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ORIGINAL ARTICLE

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

E Lapi[1,5], A Iovino[1,5], G Fontemaggi[1,2], AR Soliera[1], S Iacovelli[1], A Sacchi[1], G Rechavi[3], D Givol[4], G Blandino[1,2] and S Strano[1]

[1]*Department of Experimental Oncology, Regina Elena Cancer Institute, Rome, Italy;* [2]*Rome Oncogenomic Center, Rome, Italy;* [3]*Department of Pediatric Hematology-Oncology, Sheba Medical Center, Tel-Hashomer, Israel and* [4]*Department of Molecular Cell Biology, Weizmann Institute of Science, Rehovot, Israel*

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 *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 *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.

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Introduction

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). Unlike p53-deficient mice, which grow quite normally but undergo spontaneous tumor development, mainly sarcomas and lymphomas, p73 and p63 knockout mice exhibit severe developmental defects (Mills *et al*., 1999; Yang *et al*., 1999, 2000).

p73 and p63 share a remarkable homology in DNA sequence as well as in protein structure with p53 (Kaghad *et al*., 1997; Yang *et al*., 1998, 2002). Like p53, whose isoforms have been recently identified (Bourdon *et al*., 2005), p73 and p63 are subjected to alternative splicing, giving rise to a family of proteins, whose individual function has not been elucidated yet (De Laurenzi *et al*., 1998, 1999; Zaika *et al*., 1999; Pozniak *et al*., 2000). The ectopic expression of p73 and p63 in p53−/− and p53+/+ cells causes, similarly to p53, growth arrest, apoptosis and differentiation (Jost *et al*., 1997; Kaghad *et al*., 1997; Yang *et al*., 1998; 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 (Jost *et al*., 1997; Kaghad *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 *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 tumor suppressor genes by subtractive hybridization between normal mammary epithelial cells and breast tumor cells (Vellucci *et al*., 1995). S100 proteins are characterized by common structural motifs including two EF hands (helix–loop–helix calcium-binding domain) that are separated by a hinge region and flanked by amino- and carboxy-terminal domains. At least 10 of S100 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

Correspondence: Dr G Blandino, Department of Experimental Oncology, Regina Elena Cancer Institute, Molecular Oncogenesis Laboratory, Via delle Messi d' oro, 156, Rome RM 158, Italy.
E-mail: blandino@ifo.it
[5]These two authors contributed equally to this work.
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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. Recent report showed that some S100 proteins interact with the C-terminal domain of p53 and disrupt its tetramerization leading to interference in the cellular localization of p53 (Fernandez-Fernandez *et al*., 2005).

Here, we show that *S100A2* gene is a direct transcriptional target of ΔNp63α and p73β during keratinocyte differentiation. Unlike ΔNp63α, which is predominantly found on the regulatory regions of S100A2 in proliferating cells, p73β is recruited to S100A2 during keratinocyte differentiation of HaCaT cells. We also found that ΔNp63α and p73β exert opposite transcriptional effects on *S100A2* gene. Indeed, ΔNp63α functions as a transcriptional repressor, whereas p73β promotes the transcriptional activity of *S100A2* gene. Silencing of S100A2 impairs proper differentiation, indicating its active participation to the differentiation process. The latter is neither activated nor recruited by p73 during cisplatin-induced apoptosis of diverse cell lines. Altogether, our data contribute to identify *S100A2* gene as a direct target of p53 family members during keratinocyte differentiation.

Results

S100A2 expression is induced during keratinocyte differentiation

To search for target genes specifically activated or repressed by p73 and not by wt-p53, we have 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 S100A2 transcript and protein are induced during keratinocyte differentiation of HaCaT cells (Figure 1a and 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 (Figure 1c), is downregulated, and p73 is upregulated (De Laurenzi *et al*., 2000; Bamberger *et al*., 2002). As shown in Figure 1a–d, we found that the

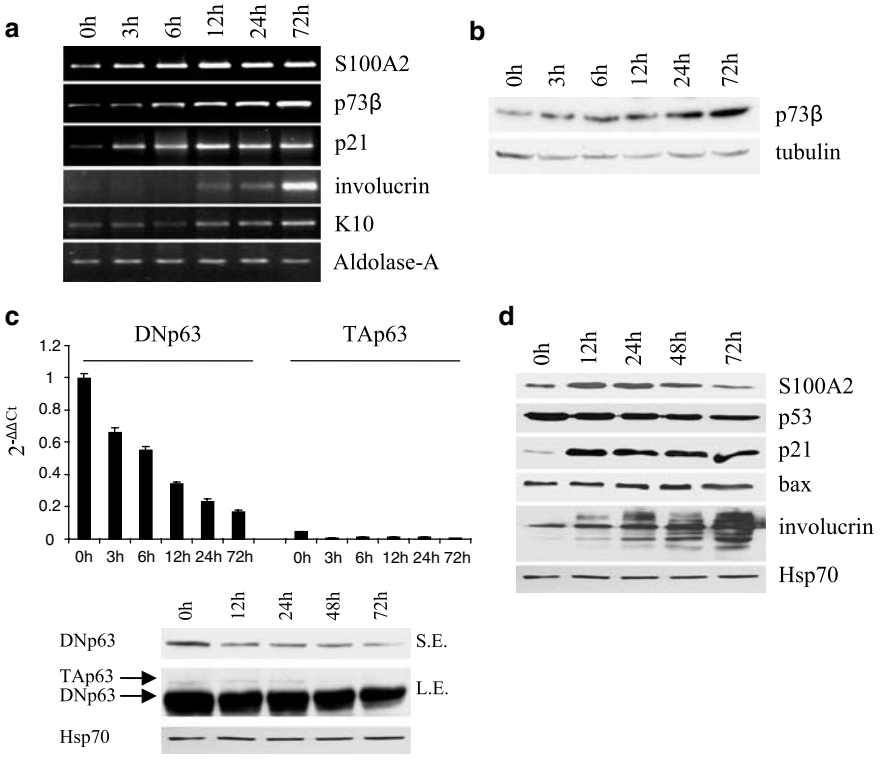


Figure 1 S100A2 expression is induced during keratinocyte differentiation. (**a**) RNA was extracted from HaCaT cells at the indicated time points after the induction of differentiation and subjected to RT–PCR analysis. Amplification of aldolase-A was used for normalization. (**b**) Detection of protein levels of p73 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 after the induction of differentiation and subjected to Western blot analysis. Equal loading of protein amount for each lane was determined by probing with anti-Hsp70 antibody.

induction of S100A2 during keratinocyte differentiation of HaCaT cells pairs with the downregulation of ΔNp63α and the upregulation of p73β. The latter is the only p73 isoform present in HaCaT cells, as determined by RT–polymerase chain reaction (PCR) using primers that discriminate between the different p73 isoforms (Figure 1a and data not shown). 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 (Figure 1a and d). The modulation of both S100A2 and p53 family members' expression was also found during differentiation of primary human epidermal keratinocytes (HEKs) (data not shown).

*S100A2 is a direct transcriptional target of p73*β *and ΔNp63*α

Next, we aimed to investigate whether S100A2 was a direct transcriptional target of p53 family members during keratinocyte differentiation. We have previously found that the second intron of human *S100A2* gene contains two inverted/repeated p53/p63/p73 consensus sites (Fontemaggi *et al*., 2002) (Figure 2a). It has also been reported that *S100A2* promoter encloses a p53/p63/p73 consensus site, which has been shown to be transcriptionally activated by wt-p53 (Tan *et al*., 1999 and data not shown) (Figure 2a). 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 p53/p63/p73 consensus of *S100A2* promoter was incubated with an *in vitro* translated p73β or ΔNp63α or TAp63α protein, respectively. As shown in Figure 2b 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 (Figure 2b).

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. To this end, the consensus site of the promoter and the two consensus enclosed in the second intron of *S100A2* gene were individually cloned into a TK-LUC reporter construct. As shown in Figure 2c, ectopic expression of p73β promotes the transcriptional activation of S100A2 regulatory regions, while ΔNp63α induces a strong transcriptional repression. Unlike ΔNp63α and p73β, p63α is unable to transcriptionally modulate *S100A2* gene, at least under our experimental conditions. Altogether, these results indicate that p73β and ΔNp63α exert opposite transcriptional effects onto S100A2 regulatory regions.

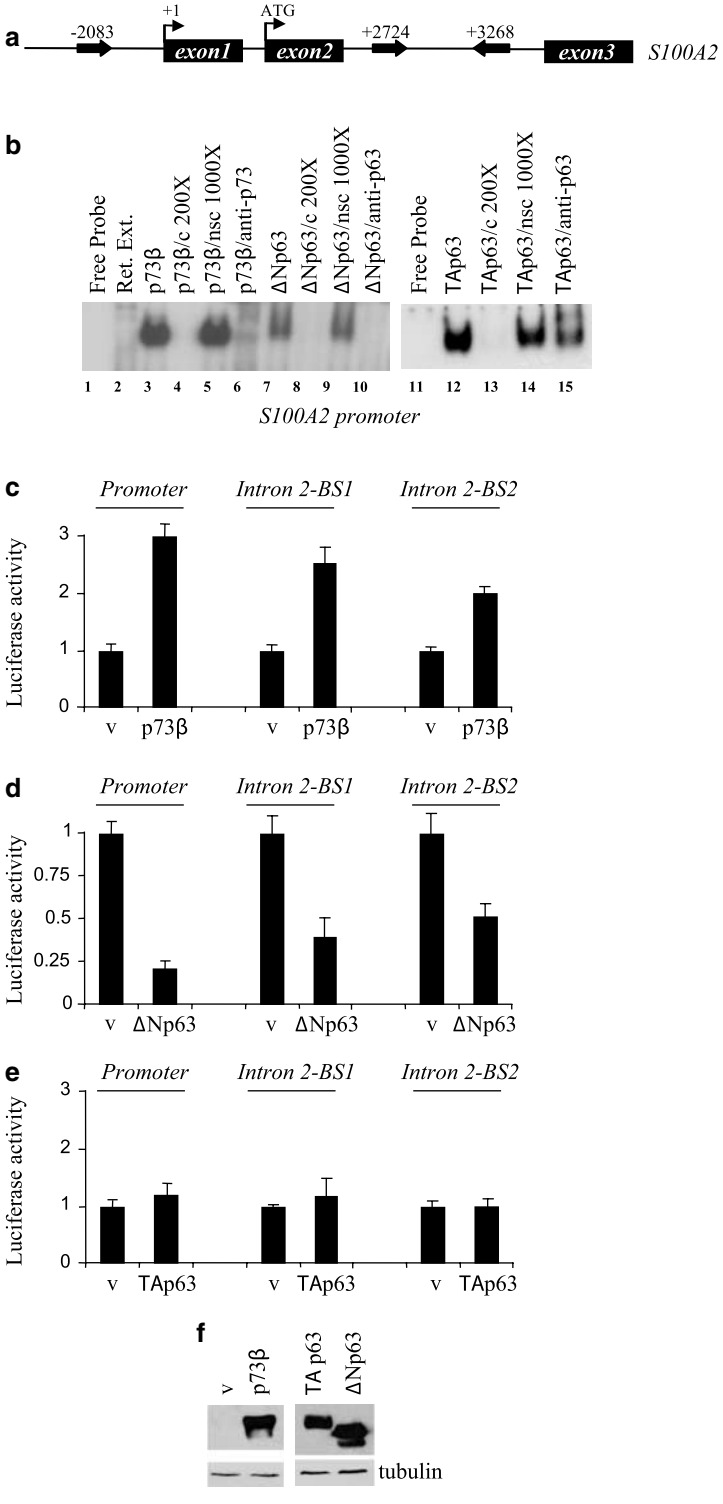
In vivo *recruitment of p73*β *and ΔNp63*α *onto the regulatory regions of S100A2 gene*

To verify whether the S100A2 upregulation during keratinocyte differentiation depends on the transcriptional activity of p53 family members, we analysed by chromatin immunoprecipitation (ChIP) the *in vivo* binding pattern of p73β and ΔNp63α onto S100A2 regulatory regions. To this end, crosslinked 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). We found that the amount of p73β bound to the regulatory regions of S100A2 is mainly increased at the level of the second intron in differentiated cells (Figure 3a). Conversely, p63 is recruited onto S100A2 promoter mainly in proliferating cells and its binding is reduced upon differentiation (Figure 3a). It is reasonable to believe that the p63 bound to S100A2 promoter is predominantly ΔNp63α, as we observed (Figure 1a) and it is 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 *S100A2* gene, suggesting that the promoter occupancy might be sufficient to exert its transcriptional repression (Figure 3a). No specific occupancy by p73β and ΔNp63 was found on the thymidine kinase (TK) promoter (Figure 3a), 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 the keratinocyte differentiation

Figure 2 ΔNp63α and p73β exert opposite transcriptional effects on S100A2 gene. (**a**) Schematic representation of the S100A2 gene. Arrows indicate p53/p63/p73 consensus 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 consensus of S100A2 promoter. p73β, ΔNp63 and TAp63 bind *in vitro* to the p53/p63/p73 consensus sites of S100A2 promoter (lanes 3, 7 and 12). The binding was competed with 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 decreased the amount of the binding (lanes 6, 10, and 15). (**c–e**) H1299 cells were transiently transfected with plasmids encoding p73β (**c**) or ΔNp63α (**d**) or TAp63α (**e**) (1 μg/60-mm dish) together with constructs carrying the luciferase reporter gene driven by S100A2 promoter or S100A2 second intron-binding site 1 (Intron 2-BS1) or S100A2 second intron-binding site 2 (Intron 2-BS2) (500 ng/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 36 h later. Luciferase activity was determined relatively to total proteins and β-gal activity. Results are presented as folds of induction over the control. Histograms show the mean of three experiments each performed in duplicate; bars indicate s.d. (**f**) Expression of p73β, TAp63 and ΔNp63 proteins in transactivation assays was assessed by Western blot analysis.

of HaCaT cells correlates with changes in the degree of histone H4 acetylation. To this end, crosslinked chromatin derived from HaCaT cells harvested at 0 and 12 h after induction of differentiation was immunoprecipitated with antiacetylated H4 histone antibody. We found an increase of H4 histone acetylation at 12 h onto the second intron of *S100A2* gene (Figure 3b). This correlates well with both S100A2 mRNA induction (Figure 1b) and the *in vivo* recruitment of p73 onto the S100A2 regulatory regions. No difference in the amount of H4 histone acetylation was found between proliferating and differentiating cells on S100A2 promoter (Figure 3b); this could be because of the significant basal expression level of S100A2 in proliferating cells (Figure 1a and b). Altogether, these results indicate that the p73 consensus sequences present on S100A2 second



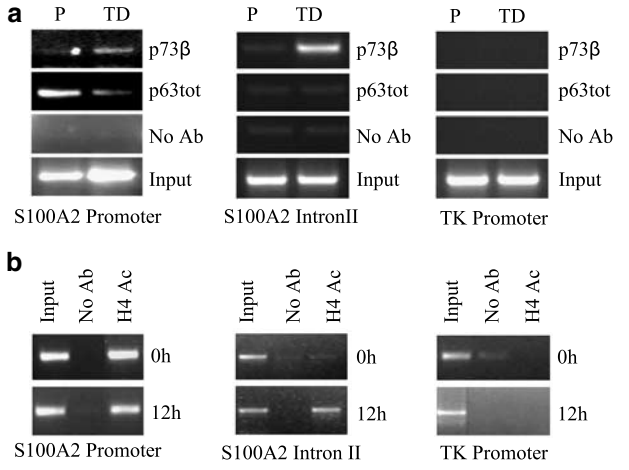


Figure 3 *In vivo* binding of p73*β* and p63 to the regulatory regions of S100A2 gene. (**a**) Crosslinked chromatin derived from proliferating (P) or terminally differentiated (TD) HaCaT cells was immunoprecipated with antibodies to p73 or p63 or in the absence of antibody and analysed by PCR with specific primers for the indicated regulatory regions. Input, non-immunoprecipitated crosslinked chromatin. (**b**) Crosslinked chromatin derived from proliferating HaCaT cells (0 h) or from HaCaT cells after 12 h of differentiation (12 h) was immunoprecipated with antiacetylated histone H4 antibody or in the absence of antibody and analysed by PCR with specific primers for the indicated regulatory regions. Input, non-immunoprecipitated crosslinked chromatin.

intron might act as enhancer elements upon induction of differentiation, allowing a full transcriptional activation of the gene.

Silencing of p73 expression impairs S100A2 upregulation during keratinocyte differentiation

To further define the contribution of p73 to the induction of S100A2 expression during keratinocyte differentiation, we knocked-down p73 expression through 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) (Figure 4b and c), and the expression of S100A2 protein was evaluated by immunofluorescence. In agreement with previous findings, S100A2 protein was predominantly localized in the nuclei even at 12 h of differentiation (Figure 4b). We found that S100A2 expression in the GFP-positive/p73siRNAs-transfected cells (right panels, arrow) was clearly diminished when compared with that of the surrounding GFP-negative/untransfected cells. The specificity of such effect was given by the observation that no modulation of S100A2 expression was seen in the cells transfected with the unrelated siRNAs (Figure 4b, left panels and Figure 4c). 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 Figure 4d, the induction of both p73 and S100A2 mRNAs in differentiating HaCaT cells transfected with p73pRS vector was reduced when compared with that of lacZpRS-transfected cells. This effect pairs a reduced induction of keratin 10 and involucrin, two well-known markers of keratinocyte differentiation (Figure 4d). 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 define whether S100A2 contributes to keratinocyte differentiation, HaCaT cells were stably transfected with either a retroviral vector S100A2pRS or with a lacZpRS. As shown in Figure 5b, the induction of S100A2 mRNA in differentiating HaCaT cells transfected with S100A2pRS vector was impaired when compared with that of lacZpRS-transfected cells. This leads to a reduced induction of keratin 10 and involucrin (Figure 5c and 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 evidences 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 within 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 different amounts of cisplatin at different time points. This treatment provokes apoptotic cell death as assessed by PARP fragmentation (Figure 6a) and subG1 fraction analyses (Figure 6b). The extent of the CDDP-induced apoptosis is dose dependent (Figure 6a and b). The protein analysis of p53 family members reveals that while p73 is accumulated, ΔNp63 expression is strongly reduced (Figure 6a). As expected for a mutant p53 protein, no modification of its expression was found in response to cisplatin (Figure 6a). 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 downregulated (Figure 6a–c). Similar findings were obtained upon treatment of HaCaT cells with doxorubicin (data not shown). This effect does not seem to be related to p53 family members activities. Upregulation of S100A2 was also not observed during p73-mediated apoptosis of HCT116 (3) cells in response to DNA damage (data not shown) (Costanzo *et al*., 2002; Mantovani *et al*., 2004; Strano *et al*., 2005).

By ChIP analysis, we found that p73 is recruited on the first intron of its proapoptotic target gene p53AIP1 only in response to cisplatin (Figure 6d). Conversely, the binding of p73 to S100A2 promoter was not modulated upon cisplatin treatment (Figure 6d).

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

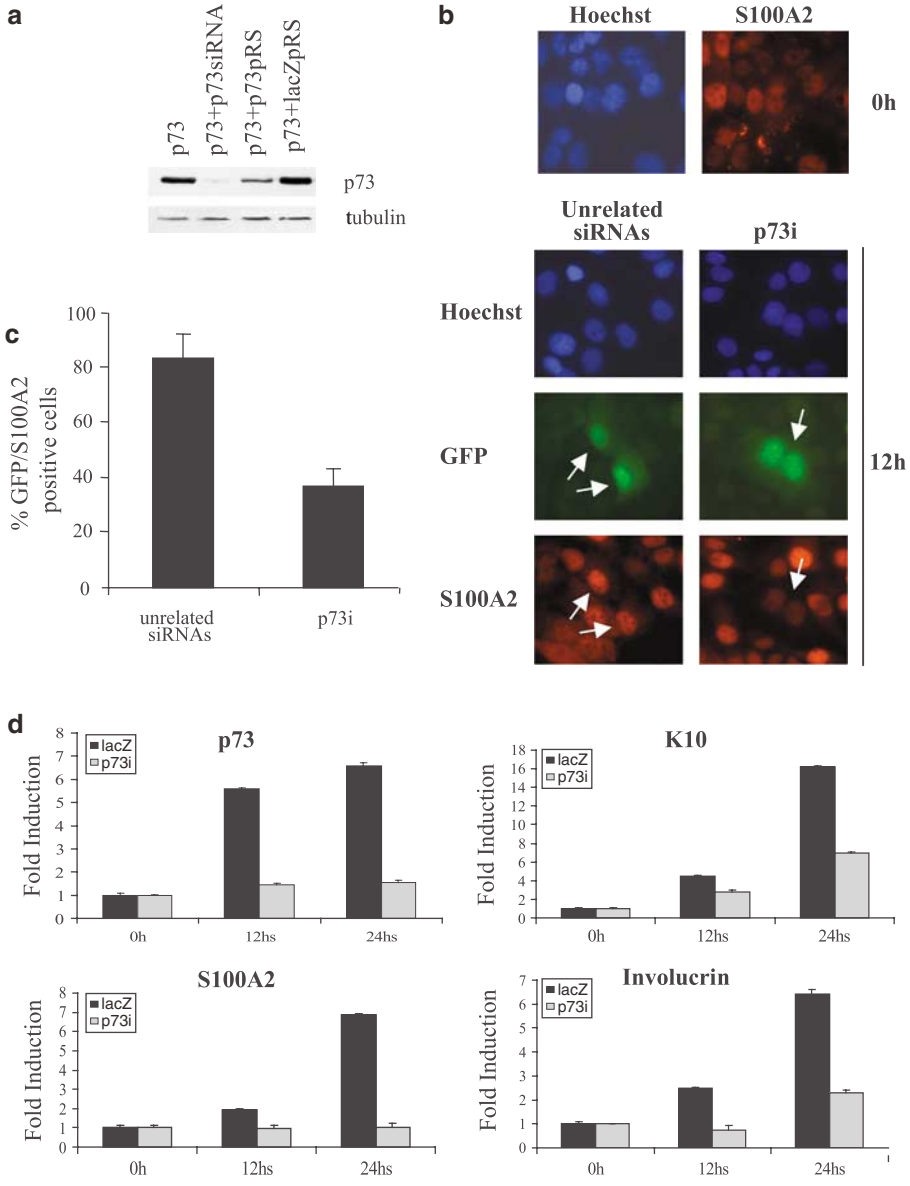


Figure 4 Silencing of p73 impairs S100A2 upregulation and keratinocyte differentiation. (**a**) The efficiency of inhibition of p73 expression by siRNAs to p73 or pRetroSuper-p73 or pRetroSuper-LacZ (negative control) was assessed by immunoblotting in H1299 cells. (**b**) Proliferating HaCaT cells were transfected with p73-specific siRNAs or unrelated siRNAs together with a GFP-expressing vector and induced to differentiate. At 12 h 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 represented as histogram. The mean of three experiments is shown. (**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 a RT–PCR analysis. Specific primers for the detection of p73, S100A2, involucrin and keratin 10 (K10) transcripts were used. Quantification by densitometry and normalization on aldolase-A expression were performed. Results represent the folds of induction over the 0 h time point. Histograms show the mean of three experiments; bars indicate s.d.

Discussion

Unlike p53-deficient mice that grow normally but undergo spontaneous tumor development, mainly sarcoma and lymphomas, p73 and p63 knockout mice exhibit severe developmental and differentiation defects (Mills *et al*., 1999; Yang *et al*., 1999, 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 tumor 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. Although 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 antitumoral effects (Blandino and Dobbelstein, 2004). As a consequence, it is reasonable to assume that the major role of p73 and p63, during

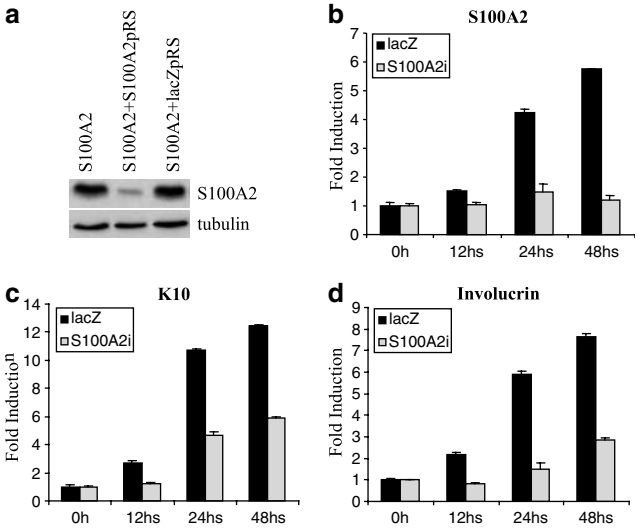


Figure 5 Silencing of S100A2 impairs keratinocyte differentiation. (**a**) The efficiency of inhibition of S100A2 expression by S100A2-pRetroSuper or LacZ-pRetroSuper (negative control) was assessed by immunoblotting in H1299 cells. (**b–d**) RNA was extracted from HaCaT cells, stably transfected with 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 S100A2, involucrin and keratin 10 (K10) transcripts were used. Quantification by densitometry and normalization on aldolase-A expression were performed. Results represent the folds of induction over the 0 h time point. Histograms show the mean of three experiments; bars indicate s.d.

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 functions in regulating *S100A2* gene at the transcriptional level. Indeed, we found that whereas p73β is a transcriptional activator of *S100A2* gene, ΔNp63α functions as a transcriptional repressor. Further support to the opposite transcriptional regulation of *S100A2* gene is provided by the differential *in vivo* recruitment during differentiation of p73β and ΔNp63α onto the regulatory regions on *S100A2* gene. ΔNp63α is found predominantly in proliferating cells, whereas p73β is recruited in differentiating cells. Altogether, our findings might contribute to define a regulatory network of p53 family members existing at the cross-road 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 switch proliferation into differentiation. The transcript of S100A2 was previously shown to be positively modulated by exogenous expression of ΔNp63α (Hibi *et al*., 2003). This opposite finding might reflect different activities of ΔNp63α related either to the different cell context or to the specific biological activity in which the latter is recruited.

Target genes of p53 family members could be hypothetically divided, at least, in 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 such case, the cell context, the type of stimulus, 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 indicate 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. *S100A2* gene was originally identified in a screen for tumor suppressor genes (Vellucci *et al*., 1995). It has been shown that the levels of S100A2 inversely correlate with tumor progression in melanoma, breast and prostate cancer (Vellucci *et al*., 1995; Maelandsmo *et al*., 1997; Gupta *et al*., 2003), thus indicating that it may play a role in suppressing tumor 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 requirements for completing the differentiation program. The proper execution of the latter can be considered a safeguard mechanism against cancer whose molecular details require additional evidence.

Materials and methods

Cell culture

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

Immunoblot analysis

Total cell lysates were prepared as previously described (Strano *et al*., 2000). 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 (Sigma) at a 1:1000 dilution.

Indirect immunofluorescence

Proliferating and differentiating HaCaT cells were fixed and processed as already described (Strano *et al*., 2001). Cells were

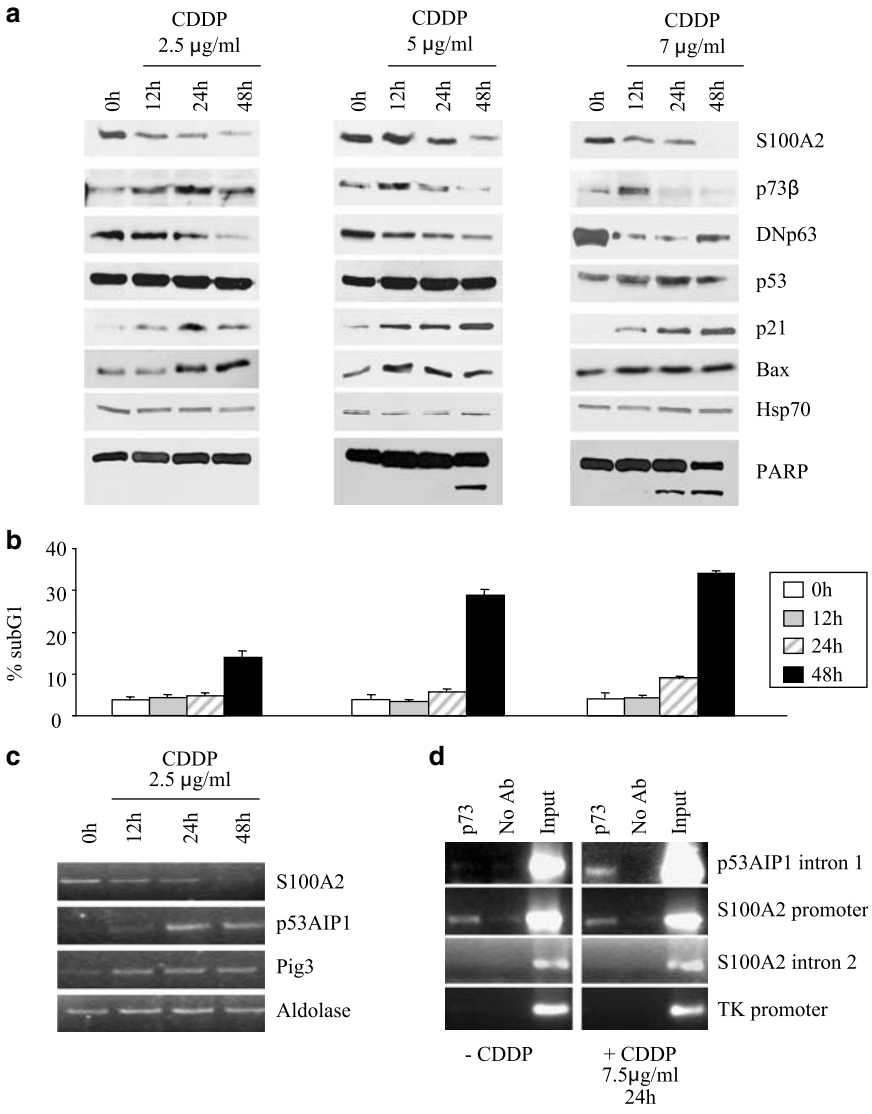


Figure 6 S100A2 gene is not induced in response to DNA damage. (**a**) Proteins were extracted from HaCaT cells at the indicated time points after the addition of cisplatin (CDDP) (2.5, 5 and 7.5 μg/ml) and subjected to Western blot analysis. Cell death was determined with PARP cleavage. Equal loading of protein amount for each lane was determined by probing with anti-Hsp70 antibody. (**b**) Percentages of subG1 fractions were quantified by cytofluorimetry in HaCaT cells treated as in (**a**). Histograms represent the mean of three experiments. (**c**) RNA was extracted from HaCaT cells at the indicated time points after treatment with CDDP 2.5 μg/ml and subjected to RT–PCR analysis. Amplification of aldolase-A was used to normalize each cDNA sample. (**d**) Crosslinked chromatin derived from HaCaT cells at 0 or 24 h after CDDP treatment was immunoprecipated with anti-p73 antibody or in the absence of antibody and analysed by PCR with specific primers for the indicated regulatory regions. Input, non-immunoprecipitated, crosslinked chromatin.

incubated with an anti-S100A2 monoclonal antibody (Transduction Laboratories) used at a 1:250 dilution for 2 h.

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 *Sal*I/*Bam*HI 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). Transient reporter assays were performed as described (Strano *et al*., 2000). Control plasmid (pCMVneo) was added to equally normalize the amount of transfected DNA for each transfection.

RNA extraction and reverse transcriptase reaction
RNAs were isolated and reverse transcribed as previously described (Fontemaggi *et al*., 2002). 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). 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, were used (Signoretti *et al*., 2000). 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 crosslinking and ChIP
ChIP experiments were performed as previously described (Fontemaggi *et al*., 2002). 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.). Enrichment in S100A2, p53AIP1 and TK 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). 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.

EMSA
The EMSA was performed as described in Fontemaggi *et al*. (2001). 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. 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. A double-stranded oligonucleotide corresponding to the p53 family consensus of S100A2 promoter was used as probe.

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 siRNAs (unrelated oligo) or with sip73 RNAs. At 24 h after the transfection, the cells were induced to differentiate as described above. SiRNAs were purchased from DHARMACON Inc. Sequences are available upon request. 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 1 μg/ml puromycin. After 10 days of culture in puromycin supplemented medium, confluent cells were induced to differentiate as described above.

Cell cycle analysis
Cells for cell cycle analysis were prepared as described in Strano *et al*. (2005) and then analysed by cytofluorimetry with an Epics-XL analyser (Coulter, Corporation). Data were analysed with the Multicycle software (Phoenix Flow System).

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