ORIGINAL ARTICLE
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Tprg, a Gene Predominantly Expressed in Skin, Is a Direct Target of the Transcription Factor p63

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p63 and *p73* are highly homologous members of the *p53* family that originated by gene duplication at the invertebrate-to-vertebrate transition. We characterize here a previously unreported gene, Transformation-related protein 63 regulated (*Tprg*), located upstream of the *p63* gene in the vertebrate genome, with striking similarity to Transformation related protein 63 regulated like (*Tprgl*), an uncharacterized gene located upstream of *p73*, suggesting that *p63*/*Tprg* and *p73*/*Tprgl* are embedded in a paralogue region originated from a single duplication event. *Tprg* is predominantly expressed in the epithelial compartment of the skin, more abundantly in differentiated cells. Consistent with its relative higher expression in differentiated keratinocytes, finely tuned p63 expression levels are required for optimal *Tprg* expression in primary keratinocytes. p63 is essential for *Tprg* expression as shown in p63-knockdown keratinocytes; however, high levels of p63 result in *Tprg* down-regulation. p63 directly binds *in vivo* to a canonical p63-binding site in an evolutionary conserved genomic region located in *Tprg* intron 4. This genomic region is sufficient to function as a p63-inducible enhancer in promoter studies. Thus, we demonstrate that the *Tprg* gene is predominantly expressed in skin, is physically associated with the *p63* gene during evolution, and directly regulated by p63 through a long-distance enhancer located within the *Tprg* locus.

Journal of Investigative Dermatology (2008) **128,** 1676–1685; doi:10.1038/jid.2008.12; published online 7 February 2008

INTRODUCTION

A number of transcription factors are known to regulate skin development and differentiation. Among these, p63 is a key modulator of these processes, as is clearly demonstrated *in vivo* by knockout studies. *p63*−/− mice fail to form a stratified epidermis, resulting in lack of barrier formation, consequent dehydration, and death within hours after birth (Mills *et al*., 1999; Yang *et al*., 1999).

p63 belongs to the *p53* gene family consisting of three genes, *p53*, *p63*, and *p73*, that share a significant sequence homology (reviewed by Yang *et al*., 2002). Each p53 family member contains a transactivation domain at the amino terminus, a DNA-binding domain, and an oligomerization domain. In addition, all family members share some common functions, and bind to a canonical p53-binding site, thus controlling the expression of a subset of p53 target genes (Yang *et al*., 2002, 2006). The use of alternative promoters and transcription start sites gives rise to two classes of *p63* transcripts, those encoding proteins with an amino-terminal transactivation domain (TA isoforms) and those encoding proteins lacking this domain (ΔN isoforms) (Yang *et al*., 1998). Three different carboxyl-termini, designated α, β, and γ, are generated by alternative splicing. The carboxyl-terminus of p63-α is the longest and contains a sterile α-motif domain and a transactivation-inhibitory domain (Chi *et al*., 1999; Thanos and Bowie, 1999; Serber *et al*., 2002). Accordingly, ΔNp63-α has been shown to act as a repressor and to display dominant-negative function against both TAp63 isoforms and p53 (Yang *et al*., 1998; Ghioni *et al*., 2002; Westfall *et al*., 2003; Chan *et al*., 2004). However, ΔNp63-α also positively regulates the expression of some target genes, such as integrins and other adhesion-associated genes (Kurata *et al*., 2004; Carroll *et al*., 2006; Truong *et al*., 2006), as well as keratin 14 (K14) (Romano *et al*., 2006).

p63 is predominantly expressed in the basal and spinous layers of the epidermis, and is downregulated upon keratinocyte differentiation both *in vitro* and *in vivo* (Parsa *et al*., 1999; Yang *et al*., 1999; Liefer *et al*., 2000; Bamberger and Schmale, 2001; Pellegrini *et al*., 2001; Westfall *et al*., 2003; Nguyen *et al*., 2006). In the basal layer, p63 is mainly

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Abbreviations: aa, amino acids; ChIP, chromatin immunoprecipitation; GFP, green-fluorescent protein; hrs, hours; PBS, phosphate-buffered saline; RT–PCR, reverse transcriptase–polymerase chain reaction; siRNA, short interfering RNA; TK, thymidine kinase; Tprg, *Transformation-related protein 63 regulated;* Tprgl, *Transformation-related protein 63 regulated like*

Received 29 June 2007; revised 23 November 2007; accepted 4 December 2007; published online 7 February 2008

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involved in maintaining cell proliferation and cell adhesion (Koster *et al.*, 2004; Deyoung *et al.*, 2006; Sbisa *et al.*, 2006; Truong *et al.*, 2006).

As keratinocytes detach from the basement membrane, they begin a program of terminal differentiation, characterized by a change in keratin expression from K5/K14 to a suprabasal pair in the spinous layer (K1/K10). Further keratinocyte differentiation leads to the expression of filaggrin, loricrin, and other cornified envelope components in the granular layer, contributing to the formation of the cutaneous barrier (reviewed by Fuchs, 1998). It has been proposed that p63 plays a dual role in keratinocyte differentiation, as it is required for initiating epithelial stratification (Koster *et al.*, 2004; Nguyen *et al.*, 2006; Truong *et al.*, 2006), whereas concurrently it inhibits the expression of some differentiation markers, at least in part through transcriptional repression of the Notch effector Hes1 (King *et al.*, 2003; Nguyen *et al.*, 2006). Interestingly, some p63 direct targets, such as *Perp*, whose expression in skin requires p63, are predominantly localized in the suprabasal layers (Ihrie *et al.*, 2005).

Here, we identify Transformation-related protein 63 regulated (*Tprg*), a previously uncharacterized gene located upstream of the genomic locus of *p63*, whose expression is regulated by p63. *Tprg* encodes for a cytoplasmic protein with a high degree of homology with the gene product of Transformation related protein 63 regulated like (*Tprgl*), located upstream of the *p73* gene. Both loci are conserved throughout vertebrate evolution upstream of *p63* and *p73*, respectively. *Tprg* is significantly expressed from embryonic day 15.5 in a cell type-specific manner in the epidermis and in the hair follicle in contrast to the ubiquitous expression of *Tprgl*. We show that *Tprg* expression is suppressed by *p63* knockdown in mouse primary keratinocytes, and is specifically affected by knockdown of the ΔNp63-α isoform. Interestingly, ΔNp63-α overexpression also negatively regulates *Tprg*, suggesting that a finely tuned p63 activity is required for optimal *Tprg* expression. p63, moreover, directly binds and activates a long-distance enhancer located in a *Tprg* intronic region. Taken together, our data indicate that *Tprg* is a previously uncharacterized gene, conserved throughout evolution in proximity of p63, and whose expression is under direct control of p63.

RESULTS

In vertebrates, p63 and p73 proteins are more closely related to one another than to p53, owing to a higher percentage of similarity in the DNA-binding domain as well as the presence of the sterile α-motif domain, absent in p53 (Saccone *et al.*, 2002; Yang *et al.*, 2002). As p63 and p73 are known to have derived from a gene duplication event, we investigated whether they are embedded in paralogous regions, which would indicate duplication of nearby genes. Interestingly, an uncharacterized gene located 260 kb upstream of the p63 gene in mouse, indicated by the full-length cDNA 5430420C16Rik, is highly homologous to another uncharacterized gene located 101 kb upstream of the p73 gene, indicated by the full-length cDNA 1200015A19Rik (Kawai *et al.*, 2001; Figure 1a). On the basis of the results described below, we have named the above two transcripts *Tprg* and *Tprgl*, respectively. They are both transcribed in the same direction as the corresponding *p63* and *p73* genes, and share 44.1% of identity at the protein level (Figure 1b). p63 and p73 share a comparable percentage of identity (56.6%), suggesting that they have evolved in a parallel manner, and may be embedded in a paralogue region originated from a single duplication event.

Tprg is transcribed in a 1,087-bp transcript originally isolated as a full-length cDNA from 6 days neonate head and adult female vagina cDNA library (Kawai *et al.*, 2001). *Tprgl* is transcribed in a 1,756-bp transcript originally isolated from a cDNA library of adult male lung (Kawai *et al.*, 2001). To characterize the expression pattern of the two genes, we measured their expression in mouse adult tissues by real time reverse transcriptase–polymerase chain reaction (RT–PCR). *Tprg* was specifically expressed in skin, and to a much lesser extent in tongue and esophagus (Figure 2a). In contrast, *Tprgl* was abundantly expressed in all tested tissues (Figure 2b). As previously reported (Yang *et al.*, 1998; Nakamuta and Kobayashi, 2003; Cam *et al.*, 2006), p63 was expressed at high levels in skin, tongue, muscles, and testis, and to a lesser extent in esophagus and heart (Figure 2c). ΔNp63 was the predominant isoform in skin, tongue, and esophagus, whereas TAp63 was highly expressed in muscle and testis (Figure 2d). Thus, Tprg expression correlates with ΔNp63 expression in adult mouse tissues. The *Tprg* gene is predicted to encode for a putative protein of 279 amino acids (aa), whereas the *Tprgl* gene is predicted to encode for a putative protein of 266 aa. Both proteins have clear orthologues in 26 annotated vertebrate genomes, including other mammals, xenopus, chicken, zebrafish, and fugu (Figure 1c), but no homology in non vertebrate genomes, indicating them as clearly vertebrate-specific proteins. The putative Tprg and Tprgl proteins share two highly conserved domains, one in the central region (78% of identity in 32 aa) and the other at the carboxyl-terminus of the protein (76% of identity in 29 aa). No known protein domains were found in their sequences. The central portion of the sequence shares a significant degree of similarity with members of the Sac family of phosphoinositide phosphatases in *Drosophila melanogaster* (Figure S1); however, the similarity occurs within uncharacterized protein domains. Moreover, mouse orthologues of these *Drosophila* genes exist and they do not display any similarity to *Tprg* and *Tprgl*. In the absence of specific antibodies, Tprg protein expression was evaluated by transient transfection of a FLAG-tagged construct. Immunoblotting analysis revealed that FLAG-Tprg protein run at an apparent molecular weight of approximately 36 kDa, consistent with the theoretical molecular weight of the wild-type protein (31 kDa) (Figure 3a). In mouse primary keratinocytes, immunofluorescence with anti-FLAG antibodies revealed that exogenous Tprg protein was localized in the cytoplasm, whereas being absent from the nucleus (Figure 3b). A similar localization was observed in both undifferentiated (0 hour) keratinocytes and in keratinocytes induced to differentiate by Ca^{2+} addition at 8 and 24 hours. Thus, *Tprg* is unlikely to be

Figure 1. ***Tprg* and *Tprgl* are evolutionarily conserved genes.** (**a**) Graphic alignment of the *p63* genomic locus in different species, and comparison with the *p73* genomic region. The indicated genes are conserved in humans, mouse, and fugu. The arrows indicate the direction of each gene. *p63* and *Tprg* are flanking genes that are highly homologous to *p73* and *Tprgl*, respectively, (dark gray arrows). (**b**) Pairwise alignment analysis of mouse Tprg and Tprgl proteins (left panel) and of p63 and of p73 (right panel) by GraphAlign (Spalding and Lammers, 2004). The graph indicates the percentage of identity in each aligned region. (**c**) Multiple protein sequence alignment of Tprg and Tprgl by ClustalW analysis (Thompson *et al.*, 1994), showing a high level of conservation among several vertebrate sequences. Similar or identical aa are highlighted in gray. Identical aa are indicated by ''*''; similar aa conserved in all sequences by '':''; and similar aa not conserved in all sequences by ''.''. Underlined are the two regions with highest degree of identity.

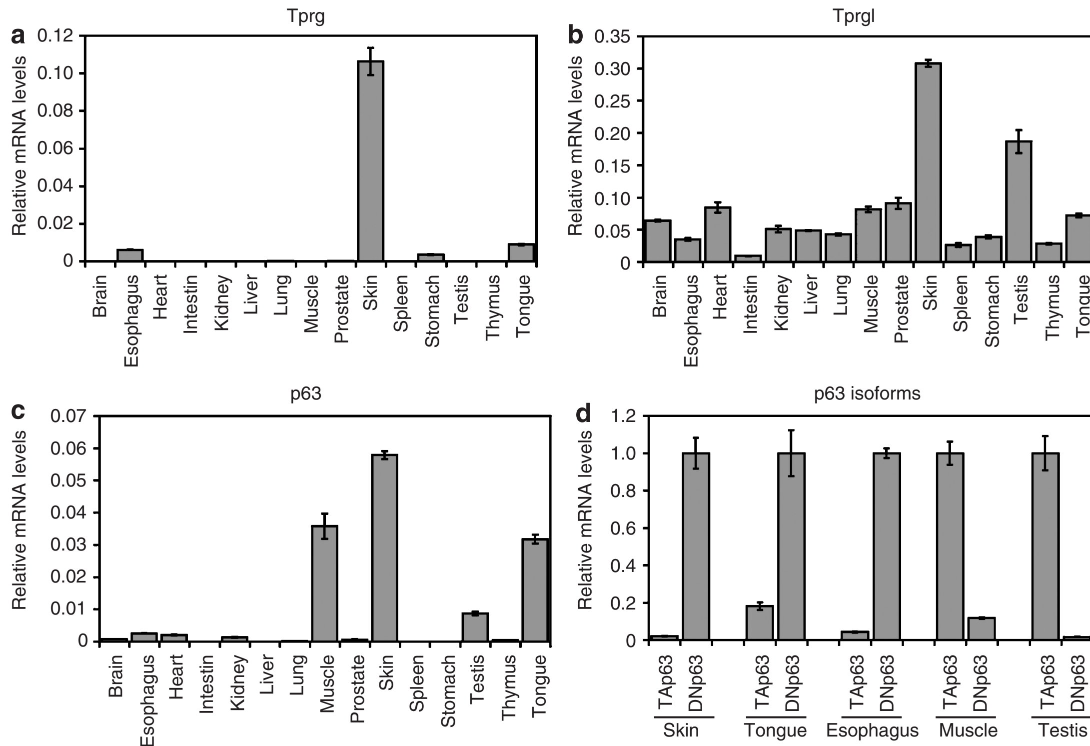

Figure 2. The *Tprg* gene is predominantly expressed in skin. Real-time RT–PCR analysis of *Tprg* (**a**), *Tprgl* (**b**), *p63* (**c**), and TA and ΔNp63 (**d**) expression in total RNA prepared from the indicated adult mouse tissues. Values are expressed as relative arbitrary units, after internal normalization for actin mRNA expression. Each condition was tested in duplicate and the standard error is indicated.

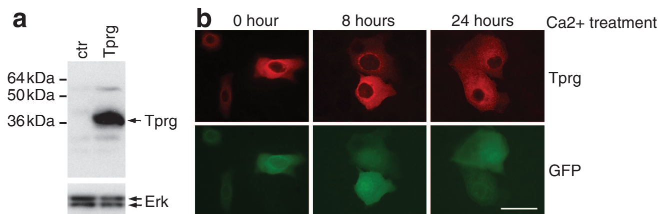

Figure 3. *Tprg* encodes for a cytoplasmic protein. (**a**) Immunoblotting analysis of the FLAG-Tprg protein transiently transfected in human embryonic kidney 293 cells. After 24 hours, cell were lysed in sample buffer and run on 12% SDS–PAGE gels. Proteins were detected with anti-FLAG monoclonal antibodies (upper panel). The molecular weight of the protein standards is indicated. Protein extracts were normalized using anti-ERK polyclonal antibodies (lower panel, ERK). (**b**) FLAG-Tprg was cotransfected with a GFP construct in mouse primary keratinocytes. Cells were treated with Ca^{2+} as a differentiating agent for 8 or 24 hours (8, 24 hours) or left untreated (0 hours). Forty-eight hours after transfection, immunofluorescence analysis was performed using an anti-FLAG-specific antibody (Tprg, upper panel). GFP staining was used to mark transfected cells (GFP, lower panel). FLAG-Tprg localizes selectively to the cytoplasm, whereas GFP is present both in the nucleus and cytoplasm. Bar = 20 μm.

a structural envelope component, or to participate in the desmosome or other cell junction formations, as the over-expressed protein remains soluble in the cytoplasm even upon Ca^{2+} induced differentiation.

Because of its specific expression in skin and its overlap with *p63* expression, we investigated *Tprg* expression in further details. During embryonic development, expression of p63 and basal layer K5 and K14 begins early around embryonic days 8.5–9.5, whereas suprabasal markers start to be expressed at embryonic day 15.5 (Byrne *et al*., 1994; Koster *et al*., 2004). Real-time RT–PCR and *in situ* hybridization analysis revealed that *Tprg* was undetectable until embryonic day 15.5 (Figure 4a, and data not shown). *Tprg* expression was detected at embryonic day 17.5 in the epidermis and in the developing hair follicle (Figure 4b). Stronger expression was obtained in skin 4 days after birth (P4) when *Tprg* was specifically expressed in the epithelial component of the skin, and it was most prominent in the differentiated layers of the epidermis (Figure 4b). A similar pattern of expression was observed in adult mouse skin, whereas in human skin expression was more broadly distributed (Figure S2).

Consistent with the pattern of expression in skin, *Tprg* mRNA was expressed in both human and mouse primary keratinocytes (Figure 4c, and data not shown). Using mouse primary keratinocytes as a model system, we measured the expression levels of *p63, Tprg, K1, and filaggrin* under basal undifferentiated conditions (0), and at different time points upon Ca^{2+}-induced differentiation. *p63* expression was reduced by Ca^{2+} addition, whereas *K1* and *filaggrin* were induced. *K1*, however, returned to basal levels by 24 hours (Figure 4c), consistent with its expression in the spinous layer but not in the granular layer. In contrast, *Tprg* was expressed under basal conditions and was progressively upregulated upon Ca^{2+}-induced differentiation (Figure 4c).

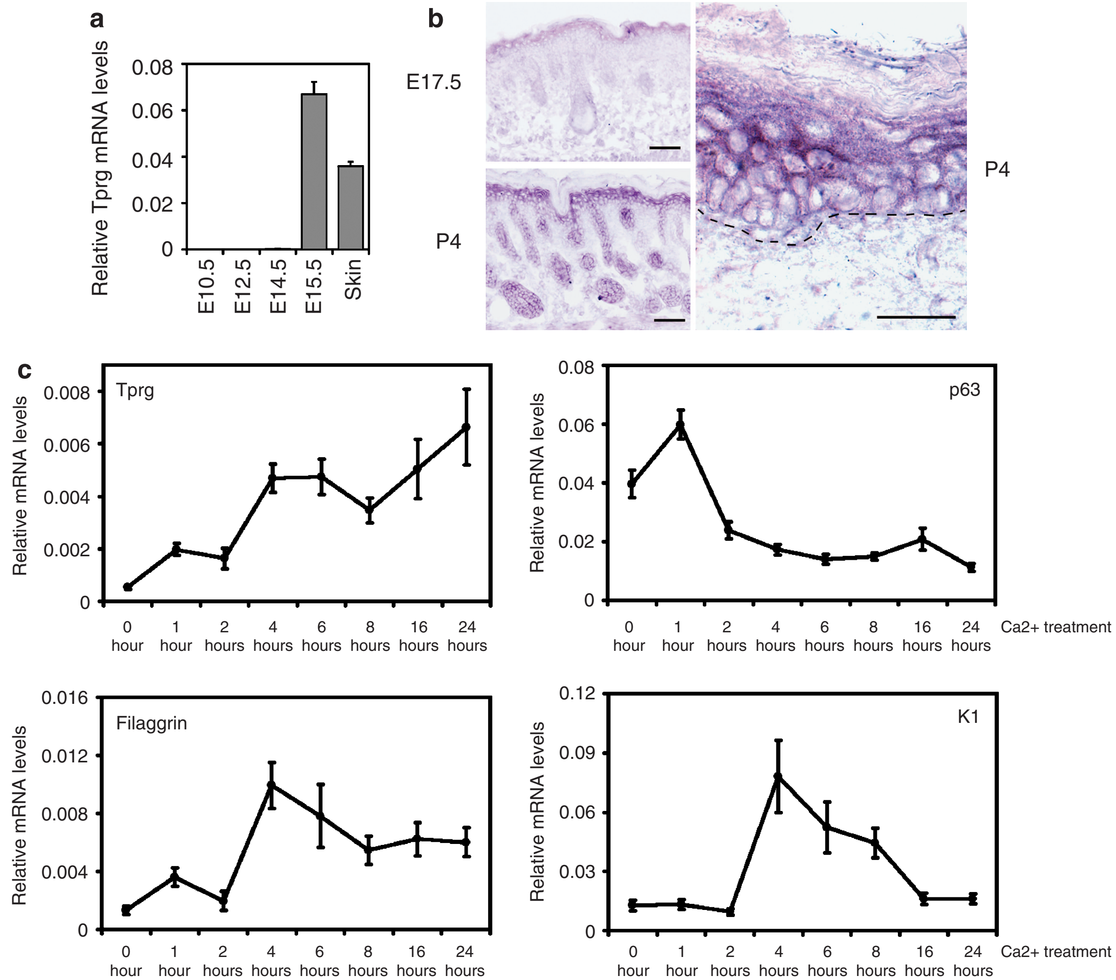

Figure 4. *Tprg* is expressed in the epidermis and in the hair follicle and is more abundant in differentiated keratinocytes. (**a**) Real-time RT–PCR analysis of whole-embryo RNA at different time points during embryonic development or in adult skin as indicated. Values are expressed and normalized as in Figure 3. (**b**) RNA *in situ* hybridization of mouse skin sections at embryonic day 17.5 (upper left panel) and at postnatal day 4 (P4, lower left panel, and right panel) using a digoxygenin-labeled antisense probe for mouse *Tprg*. The dashed line indicates the dermal–epidermal junction. Similar results were observed using an independent probe, whereas a *Tprg* sense probe gave no detectable signal under the same conditions (data not shown). Bars = 50 μm for the left panels, and 10 μm for the right panel. (**c**) Real-time RT–PCR analysis of total RNA extracted from primary mouse keratinocytes at different time points upon Ca^{2+} treatment, as indicated (hours), reveals an induction of the *Tprg* expression upon differentiation, whereas *p63* is modestly upregulated at early time points, and then strongly downregulated. Expression of filaggrin and K1 are shown for comparison. Values are expressed as described in Figure 3.

Given that *Tprg* and *p63* are predominantly expressed in skin, and their temporal patterns of expression in skin, we asked whether p63 might control *Tprg* transcription. To test this possibility, we measured the expression of *Tprg* in p63-knockdown keratinocytes, using previously characterized total *p63*-specific short interfering RNA (siRNA) (Antonini *et al.*, 2006). Forty-eight hours after transfection of *p63* siRNA in mouse primary keratinocytes, a strong reduction of *Tprg* expression was observed (Figure 5a). In contrast, all the other tested genes spanning a genomic region −1.7 Mb + 687 kb from the *p63* gene (Figure 1a) were not affected by p63 knockdown (Figure 5a). Similar results were obtained with a previously characterized independent *p63* siRNA oligonucleotide (data not shown) (Antonini *et al.*, 2006). We then asked which specific p63 isoform controls *Tprg* expression by transfection of isoform-specific siRNA oligonucleotides. Knockdown of the ΔNp63 or the α-isoforms strongly inhibited *Tprg* expression, whereas knockdown of the TA and γ-isoforms was unable to affect *Tprg*, both under basal conditions and upon Ca^{2+} induced differentiation (Figure 5b; Figure S3). Thus, ΔNp63-α is required for proper expression of the *Tprg* gene in mouse primary keratinocytes.

To further investigate the regulation of *Tprg* expression by ΔNp63-α, we infected primary keratinocytes with a retrovirus expressing ΔNp63-α protein fused to an estrogen-receptor domain (ERp63) and maintained under basal conditions in an inactive form (Nguyen *et al.*, 2006). Total RNA was prepared at early time points after ERp63 activation by tamoxifen treatment, and *Tprg* expression was measured by real-time RT–PCR. Upon ΔNp63-α activation, *Tprg* expression was significantly reduced by ERp63 between 40 minutes and 1 hour, suggesting that *Tprg* is likely to be directly regulated

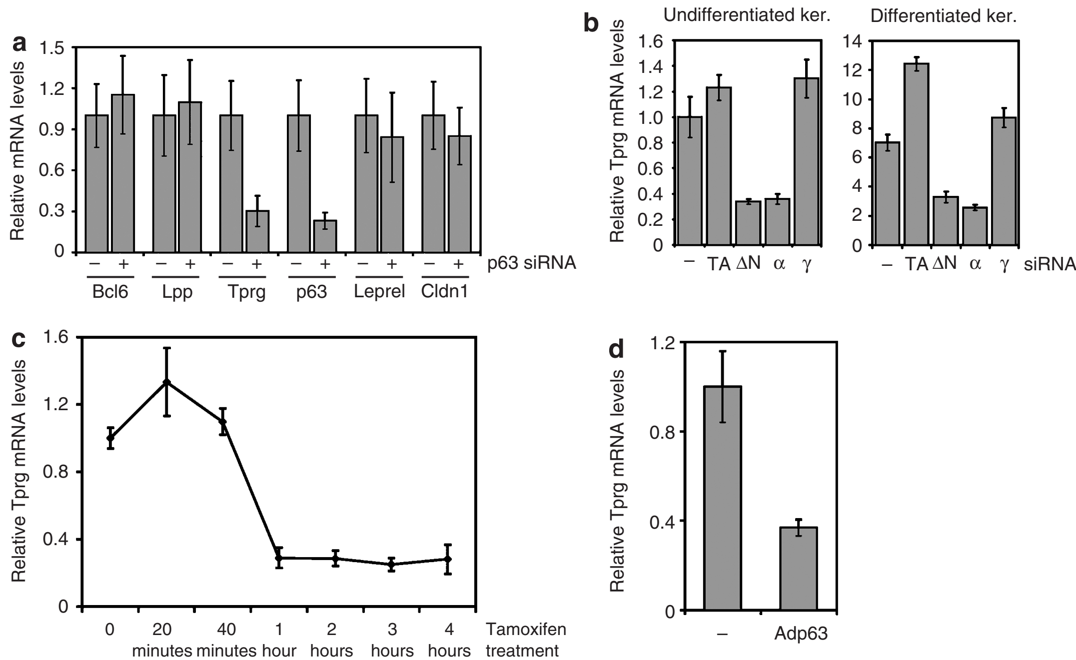

Figure 5. ***Tprg* gene expression is controlled by p63.** (**a**) Real-time RT–PCR analysis of total RNA prepared from primary mouse keratinocytes transfected with siRNA specific for *p63* (+) or with an siRNA-negative control (−) reveals a downregulation of *Tprg* that parallels *p63* expression, whereas the other indicated genes located in the *p63* locus are unaffected. Values for each gene are expressed as fold changes *versus* the siRNA-negative control set to 1. (**b**) Analysis of Tprg expression by real-time RT–PCR of total RNA prepared from primary mouse keratinocytes transfected with siRNA specific for p63α, p63γ, TAp63, and ΔNp63 isoforms, or negative control (**c**), and either grown under basal conditions (undifferentiated ker.) or induced 0.2 mM Ca^{2+} to differentiate by for 24 hours (differentiated ker.). Values are expressed as fold changes *versus* the siRNA-negative control in basal conditions set to 1. Knockdown efficiency and specificity for all p63 isoforms are shown in Figure S3. (**c**) Expression profile of *Tprg* at early time points upon induction of p63 activity. Primary mouse keratinocytes were infected with a retrovirus carrying an ER-ΔNp63-α fusion protein or empty vector control and subsequently treated with 20 nM tamoxifen for the indicated times. Total RNA was used for cDNA preparation followed by real-time RT–PCR. Values are expressed as changes in relative mRNA levels in the ER-p63-expressing *versus* control keratinocytes. (**d**) Down-modulation of *Tprg* mRNA expression by ΔNp63-α. Primary mouse keratinocytes were infected with a recombinant adenovirus expressing ΔNp63-α or a control GFP-expressing adenovirus (**c**) for 24 hours. *Tprg* mRNA levels were quantified by real-time RT–PCR. Values are expressed as relative arbitrary units, after internal normalization for GAPDH. GAPDH, glycerladehyde-3-phosphate dehydrogenase.

by p63 (Figure 5c). Interestingly, ΔNp63-α activation resulted in *Tprg* downregulation rather than induction. ΔNp63-α activation is unlikely to repress *Tprg* due to a generic squelching effect, in agreement with a global gene expression analysis, which revealed that most genes that are repressed by p63 activation are induced by p63 knockdown (Giusy Della Gatta and Caterina Missero, in preparation). A similar inhibition of *Tprg* expression was observed in primary keratinocytes infected with an adenovirus expressing ΔNp63-α (Figure 5d), suggesting that Tprg expression is finely regulated by ΔNp63-α in keratinocytes.

Regulation of *Tprg* expression by p63 could either occur directly through p63 binding to a *Tprg*-regulatory sequence or could be mediated by other mechanisms. To identify potential p63-binding sites in the *Tprg* gene, we examined the entire genomic region containing the *Tprg* locus for p63-binding sites, using a recently performed genome-wide chromatin immunoprecipitation (ChIP)-on-chip analysis in human carcinoma cells (Yang *et al.*, 2006). The *Tprg* gene spans 151 kb in the human genome and has five introns. Interestingly, two genomic regions located in human *Tprg* intron 4 displayed p63-binding activity with a significant binding score, whereas no p63 binding could be detected in the *Tprg* putative proximal promoter or in upstream regions (up to 50 kb from the transcription start site). Both genomic regions identified are conserved throughout evolution (Figure 6a, and data not shown); however, only one displays a p53/p63 consensus sequence that is conserved between human and chicken (Figure 6b). We proceeded to test whether p63 could bind *in vivo* to the latter. ChIP was performed using anti-p63-specific antibodies in human primary keratinocytes, and amplifying three sequences approximately 500 bp apart (Figure 6a). Interestingly, p63 specifically bound to the sequence corresponding to the most highly conserved region and containing the conserved p63-binding site (Figure 6c). A strong binding was obtained with the corresponding mouse sequence in mouse primary keratinocytes, where p63 bound the *Tprg*-binding site to a similar extent of as that of a high-affinity long-distance enhancer (C40 enhancer; Antonini *et al.*, 2006; Yang *et al.*, 2006; Figure 6d). This genomic region is likely to contain a functional p63-binding site and also to function as an enhancer. We tested this possibility by cloning the conserved genomic region containing the binding site upstream of a thymidine kinase (TK) minimal promoter driving the expression of a luciferase gene. Transient transfection assays in HeLa cells revealed that the *Tprg*

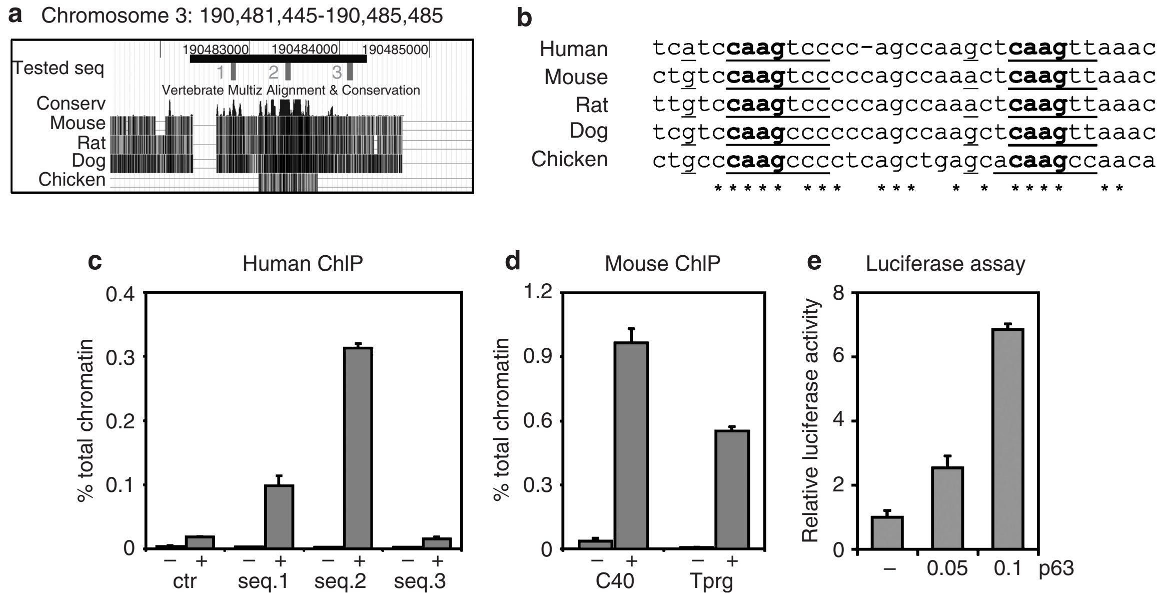

Figure 6. p63 binds to a highly conserved *Tprg* intronic region. (**a**) A 2-kb genomic region located in intron 4 and previously found to bind p63 by ChIP-on-chip (Yang *et al.*, 2006) is shown (horizontal black bar). Three sequences were selected for amplification to test p63-binding in human and mouse primary keratinocytes (Tested seq 1–3). The degree of evolutionary conservation is represented by vertical black bars and peaks. (**b**) A conserved p63-binding site is located within region 2. The predicted p63-binding site is indicated together with its precise nucleotide sequence: bold nucleotides correspond to the core nucleotide sequence required for p63-binding (el-Deiry *et al.*, 1992; Barbieri *et al.*, 2005; Ihrie *et al.*, 2005; Antonini *et al.*, 2006; Nguyen *et al.*, 2006; Yang *et al.*, 2006), while underlined nucleotides are matches in the consensus. Nucleotides identical in all species are indicated by *. (**c**) Specific binding of endogenous p63 to the human *Tprg* regions. Human primary keratinocytes under growing conditions were processed for ChIP with antibodies specific for p63 (+), or unrelated anti-ERK1 antibodies as control (−), followed by real-time PCR amplification of various regions of the *Tprg* intronic sequences indicated in the schematic above. Unprecipitated chromatin preparations were similarly analyzed and used as ''input'' control. The amount of precipitated DNA was calculated relative to the total input chromatin, and expressed as the percentage of the total (Frank *et al.*, 2001). (**d**) Specific binding of endogenous p63 to the mouse *Tprg* region corresponding to the human conserved binding site (Tprg) and to the previously characterized C40 enhancer used as control (Antonini *et al.*, 2006). ChIP was performed as described in (**c**). (**e**) The *Tprg* enhancer is responsive to p63. The *Tprg* putative enhancer region was cloned in front of a TK minimal promoter that drives the expression of the luciferase reporter. The construct was transiently transfected into HeLa cells in the absence (−) or in the presence of various concentrations of an expression construct for ΔNp63-γ as indicated (in μg). The activity of the enhancer was measured by luciferase assays and values are expressed relative to (−) set to 1.

enhancer activity was positively regulated by p63 in a dose-dependent manner (Figure 6e). Thus, p63 controls *Tprg* gene expression by directly binding to a previously unreported highly conserved long distance enhancer located at approximately 100 kb from the putative *Tprg* transcription start site.

DISCUSSION

Tprg was identified here as a previously uncharacterized gene physically located upstream of the *p63* gene in the vertebrate genomes, whose expression is regulated by p63. *Tprg* encodes for a protein highly homologous to the one encoded by the *Tprgl* gene, located upstream of the *p73* gene. A *Tprg*/*Tprgl* ancestor gene is absent in invertebrates, including in the invertebrate chordate *Ciona intestinalis* (Dario Antonini and Caterina Missero, unpublished observations). Given the similar percentage of identity at the protein level between *Tprg*/*Tprgl* and *p63*/*p73*, and the absence of genes homologous to *Tprg* in non-vertebrate genomes, we propose that *Tprg* and *Tprgl* emerged during invertebrate-to-vertebrate transition of the *p53* family, arising from the segmental duplication of the p63/p73 locus in vertebrates.

The proteins encoded by *Tprg* and *Tprgl* are highly conserved throughout the vertebrate lineage, with long stretches of aa showing very high conservation across both *Tprg* and *Tprgl* from fish to human. Surprisingly, however, these proteins do not contain any known protein domains. Even the most conserved stretches of amino-acid sequence (the DHWNNE ''signature'' around amino acid 110 and the KYDF stretch at amino acid 130) are not explained by any specific protein feature. Some similarity is observed with some phosphoinositide phosphatases, and it would be tempting to speculate a connection between the role of these proteins in Ca^{2+} mobilization from intracellular stores, or intracellular trafficking. The conservation, however, is poor, and lies outside of any known protein domains. Moreover the similarity to this family of proteins is more significant in lower organisms such as *Drosophila* and *Aspergillus*, despite the fact that these proteins do exist in mammalian genomes. Thus, this significance of this similarity will require further investigation.

We demonstrate here that p63 is required for *Tprg* expression in skin, and that p63 exerts a direct control by binding in human as well as in mouse to a genomic region in *Tprg* intron 4, which acts as a p63-dependent enhancer. Genome-wide analysis of p63-binding regions by ChIP-on-chip demonstrated that on average p63-binding sites are

38.6% nucleotide-identical between human and mouse (Yang *et al*., 2006). The p63-binding site in the *Tprg* intron 4 is conserved in mammals and in chicken, and is more than 90% identical between human and mouse, thus being among the most conserved binding regions identified to date. Interestingly, we identify that p63 binds to several other highly conserved binding sites in the genomic region encompassing *Tprg* and *p63* (Antonini *et al*., 2006; Yang *et al*., 2006, and Dario Antonini and Caterina Missero, unpublished data), and their functional relevance will require further investigation.

Although p63 is required for *Tprg* expression, *Tprg* starts to be expressed in embryonic skin much later than p63, and unlike p63, it is more abundant in differentiated keratinocytes, at least in the developing and newborn skin, suggesting either that other transcription factors may be involved in *Tprg* expression in the suprabasal compartment, or that a balance between different p63 isoforms may trigger *Tprg* expression. The expression and putative function of the various p63 isoforms in skin is controversial. It has been proposed that during embryogenesis ΔNp63-α is required to counterbalance the inhibitory effect of TAp63-α on terminal differentiation (Koster *et al*., 2004). However, ΔNp63 isoforms are highly expressed even before epidermal stratification, whereas the TAp63 isoforms are expressed at very low levels (Laurikkala *et al*., 2006). Similarly, in normal human and mouse epidermis, ΔNp63-α is the most abundant p63 splice variant, whereas very weak expression of TAp63-α and ΔNp63-γ is detected at the RNA but not at the protein level (Bamberger *et al*., 2002, 2005). In primary keratinocytes, ΔNp63-α is readily detectable at the protein level under proliferating conditions, and declines upon Ca^{2+}-induced differentiation. The onset of differentiation in human keratinocytes does not change the ratio of two other very weakly expressed isoforms (Bamberger *et al*., 2002). In mouse keratinocytes, it has been reported that the TAp63-γ isoform is induced upon Ca^{2+} addition (King *et al*., 2006), although under our culture conditions, we could not detect any significant change in TAp63 expression during differentiation (data not shown). Our knockdown studies clearly demonstrate that *Tprg* expression is dependent on ΔNp63-α, whereas the TAp63 and p63-γ isoforms do not alter *Tprg* expression under proliferating or differentiating conditions. Consistent with these data, *Tprg* expression in the adult mouse follows the tissue distribution of ΔNp63, whereas it is not expressed in tissues where TAp63 is abundant.

In contrast to *p63*, *Tprg* expression in skin and in isolated keratinocytes is higher in differentiated keratinocytes than in basal keratinocytes. Accordingly, p63 is required for *Tprg* expression in keratinocytes (Figure 5a and b), but high levels of p63 results in *Tprg* downregulation (Figure 5c and d), suggesting that optimal *Tprg* expression may require levels of p63 expression lower than those present in the basal layer. Thus, ΔNp63-α finely regulates *Tprg* expression possibly in conjunction with other transcription factors.

In conclusion, we have identified *Tprg* as a gene, predominantly expressed in skin, likely to be co-regulated with its adjacent gene *p63*. We have shown that p63 participates directly in the transcriptional control of *Tprg* expression in skin. *Tprg* gene and its paralogue *Tprgl* encode for proteins, which are specific to the vertebrate lineage and highly conserved in sequence; however, their functions will require further investigation.

MATERIALS AND METHODS

Cell cultures, transfections, and reporter assays

Mouse primary keratinocytes were isolated from 2-day-old Swiss CD1 mice and cultured under low-Ca^{2+} conditions (0.05 mM) in the presence of 4% Ca^{2+}-chelated fetal bovine serum (Invitrogen, Carlsbad, CA), and epidermal growth factor (Invitrogen), as previously described (Antonini *et al*., 2006). Terminal differentiation was induced by addition of 0.2 mM calcium chloride to the medium. Human primary keratinocytes were kindly provided by Dr GP Dotto, and cultured in keratinocyte-serum-free medium medium supplemented with bovine pituitary extracts and epidermal growth factor (Invitrogen). Human embryonic kidney 293 and HeLa cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. All cell types were transfected using Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol. Confluent mouse primary keratinocytes were transfected 5 days after plating. Keratinocytes in 60-mm dishes were transfected with 2 μg of pCMV2-FLAG-*Tprg* and pCMV2-FLAG (control) together with 0.2 μg of pCMV–GFP (green-fluorescent protein; Clontech, Palo Alto, CA), or with 200 mM of stealth siRNA for mouse p63-α, -γ, ΔNp63, Tap63, or an siRNA recognizing all isoforms (Antonini *et al*., 2006; Supplementary Material), or a control medium GC-rich stealth siRNA (Invitrogen).

HeLa cells in 12-well dishes were transfected with a construct carrying the *Tprg* enhancer and the TK minimal promoter driving the expression of the luciferase gene (see below) (0.25 μg); the ΔNp63-γ (0.05 and 0.1 μg); and CMV–*Renilla* (0.02 μg; Promega, Madison, WI). Luciferase activity was determined 48 hours after transfection with the dual-luciferase reporter assay kit (Promega). *Renilla* luciferase activity was used to normalize transfection efficiency.

Plasmids and constructs

For the retrovirus expressing inducible p63, a modified ER ligand-binding domain (Littlewood *et al*., 1995) was cloned in frame between the FLAG epitope and the ΔNp63-α cDNA lacking the first ATG, and inserted into the *Hin*dIII–*Not*I sites under the control of the CMV promoter in the PINCO retroviral vector (Nocentini *et al*., 1997). The pCMV2-FLAG Tprg expression vector was obtained by amplifying the putative coding sequence of *Tprg* lacking the ATG from mouse primary keratinocyte cDNA using the *Pfu*I polymerase (Stratagene, La Jolla, CA) with specific oligonucleotide primers (see Supplementary Material), and by cloning it in frame in pCMVFLAG2 (Sigma, St. Louis, MO) in *Not*I–*Xba*I. The construct was sequence verified and tested by immunoblotting for its ability to encode for a protein. The *Tprg* enhancer sequence (597 bp) was amplified by PCR from mouse genomic DNA using specific oligonucleotide primers (see Supplementary Material), and cloned in the pGL3-TK-Luc construct (Ohno *et al*., 1999) at the *Kpn*I site. The enhancer sequence was verified by sequencing.

Real-time RT–PCR and microarray

Mouse embryos and adult tissues derived from adult CD1 female mice were snap frozen, pulverized, and dissolved in TRIzol reagent

(Invitrogen) for RNA preparation according to manufacturer's protocol. RNA samples were treated with RNase-free DNaseI (Promega). cDNA was synthesized using Superscript III (Invitrogen). Two-step real-time RT–PCR was performed using the SYBR Green PCR core kit (Applied Biosystems, Foster City, CA). Expression of the endogenous *Tprg* transcript, as well as of the other target genes, was quantified using specific oligonucleotide primers (see Supplementary Material).

Immunostaining and immunoblotting

Forty-eight hours after transfection, mouse primary keratinocytes were fixed in methanol for 5 minutes at $-20\,^{\circ}C$, washed in phosphate-buffered saline (PBS), and permeabilized in 0.2% Triton/PBS. Fixed cells were incubated with anti-FLAG biotinylated antibodies (BioM2, 1:100; Sigma) in 0.1% Triton/5% goat serum/PBS for 1 hour at room temperature. After extensive washing in 0.1% Triton/PBS, cells were incubated with streptavidin Cy3 (1:100; Sigma), washed, and stained with 4′,6-diamidino-2-phenylindole at $100\,ng\,ml^{-1}$ in PBS. Slides were mounted using Vectashield as mounting reagent (Vector Laboratories, Burlingame, CA), and examined under an Axioplan imaging microscope (Zeiss, Micro Imaging, Thornwood, NY).

For immunoblotting, human embryonic kidney 293 cells plated in 60-mm dishes were transfected with 4 μg of pCMV2-FLAG *Tprg* or empty vector as control. Cells were lysed in sample buffer 24 hours after transfection. Protein extracts (10 μg) were run on 12% SDS–PAGE and transferred on an Immobilon-P transfer membrane (Millipore, Bedford, MA). The membrane was probed with anti-Flag monoclonal antibodies (M5, Sigma), or with anti-extracellular signal-regulated kinase (ERK) antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) as loading control.

In situ hybridization

Skin was fixed in 4% fresh paraformaldehyde overnight at $4\,^{\circ}C$, washed in PBS, incubated in 30% sucrose/PBS overnight at $4\,^{\circ}C$, and embedded in OCT compound (Sakura, Torrance, CA). *In situ* hybridization was performed as described previously (Brancaccio *et al.*, 2004). Hybridization was performed with $1\,\mu g\,ml^{-1}$ of digoxygenin-labeled *Tprg* cRNA probe corresponding to a cDNA fragment of 511 bp of coding sequence (see Supplementary Material), and cloned into pCR2.1-TOPO vector. Antisense and sense probes were transcribed from the T3 and T7 promoters, respectively, using a digoxygenin labeling kit (Roche, Basel, Switzerland) as described by manufacturer's instructions. Mice were housed and treated according to the guidelines of the local Institutional Animal Care and Use Committee. This study was conducted under approval of the Institutional Review Board and according to the Declaration of Helsinki Principles. Written informed consent was obtained from the human skin donors.

Chromatin immunoprecipitation

Approximately 3×10^{6} mouse keratinocytes were fixed with 1% formaldehyde in growth medium at $37\,^{\circ}C$ for 10 minutes. Extracts were extensively sonicated on ice to obtain DNA fragments ranging from 200 to 800 bp in length. Chromatin was immunoprecipitated following the Upstate protocol (http://www.upstate.com). Immunoprecipitation was performed using anti-p63 (H-137; Santa Cruz Biotechnology) and anti-ERK-1 (K23; Santa Cruz Biotechnology) antibodies. Real-time PCR was performed using the SYBR Green PCR master mix in an ABI PRISM 7000 (Applied Biosystems), using specific oligonucleotide sequences (see Supplementary Material).

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

This manuscript is dedicated to the memory of Parvesh Mahtani. We thank Tommaso Russo and Nicola Zambrano for critical reading of the manuscript and Diego Di Bernardo for helpful discussion. We also thank Annie Yang and co-workers for sharing ChIP-on-chip data before publication. DA is a PhD student at the European School of Molecular Medicine (SEMM), LDR is a PhD student at Genetics and Molecular Medicine of the University ''Federico II'', and GDG is a PhD student in Medical Genetics of the Second University of Napoli, Italy. This work was supported by grants from the European Union (LSHG-CT-2004-511990), the Italian Telethon Foundation (GGP06243), and the NIH (AR-01-004).

SUPPLEMENTARY MATERIAL

Supplementary Materials.

Figure S1. The TPRG central hydrophobic domain shares a significant degree of similarity with an uncharacterized region of the phosphoinositide polyphosphatases of the Sac family.

Figure S2. *Tprg* expression in mouse and human adult skin.

Figure S3. Knockdown of the p63-specific isoforms in mouse primary keratinocytes under basal and differentiating conditions.

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