Sorting Out the p63 Signaling Network

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p63 is a transcription factor required for normal epidermal development and differentiation. Because of the complexity of these processes, p63 is expected to regulate a myriad of target genes, providing impetus to many laboratories to identify these genes. p63 target genes have been shown to encode a diverse group of proteins, including structural proteins, proteins that control cell cycle withdrawal, and proteins that regulate the epidermal differentiation program. In this issue, Antonini *et al.* describe a novel p63 target gene whose evolutionary conservation suggests a critical role for this gene in the epidermis.

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Epidermal morphogenesis begins during embryonic development when cells of the single-layered surface ectoderm, which initially covers the developing embryo, adopt an epidermal fate and differentiate into actively proliferating basal keratinocytes (reviewed in Koster and Roop, 2007). The first morphological sign of stratification occurs when these basal keratinocytes give rise to a suprabasal cell layer. Cells in this so-called intermediate layer undergo a few rounds of cell division before they permanently withdraw from the cell cycle and become spinous keratinocytes. During subsequent stages of epidermal morphogenesis, spinous keratinocytes differentiate into cells of the granular and cornified cell layers, the latter of which are primarily responsible for the barrier function of the epidermis. The end point of the terminal keratinocyte differentiation program is the shedding of the dead and enucleated keratinocytes of the cornified cell layers into the environment. The embryonic differentiation program described above is maintained, with few modifications, during postnatal development. This program is essential to the continuous replacement of cells that are lost and to the maintenance of epidermal tissue architecture.

One striking difference between the terminal differentiation programs executed during embryogenesis and those executed postnatally is the mechanism by which the spinous layer is formed. Whereas this layer develops from intermediate keratinocytes during embryonic skin development, basal keratinocytes directly differentiate into spinous keratinocytes in postnatal epidermis. In addition to generating the epidermis, basal keratinocytes also produce and secrete crucial components of the basement membrane, the dermal-epidermal interface, which anchors epidermal keratinocytes to the dermis. Finally, basal keratinocytes participate in epithelial-mesenchymal interactions that are required for the formation of epithelial appendages, including teeth, hair follicles, mammary glands, and limbs.

A key role for the transcription factor p63 in the above-described processes was first inferred from the phenotype of p63-deficient mice. These mice do not develop stratified epithelia or epithelial appendages (Yang *et al.*, 1999; Mills *et al.*, 1999). Instead of an epidermis, these mice are covered with a single-layered epithelium that resembles the surface ectoderm that covers the early embryo, suggesting that loss of p63 leads to obstruction of keratinocyte commitment and differentiation and thus to aborted skin development. As a consequence, p63-deficient mice die shortly after birth owing to uncontrolled water loss caused by a lack of skin barrier function.

The p63 gene encodes six different proteins, each of which can function as a transcriptional activator or transcriptional repressor. Because p63-deficient mice lack expression of all six proteins, it is not possible to determine the isoform(s) required for normal skin and appendage development using this animal model. In late embryonic and postnatal epidermis, $\Delta Np63\alpha$ is the predominantly expressed p63 isoform, whereas expression of the other isoforms is barely detectable (Yang et al., 1998). To determine the contribution of $\Delta Np63\alpha$ to epidermal development and differentiation, two different groups downregulated $\Delta Np63$ expression in the epidermis using different approaches. We used an epidermalspecific promoter to generate transgenic mice that inducibly express a hairpin sequence consisting of sequences unique to the $\Delta Np63$ isoforms. The hairpin was designed to be processed into short interfering RNA (siRNA) sequences that subsequently degrade endogenous $\Delta Np63$ transcripts (Koster et al., 2007). Truong and colleagues (2006) took an in vitro approach by downregulating $\Delta Np63$ expression in human primary keratinocytes using an siRNA specific for $\Delta Np63$. These keratinocytes were then placed into organotypic cultures to allow them to regenerate epidermis. In both the transgenic mouse epidermis and the human skin equivalents, the spinous layer failed to develop properly, as demonstrated by a delay in the expression of a marker of the spinous layer, keratin 1 (K1). As a consequence, subsequent stages of terminal differentiation failed to proceed normally. The data summarized above clearly highlight the importance of $\Delta Np63\alpha$ in epidermal differentiation.

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COMMENTARY

How does $\Delta Np63\alpha$ regulate epidermal development and differentiation? One approach to obtaining insight into the p63 regulatory network is to identify genes whose expression is controlled by p63. Although several groups have performed microarray or chromatin immunoprecipitation (ChIP)-on-chip experiments to address this question, the use of cell lines rather than primary cultures or tissue extracts to perform these high-throughput experiments has limited their biological significance. Nevertheless, several downstream effectors of $\Delta Np63\alpha$ have been identified.

Most recently, Antonini and colleagues identified a novel target gene of Δ Np63 that they have named *Tprg* (transformation-related protein 63 regulated) (Figure 1). Unlike Δ Np63 α , *Tprg* is predominantly expressed in the suprabasal layers of the epidermis. Although the role of *Tprg* in epider-

An emerging network of signaling pathways controlled by p63.

mal development and differentiation remains unknown, the expression pattern of *Tprg* suggests a role for this gene in epidermal differentiation. In further support of this hypothesis, Tprg transcripts are first detected at the developmental stage when terminal differentiation of the epidermis initiates. Although a role for Tprg in epidermal terminal differentiation appears plausible, neither the structure nor the subcellular localization of Tprg provides any clear hints of its precise function. The extensive variety of processes that must occur during terminal differentiation leaves the possibilities open. For example, Tprg may be involved in cell cycle withdrawal, the regulation of structural proteins, and/or barrier formation. Finally, the evolutionary conservation of Tprg suggests a critical role for this protein in the epidermis. However, future experimental approaches, such as the germline deletion of Tprg, will be required to reveal its exact function.



Figure 1. $\Delta Np63\alpha$ **regulates the expression of a myriad of target genes.** These target genes encode proteins that have different functions in the epidermis. For example, within the basal layer, $\Delta Np63\alpha$ induces expression of Fras1, a critical component of the basement membrane. Another structural protein induced by $\Delta Np63\alpha$ is Perp, a desmosomal protein that is essential for keratinocyte adhesion. In addition to structural proteins, $\Delta Np63\alpha$ induces the expression of IKK α , a protein that is required for cell cycle withdrawal during spinous layer development. Finally, a novel $\Delta Np63\alpha$ target gene, *Tprg*, is expressed in the suprabasal layers of the epidermis and may have a role in epidermal terminal differentiation.

In addition to Tprg, $\Delta Np63\alpha$ also regulates the expression of other genes involved in epidermal terminal differentiation. One of these genes is $lkk\alpha$, which is a direct transcriptional target of $\Delta Np63\alpha$ in the developing epidermis (Koster et al., 2007) (Figure 1). The importance of IKKα in epidermal morphogenesis is highlighted by the phenotype of mice lacking ΙΚΚα. In ΙΚΚα-deficient mice, the intermediate cell layer forms normally. However, in the absence of IKKα, intermediate keratinocytes fail to withdraw from the cell cycle and the spinous layer therefore fails to develop. The consequent failure of the granular and cornified cell layers to develop results in compromised barrier function and early postnatal lethality. In addition to the defects in epidermal morphogenesis, development of epithelial appendages, including teeth and limbs, is aborted in IKKa-deficient mice. These defects are a direct consequence of the failure of the underdeveloped epidermis to participate in the epithelial-mesenchymal interactions required for appendage development (Sil et al., 2004). Whereas IKK α is primarily responsible for cell cycle withdrawal during keratinocyte terminal differentiation, $\Delta Np63\alpha$ also induces markers of terminal differentiation, such as K1. For example, $\Delta Np63\alpha$ was found to synergize with Notch signaling components to initiate K1 expression (Nguyen et al., 2006).

In addition to the induction of genes that are required for epidermal differentiation, $\Delta Np63\alpha$ directly induces the expression of genes required for maintaining the structural integrity of the skin. This includes Fras1, a basement membrane component, and Perp, a desmosomal component that is important for cell-cell adhesion (Ihrie *et al.*, 2005; Koster *et al.*, 2007) (Figure 1). Thus, it comes as no surprise that mice with reduced $\Delta Np63$ expression in the epidermis develop fragile skin that is easily injured, resulting in large skin erosions (Koster *et al.*, 2007).

Together, the data summarized above establish an emerging network of signaling pathways controlled by $\Delta Np63\alpha$. Due to the complexity of the p63 signaling network, highthroughput approaches may prove to be less useful in identifying components of the pathway that are active only at a certain stage of epidermal development or differentiation. That said, ongoing efforts to identify novel $\Delta Np63\alpha$ target genes are needed to further clarify the various complex roles of this intriguing transcription factor in the epidermis.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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COMMENTARY

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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, Transformationrelated 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 downregulation. 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.

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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

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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

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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 carboxylterminus 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, $\Delta Np63-\alpha$ 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, $\Delta Np63-\alpha$ 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 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 $\Delta Np63-\alpha$ 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). $\Delta Np63$ 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 $\Delta Np63$ 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

D Antonini et al. *Tprg* Is a p63 Target Gene



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 ".". 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 Δ Np63 (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 overexpressed 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 Ca²⁺-induced differentiation. *p63* expression was reduced by Ca²⁺ 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 Ca²⁺-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 Ca²⁺ 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 p63knockdown 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 p63knockdown (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 Δ Np63 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 Ca²⁺ induced differentiation (Figure 5b; Figure S3). Thus, Δ Np63- α is required for proper expression of the *Tprg* gene in mouse primary keratinocytes.

To further investigate the regulation of *Tprg* expression by Δ Np63- α , we infected primary keratinocytes with a retrovirus expressing Δ Np63- 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 Δ Np63- 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 $p63\alpha$, $p63\gamma$, TAp63, and Δ Np63 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- Δ Np63- 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 Δ Np63- α . Primary mouse keratinocytes were infected with a recombinant adenovirus expressing Δ Np63- 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, $\Delta Np63-\alpha$ activation resulted in *Tprg* downregulation rather than induction. $\Delta Np63-\alpha$ 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 $\Delta Np63-\alpha$ (Figure 5d), suggesting that Tprg expression is finely regulated by $\Delta Np63-\alpha$ 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 p63binding 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 highaffinity 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 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 Δ Np63- γ as indicated (in µg). The activity of the enhancer (–) set

enhancer activity was positively regulated by p63 in a dosedependent 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 $\dot{C}a^{2\,+}$ 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 $\Delta Np63-\alpha$ is required to counterbalance the inhibitory effect of TAp63-α on terminal differentiation (Koster et al., 2004). However, $\Delta Np63$ 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, $\Delta Np63-\alpha$ is the most abundant p63 splice variant, whereas very weak expression of TAp63- α and Δ Np63- γ is detected at the RNA but not at the protein level (Bamberger et al., 2002, 2005). In primary keratinocytes, $\Delta Np63-\alpha$ is readily detectable at the protein level under proliferating conditions, and declines upon Ca²⁺-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 $\Delta Np63-\alpha$, 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 $\Delta Np63$, 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, Δ Np63- 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-Ca²⁺ conditions (0.05 mM) in the presence of 4% Ca2+-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 , Δ Np63, 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 Δ Np63- 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 ligandbinding domain (Littlewood et al., 1995) was cloned in frame between the FLAG epitope and the $\Delta Np63-\alpha$ cDNA lacking the first ATG, and inserted into the HindIII-Notl 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 Pful 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 Notl-Xbal. 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 Kpnl 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 DNasel (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 °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 $4 \mu g$ of pCMV2-FLAG *Tprg* or empty vector as control. Cells were lysed in sample buffer 24 hours after transfection. Protein extracts ($10 \mu g$) 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°C, washed in PBS, incubated in 30% sucrose/PBS overnight at 4 °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 $1 \mu g m l^{-1}$ 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.

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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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