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Diverse p63 and p73 isoforms regulate Δ133p53 expression through modulation of the internal *TP53* promoter activity

V Marcel[1], I Petit[2], F Murray-Zmijewski[1], T Goullet de Rugy[1], K Fernandes[1], V Meuray[1], A Diot[1], DP Lane[1,3], D Aberdam[2] and J-C Bourdon*[,1]

In response to stress, p53 binds and transactivates the internal *TP53* promoter, thus regulating the expression of its own isoform, Δ133p53α. Here, we report that, in addition to p53, at least four p63/p73 isoforms regulate Δ133p53 expression at transcriptional level: p63β, ΔNp63α, ΔNp63β and ΔNp73γ. This regulation occurs through direct DNA-binding to the internal *TP53* promoter as demonstrated by chromatin immunoprecipitation and the use of DNA-binding mutant p63. The promoter regions involved in the p63/p73-mediated transactivation were identified using deleted, mutant and polymorphic luciferase reporter constructs. In addition, we observed that transient expression of p53 family members modulates endogenous Δ133p53α expression at both mRNA and protein levels. We also report concomitant variation of p63 and Δ133p53 expression during keratinocyte differentiation of HaCat cells and induced pluripotent stem cells derived from mutated p63 ectodermal dysplasia patients. Finally, proliferation assays indicated that Δ133p53α isoform regulates the anti-proliferative activities of p63β, ΔNp63α, ΔNp63β and ΔNp73γ. Overall, this study shows a strong interplay between p53, p63 and p73 isoforms to orchestrate cell fate outcome.
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The *TP53* family, composed of *TP53*, *TP63* and *TP73* genes, presents a strong homology of structures and expression patterns.[1,2] The three genes encode several protein isoforms carrying distinct N-termini (TA or Δ forms) and C-termini (α, β, γ, …), because of the use of alternative promoters, splicing sites and translational initiation sites.[3,4] The full-length proteins also share a similar protein structure with a N-terminal transactivation domain, a central DNA-binding domain (DBD) and a C-terminal oligomerisation domain.[4,5] A strong interplay has been described between p53 family members. They all bind specifically to DNA response elements (p53RE), modulate gene expression and thus cell-fate outcome.[6] Moreover, the p53-mediated apoptosis is severely impaired in the absence of p63 and p73 in response to DNA damage.[7]

The interplay between p53 family members is not limited to transcriptional modulation of common target genes; they also regulate each other's expression and activity.[6] p53, p73 and ΔNp73 transactivate the promoter of ΔNp73 isoform, which in turn, inhibits the p53- and p73-mediated transcriptional activity and apoptosis through direct competition for DNA-binding.[8–12] Similarly, p53, ΔNp63 and p73γ modulate the activity of the internal *TP63* promoter regulating ΔNp63 expression, which inhibits p53, p63 and p73 transcriptional activities.[2,6,13–15] Recently, it has been reported that p53 regulates the transcription of Δ133p53 isoform, which lacks the entire transactivation domain and part of the DBD.[16,17] p53 directly binds to p53REs located within the internal *TP53* promoter leading to an increase of Δ133p53 mRNAs. Those transcripts generate two different N-terminal p53 isoforms, Δ133p53 and Δ160p53, through the use of two alternative translational initiation sites (codons 133 or 160).[18] Δ133p53α inhibits replicative senescence, p53-mediated apoptosis and G1 arrest in response to stress, without inhibiting p53-mediated G2 cell cycle arrest.[16,19] Thus, Δ133p53α isoform is a strong modulator of p53-suppressive functions and presents similar characteristics to ΔNp63 and ΔNp73 towards their full-length form.

The interplay between p53 and p63/p73 isoforms has never been investigated. It is thus still unknown whether p63 or p73 isoforms affect the internal *TP53* promoter activity. Here, we identify four p53 family members (p63β, ΔNp63α, ΔNp63β and ΔNp73γ) able to strongly transactivate the internal *TP53* promoter. We also report that p63, p73 and Δ133p53α expressions are concomitantly regulated during skin differentiation and that Δ133p53α modulates the anti-proliferative activities of p63 and p73 isoforms, supporting the interplay between p53 family members.

Results

Transactivation of the internal *TP53* promoter by several p63 and p73 isoforms. To determine whether p53 family members can regulate the activity of the internal *TP53*

[1]Centre for Oncology and Molecular Medicine, INSERM-European Associated Laboratory, Ninewells Hospital and Medical School, Dundee, DD1 9SY, Scotland, UK; [2]INSERM U898, University of Nice-Sophia Antipolis, Nice, France and [3]p53 Laboratory (p53Lab), 8A Biomedical Grove, no. 06-06, Immunos, Singapore 138648, Singapore

*Correspondence: J-C Bourdon, Centre for Oncology and Molecular Medicine, INSERM-European Associated Laboratory, Ninewells Hospital and Medical School, Dundee, Scotland DD1 9SY, UK, Tel: +44 (0) 1382 496400; Fax: +44 (0) 1382 496363; E-mail: j.bourdon@dundee.ac.uk

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Abbreviations: DBD, DNA-binding domain; EEC, ectrodactyly, ectodermal dysplasia and cleft lip/palate; iPSC, inductible pluripotent stem cells; p53RE, p53 response elements; PEX4, polymorphism of exon 4; PIN3, polymorphism of intron 3

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promoter, we performed luciferase assays (Figure 1a). The pi3i4-Luc construct, which expresses the *Firefly* luciferase gene driven by the P2 promoter,[16] was co-transfected with vectors expressing different p53 family members. The study was conducted in two different cell lines: H1299 cells, which express *TP73* but not *TP53* and *TP63*; and MCF7 cells, which express *TP53*, *TP63* and *TP73* (Supplementary Figure 1).

We first investigated the effect of four p53 isoforms. Only p53α (i.e. p53) strongly transactivates the P2 promoter as previously described (Supplementary Figures 2a–d).[16,17] We then analysed the impact of six p63 isoforms (Figures 1b and c). Among TAp63 forms, p63β significantly increased the P2 promoter activity in both H1299 and MCF7 cells. A slight increase of promoter activity was observed in the presence of p63α in MCF7 but not in H1299 cells, and by p63γ in H1299 but not in MCF7 cells. Protein expression levels of p63α, p63β and p63γ were comparable in our experimental conditions (Figure 1d and Supplementary Figure 2e), suggesting that TAp63 forms have distinct intrinsic transcriptional activities towards the P2 promoter. This was verified by normalising their transcriptional activities with their protein expression levels (Supplementary Figure 3a). Contrary to TAp63 forms, the three ΔNp63 forms significantly increased the promoter activity in the two cell lines (Figures 1b and c), the transcriptional activities of ΔNp63α and ΔNp63β being stronger than that of ΔNp63γ. As the three ΔNp63 forms were expressed at equivalent protein levels (Figure 1d and Supplementary Figure 2e), this suggests that ΔNp63γ has a reduced transcriptional activity on the P2 promoter compared with ΔNp63α and ΔNp63β (Supplementary Figure 3a). Altogether, our data showed that p63β, ΔNp63α and ΔNp63β have the strongest transcriptional activity on the internal *TP53* promoter.

The study was then extended to seven p73 isoforms. In H1299 cells, all p73 isoforms co-transfected with the pi3i4-Luc construct significantly increased the promoter activity, with different intrinsic transcriptional activities (from $+1.9$- to $+7.3$-fold) (Figure 2a). In MCF7 cells, only the β forms (p73β and ΔNp73β) expressed at a detectable level by western blot did not change the P2 promoter activity (Figures 2b and c, Supplementary Figures 2f and g). In contrast, p73, p73γ, p73δ and ΔNp73α significantly induced the promoter activity (Figure 2b). However, the highest promoter activity

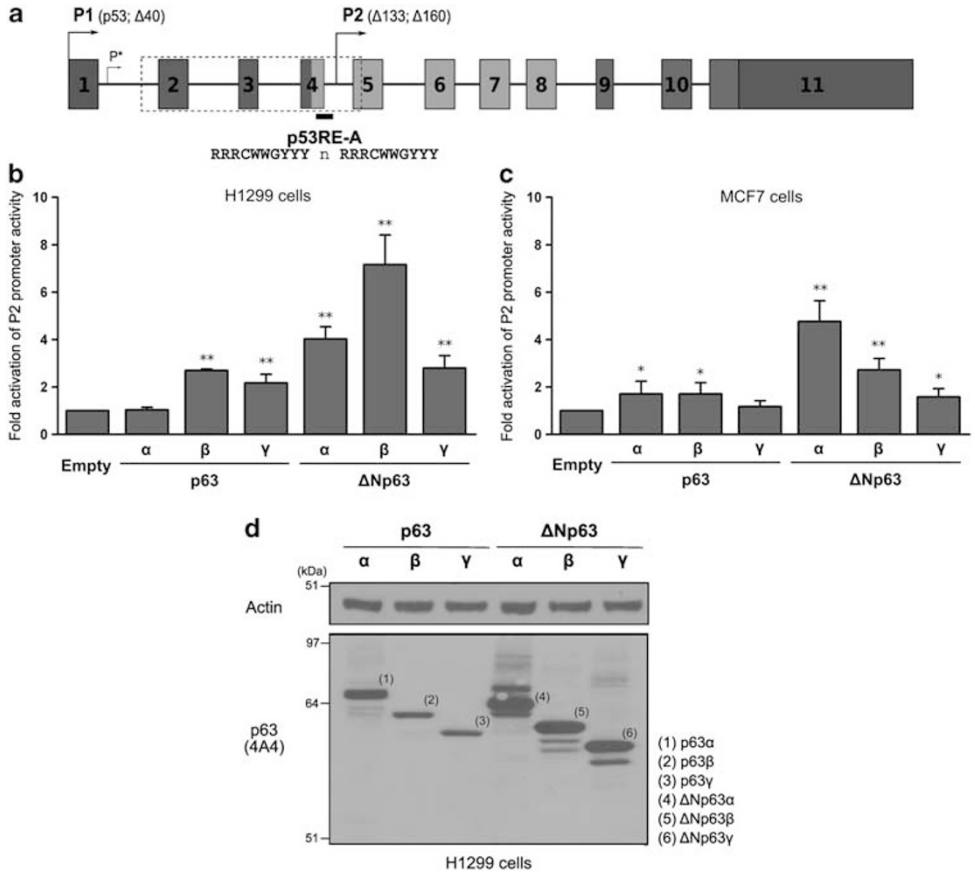

Figure 1 Regulation of the internal *TP53* promoter activity by p63 isoforms. (**a**) Schematic representation of the human *TP53* gene. In addition to the proximal promoter (P1) located upstream exon 1, regulating p53 and Δ40 forms, a second promoter (P2) has been described from the end of intron 1 to the beginning of exon 5 that regulate Δ133 and Δ160 form expression. The main p53 response elements (p53REs) have been described at the exon 4/intron 4 junction. Dotted box: internal *TP53* promoter introduced in pi3i4-Luc construct; P*: promoter in *TP53* intron 1 identified by Reisman *et al.*[40,41] that regulates the expression of an unrelated p53 transcript encoded by *TP53* intron 1. (**b** and **c**) Impact of p63 isoforms on the internal *TP53* promoter activity in H1299 (**b**) and in MCF7 cells (**c**). Luciferase assays were performed in the presence of p63 isoforms using pi3i4-Luc construct. Among p63 isoforms, p63β, ΔNp63α and ΔNp63β strongly increased luciferase activity. $^*P<0.05$; $^{**}P<0.005$. (**d**) Expression levels of ectopic p63 isoforms in H1299 cells analyzed by western blot. 4A4: antibody specific for all p63 isoforms; Actin: loading control

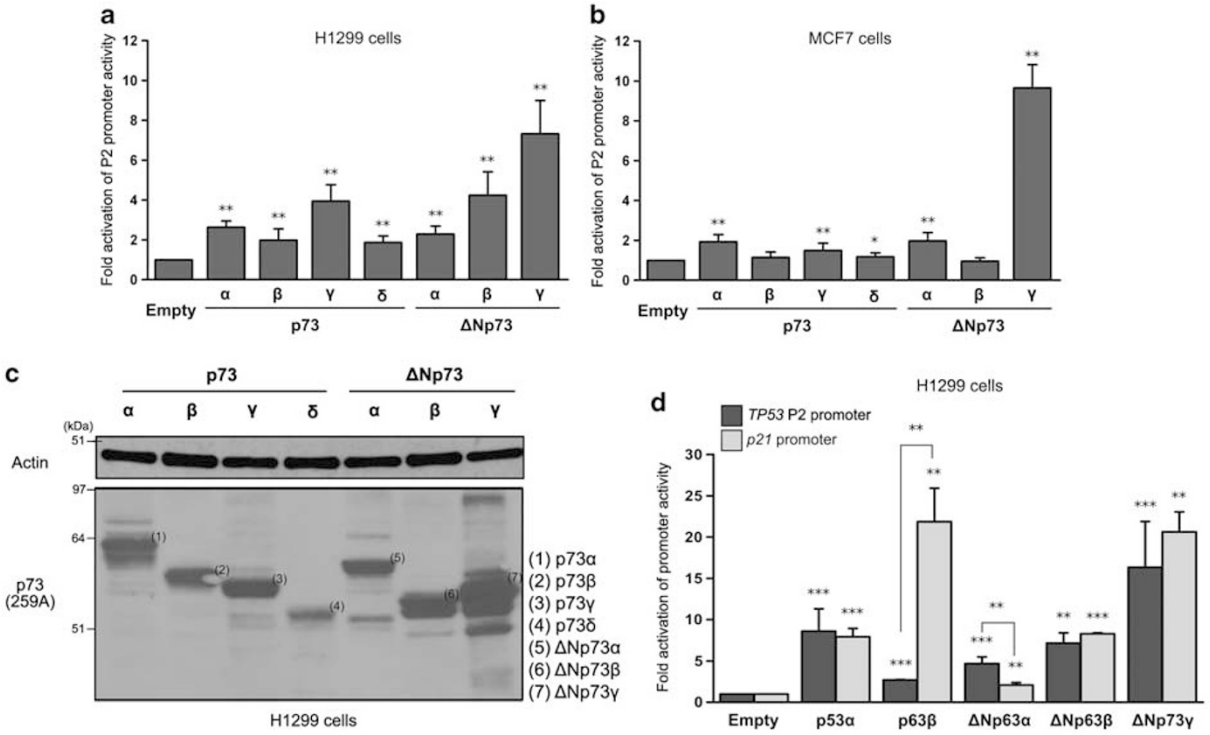

Figure 2 Regulation of the internal *TP53* promoter activity by p73 isoforms. (**a** and **b**) Impact of p73 isoforms on the internal *TP53* promoter activity in H1299 (**a**) and in MCF7 cells (**b**). Luciferase assays were performed in presence of p73 isoforms using pi3i4-Luc construct. Among p73 isoforms, only ΔNp73γ strongly induced the activity of the internal *TP53* promoter. *$P<0.05$; **$P<0.005$. (**c**) Expression levels of ectopic p73 isoforms in H1299 cells analyzed by western blot. 259A: antibody specific for all p73 isoforms; Actin: loading control. (**d**) Comparison of the transcriptional activities of p53 family members with the internal *TP53* and the *p21* promoters. The p53 family members showing the strongest transactivation activity on the internal *TP53* promoter were used. The same range of activation was observed for p53α, ΔNp63α, ΔNp63β and ΔNp73γ on the two different promoters in H1299 cells. **$P<0.005$; ***$P<0.0005$

was observed in the presence of ΔNp73γ, in both H1299 and MCF7 cells (Figures 2a and b). The expression level of ΔNp73γ protein was comparable to the other p73 isoforms (Figure 2c, Supplementary Figure 2f and g), indicating that, among p73 isoforms, ΔNp73γ isoform has one of the strongest intrinsic transcriptional activities on the P2 promoter (Supplementary Figure 3b). These results suggest that several p73 isoforms, including ΔNp73γ, can transactivate the internal *TP53* promoter.

We finally compared the transactivation levels of each p53 family member with the internal *TP53* and the *p21* promoters, a p53-/p63-/p73-target gene.[6,20] This analysis was focused on the strongest activators identified above (p53α, p63β, ΔNp63α, ΔNp63β and ΔNp73γ) (Figure 2d). The transactivation mediated by p53α, ΔNp63β and ΔNp73γ showed similar levels on the P2 and *p21* promoters. Interestingly, ΔNp63α had a significantly stronger transactivation activity on the P2 promoter than on the *p21* promoter. Only p63β showed a significantly stronger transactivation activity on the *p21* promoter than on the P21 promoter. Overall, several p53 family members transactivate both the internal *TP53* and *p21* promoters, suggesting that their transactivation can be regulated by p53 family members.

Identification of nucleotide sequences involved in the transactivation mediated by p53 family members. The regions of the internal *TP53* promoter involved in the transactivation mediated by p63 and p73 isoforms were investigated by luciferase assays using deleted versions of the pi3i4-Luc construct (Figure 3).[16] In MCF7 cells, the deleted constructs presented distinct basal luciferase activities (Figure 3b). The higher basal activities were observed for the deleted promoter constructs pi3i4-Luc(C) and (D). This indicates that the regions 1-723 and 953-1042 contain negative regulatory elements (silencers), whereas the region 723-953 encompassing p53RE-A contains a positive regulatory element (enhancer), as previously described (Figure 3a).[16,17] To identify the regions involved in the p63-/p73-mediated transactivation using the deleted pi3i4-Luc constructs, the promoter activity induced by p53 family members was normalised to its respective basal activity (Figures 3c–f). Compared with the empty vector, p63β, ΔNp63α, ΔNp63β or ΔNp73γ significantly increased the P2 promoter activity. For p63β, the increased luciferase activity of pi3i4-Luc(C) is significantly different from that of pi3i4-Luc(D) but not from that of pi3i4-Luc(G), indicating that the deleted region 723-1042 encompassing p53RE-A is important for the p63β-mediated transactivation in MCF7 cells (Figure 3c). Using similar experiments for ΔNp63α and ΔNp63β (Figures 3d and e), we observed that induction of pi3i4-Luc(G) was significantly stronger than that of pi3i4-Luc(D), indicating that the region 953-1042 lacking p53RE-A is required to induce the maximal transactivation mediated by ΔNp63α or ΔNp63β. Regarding ΔNp73γ (Figure 3f),

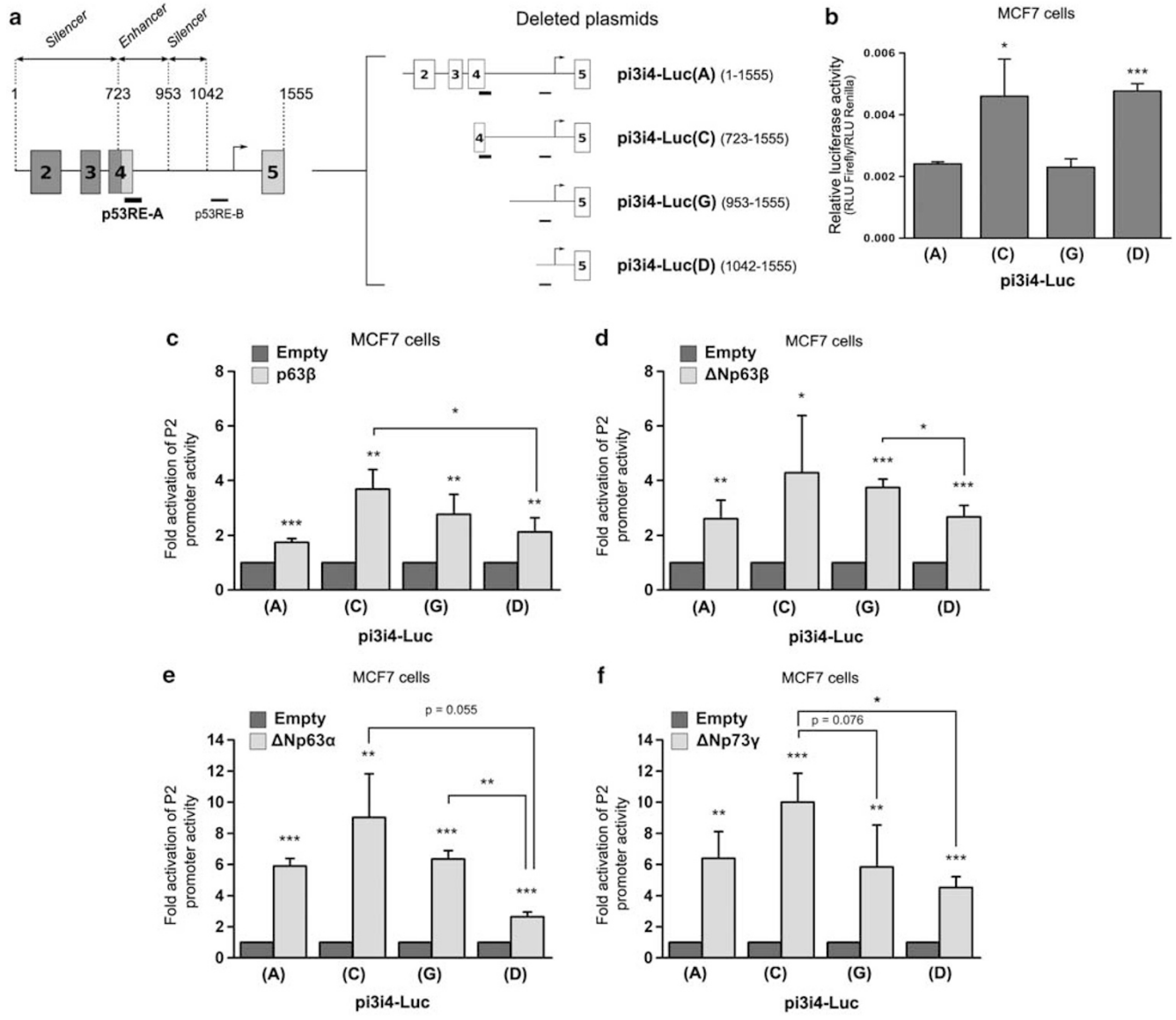

Figure 3 Identification of internal *TP53* promoter regions involved in the transactivation mediated by p53 family members. (**a**) Schematic representation of the sequential deletions introduced in the full-length pi3i4-Luc(A) construct to generate three deleted pi3i4-Luc vectors (C, G, and D). Only pi3i4-Luc(A) and pi3i4-Luc(C) retain the main p53REs. Arrow: initiation site of transcription; nucleotide number 1: corresponds to nucleotide +11523 – accession no. X54156, NCBI. (**b**) Basal luciferase activity of the deleted pi3i4-Luc constructs in MCF7 cells. As compared with the entire pi3i4-Luc(A) construct, the pi3i4-Luc(C) and pi3i4-Luc(D) showed a significant increase in basal luciferase activity, suggesting the presence of different regulatory elements on the internal *TP53* promoter (silencers and enhancers). (**c–f**) Regions of the internal *TP53* promoter involved in the transactivation mediated by p63β (**c**), ΔNp63β (**d**), ΔNp63α (**e**) and ΔNp73γ (**f**). The differences in basal activities have been normalized to evaluate the activation induced by p53 family members in MCF7 cells. Nucleotides 753–1042 are involved in the transactivation mediated by p63β, 953–1042 in the one mediated by ΔNp63α and ΔNp63β, 723–953 in the one mediated by ΔNp73γ. *$P<0.05$; **$P<0.005$; ***$P<0.0005$

the induced luciferase activity of pi3i4-Luc(C) was significantly different from that of pi3i4-Luc(G) and pi3i4-Luc(D), suggesting that the region 723-953 encompassing p53RE-A is important for the ΔNp73γ-mediated transactivation. These data indicated that each p63 and p73 isoforms required distinct regions to regulate the internal *TP53* promoter activity.

To determine the importance of p53RE-A located in exon 4 in the p63-/p73-mediated transactivation of the internal *TP53* promoter, we performed luciferase assays in H1299 and MCF7 cells using the mutant p53RE-A1/A2 pi3i4-Luc construct (Figures 4a–c).[16] As previously described, introduction of mutations in p53RE-A1 and p53RE-A2 significantly reduced the responsiveness to p53α, confirming that p53RE-A1 and p53RE-A2 are required for the p53α-mediated transactivation (Figures 4b and c).[16,17] The same result was observed for ΔNp73γ; the introduction of mutations in p53RE-A1/2 reduced the ΔNp73γ-mediated transactivation by 50% in H1299 cells and by 40% in MCF7 cells. This suggests that ΔNp73γ regulates the internal *TP53* promoter activity using the p53RE-A1/A2. However, no significant difference in luciferase activity was observed in the presence of p63

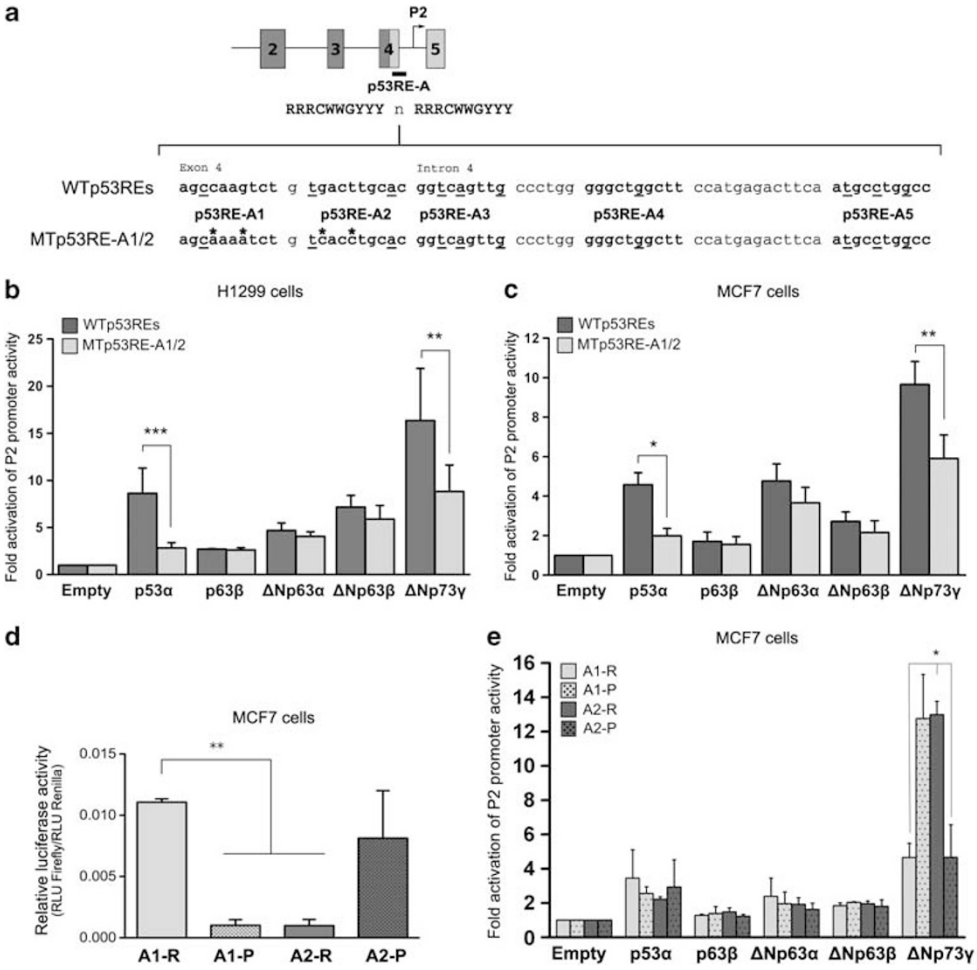

Figure 4 Role of *cis*-elements in the transactivation mediated by p53 family members. (**a**) Five p53 response elements (p53RE-A1 to -A5) have been identified at exon 4/intron 4 junction. In pi3i4-Luc construct, point mutations have been introduced in p53RE-A1 and -A2 to abolish their usage. WTp53REs: WT sequence of p53REs; MTp53RE-A1/2: mutant sequence of p53REs; bold: p53REs; underlined: mismatch between consensus p53RE and p53REs of the internal *TP53* promoter; star: point mutations introduced by site-directed mutagenesis. (**b** and **c**) Impact of mutations within p53REs on the transactivation mediated by p53 family members on the internal *TP53* promoter activity in H1299 (**b**) and in MCF7 cells (**c**). Luciferase assays were performed in the presence of p53 family members using pi3i4-Luc constructs carrying WTp53REs or MTp53RE-A1/2. Only p53α and ΔNp73γ were affected by mutations introduced in p53RE-A1 and p53RE-A2. *$P<0.05$; **$P<0.005$; ***$P<0.0005$. (**d**) Impact of *TP53* polymorphisms on the basal promoter activity. Luciferase assays were performed in MCF7 cells using pi3i4-Luc construct carrying four different haplotypes mimicking haplotypes derived from *TP53* PIN3 (rs17878362 in intron 3: A1: non-duplicated; A2: 16-bp duplication) and from *TP53* PEX4 (rs1042522, G>C in exon 4: R: arginine at codon 72; P: proline at codon 72). A1-R and A2-P presented the strongest intrinsic luciferase activity compared with A2-R and A1-P. **$P<0.005$. (**e**) Impact of *TP53* polymorphisms on the transactivation of the internal *TP53* promoter mediated by p53 family members. Luciferase assays were performed in MCF7 cells using the four polymorphic pi3i4-Luc constructs in the presence of p53 family isoforms. Differences in basal activities have been normalized to evaluate the activation induced by p53 family isoforms. *TP53* PIN3 and PEX4 significantly affect ΔNp73γ-mediated transactivation. *$P<0.05$

isoforms between wild-type (WT) and mutant p53RE-A1/A2 plasmids, indicating that p53RE-A1 and p53RE-A2 have no or only limited roles in the transactivation mediated by p63β, ΔNp63α and ΔNp63β.

We finally investigated the impact of two *TP53* polymorphisms on the transactivation mediated by p53 family members: PIN3 (rs17878362), a 16-bp duplication in intron 3 (non-duplicated A1 allele *versus* duplicated A2 allele) and PEX4 (rs1042522, G>C), a substitution of an arginine (R) for a proline (P) at codon 72 (R72 *versus* 72P).[21,22] We confirmed that Δ133p53 expression is under genetic control as reported by Bellini et *al.*,[23] because constructs carrying A2-R and A1-P haplotypes had a stronger and significantly higher intrinsic luciferase activity than that of A1-R and A2-P haplotypes (Figure 4d). Although no difference was observed for p53α, p63β, ΔNp63α and ΔNp63β, these two polymorphisms modulate the ΔNp73γ-mediated transactivation (Figure 4e). Altogether, these observations indicate that distinct regions are involved in the p63- and p73-mediated transactivation of the internal *TP53* promoter. In particular, the regions encompassing p53RE-A, PIN3 and PEX4 affect only the ΔNp73γ-mediated transactivation, and thus may affect the expression levels of Δ133p53 isoforms.

Regulation of the internal *TP53* promoter through direct binding of p63 and p73 isoforms. *TP63* mutations have been associated with EEC syndrome.[24] Like *TP53*, *TP63* mutations mainly occur in the DBD impairing p63

DNA-binding and transactivation activities.[25,26] We took advantage of these physiological mutants to investigate whether the p63-mediated transactivation of the internal *TP53* promoter involved the p63 DBD. In addition to the WT ΔNp63α isoform, two distinct ΔNp63α mutants were co-transfected with the pi3i4-Luc(A) construct in H1299 cells (Figure 5a): a conformational (C306R) and a DNA-contact mutant (R279H). As observed above, WT ΔNp63α is a strong inducer of the P2 promoter. However, presence of a single mutation in the p63 DBD abolished (R279H) or significantly impaired (C306R) the ΔNp63α-mediated transactivation of the P2 promoter. This indicates that the DBD of ΔNp63α is required to transactivate the internal *TP53* promoter.

We then performed chromatin immunoprecipitation (ChIP) assay to assess whether p63 and p73 isoforms regulate the transactivation of the internal *TP53* promoter through direct DNA-binding (Figures 5b and c, Supplementary Figures 4a and b). ChIPs were performed in HaCat cells, which physiologically express high levels of *TP63* and *TP73*, using antibodies specific for all isoforms and sets of primers/probes specific for the internal *TP53* promoter (exon 4/intron 4 junction), of *TP53* intron 8 (negative control) and of the *p21* promoter (positive control).[16] The P2 promoter was specifically immunoprecipitated with 4A4 and IMG-259 antibodies in HaCat, suggesting that p63 and p73 isoforms bind to the P2 promoter (Figures 5b and c, Supplementary Figure 4a). In addition, p73 binding to the P2 promoter was also observed in MCF7 cells (Supplementary Figure 4b). Altogether, the transactivation mediated by p63 and p73 occurs through direct binding to the internal *TP53* promoter.

Increased expression of endogenous Δ133p53α by ectopic expression of p63 isoforms. The above results suggest that p63 and p73 isoforms transactivate the internal *TP53* promoter through direct binding. To determine whether p63 and p73 isoforms modulate endogenous expression of Δ133p53, we transiently transfected p63 and p73 expression vectors in mutant p53 MDA-MB-231 cells to avoid the regulation of endogenous Δ133p53 expression by WT p53α (Figures 6a and b). Ectopic expression of p63β, ΔNp63α and ΔNp63γ significantly induced endogenous Δ133p53 mRNA expression, which is associated with an increase of Δ133p53α protein level. In the presence of ectopic p63 isoforms, the fold induction of Δ133p53 mRNA levels varied from +1.5 to +1.7, this range of induction being consistent with the +1.5-fold induction previously reported in the presence of ectopic p53α expression.[17] Contrary to p63 isoforms, ectopic expression of ΔNp73γ did not affect endogenous Δ133p53 mRNA and protein expression levels in mutant p53 MDA-MB-231 cells. Overall, we showed that p63 isoforms can modulate endogenous Δ133p53 expression at both mRNA and protein levels.

Role of Δ133p53α on anti-proliferative activities of p63 and p73 isoforms. To determine whether regulation of Δ133p53α expression by p63/p73 isoforms may affect their biological activities independently of p53α, we performed cell proliferation assay in p53-null H1299 cells co-transfected with Δ133p53α and some p63/p73 isoforms (p63β, ΔNp63α, ΔNp63β and ΔNp73γ) (Figure 6c). Cell proliferation was assessed after 15 days of neomycin selection by determining the amount of the protein-binding dye sulforhodamine B, which is directly correlated to the number of cells. In addition, ectopic expression of p63, p73 and Δ133p53α was determined by western blot (Supplementary Figures 5a and b). Ectopic expression of Δ133p53α significantly reduced by 30% the cell number compared with the empty expression vector, suggesting that Δ133p53α can inhibit cell proliferation in the absence of p53α as previously described (Figure 6c).[17] Likewise, ectopic expression of ΔNp63α, alone or in combination with Δ133p53α, decreased by 30% the cell number, indicating that ΔNp63α inhibits cell proliferation independently of Δ133p53α.

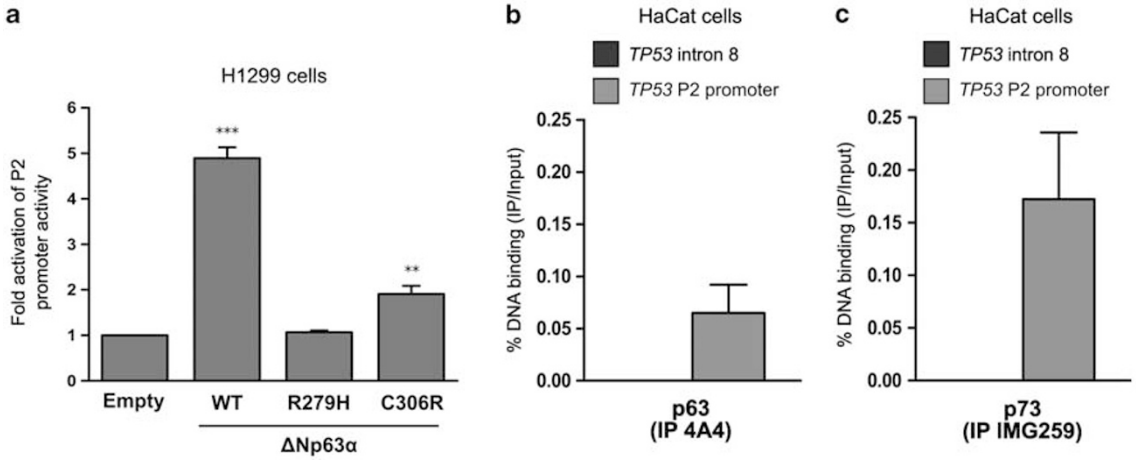

Figure 5 Direct DNA-binding of p63 and p73 isoforms to the internal *TP53* promoter. (**a**) Impact of WT and mutant ΔNp63α isoforms on the internal *TP53* promoter. Luciferase assays were performed in H1299 cells using the pi3i4-Luc construct in the presence of WT or mutant ΔNp63α (contact mutation R279H and conformational mutation C306R). Presence of a single mutation in the DNA-binding domain of ΔNp63α alters the ΔNp63α-mediated transactivation on the internal *TP53* promoter. $^{**}P<0.005$; $^{***}P<0.0005$. (**b** and **c**) Direct binding of p63 and p73 isoforms to the internal *TP53* promoter. CHiPs were performed in HaCat cells using the 4A4 (p63 isoforms, B) or IMG-259 (p73 isoforms, C) antibodies. Two set of primers/probe were used: one hybridizing the exon 4/intron 4 junctions specific for the internal *TP53* promoter and one hybridizing the *TP53* intron 8 used as negative control. A representative experiment was illustrated

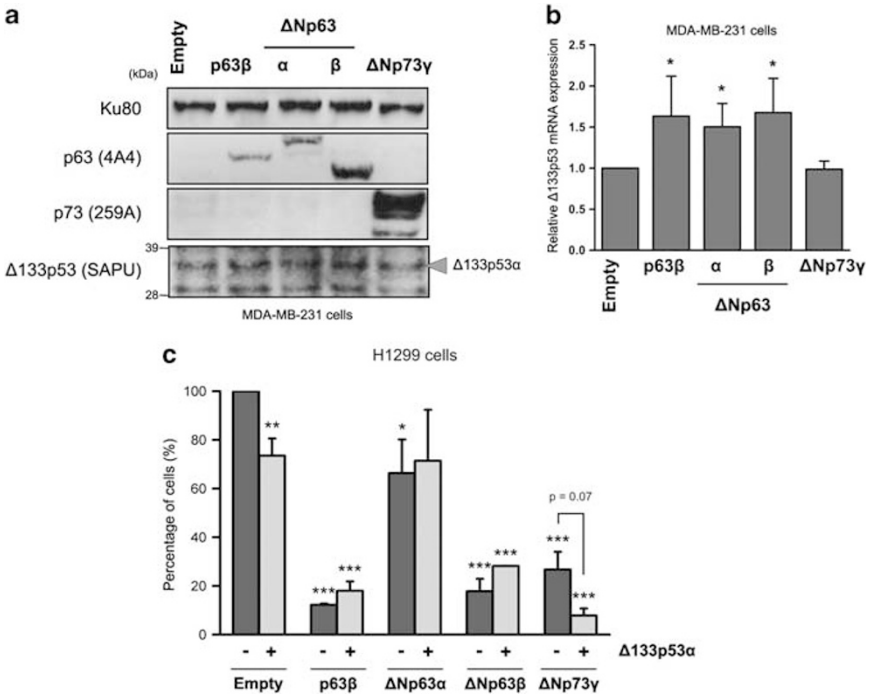

Figure 6 Modulation of Δ133p53α isoform expression and biological functions in response to ectopic expression of p63/p73 isoforms. (**a** and **b**) Variation of endogenous Δ133p53 expression in response to ectopic expression of p63/p73 isoforms. In mutant p53 MDA-MB-231 cells, introduction of p63/p73 isoforms resulted in a weak expression of Δ133p53 protein level (**a**) and in a significant increase of Δ133p53 mRNA level (**b**). Ku80: loading control; $^{*}P<0.05$. (**c**) Proliferation assays in the presence of p53 family members. Cell numbers have been determined on three independent experiments by measuring sulforhodamine B staining using spectroscopy. Statistical analyses showed that p63β, ΔNp63β and ΔNp73γ had growth suppressive activities, which can be modulated by introduction of Δ133p53α. $^{*}P<0.05$; $^{**}P<0.005$ and $^{***}P<0.0005$

Compared with the empty expression vector, p63β significantly reduced the cell number (−85%) (Figure 6c). Co-transfection of Δ133p53α with p63β slightly increased the cell number, indicating that Δ133p53α may inhibit the anti-proliferative activity of p63β. Interestingly, ectopic ΔNp63β expression decreased cell numbers by 80%, suggesting that ΔNp63β has also anti-proliferative activity. Compared with ectopic expression of ΔNp63β alone, co-transfection of Δ133p53α and ΔNp63β increased the cell percentage, indicating that Δ133p53α may inhibit ΔNp63β-mediated growth suppression. Like p63 isoforms, ΔNp73γ presented anti-proliferative activity and addition of Δ133p53α reduces further the cell number, suggesting that Δ133p53α increases ΔNp73γ-mediated growth-suppressive capacity. Overall, our data indicate that Δ133p53α, ΔNp63α, ΔNp63β or ΔNp73γ on their own inhibit cell proliferation independently of p53α. Interestingly, Δ133p53α inhibits the anti-proliferative activities of p63β and ΔNp63β, whereas it enhances the anti-proliferative activity of ΔNp73γ. Therefore, Δ133p53α does not systematically inhibit the activities of p63 and p73 isoforms. Rather, it suggests that cell proliferation is regulated by a subtle interplay between p53 family members.

Dynamic expression of Δ133p53α, p63 and p73 isoforms during keratinocyte differentiation. Endogenous variation of p63 and p73 isoform expression has been observed during keratinocyte differentiation.[26,27] We thus investigated whether physiological modulation of p63 and p73 expression may have an impact on Δ133p53α expression during keratinocyte differentiation of HaCat cells.[28] First, HaCat cells were maintained at low calcium concentration for 10 days to allow their de-differentiation, as indicated by the lost of keratin-10 expression, a marker of squamous differentiation (Figure 7a).[28] HaCat de-differentiation was also associated with an increased expression of p63 and, interestingly, with a concomitant decrease of p73 and Δ133p53α expression at mRNA and protein levels (Figures 7a and b). After 10 days of calcium deprivation, calcium was added to induce HaCat cell differentiation, as assessed by keratin-10 expression. Differentiation was associated with a repression of p63 expression concomitantly with an induction of both p73 and Δ133p53α expression at mRNA and protein levels. Therefore, Δ133p53α, p63 and p73 isoforms are differentially regulated during keratinocyte differentiation.

ΔNp63α has also been shown to induce epidermal commitment of pluripotent stem cells, and a single mutation found in patients affected by ectodermal dysplasia syndromes could prevent it.[29,30] Therefore, to gain further insight into the regulation of Δ133p53 expression during keratinocyte differentiation, we used induced pluripotent stem cell (iPSC) lines derived from EEC patients and control individuals. Skin fibroblasts from two patients carrying R304W or R204W single mutation in the p63 DNA-binding domain were reprogrammed into iPSCs (Petit *et al.*, manuscript in preparation). Control and EEC iPSCs were induced to epidermal fate for 15 days and expression of p63, Δ133p53 and K14, a marker of keratinocytes, was monitored by real time PCR (Figure 7c). During epidermal differentiation, control

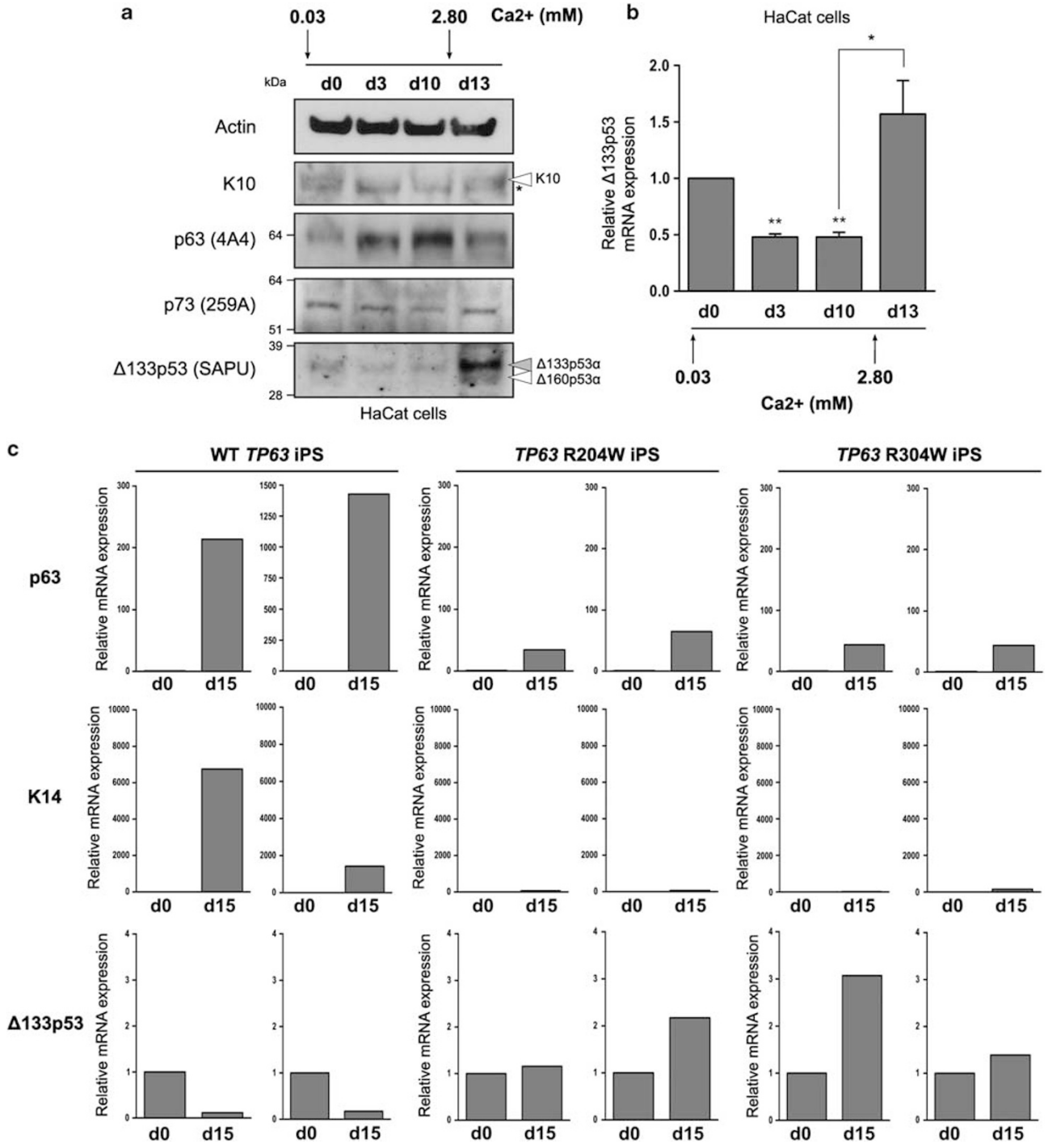

Figure 7 Concomitant variation of p63, p73 and Δ133p53α expression during keratinocyte differentiation. (**a** and **b**) Differentiation of HaCat cells by increased concentration of calcium and analysis of the expression of p53 family members at both protein (**a**) and mRNA (**b**) levels. Forced differentiation of mutant p53 HaCat cells by addition of calcium decreased ΔNp63α and increased p73β expression. In parallel, an increased expression of Δ133p53α was observed at both mRNA and protein levels. d: days; *nonspecific band; Actin: loading control; $*P<0.05$; $**P<0.005$. (**c**) Epidermal differentiation of six iPSC lines expressing a WT or a mutant *TP63* gene. Because of variability of epidermal fate efficiency between experiments, we illustrated here two representative experiments for control and mutated iPS individuals. In WT cells, differentiation was associated with an increased expression of p63 and a decreased expression of Δ133p53 at mRNA levels, whereas in mutant cells, only the increase of p63 was observed. WT: cells expressing WT *TP63* gene; MT, cells expressing mutant *TP63* gene (R304W or R204W)

and EEC iPSCs expressed p63, although at a different level. However, EEC iPSCs failed to differentiate into keratinocytes, as illustrated by a marked reduction in K14 expression as compared with control cells. Interestingly, the strong increase of p63 mRNA level during epidermal commitment of control iPSCs was concomitant to a significant decreased expression

level of Δ133p53 mRNA. In contrast, although p63 mRNA level increased in mutant *TP63* EEC cells, however to a lesser extend than in WT cells, no significant change in Δ133p53 mRNA expression was observed during differentiation. Overall, it suggests that, like HaCat cells, differentiation is associated with an inverse evolution of p63 and Δ133p53 mRNA expression that is lost in mutant *TP63* cells.

Discussion

The *TP53* family members regulate each other's functions and expressions through modulation of each other's internal promoters.[4–6,16,17] Using luciferase assays in two different cell lines, H1299 and MCF7 cells, we identified five p53 family members that strongly transactivate the internal *TP53* P2 promoter: p53α, p63β, ΔNp63α, ΔNp63β and ΔNp73γ. The transactivation activities were similar or stronger on the P2 promoter than on the *p21* promoter for most of the p53 family members (Table 1). In addition, we determined that the P2 promoter is differentially responsive to each p53 family members. It has been reported that ΔNp63β retains the capacity to transactivate p53-target genes, whereas ΔNp63α does not.[2,31,32] In our study, both ΔNp63α and ΔNp63β transactivate the P2 promoter, indicating that ΔNp63α and ΔNp63β act in a promoter-dependent manner. Compared with ΔNp63α and ΔNp63β, ΔNp63γ induced a weak transactivation level on the P2 promoter, as already observed towards the *p21* and others promoter.[2,31] Thus, p63 C-terminal domains produced by alternative splicing may confer distinct and specific transcriptional activity. In addition, we determined that the responsiveness of the P2 promoter to each p53 family members is cell-type-dependent. In our conditions, p53α, p63β, ΔNp63α, ΔNp63β and ΔNp73γ induced a higher transactivation level in p53-/p63-null H1299 cells than in MCF7 cells, which express all the three *TP53* family genes. This suggests that the cellular context, including the expression of p53 family members, modulates the activities of each p53 family members on the internal *TP53* promoter.

ChIP and luciferase assays showed that the transactivation mediated by p53 family members occurs through their direct binding to specific and distinct DNA regions of the internal *TP53* promoter (Table 1). Like p53α, the p53RE-A located at exon 4/intron 4 junction is important but not sufficient for the ΔNp73γ-mediated transactivation. Indeed, deletion of the promoter region 753-953 encompassing the p53RE-A1/A2 and introduction of point mutations within p53RE-A1/A2 significantly reduced, without abolishing, the transactivation level induced by ΔNp73γ. Other active p53REs have been identified within the internal *TP53* promoter that may be used by ΔNp73γ (p53RE-A3/A4/A5 and p53RE-B).[17] Interestingly, the ΔNp73γ-mediated transactivation is altered by the polymorphisms *TP53* PIN3 and PEX4 (codon 72/R72P), which affect the basal activity of the internal *TP53* promoter as already described.[23] This suggests a cooperation between ΔNp73γ and another transcription factor that is able to bind to the response elements encompassing those two SNPs, such as ZNF143, known to interact with p73 protein,[33] and for which several predicted binding sites are overlapping with PIN3.[34]

For p63β, we observed that p53RE-A1/A2 is not essential, whereas the region 753-1042 is required to obtain the maximal p63β-mediated transactivation. The region encompassing p53RE-A1/A2 contains additional active p53REs (p53RE-A3/A4/A5), suggesting that to modulate the internal *TP53* promoter activity, p63β may compete with p53 and/or

Table 1 Impact of p63 and p73 isoform expression on the internal *TP53* promoter activity and Δ133p53 expression.

	Transactivation activity[a]		DNA-binding activity[b]				Growth suppression[f]	
Isoforms	***p21* promoter**	**Internal *TP53* promoter**	**Internal *TP53* promoter**	**Region of the internal *TP53* promoter involved[c]**	**Impact of ectopic expression on endogenous Δ133p53α expression[d]**	**Correlation expression with Δ133p53α during keratinocyte differentiation[e]**	**Alone**	**+ Δ133p53**
p63								
α		+	Yes[C,M]			Inverse		
β	++	++		5′ intron 4 (p53RE-A3/A4?)	↑		+++	+++
γ		+						
Δ-α	+++	++		3′ intron 4	↑		+	+
Δ-β	+++	+++		3′ intron 4	↑		+++	++
Δ-γ		++						
p73								
α		++	Yes[C]			Similar		
β		+						
γ		++						
δ		+						
Δ-α		++						
Δ-β		++						
Δ-γ	+++	+++		p53RE-A1/A2	=		++	+++

[a]Grey: not determined; +: 1.2–2.0 fold induction; ++: 2.0–4.5 fold induction; +++: >4.5 fold induction [b]C: ChIP assays; M: use of DNA-binding mutants [c]Grey: not determined; p53REs: p53 response elements (see Figures 1A and 3A for location of p53REs) [d]Grey: not determined; ↑: increased Δ133p53α expression at both mRNA and protein levels; =: no variation of Δ133p53α expression at both mRNA and protein levels [e]Inverse or similar correlation of expression at both mRNA and protein levels [f]Grey: not determined; +: 20–40% suppression; ++: 40–75% suppression; +++: >75% suppression

p63 to bind to p53RE-A3/A4/A5, as already proposed.[6] In contrast, our results indicate that ΔNp63α- and ΔNp63β-mediated transactivations are independent of p53RE-A. Nevertheless, EEC-derived mutant ΔNp63α (R279H and C306, DNA-binding contact and conformational mutant, respectively) cannot transactivate the P2 promoter,[24] suggesting that both WT conformation and direct DNA contact of ΔNp63α to the region 953-1042 are required to transactivate the P2 promoter. This observation is consistent with our ChIP experiments showing a direct interaction between p63 and the P2 promoter. Thus, ΔNp63α can regulate the internal *TP53* promoter activity through direct binding to response elements different from the p53REs already identified.[16,17]

Our data indicate that overexpressed p53 family members can transactivate the internal *TP53* promoter independently of p53α. Indeed, ectopic expression of p63 isoforms induced Δ133p53 expression at both mRNA and protein levels in mutant p53 MDA-MB-231 cells. Unexpectedly during keratinocyte differentiation, we observed an inverse correlation between Δ133p53α and p63 expression in both HaCat and iPSCs derived from WT *TP63* patients. As no correlation was observed in mutant *TP63* iPSC, the repression of Δ133p53α during keratinocyte differentiation is, at least in part, p63-dependent. Knowing that the ratio of p63 and p73 isoform expression is modulated during keratinocyte differentiation and that WT p53 can also induce Δ133p53 expression, we hypothesise that Δ133p53α expression results from the interplay between p53 family members and the P2 promoter during keratinocyte differentiation. Altogether, our data illustrate the existence of a strong interplay between p53, p63 and p73 isoforms to regulate the expression of Δ133p53α and orchestrate cell-fate outcome in response to stress and in cell differentiation.[16,19,35,36] Further studies will allow clarifying the role of each p53 family members in the orchestration of an integrated cellular response.

Materials and Methods

Plasmids. Activity of the internal *TP53* promoter was analyzed using a luciferase reporter assay.[16] A large *TP53* fragment (+ 11523 to + 13076 bp – accession no. X54156, NCBI) was cloned into pGL3-basic plasmid upstream of the *Firefly* luciferase gene (pi3i4-Luc) (Figure 1a, dotted box). The p21-luciferase reporter assay was used as a positive control.[37] The empty pGL3-basic plasmid was used to normalize the results between the independent experiments, and the plasmid expressing the *Renilla* luciferase gene was used as an internal control.

Three deleted constructs were derived from the full-length pi3i4-Luc (termed A) (Figure 3a): pi3i4-Luc(C), which retains a DNA fragment from nucleotides 723 to 1555 (where 1 corresponds to nucleotide + 11523 – accession no. X54156, NCBI); pi3i4-Luc(G) (953-1555) and pi3i4-Luc(D) (1042-1555).[16] Point mutations were introduced by site-directed mutagenesis in p53RE-A (WTp53REs or MTp53RE-A1/2) (Figure 4a).[16,17] Four polymorphic versions of pi3i4-Luc were constructed to mimic the combination of two *TP53* polymorphisms, *TP53* PIN3 (non-duplicated A1 allele *versus* 16-bp duplicated A2 allele) and *TP53* PEX4 (R *versus* P at codon 72): pi3i4-Luc(A1-R), corresponding to pi3i4-Luc WTp53REs; pi3i4-Luc(A1-P); pi3i4-Luc(A2-R); and pi3i4-Luc(A2-P).[21,22]

The cDNAs encoded by *TP53* family members were cloned into pcDNA3 expressing vector. The pcDNA3-empty vector was used as a negative control. The missense mutants ΔNp63α was kindly provided by L Guerrini (Milan, Italy).

Cell lines and differentiation protocols. The human H1299, MCF7, MDA-MB-231 and HaCat cell lines were maintained at 37 °C in DMEM medium supplemented by 10% foetal calf serum and 0.5%. gentamycin (except HaCat cells), under 5% CO_2 atmosphere. HaCat cells, kindly provided by C. Pourreyron (Dundee, UK), were maintained in low or high calcium concentration to induce differentiation, as previously described.[28] Skin fibroblasts from two EEC patients carrying R304W and R204W mutations in *TP63* and one healthy control were reprogrammed into iPSC by lentivirus-mediated over-expression of Oct3/4, Sox2, Klf4 and c-myc. Protocol for epidermal differentiation was adapted from Aberdam *et al.*[38] and Guenou *et al.*[39] (Petit *et al.*, manuscript in preparation).

Luciferase assays. Twenty-four hours before transfection, 3×10^4 H1299 and MCF7 cells were seeded in 24-well plates. Co-transfections were performed using Fugene reagent (Roche, Mannheim, Germany), as described by the manufacturer, to introduce 500 ng of Firefly luciferase construct, 50 ng of Renilla luciferase plasmid and 200 ng of pcDNA3 expressing vector. Each transfection was performed in triplicate. Cells were harvested 24 h post-transfection to perform luciferase assays using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) as described by the supplier.

Western blots. Proteins were extracted by scrapping and syringing cells in 1X NuPAGE LDS buffer (Invitrogen, Carlsbad, CA, USA) and were separated on precast 10% NuPAGE gels (Invitrogen). Several antibodies have been used: DO-1 (anti-p53); SAPU (anti-p53, all isoforms); 4A4 (anti-p63, all isoforms) (Imgenex, San Diego, CA, USA; IM-80212); 259A (anti-p73, all isoforms) (Imgenex IM-259A); 4B2 (anti-Hdm2); keratin-10 (anti-cytokeratin 10) (Abcam, Cambridge, UK; ab76318). The Ku80 or actin monoclonal antibodies were used as loading controls. Amount of protein was determined by densitometry using ImageJ software (NIH).

CHiP. Cells were seeded in 15-cm plate (2×10^6) and fixed with 1% formaldehyde 24 h after seeding, scraped and washed using 1X PBS. Immunoprecipitation was carried out as previously described using Dynabeads and different antibodies: non-immunized mouse IgG (negative control), 4A4 and IMG-249. Immunoprecipitated DNA was analysed by real time PCR.[16] Of note, immunoprecipitation buffers used to immunoprecipitate 4A4 antibodies were used at pH 8.

Real time PCR. Twenty-four hours before transfection, $5 \times 10^{4.}$ MDA-MB-231 cells were seeded in 6-well plates. Transfections were performed using Fugene reagent (Roche) to introduce 1 μg of p63 or p73 pcDNA3 expressing vector. Cells were harvested 72 h post-transfection to validate p63/p73 isoform expression by western blot and to extract total RNAs using the RNeasy Mini Kit (Qiagen, Crawley, UK). Reverse transcription was performed on 1 μg of total RNAs using the SuperScriptII (Invitrogen) and quantification of Δ133p53, p63 and K14 mRNA levels was carried out by real time PCR on MX3000P apparatus (Stratagene, La Jolla, CA, USA).

Proliferation assays. Proliferation assays were performed in $5 \times 10^{3.}$ H1299 cells, seeded in 6-well plates 24 h before transfection. Fugene reagent (Roche) was used to introduce 1 μg of each p53/p63/p73 pcDNA3 expressing vectors completed by 1 μg of pcDNA3-empty vector when required. Treatment for 14 days with 2 mg/ml G418 (Sigma, Dorset, UK) allowed the selection of transfected cells, which were fixed by addition of methanol and stained by 0.4% sulforhodamine B solution. Bound stain was solubilised by 10 mM TrisBase and quantified by spectroscopy at 515 nm.

Statistical analyses. Results are shown as an average of at least three independent experiments and error bars correspond to S.D.. Statistical analyses were performed using the student's *t*-test. $^*P<0.05$; $^{**}P<0.005$; $^{***}P<0.0005$.

Conflict of Interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Cell Death and Differentiation website (http://www.nature.com/cdd)