Functional interplay between p63 and p53 controls RUNX1 function in the transition from proliferation to differentiation in human keratinocytes

I Masse*1, L Barbollat-Boutrand1, M Molina1, O Berthier-Vergnes1, N Joly-Tonetti1, MT Martin2, C Caron de Fromentel3, J Kanitakis4 and J Lamartine*1

The interfollicular epidermis is continuously renewed, thanks to a regulated balance between proliferation and differentiation. The ΔNp63 transcription factor has a key role in the control of this process. It has been shown that ΔNp63 directly regulates Runx-related transcription factor 1 (RUNX1) transcription factor expression in mouse keratinocytes. The present study showed for the first time that RUNX1 is expressed in normal human interfollicular epidermis and that its expression is tightly regulated during the transition from proliferation to differentiation. It demonstrated that ΔNp63 directly binds two different RUNX1 regulatory DNA sequences and modulates RUNX1 expression differentially in proliferative or differentiated human keratinocytes. It also showed that the regulation of RUNX1 expression by ΔNp63 is dependent on p53 and that this coregulation relies on differential binding and activation of RUNX1 regulatory sequences by ΔNp63 and p53. We also found that RUNX1 inhibits keratinocyte proliferation and activates directly the expression of KRT1, a critical actor in early keratinocyte differentiation. Finally, we described that RUNX1 expression, similar to ΔNp63 and p53, was strongly expressed and downregulated in basal cell carcinomas and squamous cell carcinomas respectively. Taken together, these data shed light on the importance of tight control of the functional interplay between ΔNp63 and p53 in regulating RUNX1 transcription factor expression for proper regulation of interfollicular epidermal homeostasis.

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The epidermal homeostasis relies on proper control of proliferation and differentiation programs within epidermal cells. This differentiation process requires a precise coordination of the molecular events maintaining functional epidermis. p63 belongs to the p53/p63/p73 family of transcription factors (TFs) that share a conserved DNA-binding domain and similar DNA target sites in regulatory sequences of eukaryotic genes. p63 is essential for the development and maintenance of pluristratified epithelia, including epidermis. Mice lacking p63 display profound defects in epidermis formation and died shortly after birth due to dehydration. p63 is also critical for postnatal proliferation in basal keratinocytes and epidermal differentiation. Several p63 proteins have been described, resulting from two distinct promoters, TAp63 and ΔNp63, and from alternative mRNA splicing at the 3’ end of the gene. The major isoform present in adult human epidermis is ΔNp63x, expressed at high levels in the basal layer and at lower levels in suprabasal keratinocytes. p63 is overexpressed in many epithelial tumors, namely basal cell carcinoma (BCCs) and squamous cell carcinomas (SCCs) and is mutated in 8% of head and neck SCCs. Runx-related transcription factor 1 (RUNX1) has been identified among the direct p63 transcriptional targets in epidermis. RUNX1 belongs to the RUNX TFs family, which have an essential role in the balance between cell proliferation and differentiation in animal tissue development. RUNX family genes share a region, the Runt domain, which, with its partner subunit core-binding factor beta (CBFβ), is responsible for specific DNA binding and heterodimerization and activates or represses transcription through the recruitment of various coactivators and corepressors depending on the cellular context. The RUNX genes also have a complex role in cancer, acting as either tumor suppressors or dominant oncogenes. RUNX1 is a master regulator in the development, growth and differentiation of hematopoietic, muscular and nerve cells. In mouse skin, RUNX1 expression was found in hair follicles, and its specific disruption in mouse epidermal cells impaired hair follicle homeostasis and long-term differentiation. In human skin, RUNX1 is also highly expressed in hair follicles and involved in KAP5 regulation, a keratin-associated protein expressed in the cuticle layer of anagen hair follicles.

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1Centre de Génétique et de Physiologie Moléculaires et Cellulaires, CNRS UMR5534—Université Lyon I, 43 Bd du 11 Novembre 1918, F-69622 Villeurbanne, France; 2Laboratoire de Génomique et Radiobiologie de la Kératinopoiesèse, CEA, IRCM, Evry F-91000, France; 3Centre de Recherche en Cancérologie—Lyon Est, INSERM, U1052, CNRS, UMR2886, F-69006 Lyon, France and 4Department of Dermatology, Edouard Herriot Hospital Group (Pavillon R), F-69008 Lyon, France

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Abbreviations: RUNX1, Run-related transcription factor 1; TF, transcription factor; BCC, basal cell carcinoma; SCC, squamous cell carcinoma; CBFI, core-binding factor beta; KAP5, keratin-associated protein 5; CHIP, chromatin immunoprecipitation; QPCR, quantitative PCR; siRNA, small interfering RNA; PCNA, proliferation cell nuclear antigen; KLFI, Kruppellike factor 4

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To date, RUNX1 function has never been explored in human interfollicular epidermis. We therefore investigated RUNX1 expression pattern in human normal skin, revealing expression in the interfollicular epidermis. Our data shed light on the complex coregulation of RUNX1 by ΔNp63 and p53, leading to a role for RUNX1 in the switch from proliferation to differentiation by repressing the cell cycle and activating a marker of early keratinocytes differentiation. It also showed that RUNX1 expression is dysregulated in human cutaneous BCCs and SCCs.

Results

RUNX1 is expressed in normal human interfollicular epidermis and tightly regulated in primary keratinocytes. RUNX1 gene expression is restricted to hair follicles in mouse skin and is strongly expressed in human hair follicles. We therefore investigated whether RUNX1 was also expressed in human interfollicular epidermis, using a RUNX1-specific antibody on sections of normal human skin (Figure 1). As previously described, a strong RUNX1 expression was observed in cell nuclei from both the outer root sheath and the inner root sheath of human anagen hair follicles (Figures 1a and b). RUNX1 was also detected at lower but consistent levels in interfollicular epidermis: most of the basal keratinocytes exhibited specific nuclear labeling (Figures 1c and d) that decreased in suprabasal layers, when compared with negative control (Figure 1e). This staining pattern was observed in foreskin, facial, breast and abdominal skin.

To investigate RUNX1 expression more precisely in normal human interfollicular keratinocytes, we next used a model of Ca²⁺-induced differentiation of human primary keratinocytes (HPK) in culture, monitored by induction of keratin1 (Figure 2a), keratin10 (Figure 2b), involucrin (Figure 2c) and loricrin (Figure 2d), widely used markers of epidermal differentiation. At both mRNA (Figure 2e) and protein (Figure 2f) levels, RUNX1 was tightly regulated along the

Figure 1  RUNX1 expression in human skin. Sections of paraffin-embedded normal human facial skin stained with anti-RUNX1 polyclonal (a–d) or control rabbit IgG (e) antibodies. The arrow shows RUNX1 nuclear labeling in longitudinal (a) and transversal (b) sections of a hair follicle. In the interfollicular epidermis (c and d), RUNX1 is essentially expressed in keratinocytes of the basal layer and its expression strongly decreases in suprabasal layers. The square in (c) represents the area of magnification shown in the (d). Scale bar = 50 μm
transition from proliferation to differentiation: in keratinocytes from three different donors, RUNX1 was strongly expressed in semi-confluent cells, then gradually declined when cells reached confluence and was weakly but reproducibly reactivated at the onset of differentiation. So, RUNX1 is expressed in human interfollicular epidermis and tightly regulated during the transition from proliferation to differentiation.

**ΔNp63 modulates RUNX1 expression differentially in proliferative or differentiated human keratinocytes via two DNA regulatory sequences.** RUNX1 has been recently identified by chromatin immunoprecipitation (ChIP) screens as a direct transcriptional target gene of ΔNp63 in subconfluent mouse keratinocytes and HaCaT cell line. These studies revealed that ΔNp63 binds an intronic region and one part of exon 1 of the RUNX1 gene that are highly conserved between species, and that p63 is able to transactivate Runx1 expression. ChIP-quantitative PCR (QPCR) confirmed that RUNX1 exon 1 and intron 5 sequences are indeed significantly bound by p63 in HPK (Figure 3a).

The fact that p63 can bind two distinct regulatory DNA sequences of RUNX1 gene suggested a more complex modulation of RUNX1 expression in human than that was previously proposed in mouse. Because RUNX1 expression is tightly regulated at the transition from proliferation to differentiation (Figures 2e and f) and ΔNp63 can exert separate control over keratinocyte proliferation and differentiation, we hypothesized that RUNX1 expression can be differentially modulated by ΔNp63 in cells that stopped proliferation and began a differentiation program, versus still nonconfluent proliferating keratinocytes (Materials and Methods). RUNX1 expression was analyzed after ΔNp63-specific inactivation by RNA interference in these human-differentiated or proliferative keratinocytes. ΔNp63 silencing was very efficient in both proliferating and differentiated keratinocytes at the mRNA (Figure 3b, left panel) and protein (Figure 3c) levels. When ΔNp63 was specifically knocked down in proliferative cells, RUNX1 expression was significantly decreased (Figure 3b right panel and 3c left panel), suggesting that ΔNp63 positively regulates RUNX1 expression in proliferative human keratinocytes, as also observed in proliferative mouse keratinocytes. In contrast, reducing ΔNp63 expression in differentiated keratinocytes strongly increased RUNX1 expression (Figures 3b and c, right panels), showing that ΔNp63 negatively controls RUNX1 expression in

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**Figure 2** RUNX1 expression in cultured human keratinocytes. (a-e) Keratin1 (a), keratin10 (b), involucrin (c), loricrin (d) and RUNX1 (e) transcripts were quantified by QPCR in semi-confluent, confluent or Ca²⁺-induced differentiating keratinocyte cultures (D1, D2, D3) from three different donors (HPK1, HPK2, HPK3). Mean expression is indicated for (a-d); detailed expression for each culture is given for (e). (f) RUNX1 protein expression was analyzed from keratinocyte cultures established at the same stages.
cells that stopped proliferating and began differentiating. Taken together, these data showed that ΔNp63 differentially modulates RUNX1 expression depending on the proliferative or differentiation-committed state of human keratinocytes.

We then postulated that this differential regulation of RUNX1 expression could be linked to ΔNp63 differential binding onto RUNX1 regulatory DNA sequences. ChIP-QPCR was therefore performed in HPK from three different donors in a proliferative or 3-day differentiated state. p63 was systematically bound (1.5–4-fold relative to the three donors) to the RUNX1 intron 5 sequence in both proliferative and differentiated keratinocytes (Figure 3d, left panel), but was able to bind exon 1 only in differentiated cells (1.6–2-fold; Figure 3d, right panel).

Overall, these results strongly suggest that ΔNp63 differentially regulates RUNX1 expression along the transition from proliferation to differentiation by differential binding of its regulatory sequences, highlighting the complexity of direct RUNX1 transcriptional regulation by ΔNp63 in human interfollicular keratinocytes.

p53 binds the same RUNX1 regulatory sequences and cooperates with ΔNp63 for regulation of RUNX1 expression. We postulated that other p53 family members, which are able in some specific cases to directly regulate the same transcriptional target genes, in either a similar or a converse way,21,22 could likewise modulate the ΔNp63 action on RUNX1 regulation by binding its regulatory sequences. We therefore questioned whether the long p63 isoform, TAp63, as well as the p53 and/or p73 TFs could regulate RUNX1 expression in human interfollicular keratinocytes, given that they are expressed along the transition from proliferation to differentiation (data not shown).22 However, modulating TAp63 or p73 expression in primary keratinocytes did not affect RUNX1 expression levels (data not shown). In contrast, p53 was found to cooperate with ΔNp63 for RUNX1 regulation in HPK (Figure 4). Silencing of p53 alone in human keratinocytes had no effect on RUNX1 expression (Figure 4a). However, when both p53 and ΔNp63 expression was decreased by small interfering RNA (siRNA), RUNX1 expression was not repressed anymore.

Figure 3 ΔNp63 can bind RUNX1 intron 5 and exon 1 DNA sequences and regulates its expression differentially in proliferating and differentiated keratinocytes. (a) Human RUNX1 gene map (upper panel) showing the exon–intron structure: arrows represent the DNA regions in exon 1 and intron 5 amplified by QPCR after ChIP experiments (lower panel). p33-ChIP assays were performed in three cultures of differentiated HPK. Enrichment of RUNX1 exon 1 and intron 5 regions was analyzed in comparison with a control promoter region. (b) ΔNp63 (left panel) and RUNX1 (right panel) transcript levels were quantified by RT-QPCR after treatment of proliferative or differentiated HPK with control or ΔNp63 siRNA. (c) p63 and RUNX1 protein levels were assessed by immunoblotting after treatment of proliferative (left panel) or differentiated (right panel) HPK with control or ΔNp63 siRNA. (d) p63-ChIP assays were performed in proliferative or 3-day differentiated HPK from three different donors (HPK1, HPK2, HPK3). Enrichment of the RUNX1 intron 5 (left panel) and exon 1 (right panel) regions was analyzed in comparison with a control promoter region by QPCR.
in proliferative keratinocytes (Figure 4b left panel and 4c upper panel), compared with what was observed after ΔNp63 inhibition alone (Figure 3b right panel and 3c left panel). So ΔNp63 needs p53 expression for RUNX1-positive regulation in proliferative keratinocytes. However, in differentiated keratinocytes, inhibition of both p53 and ΔNp63 did not disturb the ΔNp63 silencing-mediated effect on RUNX1 expression (compare Figure 4b right panel with 3b right panel and Figure 4c lower panel with Figure 3c right panel). These results are confirmed by ΔNp63 silencing in the human HaCaT keratinocyte cell line (Figure 4d), known to express a mutated form of p53 that is transcriptionally inactive.23 Indeed, RUNX1 expression was not significantly modified in proliferative HaCaT cells when ΔNp63 expression was decreased, conversely to what was observed in HPK that express a functional p53 protein (compare Figures 4d with 3b). These results were strengthened by studying RUNX1 expression profile in HaCaT cells: RUNX1 levels significantly decreased between proliferation and 1 day of differentiation but did not significantly increase again between 1 and 3 days of differentiation, conversely to what was observed in normal HPK (compare Figures 4e with 2e and f). Thus, a functional p53 protein is necessary to properly regulate RUNX1 expression, in cooperation with ΔNp63, during the switch from proliferation to differentiation.

We confirmed by ChIP-QPCR that the p53 role in RUNX1 regulation depends on direct binding of p53 protein onto RUNX1 intron 5 and exon 1 (1.5–3.5-fold relative to the different donors; Figure 5a), whatever the state (proliferative or differentiated) of HPK.

To further investigate the specific role of each of the two DNA regulatory sequences of RUNX1 in ΔNp63 and p53 regulation, the parts of RUNX1 exon 1 or intron 5 that are bound in vivo by these two TFs were individually cloned into a luciferase reporter vector. The p53 and ΔNp63 overexpression effect (Figure 5b, left panel) on RUNX1 exon 1 and intron 5 transcriptional regulation was then analyzed by transactivation assays and revealed opposite transcriptional effects on the RUNX1 gene; p53 overexpression significantly activated luciferase activity via RUNX1 intron 5 without influencing exon 1 activity, whereas ΔNp63 overexpression did not affect intron 5 regulation but significantly repressed luciferase activity via RUNX1 exon 1.

**Figure 4** p53 cooperates with ΔNp63 to regulate RUNX1 in HPK. (a) RUNX1 transcript levels was quantified by RT-QPCR after treatment of proliferative or differentiated HPK with control or p53 siRNA. The effect of p53 siRNA on p53 expression was shown in Figure 2b. (b) p53, ΔNp63 and RUNX1 transcript levels were quantified by RT-QPCR after treatment of proliferative (left panel) or differentiated (right panel) HPK with control or p53 and ΔNp63 siRNA. (c) RUNX1 protein level was assessed by WB 48 h after treatment of proliferative (upper panel) or differentiated (lower panel) HPK with both p53 and ΔNp63 siRNA. (d) ΔNp63 (left panel) and RUNX1 (right panel) transcripts levels were quantified by RT-QPCR after treatment of proliferative or differentiated HaCaT cells with control or ΔNp63 siRNA. (e) RUNX1 transcripts were measured by QPCR at different stages of the HaCaT cell cultures (left panel). Involucrin (INV) and RUNX1 proteins expression was assessed by immunoblotting at different stages of the HaCaT culture (right panel).
activity via the RUNX1 exon 1 (Figure 5b, right panel). Moreover, additional experiments showed that ΔNp63 was able to compete with p53 on RUNX1 intron 5 (Figure 5c), in agreement with in vivo ChIP experiments. These data suggested that ΔNp63 could repress RUNX1 expression by binding to its exon 1 regulatory sequence, whereas p53 could induce RUNX1 expression by binding to its intron 5 DNA sequence.

RUNX1 modulates keratinocyte proliferation and directly regulates KRT1 gene expression. As ΔNp63 has a critical function in the regulation of entry into differentiation and is involved in RUNX1 coregulation along with p53, we investigated whether RUNX1 may also have a key role in the regulation of proliferation and differentiation processes in human interfollicular epidermis.

To this end, the RUNX1 role in proliferation was explored in proliferating HPK. RUNX1 overexpression under exponential growth conditions led to a strong increase in mRNA (data not shown) and protein RUNX1 levels (Figure 6a, left panel) associated with a significant decrease in cell number compared with an empty vector control (Figure 6a, right panel). Conversely, RUNX1 knockdown (Figure 6b, left panel), by transfecting a validated specific siRNA, significantly increased cell number (Figure 6b, right panel). This modification of cell number is due to a higher proliferation rate (Figure 6c). In fact, RUNX1 inhibition by siRNA increased the percentage of BrdU-positive keratinocytes (R2 region) and concomitantly decreased the percentage of keratinocytes in the G1 phase (R1 region) (Figure 6d, left panel). This significant RUNX1 effect on cell cycle modulation was consistently found in four independent experiments using keratinocytes from various donors (Figure 6d, right panel). Interestingly, we found that RUNX1 depletion by siRNA led to a significant decrease of p21 expression, a negative regulator of cell cycle, as well as to a significant increase of proliferation.
cell nuclear antigen (PCNA) and Ki67, two well-known markers of proliferation (Figure 6e). Taken together, these data indicate that RUNX1 is a negative regulator of cell proliferation in cultured human keratinocytes.

We also investigated whether RUNX1 has a role in regulating the early differentiation process, as RUNX1 and its regulator ΔNp63 are both expressed in the first differentiated layers of the human epidermis. A bioinformatic screen for RUNX1 binding sites in promoter regions of 27 genes selected for their strong expression in human granular and spinous keratinocytes revealed highly conserved RUNX1 binding sites (score >0.9) in the promoter sequences of 3 genes. One site in the promoter (−577 to −563) and another in the first exon (−186 to +190) was identified for KRT1 (encoding keratin1), whereas TGM1 and LOR (respectively encoding transglutaminase1 and loricrin) exhibited only one site (−508 to −494 and −323 to −309, respectively). As the presence of a consensus binding site is merely suggestive of a possible regulation, RUNX1 depletion was conducted by siRNA transfection. It led to reduction of KRT1 and LOR mRNA levels by >30% whereas TGM1 mRNA was not significantly modified (Figure 7a), showing that KRT1 and LOR are RUNX1 downstream targets in human keratinocytes. To investigate whether RUNX1 directly regulates KRT1 and LOR expression, the effect of RUNX1 and/or its cofactor CBFβ on the promoter regulation of these two genes was examined by luciferase assay (Figure 7b). RUNX1 overexpression did not modify the levels of LOR reporter activity, whereas it reproducibly and significantly increased KRT1 reporter activity. When one of the two potential RUNX1 binding sites was deleted, basal luminescence intensity and induced luciferase activity after RUNX1 and/or CBFβ transfection were abolished. Taken together, these findings indicate that RUNX1 positively regulates KRT1 expression by directly binding to its promoter. Overexpression or inhibition of RUNX1 in HPK led to a correlated regulation of endogenous KRT1 protein (Figure 7c), confirming the positive regulation exerted by RUNX1 on KRT1 protein. Finally, ChIP-QPCR experiments using three different anti-RUNX1 antibodies showed strongly enriched KRT1 promoter sequences in the immunoprecipitated fraction, (3- to 13-fold) whereas LOR and TGM1 target sequences were not enriched.
RUNX1 directly binds the KRT1 promoter sequence and validate KRT1 as a direct RUNX1 transcriptional target in these cells.

Taken together, these data showed that RUNX1, tightly regulated by \( \Delta Np63 \) and p53 during the transition from proliferation to differentiation in human interfollicular keratinocytes, is able to modulate both the proliferation process and the expression of early differentiation markers.

**RUNX1 expression is dysregulated in human skin carcinomas.** BCCs and SCCs are the two commonest nonmelanoma cancers of epithelial origin in the skin. BCCs are derived from or closely related to long-term resident progenitor cells of the interfollicular epidermis, whereas SCCs might originate from both hair follicles and interfollicular epidermis. Missense mutations in the tumor suppressor gene p53 are a common genetic feature of these tumors, as a result of a complex sequence of events initiated by exposure to UV light. BCCs and SCCs are also characterized by \( \Delta Np63 \) protein upregulation. As RUNX1 expression is tightly regulated by differential interplay between p53 and \( \Delta Np63 \), we analyzed RUNX1 expression in a panel of BCCs and SCCs (Figure 8). All BCCs overexpressed RUNX1, compared with RUNX1 expression level in the basal layer of surrounding epidermis (Figure 8a), but to varying degrees: 3/18 showed low RUNX1 expression with <10% immunostained cells, 6/18 had a moderate immunostaining with 40–80% positive cells, and 9/18 showed a high RUNX1 expression with over 80% immunostained cells (Figure 8c). By contrast, only 9 out of 15 SCCs presented RUNX1 immunostaining (Figures 8b and c). The majority exhibited a low to moderate RUNX1 staining.

In conclusion, these data support the contention that RUNX1 is dysregulated in nonmelanoma skin tumors and is expressed at higher levels in BCCs than in SCCs.

**Discussion**

The present study shows for the first time that RUNX1 is expressed in human interfollicular epidermis, while being restricted to hair follicles in mouse skin. These results are in line with the strong \( \Delta Np63 \) expression in human interfollicular epidermis, shown as a direct transcriptional regulator of RUNX1. We have also demonstrated that RUNX1 is tightly regulated by a complex mechanism involving \( \Delta Np63 \) and p53, and control cell proliferation and onset of differentiation.

Among the members of the p53/p63/p73 TFs family, TAp63 and the short (\( \Delta N \)) and long (TA) isoforms of p73 are also expressed in human interfollicular keratinocytes. However, the \( \Delta Np63 \) isoform was shown as the main p63 mediator regulating the switch from proliferation to differentiation, whereas several studies involved TAp63 and p73 isoforms in the development of the granular and horny layers. These data agree with our results, suggesting that TAp63 and p73 are dispensable for RUNX1 expression in human interfollicular epidermis.

Only p53 cooperates with \( \Delta Np63 \) during the transition from proliferation to differentiation of human interfollicular keratinocytes. \( \Delta Np63 \) would have a key role for the entry into differentiation by inhibiting RUNX1 expression, whereas, in proliferating keratinocytes, p53 would overcome the \( \Delta Np63 \)-negative regulatory effect to activate RUNX1. Our results reinforce and complete the study conducted in human keratinocytes by Truong et al. that clearly demonstrated a role for p53 in epidermal proliferation but not in the
differentiation process. Moreover, the importance of p53 for regulation by p63 of another TF, Krueppel-like factor 4 (KLF4), was recently shown in proliferative epidermal cells. Collectively, our data of expression, ChIP and reporter assays allows us to propose a model for the regulation of RUNX1 expression: in proliferating keratinocytes, both DNp63 and p53 would bind RUNX1 intron 5, that in turn leads to RUNX1 activation by p53 via this positive regulation element. In keratinocytes that reach confluence, stop proliferating and begin differentiating, DNp63 would join p53 onto RUNX1 exon 1 regulatory sequence that has for consequence RUNX1 repression by DNp63. Therefore, the tight control of RUNX1 expression by this functional interplay between the two TFs DNp63 and p53 could lead to a fine regulation of downstream molecular events to allow a proper transition from proliferation to differentiation in human interfollicular keratinocytes.

Indeed, we showed that the tight regulation of RUNX1 expression leads to the regulation of both proliferation and early differentiation. RUNX1 inhibits human interfollicular keratinocyte proliferation. This antiproliferative effect seems to be specific to this cell type, as RUNX1 is known to be a positive regulator of G1-to-S cell cycle progression in hematopoietic tissue, neural progenitors and, more recently, in mouse hair follicle stem cells. However, a strong correlation between negative cell cycle regulators and RUNX1 expression was observed in endometrial carcinoma, underlying the complexity of RUNX1 cell growth regulation, in line with the fact that RUNX1 can act as both a transcriptional repressor and activator. We also showed that RUNX1 could activate the KRT1 gene in human keratinocytes through direct binding to its promoter region. KRT1 induction is one of the earliest events in keratinocyte differentiation, as it occurs in basal keratinocytes that have ceased mitotic activity. These results are even more interesting as DNp63 is also responsible for KRT1 regulation via c-Jun N-terminal kinase/AP1 activation. Thus, RUNX1 appears to have a key role together with DNp63 in the switch from proliferation to differentiation in the basal layer, by downregulating the cell cycle and inducing early differentiation markers such as KRT1.

Finally, we showed that RUNX1 expression is dysregulated in the two most widespread nonmelanoma skin cancers of epithelial origin, BCCs that are locally destructive but metastasizes only exceptionally, and SCCs with high metastatic potential. RUNX1 seems to be globally more expressed in BCC cells, which are phenotypically closed to basal proliferating keratinocytes, than in SCC cells, which appear as more differentiated. This RUNX1 pattern is not identical with that of DNp63 expression, although DNp63 is also more strongly expressed in BCCs compared with SCCs (data not shown). Conversely, our data and those of the literature show a better correlation between RUNX1 and p53 expression. As p53 is mutated and highly expressed in SCC cells (whereas expression is lower in BCCs), it could lead to a lack of RUNX1 activation in differentiated SCC cells, which in turn would be silenced by DNp63 overexpression, according to our proposed model. This model is strengthened by the fact that clusters of cells overexpressing a mutated form of p53, considered as potential precursors of SCCs, can be detected in the interfollicular epidermis before the skin tumors arise and that the negative regulation of KLF4, another target of p63, is subverted by p53 mutations commonly found in SCC. The fact that only part of SCCs are positive for RUNX1...
expression suggest that it may reflect varying carcinogenic insults and may be linked in particular with differential p53 mutations.

In summary, we present evidence that RUNX1 TF expression is regulated by a functional interplay between ΔNp63 and p53, and that RUNX1 is involved in the control of the transition between proliferation and early differentiation in human keratinocytes. The use of large-scale genomic approaches such as ChIP-chip or ChIP-seq would be of great interest for identifying novel RUNX1 target genes in proliferating versus differentiated keratinocytes in order to elucidate its role in the regulation of epidermal homeostasis.

Materials and Methods

Cell cultures. HPK, isolated from neonatal foreskin, are cultured in KGM2 medium (PromoCell, Heidelberg, Germany) as previously described.28 This study was approved by the ethical research committee Comité de Protection des Personnes Sud-Est-II (CODECOH Number DC-2008-262). A written informed consent was obtained from infants’ parents according to the French bioethical law for cell cultures.

HPK or HaCaT cells were differentiated in KGM2 medium (PromoCell, Heidelberg, Germany) as previously described.38 This study was approved by the ethical research committee Comité de Protection des Personnes Sud-Est-II (CODECOH Number DC-2008-262). A written informed consent was obtained from infants’ parents according to the French bioethical law for cell cultures.

Protein extraction and blotting were performed as described.39 Actin was used as a loading control. Results were obtained from three independent experiments with unpaired samples. Mean differences were considered to be significant when P < 0.05 (*P < 0.05; **P < 0.01; ***P < 0.001; ns, not significant).

Statistical analysis. All data are represented as a mean for at least three experiments. Statistical significance was calculated by a two-tailed Student’s t-test for unpaired samples. Mean differences were considered to be significant when P < 0.05 (*P < 0.05; **P < 0.01; ***P < 0.001; ns, not significant).

Conflict of Interest

The authors declare no conflict of interest.

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RUNX1 regulation by p63/p53 in human keratinocytes

I Masse et al

In summary, we present evidence that RUNX1 TF expression is regulated by a functional interplay between ΔNp63 and p53, and that RUNX1 is involved in the control of the transition between proliferation and early differentiation in human keratinocytes. The use of large-scale genomic approaches such as ChIP-chip or ChIP-seq would be of great interest for identifying novel RUNX1 target genes in proliferating versus differentiated keratinocytes in order to elucidate its role in the regulation of epidermal homeostasis.

Promoter analysis. 5’ regulatory sequences (–750, +250 from TSS) were retrieved from the promoter database at http://rulai.cshl.edu/cgi-bin/TRED/ and screened for RUNX1 consensus sites using the MatInspector promoter analysis tool. For reporter gene assay, 251 bp of human RUNX1 intron 5 or 312 bp of RUNX1 exon 1 were cloned by PCR ligation into the pGL3 promoter vector (Promega, Madison, WI, USA) upstream of SV40 promoter. The KRT1 (–846/ +257) and LOR (–1370/+9) promoter segments were cloned into pGL4-basic vector (Promega). Deletion of the distal (D) or proximal (P) RUNX1 binding sites was performed by PCR/digestion from the full KRT1 promoter into pGL4-basic vector to construct the P-site KRT1 promoter (–546/+257) and the D-site KRT1 promoter (–846/+28) vectors, respectively. Transient transfections were carried out four times in duplicate on HEK293 cells using JetPei reagent (Polyplus). Luciferase assays were performed using Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer instructions. The ratio of luciferase activity for intron5-pGL3 promoter and exon1-pGL3 promoter was calculated 24 h after transfection with empty pCMV, pCMV-p53, empty pcDNA3 or pcDNA3-ΔNp63 relative to pGL3 promoter empty vector. For RUNX1 transfections, the luciferase activity ratio was calculated 24 h after transfection by pCMV-CBFβ, pCMV-RUNX1 or both relative to pGL4-basic empty vector. Data were normalized to the transfection efficiency assessed by pCMV-RL vector cotransfection.

Western blot, immunofluorescence and immunohistochemistry.

Protein extraction and blotting were performed as described.39 Actin was used as a loading control. Results were obtained from three independent experiments with unpaired samples. Mean differences were considered to be significant when P < 0.05 (*P < 0.05; **P < 0.01; ***P < 0.001; ns, not significant).

Transient transfection. RUNX1 was overexpressed using JetPei transfection (PolyPlus, Illkirch, France) with 1 μg of pCNeo-RUNX1 or pCMV-RUNX1 (provided by Dr. Mouchiroud, CNRS, Lyon) following the manufacturer’s instructions. For silencing experiments, keratinocytes were transfected at 20 nM final concentration using Hepes transfection reagent (Qiagen). Western blot analysis was performed on a FACS Calibur using Cellquest software (BD Biosciences, Franklin Lakes, NJ, USA) and control immunoglobulins (Zymed, South San Francisco, CA, USA). The primary antibodies were precleared with proteinG-agarose/salmon sperm DNA beads, washed, extracted in 1% SDS, 0.1 M NaHCO3 and immunoglobulins. Immune complexes were collected with proteinG-agarose/salmon sperm DNA beads (Upstate, Darmstadt, Germany) and control immunoglobulins (Zymed, South San Francisco, CA, USA), incubated overnight at 4 °C with 2 μg anti-p63 4a4 (sc-6431 Santa Cruz, Santa Cruz, CA, USA), anti-p53 DO-1 (sc-126 Santa Cruz) or control mouse immunoglobulins. Immune complexes were collected with proteinG-agarose/salmon sperm DNA beads, washed, extracted in 1% SDS, 0.1 M NaHCO3 and treated with RNase A and proteinase K. Protein crosslink was reverted by heating at 65 °C for at least 5 h. DNA was purified using Qiaquick PCR-Purification Kit (Qiagen). Western blot analysis was performed on a FACS Calibur using Cellquest software (BD Biosciences, Franklin Lakes, NJ, USA).

Chromatin immunoprecipitation. Equivalent numbers of HPK in a proliferative or 3-day differentiated state were lysed after crosslinking with 1% formaldehyde (Sigma, St. Louis, MO, USA). Chromatin was sheared by sonication, preliminary p33/p50 or p63/p53.
Supplementary Information accompanies the paper on Cell Death and Disease website (http://www.nature.com/cddis)