heterodimerization with PML-RARα, but does not confer DNA-binding capability to PML (Zhong *et al.*, 2000b; Jensen *et al.*, 2001).

Multiple PML isoforms have been identified due to alternative splicing of the carboxyl (C)-terminal exons (Jensen *et al.*, 2001). The biological relevance of the various PML isoforms has been recently underscored by the recognition that PML C-termini dictate PML protein–protein interaction

specificity. For instance, only one PML C-terminus mediates PML/p53 physical association (PML IV according to Fogal *et al.*, 2000; Guo *et al.*, 2000; Jensen *et al.*, 2001). Furthermore, while PML is mostly associated with the NB and the nuclear matrix some of these isoforms are found to accumulate into the cytosolic fraction (Jensen *et al.*, 2001), therefore suggesting that PML might have additional as yet unidentified functions independent from the PML-NB.

Many *in vivo* and *in vitro* data have directly implicated PML overexpression in growth suppression, apoptosis and replicative senescence (Mu *et al.*, 1994; Quignon *et al.*, 1998; Pearson and Pelicci, 2001; Salomoni and Pandolfi, 2002), but the direct demonstration for a physiological role of PML in apoptosis control came from the phenotypic analysis of $PML^{-/-}$ mice (Wang *et al.*, 1998a, 1998b). Cells derived from $PML^{-/-}$ mice presented defects in apoptosis induced by Fas, TNF, interferons and ceramides. Apoptosis induction was reduced, but not abrogated, implying a role for PML as a modulator, rather than as an essential trigger. Intriguingly, despite the number of abnormalities described in $PML^{-/-}$ cells, $PML^{-/-}$ mice do quite well (Wang *et al.*, 1998a, 1998b) and no gene homologous to PML has been found in *Drosophilia melanogaster* nor in *Xenopus laevis*. Yet, $PML^{-/-}$ mice are more sensitive to cancer-promoting drugs and conversely more resistant to γ-irradiation, due to defects in the apoptosis process. Finally, expression studies have shown that in normal human tissues, PML expression is restricted to some myeloid and endothelial cells (Flenghi *et al.*, 1995; Koken *et al.*, 1995). However, PML expression pops up in response to a number of stresses (Koken *et al.*, 1995; Terris *et al.*, 1995). All of these observations, which suggest a role of PML˜ and hence PML bodies˜ in stress responses, should be kept in mind when thinking about PML bodies.

Several models have been put forward to assign a function to PML bodies. All these models have to take into account the number and striking variety of partner proteins, as well as the unessential nature of the PML gene and NBs. PML bodies were proposed to be: (i) active sites for some enzymatic modifications of partner proteins, in particular sumolation, but also ubiquitination or acetylation (Everett, 2000); (ii) sites of transient accumulation of sequestered proteins, in particular transcription factors, coactivators or corepressors such as Daxx (Li *et al.*, 2000a; Lehembre *et al.*, 2001); (iii) sites of degradation of proteins misfolded or tagged for degradation (Anton *et al.*, 1999; Lallemand-Breitenbach *et al.*, 2001; Lafarga *et al.*, 2002). It is also possible that some functions of PML are independent

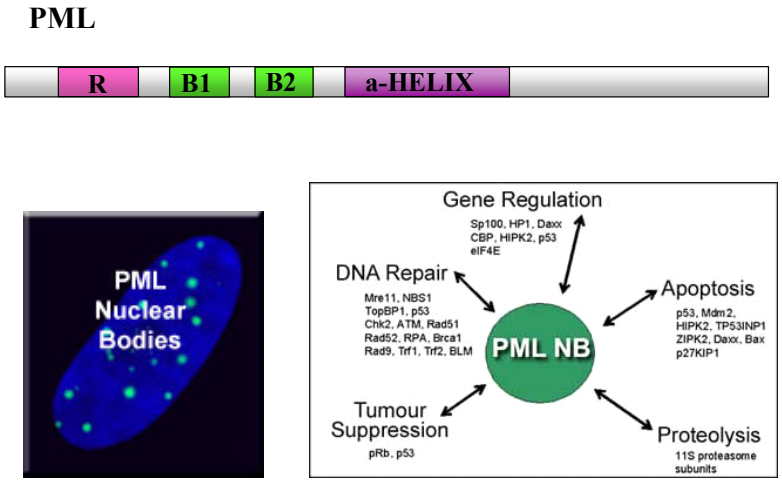


Fig.6. PML structure and PML nuclear bodies.

PML protein contains a zinc finger domain termed RING, two zinc fingers termed B-boxes and an a-helical coiled coiled motif (RBCC) that mediate protein-protein interaction.
The figure summarises the many diverse cellular functions attributed to PML nuclear bodies and lists the proteins implicated in those processes that localize at PML NBs or associate with PML directly.

of its ability to form nuclear bodies, as proposed by a recent study (Bischof *et al.*, 2002).

PML modification by the ubiquitin-like protein SUMO1 is an essential prerequisite for proper NB formation (Zhong *et al.*, 2000a). Indeed, while PML-NBs fail to form in $PML^{-/-}$ cells, exogenous expression of PML can nucleate their formation; however, exogenous expression of a PML that is mutated at its three SUMO modification sites is unable to nucleate PML-NB formation (Ishov *et al.*, 1999 and Zhong *et al.*, 2000a).

This observation allowed proposing a working model by which PML has to be at first sumoylated to be able to recruit other NB components in a mature and properly formed PML-NB (Zhong *et al.*, 2000b). Also in agreement with this model, time-lapse imaging after photobleaching reveals that NB components including PML are dynamically recruited in the PML-NB (Wiesmeijer *et al.*, 2002). The picture is further complicated by the fact that sumoylation is a reversible process. The identification of enzymes that can remove the SUMO moiety from its substrates such as ULP1 and SENP1, in fact renders sumoylation-dependent processes dynamic and possibly subject to tight regulation (Zhong *et al.*, 2000b). In this respect, PML desumoylation could induce the release and mobilization of PML and NB components from the PML-NB for transcriptional regulation and/or checkpoint/DNA repair in response to apoptotic stimuli. Indeed, a PML desumoylase, SUMO protease 1 (SUPR-1), has been recently identified and as anticipated, its forced overexpression induces a mobilization of PML and NB components (e.g. CBP) from the NB (Best *et al.*, 2002). PML sumoylation and desumoylation may therefore regulate the function of multiple NB proteins by regulating their in- and out-flow from the NB upon apoptotic stimuli. For example, while p53 is recruited in the PML-NB upon γ-irradiation for acetylation, transcriptional activation and possibly stabilization, it is possible that, once activated, p53 is released from the PML-NB in a SUPR-1-PML-desumoylation-dependent manner for DNA-binding and transcription regulation. This would also explain why nascent mRNAs and active transcription are not detected in the PML-NBs, but rather in their vicinity (Grande *et al.*, 1996).

The work reported by Shen *et al.* in a recent issue of *Molecular Cell* (Shen *et al.*, 2006) provides an attractive new model that helps to explain the essential role that PML plays in regulating PML-NB formation and why SUMO modification is essential to this process. Critical to this new model is the discovery that PML contains a SUMO binding motif that enables it to interact noncovalently with SUMO. The motif in PML resembles a common

SUMO binding motif recently identified in other proteins shown to bind SUMO noncovalently (Minty *et al.*, 2000 and Song *et al.*, 2004). Shen *et al.* found that mutating the SUMO binding motif in PML did not affect the ability of PML to be SUMO modified, but it did affect the ability of PML to nucleate the formation of PML-NBs in $PML^{-/-}$ cells. Thus, for PML to nucleate the formation of PML-NBs, it must be SUMO modified and it must also be able to interact noncovalently with SUMO. These findings suggest a model whereby PML-NB formation is dependent on a network of noncovalent interactions between the SUMOs on SUMO-modified PMLs and the SUMO binding motifs on PML. Many other proteins in PML-NBs are also SUMO modified or contain SUMO-binding motifs, further suggesting that PML-NB assembly occurs through a complex network of noncovalent interactions between modified substrates and SUMO-binding proteins, as recently proposed (Takahashi *et al.*, 2005). One question that is still not fully understood is why the PML protein itself is so critical for PML-NB formation, but Shen *et al.* provide one tantalizing possibility. They demonstrate that the RING domain of PML is required for efficient SUMO modification of PML and also for PML-NB formation. Thus, they propose that PML may function as a SUMO E3 ligase to catalyze its own SUMO modification and possibly the SUMO modification of other PML-NB-associated proteins. Moreover, recently has been demonstrated that PML stimulates SUMO conjugation in yeast (Quimby *et al.*, 2006).

If the formation of the structure is linked to function, then external factors that influence NBs are likely to provide insights into their function. In that respect, PML localization and sumolation are regulated by phosphorylation, heat shock and exposure to proteasome inhibitors or arsenic (Muller *et al.*, 1998; Lallemand-Breitenbach *et al.*, 2001; Negorev and Maul, 2001; Pokrovskaja *et al.*, 2001; Zhu *et al.*, 2002). The latter observation is particularly interesting, because arsenic trioxide induces clinical remissions in acute promyelocytic leukaemia (Zhu *et al.*, 1997, 2002), a disease in which PML is fused to RARA (de Thé *et al.*, 1990, 1991; Warrell *et al.*, 1993). Arsenic triggers a rapid PML or PML/RARA sumolation, followed by proteasome-dependent catabolism (Zhu *et al.*, 1997; Muller *et al.*, 1998; Lallemand-Breitenbach *et al.*, 2001). PML nuclear matrix targeting, the first step of NB formation, is dependent on dephosphorylations (Muller *et al.*, 1998; Lallemand-Breitenbach *et al.*, 2001), whereas the second step–NB maturation with recruitment of partner proteins – is dependent on PML sumolation (Ishov *et al.*, 1999; Zhong *et al.*, 2000a). After mitosis, PML aggregates in the cytoplasm without any NB-associated proteins (Koken *et*

al., 1995), reflecting PML phosphorylation and desumolation (Everett *et al.*, 1999). In contrast to As_2O_3, which induces recruitment of partner proteins through an induction of PML sumolation, cellular stresses (including heat shock) induces desumolation and release of partner proteins from PML NBs (Maul *et al.*, 1995).

Role of PML in multiple apoptotic pathways

PML is ubiquitously expressed, albeit at very low levels and PML-NBs are detected in almost any cell of the developing embryo or the adult organism. Nonetheless, PML is markedly upregulated upon a number of cellular stresses including inflammation, oncogenic transformation and proapoptotic stimuli such as for instance ionizing radiation (Terris *et al.*, 1995; Ferbeyre *et al.*, 2000; Pearson *et al.*, 2000; Carbone *et al.*, 2002). Under these conditions, the number and the size of the PML-NBs (and hence the matrix-associated PML fraction), as well as the soluble nonmatrix PML nuclear and cytosolic fractions increase (Salomoni, Bernardi and Pandolfi, unpublished observation). These stimuli also trigger a dynamic reorganization of the PML-NB with transient recruitment and release of proteins from these nuclear organelles.

Definitive proof of the importance of PML in modulating programmed cell death programs comes from studies conducted in *Pml*$^{-/-}$ mice and cells. *Pml*$^{-/-}$ mice are resistant to the lethal effects of both γ-irradiation and CD95/Fas (Wang *et al.*, 1998b), while primary *Pml*$^{-/-}$ splenocytes, thymocytes mouse embryonic fibroblasts (MEFs) and hematopoietic cells are resistant to apoptosis induced by either CD95/Fas or γ-irradiation, as well as by ceramide, TNF and IFN (Wang *et al.*, 1998b; Wu *et al.*, 2003). Caspase activation upon these stimuli is invariably impaired in *Pml*$^{-/-}$ cells (Wang *et al.*, 1998b; Wu *et al.*, 2003). PML is therefore essential for both p53-dependent (e.g. apoptosis of primary thymocytes upon γ-rays: Wang *et al.*, 1998b) as well as p53-independent apoptotic programs (e.g. apoptosis of activated splenocytes or hepatocytes upon CD95/Fas; Wang *et al.*, 1998b; Zhong *et al.*, 2000c).

Strong evidence obtained in *Pml* null primary cells of various histological origins has recently established a key role for PML and the PML-NB in the regulation and functional activation of a number of proapoptotic/tumor suppressive transcription factors. These transcription factors are known to be

essential for the induction of apoptosis in response to the same various stimuli that depend on PML to be properly executed. These findings on the one hand, point to the PML-NBs as general sites of transcriptional regulation, on the other explain how PML could coordinate both p53-dependent and -independent apoptotic responses.

PML and p53

Pml$^{-/-}$ mice are resistant to the lethal effects of γ-radiation, thus demonstrating that PML is involved in modulating the apoptotic response to DNA damage. The tumor suppressor p53 plays a key role in this process. In particular, *p53*$^{-/-}$ thymocytes are completely resistant to γ-radiation-induced apoptosis, indicating that normal p53 function is absolutely required for DNA damage-induced apoptosis in this cell type. *Pml*$^{-/-}$ thymocytes are also resistant to γ-radiation-induced apoptosis, although to a lesser extent than *p53*$^{-/-}$ thymocytes, suggesting that Pml may be important in mediating proper p53 proapoptotic functions *in vivo* (Guo *et al.*, 2000). In support of this notion, protection from apoptosis in *Pml*$^{-/-}$ thymocytes correlates with impaired induction of *bona fide* p53 target genes, such as the proapoptotic gene *bax* and the inhibitor of cell cycle *p21* (Guo *et al.*, 2000). Indeed, PML directly interacts with the DNA-binding domain of p53, colocalizes with p53 in the PML-NB and acts as a p53 transcriptional coactivator (Fogal *et al.*, 2000; Guo *et al.*, 2000; Pearson *et al.*, 2000). PML specifically interacts with p53 through the C-terminal domain of one of the NB-specific isoforms of PML (PML IV; Fogal *et al.*, 2000; Guo *et al.*, 2000). In *Pml*$^{-/-}$ cells, the γ-radiation-induced acetylation of p53 is impaired, indicating that PML might regulate p53 transcriptional function favoring its acetylation (Guo *et al.*, 2000). PML has also been shown to regulate p53 activity by promoting its acetylation when premature cellular senescence is triggered by oncogenic Ras^{Val12} overexpression (Pearson *et al.*, 2000). Although PML does not possess intrinsic acetyltransferase activity, it directly interacts with the acetyltransferase CBP and colocalizes with CBP/p300 and p53 in the PML-NB (Guo *et al.*, 2000; Pearson *et al.*, 2000). Strikingly, the ability of PML to activate p53 transcription is PML-NB dependent, since a mutant of PML that does not localize in the PML-NB, but still interacts with p53 and CBP, is unable to coactivate p53 transcription (Guo *et al.*, 2000).

The ability of PML and the PML-NB to potentiate p53 function goes beyond the sole regulation of p53 acetylation. Recent reports identified in the

PML-NB two additional important p53 regulators: HIPK2 a p53 kinase and HAUSP, a p53 deubiquitilase. The homeodomain-interacting protein kinase-2 (HIPK2) directly interacts with and phosphorylates p53 at serine 46, resulting in the activation of p53 proapoptotic transcription (D'Orazi *et al.*, 2002; Hofmann *et al.*, 2002).

The Herpesvirus-associated ubiquitin-specific protease (HAUSP) interacts with and strongly stabilizes p53 even in the presence of excess Mdm2 and induces p53-dependent apoptosis (Li *et al.*, 2002). HAUSP has an intrinsic enzymatic activity that specifically deubiquitinates p53 both *in vitro* and *in vivo*, therefore inducing p53 stabilization and activation. Thus, acetylation, phosphorylation and deubiquitination of p53 could occur in the PML-NB.

PML and p73

In the first publication on p73, a speckled distribution of p73 within the nucleus was described (Kaghad *et al.*, 1997) without further specification. Now, a more recent report (Bernassola *et al.*, 2004) describes a partial co-localization of p73 with the PML protein. Moreover, cells with a genetic deletion of *PML* are defective for p73-induced apoptosis. The authors suggest a mechanism for this phenomenon: p73 first needs to be phosphorylated through a p38-dependent kinase pathway, in order to be recruited to the PODs. Conversely, POD-association is a prerequisite for the acetylation of p73 by histone acetyltransferase p300. Finally, p73 acetylation inhibits the conjugation of p73 to ubiquitin, thereby stabilizing p73 and increasing p73-induced transcription and apoptosis.

An important POD-associated posttranslational modification consists in the conjugation of proteins with the small ubiquitin-like modifier (SUMO). In fact, most of the known principal components of PODs are SUMOylated (Seeler *et al.*, 2001). Intriguingly, a proportion of p73 molecules are conjugated with SUMO (Minty *et al.*, 2000). Taken together, it might be the POD-associated fraction of p73 that is SUMOylated. Initial evidence did not support the idea that SUMOylation might alter p73-induced transcription (Minty *et al.*, 2000), but further experiments will be needed to definitely rule out such a possibility. Since p73 was found to be destabilized by SUMO (Minty *et al.*, 2000), and since stabilization vs. degradation also appears as a principal mechanism of regulating p73-mediated apoptosis by PML (Bernassola *et al.*, 2004), a positive or negative role of SUMO in p73-induced apoptosis at least deserves more detailed investigation.

Daxx and PML

PML is necessary for FAS- and TNF-induced apoptosis, thus indicating that PML can also modulate p53-independent proapoptotic pathways. However, unlike p53-dependent apoptosis, which is triggered from the nucleus, FAS/TNF apoptotic signals are initiated by the death-inducing signaling complex (DISC) at the plasma membrane and are then transduced to a caspase proteolytic cascade. Thus, the proapoptotic signal would need to be transduced from the plasma membrane to the PML-NB. One of the most relevant candidates to serve this bridging function between the DISC and the PML-NB is DAXX, originally cloned as a FAS-interacting protein and found to act as a positive mediator of FAS- and TGFβ-induced apoptosis (Perlman *et al.*, 2001). DAXX directly interacts both with PML in the PML-NB as well as PML-RARα (Torii *et al.*, 1999; Zhong *et al.*, 2000b). PML and DAXX cooperate in potentiating FAS-induced apoptosis (Torii *et al.*, 1999). In the absence of Pml, DAXX is delocalized from the PML-NB and accumulates in chromatin-dense nuclear regions (Zhong *et al.*, 2000b). Furthermore, in *Pml*$^{-/-}$ cells the ability of DAXX to trigger apoptosis and to potentiate the FAS proapoptotic signal is markedly impaired (Zhong *et al.*, 2000b). DAXX is a transcriptional repressor and its sequestration in the PML-NB has been shown to block this activity. Conversely, the expression of PML-RARα results in the delocalization of DAXX from the PML-NB, in turn enhancing DAXX transcriptional repression (Li *et al.*, 2000). Recently, it has been shown that DAXX movement from the PML-NB can be regulated by the homeodomain-interacting protein kinase-1 (HIPK1) through physical association. However, subsequent phosphorylation of Daxx on Ser 669 by this kinase attenuates DAXX transcriptional repression (Ecsedy *et al.*, 2003). Taken together, all these observations may suggest a model by which DAXX represses antiapoptotic genes and PML would antagonize this activity through titration of DAXX in the NBs leading to potentiation of apoptosis.

Recently the work by Lin *et al.* demonstrates that Daxx contains a SUMO-binding motif that, like PML's SUMO-binding motif, is essential for binding to SUMO-modified PML and for its targeting to PML-NBs (Lin *et al.*, 2006). This same SUMO-binding motif is also shown to be essential for interactions between Daxx and SUMO-modified transcription factors and essential for Daxx's ability to repress transcription. In vitro binding studies indicate that SUMO-modified PML is able to compete for interactions between Daxx and SUMO-modified glucocorticoid receptor,

suggesting that competition for the Daxx SUMO-binding motif regulates its partitioning between PML-NBs and transcription sites in the nucleoplasm. Consistent with this model, increasing the levels of SUMO-modified PML in the nucleus by exogenous expression of PML or by treatment of cells with arsenic trioxide results in increased sequestration of Daxx in PML-NBs and relief of transcriptional repression of Daxx regulated genes.

NF-κB/Rel and PML

As aforementioned, *Pml* inactivation also protects cells from TNF-induced apoptosis in the bone marrow (Wang *et al.*, 1998b). Conversely, it was recently shown that ectopic expression of PML sensitizes to TNF-induced apoptosis TNF-resistant cell lines such as U2OS and other cell lines (Wu *et al.*, 2003). Once again, at the transcription level PML was found to modulate a survival pathway negatively, which in turn antagonizes TNF proapoptotic activity. PML has been shown to act as a transcriptional repressor of NF-κB by physically interacting with RelA/p65, one of the two subunits of NF-κB, and inhibiting its DNA-binding potential. PML physically interacts with RelA/p65 *in vivo* and sequesters it in the PML-NB. Indeed, overexpression of NF-κB antagonizes cell death induced by PML/TNF (Wu *et al.*, 2003). In agreement with these findings, PML was found to repress the TNF-dependent induction of the zinc finger protein A20. A20 is an NF-κB target, inducible by TNF, and it is a potent inhibitor of TNF-induced apoptosis (Wu *et al.*, 2002).

AIM OF THE WORK

The recently established p53 family is heavily involved in the transcriptional control of many biological processes ranging from growth arrest and apoptosis to development and differentiation (Blandino and Dobbelstein, 2004). p53 deficient mice develop quite normally but undergo spontaneous tumours, mainly sarcomas and lymphomas. In contrast, p73 and p63 knock-out mice exhibit severe developmental defects (Mills *et al.*, 1999; Yang *et al.*, 1999; Yang *et al.*, 2000). The ectopic expression of p73 and p63 in p53 -/- and p53 +/+ cells causes, similarly to p53, growth arrest, apoptosis and differentiation (Kaghad *et al.*, 1997; Yang *et al.*, 1998; Jost *et al.*, 1997; De Laurenzi *et al.*, 2000; Fontemaggi *et al.*, 2002; Strano *et al.*, 2005). These effects are achieved mainly through the activation of a plethora of specific target genes. Several reports have shown that p73 and p63 bind to p53-binding sites, *in vitro* and *in vivo*, and consequently activate p53 target genes (Kaghad *et al.*, 1997; Jost *et al.*, 1997). Thus, transcriptional activation and repression of specific sets of target genes or the recruitment of specific co-activators and co-repressors might explain the major differences between p53 family members when their respective gene targeting phenotypes are compared. We have previously shown through microarray analysis that inducible expression of p53 or p73α promotes the transcriptional activation or repression of common, as well as quite distinct, patterns of direct target genes (Fontemaggi *et al.*, 2002). Among them we found that the S100A2 gene was induced by p73α but not by p53. The gene product of S100A2 is a member of the calcium-binding S100 family of proteins and was identified in a screen for tumour suppressor genes by subtractive hybridization between normal mammary epithelial cells and breast tumour cells (Vellucci *et al.*, 1995). At least 10 of the S100 group of proteins are clustered in the epidermal differentiation complex (EDC) located on human chromosomal band 1q21 (Mischke *et al.*, 1996; Eckert et al, 2004). The EDC also contains the genes encoding loricrin, involucrin, filaggrin, trichohyalin and several small proline-rich region proteins known to play important structural roles in keratinocyte terminal differentiation, including keratin filament reorganization, chromatin condensation and cornified envelope formation (Dale *et al.*, 1997; Steinert and Marekov, 1997). S100 proteins appear to influence cell shape and motility via calcium-dependent interactions with cytoskeletal proteins and modulation of protein kinase activity (Heierhorst *et al.*, 1996; Donato 2003), suggesting that they may mediate at least some of the protean effects of calcium on epithelial differentiation.

The aim of the first part of this work is to investigate the functional relationship between p73/p63 and S100A2 during keratinocyte

differentiation. Interestingly, it has been demonstrated that the expression of p53 family members is modulated during keratinocyte differentiation and, in particular, it has been reported that ΔNp63 is downregulated and p73 is upregulated (De Laurenzi *et al.*, 2000; Bamberger *et al.*, 2002).

Despite the recent identification of specific p73-target genes by genome-wide expression profile techniques (Fontemaggi *et al.*, 2002), p73-mediated apoptosis occurs mostly through the activation of a set of genes that were originally found to be activated by p53 (Costanzo *et al.*, 2002, Irwin *et al.*, 2003 and Bergamaschi *et al.*, 2003). This suggests that promoter selectivity by both p53 and p73 might be the result of biochemical events such as post-translational modifications and specific protein-protein interactions (Costanzo *et al.*, 2002; Agami *et al.*, 1999; Gong *et al.*, 1999, Yuan *et al.*, 1999; Strano *et al.*, 2001). An important cofactor involved in the full activation of p73 in response to DNA damage and other stressors is the prolyl isomerase Pin1 (Mantovani *et al.*, 2004). Pin1 activity is also involved in DNA damage-induced activation of p53 (Zacchi *et al.*, 2002 ; Zheng *et al.*, 2002) and therefore cannot dictate the final biological outcome of the cell response to DNA damage, i.e., growth arrest versus apoptosis. Selective activation of specific subsets of target genes is increasingly recognized as a crucial event in the choice between p73-mediated growth arrest or apoptosis in response to DNA damage (Costanzo *et al.*, 2002; Bergamaschi *et al.*, 2003). Our recent demonstration that the transcriptional coactivator YAP (Sudol and Hunter, 2000) determines p73 gene targeting in response to DNA damage (Strano *et al.*, 2005) is one such example.
The aim of the second part of this work is to gain insight into the apoptotic response specifically triggered by the p73/YAP complex. Specific target genes, whose transcriptional activation upon cisplatin treatment requires both p73 and YAP, will be identified by microarray analysis.

MATERIALS AND METHODS

Cell culture

Transformed human HaCaT keratinocytes were cultured in Dulbecco's modified medium (DMEM) with 10% fetal bovine serum (FBS); differentiation was induced by removal of growth factors and addition of 2mM $CaCl_2$. Human epithelial non-small cell lung carcinoma (NSCLC) cell line H1299 was maintained in RPMI supplemented with 10% fetal calf serum (FCS). Human colon carcinoma cell line HCT116, mouse embryo fibroblasts MEFs and human breast cancer cell line SKBR3 were cultured in Dulbecco's modified medium (DMEM) with 10% fetal bovine serum (FBS).

Immunoprecipitation and Western Blot Analysis

Cells were lysed with 50 mM Tris, pH 8, 100 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM EDTA, 100 mM NaF, 1 mM $MgCl_2$, 2 mM phenylmethylsulfonyl fluoride, and protease inhibitors, and the extracts were sonicated for 10 s and centrifuged at 14000 × rpm for 10 min to remove cell debris. Protein concentrations were determined by a colorimetric assay (Bio-Rad). After preclearing for 60 min at 4 °C with protein G-agarose (KPL, Guilford, CA), immunoprecipitations were performed by incubating 1 mg of whole-cell extract with anti-PML monoclonal antibody PG-M3 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or with anti-YAP policlonal antibody H-125 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA)or with unspecific IgG, with rocking at 4 °C for 2 h. Immunocomplexes were precipitated with protein G-agarose (KPL, Guilford, CA). The immunoprecipitates were washed three times with 1 ml of wash Net-gel buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.25% gelatin, 0.1% Nonidet P-40). The excess liquid was aspirated, and 20 µl of 1X sample buffer was added. Immunoprecipitates as well as 50µg of each extract were resolved by SDS-10% PAGE. Protein gels were transferred to nitrocellulose membranes (Bio-Rad). For immunoblotting, the following antibodies were used: anti-p73 monoclonal antibody (Ab4, Neomarker) at a 1:200 dilution; anti-S100A2 monoclonal antibody (Transduction Laboratories) at a 1:1000 dilution; anti-p63 monoclonal antibody (4A4, Santa Cruz) at a 1:500 dilution; anti-p53 monoclonal antibody (DO1) at a 1:40 dilution; anti-p21 polyclonal antibody (C19, Santa Cruz) at a 1:200 dilution; anti-bax polyclonal antibody (N20, Santa Cruz) at a 1:200 dilution; anti-involucrin monoclonal antibody (from

Dr.Costanzo) at a 1:200 dilution; anti-Hsp70 monoclonal antibody (StressGen) at a 1:1000 dilution; anti-PARP p85 fragment polyclonal antibody (Promega) at a 1:400 dilution; anti-tubulin monoclonal antibody (Sigma) at a 1:1000 dilution; anti-YAP polyclonal antibody (H125, Santa Cruz) at a 1:1000 dilution; anti-PML monoclonal antibody (PG-M3, Santa Cruz) at a 1:1000 dilution; anti-GFP monoclonal antibody (Invitrogen) at a 1:5000 dilution and anti-HA monoclonal antibody (Covance) at a 1:2000 dilution. Western blot analysis was performed with the aid of the enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotech, Inc.).

Indirect immunofluorescence

HaCaT, HCT116 and H1299 cells were first incubated for 5 min at room temperature with a solution containing 10% bovine serum albumin, 0.5% Tween 20 in 1× PBS (PAT solution) and then fixed in 4% PBS-paraformaldeyde plus 0.1% Triton-100 for 10 min on ice. After rehydration with PAT for 5 min, the cells were incubated in 50% PAT and then stained for 1 h with an anti-S100A2 monoclonal antibody (Transduction Laboratories) used at a 1:250 dilution or with an anti-PML monoclonal antibody (PG-M3, Santa Cruz) used at a 1:400 dilution for 2 hrs. Staining with the secondary antibody and with Hoechst was performed as described before (Blandino et al 1999), followed by visualization under a fluorescence microscope.

Plasmids

Three S100A2 regulatory regions (one located in the promoter and two in the second intron) enclosing p53 family consensus sequences were amplified by PCR and cloned in SalI/BamHI sites of TK-LUC reporter vector (Fontemaggi *et al.*, 2001) using the following oligonucleotides: S100A2 promoter, sense CCG ACC CGT TGT CTC GGT TC, antisense CAA GGG GGA AGG CCC AGA GA; S100A2 second intron (binding site 1), sense GTG TTC AAA GCC TGA CAC CTA ACT T, antisense TGG ATC ATA GCT CAC TGT AAT CTC G; S100A2 second intron (binding site 2), sense AAG TAG CTG GGA CTA CAA GCG TAT G, antisense GGG ATA GAA AAG CCC AGC TAA GAT A. Oligonucleotides, derived from p73 and

S100A2, enclosing the following target sequences, were ligated into pRetro-Super expression plasmids as described previously (Brummelkamp *et al.*, 2002): TAp73, CCA GAC AGC ACC TAC TTC G; S100A2, CCA CAG TGA GCA GGT GGA C.

Transfections and luciferase assays

Transient transfections were performed using either the calcium phosphate precipitation method (BES) or the Lipofectamine Plus reagent (GIBCO-BRL). H1299 cells were transfected with reporter plasmid together with the indicated expression plasmid combinations. 36 h later, cells were rinsed with cold phosphate-buffered saline, resuspended in cell lysis buffer (Promega Corp., Madison, WI), and incubated for 10 min at room temperature. Insoluble material was spun down, and luciferase activity was quantitated using a commercially available kit (Promega) with the aid of a TD-20E luminometer (Turner). Control plasmid (pCMVneo) was added to equally normalize the amount of transfected DNA for each transfection.

RNA extraction and reverse transcriptase reaction

Cells were harvested in TRIzol reagent (Invitrogen) and total RNA was isolated as per the manufacturer's instructions. Five micrograms of total RNA was reverse-transcribed at 37 °C for 45 min in the presence of random hexamers and Moloney murine leukemia virus reverse transcriptase (Invitrogen). Polymerase Chain Reaction (PCR) analyses were carried out by using oligonucleotides specific for the following genes: S100A2 (down 5'- GTA AGG GGG AAA TGA AGG AAC TTC T; up 5'- ACA AAA CTC AAA GGC ATC AAC AGT C); p73 (down 5'- TCA GGA TTT GAA CCC AGA CC; up 5'- CTT TGC ACC ACT GAC CTT GA); involucrin (down 5'- TAG AGG AGC AGG AGG GAC AA; up 5'- AGG GCT GGT TGA ATG TCT TG); K10 (down 5'- GCT TCA GAT CGA CAA TGC AA; up 5' – AGC ATC TTT GCG GTT TTG TT); Pig3 (down 5'- CCG GAA AAC CTC TAC GTG AA; up 5'- CTC TGG GAT AGG CAT GAG GA); p53AIP1 (down 5'- TCA GGA TTT GAA CCC AGA CC; up 5'- CTT TGC ACC ACT GAC CTT GA); $p21^{waf1}$ (down 5'- CCT CTT CGG CCC GGT GGA C; up 5'- CCG TTT TCG ACC CTG AGA G); hPML (down 5'- AGT CGG TGC GTG AGT TCC T; up 5'- GGA ACA TCC TCG GCA GTA

GA); mPML (down 5'- AAA AGA TCC TCC TGC CCA AC; up 5'- CAG ATT CTC GGT GTC CGA AT). The housekeeping aldolase A mRNA, used as an internal control, was amplified from each cDNA reaction mixture using the following specific primers: down 5'- CGC AGA AGG GGT CCT GGT GA; up 5'- CAG CTC CTT CTT CTG CTG CG.

Real-Time RT-PCR

PCR was performed on the cDNA samples using an ABI PRISM 7500 Sequence Detector (PE Applied Biosystems). Specific primers for *TAp63* and *ΔNp63* isoforms, with similar amplification efficiencies (Signoretti *et al.*2000) and specific primers for *hPML* and *mPML* were used. The housekeeping glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as endogenous control to standardize the amount of RNA in each reaction (Taqman GAPDH control reagents).

Formaldehyde cross-linking and chromatin immunoprecipitation

DNA and proteins were cross-linked by the addition of formaldehyde (1% final concentration) 10 min before harvesting, and cross-linking was stopped by the addition of glycine pH 2.5 (125 µM final concentration) for 5 min at room temperature. Cells were scraped off the plates, resuspended in hypotonic buffer, and passed through a 26-gauge needle. Nuclei were spun down, resuspended in 300 µl of SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8, and a protease inhibitor mixture), and sonicated to generate 500-2000-bp fragments. After centrifugation, the cleared supernatant was diluted 10-fold with immunoprecipitation buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40). The cell lysate was precleared by incubation at 4 °C with 15 µl of protein G beads preadsorbed with sonicated single-stranded DNA and bovine serum albumin. The cleared lysates were incubated overnight with specific antibodies or without any antibody. Immune complexes were precipitated with 30 µl of protein G beads preadsorbed with sonicated single-stranded DNA and bovine serum albumin. After centrifugation the beads were washed, and the antigen was eluted with 1% SDS, 100 mM sodium carbonate. DNA-protein cross-links were reversed by heating at 65 °C for 4-5 h, and DNA was phenol-extracted and ethanol-precipitated. The following antibodies were used: a

mixture of anti-p73 polyclonal antibodies (sc-7237 and sc-7238, Santa Cruz); anti-p63 polyclonal antibody (sc-8343, Santa Cruz); anti-acetyl-histone H4 (cat. n. 06-866, Upstate Biotechnology, Inc.); anti-YAP (H125, Santa Cruz); a mixture of anti-p300 polyclonal antibody (sc-584 and sc-585, Santa Cruz). Enrichment in S100A2, p53AIP1, TK and PML sequences was evaluated by PCR. The following specific oligonucleotides were used: S100A2 promoter (sense 5'- GCT CAC TAT GTG GGG TGA GG, antisense 5'- CTG CGT GTC CCT TAA ACA CA); S100A2 second intron (sense 5'- CTC AAG CAA TCC TCC CAA GT, antisense 5'- CAG CAA TCC TCC CAC CTT TA); p53AIP1 first intron (sense 5'- TGG GTA GGA GGT GAT CTC ACC, antisense 5'- GAG CAG CAC AAA ATG GAC TGG G); PML promoter (sense 5'- TTT GAG GCT GCA GTG AGC TA, antisense 5'- GGC CAG GCC ATT ACT TAA CA); PML first intron (sense 5'- GGA GAG GAG CTG TTT GGT GA, antisense 5'- GGG GAA GGA GAA AAT GAG GA). Oligonucleotides specific for TK promoter (down 5'- GTG AAC TTC CCG GAG GCG CAA ; up 5'- GCC CCT TTA AAC TTG GTG GGC) were used as negative control.

Electrophoretic mobility shift assay

Recombinant p73β or ΔNp63 or TAp63 proteins were produced from plasmids carrying the cDNAs under the control of T7 promoter using TnT Coupled Reticulocyte Lysate Systems (Promega), according to the manufacturer's protocol. Electrophoretic mobility shift assays (EMSAs) were performed on a 25-µl DNA binding reaction mixture which contained in vitro translated p73β or ΔNp63 or TAp63 proteins, 4 fmol of labeled duplex oligonucleotides, binding buffer (20 mM Tris-HCl [pH 7.8], 60 mM KCl, 0.5 mM EDTA, 0.1 mM dithiothreitol, 3 mM $MgCl_2$), 1.5 µg of poly(dI-dC), 10 mM spermidine, and 100 to 400 ng of salmon sperm. The reaction was carried out at room temperature for 15 min, and the protein-DNA complexes were subjected to native electrophoresis on 5% polyacrylamide-0.5× TBE gels. A double-stranded oligonucleotide corresponding to the p53 family consensus of S100A2 promoter was used as probe. In supershift experiments antibodies were added to the mixture before the labeled oligonucleotides and the mixture was incubated for 10 min at room temperature. For supershift analysis anti-p73 (sc-7237 and sc-7238 from Santa Cruz) and anti-p63 (sc-8343 from Santa Cruz) polyclonal antibodies were used.

siRNA studies

HaCaT cells were transiently transfected using the Lipofectamine Plus Reagent (Invitrogen) according to the manufacturer's protocol with 2 µg of GFP expressing vector, along with either 1 µg of the control small interfering RNAs (unrelated oligo) or with sip73 RNAs. 24 hours after the transfection the cells were induced to differentiate as described above. Small Interfering RNAs were purchased from DHARMACON Inc. Stable expression of siRNAs directed against LacZ, p73 and S100A2 was obtained by transfection of HaCaT cells with pRetroSuper-LacZ (negative control), pRetroSuper-p73 or pRetroSuper-S100A2 vectors followed by selection with 1µg/ml puromycin. After 10 days of culture in puromycin supplemented medium, confluent cells were induced to differentiate as described above.

HCT116 cells and SKBR3 cells were traniently transfected using the Lipofectamine Plus Reagent (Invitrogen) according to the manufacturer's protocol with either 1 µg of the control small interfering RNAs (unrelated oligo, AA/GUUCAGCGUGUCCGGGGAG) or with sip73 RNAs (AA/CCAUCCUGUACAACUUCAUGU) or with siYAP RNAs

(AA/GACAUCUUCUGGUCAGAGA). 24 hours after the transfection the cells were treated with cisplatin (7.5 μg/ml).

Cell cycle analysis

HaCaT cells were treated with cisplatin (2.5 μg/ml, 5 μg/ml and 7.5 μg/ml) and were collected at the indicated time points. Cells were then washed with 1x PBS and resuspended in 1x PBS/Triton X-100, 0.1% PI 0.1 mg/ml and the percentages of apoptotic cells were evaluated with an Epics-XL analyzer (Coulter, Corporation). Data were analysed with the Multicycle software (Phoenix Flow System).

RESULTS

S100A2 gene is a direct transcriptional target of p53 homologues during keratinocyte differentiation.

S100A2 expression is induced during keratinocyte differentiation.

To search for target genes specifically activated or repressed by p73 and not by wt-p53 we previously performed a DNA microarray analysis (Fontemaggi *et al.*, 2002) using a cell line whose ectopic inducible expression of either p73α or p53 was under the control of ponasterone A. S100A2 was found to be a transcriptional target of p73 but not of p53 (Fontemaggi *et al.*, 2002). Here we show that the S100A2 transcript and protein are induced during keratinocyte differentiation of HaCaT cells (Fig.7A,D). It has previously been reported that the expression of p53 family members is modulated during keratinocyte differentiation. In particular, ΔNp63, which is the most abundant p63 isoform in HaCaT cells (Fig.7C), is down-regulated, and p73 is up-regulated (De Laurenzi *et al.*, 2000; Bamberger *et al.*, 2002). As shown in Fig. 7A-D, we found that induction of S100A2 during keratinocyte differentiation of HaCaT cells coincides with the down-regulation of ΔNp63α and up-regulation of p73β. The latter is the only p73 isoform present in HaCaT cells, as determined by RT-PCR using primers that discriminate between the different p73 isoforms (Fig.7A). The analysis of $p21^{waf1}$, involved in the exit from cell-cycle, and two differentiation markers (involucrin and keratin 10), confirmed the proper differentiated status of HaCaT cells (Fig.7A,D).

S100A2 is a direct transcriptional target of p73β and ΔNp63α.

Next, we investigated whether S100A2 was a direct transcriptional target of p53 family members during keratinocyte differentiation. We have previously found that the second intron of the human S100A2 gene contains two inverted/repeated p53/p63/p73 consensus sites (Fontemaggi *et al.*, 2002) (Fig.2A). It has also been reported that the S100A2 promoter contains a p53/p63/p73 consensus site that has been shown to be transcriptionally activated by wt-p53 (Tan *et al.*, 1999) (Fig.8A). To assess the ability of p53 family members to directly bind *in vitro* the p53/p63/p73 binding site of S100A2 promoter, we performed an Electrophoretic Mobility Shift Assay (EMSA). To this end, a phospholabeled oligonucleotide encompassing the S100A2 promoter's p53/p63/p73 binding site was incubated with *in vitro*

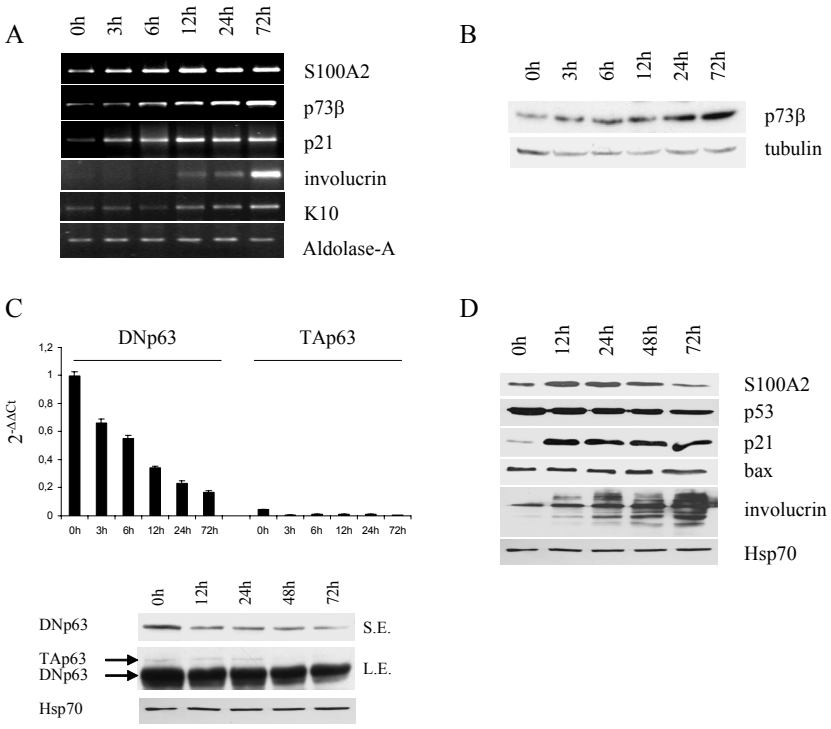


Fig.7. S100A2 expression is induced during keratinocyte differentiation.

(A) RNA was extracted from HaCaT cells at the indicated time points following induction of differentiation and subjected to RT-PCR analysis. Amplification of aldolase-A was used for normalization.
(B) Detection of p73 protein levels during HaCaT differentiation.
(C) Quantitative analysis of TAp63 and ΔNp63 transcripts during differentiation of HaCaT cells. Protein levels of TAp63 and ΔNp63 during differentiation are also shown. TAp63 protein is detectable only after long exposure (L.E.). S.E.: short exposure.
(D) Proteins were extracted from HaCaT cells at the indicated time points following induction of differentiation and subjected to western blot analysis. Equal protein loading was determined by probing with anti-Hsp70 antibody.

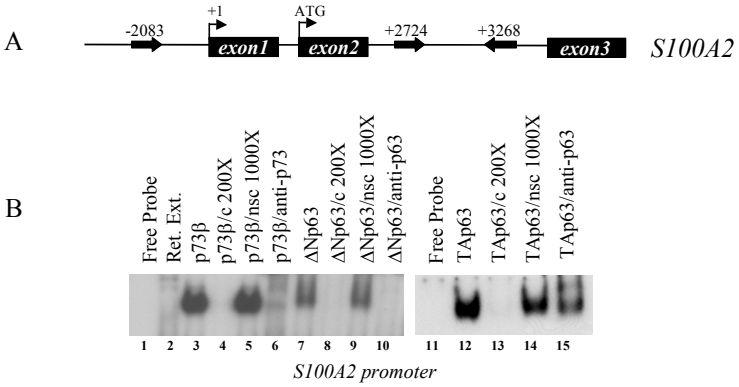


Fig.8. p73 and p63 bind *in vitro* to S100A2 promoter.

(A) Schematic representation of the S100A2 gene. Arrows indicate p53/p63/p73 consensus binding sites of S100A2 promoter and second intron; positions are relative to the transcription start site.
(B) Gel shift assay was performed using a probe resembling the p53/p63/p73 binding sites of the S100A2 promoter. p73β, ΔNp63 and TAp63 bind *in vitro* (lanes 3, 7 and 12 respectively). The binding was disrupted using a 200-fold molar excess of unlabeled double-stranded probe (lanes 4, 8 and 13) but not with a 1000-fold molar excess of an unrelated oligonucleotide (lanes 5, 9 and 14). The addition of anti-p73 or anti-p63 antibodies to the reaction mixture reduced the binding (lanes 6, 10, and 15).

translated p73β, ΔNp63α or TAp63α protein. As shown in Fig.8B, p73β ΔNp63α and TAp63α were able to bind the consensus of S100A2 promoter. The addition of anti-p73 or anti-p63 antibodies to the reaction mixture decreased (lanes 6, 10 and 15) or weakly supershifted (lane 15) the binding, thus confirming the specificity of these DNA/protein complexes (Fig.8B).

To further investigate whether the above-mentioned binding sites contained in the regulatory regions of S100A2 gene confer p73/p63-dependent transcriptional activity we performed transactivation assays. The consensus site of the promoter and the two consensus sites enclosed in the second intron of S100A2 gene were individually cloned into a TK-LUC reporter construct. As shown in Fig. 9 ectopic expression of p73β promoted the transcriptional activation of S100A2 regulatory regions, while ΔNp63α induced a strong transcriptional repression. Unlike ΔNp63α and p73β, p63α was unable to transcriptionally modulate the S100A2 gene, at least under our experimental conditions. Altogether these results indicate that p73β and ΔNp63α exert opposite transcriptional effects upon the S100A2 regulatory regions.

In vivo recruitment of p73β and ΔNp63α onto the regulatory regions of the S100A2 gene.

To verify whether S100A2 upregulation during keratinocyte differentiation depends on the transcriptional activity of p53 family members, we analyzed the *in vivo* binding pattern of p73β and ΔNp63α onto the S100A2 regulatory regions by Chromatin Immunoprecipitation (ChIP). To this end, cross-linked chromatin derived from equivalent numbers of proliferating (P) and terminally differentiated (TD) HaCaT cells was immunoprecipitated by using antibodies against p73 and p63 (TAp63 and ΔNp63). The amount of p73β bound to the regulatory regions of S100A2 was mainly increased at the level of the second intron in differentiated cells (Fig. 10A). Conversely, p63 was recruited onto the S100A2 promoter mainly in proliferating cells and its binding was reduced upon differentiation (Fig.10A). It is reasonable to assume that the p63 bound to the S100A2 promoter is predominantly ΔNp63α since we observed (fig.7A) and it has been reported (Bamberger *et al.* 2002) that ΔNp63α is the most abundant p63 isoform present in human keratinocytes. p63 was not recruited onto the second intron of the S100A2

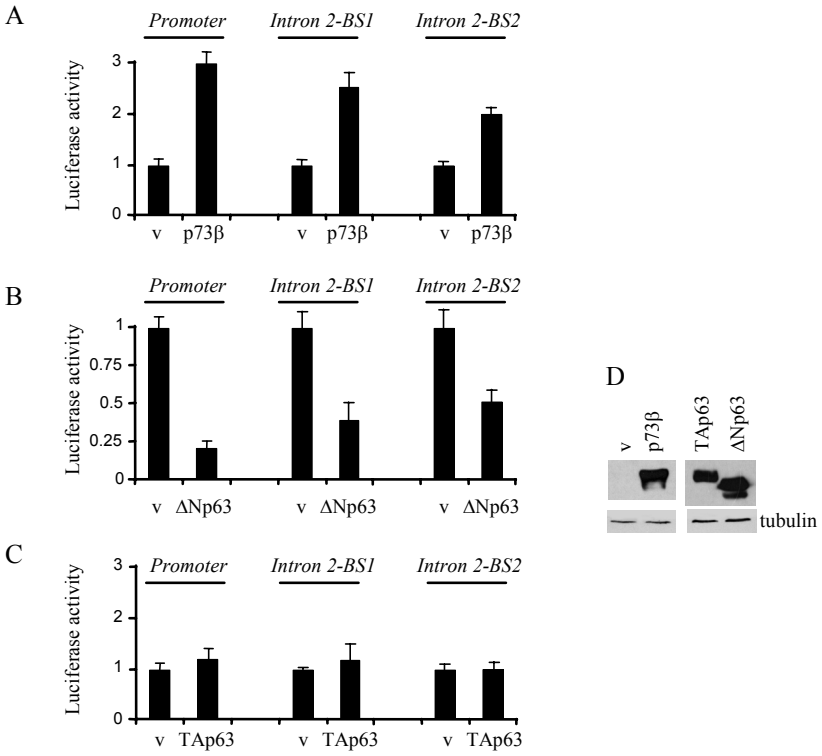


Fig.9. ΔNp63α and p73β exert opposite transcriptional effects on the S100A2 gene.

(A-D) H1299 cells were transiently transfected with plasmids encoding p73β (A), ΔNp63α (B) or TAp63α (C) (1μg/60-mm dish) together with plasmids encoding the luciferase reporter gene driven by the S100A2 promoter, S100A2 second intron-binding site 1 (Intron 2-BS1) or S100A2 second intron-binding site 2 (Intron 2-BS2) (500ng/60-mm dish). The total amount of transfected DNA in each dish was kept constant by the addition of empty vector. An equal amount of CMV-βgal was added to each transfection. Cell extracts were prepared 36h later. Luciferase activity was determined relative to total proteins and β-gal activity. Results are presented as fold induction over the control. Histograms show the mean of three experiments each performed in duplicate; bars indicate S.D.
(D) Expression of p73β, TAp63 and ΔNp63 proteins in transactivation assays was assessed by western blot analysis.

gene, suggesting that the promoter occupancy might be sufficient to exert its transcriptional repression (Fig.10A). No specific occupancy by p73β and ΔNp63 was found on the thymidine kinase (TK) promoter (fig.10A), which does not contain any p73/p63-binding site. The induction of S100A2 transcript during keratinocyte differentiation might result from the combinatorial activity of ΔNp63 and p73β. In particular, while ΔNp63 downregulation releases its repressive activity, p73β upregulation might be involved in the full transcriptional activation of S100A2 during differentiation.

We next verified whether the transcriptional activation of S100A2 during keratinocyte differentiation of HaCaT cells correlates with changes in the degree of histone H4 acetylation. Cross-linked chromatin derived from HaCaT cells harvested at 0h and 12h after induction of differentiation was immunoprecipitated with anti-acetylated H4 histone antibody. We found an increased H4 histone acetylation on the second intron of S100A2 gene at 12h (Fig. 10B). This correlates well with both S100A2 mRNA induction (Fig. 7B) and the *in vivo* recruitment of p73 onto S100A2 regulatory regions. No difference in the amount of H4 histone acetylation was detected between proliferating and differentiating cells on S100A2 promoter (Fig. 10B); this could be due to the significant basal expression level of S100A2 in proliferating cells (fig.7A-B). Altogether these results indicate that the p73 consensus sequences present on the second intron of the S100A2 gene might act as enhancer elements upon induction of differentiation, allowing full transcriptional activation of the gene.

Silencing of p73 expression impairs S100A2 upregulation during keratinocyte differentiation.

To further define the contribution of p73 in the induction of S100A2 expression during keratinocyte differentiation, we knocked-down p73 expression by RNA interference. Two different experimental approaches were used. First, HaCaT cells were transfected either with p73-specific small interfering RNAs (siRNAs) or unrelated siRNAs along with a vector encoding a green fluorescent protein (GFP) (fig.11B-C). The expression of S100A2 protein was evaluated by immunofluorescence. In agreement with previous findings, S100A2 protein was predominantly localized in the nuclei even at 12h of differentiation (fig.11B). S100A2 expression in the GFP positive/p73siRNAs-transfected cells (Fig. 11C, right panels, arrow) was

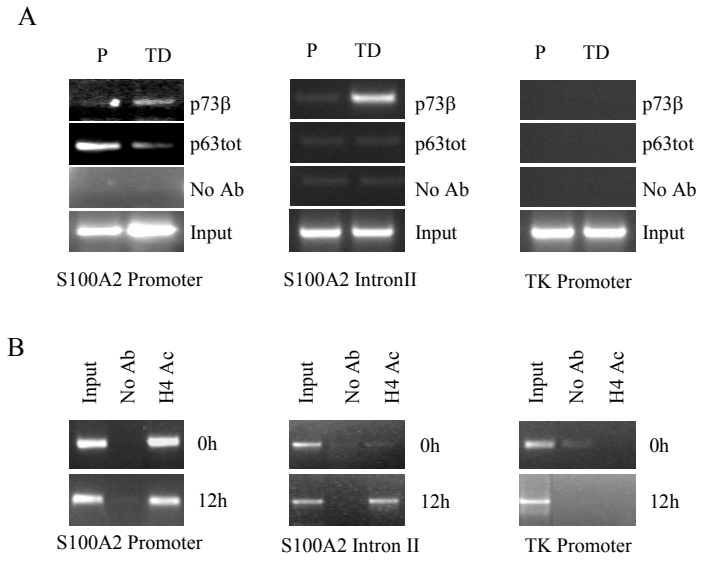


Fig.10. *In vivo* binding of p73β and p63 to the regulatory regions of S100A2 gene.

(A) Cross-linked chromatin derived from proliferating (P) or terminally differentiated (TD) HaCaT cells was immunoprecipated with antibodies to p73 or p63 or in the absence of antibody and analyzed by PCR with specific primers for the indicated regulatory regions. Input, non-immunoprecipitated cross-linked chromatin.
(B) Cross-linked chromatin derived from proliferating HaCaT cells (0h) or from HaCaT cells after 12h of differentiation (12h) was immunoprecipated with anti-acetylated histone H4 antibody or in the absence of antibody and analyzed by PCR with specific primers for the indicated regulatory regions. Input: non-immunoprecipitated cross-linked chromatin.

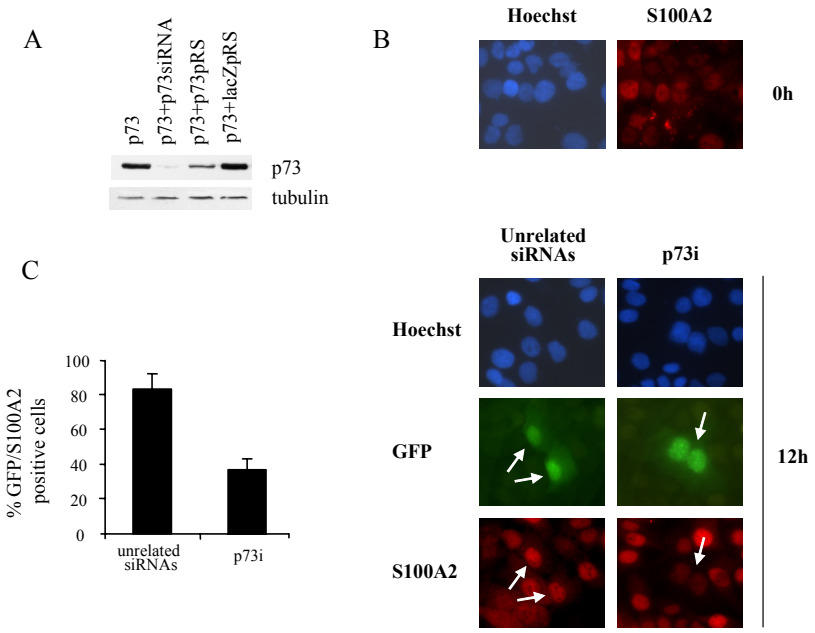


Fig.11. Silencing of p73 impairs S100A2.

(A) The efficiency of inhibition of p73 expression by siRNAs directed against p73, pRetroSuper-p73 or pRetroSuper-LacZ (negative control) was assessed by immunoblotting in H1299 cells.
(B) Proliferating HaCaT cells were transfected with p73-specific siRNAs or unrelated siRNAs together with a GFP expressing vector and induced to differentiate. At 12hs of differentiation cells were fixed and stained with an anti-S100A2 monoclonal antibody. Staining of S100A2 in GFP positive cells (arrows) was assessed.
(C) GFP/S100A2 double positive HaCaT cells described in (B) were counted and data is represented in the histogram. The mean of three experiments is shown.

clearly diminished when compared to that of the surrounding GFP negative/untransfected cells. This effect was specific, given that no modulation of S100A2 expression was seen in the cells transfected with the unrelated siRNAs (fig.11C, left panels). In the second approach, HaCaT cells were stably transfected with either a retroviral vector p73pRetroSuper (p73pRS), expressing siRNAs against p73, or with a control vector lacZpRS. As shown in Fig.12, the induction of both p73 and S100A2 mRNAs in differentiating HaCaT cells transfected with p73pRS vector was reduced when compared with lacZpRS transfected cells. This effect coincides with a reduced induction of keratin 10 and involucrin, two well-known markers of keratinocyte differentiation (fig.12). These findings indicate that p73 expression is required for S100A2 upregulation during keratinocyte differentiation and for the proper expression of differentiation markers.

Silencing of S100A2 impairs keratinocyte differentiation.

To identify whether S100A2 contributes to keratinocyte differentiation, HaCaT cells were stably transfected with either a retroviral vector S100A2pRS or with lacZpRS. As shown in Fig.13, the induction of S100A2 mRNA in differentiating HaCaT cells transfected with S100A2pRS was impaired when compared to that of lacZpRS transfected cells. This leads to a reduced induction of keratin 10 and involucrin (Fig.13C-D). Altogether our findings indicate that S100A2 participates actively in proper keratinocyte differentiation.

S100A2 gene is not induced in response to DNA damage.

A growing number of studies have shown that p53 family members are transcription factors heavily involved in the apoptosis induced by common anticancer treatments. This effect occurs mainly through the transcriptional activation of a plethora of target genes that were originally identified as components of p53-mediated apoptotic processes. To assess whether S100A2 is a target of p53 family members not only during keratinocyte differentiation but also upon DNA damage-induced apoptosis, we treated HaCaT cells with cisplatin (CDDP) in a time and dose-dependent manner. Cisplatin treatment induced apoptotic cell death as assessed by PARP

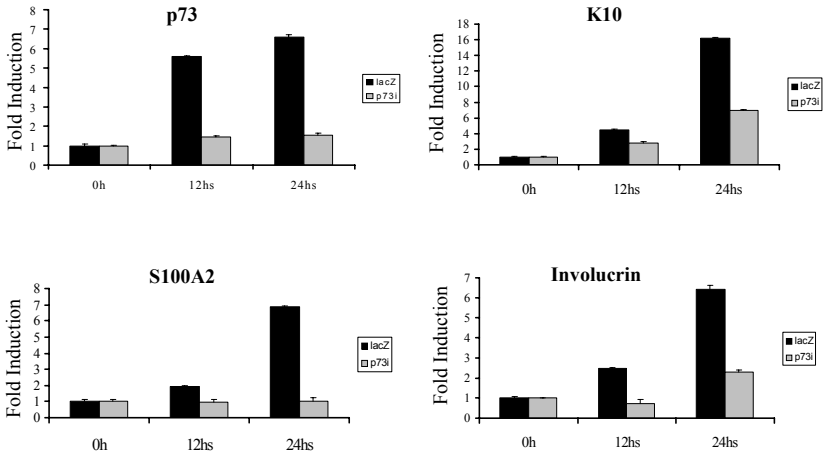


Fig.12. Silencing of p73 impairs S100A2 upregulation and keratinocyte differentiation.

RNA was extracted from HaCaT cells, stably transfected with p73-pRetroSuper or control lacZ-pRetroSuper, at the indicated time points after the induction of differentiation, and subjected to RT-PCR analysis. Specific primers for the detection of S100A2, involucrin and keratin 10 (K10) transcripts were used. Quantification by densitometry and normalization based on aldolase-A expression were performed. Results represent fold induction over the 0h time point. Histograms show the mean of three experiments; bars indicate S.D.

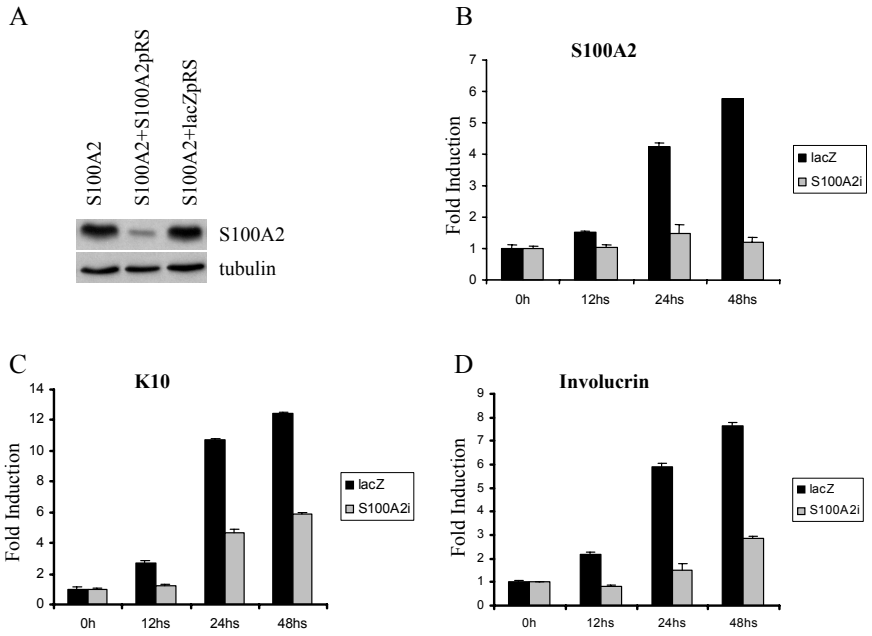


Fig.13. Silencing of S100A2 impairs keratinocyte differentiation.

(A) The efficiency of inhibition of S100A2 expression by S100A2-pRetroSuper or LacZ-pRetroSuper (negative control) was assessed by immunoblotting in H1299 cells.
(B-D) RNA was extracted from HaCaT cells, stably transfected with p73-pRetroSuper or control lacZ-pRetroSuper, at the indicated time points after the induction of differentiation, and subjected to RT-PCR analysis. Specific primers for the detection of p73, S100A2, involucrin and keratin 10 (K10) transcripts were used. Quantification by densitometry and normalization based on aldolase-A expression were performed. Results represent fold induction over the 0h time point. Histograms show the mean of three experiments; bars indicate S.D.

fragmentation (Fig.14A) and subG1 fraction analyses (fig.14B). The extent of the cisplatin-induced apoptosis is dose-dependent (fig.14A-B). Analysis of protein levels of p53 family members reveals that while p73 accumulates, ΔNp63 expression is strongly reduced (fig. 14A). As expected no modification of mutant p53 expression was observed in response to cisplatin (fig.14A). We found that while p73-mediated growth arrest and apoptotic target genes $p21^{waf1}$, bax, p53AIP1, pig-3 were induced upon cisplatin treatment, S100A2 mRNA and protein, in agreement with previously reported findings (Dazard *et al.*, 2003), were down-regulated (fig.14-15A). This effect does not seem to be related to the activity of p53 family members. Up-regulation of S100A2 was also not observed during p73-mediated apoptosis of HCT116(3) cells in response to DNA damage (Costanzo *et al.*, 2002; Mantovani *et al.*, 2004; Strano *et al.*, 2005).

By chromatin immunoprecipitation analysis we found that p73 is recruited on the first intron of its pro-apoptotic target gene p53AIP1 only in response to cisplatin (fig.15B). Conversely, the binding of p73 to the S100A2 promoter was not modulated upon cisplatin treatment (fig.15B).

Altogether these findings highlight S100A2 as a transcriptional target that might be selectively activated by p53 family members during differentiation.

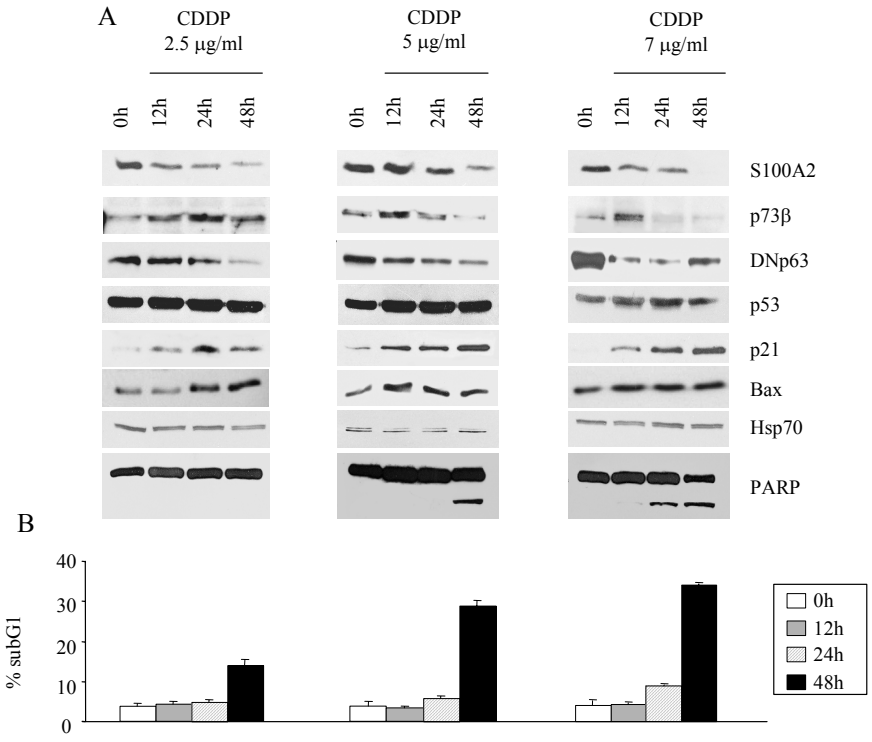


Fig.14. S100A2 gene is not induced in response to DNA damage.

(A) Proteins were extracted from HaCaT cells at the indicated time points after the addition of cisplatin (CDDP) (2.5 μg/ml, 5 μg/ml and 7.5 μg/ml) and subjected to western blot analysis. The extent of cell death was determined by PARP cleavage. Equal protein loading was determined by probing with anti-Hsp70 antibody.
(B) The percentage of sub-G1 cells were quantified by cytofluorimetry in HaCaT cells treated as in (A). Histograms represent the mean of three experiments.

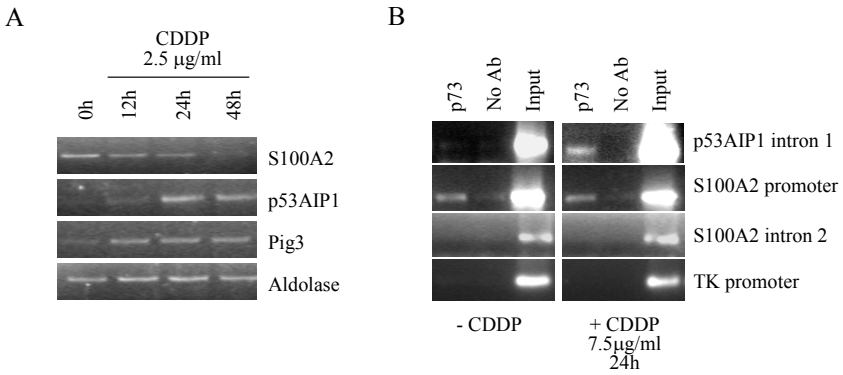


Fig.15. S100A2 gene is not induced in response to DNA damage.

(A) RNA was extracted from HaCaT cells at the indicated time points after treatment with 2.5 µg/ml CDDP and subjected to RT-PCR analysis. Amplification of aldolase-A was used to normalize each cDNA sample.
(B) Cross-linked chromatin derived from HaCaT cells at 0h or 24h following CDDP treatment was immunoprecipated with anti-p73 antibody, or in the absence of antibody, and analyzed by PCR with specific primers for the indicated regulatory regions. Input: non-immunoprecipitated cross-linked chromatin.

The protein complex p73/YAP is a transcriptional regulator of PML.

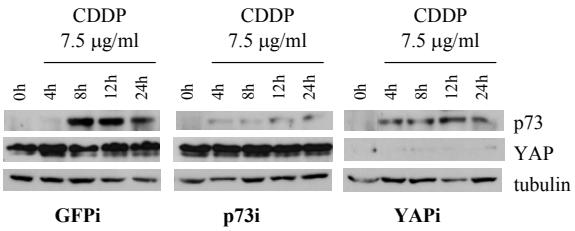
Search for genes modulated by the protein complex p73/YAP.

We have previously shown that YAP is a transcriptional co-activator that imparts selectivity to p73-gene target specificity within DNA-damage induced apoptosis (Strano *et al.*, 2005). To identify specific target genes whose transcriptional activation requires both p73 and YAP, we performed microarray analysis of cisplatin-treated HCT116(3) cells following siRNA-mediated interference of p73 and YAP. To this end, RNA preps were prepared from HCT116(3) cells transduced either with specific p73 or YAP siRNAs and starved at different time points following the addition of CDDP (Fig 16). Identical cells transduced with unrelated oligonucletides (GFP) and treated with CDDP were used as a control (Fig 16). cDNA preps were used to probe Affymetrix chips.
HCT116(3) cells transfected either with p73-specific, YAP-specific or unrelated siRNAs, were treated with 7.5 μg/ml of cisplatin and were harvested in TRIzol reagent after 0, 4, 8, 12 and 24 hours. Fig 16 shows the reduction in the levels of p73 and YAP after RNA interference (Fig 16A) by western blot and the percentage of apoptotic cells in those samples (Fig 16B).
To eliminate background noise in the analysis of the microarray experiments, we chose a very stringent filter. We considered only those genes that showed more than two-fold induction at 3 or more time points in the GFP-interfered samples over the 0h time point, but didn't show this induction in both p73 and YAP interfered samples. Using these criteria we generated a list containing 208 genes. Table 1 shows the 100 most differentially expressed genes, in decreasing order.

p73 and YAP are required for PML induction after treatment with cisplatin.

The importance of PML in p73-mediated apoptosis has already been demonstrated (Strano *et al.*, 2005).
As we found PML between those genes upregulated upon cisplatin treatment and with low expression in YAP and p73 interfered cells, we set out to test

A



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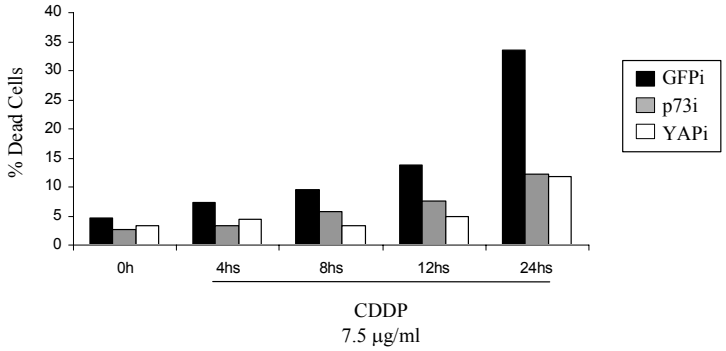

Fig.16. PML expression is induced upon treatment with cisplatin.

(A) Specific anti-p73, anti-YAP and control anti-GFP siRNAs were transfected into HCT116(3) cells. Proteins were extracted from cells at the indicated time points following treatment with 7.5 µg/ml cisplatin (CDDP) and subjected to western blot analysis. Equal protein loading was determined by probing with anti-tubulin antibody.
(B) Floating and adherent HCT116(3) cells, transfected with specific anti-p73, anti-YAP and control anti-GFP siRNAs and exposed to apoptotic dosage of CDDP, were collected and counted with a Thoma's hemocytometer. Cell viability was determined by the ability to exclude trypan blue.

Table 1

#	Symbol	Name
1	DNAJB5	"DnaJ (Hsp40) homolog, subfamily B, member 5"
2	SOX15	SRY (sex determining region Y)-box 15
3	ABCA2	"ATP-binding cassette, sub-family A (ABC1), member 2"
4	VAMP2	vesicle-associated membrane protein 2 (synaptobrevin 2)
5	PIM2	pim-2 oncogene
6	EFNA3	ephrin-A3
7	C20orf67	chromosome 20 open reading frame 67
8	MUC3B	mucin 3B
9	DKFZp434F054	hypothetical protein DKFZp434F054
10	LOC150946	hypothetical protein LOC150946
11	GPR3	G protein-coupled receptor 3
12	EVI5L	ecotropic viral integration site 5-like
13	FLJ37970	hypothetical protein FLJ37970
14	ANGPTL4	angiopoietin-like 4
15	DPF1	"D4, zinc and double PHD fingers family 1"
16	FLJ25467	hypothetical protein FLJ25467
17	LOC112703	Hypothetical protein BC004941
18	D4ST1	dermatan 4 sulfotransferase 1
19	LOC284628	Hypothetical protein LOC284628
20	KIAA1036	KIAA1036
21	TNK1	"tyrosine kinase, non-receptor, 1"
22	TYSND1	trypsin domain containing 1
23	C6orf110	Chromosome 6 open reading frame 110
24	---	Similar to Serine/threonine-protein kinase PLK1 (Polo-like kinase 1) (PLK-1) (Serine-threonine protein kinase 13) (STPK13)
25	EPHX1	"epoxide hydrolase 1, microsomal (xenobiotic)"
26	PML	promyelocytic leukemia
27	CFLAR	CASP8 and FADD-like apoptosis regulator
28	NFKBIE	"nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, epsilon"
29	RPP25	ribonuclease P 25kDa subunit
30	DYRK3	dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 3
31	LOC90379	Hypothetical protein BC002926
32	RFX1	"regulatory factor X, 1 (influences HLA class II expression)"
33	UNC93B1	unc-93 homolog B1 (C. elegans) /// unc-93 homolog B1 (C. elegans)

34	BMP1	bone morphogenetic protein 1
35	USP19	ubiquitin specific protease 19
36	HSPA2	heat shock 70kDa protein 2
37	APOL2	"apolipoprotein L, 2 /// apolipoprotein L, 2"
38	FLJ14299	hypothetical protein FLJ14299
39	---	---
40	ADRBK1	"adrenergic, beta, receptor kinase 1"
41	MAGEA5	"melanoma antigen, family A, 5"
42	---	"Transcribed locus, weakly similar to XP_375099.1 hypothetical protein LOC283585 [Homo sapiens]"
43	TREX1	three prime repair exonuclease 1
44	SSBP4	single stranded DNA binding protein 4
45	RUNDC1	RUN domain containing 1
46	---	---
47	FSD1	fibronectin type 3 and SPRY domain containing 1
48	GRIPAP1	GRIP1 associated protein 1
49	RRAD	Ras-related associated with diabetes
50	OGFR	opioid growth factor receptor
51	MGC20806	hypothetical protein MGC20806
52	LOC146206	similar to CG1399-PB
53	ATF5	activating transcription factor 5
54	SREBF1	Sterol regulatory element binding transcription factor 1
55	NDRG2	NDRG family member 2
56	CBX4	"chromobox homolog 4 (Pc class homolog, Drosophila)"
57	TYRO3	TYRO3 protein tyrosine kinase
58	PPP1R15A	"protein phosphatase 1, regulatory (inhibitor) subunit 15A"
59	ZFYVE19	"zinc finger, FYVE domain containing 19"
60	SF4	splicing factor 4
61	DUSP18	dual specificity phosphatase 18
62	BAT4	HLA-B associated transcript 4
63	FBXW5	F-box and WD-40 domain protein 5
64	BMP1	bone morphogenetic protein 1
65	TOP3B	topoisomerase (DNA) III beta
66	FLJ12528	threonyl-tRNA synthetase
67	---	---
68	---	---
69	MAPK8IP2	mitogen-activated protein kinase 8 interacting protein 2
70	PDCD5	Programmed cell death 5
71	SNIP1	Smad nuclear interacting protein
72	---	---

73	SHANK3	SH3 and multiple ankyrin repeat domains 3
74	ESPN	espin
75	ZNF307	zinc finger protein 307
76	ZNF580	zinc finger protein 580
77	---	---
78	---	---
79	EPN2	epsin 2
80	LTB4R	leukotriene B4 receptor
81	---	---
82	SHD1	likely ortholog of mouse Sac3 homology domain 1 (S. cerevisiae)
83	DES /// FAM48A	"desmin /// family with sequence similarity 48, member A"
84	ORMDL3	ORM1-like 3 (S. cerevisiae)
85	DOK4	docking protein 4
86	LOC89944	hypothetical protein BC008326
87	OPN3	"opsin 3 (encephalopsin, panopsin)"
88	GATA2	GATA binding protein 2
89	HLA-B	"major histocompatibility complex, class I, B"
90	---	---
91	MGC2752	hypothetical protein MGC2752
92	PARD6A	par-6 partitioning defective 6 homolog alpha (C.elegans)
93	HTPAP	HTPAP protein
94	UBCE7IP5	likely ortholog of mouse ubiquitin conjugating enzyme 7 interacting protein 5
95	---	---
96	LOC92154	hypothetical protein BC002770
97	AGRN	agrin
98	FZR1	fizzy/cell division cycle 20 related 1 (Drosophila)
99	LENG1	leukocyte receptor cluster (LRC) member 1
100	CENTG1	"Centaurin, gamma 1"

whether p73/YAP complex plays a role in PML induction in that specific apoptotic response. To confirm the microarray data, quantitative real-time RT-PCR analysis of PML was performed using cDNA from an aliquot of the RNA used in the DNA chip analysis (Fig 17A). After treatment with CDDP there was an up-regulation of PML expression in the control samples, which was reduced after interference of both p73 and YAP.
The up-regulation of PML mRNA after treatment with cisplatin correlates with the formation of PML nuclear bodies (Fig 17B) and the accumulation of PML protein in the HCT116 cells, as shown by the western blot in Fig. 17C. This accumulation was impaired after the abrogation of p73 or YAP expression by RNA interference.

PML is induced by p73.

To provide genetic evidence for PML as a transcriptional target of p73 upon DNA damage, we treated wild-type MEFs, p73-/-MEFs and p73-/-MEFs reconstituted with p73β with CDDP. As shown in Fig.18A the upregulation of PML in wt MEFs after DNA damage was impaired in p73-/-MEFs and was partially restored in p73βMEFs.
It has been previously demonstrated that PML is a p53 target gene (de Stanchina *et al.*, 2004), so it is not surprising that PML upregulation in p53-/- MEF was also impaired. But, to underline the importance of the p73/YAP mediated induction of PML upon cisplatin treatment, we asked whether PML is induced in cells that lacks p53 or in cells expressing mutant p53. We used H1299 cells (p53 null) with ponasterone A inducible p73 expression (H1299#9) and H1299 cells stably transfected with the pIND vector (H1299 pIND) as a control. Cells were treated with ponasterone A and PML expression was evaluated by immunofluorescence (Fig. 18B). In agreement with previous findings, H1299 cells show a very low level of PML expression that is clearly increased by overexpression of p73, indicating that overexpression of p73 alone is sufficient to induce PML expression and nuclear bodies (NBs) formation. To investigate whether this pathway also works in cells expressing mutant p53, we transfected SKBR3 cells, which express the p53R175H mutant, either with p73-specific, YAP-specific or unrelated siRNAs and treated them with cisplatin. As shown in Fig. 18C the induction of PML in p73 or YAP interfered cells was reduced when compared with the control cells.

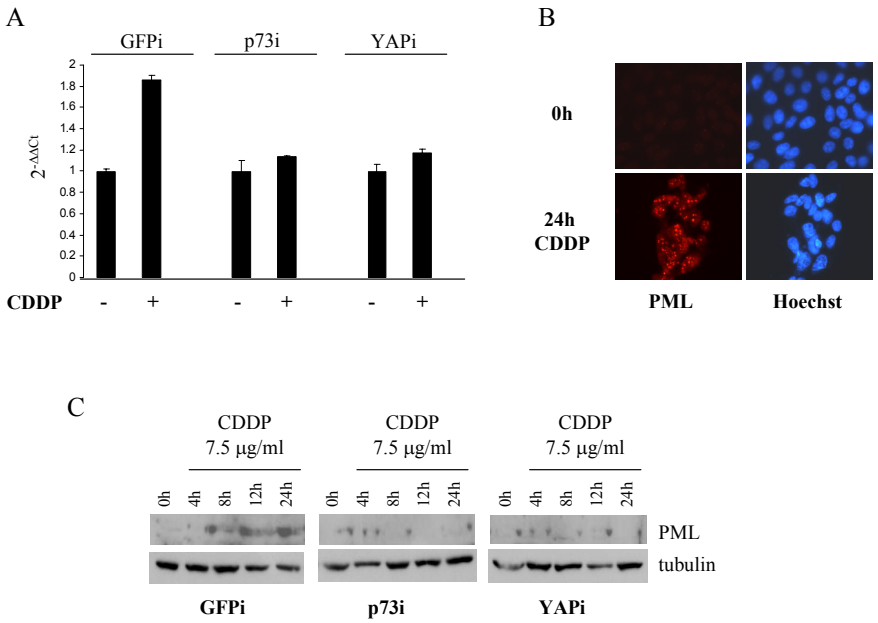

Fig.17. p73 and YAP are required for PML induction after treatment with cisplatin.

(A) Quantitative analysis of PML transcripts in HCT116(3) cells transfected with specific anti-p73, anti-YAP and control anti-GFP siRNAs and exposed to apoptotic dosage of CDDP for 24h.
(B) HCT116(3) cells after 24h of CDDP treatment were fixed and stained with an anti-PML antibody.
(C) Proteins were extracted from HCT116(3) cells transfected with specific anti-p73, anti-YAP and control anti-GFP siRNAs, at the indicated time points following treatment with 7.5 µg/ml cisplatin (CDDP) and subjected to western blot analysis. Equal protein loading was determined by probing with anti-tubulin antibody.

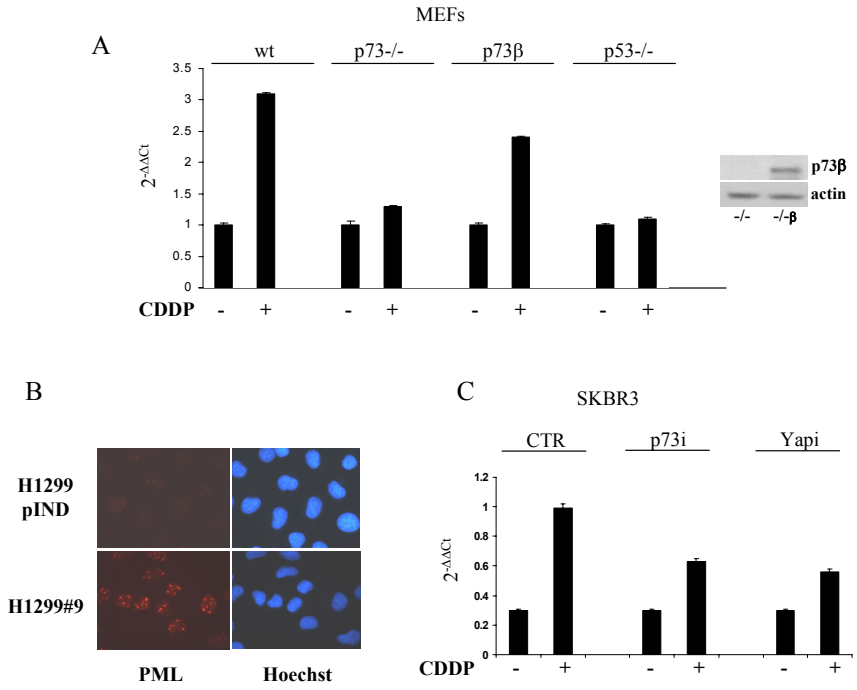

Fig.18. PML is induced by p73.

(A) Quantitative analysis of PML transcripts in wild-type, p73-/-, p73-/- reconstituted with p73β and p53-/- MEFs exposed to apoptotic dosage of CDDP for 24h.
(B) H1299 cells with ponasterone A inducible p73 expression (H1299#9) and H1299 stably transfected with the pIND control vector (H1299 pIND) were treated with 2.5mM ponasterone A for 24h and were fixed and stained with an anti-PML antibody.
(C) Quantitative analysis of PML transcripts in SKBR3 cells transfected with specific anti-p73, anti-YAP and control anti-GFP siRNAs and exposed to apoptotic dosage of CDDP for 24h.

PML is a direct transcriptional target of p73 and YAP.

Using Mat-Inspector Professional software (genomatrix.gaf.de) to analyze the TRANSFAC 5.0 database (transfac.gbf.de/TRANSFAC) we examined whether p53/p73 consensus sites were contained within the promoter region or the first intron of the PML gene. As shown in Fig. 19A, the PML promoter and first intron contain several p53/p73 binding sites.
To investigate whether the above-mentioned binding sites contained in the regulatory regions of the PML gene confer p73/YAP-dependent transcriptional activity we performed transactivation assays. As shown in Fig. 19B ectopic expression of p73 promoted the transcriptional activation of PML regulatory regions, which were further activated by the ectopic expression of YAP.
To verify whether PML upregulation during cisplatin-induced apoptosis depends on the transcriptional activity of p73 and YAP, we analyzed the *in vivo* binding pattern of p73 and YAP onto the PML regulatory regions by ChIP (Fig. 20A). To this end, cross-linked chromatin derived from equivalent numbers of untreated HCT116 cells (0h) and cells harvested after a 12h treatment with cisplatin (12h) was immunoprecipitated by using antibodies against p73, YAP, p300 and anti-acetylated H4 histone antibody. We found that there is binding of p73 and YAP to the regulatory regions of PML only after treatment with cisplatin and that this correlates with an increased binding of p300 and with an increase of H4 histone acetylation. A ChIP assay was performed in which cells were transfected with a costitutively active mutant form of AKT, capable of phosphorylating YAP in a costitutive manner, thus sequestring it to the cytoplasm. Interestingly, we found no changes in the degree of histone H4 acetylation of the regulatory regions of PML after DNA damage and we found less p300 bound onto these regulatory regions (Fig. 20B). This underlines once again that YAP, together with p73, is essential for the activation of PML after treatment with CDDP.

YAP and PML physically interact in vivo.

We have previously shown by confocal microscopy that exogenously expressed PML and GFP-tagged YAP colocalize in NBs (Strano *et al.*, 2005).

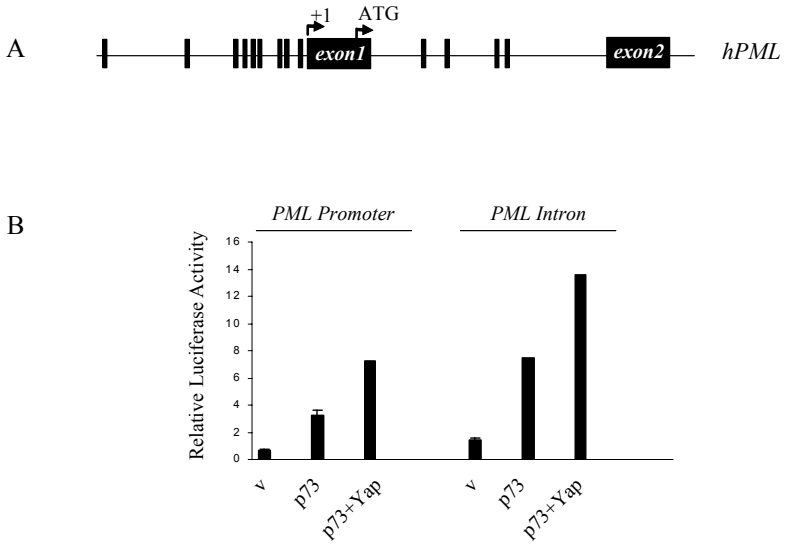

Fig.19. PML is a direct transcriptional target of p73 and YAP.

(A) Schematic diagram depicting the genomic regions spanning 5000 bp upstream of the predicted PML transcriptional start site and 5000 bp into the first and the second exons of human PML. Predicted p53 responsive elements, identified using Mat-Inspector Professional software, are represented by black boxes.
(B) H1299 cells were transiently transfected with plasmids encoding p73β and YAP together with plasmids encoding the luciferase reporter gene driven by the human PML promoter or the first intron of PML. The total amount of transfected DNA in each dish was kept constant by the addition of empty vector. An equal amount of CMV-βgal was added to each transfection. Cell extracts were prepared 36h later. Luciferase activity was determined relatively to total proteins and β-gal activity. Results are presented as fold induction over the control. Histograms show the mean of three experiments each performed in duplicate; bars indicate S.D.

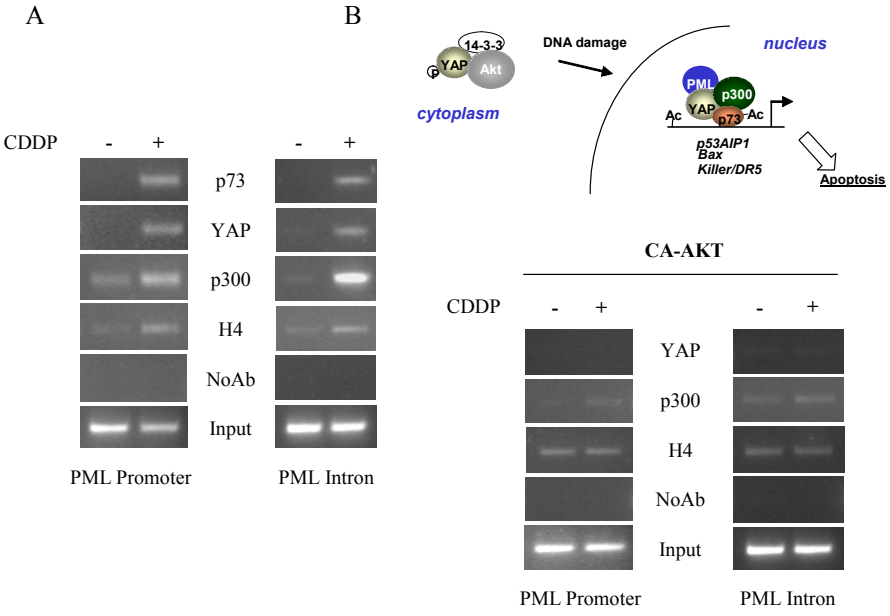

Fig.20. PML is a direct transcriptional target of p73 and YAP.

(A) Cross-linked chromatin derived from HCT116(3) cells untreated or treated with 7.5μg/ml CDDP for 24h was immunoprecipated with antibodies against p73, YAP, p300, acetylated histone H4 or in the absence of antibody and analyzed by PCR with specific primers for the indicated regulatory regions. Input, non-immunoprecipitated cross-linked chromatin.
(B) Cross-linked chromatin derived from HCT116(3) cells, stably transfected with a costitutively active mutant form of AKT (CA-AKT), untreated or treated with 7.5 βg/ml CDDP for 24h was immunoprecipated with antibodies against YAP, p300, acetylated histone H4 or in the absence of antibody and analyzed by PCR with specific primers for the indicated regulatory regions. Input, non-immunoprecipitated cross-linked chromatin.

We therefore now asked whether there is direct binding between these two proteins. H1299 cells were transiently transfected with expression vectors encoding PML along with GFP-YAP. Complexes immunoprecipitated with anti-PML antibody were found to contain YAP (Fig. 21A). To verify whether the association of YAP and PML occurs under physiological conditions, lysates of HCT116 cells were immunoprecipitated with anti-YAP antibody. As shown in Fig. 21B endogenous PML was co-immunoprecipitated by anti-YAP. As PML was a candidate identified in a WW domain-interacting protein screen (Sudol personal communication), we investigated whether the YAP WW domain is involved in the binding between YAP and PML. We expressed PML along with GFP-YAP or a YAP mutant lacking the WW domain (GFP-YAPΔWW) in p73-/- MEF. We observed that the absence of p73 did not influence the ability of YAP to associate with PML (Fig. 21C), indicating that, even if p73 is known to interact either with YAP and PML (Strano *et al.*, 2001; Bernassola *et al.*, 2005), it does not mediate the binding between these two proteins. Moreover the YAP mutant lacking the WW domain lost the ability to interact with PML (Fig. 21C), indicating that the WW domain is responsible for the interaction between YAP and PML.

YAP degradation occurs through the ubiquitin-proteasome pathway and is negatively regulated by PML.

PML isoform IV protects both p53 and p73 from proteasome-mediated degradation. We therefore investigated whether PML might regulate YAP stability. For this purpose we followed YAP half-life in PML-/- and wild-type MEFs upon treatment with cycloheximide. We observed that YAP half-life was markedly shortened in PML-/- compared with wild-type MEFs (Fig. 22A).
Next, we asked whether YAP turnover is directly regulated by ubiquitin-mediated proteolysis. We transfected H1299 cells with GFP-YAP and we treated the cells with MG132, a proteasome inhibitor. As can be seen in Fig. 22B there was a clear increase in the levels of GFP-YAP upon proteasome inhibitor treatment and the appearance of a ladder of higher molecular weight bands, probably containing ubiquitin, in the endogenous YAP.

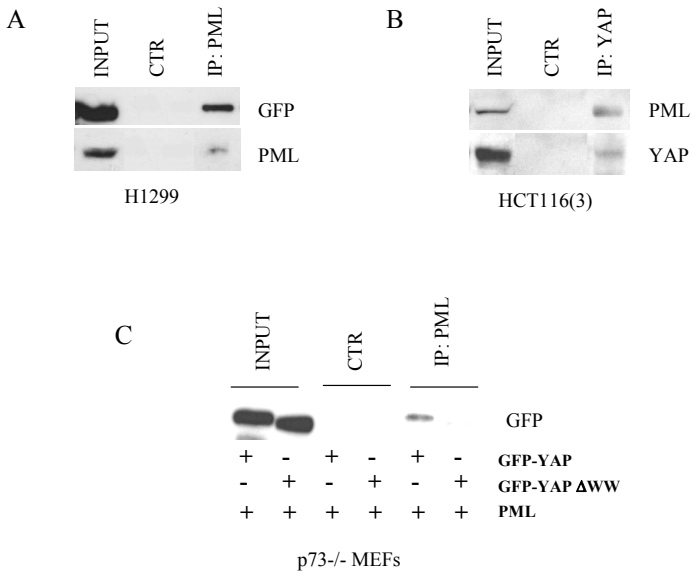

Fig.21. PML and YAP physically interact *in vivo*.

(A) H1299 cells were transiently transfected with a vector encoding PML along with GFP-YAP. YAP-PML binding was analyzed by immunoprecipitation (IP) using a monoclonal anti-PML antibody followed by immunoblotting (IB) with anti-GFP antibody.
(B) HCT116(3) cells were lysed and subjected to IP using a polyclonal anti-YAP antibody and the immunoprecipitate was analyzed by IB using anti-PML antibody.
(C) p73-/- MEFs were cotransfected with PML and GFP-YAP or GFP-YAPDWW, a mutant lacking the WW domain. Cell lysates were immunoprecipitated with anti-PML antibody and the immunoprecipitate analyzed by IB using anti-GFP antibody.

To assess whether YAP is ubiquitinylated and whether PML interferes with the ubiquitinylation, H1299 cells were transfected with pCDNA3-YAP, HA-Ubiquitin and PML and immunoprecipitated with anti-YAP antibody. YAP-ubiquitin immunocomplexes were analyzed by immunoblotting with anti-HA antibody (Fig 22C). We observed that YAP was clearly polyubiquitinylated *in vivo* and its ubiquitinylation levels were significantly reduced in PML-overexpressing cells (Fig. 22C). PML also prevented YAP ubiquitinylation in MG132-treated cells, indicating that PML acts upstream of the proteasome degradation process. These findings indicate that YAP turnover is directly regulated by ubiquitin-mediated degradation and that PML plays a role in protecting YAP from ubiquitinylation.

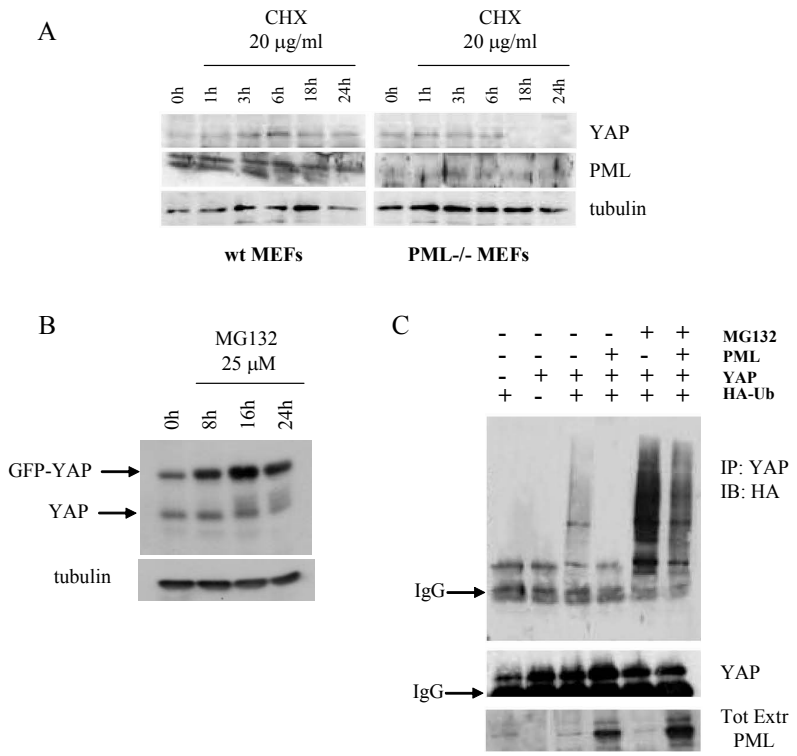

Fig.22. YAP degradation occurs through the ubiquitin-proteasome pathway and is negatively regulated by PML.

(A) Proteins were extracted from wild-type and PML-/- MEFs at the indicated time points after the addition of cycloheximide (CHX) (20 μg/ml) and subjected to western blot analysis. Equal protein loading was determined by probing with anti-tubulin antibody.
(B) H1299 cells were transiently transfected with a plasmid encoding GFP-YAP and proteins were extracted at the indicated time points after the addition of MG132 (25 μM) and subjected to western blot analysis. Levels of GFP-YAP and endogenous YAP were detected using an anti-YAP antibody. Equal protein loading was determined by probing with anti-tubulin antibody.
(C) H1299 cells were cotransfected with the indicated plasmid for 24h, incubated with or without MG132 (25 μM) for 12h, and subjected to IP using a polyclonal anti-YAP antibody. YAP-ubiquitin (Ub) immunocomplexes were analyzed by western blot with anti-HA (top panel) and anti-YAP (middle panel) antibodies. The expression of PML was analyzed by western blot on aliquots containing 50 mg of total protein from unprocessed lysates (bottom panel).

DISCUSSION

Unlike p53 deficient mice that develop normally but undergo spontaneous tumors, mainly sarcoma and lymphomas, p73 and p63 knock-out mice exhibit severe developmental and differentiation defects (Mills *et al.*, 1999; Yang *et al.*, 1999; Yang *et al.*, 2000).

Different p73 and p63 isoforms, either derived from alternative splicing or by a cryptic promoter located in the third intron of the genes, are present in normal and tumour cells. Altogether they give rise to a network of proteins whose fine-tuning dictates biological activities ranging from development and differentiation to growth arrest and apoptosis. While the alternative splicing products lack different portions of the C-terminus, those originating by an intrinsic promoter are N-terminal truncated and have been shown to counteract p53- and p73-induced anti-tumoral effects (Blandino and Dobbelstein, 2004). As a consequence, it is reasonable to assume that the major role of p73 and p63, during differentiation/development processes, is the transcriptional activation or repression of specific sets of genes. Here we show that S100A2 gene is an *in vivo* direct transcriptional target of p53 family members during keratinocyte differentiation of HaCaT cells. Interestingly, p73β and ΔNp63α play opposite roles in regulating the S100A2 gene at the transcriptional level. Indeed, we found that while p73β is a transcriptional activator of S100A2 gene, ΔNp63α functions as a transcriptional repressor. Further support to the opposite transcriptional regulation of the S100A2 gene is provided by the differential *in vivo* recruitment during differentiation of p73β and ΔNp63α onto the regulatory regions of the S100A2 gene. ΔNp63α is found predominantly in proliferating cells while p73β is recruited in differentiating cells. Altogether our findings define a regulatory network of p53 family members existing at the cross-roads between proliferation and differentiation that controls the activation or repression of selected target genes at the transcriptional level. Furthermore, our findings identify S100A2 as one of those genes whose activation contributes to the switch between proliferation and differentiation. The S100A2 transcript was previously shown to be positively modulated by exogenous expression of ΔNp63α (Hibi *et al.*, 2003). This opposite finding suggests there may be different activities of ΔNp63α related either to cell context or to the specific biological activity to which it is recruited.

Target genes of p53 family members could hypothetically be divided into two large categories. The first one includes genes that are activated by all the p53 family members and broadly involved in growth arrest, apoptosis and differentiation (el-Deiry *et al.*, 1993; Attardi *et al.*, 2000; Ihrie *et al.*, 2005). In this case the cell context, type of stimulus and the quantitative balance

between the diverse p53 family members could be some of the key determinants in dictating the final biological output. The second class includes target genes that are selectively activated by each of the p53 family members, comprising p53, p73, p63 and their related isoforms, and mediate a specific biological activity. The findings that S100A2 gene is not triggered in response to different types and amounts of DNA damage in diverse cell contexts suggest that it might fall in this last category of genes.

Unbalanced proliferation and lack of proper differentiation are two hallmarks of many types of cancer cells. The S100A2 gene was originally identified in a screen for tumour suppressor genes (Vellucci *et al.*, 1995). It has been shown that the levels of S100A2 inversely correlate with tumour progression in melanoma, breast and prostate cancer (Gupta *et al.*, 2003; Maelandsmo *et al.*, 1997; Vellucci *et al.*, 1995) thus indicating that it may play a role in suppressing tumour cell growth.

Here we provide evidence that might support a model of a cooperative activation of the tumor suppressor genes p73 and S100A2, whose spatially and temporally coordinated activities contribute to fulfill the differentiation program. The proper execution of this process can be considered as a safeguard mechanism against cancer whose molecular details require further investigation.

As mentioned previously, p73-mediated apoptosis occurs mostly through the activation of a set of genes that were originally found to be activated by p53 (Costanzo *et al.*, 2002; Irwin *et al.*, 2003; Bergamaschi *et al.*, 2003). This suggests that promoter selectivity by both p53 and p73 might be the result of biochemical events such as post-translational modifications and specific protein-protein interactions (Costanzo *et al.*, 2002; Agami *et al.*, 1999; Gong *et al.*, 1999; Yuan *et al.*, 1999; Strano *et al.*, 2001). We have previously shown that the transcriptional coactivator YAP (Sudol and Hunter, 2000) determines p73 gene targeting in response to DNA damage (Strano *et al.*, 2005). In this work we analysed, by RNA interference and microarray analysis, the apoptotic response of HCT116(3) cells treated with CDDP, and identified target genes specifically regulated by p73/YAP complex. We identified a set of genes whose expression, during the apoptotic response, requires the presence of both p73 and YAP. Further studies are required to determine which specific pathways these genes belong to. In this study, we focused our attention on one particular gene, PML and our findings have lead to two major conclusions.

Firstly, PML is a direct target gene of the p73/YAP complex during apoptosis triggered by CDDP treatment in HCT116(3) cells. We found that PML belongs to a group of genes whose expression was upregulated by CDDP treatment in HCT116(3) cells transfected with control siRNA, but remained constant in both p73 and YAP interfered cells.
PML is not a specific p73 target gene, since it has also been demonstrated to be a p53 target gene (de Stanchina *et al.*, 2004). But, interestingly, we found that in specific cellular contexts where p53 is absent or mutated, p73 alone is able to induce PML expression and NBs formation. This finding could highlight a potential tumour suppressor pathway that works in cells lacking functional p53 protein. Moreover we underlined the importance of YAP as a coactivator of p73 in the transactivation of PML. We have previously demonstrated that YAP is required for p300 recruitment onto the regulatory regions of the apoptotic target gene p53AIP1 (Strano *et al.*, 2005). Here we show that when YAP is sequestred into the cytoplasm by a constitutively active mutant of AKT there is a reduction of p300 recruitment onto the PML regulatory regions and this correlates with a reduction in histone acetylation and a reduction in PML expression.
Second, our data suggest a role for PML in the regulation of YAP stability. YAP is becoming a very intriguing protein due to its critical role in regulating p73 accumulation and function following DNA damage, but very little is known about its regulation. Here we show that YAP is polyubiquitinylated *in vivo* and degraded through the ubiquitin-proteasome pathway. We found that YAP and PML physically interact through the YAP WW domain and that the binding does not occur through the mediation of p73. We also showed that PML plays a role in the regulation of YAP half-life, preventing its ubiquitinylation and subsequent degradation.
It is becoming apparent that the PML-NBs can be regarded as factories for post-translational modifications. It has been demonstrated that PML enhances p53 and p73 acetylation, leading to the potentiation of p53 transcriptional activity (Guo *et al.*, 2000; Pearson *et al.*, 2000) and to the inhibition of p73 ubiquitin-dependent degradation (Bernassola *et al.*, 2005). Moreover it has been shown that PML contains a SUMO binding motif that mediates PML-SUMO1 interaction independently of sumoylation (Shen *et al.*, 2006) and that PML stimulates SUMO conjugation in yeast (Quimby *et al.*, 2006). Competition between ubiquitinylation, sumoylation and acetylation of overlapping lysine residues constitutes a novel mechanism to regulate protein stability. It remains to be established whether YAP can be acetylated or sumoylated and whether PML has a role in modulating YAP posttranslational modifications.

So far, our findings suggest the existence of a positive regulatory loop between the p73/YAP protein complex and PML during apoptosis triggered by CDDP in HCT116(3) cells. It has been previously demonstrated that YAP requires PML and NBs localization to coactivate p73. Here we show that p73 and YAP are required for the transcriptional activation of PML during the apoptotic response and for the subsequent accumulation of PML protein and formation of nuclear bodies. As a consequence, PML can contribute to the p73-dependent apoptotic response both promoting p300-mediated acetylation of p73 and inhibiting YAP ubiquitin-mediated degradation.

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REFERENCES

- **Ambartsumian, J. Klingelhofer, M. Grigorian, C. Christensen, M. Kriajevska, E. Tulchinsky, G. Georgiev, V. Berezin, E. Bock, J. Rygaard, R. Cao, Y. Cao and E. Lukanidin,** The metastasis-associated Mts1(S100A4) protein could act as an angiogenic factor, *Oncogene* **20** (2001), pp. 4685–4695.
- **Agami, G. Blandino, M. Oren and Y. Shaul,** Interaction of c-Abl and p73α and their collaboration to induce apoptosis, *Nature* **399** (1999), pp. 809–813.
- **Anton LC, Schubert U, Bacik I, Princiotta MF, Wearsch PA, Gibbs J, Day P, Realini C, Rechsteiner M, Bennink J and Yewdell J**. (1999). *J. Cell Biol.*, **146**, 113–124.
- **Appella E.** Modulation of p53 function in cellular regulation. Eur J Biochem 2001;268:2763.
- **Arumugam, D.M. Simeone, A.M. Schmidt and C.D. Logsdon.** S100P stimulates cell proliferation and survival via receptor for activated glycation end products (RAGE), *J. Biol. Chem.* **279** (2004), pp. 5059–5065.
- **Asher G, Lotem J, Sachs L, Kahana C, Shaul Y.** Mdm-2 and ubiquitin-independent p53 proteasomal degradation regulated by NQO1. Proc Natl Acad Sci USA 2002;99:13125-30.
- **Balint E, Bates S, Vousden KH.** Mdm2 binds p73 αwithout targeting degradation. Oncogene 1999;18:3923-9.
- **Banelli B, Casciano I, Romani M.** Methylation-independent silencing of the p73 gene in neuroblastoma. *Oncogene* 2000; 19: 4553–6.
- **Barrera FN, Poveda JA, Gonzalez-Ros JM, Neira JL.** Binding of the C-terminal sterile αmotif (SAM) domain of human p73 to lipid membranes. J Biol Chem 2003;278:46878-85.
- **Basu, N.F. Totty, M.S. Irwin, M. Sudol and J. Downward.** Akt phosphorylates the Yes-associated protein, YAP, to induce interaction with 14–3-3 and attenuation of p73-mediated apoptosis, *Mol. Cell* **11** (2003), pp. 11–23.
- **Baudier J, Delphin C, Grunwald D, Khochbin S, Lawrence JJ.** 1992. Characterization of the tumor suppressor protein p53 as a protein kinase C substrate and an S100β binding protein. *Proc Natl Acad Sci USA* **89**: 11627-11631.
- **Baudier J, Bergeret E, Bertacchi N, Weintraub H, Gagnon J, Garin J.** 1995. Interaction of bHLH transcription factors with

calcium-binding calmodulin and S100a(αα) proteins. *Biochemistry* **34**: 7834-7846.

- **Bergamaschi D, Samuels Y, Jin B, Duraisingham S, Crook T, Lu X.** ASPP1 and ASPP2: common activators of p53 family members. Mol Cell Biol 2004;24:1341-50.
- **Bergamaschi D, Gasco M, Hiller L, *et al.*** p53 polymorphism influences response in cancer chemotherapy via modulation of p73-dependent apoptosis. Cancer Cell 2003;3:387-402.
- **Bernassola, P. Salomoni, A. Oberst, C.J. Di Como, M. Pagano, G. Melino and P.P. Pandolfi,** Ubiquitin-dependent degradation of p73 is inhibited by PML, *J. Exp. Med.* **199** (2004), pp. 1545–1557.
- **Best JL, Ganiatsas S, Agarwal S, Changoo A, Salomoni P, Shirihai O, Meluh PB, Pandolfi PP, and Zon LI**. (2002). *Mol. Cell*, **10**, 843–855.
- **Bischof O, Kirsh O, Pearson M, Itahana K, Pelicci PG and Dejean A**. (2002). *EMBO J.*, **21**, 3358–3369.
- **Blandino G, Levine AJ, Oren M.** Mutant p53 gain of function: differential effects of different p53 mutants on resistance of cultured cells to chemotherapy. Oncogene 1999;18:477-85.
- **Boni R, Burg G & Doguoglu A *et al.*** Immunohistochemical localization of the Ca2+ binding S100 proteins in normal human skin and melanocytic lesions. *Br J Dermatol* (1997) **137**: 39–43.
- **Broome AM, Ryan D & Eckert RL.** S100 protein subcellular localization during epidermal differentiation and psoriasis. *J Histochem Cytochem* (2003) **51**: 675–685.
- **Brunner HG, Hamel BC, Bokhoven Hv H.** P63 gene mutations and human developmental syndromes. Am J Med Genet 2002;112:284-90.
- **Cai YC, Yang GY, Nie Y, *et al.*** Molecular alterations of p73 in human esophageal squamous cell carcinomas: loss of heterozygosity occurs frequently; loss of imprinting and elevation of p73 expression may be related to defective p53. Carcinogenesis 2000;21:683-9.
- **Carbone R, Pearson M, Minucci S and Pelicci PG**. (2002). *Oncogene*, **21**, 1633–1640.
- **Casciano I, Mazzocco K, Boni L, *et al.*** Expression of ΔNp73 is a molecular marker for adverse outcome in neuroblastoma patients. Cell Death Differ 2002;9:246-51.

- **Chen CL, Ip SM, Cheng D, Wong LC, Ngan HY.** P73 gene expression in ovarian cancer tissues and cell lines. Clin Cancer Res 2000;6:3910-5.
- **Chen X, Liu G, Zhu J, Jiang J, Nozell S, Willis A.** Isolation and characterization of fourteen novel putative and nine known target genes of the p53 family. Cancer Biol Ther 2003;2:55-62.
- **Chi SG, Chang SG, Lee SJ, Lee CH, Kim JI, Park JH.** Elevated and biallelic expression of p73 is associated with progression of human bladder cancer. Cancer Res 1999;12:2791-3.
- **Chi SW, Ayed A, Arrowsmith CH.** Solution structure of a conserved C-terminal domain of p73 with structural homology to the SAM domain. EMBO J 1999;18:4438-45.
- **Choi HR, Batsakis JG, Zhan F, Sturgis E, Luna MA, El-Naggar AK.** Differential expression of p53 gene family members p63 and p73 in head and neck squamous tumorigenesis. Hum Pathol 2002;33:158-64.
- **Concin N, Becker K, Slade N, Erster S, *et al.*** Transdominant DeltaTAp73 isoforms are frequently up-regulated in ovarian cancer. Evidence for their role as epigenetic p53 inhibitors in vivo. Cancer Res 2004;7:2449-60.
- **Corn PG, Kuerbitz SJ, van Noesel MM, *et al.*** Transcriptional silencing of the p73 gene in acute lymphoblastic leukemia and Burkitt's lymphoma is associated with 5' CpG island methylation. Cancer Res 1999;59:3352-6.
- **Costanzo, P. Merlo, N. Pediconi, M. Fulco, V. Sartorelli, P.A. Cole, G. Fontemaggi, M. Fanciulli, L. Schiltz and G. Blandino *et al.*,** DNA damage-dependent acetylation of p73 dictates the selective activation of apoptotic target genes, *Mol. Cell* **9** (2002), pp. 175–186.
- **Crook T, Nicholls JM, Brooks L, O'Nions J, Allday MJ.** High level expression of ΔN'p63: a mechanism for the inactivation of p53 in undifferentiated nasopharyngeal carcinoma (NPC)? Oncogene 2000;19:3439-44.
- **D'Orazi G, Cecchinelli B, Bruno T, Manni I, Higashimoto Y, Saito S, Gostissa M, Coen S, Marchetti A, Del Sal G, Piaggio G, Fanciulli M, Appella E and Soddu S**. (2002). *Nat. Cell Biol.*, **4**, 11–19.

- **Davis LD, Zhang W, Merseburger A, *et al.*** p63 expression profile in normal and malignant prostate epithelial cells. Anticancer Res 2002;22:3819-25.
- **Davies MPA, Rudland PS, Robertson L, Parry EW, Jolicouer P, Barraclough R.** 1996. Expression of the calcium-binding protein S100S4 (p9Ka) in MMTV-neu transgenic mice induces metastasis of mammary tumours. *Oncogene* **13**: 1631-1637.
- **De Laurenzi V, Raschella G, Barcaroli D, *et al.*** Induction of neuronal differentiation by p73 in a neuroblastoma cell line. J Biol Chem 2000;275:15226-31.
- **De Laurenzi V, Rossi A, Terrinoni A, *et al.*** p63 and p73 transactivate differentiation gene promoters in human keratinocytes. Biochem Biophys Res Commun 2000;273:342-6.
- **De Laurenzi V, Costanzo A, Barcaroli D, *et al.*** Two new p73 splice variants, 7and 5, with different transcriptional activity. J Exp Med 1998;188:1763-8.
- **de Thé H, Chomienne C, Lanotte M, Degos L and Dejean A**. (1990). *Nature*, **347**, 558–561.
- **de Thé H, Lavau C, Marchio A, Chomienne C, Degos L and Dejean A**. (1991). *Cell*, **66**, 675–684.
- **Deshpande R, Woods TL & Fu J *et al.*** Biochemical characterization of S100A2 in human keratinocytes: Subcellular localization, dimerization, and oxidative cross-linking. *J Invest Dermatol* (2000) **115**: 477–485.
- **Di Como CJ, Urist MJ, Babayan I, *et al.*** p63 expression profiles in human normal and tumor tissues. Clin Cancer Res 2002;8:494-501.
- **Di Como CJ, Gaiddon C, Prives C.** p73 function is inhibited by tumor-derived p53 mutants in mammalian cells. Mol Cell Biol 1999;19:1438-49.
- **Dittmer D, Pati S, Zambetti G, *et al.*** Gain of function mutations in p53. Nat Genet 1993;4:42-6.
- **Dobbelstein M, Wienzek S, Konig C, Roth J.** Inactivation of the p53-homologue p73 by the mdm2-oncoprotein. Oncogene 1999;18:2101-6.
- **Donato R.** 1999. Functional roles of S100 proteins, calcium-binding proteins of the EF-hand type. *Biochim Biophys Acta* **1450**: 191-231.

- **Donato R.** 2001. S100: a multigenic family of calcium-modulated proteins of the EF-hand type with intracellular and extracellular functional roles. *Int J Biochem Cell Biol* **33**: 637-668.
- **Donehower LA, Harvey M, Slagle BL, *et al.*** Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumors. Nature 1992;356:215-21.
- **Douc-Rasy S, Barrois M, Echeynne M, *et al.*** ΔN'p73α accumulates in human neuroblastic tumors. Am J Pathol 2002;160:631-9.
- **Duda T, Goraczniak RM, Sharma RK.** 1996. Molecular characterization of S100A1-S100B protein in retina and its activation mechanism of bovine photoreceptor guanylate cyclase. *Biochemistry* **35**: 6263-6266.
- **Eberhard DA, Karns LR, VandenBerg SR, Creutz CE.** 2001. Control of the nuclear-cytoplasmic partitioning of annexin II by a nuclear export signal and by p11 binding. *J Cell Sci* **114**: 3155-3166.
- **Ecsedy JA, Michaelson JS and Leder P**. (2003). *Mol. Cell. Biol.*, **23**, 950–960.
- **El-Rifai, C.A. Moskaluk, M.K. Abdrabbo, J. Harper, C. Yoshida, G.J. Riggins, H.F. Frierson Jr. and S.M. Powell,** Gastric cancers overexpress S100A calcium-binding proteins, *Cancer Res.* **62** (2002), pp. 6823–6826.
- **Everett RD**. (2000). *J. Virol.*, **74**, 9994–10005.
- **Everett RD, Lomonte P, Sternsdorf T, van Driel R and Orr A**. (1999). *J. Cell Sci.*, **112**, 4581–4588.
- **Ferbeyre G, de Stanchina E, Querido E, Baptiste N, Prives C and Lowe SW**. (2000). *Genes Dev.*, **14**, 2015–2027.
- **Field SJ, Tsai FY, Kuo F, *et al.*** E2F-1 functions in mice to promote apoptosis and suppress proliferation. Cell 1996;85:549-61.
- **Fillippovich I, Sorokina N, Gatei M, *et al.*** Transactivation-deficient p73α (p73Δexon2) inhibits apoptosis and competes with p53. Oncogene 2001;514-22.
- **Flenghi L, Fagioli M, Tomassoni L, Pileri S, Gambacorta M, Pacini R, Grignani F, Casini T, Ferrucci PF, Martelli MF, Pelicci P-G and Falini B**. (1995). *Blood*, **85**, 1871–1880.
- **Flores ER, Tsai KY, Crowley D, *et al.*** p63 and p73 are required for p53-dependent apoptosis in response to DNA damage. Nature 2002;416:560-4.

- **Fogal V, Gostissa M, Sandy P, Zacchi P, Sternsdorf T, Jensen K, Pandolfi PP, Will H, Schneider C and Del Sal G**. (2000). *EMBO J.*, **6**, 185–195.
- **Fontemaggi G, Gurtner A, Strano S *et al.*** The transcriptional repressor ZEB regulates p73 expression at the crossroad between proliferation and differentiation. *Mol Cell Biol* 2001; 21: 8461–70.
- **Fujii T, Machino K, Andoh H, Satoh T, Kondo Y.** 1990. Calcium-dependent control of caldesmon-actin interaction by S100 protein. *J Biochem* **107**: 761-764.
- **Gaiddon C, Lokshin M, Ahn J, Zhang T, Prives C.** A subset of tumor-derived mutant forms of p53 down-regulate p63 and p73 through a direct interaction with the p53 core domain. Mol Cell Biol 2001;21:1874-87.
- **Gaiddon C, Lokshin M, Gross I *et al.*** Cyclin-dependent kinases phosphorylate p73 at threonine 86 in a cell cycle-dependent manner and negatively regulate p73. *J Biol Chem* 2003; 278: 27421–31.
- **Garraway LA, Lin D, Signoretti S, *et al.*** Intermediate basal cells of the prostate: in vitro and in vivo characterization. Prostate 2003;55:206-18.
- **Gerke V, Weber K.** 1985. The regulatory chain in the p36-kd substrate complex of viral tyrosine-specific protein kinases is related in sequence to the S100 protein of glial cells. *EMBO J* **4**: 2917-2920.
- **Goldschneider D, Blanc E, Raguenez G *et al.*** When p53 needs p73 to be functional-forced p73 expression induces nuclear accumulation of endogenous p53 protein. *Cancer Lett* 2003; 197: 99–103.
- **Gong, A. Costanzo, H.Q. Yang, G. Melino, W.G. Kaelin, M. Levrero and J.Y.J. Wang,** The tyrosine kinase c-Abl regulates p73 in apoptotic response to cisplatin-induced DNA damage, *Nature* **39** (1999), pp. 806–809.
- **Gonzalez S, Prives C, Cordon-Cardo C.** p73α regulation by Chk1 in response to DNA damage. *Mol Cell Biol* 2003; 23: 8161–71.
- **Grande MA, van der Kraan I, van Steensel B, Schul W, de The H, van der Voort HT, de Long L and Driel R**. (1996). *J. Cell. Biochem.*, **63**, 280–291.
- **Grigorian M, Andresen S, Tulchinsky E, Kriajevska M, Carlberg C, Kruse C, Cohn M, Ambartsumian N, Christensen A, Selivanova G, Lukanidin E.** 2001. Tumor suppressor p53 protein is a new target for the metastasis-associated Mts1/S100A4

protein: functional consequences of their interaction. *J Biol Chem* **276**: 22699-22708.

- **Grob TJ, Novak U, Maisse C, *et al.*** Human ΔNp73 regulates a dominant negative feedback loop for TAp73 and p53. Cell Death Differ 2001;8:1213-23.
- **Gu J, Chen D, Rosenblum J, Rubin RM, Yuan ZM.** Identification of a sequence element from p53 that signals for Mdm2-targeted degradation. Mol Cell Biol 2000;20:1243-53.
- **Guan M, Peng HX, Yu B, Lu Y.** p73 Overexpression and angiogenesis in human colorectal carcinoma. Jpn J Clin Oncol 2003;33:215-20.
- **Guerreiro Da Silva, Y.F. Hu, I.H. Russo, X. Ao, A.M. Salicioni, X. Yang and J. Russo,** S100P calcium-binding protein overexpression is associated with immortalization of human breast epithelial cells in vitro and early stages of breast cancer development in vivo, *Int. J. Oncol.* **16** (2000), pp. 231–240.
- **Guo A, Salomoni P, Luo J, Shih A, Zhong S, Gu W and Pandolfi PP**. (2000). *Nat. Cell Biol.*, **2**, 730–736.
- **Halevy O, Michalovitz D, Oren M.** Different tumor-derived p53 mutants exhibit distinct biological activities. Science 1990;250:113-6.
- **Hanamoto T, Ozaki T, Furuya K *et al.*** Identification of protein kinase A catalytic subunit β as a novel binding partner of p73 and regulation of p73 function. *J Biol Chem* 2005; 280: 16665–75.
- **Hardas BD, Zhao X & Zhang J *et al.*** Assignment of psoriasin to human chromosomal band 1q21: Coordinate overexpression of clustered genes in psoriasis. *J Invest Dermatol* (1996) **106**: 753–758.
- **Heierhorst J, Kobe B, Feil SC, Parker MW, Benians GM, Weiss KR, Kemp BE.** 1996. Ca^{2+}/S100 regulation of giant protein kinases. *Nature* **380**: 636-639.
- **Heighway, T. Knapp, L. Boyce, S. Brennand, J.K. Field, D.C. Betticher, D. Ratschiller, M. Gugger, M. Donovan, A. Lasek and P. Rickert,** Expression profiling of primary non-small cell lung cancer for target identification, *Oncogene* **21** (2002), pp. 7749–7763.
- **Heizmann CW & Cox JA.** New perspectives on S100 proteins: A multi-functional Ca(2+)-, Zn(2+)- and Cu(2+)-binding protein family. *Biometals* (1998) **11**: 383–397.
- **Herranz M, Santos J, Salido E, Fernandez-Piqueras J, Serrano M.** Mouse p73 gene maps to the distal part of chromosome 4 and

might be involved in the progression of γ-radiation-induced T-cell lymphomas. Cancer Res 1999;59:2068-71.

- **Hibi K, Trink B, Patturajan M, *et al.*** AIS is an oncogene amplified in squamous cell carcinoma. Proc Natl Acad Sci USA 2000;97:5462-7.
- **Hofmann, S. Drury, C. Fu, W. Qu, A. Taguchi, Y. Lu, C. Avila, N. Kambham, A. Bierhaus, P. Nawroth, M.F. Neurath, T. Slattery, D. Beach, J. McClary, M. Nagashima, J. Morser, D. Stern and A.M. Schmidt,** RAGE mediates a novel proinflammatory axis: a central cell surface receptor for S100/calgranulin polypeptides, *Cell* **97** (1999), pp. 889–901.
- **Hofmann TG, Moller A, Sirma H, Zentgraf H, Taya Y, Droge W, Will H and Schmitz ML**. (2002). *Nat. Cell Biol.*, **4**, 1–10.
- **Hosoda M, Ozaki T, Miyazaki K *et al.*** UFD2a mediates the proteasomal turnover without promoting p73 ubiquitination. *Oncogene* 24 (2005), pp. 7156-69.
- **Hsieh, B.W. Schäfer, N. Sasaki and C.W. Heizmann,** Expression analysis of S100 proteins and RAGE in human tumors using tissue microarrays, *Biochem. Biophys. Res. Commun.* **307** (2003), pp. 375–381.
- **Hu H, Xia SH, Li AD, *et al.*** Elevated expression of p63 protein in human esophageal squamous cell carcinomas. Int J Cancer 2002;102:580-3.
- **Huttunen, J. Kuja-Panula, G. Sorci, A.L. Agneletti, R. Donato and H. Rauvala,** Coregulation of neurite outgrowth and cell survival by amphoterin and S100 proteins through receptor for advanced glycation end products (RAGE) activation, *J. Biol. Chem.* **275** (2000), pp. 40096–40105.
- **Ikawa S, Nakagawara A, Ikawa Y.** p53 family genes: structural comparison, expression and mutation. Cell Death Differ 1999;6:1154-61.
- **Ilg EC, Schäfer BW, Heizmann CW.** 1996. Expression pattern of S100 calcium-binding proteins in human tumors. *Int J Cancer* **68**: 325-332.
- **Irwin M, Marin MC, Phillips AC, *et al.*** Role for the p53 homologue p73 in E2F-1-induced apoptosis. Nature 2000;407:645-8. Wan YY, DeGregori J. The survival of antigen-stimulated T cells requires NFκB-mediated inhibition of p73 expression. Immunity 2003;18:331-42.

- **Irwin MS, Kondo K, Marin MC, Cheng LS, Hahn WC, Kaelin WG.** Chemosensitivity linked to p73 function. Cancer Cell 2003;3:403-10.
- **Irwin, K. Kondo, M.C. Marin, W.C. Hahan and W.G. Kaelin Jr.,** Chemosensitivity linked to p73 function, *Cancer Cell* **3** (2003), pp. 403–410.
- **Irwin MS, Kaelin WG Jr.** p53 family update: p73 and p63 develop their own identities. *Cell Growth Differ* 2001; 12: 337–49.
- **Irwin M, Marin MC, Phillips AC *et al.*** Role for the p53 homologue p73 in E2F-1-induced apoptosis. *Nature* 2000; 407: 645–8.
- **Ishida S, Yamashita T, Nakaya U, Tokino T.** Adenovirus-mediated transfer of p53-related genes induces apoptosis of human cancer cells. Jpn J Cancer Res 2000;91:174-80.
- **Ishimoto O, Kawahara C, Enjo K, Obinata M, Nukiwa T, Ikawa S.** Possible oncogenic potential of ΔNp73: a newly identified isoform of human p73. Cancer Res 2002;62:636-41.
- **Ishov, A.G. Sotnikov, D. Negorev, O.V. Vladimirova, N. Neff, T. Kamitani, E.T. Yeh, J.F. Strauss 3rd and G.G. Maul,** *J. Cell Biol.* **147** (1999), pp. 221–234.
- **Jacks T, Remington L, Williams BO, *et al.*** Tumor spectrum analysis in p53-mutant mice. Curr Biol 1994;4:1-7.
- **Jensen K, Shiels C and Freemont PS**. (2001). *Oncogene*, **20**, 7223–7233.
- **Johnson DG, Schwarz JK, Cress WD, Nevins JR.** Expression of transcription factor E2F1 induces quiescent cells to enter S phase. *Nature* 1993; 365: 349–52.
- **Jones SN, Roe AE, Donehower LA, Bradley A.** Rescue of embryonic lethality in Mdm2-deficient mice by absence of p53. Nature 1995;378:206-8.
- **Jost CA, Marin MC, Kaelin WG Jr.** p73 is a simian [correction of human] p53-related protein that can induce apoptosis. Nature 1997;389:191-4.
- **Kaghad, H. Bonnet, A. Yang, L. Creancier, J.C. Biscan, A. Valent, A. Minty, P. Chalon, J.M. Lelias, X. Dumont, P. Ferrara, F. McKeon and D. Caput,** Monoallelically expressed gene related to p53 at 1p36, a region frequently deleted in neuroblastoma and other human cancers, *Cell* **90** (1997), pp. 809–819.

- **Kawano S, Miller CW, Gombart AF, *et al.*** Loss of p73 gene expression in leukemias/lymphomas due to hypermethylation. Blood 1999;94:1113-20.
- **Kanai, P.A. Marignani, D. Sarbassova, R. Yagi, R.A. Hall, M. Donowitz, A. Hisaminato, T. Fujiwara, Y. Ito, L.C. Cantley and M.B. Yaffe,** TAZ: a novel transcriptional co-activator regulated by interactions with 14–3-3 and PDZ domain proteins, *EMBO J.* **19** (2000), pp. 6778–6791.
- **Kang MJ, Park BJ, Byun DS, *et al.*** Loss of imprinting and elevated expression of wild-type p73 in human gastric adenocarcinoma. Clin Cancer Res 2000;6:1767-71.
- **Kartasheva NN, Contente A, Lenz-Stoppler C, Roth J, Dobbelstein M.** p53 induces the expression of its antagonist p73Δ N, establishing an autoregulatory feedback loop. Oncogene 2002;21:4715-27.
- **Koken MHM, Linares-Cruz G, Quignon F, Viron A, Chelbi-Alix MK, Sobczak-Thépot J, Juhlin L, Degos L, Calvo F and de Thé H**. (1995). *Oncogene*, **10**, 1315–1324.
- **Koster MI, Kim S, Mills AA, DeMayo FJ, Roop DR.** p63 is the molecular switch for initiation of an epithelial stratification program. Genes Dev 2004;18:126-31.
- **Kovalev S, Marchenko N, Swendeman S, LaQuaglia M, Moll UM.** Expression level, allelic origin, and mutation analysis of the p73 gene in neuroblastoma tumors and cell lines. Cell Growth & Differ 1998;9:897-903.
- **Kramer S, Ozaki T, Miyazaki K, Kato C, Hanamoto T, Nakagawara A.** Protein stability and function of p73 are modulated by a physical interaction with RanBPM in mammalian cultured cells. *Oncogene* 2005; 24: 938–44.
- **Kress M, May E, Cassigena R and May P.** Simian virus 40-transformed cells express new species of proteins precipitable by anti-simian virus 40 tumor serum. J Virol. 1979; 2: 472-83
- **Kriajevska M, Tarabykina S, Bronstein I, Maitland N, Lomonosov M, Hansen K, Georgiev G, Lukanadin E.** 1998. Metastasis-associated 1 (S100A4) protein modulates protein kinase C phosphorylation of the heavy chain of nonmuscle myosin. *J Biol Chem* **273**: 9852-9856.

- **Lafarga M, Berciano MT, Pena E, Mayo I, Castano JG, Bohmann D, Rodrigues JP, Tavanez JP and Carmo-Fonseca M**. (2002). *Mol. Cell. Biol.*, **13**, 2771–2782.
- **Lagasse E, Clerc RG.** 1988. Cloning and expression of two human genes encoding calcium-binding proteins that are regulated during myeloid cell differentiation are expressed by subsets of macrophages in inflammatory tissues. *Mol Cell Biol* **8**: 2402-2410.
- **Lallemand-Breitenbach V, Zhu J, Puvion F, Koken M, Honore N, Doubeikovsky A, Duprez E, Pandolfi PP, Puvion E, Freemont P and de The H**. (2001). *J. Exp. Med.*, **193**, 1361–1372.
- **Lane DP and Crawford LV** 1979. T antigen is bound to a host protein in SV40-transformed cells. *Nature* **278**: 261-3.
- **Lee SW, Tomasetto C, Swisshelm K, Keyomarsi K, Sanger R.** 1992. Down-regulation of a member of the S100 gene family in mammary carcioma cells and re-expression by azadeoxycytidine treatment. *Proc Natl Acad Sci USA* **89**: 2504-2508.
- **Lee H, Kimelman D.** A dominant-negative form of p63 is required for epidermal proliferation in zebrafish. Dev Cell 2002;2:607-16.
- **Lee CW, La Thangue NB.** Promoter specificity and stability control of the p53-related protein p73. Oncogene 1999;18:4171-81.
- **Lehembre F, Muller S, Pandolfi PP and Dejean A**. (2001). *Oncogene*, **20**, 1–9.
- **Levine AJ.** p53, the cellular gatekeeper for growth and division. *Cell.* 1997 88: 323-31.
- **Levy D, Adamovich Y, Reuven N and Shaul Y.** (2006). *Cell Death Differ.* In press
- **Li H, Leo C, Zhu J, Wu X, O'Neil J, Park EJ and Chen JD**. (2000). *Mol. Cell. Biol.*, **20**, 1784–1796.
- **Li M, Chen D, Shiloh A, Luo J, Nikolaev AY, Qin J and Gu W**. (2002). *Nature*, **416**, 648–653.
- **Lin J, Blake M, Tang C, Zimmer D, Rustandi RR, Weber DJ, Carrier F.** 2001. Inhibition of p53 transcriptional activity by the S100B calcium-binding protein. *J Biol Chem* **276**: 35037-35041.
- **Lin, M.Z. Lai, D.K. Ann and H.M. Shih,** *J. Biol. Chem.* **278** (2003), pp. 15958–15965.
- **Lin, O. Barbash, K.G.S. Kumar, J.D. Weber, J.W. Harper, A.J.P. Klein-Szanto, A. Rustgi, S.Y. Fuchs and J.A. Diehl,** *Mol. Cell* **24** (2006), pp. 355–366.

- **Linzer DI and Levine AJ.** Characterization of a 54K Dalton cellular SV40 tumor antigen present in SV40-transformed cells and uninfected embryonal carcinoma cells. *Cell* 1979; 17: 43-52.
- **Lissy NA, Davis PK, Irwin M, Kaelin WG, Dowdy SF.** A common E2F-1 and p73 pathway mediates cell death induced by TCR activation. Nature 2000;407:642-5.
- **Liu M, Taketani T, Li R, *et al.*** Loss of p73 gene expression in lymphoid leukemia cell lines is associated with hypermethylation. Leuk Res 2001;25:441-7.
- **Mai M, Yokomizo A, Qian C, *et al.*** Activation of p73 silent allele in lung cancer. Cancer Res 1998;58:2347-49.
- **Mandinova, D. Atar, B.W. Schäfer, M. Spiess, U. Aebi and C.W. Heizmann,** Distinct subcellular localization of calcium binding S100 proteins in human smooth muscle cells and their relocation in response to rises in intracellular calcium, *J. Cell Sci.* **111** (1998), pp. 2043–2054.
- **Mantovani F, Piazza S, Gostissa M *et al.*** Pin1 links the activities of c-Abl and p300 in regulating p73 function. *Mol Cell* 2004; 14: 625–36.
- **Marin MC, Jost CA, Irwin MS, *et al.*** Viral oncoproteins discriminate between p53 and the p53 homolog p73. Mol Cell Biol 1998;18:6316-24.
- **Marin MC, Jost CA, Brooks LA, *et al.*** A common polymorphism acts as an intragenic modifier of mutant p53 behavior. Nat Genet 2000;25:47-54.
- **Maul GG, Yu E, Ishov AM and Epstein AL**. (1995). *J. Cell. Biochem.*, **59**, 498–513.
- **McClintock KA, Van Eldik LJ, Shaw GS.** 2002. The C-terminus and linker region of S100B exerts dual control on protein-protein interactions with TRTK-12. *Biochemistry* **41**: 5421-5428.
- **Melnick A and Licht JD**. (1999). *Blood*, **93**, 3167–3215.
- **Mills AA, Zheng B, Wang XJ, Vogel H, Roop DR, Bradley A.** p63 is a p53 homologue required for limb and epidermal morphogenesis. Nature 1999;398:708-13.
- **Millward TA, Heizmann CW, Schäfer BW, Hemmings BA.** 1998. Calcium regulation of Ndr protein kinase mediated by S100 calcium-binding proteins. *EMBO J* **17**: 5913-5922.
- **Minty A, Dumont X, Kaghad M, Caput D.** Covalent modification of p73α by SUMO-1. Two-hybrid screening with p73 identifies

novel SUMO-1-interacting proteins and a SUMO-1 interaction motif. J Biol Chem 2000;275:36316-23.

- **Miro-Mur F, Meiller A, Haddada H, May E.** p73α expression induces both accumulation and activation of wt-p53 independent of the p73α transcriptional activity. *Oncogene* 2003; 22: 5451–6.
- **Mischke D, Korge BP & Marenholz I *et al.*** Genes encoding structural proteins of epidermal cornification and S100 calcium-binding proteins form a gene complex ("epidermal differentiation complex") on human chromosome 1q21. *J Invest Dermatol* (1996) **106**: 989–992.
- **Miyazaki K, Ozaki T, Kato C *et al.*** A novel HECT-type E3 ubiquitin ligase, NEDL2, stabilizes p73 and enhances its transcriptional activity. *Biochem Biophys Res Commun* 2003; 308: 106–13.
- **Montes de Oca Luna R, Wagner DS, Lozano G.** Rescue of early embryonic lethality in mdm2-deficient mice by deletion of p53. Nature 1995;378:203-6.
- **Moore B.V.** A soluble protein characteristic of the nervous system. *Biochem. Biophys. Res.Commun.*(1965) **19**: 739-744.
- **Mu ZM, Chin KV, Liu JH, Lozano G and Chang KS**. (1994). *Mol. Cell. Biol.*, **14**, 6858–6867.
- **Muller S, Matunis MJ and Dejean A**. (1998). *EMBO J.*, **17**, 61–70.
- **Murao S, Collart FR, Huberman E.** 1989. A protein containing the cystic fibrosis antigen is an inhibitor of protein kinases. *J Biol Chem* **264**: 8356-8360.
- **Naar, B.D. Lemon and R. Tijian,** Transcriptional co-activator complexes, *Annu. Rev. Biochem.* **70** (2001), pp. 475–501.
- **Nagy, D. Hoyaux, I. Gielen, B.W. Schäfer, R. Pochet, C.W. Heizmann, R. Kiss, I. Salmon and C. Decaestecker,** The Ca^{2+}-binding S100A2 protein is differentially expressed in epithelial tissue of glandular or squamous origin, *Histol. Histopathol.* **17** (2002), pp. 123–130.
- **Nakagawa T, Takahashi M, Ozaki T, *et al.*** Autoinhibitory regulation of p73 by ΔNp73 to modulate cell survival and death through a p73-specific target element within the ΔNp73 promoter. Mol Cell Biol 2002;22:2575-85.

- **Nakano K, Balint E, Ashcroft M, Vousden KH.** A ribonucleotide reductase gene is a transcriptional target of p53 and p73. Oncogene 2000;19:4283-9.
- **Negorev D and Maul GG**. (2001). *Oncogene*, **20**, 7234–7242.
- **Newton and N. Hogg,** The human S100 protein MRP-14 is a novel activator of the β2 integrin Mac-1 on neutrophils, *J. Immunol.* **160** (1998), pp. 1427–1435.
- **Ng SW, Yiu GK, Liu Y, *et al.*** Analysis of p73 in human borderline and invasive ovarian tumor. Oncogene 2000;19:1885-90.
- **Novak U, Grob TJ, Baskaynak G, *et al.*** Overexpression of the p73 gene is a novel finding in high-risk B-cell chronic lymphocytic leukemia. Ann Oncol 2001;12:981-6.
- **Nylander K, Coates PJ, Hall PA.** Characterization of the expression pattern of p63α and ΔNp63α in benign and malignant oral epithelial lesions. Int J Cancer 2000;87:368-72.
- **Ohtsuka T, Ryu H, Minamishima YA, Ryo A, Lee SW.** Modulation of p53 and p73 levels by cyclin G: Implication of a negative feedback regulation. *Oncogene* 2003; 22: 1678–87.
- **Onions J, Hermann S, Grundström T.** 1997. Basic helix-loop-helix protein sequences determining differential inhibition by calmodulin and S100 proteins. *J Biol Chem* **272**: 23930-23937.
- **Ozaki T, Watanabe K, Nakagawa T *et al.*** Function of p73, but not of p53, is inhibited by the physical interaction with RACK1 and its inhibitory effect is counteracted by pRB. *Oncogene* 2003; 22: 3231–42.
- **Pandolfi PP**. (2001). *Oncogene*, **20**, 5726–5735.
- **Parsa R, Yang A, McKeon F, Green H.** Association of p63 with proliferative potential in normal and neoplastic human keratinocytes. J Invest Dermatol 1999;113:1099-105.
- **Park BJ, Lee SJ, Kim JI, *et al.*** Frequent alteration of p63 expression in human primary bladder carcinomas. Cancer Res 2000;60:3370-4.
- **Pearson M, Carbone R, Sebastiani C, Cioce M, Fagioli M, Saito S, Higashimoto Y, Appella E, Minucci S, Pandolfi PP and Pelicci PG**. (2000). *Nature*, **406**, 207–210.
- **Pearson M and Pelicci PG**. (2001). *Oncogene*, **20**, 7250–7256.
- **Pellegrini G, Dellambra E, Golisano O, *et al.*** p63 identifies keratinocyte stem cells. Proc Natl Acad Sci USA 2001;98:3156-61.

- **Perry WL, Hustad CM, Swing DA, O'Sullivan TN, Jenkins NA and Copeland NG.** (1998). Nat Genet, **18**, 143-146.
- **Perlman R, Schiemann WP, Brooks MW, Lodish HF and Weinberg RA**. (2001). *Nat. Cell Biol.*, **3**, 708–714.
- **Peters UR, Tschan MP, Kreuzer KA, *et al.*** Distinct expression patterns of the p53-homologue p73 in malignant and normal hematopoiesis assessed by a novel real-time reverse transcription-polymerase chain reaction assay and protein analysis. Cancer Res 1999;59:4233-6.
- **Piazza F, Gurrieri C and Pandolfi PP**. (2001). *Oncogene*, **20**, 7216–7222.
- **Pignatelli M, Luna-Medina R, Perez-Rendon A, Santos A, Perez-Castillo A.** The transcription factor early growth response factor-1 (EGR-1) promotes apoptosis of neuroblastoma cells. Biochem J 2003;373:739-46.
- **Pokrovskaja K, Mattsson K, Kashuba E, Klein G and Szekely L**. (2001). *J. Gen. Virol.*, **82**, 345–358.
- **Pozniak CD, Radinovic S, Yang A, McKeon F, Kaplan DR, Miller FD.** An anti-apoptotic role for the p53 family member, p73, during developmental neuron death. Science 2000;289:304-6.
- **Pozdnyakoz N, Goraczniak R, Margulis A, Duda T, Sharma RK, Yoshida A, Sitaramayya A.** 1997. Structural and functional characterization of retinal calcium-dependent guanylate cyclase activator protein (CD-GCAP): identity with S100β protein. *Biochemistry* **36**: 14159-14166.
- **Pritchard K, Martson SB.** 1991. Ca^{2+}-dependent regulation of vascular smooth-muscle caldesmon by S100 and related smooth-muscle proteins. *Biochem J* **277**: 819-824.
- **Puig P, Capodieci P, Drobnjak M, *et al.*** p73 Expression in human normal and tumor tissues: loss of p73α expression is associated with tumor progression in bladder cancer. Clin Cancer Res 2003;9:5642-51.
- **Putzer BM, Tuve S, Tannapfel A, Stiewe T.** Increased ΔN'p73 expression in tumors by upregulation of the E2F1-regulated, TA-promoter-derived ΔN'-p73 transcript. Cell Death Differ 2003;10:612-4.
- **Quignon F, de Bels F, Koken M, Feunteun J, Ameisen J-C and de Thé H**. (1998). *Nat. Genet.*, **20**, 259–265.

- **Reis-Filho JS, Milanezi F, Amendoeira I, Albergaria A, Schmitt FC.** Distribution of p63, a novel myoepithelial marker, in fine-needle aspiration biopsies of the breast: an analysis of 82 samples. Cancer 2003;99:172-9.
- **Ren J, Datta R, Shioya H *et al.*** p73β is regulated by protein kinase Cδ catalytic fragment generated in the apoptotic response to DNA damage. *J Biol Chem* 2002; 277: 33758–65.
- **Ribeiro-Silva A, Zambelli Ramalho LN, Britto Garcia S, Zucoloto S.** The relationship between p63 and p53 expression in normal and neoplastic breast tissue. Arch Pathol Lab Med 2003;127:336-40.
- **Rossi M, De Laurenzi V, Munarriz E *et al.*** The ubiquitin-protein ligase Itch regulates p73 stability. *EMBO J* 2005; 24: 836–48.
- **Rustandi RR, Baldisseri DM, Weber DJ.** 2000. Structure of the negative regulatory domain of p53 bound to S100B(ββ). *Nat Struct Biol* **7**: 570-574.
- **Roth J, Konig C, Wienzek S *et al.*** Inactivation of p53 but not p73 by adenovirus type 5 E1B 55-kilodalton and E4 34-kilodalton oncoproteins. *J Virol* 1998; 72: 8510–6.
- **Sakaguchi M, Miyazaki M, Inoue Y, Tsuji T, Kouchi H, Tanaka T, Yamada H, Namba M.** 2000. Relationship between contact inhibition and intranuclear S100C of normal human fibroblasts. *J Cell Biol* **149**: 1193-1206.
- **Salimath B, Marme D, Finkenzeller G.** Expression of the vascular endothelial growth factor gene is inhibited by p73. Oncogene 2000;19:3470-6.
- **Salomoni P and Pandolfi PP**. (2002). *Cell*, **108**, 165−170.
- **Samuels-Lev Y, O'Connor DJ, Bergamaschi D, *et al.*** ASPP proteins specifically stimulate the apoptotic function of p53. Mol Cell 2001;8:781-94.
- **Sayan AE, Sayan BS, Findikli N, Ozturk M.** Acquired expression of transcriptionally active p73 in hepatocellular carcinoma cells. Oncogene 2001;20:5111-7.
- **Scaruffi P, Casciano I, Masiero L, Basso G, Romani M, Tonini GP.** Lack of p73 expression in mature B-ALL and identification of three new splicing variants restricted to pre B and C-ALL indicate a role of p73 in B cell ALL differentiation. Leukemia 2000;14:518-9.

- **Schäfer BW, Heizmann CW.** 1996. The S100 family of EF-hand calcium-binding proteins: functions and pathology. *Trends Biochem Sci* **21**: 134-140.
- **Schmidt-Hansen, J. Klingelhofer, B. Grum-Schwensen, A. Christensen, S. Andresen, C. Kruse, T. Hansen, N. Ambartsumian, E. Lukanidin and M. Grigorian,** Functional significance of metastasis-inducing S100A4(Mts1) in tumor-stroma interplay, *J. Biol. Chem.* (2004).
- **Scotto C, Deloulme JC, Rousseau D, Chambaz E, Baudier J.** 1998. Calcium and S100B regulation of p53-dependent cell growth arrest and apoptosis. *Mol Cell Biol* **18**: 4272-4281.
- **Seeler and A. Dejean,** SUMO: of branched proteins and nuclear bodies, *Oncogene* **20** (2001), pp. 7243–7249.
- **Selinfreund RH, Barger SW, Welsh MJ, Van Eldik LJ.** 1990. Antisense inhibition of glial S100β production results in alteration in cell morphology, cytoskeletal organization, and cell proliferation. *J Cell Biol* **111**: 2021-2028.
- **Serber Z, Lai HC, Yang A, *et al.*** A C-terminal inhibitory domain controls the activity of p63 by an intramolecular mechanism. Mol Cell Biol 2002;22:8601-11.
- **Shaulsky G, Goldfinger N, Rotter V.** Alterations in tumor development in vivo mediated by expression of wild type or mutant p53 proteins. Cancer Res 1991;51:5232-7.
- **Shen, H.-K. Lin, P.P. Scaglioni, T.M. Yung and P.P. Pandolfi,** *Mol. Cell* **24** (2006), pp. 331–339
- **Shrestha P, Muramatsu Y & Kudeken W *et al.*** Localization of Ca(2+)-binding S100 proteins in epithelial tumours of the skin. *Virchows Arch* (1998) **432**: 53–59.
- **Song, L.K. Durrin, T.A. Wilkinson, T.G. Krontiris and Y. Chen,** *Proc. Natl. Acad. Sci. USA* **101** (2004), pp. 14373–14378.
- **Sorci, F. Riuzzi, A.L. Agneletti, C. Marchetti and R. Donato,** S100B inhibits myogenic differentiation and myotube formation in a RAGE-independent manner, *Mol. Cell. Biol.* **23** (2003), pp. 4870–4881.
- **South AP, Cabral A & Ives JH *et al.*** Human epidermal differentiation complex in a single 2.5 Mbp long continuum of overlapping DNA cloned in bacteria integrating physical and transcript maps. *J Invest Dermatol* (1999) **112**: 910–918.

- **Skripnikowa EV, Gusev NB.** 1989. Interaction of smooth muscle caldesmon with S100 protein. *FEBS Lett* **257**: 380-382.
- **Steegenga WT, Shvarts A, Riteco N, Bos JL, Jochemsen AG.** Distinct regulation of p53 and p73 activity by adenovirus E1A, E1B, and E4orf6 proteins. Mol Cell Biol 1999;19:3885-94.
- **Stiewe T, Putzer BM.** Role of the p53-homologue p73 in E2F1-induced apoptosis. Nat Genet 2000;26:464-9.
- **Stiewe T, Putzer BM.** Role of p73 in malignancy: tumor suppressor or oncogene? Cell Death Differ 2002;9:237-45.
- **Stiewe T, Theseling CC, Putzer BM.** Transactivation-deficient Δ TA-p73 inhibits p53 by direct competition for DNA binding: implications for tumorigenesis. J Biol Chem 2002;277:14177-85.
- **Stiewe T, Zimmermann S, Frilling A, Esche H, Putzer BM.** Transactivation-deficient ΔTA-p73 acts as an oncogene. Cancer Res 2002;62:3598-602.
- **Stoll SW, Chia NV & Nair RP *et al.*** S100A2 coding sequence polymorphism: Characterization and lack of association with psoriasis. *Clin Exp Dermatol* (2001) **26**: 79–83.
- **Strano, S. Munarriz, M. Rossi, L. Castagnoli, Y. Shaul, A. Sacchi, M. Oren, M. Sudol, G. Cesareni and G. Blandino,** Physical interaction with Yes-associated protein enhances p73 transcriptional activity, *J. Biol. Chem.* **276** (2001), pp. 15164–15173.
- **Strano S, Monti O, Pediconi N, Baccarini A, Fontemaggi G, Lapi E, Mantovani F, Damalas A, Citro G, Sacchi A, Del Sal G, Levrero M and Blandino G.** The transcriptional coactivator Yes-Associated Protein drives p73 gene target specificity in response to DNA damage. *Mol. Cell* **18** (2005), pp. 447-459.
- **Sun XF.** p73 overexpression is a prognostic factor in patients with colorectal adenocarcinoma. Clin Cancer Res 2002;8:165-70.
- **Sunahara M, Ichimiya S, Nimura Y, *et al.*** Mutational analysis of the p73 gene localized at chromosome 1p36.3 in colorectal carcinomas. Int J Oncol 1998;13:319-23.
- **Sudol,** Yes-associated protein (YAP65) is a proline-rich phosphoprotein that binds to the SH3 domain of the Yes proto-oncogene product, *Oncogene* **9** (1994), pp. 2145–2152.
- **Sudol and T. Hunter,** NeW wrinkles for an old domain, *Cell* **103** (2000), pp. 1001–1004.
- **Takahashi, S. Hatakeyama, H. Saitoh and K.I. Nakayama,** *J. Biol. Chem.* **280** (2005), pp. 5611–5621.

- **Tannapfel A, Wasner M, Krause K, *et al.*** Expression of p73 and its relation to histopathology and prognosis in hepatocellular carcinoma. J Natl Cancer Inst 1999;91:1154-8.
- **Tannapfel A, Engeland K, Weinans L, *et al.*** Expression of p73, a novel protein related to the p53 tumor suppressor p53, and apoptosis in cholangiocellular carcinoma of the liver. Br J Cancer 1999;80:1069-74.
- **Terris B, Baldin V, Dubois S, Degott C, Flejou JF, Henin D and Dejean A**. (1995). *Cancer Res.*, **55**, 1590–1597.
- **Toh WH, Siddique MM, Boominathan L, Lin KW, Sabapathy K.** c-Jun regulates the stability and activity of the p53 homologue, p73. *J Biol Chem* 2004; 279: 44713–22.
- **Tokuchi YHT, Kobayashi Y, Hayashi M, *et al.*** The expression of p73 is increased in lung cancer, independent of p53 gene alteration. Br J Cancer 1999;80:1623-9.
- **Torii S, Egan DA, Evans RA and Reed JC**. (1999). *EMBO J.*, **18**, 6037–6049.
- **Tschan MP, Grob TJ, Peters UR, *et al.*** Enhanced p73 expression during differentiation and complex p73 isoforms in myeloid leukemia. Biochem Biophys Res Commun 2000;277:62-5.
- **Tsoporis JN, Marks A, Kahn HJ, Butany JW, Liu PP, O'Hanlon D, Parker TG.** 1997. S100β inhibits α_1-adrenergic induction of the hypertrophic phenotype in cardiac myocytes. *J Biol Chem* **272**: 31915-31921.
- **Tsoporis JN, Marks A, Kahn HJ, Butany JW, Liu PP, O'Hanlon D, Parker TG.** 1998. Inhibition of norepinephrine-induced cardiac hypertrophy in S100β transgenic mice. *J Clin Invest* **102**: 1609-1616.
- **Urist MJ, Di Como CJ, Lu ML, *et al.*** Loss of p63 expression is associated with tumor progression in bladder cancer. Am J Pathol 2002;161:1199-206.
- **Vassilev, K.J. Kaneko, H. Shu, Y. Zhao and M.L. DePamphilis,** TEAD/TEF transcription factors utilize the activation domain of YAP65, a Src/Yes-associated protein localized in the cytoplasm, *Genes Dev.* **15** (2001), pp. 1229–1241.
- **Vikhanskaya F, D'Incalci M, Broggini M.** p73 competes with p53 and attenuates its response in a human ovarian cancer cell line. *Nucl Acids Res* 2000; 28: 513–9.

- **Volz A, Korge BP & Compton JG *et al.*** Physical mapping of a functional cluster of epidermal differentiation genes on chromosome 1q21. *Genomics* (1993) **18**: 92–99.
- **Vossio S, Palescandolo E, Pediconi N, *et al.*** DN-p73 is activated after DNA damage in a p53-dependent manner to regulate p53-induced cell cycle arrest. Oncogene 2002;21:3796-803.
- **Waltermann A, Kartasheva NN, Dobbelstein M.** Differential regulation of p63 and p73 expression. Oncogene 2003;22:5686-93.
- **Wang WK, Bycroft M, Foster NW, Buckle AM, Fersht AR, Chen YW.** Structure of the C-terminal sterile α-motif (SAM) domain of human p73α. Acta Crystallogr D Biol Crystallogr 2001;57:545-51.
- **Wang XQ, Ongkeko WM, Lau AW, Leung KM, Poon RY.** A possible role of p73 on the modulation of p53 level through MDM2. Cancer Res 2001;61:1598-603.
- **Wang ZG, Ruggero D, Ronchetti S, Zhong S, Gaboli M, Rivi R and Pandolfi PP**. (1998b). *Nat. Genet.*, **20**, 266–271.
- **Wang ZG, Delva L, Gaboli M, Rivi R, Giorgio M, Cordon-Cardo C, Grosveld F and Pandolfi PP**. (1998b). *Science*, **279**, 1547–1551.
- **Warrell R, de Thé H, Wang Z and Degos L**. (1993). *N. Engl. J. Med.*, **329**, 177–189.
- **Watanabe K, Ozaki T, Nakagawa T *et al.*** Physical interaction of p73 with c-Myc and MM1, a c-Myc-binding protein, and modulation of the p73 function. *J Biol Chem* 2002; 277: 15113–23.
- **Weber MKZ, Clavien PA.** Low recurrence rate of hepatocellular carcinoma after liver transplantation: better patient selection or lower immunosuppression? Transplantation 2002;74:1664-5.
- **Weber A, Bellmann U, Bootz F, Wittekind C, Tannapfel A.** Expression of p53 and its homologues in primary and recurrent squamous cell carcinomas of the head and neck. Int J Cancer 2002;99:22-8.
- **Westfall MD, Mays DJ, Sniezek JC, Pietenpol JA.** The ΔNp63α phosphoprotein binds the p21 and 14-3-3σ promoters in vivo and has transcriptional repressor activity that is reduced by Hay-Wells syndrome-derived mutations. Mol Cell Biol 2003;23:2264-76.
- **Wicki R, Franz C, Scholl FA, Heizmann CW, Schäfer BW.** 1997. Repression of the candidate tumor suppressor gene S100A2 in breast

cancer is mediated by site-specific hypermethylation. *Cell Calcium* **22**: 243-254.

- **Wicki R, Marenholz I & Mischke D *et al.*** Characterization of the human S100A12 (calgranulin C, p6, CAAF1, CGRP) gene, a new member of the S100 gene cluster on chromosome 1q21. *Cell Calcium* (1996) **20**: 459–464.
- **Wiesmeijer K, Molenaar C, Bekeer IM, Tanke HJ and Dirks RW**. (2002). *J. Struct. Biol.*, **140**, 180–188.
- **Wolf R, Mirmohammadsadegh A & Walz M *et al.*** Molecular cloning and characterization of alternatively spliced mRNA isoforms from psoriatic skin encoding a novel member of the S100 family. *FASEB J* (2003) **17**: 1969–1971.
- **Wu T, Angus W, Yao X-L, Logun C, Shelhamer JH.** 1997. p11, a unique member of the S100 family of calcium-binding proteins, interacts with and inhibits the activity of the 85-kDa cytosolic phospholipase A_2. *J Biol Chem* **272**: 17145-17153.
- **Wu L, Zhu H, Nie L, Maki CG.** A link between p73 transcriptional activity and p73 degradation. *Oncogene* 2004; 23: 4032–6.
- **Wu WS, Xu ZX and Chang KS**. (2002). *J. Biol. Chem.*, **277**, 31734–31739.
- **Wu WS, Xu ZX, Hittelman WN, Salomoni P, Pandolfi PP and Chang KS**. (2003). *J. Biol. Chem.*, **278**, 12294–12304.
- **Xia L, Stoll SW & Liebert M *et al.*** CaN19 expression in benign and malignant hyperplasias of the skin and oral mucosa: Evidence for a role in regenerative differentiation. *Cancer Res* (1997) **57**: 3055–3062.
- **Yagi, L.F. Chen, K. Shigesada, Y. Murakami and Y. Ito,** A WW domain-containing yes-associated protein (YAP) is a novel transcriptional co-activator, *EMBO J.* **18** (1999), pp. 2551–2562.
- **Yamasaki R, Berri M, Wu Y, Trombitas K, McNabb M, Kellermayer MS, Witt C, Labeit D, Labeit S, Greaser M, Granzier H.** 2001. Titin-actin interaction in mouse myocardium: passive tension modulation and its regulation by calcium/S100a1. *Biophysiology* **81**: 2297-2313.
- **Yamasaki L, Jacks T, Bronson R, Goillot E, Harlow E, Dyson NJ.** Tumor induction and tissue atrophy in mice lacking E2F-1. Cell 1996;85:537-48.

- **Yang A, Walker N, Bronson R, *et al.*** p73-deficient mice have neurological, pheromonal and inflammatory defects but lack spontaneous tumors. Nature 2000;404:99-103.
- **Yang A, Schweitzer R, Sun D, *et al.*** p63 is essential for regenerative proliferation in limb, craniofacial and epithelial development. Nature 1999;398:714-8.
- **Yang A, Kaghad M, Wang Y, *et al.*** p63, a p53 homolog at 3q27-29, encodes multiple products with transactivating, death-inducing, and dominant-negative activities. Mol Cell 1998;2:305-16.
- **Yokomizo A, Mai M, Tindall DJ, *et al.*** Overexpression of the wild type p73 gene in human bladder cancer. Oncogene 1999;18:1629-33.
- **Yu WH, Fraser PE.** 2001. S100β interaction with tau is promoted by zinc and inhibited by hyperphosphorylation in Alzheimer's disease. *J Neurosci* **21**: 2240-2246.
- **Yuan, H. Shioya, T. Ishiko, X. Sun, J. Gu, Y.Y. Huang, H. Lu, S. Kharbanda, R. Weichselbaum and D. Kufe,** p73 is regulated by tyrosine kinase c-Abl in the apoptotic response to DNA damage, *Nature* **399** (1999), pp. 814–817.
- **Zacchi, M. Gostissa, T. Uchida, C. Salvagno, F. Avolio, S. Volinia, Z. Ronai, G. Blandino, C. Schneider and G. Del Sal,** The prolyl isomerase Pin1 reveals a mechanism to control p53 functions after genotoxic insults, *Nature* **419** (2002), pp. 853–857.
- **Zaika AI, Kovalev S, Marchenko ND, Moll UM.** Overexpression of the wild type p73 gene in breast cancer tissues and cell lines. Cancer Res 1999;59:3257-63.
- **Zaika AI, Slade N, Erster SH, *et al.*** ΔNp73, a dominant-negative inhibitor of wild-type p53 and TAp73, is up-regulated in human tumors. J Exp Med 2002;196:765-80.
- **Zaika A, Irwin M, Sansome C, Moll UM.** Oncogenes induce and activate endogenous p73 protein. J Biol Chem 2001;276:11310-6.
- **Zeng X, Chen L, Jost CA, *et al.*** MDM2 suppresses p73 function without promoting p73 degradation. Mol Cell Biol 1999;19:3257-66.
- **Zeng X, Li X, Miller A *et al.*** The N-terminal domain of p73 interacts with the CH1 domain of p300/CREB binding protein and mediates transcriptional activation and apoptosis. *Mol Cell Biol* 2000; 20: 1299–310.
- **Zhang T, Woods TL & Elder JT.** Differential responses of S100A2 to oxidative stress and increased intracellular calcium in

normal, immortalized, and malignant human keratinocytes. *J Invest Dermatol* (2002) **119**: 1196–1201.

- **Zheng, H. You, X.Z. Zhou, S.A. Murray, T. Uchida, G. Wulf, L. Gu, X. Tang, K.P. Lu and Z.X. Xiao,** The prolyl isomerase Pin1 is a regulator of p53 in genotoxic response, *Nature* **419** (2002), pp. 849–853.
- **Zhu J, Jiang J, Zhou W, Chen X.** The potential tumor suppressor p73 differentially regulates cellular p53 target genes. Cancer Res 1998;58:5061-5.
- **Zimmer DB, Landar A.** 1995. Analysis of S100A1 expression during skeletal muscle and neuronal cell differentiation. *J Neurochem* **64**: 2727-2736.
- **Zimmer DB, Cornwall EH, Reynolds PD, Donald CM.** 1998. S100A1 regulates neurite organization, tubulin levels, and proliferation in PC12 cells. *J Biol Chem* **273**: 4705-4711.
- **Zhong S, Muller S, Freemont PS, Dejean A and Pandolfi PP**. (2000a). *Blood*, **95**, 2748–2753.
- **Zhong S, Salomoni P and Pandolfi PP**. (2000b). *Nat. Cell Biol.*, **2**, E85–E90.
- **Zhong S, Salomoni P, Ronchetti S, Guo A, Ruggero D and Pandolfi PP**. (2000c). *J Exp. Med.*, **191**, 631–640.
- **Zwadlo G, Brüggen J, Gerhards G, Schlegel R, Sorg C.** 1988. Two calcium-binding proteins associated with specific stages of myeloid cell differentiation are expressed by subsets of macrophages in inflammatory tissues. *Clin Exp Immunol* **72**: 510-515.
- **Zwahlen D, Tschan MP, Grob TJ, *et al.*** Differential expression of p73 splice variants and protein in benign and malignant ovarian tumors. Int J Cancer 2000;88:66-70.