Peroxisome Proliferator-Activated Receptor-α Is a Functional Target of p63 in Adult Human Keratinocytes

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p63 is a master switch in the complex network of signaling pathways controlling the establishment and maintenance of stratified epithelia. We provide evidence that peroxisome proliferator-activated receptor-α (PPARα), a ligand-activated nuclear receptor that participates in the skin wound healing process, is a target of p63 in human keratinocytes. Silencing of p63 by RNA interference and transient transfections showed that p63 represses PPARα through a functional region of promoter B. Chromatin immunoprecipitation analyses indicate that p63 is bound to this region, in the absence of a recognizable p63-binding motif, suggesting that it acts through interactions with other transcription factors (TFs). Distinct PPARα transcripts are differentially regulated by p63, indicating a bimodal action in promoter and/or transcription start specification. PPARα repression is consistent with lack of expression in the interfollicular epidermis under physiological conditions. Furthermore, we show that PPARα is a negative regulator of ΔNp63α levels and that it also binds to a functional region of the ΔNp63 promoter that lacks PPRE motifs. Therefore, the reciprocal regulation is exerted either through binding to non-consensus sites or through interactions with other DNA-bound TFs. In conclusion, our data establish a link between two TFs intimately involved in the maintenance of skin homeostatic conditions.

Journal of Investigative Dermatology (2009) **129,** 2376–2385; doi:10.1038/jid.2009.92; published online 21 May 2009

INTRODUCTION

p63, a transcription factor (TF) member of the p53 family, plays a unique role in skin development as lack of this protein in mice causes perinatal lethality due to several abnormalities, including the absence of stratified epithelia and their appendages (Mills *et al.*, 1999; Yang *et al.*, 1999). p63 exerts its indispensable biological role through a number of protein variants displaying opposite functions, all possessing a DNA-binding domain and an oligomerization domain (Yang *et al.*, 1998; Ghioni *et al.*, 2002; Westfall *et al.*, 2003). p63 diversity is achieved through differential promoter usage, yielding proteins either possessing (TA) or lacking (ΔN) a transactivation domain. Moreover, alternative splicing of TAp63 and ΔNp63 proteins at the C terminus gives rise to α-, β-, and γ-isoforms (Yang *et al.*, 1998), all of which possess different, if not divergent, transactivating properties.

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Abbreviations: ChIP, chromatin immunoprecipitation; KC, first-passage adult human keratinocyte; PPAR, peroxisome proliferator-activated receptor; RNAi, RNA interference; TF, transcription factor; TSS, transcription start site

Received 23 April 2008; revised 15 January 2009; accepted 5 February 2009; published online 21 May 2009

Compelling genetic analyses support the hypothesis that ΔNp63 is essential for the proliferative potential of cells in stratified epithelia (Parsa *et al.*, 1999; Yang *et al.*, 1999; Senoo *et al.*, 2007). The presence of a pool of keratinocytes capable of self-renewal is paramount for skin homeostasis, including the ability to intervene when a dermal/epidermal injury occurs. As a counterproof, transgenic mice in which epidermal-specific ΔNp63α knockdown was induced after birth were characterized by severe skin fragility and displayed an impaired ability to heal skin wounds (Koster *et al.*, 2007).

Studies conducted on both rodent and human models indicate that peroxisome proliferator-activated receptors (PPARs) are key players in the skin wound healing process. Three distinct PPARs—α, β/δ, and γ, exhibiting distinct tissue distribution and different ligand specificities—have been described (Kliewer *et al.*, 1994; Mukherjee *et al.*, 1994; Auboeuf *et al.*, 1997). In mouse and human keratinocytes, PPARβ/δ is the most abundant isoform in adults, whereas γ is not expressed (Braissant and Wahli, 1998; Michalik *et al.*, 2001; Westergaard *et al.*, 2001). PPARα, expressed mainly in tissues with a high rate of β-oxidation such as liver, brown fat, and skeletal muscle (Gonzalez, 1997 and references within), is detectable in the hair follicles (Braissant and Wahli, 1998; Michalik *et al.*, 2001) but barely traceable in the interfollicular epidermis (Westergaard *et al.*, 2001). However, upregulation of PPARα and PPARβ/δ expression can be observed in the interfollicular epidermis upon proliferation

stimuli or hair plucking and during wound healing (Michalik *et al.*, 2001).

Although targeted disruption of PPARα in mouse models had no significant consequences on the skin phenotype (Lee *et al.*, 1995; Komuves *et al.*, 2000), the onset of the skin wound healing process was transiently delayed both in mice lacking PPARα (Michalik *et al.*, 2001) and in those engineered to express a dominant negative PPARα in the epidermal compartment (Michalik *et al.*, 2005). Conversely, PPARα constitutive activation targeted to the mouse epidermis and other stratified epithelia led to dramatic phenotypic changes in the skin and other ectodermally derived epithelia, namely the mammary gland and tongue, with pups dying within 2 days after birth (Yang *et al.*, 2006a).

Thus, p63 and PPARα are key regulators of skin homeostasis in mammals. The finding of p63 at the injured site during the healing process (Noszczyk and Majewski, 2001; Bamberger *et al.*, 2005; Kurokawa *et al.*, 2006), as well as expanding knowledge of p63 transcriptional targets (Yang *et al.*, 2006b; Birkaya *et al.*, 2007; Viganò and Mantovani, 2007), led us to investigate whether there is a direct interplay between p63 and PPARα.

RESULTS

p63 is a negative regulator of PPARα expression in adult human keratinocytes

Results from Affymetrix expression profiling of HaCaT cells that were untreated or transiently silenced for p63 (Testoni *et al.*, 2006a) prompted us to investigate the correlation between p63 and PPARα, as inactivation of p63 led to an increase in PPARα expression. We sought to confirm this in HaCaT and first-passage primary human keratinocytes (KCs). RNA interference (RNAi) of p63, targeting the DNA-binding domain common to TA/ΔNp63α, β, and γ, led to an overall increase in PPARα splicing isoforms compared with cells transfected with a green fluorescent protein control RNAi; the mRNA was checked by semiquantitative and quantitative real-time RT–PCR (Figure 1a), and the protein levels were examined by Western blot (Figure 1b). Ectopic expression of different human PPAR subtypes in COS cells probed with the same anti-PPARα antibody ensured that the signal detected was specific for the α subtype (Figure 1c). The larger than expected size of PPARα, observed in HepG2 and COS cells upon PPARα overexpression, is consistent with the latter being phosphorylated *in vivo* (Gervois *et al.*, 1999).

Differential promoter usage and alternative splicing at the N-terminus have been described for PPARα (Figure 1d; Pineda-Torra *et al.*, 2002; Chew *et al.*, 2003, 2007), resulting in several splicing isoforms sharing a common open reading frame (Vohl *et al.*, 2000). We explored whether distinct PPARα isoforms contributed equally to the observed overall increase: the most prominent feature was an increase in PPARα-4 on p63 removal, which was paralleled by a decline in PPARα-6, in both HaCaT and KCs (Figure 1a, bottom left). Note that the two splicing isoforms are generated by promoter B but do not share a common transcription start site (TSS) (Chew *et al.*, 2003 and Figure 1d). PPARα-5 and -2 levels, driven by promoters A and C, respectively, were not significantly affected in KCs. Finally, confocal analysis of human skin from healthy donors stained with anti-p63 and anti-PPARα antibodies showed the absence of PPARα expression in the intact interfollicular epidermis (data not shown), consistent with previous reports (Westergaard *et al.*, 2001). In conclusion, in keeping with our profiling experiments, PPARα expression is influenced by p63 levels.

PPARα is a direct target of p63

Modulation of PPARα expression could be either a secondary event mediated by other TFs or a primary one directly exerted by p63 bound to regulatory elements on the PPARα locus. To address this point, we performed *in silico* analysis of the PPARα gene searching for p63-, p53-, and PPARα-like consensus sites. We scanned the human PPARα gene by combining sequence-specific binding requirements for each TF (Juge-Aubry *et al.*, 1997; Ortt and Sinha, 2006; Perez *et al.*, 2007) and bioinformatic tools—ConSite and rVista—and identified several putative sequences (Figure 2a). Chromatin immunoprecipitation (ChIP) in HaCat with two different antibodies directed against p63 revealed *in vivo* binding to several regions upstream of TSS (Figure 2b, left panels). Furthermore, ChIPs with antibodies against PPARα and its heterodimeric partner RXR indicate that the two TFs are present on some, but not all, regions positive for p63, notably promoters A and B (Figure 2b, right panels). Several genomic regions were negative for all antibodies. The FDXR promoter region, used as a positive control for p63 binding (Viganò *et al.*, 2006), was indeed positive for p63, but not for PPARα or RXR, whereas a previously identified PPARα locus, corresponding to amplicon 2 (Pineda-Torra *et al.*, 2002), was positive for both PPARα and RXR. Finally, we performed the same assays in primary keratinocytes and indeed found similar results, notably on amplicons in promoter A and promoter B (Figure 2c). Overall, these data indicate that p63 occupies several binding sites *in vivo* in keratinocytes and suggest that PPARα/RXR might be involved in an auto-regulatory process, as previously hypothesized (Pineda-Torra *et al.*, 2002).

p63 represses PPARα promoter B

Next, we tested the transcriptional activity of p63 on reporter plasmids containing PPARα promoter A −1664/+81 or B −1147/+34. PPARα promoter A −1664/+81 contains the TSS for both PPARα isoforms −5 and −3 (Figure 3a). Transient transfections were carried out in U2OS, which lack p63, and in HaCaT, which express almost exclusively ΔNp63α (Yang *et al.*, 1999). Expression of TAp63α, -β, or -γ or ΔNp63α or -γ in U2OS cells significantly ($P<0.5$ and $P<0.02$) downregulated promoter A activity, whereas expression of ΔNp63β led to a modest increase ($P<0.05$) (Figure 3b). Note that the increases in expression observed with low doses of TAp63α and TAp63γ were not statistically significant. Under similar conditions, promoter A activity was unchanged in HaCaT cells (Figure 3c). In keeping with this result, knockdown of p63 by RNAi had negligible

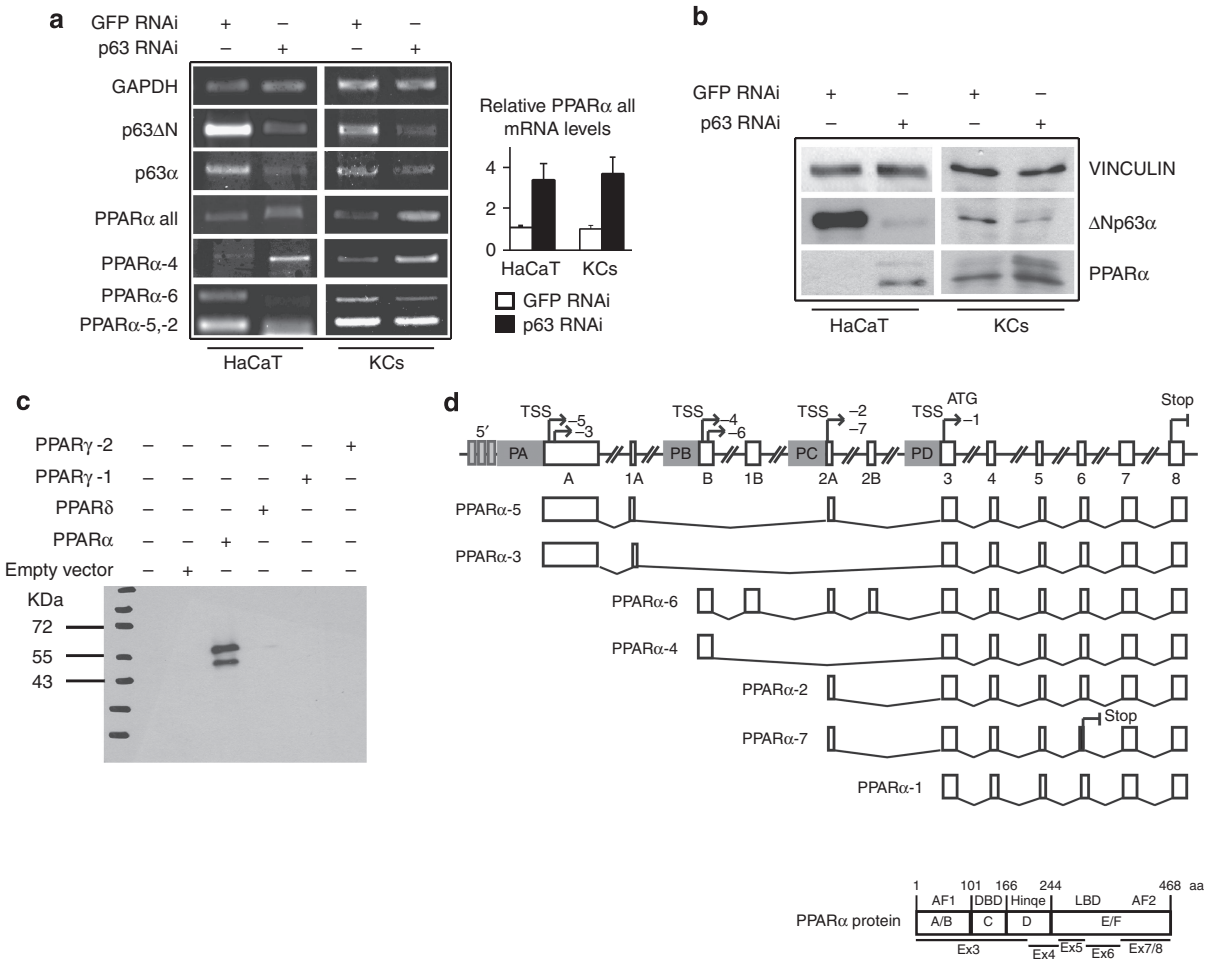

Figure 1. p63 is a negative regulator of PPARα expression in adult human keratinocytes. (**a**) RT–PCR (left) and qRT–PCR (right) analyses of PPARα expression in HaCaT cells and KCs transiently (96 hours) transfected with 3 μg of either a plasmid encoding a short hairpin targeting GFP as a control (GFP RNAi) or one targeting all p63 isoforms (p63 RNAi). cDNAs were normalized *versus* GAPDH levels. Results are representative of at least three independent experiments. (**b**) Western blotting analysis of PPARα protein expression in HaCaT and KCs as described in (**a**). Protein loading was normalized versus vinculin. (**c**) Western blotting proving the specificity of the anti-PPARα antibody by ectopic expression of human PPAR subtypes (1.5 μg each) in COS cells. (**d**) PPARα gene structure (adapted from Chew *et al.*, 2003), splicing isoforms, and protein. PA, PB, PC, and PD represent the four characterized promoters.

effects on the transcription of promoter A in HaCaT cells (Figure 3d).

PPARα promoter B −1147/+34, containing the TSS of PPARα-4, but not that for PPARα-6 (Figure 3e), in U2OS cells was repressed by ectopic p63 in a dose-dependent manner, with a stronger effect ($P<0.02$) in the presence of TAp63β and -γ and ΔNp63α and -γ (Figure 3f). In HaCaT cells, only ΔNp63α significantly ($P<0.02$) repressed promoter B, with ΔNp63β and -γ showing modest ($P<0.05$) activation (Figure 3g). Indeed, functional inactivation of endogenous ΔNp63α in the same cells enhanced ($P<0.02$) the activity of promoter B (Figure 3h). Amplicon 4 of promoter B was positive for binding *in vivo* (Figure 2) and included in the −1147/+34 construct. To ascertain whether this p63 location was implicated in promoter B regulation, we generated 5′ deletion constructs comprising −500/+34 or lacking −200/+34 such region (Figure 3i, left). Transient transfection in HaCaT cells showed a significant ($P<0.02$) decrease of the promoter B −500/+34 construct with increasing amounts of ΔNp63α (Figure 3i, right). In contrast, the activity of the −200/+34 construct was not changed by p63 overexpression. Altogether, these data strongly suggest that the *in vivo* binding of p63 to amplicon 4 mediates PPARα promoter B downregulation.

To further validate the role of p63 on PPARα promoter B activity, we overexpressed different isoforms of p63 in HaCaT and KCs (Figure 4a). To this aim, we monitored endogenous PPARα-4 mRNA, as scored by semiquantitative RT–PCR; in both HaCaT and KCs the drop was substantial (Figure 4a). In addition, ectopic expression of ΔNp63α and -γ led to a decrease in PPARα-6 mRNA levels, also generated by promoter B, in HaCaT cells but not in KCs (Figure 4a, left and right, respectively). Total PPARα mRNA levels were repressed by all ectopically expressed p63 isoforms in HaCaT cells as well as in KCs (Figure 4b). However, only ectopic expression of ΔNp63α led to a significant repression of PPARα protein levels (Figure 4c). Taken together, these data provide evidence that p63, and ΔNp63α in particular, is

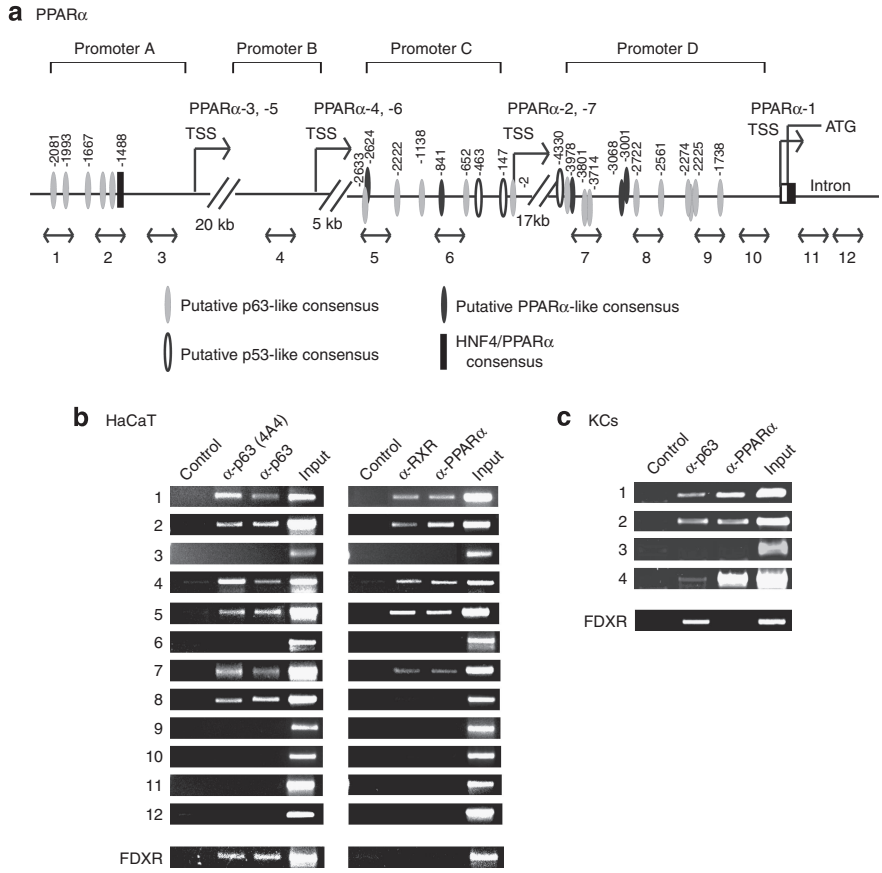

Figure 2. PPARα is a p63 target. (**a**) Schematic representation of human PPARα genomic region. Transcription start sites (TSS) for each promoter and *in silico* identified consensus for TFs are shown. Double arrows represent the positions of amplicons generated by PCR after chromatin IP (ChIP) assay, identified by numbers on the bottom. Region 2 comprises a consensus for hepatocyte nuclear factor 4 (HNF4), which is a positive control for PPARα binding (Pineda-Torra *et al.*, 2002). (**b**) Amplification of genomic regions, numbered as in (**a**), after ChIP, showing the *in vivo* binding of p63 and PPARα, as well as its heterodimeric partner RXR, to PPARα. The amplified FDXR promoter region is a positive control for p63 binding in human KCs (Viganò *et al.*, 2006). Results are representative of three to five independent ChIP assays. (**c**) ChIPs performed on KCs.

a repressor of PPARα transcription, specifically targeting a functional element between −469 and −255 of promoter B that drives PPARα-4 expression in adult human keratinocytes.

PPARα is a negative regulator of p63

Recently, a TF regulated by p63, C/EBP delta, was shown to affect the expression levels of p63 isoforms (Borrelli *et al.*, 2007). We then explored the possibility that there is also a reciprocal interplay between p63 and PPARα; HaCaT cells were infected with lentivirus expressing short hairpin RNA against PPARα and LacZ (negative control). Stable knockdown of PPARα by three different lentiviral constructs (#1, #2, #3) led to an increase in ΔNp63 mRNA, detected by quantitative real-time RT–PCR (Figure 5a, left); the levels of the ΔNp63α protein were also positively regulated in western blot analysis (Figure 5a, right). To determine whether PPARα regulation of the ΔNp63α promoter is direct, we scanned for the presence of PPARα-binding sites in ΔNp63 regulatory regions (Figure 5b, left): the proximal promoter (Romano *et al.*, 2006) and the evolutionarily conserved enhancer located in the fifth intron (Antonini *et al.*, 2006). ChIP analysis indeed detected PPARα and RXR binding to the ΔNp63 promoter and 1 kb downstream of the TSS in HaCaT cells (Figure 5b, right panels). p63 was bound to its own promoter and downstream regions, as previously assessed (Antonini *et al.*, 2006; Borrelli *et al.*, 2007; Romano *et al.*, 2006, and Figure 5b, left panels). Upstream regions (from −1.5 to −3 kb) were negative for p63 and PPARα binding (data not shown).

To understand whether PPARα binding to ΔNp63 promoter regions was involved in transcriptional downregulation, we used three ΔNp63 constructs differing at the 5′ end (Figure 5c, left), with partial overlap of the two amplicons (A and B) bound *in vivo* by PPARα. On transient transfection in a p63-negative background—COS cells—the basal activity of the two longer constructs, −1584/+69 and −736/+69, was similar, whereas the shorter core promoter construct −164/+69 showed a twofold drop. However, the activity of the longer ΔNp63 constructs, but not of the core promoter, was significantly ($P<0.05$ and $P<0.02$) downregulated by increasing amounts of ectopic PPARα, either alone or in the presence of RXRα (Figure 5c, right). In essence, these data support the hypothesis that PPARα behaves as a

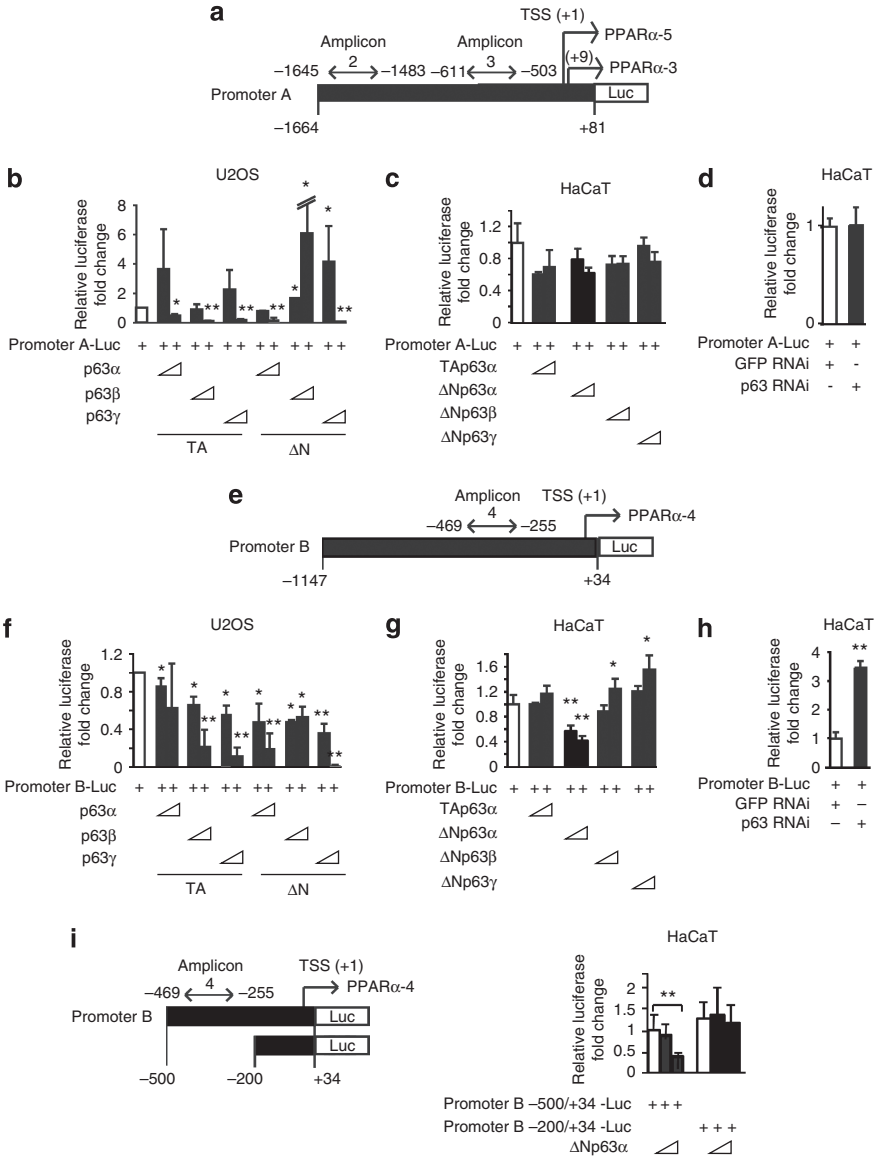

Figure 3. p63 represses PPARα promoter B. (**a**) Schematic diagram of PPARα promoter A-luciferase construct −1664/+81, used in transient transfections. The position of amplicons 2 and 3 generated after ChIP are indicated by double arrows. (**b**) U2OS cells, devoid of endogenous p63, were exposed to increasing amounts (70–210 ng) of p63 expression constructs for 24 hours. (**c**) HaCaT cells, expressing high levels of ΔNp63α, were transfected with increasing amounts (50–100 ng) of Tap63α or ΔNp63α, −β, and −γ encoding plasmids or (**d**) transiently knocked down (72 hours) for GFP (GFP RNAi) as a control or p63 (p63 RNAi) before transfection with 1.2 μg of the reporter construct for an additional 24 hours. (**e**) Schematic diagram of PPARα promoter B-luciferase construct −1147/+34 used in transient transfections as described in (**b**). The position of amplicon 4 generated after ChIP is indicated by double arrows. (**f**, **g**, and **h**) Transient transfection of U2OS and HaCaT cells as described in (**b**, **c** and **d**) using the PPARα promoter B-luciferase −1147/+34 construct. (**i**) Left: schematic diagram of 5′ deletions of PPARα promoter B, −500/+34 and −200/+34, used in transient transfection in HaCaT cells as described above. The position of amplicon 4 generated after ChIP is indicated by double arrows. All results, shown as mean ± SD of three independent experiments, each conducted in triplicate, are expressed as fold variation over the promoter-only construct, arbitrarily set as 1. Values were normalized by co-transfection with the 200 ng CMV-βGal construct. Statistical significance was calculated using the Student's *t*-test (*$P<0.5$; **$P<0.01$).

transcriptional regulator of ΔNp63α expression in adult human keratinocytes.

DISCUSSION

In this paper, we show that ΔNp63α and PPARα, two TFs that are paramount for skin homeostasis in mammals, impinge on each other's expression in human keratinocytes. Silencing of p63 resulted in a dramatic increase in PPARα expression. This effect is promoter- and isoform-specific, and it is mediated, at least in part, by the direct binding of p63 to the regulatory elements in the PPARα promoter B region. Similarly, knockdown of PPARα induces ΔNp63 mRNA and protein expression.

Besides being essential for skin stratification and correct appendage formation during embryonic development (Mills *et al.*, 1999; Yang *et al.*, 1999), p63 is the gatekeeper for the

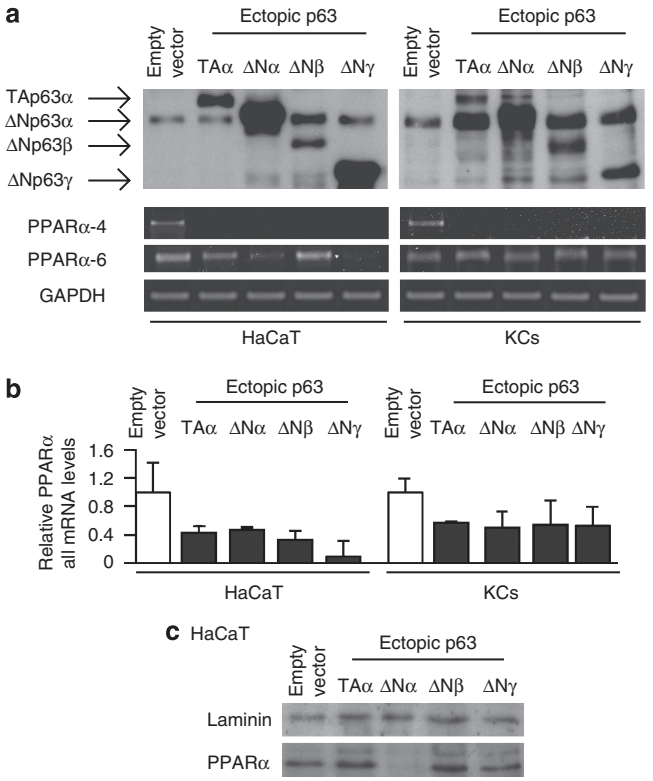

Figure 4. Negative regulation of PPARα by p63 overexpression. (**a**) Western blotting (top panels) proving the efficiency of ectopic p63 expression in HaCaT (left) and KCs (right) and semiquantitative RT–PCRs (bottom panels) showing PPARα-4 and -6 expression driven by promoter B. Results are representative of two independent experiments. Amplification of GAPDH ensured cDNA normalization. (**b**) qRT–PCR of global PPARα mRNA expression levels in HaCaT (left) and KCs (right) upon ectopic p63. (**c**) Western blotting showing PPARα protein levels in HaCaT after p63 ectopic expression. Protein loading was normalized *versus* laminin.

balance between self-renewal and differentiation in multilayered epithelia. In particular, the role in skin homeostasis of the major isoform expressed in keratinocytes—ΔNp63α—is well established. Regulation of PPARα expression is stunningly complex, as it involves at least four promoters and a plethora of splicing isoforms. A clear indication of the complexity of regulation of the PPARα locus by p63 stems from the ChIP analysis, which pinpoints binding to six different locations, mapping on all four promoters. Although we obtained clear evidence for a p63 function only on promoter B, other units could be regulated under conditions different from the ones used here in growing keratinocytes. This complexity is further reflected by the bimodal activity of ΔNp63α on the various isoforms present in human keratinocytes; the mutual exclusivity of PPARα-6 and -4 suggests that p63 might be involved in the choice of the TSS and/or in subsequent splicing events. This is not unprecedented, as we have recently shown that another p63 target, the antiapoptotic FLIP, is more controlled at the level of splicing isoform selection than overall mRNA production (Borrelli *et al.*, 2009). The precise mechanisms of these activities are currently unknown. In particular, it is unclear whether this is the result of selection of particular PolII-associated complexes loaded differentially onto the promoters or of activation of distinct splicing factors by p63. We note that p63 ChIP-on-chip analysis retrieved statistically enriched GO terms of genes controlling mRNA metabolism (Viganò *et al.*, 2006 and Pozzi *et al.*, 2009).

ΔNp63α-negative regulation of PPARα in human keratinocytes is consistent with PPARα being scarcely detectable in the lower layers of adult interfollicular epidermis under physiological conditions (Westergaard *et al.*, 2001). This is not always the case, as prolonged exposure to PPARα activators (natural and synthetic fatty acid derivatives) is able to induce differentiation, improve epidermal barrier function, and counteract hyperproliferation in several mouse models of skin disorders displaying features of exacerbated inflammation (Michalik and Wahli, 2007; Schmuth *et al.*, 2008). In general, PPARα is a key negative modulator of the inflammatory response in the skin compartment. Furthermore, PPARα mRNA levels are reduced in psoriatic lesions and atopic dermatitis in humans (Westergaard *et al.*, 2003; Michalik and Wahli, 2007; Staumont-Salle *et al.*, 2008), whereas p63 levels tend to be maintained high and possibly higher in psoriasis (Shen *et al.*, 2005; Gu *et al.*, 2006).

The negative regulation of ΔNp63 by PPARα, as evidenced by the increase in mRNA and protein levels upon RNAi inactivation of PPARα and ChIP assays, suggests a complex feedback loop between the two TFs. One physiological response in which the two TFs play a role is the reepithelialization of a wound, which initially involves the migration of basal keratinocytes from the injury edges and hair follicles and their proliferation, stratification, and differentiation. Transient expression of PPARα is induced after an injury, and PPARα-deficient mice are characterized by a transient delay in the healing process, overlapping with the temporal window of the inflammatory response. As for the p63 behavior in this process, two mouse models indicate a relevant role: (i) skin lesions of transgenic mice with constitutive expression of ΔNp63α targeted to the epidermal basal layer are not healed (Sommer *et al.*, 2006) and (ii) conditional p63 knockout mice are severely impaired in skin wound healing (Koster *et al.*, 2007). The kinetics of ΔNp63α expression are consistent with a role in the process, as it is transiently downregulated in mouse and human skin at early stages of wound healing and rebounds for several days after complete wound closure (Noszczyk and Majewski, 2001; Bamberger *et al.*, 2005; Kurokawa *et al.*, 2006). At present, it is unclear which mechanisms are responsible for this repression *in vivo*, but the identification here of PPARα makes it an excellent candidate for mediating this process on the ΔNp63 promoter during wound healing.

Interestingly, ChIP analysis convincingly shows interactions of p63 on PPARα promoter B and of PPARα on the ΔNp63 promoter, yet bioinformatic analysis fails to show the presence of canonical binding sites for the two TFs; thus, the reciprocal regulation is apparently exerted either through binding to non-canonical sequences or through association with undefined TFs binding to the two regions. This mechanism of regulation is unprecedented for PPARs, but not for p63; indeed, the latter has been shown to negatively

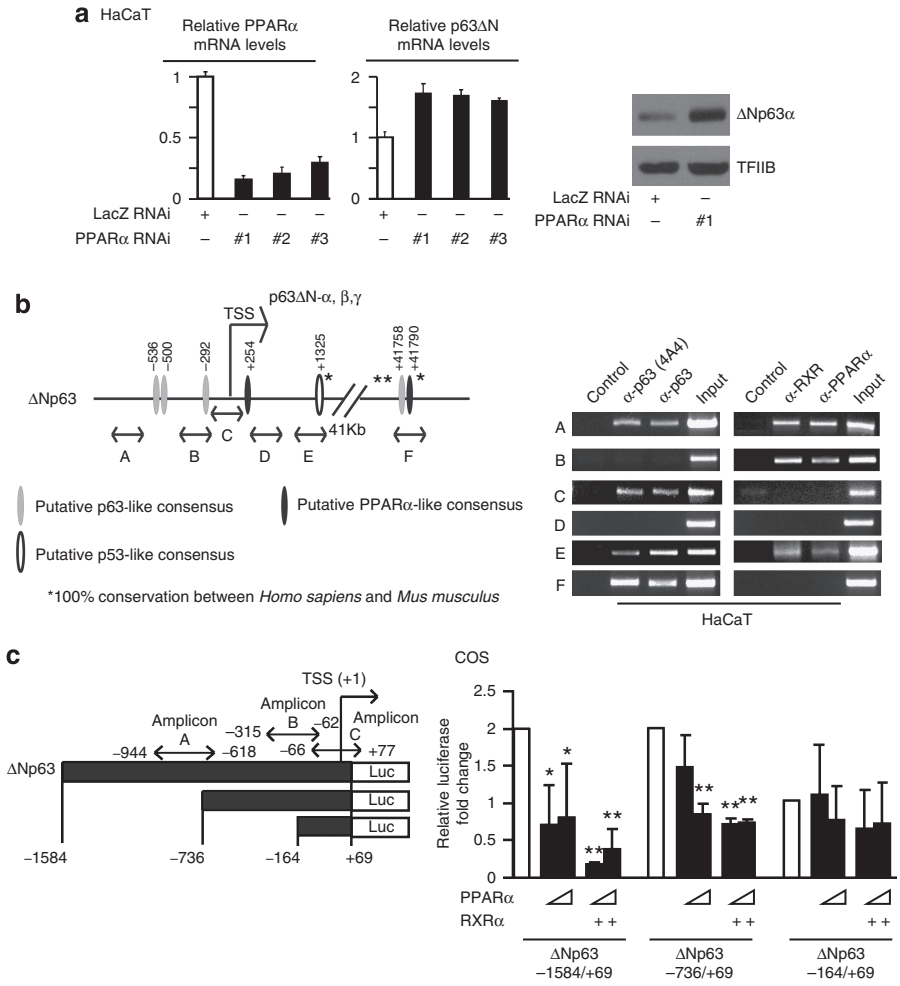

Figure 5. PPARα is a transcriptional regulator of ΔNp63α. (**a**) Stable knockdown of endogenous PPARα by lentiviral RNAi in HaCaT cells results in increased levels of ΔNp63α, as detected by qRT–PCR (left) and western blotting (right). HaCaT cells were infected with lentivirus expressing shRNA against LacZ or three different targets within the PPARα coding sequence. Cells were harvested after 10 days of puromycin selection and used for preparation of RNA and whole-cell lysates. (**b**) Left: schematic diagram of ΔNp63 regulatory regions, including the C40 enhancer in the fifth intron (Antonini *et al.*, 2006). *In silico* identified consensus for the transcription factors is shown. Position of amplicons generated by PCR after ChIP assay are identified by double arrows and letters on the bottom. Right: p63, PPARα, and RXR *in vivo* binding to the ΔNp63 promoter and downstream regions, as detected by PCR after ChIP analysis. Results are representative of three to four independent ChIP experiments. (**c**) Schematic diagram of sequential 5′ deletions of p63 promoter constructs (left) used in transient transfections. The transcription start site (TSS), *in silico*-identified consensus for the transcription factors, and position of the amplicons, as in (**b**), are indicated. COS cells were transfected with increasing amounts (50–100 ng) of PPARα-encoding plasmid, either in the absence or in the presence of RXRα (10 ng), and 1.2 μg of ΔNp63 promoter constructs (right). Results, shown as mean ± SD of three to five independent experiments, each conducted in triplicate, are expressed as fold variation over the shorter promoter-only construct (−164/+69), arbitrarily set as 1. Values were normalized by co-transfection with the 200 ng CMV-βGal construct. Statistical significance was calculated using the Student's *t*-test (*$P<0.5$; **$P<0.02$).

regulate promoters specifically active in the G2/M phase of the cell cycle through CCAAT boxes and by direct association with the CCAAT-binding TF NF-Y (Testoni and Mantovani, 2006b). In fact, NF-Y has been shown to be the intended target for other TFs. Interestingly, the ΔNp63 promoter does contain two key CCAAT boxes, but a deletion mutant containing only this region lost the capacity to be regulated by PPARα, implying that NF-Y is not the intended target. Additional work must be performed to identify the molecular details of this regulation on the two promoters. In general, work originally done on nuclear receptors in the early 1990s showed that regulation—positive and negative—of a TF on a particular gene can be exerted not through binding to a consensus site, but through association with another TF (Gaub *et al.*, 1990; Shemshedini *et al.*, 1991); this concept has since been extended for several TFs and further reinforced by dozens of ChIP-on-chip studies, whereby a large proportion of TF locations lack any visible recognition motif. In the most extreme case, E2F1, only a small minority <20% of promoter locations contain a recognizable motif (Bieda *et al.*, 2006).

PPARα mediates adaptation to intermittent nutritional deprivation by stimulating the transcription of genes critical for fatty acid oxidation (Reddy, 2001). Fatty acid synthase converts dietary carbohydrates into saturated fatty acid (Semenkovich, 1997) and is a transcriptional target of p63

in human keratinocytes (D'Erchia *et al.*, 2006). Finally, other p63 targets emerging from ChIP-on-chip experiments are CITED2 (Viganò *et al.*, 2006), a well-known PPARα co-regulator (Tien *et al.*, 2004), and PPARβ/δ (Pozzi *et al.*, 2009), which is paramount for skin wound healing (Michalik and Wahli, 2007 and references therein). It is therefore conceivable that p63 and PPARα cooperate in the physiological response to nutrient and oxygen depletion following an epidermal wound. In general, TF genes are among the preferred targets of p63 (Pozzi *et al.*, 2009). Intuitively, a network of reciprocal regulatory interactions could be established to drive and control the different features of a dynamic tissue undergoing continuous, controlled growth and terminal differentiation. Our current hypothesis is that p63 makes key connections with, in particular, regulators of specific functions that would constitute ''hubs'' with further fine-regulatory missions. While adding a new hit to the rapidly increasing list of p63 targets, we trace a direct connection between p63, the skin repair process, and the inflammatory phase, an essential ''hub'' in tissue integrity and protection against external stresses, of which PPARα is an important regulator. Further work is required to identify the network of co-regulated targets during the process.

MATERIALS AND METHODS

Cell culture, transfection, and reporter assay

First-passage primary human keratinocytes derived from healthy individuals were grown on a feeder layer of lethally irradiated human fibroblasts as previously described (Viganò *et al.*, 2006). HaCaT, U2OS, and COS cells were grown in DMEM supplemented with 10% fetal calf serum. All transfections were carried out using Lipofectamine 2000 (Invitrogen, Milan, Italy), following the manufacturer's instructions. Transient transfections for reporter assay were carried out three times in triplicate, and CMV-βGal vector was co-transfected as an internal control for transfection efficiency. Plasmid DNAs encoding a hairpin targeting green fluorescent protein (a kind gift of S. Oliviero, Siena, Italy) were used as a negative control along with that targeting the DNA-binding domain common to all p63 isoforms (Sigma, Milan, Italy). PPARα promoter A −1664/+81 and B −1147/+34 reporter constructs were generous gifts from B. Staels (Lille, France) and T.S. Tengku-Muhammad (Penang, Malaysia), respectively. PPARα promoter B 5′ deletions were generated by amplification from the original construct −1147/+34 and cloned into pGL3-basic vector (Promega, Milan, Italy) using restriction sites for *Xho*I and *Hin*dIII introduced by PCR.

Lentiviral vector construction, virus production, and infection

The software program Psicoligomaker was used to select 19-mer short hairpin RNA sequences and design sense and antisense oligos. Sequences were BLASTed to confirm target specificity. The annealed and phosphorylated oligos were cloned into the pSicoR puromycin lentiviral vector (Ventura *et al.* 2004) using the *Hpa*I and *Xho*I restriction sites. Short hairpin RNA target sequences were as follows: shPPARα no. 1: 5′-GACTCAAGCTGGTGTATGA-3′; shPPARα no. 2: 5′-GAAGAGTTCCTGCAAGAAA-3′; shPPARα no. 3: 5′-GAATACCA GTATTTAGGAA-3′; and shLacZ: 5′-GAAGGCCAGACGCGAATTA-3′. Lentiviral particles were produced by co-transfecting 293T cells with pSicoR puromycin (10 μg) and the lentiviral packaging vectors pMDL/RRE, pRSV-Rev, and pMD2G-VSVG (5 μg each) using metafectene (Biontex Laboratories, GmbH, Planegg, Germany). The medium was harvested after 48 and 72 hours, pooled, and passed through a 0.45 μm Millex-HV filter (Millipore, Copenhagen, Denmark) before concentration by ultracentrifugation at 26,000 rpm for 2 hours at 4°C and resuspension in pure DMEM. HaCaT cells were infected with lentivirus for 24 hours in the presence of 8 μg ml^{-1} Polybrene and subsequently placed under puromycin selection (1 μg ml^{-1}) for 10 days before harvest of RNA and proteins.

RT–PCR and western blotting

Total RNA was isolated using Trizol (Invitrogen) and retrotranscribed with the SuperScript First-Strand Synthesis System (Invitrogen). Sequences of primer pairs are available upon request. cDNAs were normalized versus glyceraldehyde-3-phosphate dehydrogenase and/or TATA binding protein levels. For semiquantitative RT–PCRs, several amplifications were carried out to ensure that cDNA amplification was in the linear range. Whole-cell extracts were lysed in RIPA buffer. Twenty to 40 μg of lysates were separated on 10% SDS–PAGE, transferred onto a nitrocellulose membrane (Protran, Whatman, Maidstone, UK), and probed using the following antibodies: anti-p63 (clone 4A4, Santa Cruz, Santa Cruz, CA), anti-PPARα (Santa Cruz, CA), anti-vinculin (clone VIN-11-5, Sigma), anti-TFIIB (Santa Cruz), and anti-laminin (Santa Cruz).

ChiP assay

ChIP analysis was carried out essentially as previously described (Testoni *et al.*, 2006a). In brief, 2.5×10^6 cell equivalents of chromatin were immunoprecipitated with 5 μg of the following antibodies: anti-p63 (clone 4A4, Sigma; Beretta *et al.*, 2005), anti-PPARα and anti-RXR (Santa Cruz), and anti-flag (clone M1, Sigma) as a negative control. The location of potential p53-, p63-, and PPARα-binding sites was performed by *in silico* analysis (Transfac and rVista; cutoff settings: 85–90%) applying single TF-specific criteria (Juge-Aubry *et al.*, 1997; Ortt and Sinha, 2006; Perez *et al.*, 2007). When KCs were used, equivalent amounts of crosslinked proteins/DNA from three different individuals were pooled together. Sequences of primer pairs are available upon request.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

We thank S. Oliviero, B. Staels, and T.S. Tengku-Muhammad for constructs; B. Testoni for initial screening of p63 targets; D. Alotto and C. Castagnoli for KCs; and M. Callari for help with *in silico* analysis of PPARα promoters. We are most grateful to M.A. Viganò and N. Cordani for critical discussion. This work was supported by EU EPISTEM LSHB-CT-2005-019067 and CARIPLO grants to RM.

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