A Functional Enhancer of Keratin14 Is a Direct Transcriptional Target of ΔNp63

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Keratin14 (K14) is a prototypic marker of dividing basal keratinocytes where its gene is transcribed at high levels. Transcriptional regulation of K14 is governed by an evolutionarily conserved functional enhancer marked by DNase 1 hypersensitive sites present upstream of the gene. This enhancer is sufficient to confer epidermal-specific gene expression, which is mediated in part by binding of members of activator protein-2 (AP)-2, AP-1, Ets, and Sp1 families of transcription factors. Here we provide evidence that a keratinocyte-specific nuclear protein identified as ΔNp63 binds to a conserved motif within this enhancer. Interestingly, the selective expression profile of ΔNp63 in various cell lines correlates with both the nuclear complex and the expression of K14. Biochemical studies reveal that ΔNp63 can bind to a specific DNA sequence present in the K14 enhancer and this binding leads to transactivation. In addition, chromatin immunoprecipitation experiments with ΔNp63-specific antibodies demonstrate that the enhancer is occupied by ΔNp63 in cultured keratinocytes and in mouse skin epidermal cells *in vivo*. Finally, we show that ectopic expression of either p63 isoform (ΔN or TA) can induce *de novo* expression of K14. These studies provide a potential mechanism by which ΔNp63 directly governs the expression of K14 in a keratinocyte-specific manner.

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INTRODUCTION

Keratins constitute the largest subgroup of the family of intermediate filaments and represent the most abundant proteins in epithelial cells (Fuchs and Weber, 1994). These proteins are divided into type I (acidic) and type II (basic and neutral) classes based on their sequence. Keratins from these two classes form obligate heterodimers and are expressed in tissue- and differentiation-specific manners in epithelial tissues of various types including simple epithelia, internal stratified epithelia, outer stratified epithelia, and hair follicles (Coulombe and Omary, 2002). This distinctive expression of specific keratin pairs depends largely on the tissue-type, differentiation status, and the physiological state. For example, dividing basal keratinocytes of the skin epidermis express keratin5 (K5) and keratin14 (K14). As these cells exit the cell cycle and embark on a program of differentiation, expression of K5 and K14 is downregulated and a new set of keratins, keratin1 and keratin10 are expressed in the suprabasal spinous layer (Byrne *et al.*, 1994). The differential expression of keratins has been useful in marking the boundaries of the different layers of the skin epidermis and has often served as surrogate markers to distinguish proliferation and differentiation states of the skin epidermis. Therefore, deciphering the molecular mechanisms governing keratin gene expression is important for better understanding the regulatory network that underlies epidermal development and homeostasis.

To characterize transcriptional regulatory mechanisms that govern the expression of keratin genes, many studies have focused on the identification of *cis* and *trans* regulatory elements (Byrne, 1997; Eckert *et al.*, 1997). The restricted expression of K14 in the basal layer of the skin epidermis is primarily controlled at the level of transcription and regulation of this process has been extensively studied using various complementary approaches. DNAse I hypersensitive site (Hs) mapping of the human *K14* gene has identified several Hs in the 5′ region that are present selectively in keratinocytes (Sinha *et al.*, 2000; Sinha and Fuchs, 2001). Among these Hs elements are those which correspond to the minimal promoter (Hs I) and two closely spaced Hs (Hs II and III) at the –1700 and –1400 region. Interestingly, the Hs elements are organized similarly in the mouse *K14* gene, and there is high level of sequence conservation in the Hs regions, further supporting the functional relevance and importance of these elements in controlling K14 expression. This has been experimentally proven by extensive analysis of these Hs segments by reporter gene assays in keratinocytes grown in culture as well as in transgenic mice. Indeed these studies have clearly demonstrated that the K14 Hs II and III regions act as an enhancer element that confers high levels of gene expression selectively

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Abbreviations: AP-1, activator protein-21; MK, mouse keratinocyte; ChIP, chromatin immunoprecipitation; GST, glutathione S-transferase; Hs, hypersensitive site; KSC, keratinocyte-specific complex; PBS, phosphate-buffered saline; TK, thymidine kinase

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in keratinocytes when assayed by reporter gene assays in transient transfection experiments. More importantly, this enhancer can target basal keratinocyte-specific expression of a reporter gene in transgenic mice. Similar investigative studies of the K14 partner gene, K5 have also lead to the identification of several Hs elements that function as critical regulatory modules, some of which have been characterized in detail (Kaufman *et al.*, 2002). Collectively these studies have led to the conclusion that the epidermal and differentiation-specific transcription of these two keratin genes is governed by complex regulatory elements.

To identify transcription factors that mediate the expression of the basal keratin genes, these Hs elements have been subjected to biochemical analysis. These studies show that basal-specific and differentiation-specific gene expression in keratinocytes relies on a complex array of regulatory elements that bind diverse transcription factors belonging to many families including activator protein-2 (AP)-2, AP-1, ETS, Sp1/Sp3 and as yet unidentified factors. Many of the transcriptional activators and repressors that interact with these Hs sites are quite ubiquitous in their expression pattern and only a few exhibit some level of keratinocyte-restricted expression. This has raised an important question, that is, how is epidermal-specific gene expression achieved? One hypothesis is that gene expression in basal epidermis is governed by broadly expressed factors working in a combinatorial manner. Alternatively, a mechanism may exist that confers keratinocyte-specific activity on an otherwise ubiquitously expressed transcription factor by virtue of specific post-translational modification or interaction with a co-activator. Finally, it is possible that transcription factors that are generally restricted to keratinocytes interact directly with these regulatory elements and mediate cell-type specific gene expression.

p63 is a p53 homolog that is critical for the development of the stratified epithelium of the skin as shown by several recent studies (Mills *et al.*, 1999; Yang *et al.*, 1999, 2002). Indeed, both loss-of-function and gain-of-function experiments have demonstrated that p63 is important for maintaining epithelial homeostasis (Koster and Roop, 2004b). One complicating issue in deciphering the biological role of p63 is the fact that the *p63* gene contains two distinct promoters, resulting in the expression of two major classes of proteins, one with an amino-terminal trans-activation domain (TAp63) and one without (ΔNp63) (Yang *et al.*, 1998). Knockout studies in mice so far have not examined the relative contribution of these two major isoforms in orchestrating the balance between keratinocyte proliferation and differentiation. However, several recent studies utilizing expression analysis and ectopic overexpression experiments have led to the postulation of contradictory roles for the TAp63 and ΔNp63 isoforms (Koster *et al.*, 2004; Candi *et al.*, 2006; Laurikkala *et al.*, 2006). What is unequivocally clear however, is the fact that epidermal cells that lack p63 exhibit dramatic downregulation in the levels of K14. Although, there has been a recent report suggesting that loss of expression of K14 in the absence of p63 is indirectly mediated by the transcription factor AP-2γ, the possibility that p63 can directly target K14 gene expression has not been examined thoroughly (Koster *et al.*, 2006).

We describe here a nuclear complex that is restricted to keratinocytes and binds to a specific conserved sequence present in the enhancer segment corresponding to Hs II of the *K14* gene. We provide *in vitro* and *in vivo* evidence that ΔNp63 is a component of this unique complex and that ΔNp63 binding is important for the K14 enhancer activity. Furthermore, our data suggests that contrary to published data, not only TAp63 but also all isoforms of ΔNp63 are capable of inducing K14 gene expression, when ectopically expressed in cells that normally do not express p63 or K14. The fact that ΔNp63 is the predominant isoform expressed both in cultured keratinocytes and in skin epidermis suggests it is the critical p63 isoform that governs K14 gene expression. Our data strongly argues that transcription factors working in a combinatorial manner govern gene expression in epidermis and that p63 is a keratinocyte-specific transcription factor that potentially serves an important role as a master regulator in this process.

RESULTS

A novel KSC binds to the K14 enhancer

Previously we characterized an enhancer element located ~1.4 kb upstream of the *K14* gene, delineated by a keratinocyte-specific DNAse I Hs site (Sinha *et al.*, 2000). We showed that binding of AP-1, AP-2, and the Ets family of proteins was important for enhancer activity. We identified an additional nuclear complex binding to an oligonucleotide containing K14 enhancer sequences from −1433 to −1407 that showed an intriguing behavior in electrophoretic mobility shift assay (EMSA) (Figure 1a). We named this slow migrating band keratinocyte-specific complex (KSC), as it was present only in nuclear extracts from mouse and human keratinocytes and not from any other cell types including HepG2 (liver), HeLa (epithelial), NIH3T3 (fibroblast), or B16 (melanoma). The nuclear extracts from the different cell types were prepared in a similar manner and showed comparable DNA–protein complexes when tested with other oligonucleotides, suggesting that the extracts were functionally similar (data not shown). Additional faster moving complexes that bound to the oligonucleotide containing the KSC-binding sequence were either also present in other cell types or were nonspecific as they disappeared in competition experiments with random oligonucleotides or with high amounts of competitor DNA. Additionally, KSC binding was stable in the presence of 0.5 M NaCl suggesting that there is a tight interaction between KSC and its DNA-binding site (data not shown).

Binding of KSC is dependent upon specific DNA sequences

To precisely identify the residues that were critical for DNA binding of KSC, we generated several mutant oligonucleotides (MTs) and performed direct EMSA and competition assays (see Figure S1 for oligonucleotide sequences). Two of the single base pair mutations (MT5: GGGCCTG > GGG*A*CTG) and (MT8: CCTGTCT > CCT*A*TCT) failed to bind KSC when using either HaCaT or MK nuclear extracts in

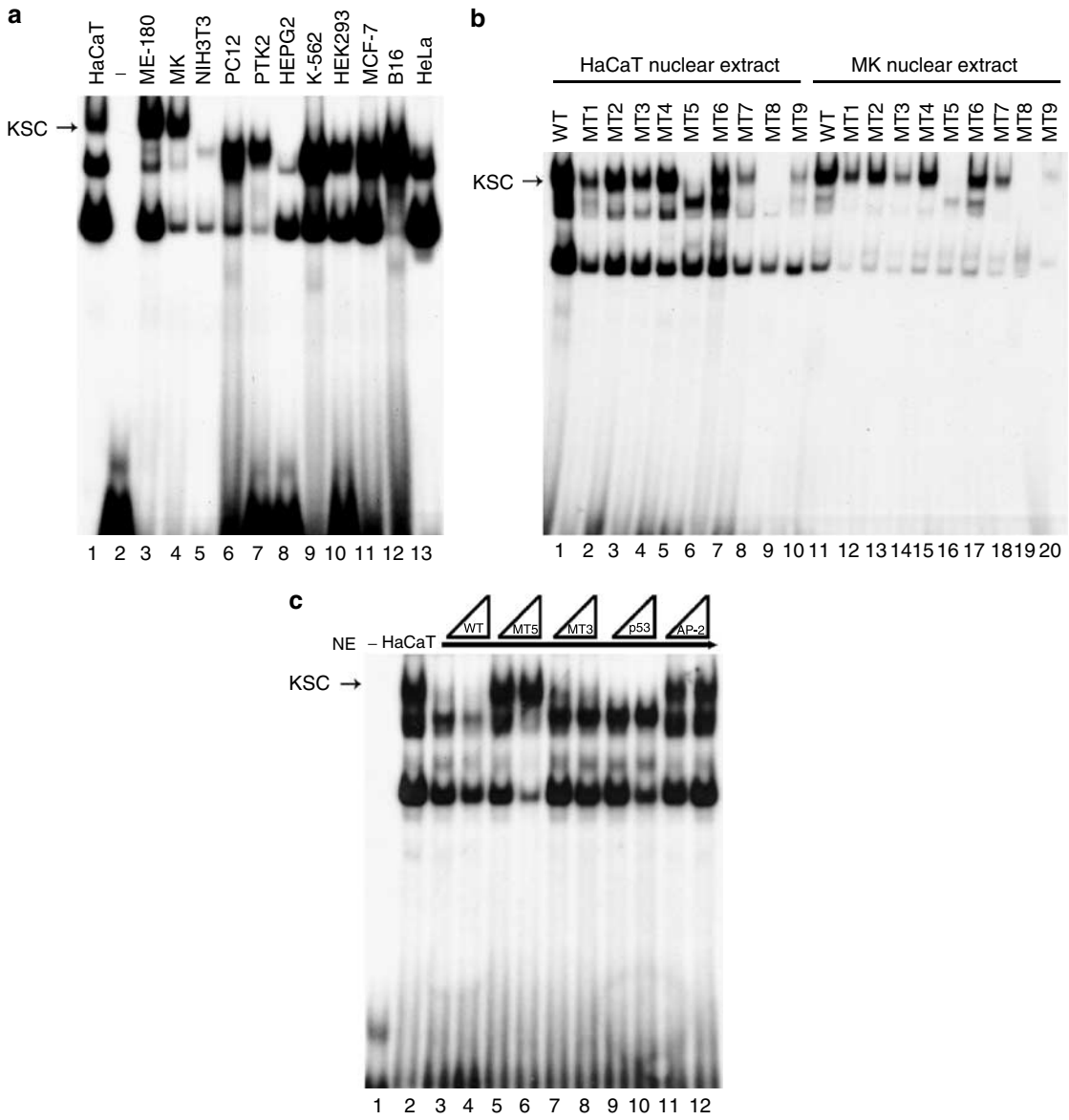

Figure 1. EMSA reveals the presence of specific nuclear complex (KSC) in keratinocytes. (**a**) EMSA was performed with nuclear extracts from various cell lines as indicated above each lane. The radiolabeled oligonucleotide probe contained the K14 enhancer sequence (−1433 to −1407). Arrow indicates KSC (**b**) Mutational analysis of the KSC-binding site identifies critical residues for KSC binding. EMSA was performed with nuclear extracts from HaCaT (lanes1–10) and MK (lanes 11–20) cells and wild type and various MT containing single base pair substitutions. See Figure S1 for oligonucleotide sequences used in EMSA. (**c**) Competition assays show that KSC binds to a p53-like consensus sequence. Wild-type K14 enhancer (lanes 3 and 4), MT3 (lanes 7 and 8), and p53 consensus (lanes 9 and 10) oligonucleotides abrogated KSC binding (indicated by the arrow). In contrast, oligonucleotides corresponding to MT5 (lanes 5 and 6) and AP2 consensus sequence (lanes 11 and 12) did not affect KSC binding.

EMSA analysis (Figure 1b). Other mutations either showed decreased binding to varying degrees (MT1 and MT9) or showed binding of KSC that was comparable to that of wild-type oligonucleotides (MT2, MT3, and MT4). These data suggested that KSC binds to a specific sequence, GGGCCTGTCTG and that this interaction could be abrogated with a single base pair mutation.

To further examine the DNA-binding characteristics of KSC, we performed competition experiments with MT3 and MT5 oligonucleotides as well as oligonucleotides containing consensus DNA-binding sequences for two known transcription factors (Figure 1c). In support of the direct EMSA experiments, MT5 did not compete for KSC binding, whereas MT3 competed to the same extent as that of the wild-type oligonucleotides. Surprisingly, KSC was also competed by oligonucleotides containing a p53-consensus sequence, whereas similar levels of oligonucleotides containing AP-2-binding sites had no effect. Upon closer examination, the KSC site showed considerable sequence similarity with the p53-consensus sequence RRRCWWGYYY and with the recently described optimum p63-binding sequence (Ortt and Sinha, 2006).

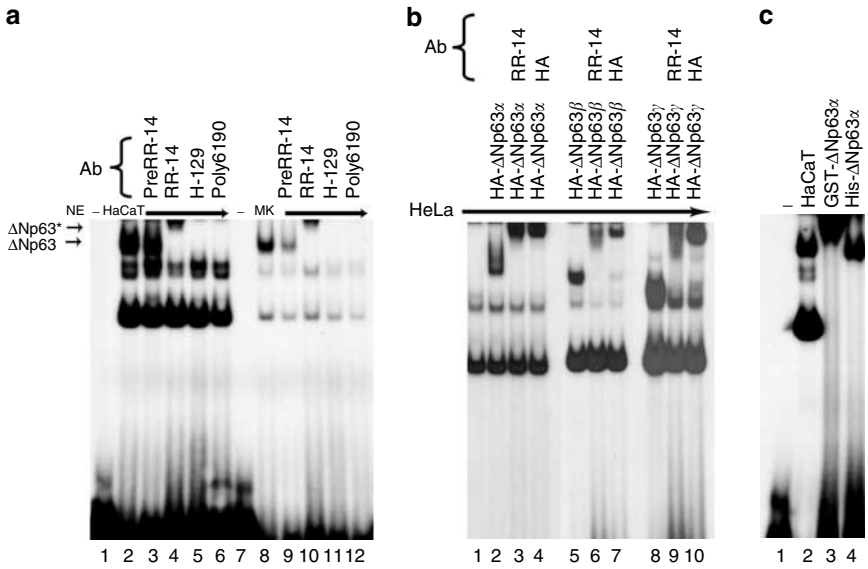

Figure 2. The KSC consists of ΔNp63. (**a**) KSC in both HaCaT (lanes 2–6) and MK (lanes 8–12) nuclear extracts is supershifted with antibodies against various domains of ΔNp63, whereas corresponding pre-immune serum has no effect (lanes 3 and 9). Lanes 1 and 7 correspond to free probe. (**b**) HeLa cells (lane 1) were transfected with HA-tagged ΔNp63α (lane 2), ΔNp63β (lane 5), and ΔNp63γ (lane 8). A complex similar to KSC is observed in transfected cells and it is supershifted with both anti-ΔNp63 and anti-HA antibodies. (**c**) Binding of recombinant p63 to the K14 enhancer. Lane 1, free probe; lane 2, HaCaT nuclear extract; lanes 3 and 4 GST-tagged ΔNp63α; and His-tagged ΔNp63α, respectively.

ΔNp63 protein is present in the KSC

The fact that binding of KSC could be competed by a p53-binding site prompted us to test if this complex contained any of the p53 family members. In particular we were interested in potential binding by p63, as it is highly expressed in keratinocytes. To test this, we used a panel of commercial antibodies as well as a polyclonal anti ΔNp63 antibody (RR-14) that has been described previously (Romano *et al.*, 2006). Interestingly, while the corresponding pre-immune serum had no effect on the KSC complex present in both HaCaT and MK nuclear extracts, the addition of RR-14 to the DNA-binding reaction led to the disappearance of the complex and a clear supershift (Figure 2a). Similar results were also obtained with the antibody H-129, targeted against the C-terminus of the p63 protein and antibody Poly6190, which is a commercial antibody specific for ΔNp63, similar to RR-14. On the other hand, antibodies that specifically recognize the TA isoforms failed to have any effect on the complex, in agreement with the fact that there is little or no expression of TAp63 proteins in these cells (data not shown). Taken together, our data utilizing the various anti-p63 antibodies clearly demonstrate that the KSC complex contains ΔNp63.

To further demonstrate binding of ΔNp63 proteins to K14 enhancer sequences, we ectopically expressed hemagglutinin (HA) epitope-tagged ΔNp63α, ΔNp63β, and ΔNp63γ in HeLa cells, which do not contain KSC. When nuclear extracts from HeLa cells were tested with KSC oligonucleotides, a complex appeared specifically in cells transfected with individual isoforms of ΔNp63 (Figure 2b). The complex formed with ΔNp63α migrated at the same level as the KSC complex, suggesting that KSC most likely represents the ΔNp63α isoform. On the other hand, HeLa cells transfected with plasmids that expressed ΔNp63β and ΔNp63γ resulted in complexes that migrated faster than that present in ΔNp63α-transfected cells, presumably, because of the smaller size of the proteins (compare lanes 2, 5, and 8). The fact that these complexes were indeed formed by the ectopically expressed p63 proteins was verified by their disappearance and supershift with specific antibodies against both p63 and the HA epitope-tag (lanes 3–4, 6–7, and 9–10). In summary, these experiments strongly suggest that ΔNp63, particularly ΔNp63α, is responsible for the KSC-binding activity present in nuclear extracts, although other p63 isoforms can also potentially bind to the K14 enhancer.

It is possible that KSC contains additional proteins present in the nuclear extract, which help to mediate the binding of p63 to the K14 enhancer site. To test if recombinant p63 can directly bind to the KSC site, we expressed and purified ΔNp63α from bacteria as a His- or glutathione *S*-transferase (GST)-tagged protein. Both of these proteins showed strong binding to the oligonucleotide containing the KSC site as shown by EMSA (Figure 2c). Interestingly, the migration of the His-ΔNp63α was faster than the native complex present in keratinocyte extracts. This altered migration pattern could be a reflection of the fact that over expressed p63 protein may not be folded properly in bacteria or that *in vivo* p63 is post-translationally modified causing it to migrate slowly on a non-denaturing gel. Alternatively it is possible that the KSC-binding activity consists of p63 in a complex with another unknown protein(s).

Co-expression of ΔNp63 in cell types that co-express K14 and *in vivo* occupancy of K14 enhancer by ΔNp63

Having shown that p63 can bind to the K14 enhancer, our next objective was to examine if the expression of p63 correlated with that of the expression of K14. For this

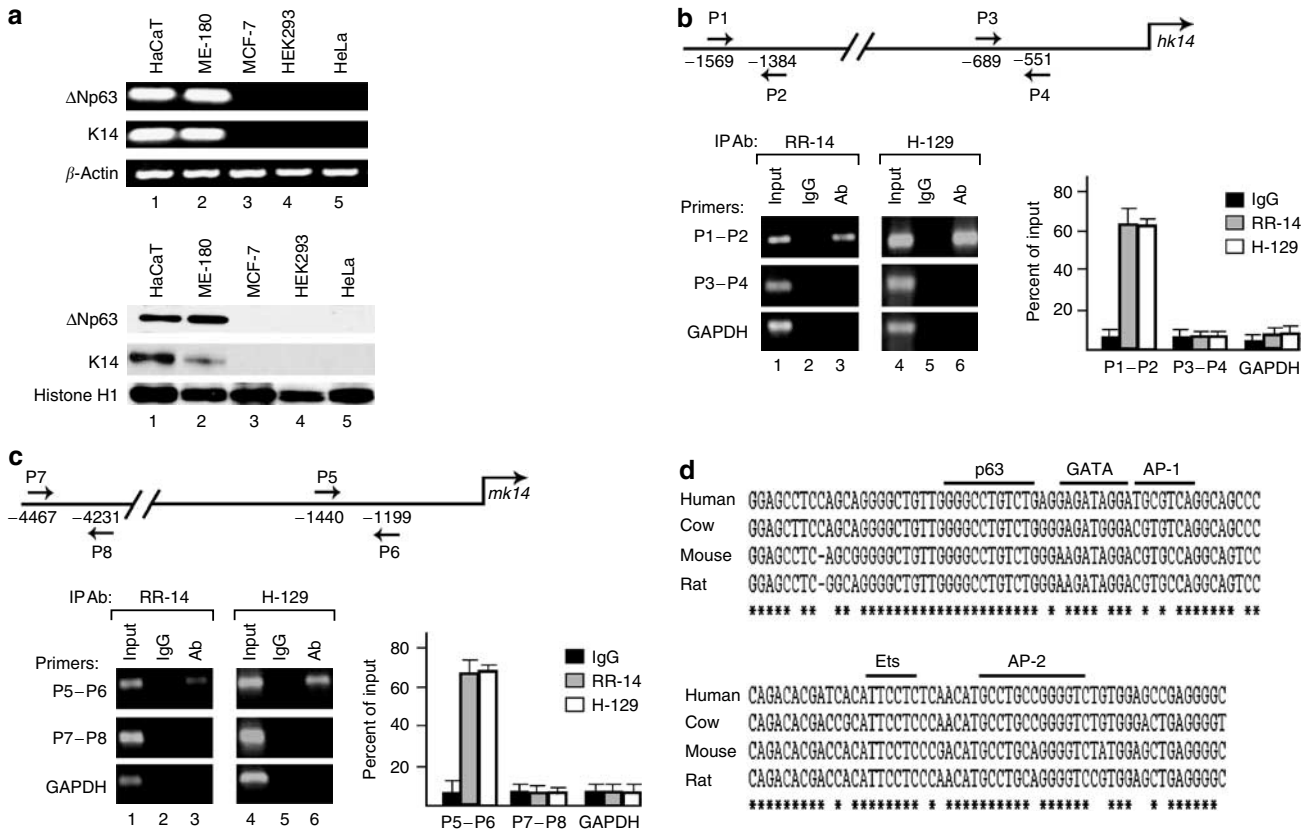

Figure 3. Expression of ΔNp63 correlates with the expression profile of K14 and ΔNp63 occupies the K14 Hs II region. (**a**) In the upper panel, total RNA was isolated from various epithelial cell lines and RT-PCR was performed using primers, which specifically amplify ΔNp63 and K14. β-Actin serves as a control. In lower panel total protein was isolated and protein levels of ΔNp63 and K14 were examined by Western Blot. Histone H1 serves as a loading control. (**b**) ChIP was performed on HaCaT cells using two separate antibodies recognizing ΔNp63α as well as a nonspecific IgG, as indicated. Primer set 1 (P1–P2) amplifies the K14 enhancer. Primer set 2 (P3–P4), amplifies a region that is not conserved and serves as a negative control. Input represents PCR amplification of 1% of the genomic DNA. Ab, antibody. On left is shown a representative result of PCR products from ChIPed samples run on an agarose gel, on right are results obtained from real-time PCR experiments. (**c**) ChIP was performed on mouse skin similar to that described in (**b**). Primer set P5–P6 amplifies the mouse K14 enhancer whereas primer set P7–P8, amplifies a region further upstream that is not conserved. Input represents PCR amplification of 1% of the genomic DNA. Ab, antibody. On left is shown a representative result of PCR products from ChIPed samples run on an agarose gel, on right are results obtained from real-time PCR experiments. (**d**) The sequence of the K14 enhancer from various species was obtained from the respective genome database and aligned using the ClustalW program. The K14 enhancer shows remarkable sequence conservation among the four species examined as indicated by *. The putative binding sites for various transcription factors are denoted by a horizontal bar.

purpose, we chose two keratinocyte cell lines ME180 and HaCaT, and three other epithelial cell lines HeLa, MCF-7, and HEK293 that did not show any detectable KSC binding in EMSA. Both RT-PCR and Western blot analysis showed that as expected, both K14 transcripts and protein were expressed in the keratinocyte cell line HaCaT and ME-180 but not in the other three cell lines tested (Figure 3a). The expression profile of ΔNp63 showed an identical pattern, suggesting that there is a clear correlation between p63 and K14. Our data confirm that expression of ΔNp63 is quite limited and restricted to epithelial cells derived from stratified epithelia but not in cells derived from single-layered epithelia.

We further tested the binding of ΔNp63α to the K14 enhancer in keratinocytes *in vivo* by performing chromatin immunoprecipitation (ChIP) experiments. For the ChIP assays, crosslinked chromatin from HaCaT cells was immunoprecipitated with either RR-14 or H-129 anti-p63 antibodies and the co-precipitation of the K14 enhancer region was ascertained by PCR. We utilized a set of primers (P1 and P2) that amplify the enhancer region of K14 and as a control we used a set of primers (P3 and P4) that amplify a segment of K14 that corresponds to no known Hs and has no reported regulatory role. As an additional negative control we used primers that amplify a genomic segment corresponding to glyceraldehyde-3-phosphate dehydrogenase. As shown in Figure 3b, after immunoprecipitation of cross-linked chromatin we found that there was specific enrichment of ΔNp63 to a DNA segment that corresponds to the Hs II with both antibodies against p63 compared to IgG control. In contrast, we did not observe any localization of ΔNp63 to the control K14 segment or glyceraldehyde-3-phosphate dehydrogenase as demonstrated by the negative PCR results. Similar results were obtained from three independent ChIP experiments. As our experiments thus far were performed with keratinocytes

grown in culture, we asked if ΔNp63α is also present at the K14 enhancer in mouse skin epidermal cells. For this purpose, we repeated ChIP experiments with several independently isolated newborn mouse skin samples. Similar to data obtained from HaCaT cells, PCR with primers P5 and P6 corresponding to the mouse K14 enhancer region, showed specific enrichment with immunoprecipitated DNA from two anti-p63 antibodies and not with control IgG (Figure 3c). These PCR products were significantly stronger than those obtained under similar conditions with control primers P7 and P8 that amplified a segment further upstream of the mouse K14 gene or with primers that amplified a genomic segment of mouse glyceraldehyde-3-phosphate dehydrogenase. Taken together these experiments strongly support the notion that both in human keratinocytes in culture and in mouse skin *in vivo*, ΔNp63α is present at the K14 enhancer segment.

Interestingly, the p63-binding site in the K14 enhancer sequence shows a high degree of sequence conservation between human, cow, mouse, and rat sequences; 24 out of the 27 nucleotides are identical in this stretch, further validating the functional significance of this element (Figure 3d).

p63 can activate the K14 enhancer in keratinocytes

Our previous studies with the K14 enhancer segment have shown that individual transcription factor binding motifs such as AP-1, AP-2, and Ets exhibited activity in reporter gene assays in transient transfection experiments. To test the potential activation properties of p63-binding sites, we generated two constructs, $(p63)_2$K14Luc and $(p63)_3$K14Luc containing two and three copies, respectively, of the p63-binding site (−1433 to −1407 of the human *K14* gene) upstream of the K14 minimal promoter and luciferase reporter gene. In transient transfection experiments in keratinocytes, $(p63)_2$K14Luc and $(p63)_3$K14Luc showed 3–7-fold more activity of luciferase as compared to the construct K14Luc, containing just the minimal K14 promoter (Figure 4a). To test if the transcriptional activation by the p63-binding site was also observed with a heterologous promoter, we generated an additional construct $(p63)_3$TKLuc, containing three copies of the −1433 to –1407 segment of the human *K14* gene upstream of the minimal promoter of thymidine kinase (TK). To address specificity, we also generated a similar construct, $(p63MT)_3$TKLuc containing three copies of a mutated p63-binding site (MT5 as described previously). As shown in Figure 4a, the $(p63)_3$TKLuc construct showed strong activation in keratinocytes (12-fold) compared to the TK promoter. This activation was significantly reduced with the mutant construct.

Finally, to examine the activation properties of p63, we performed transient transfection experiments in Ptk2 cells to test if p63 activates the K14 enhancer. Ptk2 cells have no detectable p63 or K14 expression as described previously and supported by the lack of p63 DNA-binding activity in EMSA as shown in Figure 1. Our prior data showed that a 700 bp K14 enhancer segment is highly active in keratinocytes and not in many other cell types (Sinha *et al.*, 2000). The K14 enhancer construct 700TK was transfected into Ptk2 cells along with either control expression plasmid pCMVHA or plasmids that expressed various isoforms of p63. A CMVLacZ plasmid was co-transfected to serve as an internal control for transfection efficiency. As data presented in Figure 4b indicate, expression of both ΔNp63α and ΔNp63γ isoforms led to higher transcriptional activity of the K14 enhancer element (~6-fold compared to the empty vector). Similarly, the TA isoform also activated 700TK construct and the induction was similar to that of the ΔN isoforms. This effect was specific because the p63 proteins failed to activate the 700TKMT reporter plasmid, which contained a mutation in the p63-binding site (MT5). These data suggest that ΔNp63 regulates the expression of the K14 enhancer at the transcriptional level.

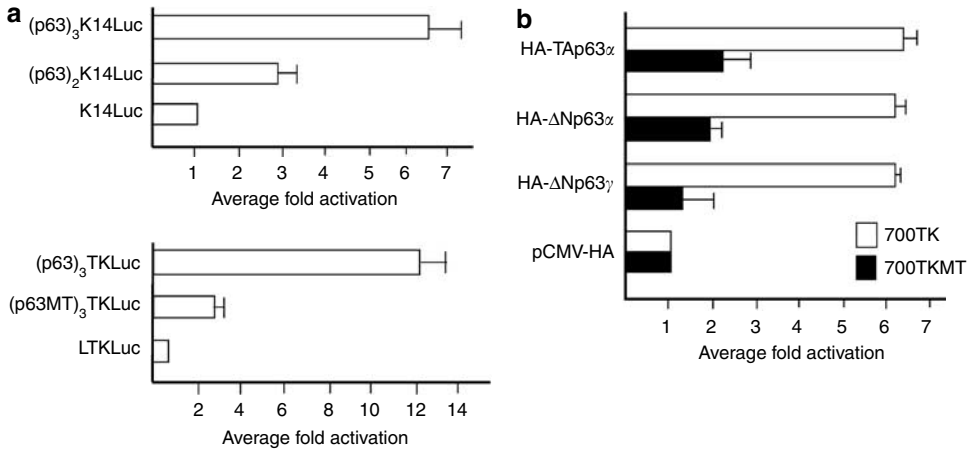

Figure 4. Various p63 isoforms can activate the K14 enhancer in reporter gene assays. (**a**) In the upper panel, a luciferase construct containing two and three copies of the p63-binding site upstream of the K14 minimal promoter is highly active in keratinocytes. In the lower panel, constructs containing three copies of the p63-binding site upstream of a heterologous TK promoter show high levels of activity. In contrast, $(p63MT)_3$TKLuc containing three copies of a mutated p63-binding site is weakly active. (**b**) Various isoforms of p63 can activate the K14 enhancer. 700TK and 700TKMT were co-transfected with expression plasmids encoding various isoforms of p63 into Ptk2 cells. Luciferase values were determined and normalized against β-galactosidase values. The corrected luciferase values for cells transfected with empty vector pCMV-HA were set at 1.

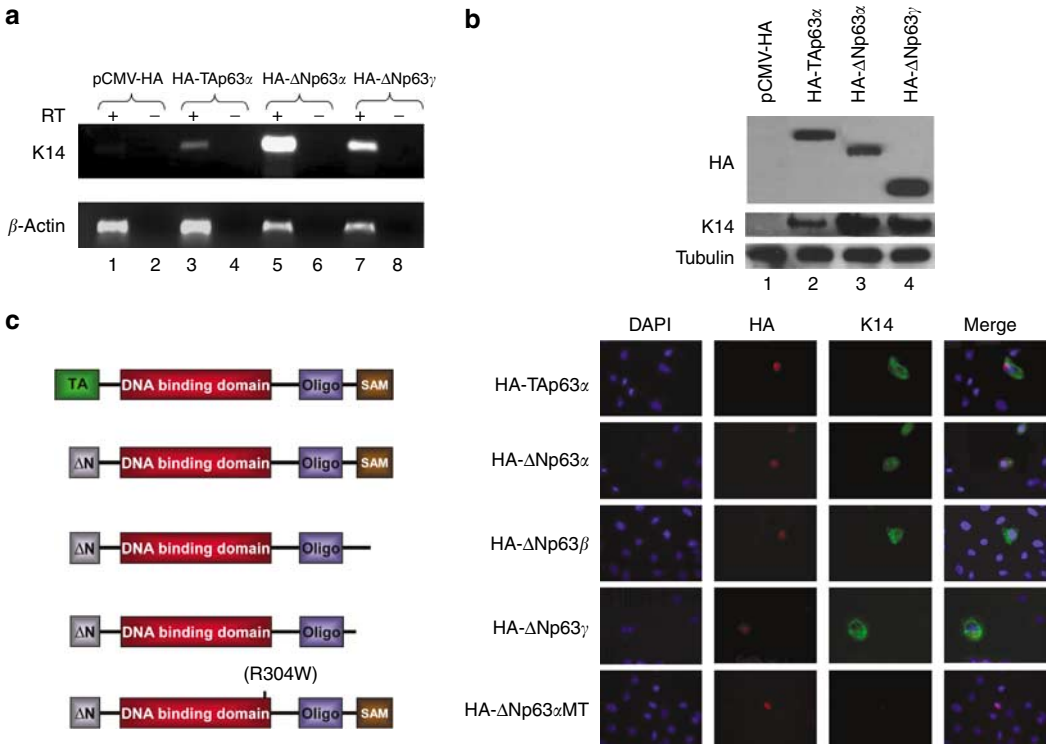

Figure 5. Expression of K14 can be induced by both TAp63 and ΔNp63. (**a**) Ptk2 cells were transfected with HA-epitope tagged plasmids encoding various isoforms of p63. Total RNA was isolated and RT-PCR was performed using primers, which specifically amplify K14. β-Actin serves as a control. (**b**) Ptk2 cells were transfected as described above. Total protein was extracted and Western blot was performed. Top panel demonstrates protein expression levels of various HA-tagged p63 isoforms and middle panel shows expression of K14. Tubulin in the lower panel serves as a loading control. (**c**) Ptk2 cells were transfected with plasmids encoding various isoforms of p63 as depicted on left. Cells were stained with antibodies detecting HA (red), and K14 (green). Nuclei are stained with 4′,6-diamidino-2-phenylindole (blue). A merge is shown on right.

Ectopic expression of multiple isoforms of p63 can induce *de novo* expression of K14

Prior studies have shown that only the TA isoform of p63 can induce K14 expression when ectopically expressed in epithelial cells (Koster *et al.*, 2004). Based on our EMSA, ChIP, and transfection data we postulated that ΔNp63 is also capable of acting as a transactivator of K14 gene expression. To test this experimentally, we transfected Ptk2 cells with various expression plasmids encoding ΔNp63 and TAp63. Initially we examined whether K14 messenger RNA is induced by ΔNp63α and ΔNp63γ by performing RT-PCR (Figure 5a). Unlike control cells transfected with an empty vector, cells that expressed either of these isoforms or TAp63α gave rise to the expected PCR product. Sequencing the PCR product confirmed that indeed it was the rat kangaroo isoform of K14 (data not shown). Western blot analysis on the transfected cells with antibodies against K14 and the epitope-tag present on p63 corroborated that the various isoforms of p63 are capable of inducing K14 (Figure 5b). We further verified our observation of K14 induction by performing immunofluorescence studies with the three major isoforms of ΔNp63 (ΔNp63α, ΔNp63β, and ΔNp63γ) and TAp63α. As illustrated in Figure 5c, in cells that express p63, as shown by anti-HA nuclear staining, a cytoplasmic K14 network was also observed. Although we believe that the induction of K14 is mediated by direct DNA-binding activity of p63, it is also possible that it could be caused by an indirect effect. To test this we generated an expression construct, ΔNp63αMT that contained a mutation in the conserved DNA-binding region (R304W). This p63 mutation is found in a subset of human patients with the ectrodactyly-ectodermal dysplasia-clefting syndrome (van Bokhoven and McKeon, 2002). As expected, recombinant p63 protein containing this mutation failed to bind to DNA when tested by EMSA (data not shown). As our immunofluorescence studies clearly indicate, although ΔNp63αMT protein was expressed and localized to the nuclei, K14 induction was not observed (Figure 5c). Taken together, our studies demonstrate that ectopic expression of all major isoforms of p63 induces K14 expression and that this induction is dependent upon the DNA-binding activity of p63.

DISCUSSION

K14 is a predominant cytoplasmic intermediate filament protein that is expressed at high levels in the proliferating basal cells of the mature adult epidermis. In addition, it is among the earliest epidermal markers, whose expression coincides with commitment of the single-layered surface ectoderm to an epidermal cell fate and stratification during mouse embryonic skin development (Byrne *et al.*, 1994). Multiple signaling pathways and transcription factors ensure the proper spatial-temporal expression of specific markers such as K14, during the carefully orchestrated epidermal morphogenesis program. Transgenic and knockout mice studies have clearly demonstrated that p63, a member

of the p53 family of proteins, is one such factor that plays an important role during this process (Koster and Roop, 2004a).

During the course of our investigation of a functional enhancer that is important for the transcriptional regulation of the *K14* gene, we identified a DNA-binding complex that binds to a conserved sequence and is highly restricted to keratinocytes. Here we provide evidence that this complex is p63, specifically the ΔNp63α isoform. As supported by our EMSA, keratinocytes express robust amounts of ΔNp63 and in nuclear extracts, ΔNp63α accounts for the majority of the DNA-binding activity. In view of the ambiguity regarding the expression levels of the various p63 isoforms *in vivo*, our data provides valuable information regarding the relative functional status of these proteins as judged by DNA-binding activity to a specific sequence. Furthermore, as evident by our data presented here and published studies on several p63 target genes, it is becoming increasingly clear that ΔNp63 isoforms do not function merely as dominant negatives, but that similar to their TA counterparts, given the right cellular and target context these isoforms can also activate transcription (Barbieri and Pietenpol, 2006).

The fact that ΔNp63 and K14 are co-expressed in keratinocyte cell lines and that ΔNp63 activates the K14 enhancer suggests that ΔNp63 is an important transcriptional regulator of K14. This is further supported by the complete absence of K14 in p63 null keratinocytes and the fact that knockdown of p63 in keratinocytes leads to downregulation of K14 expression (Figure S2) (Koster *et al.*, 2004). However, the regulatory mechanisms that govern K14 gene expression may be more complex in skin epidermis *in vivo* as compared to cells grown in culture. For example, although there is clear and dramatic downregulation of K14 messenger RNA and protein levels in p63 null embryonic and new born skin, in adult animals the situation appears to be different. In a tissue-specific knockout mouse model, there is persistent K14 expression in the basal cells of the epidermis despite the ablation of p63 in adult skin (Keyes *et al.*, 2005). One explanation for this phenomenon is that in this case, Cre-mediated p63 knockout in adult skin might not be complete as evident by the presence of persistent cells that express detectable levels of p63. These low levels of p63 may be sufficient to sustain some level of K14 expression. Alternatively in the absence of p63, it is probable that other transcription factors may function in a compensatory manner allowing at least partial but detectable K14 expression. Finally it is also possible that K14 gene expression during early embryonic skin development, a period of dramatic proliferative activity and commitment to the epidermal lineage, may be more dependent upon the levels of p63 and that these requirements are relaxed in adult skin epidermis.

It is interesting to note that in the skin-specific p63 knockout animals, the expression of K14 in skin epidermis was examined only by immunofluorescence studies. It is likely even major changes in the transcript levels may not always lead to dramatic difference in protein levels that are easily detectable. Indeed, our knockdown experiments in cell culture seem to point in that direction. Interestingly, whereas we detect a significant loss of K14 messenger RNA levels upon p63 knockdown by small interfering RNA (90%), protein levels are down only 30% (Figure S2). Taken together, these studies suggest that the regulation of K14 gene expression by p63 is a complex process that should be examined in a context-dependent manner.

What is clear however is that the expression of K14 is governed by a network of transcription factors that bind to conserved regions including p63-responsive elements that are located in the keratinocyte-specific Hs II region. It is likely that Hs regions other than the one described in this study may also harbor functional p63-binding sites. Indeed, our preliminary *in silico* sequence examination and biochemical studies have shown that that the proximal promoter of K14 (corresponding to Hs I) contains at least two p63-binding sites (data not shown). This is in agreement with a recent report that shows ΔNp63 is recruited to the K14 promoter by ChIP experiments (Candi *et al.*, 2006). On the other hand, unlike the observations of Candi *et al.* (2006) who do not detect p63 localization to the K14 enhancer element, our ChIP experiments clearly demonstrate the interaction of p63 with K14 enhancer element. This disparity is likely due to variations in experimental conditions such as choice of antibodies utilized; we used two different antibodies (RR-14 and H-129) proven to be very effective in ChIP experiments, whereas experiments by Candi *et al.* (2006) were performed with a different commercial antibody. The fact that we have obtained similar ChIP results for both the mouse and human K14 enhancer and the additional biochemical evidence presented in this paper strongly supports the idea that ΔNp63 plays an important role in regulating the expression of K14 through binding to both the enhancer and promoter elements.

It will be interesting to examine if ΔNp63 can also regulate the expression of K5, the K14 partner gene that is highly expressed in basal keratinocytes. Underscoring this prospect is the fact that p63 null keratinocytes also demonstrate loss of K5 expression. However, while transient transfection studies have clearly shown that K14 gene expression can be strongly induced by p63, under similar conditions K5 induction is weak. One explanation could be that the induction of K5 may require additional cofactors, possibly in conjunction with p63, which are limiting in the cell lines that were utilized for these experiments. On the other hand, it is possible that the transcriptional regulatory mechanisms are different for K5, as supported by the fact that the appearance of K5 precedes that of K14 during mouse skin development (Lu *et al.*, 2005). The hypothesis currently under investigation in our laboratory is that the regulation of K5 is also directly governed by ΔNp63 through potential p63-responsive elements in the previously described Hs elements of the K5 gene.

The data presented in this paper complements and augments recent studies by the Roop laboratory, which suggests that induction of K14 expression during epidermal morphogenesis requires an additional transcription factor, AP-2γ. According to their model, TAp63 specifically associates with the AP-2γ promoter at E8.5 day as demonstrated by

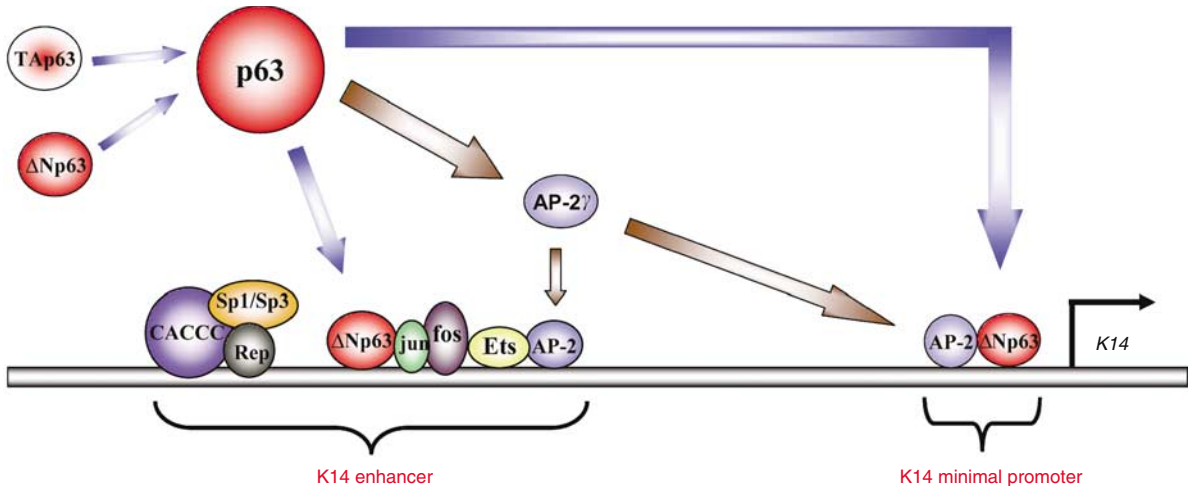

Figure 6. Model depicting the roles various p63 isoforms play in regulating K14 gene expression. Recently, Koster *et al.* (2004, 2006) have demonstrated that TAp63 can indirectly regulate K14 gene expression by upregulating AP-2γ. In addition, Candi *et al.* (2006) have determined that ΔNp63 is recruited to the minimal promoter region of K14. We propose that ΔNp63α binds directly to the K14 enhancer inducing *de novo* expression of K14 in partnership with other transcription factors as described previously (Sinha *et al.*, 2000).

ChIP experiments (Koster *et al.*, 2006). Moreover, when over expressed, TAp63 induces the expression of AP-2γ and the resulting induction of AP-2γ by TAp63 is required for expression of K14 during embryonic skin formation. Our studies using the K14 enhancer reaffirm the idea that p63 and AP-2 proteins both directly or indirectly play a critical role in governing K14 expression as illustrated in our model (Figure 6). Interestingly, the interplay between these two proteins is also evident in the study of a functional enhancer for the p63 gene, where AP-2 binds to this conserved element and cooperates with p63 to induce its activity (Antonini *et al.*, 2006). In view of the recent data demonstrating direct functional interaction between p53 and AP-2, it is tempting to speculate that a complex and interactive network between these two families of proteins may play an important role in integrating developmental cues (Li *et al.*, 2006; Stabach *et al.*, 2006).

One aspect of p63 biology that remains unsettled is the conflicting data from different laboratories regarding the expression profile of TA and ΔNp63 isoforms, particularly during embryonic development (Koster *et al.*, 2004; Laurikkala *et al.*, 2006). It is possible that the variable results reflect the complex expression pattern of these proteins, lack of reagents such as isoform-specific antibodies and the difficulties associated with manipulation of mouse embryos at early stages of development. What is clear however, from data obtained by many laboratories, is that the TA and ΔNp63 proteins may have overlapping as well as unique functions in keratinocyte development and differentiation. Teasing out the relative contribution of each of these isoforms is an interesting challenge for the future and awaits the development of new tools and strategies.

MATERIALS AND METHODS

Plasmids

Recombinant plasmids were constructed using standard molecular biology protocols. To generate expression plasmids for the various p63 isoforms, the complementary DNAs were cloned into the *Bgl*II and *Xho*I sites of the pCMV-HA vector (Clontech-BD Biosciences, Palo Alto, CA). To facilitate expression in bacteria, mouse ΔNp63α was subcloned into *Bam*HI and *Xho*I restriction enzyme sites of the pGEX-5X-1 (Amersham, Pistcataway, NJ) vector, and *Bam*HI and *Hind*III restriction enzyme sites of the pCOLD vector (Takara Mirius Bio, Madison, WI). Each construct was sequence verified to ensure that the construct expressed p63 as an in-frame fusion protein with GST and His tags. The LTK and LK14 constructs containing the TK and the K14 minimal promoters in the luciferase reporter pGL3-basic vector (Promega, Madison, WI) have been described before. A K14 enhancer construct, 700TK, which contains a –2000 to –1300 segment of the K14 5′ region was used as a template to generate 700TKMT by using a two-step PCR approach. Multimerized constructs $(p63)_2$K14Luc, $(p63)_3$K14Luc, $(p63)_3$TKLuc, and $(p63MT)_3$TKLuc were generated by cloning oligonucleotides containing two or three copies of the wild type or the mutant p63-binding sequences in the LTK or the LK14 plasmids.

Expression of GST and His-p63 fusion proteins in bacteria

GST-ΔNp63α and His-ΔNp63α expression plasmids were transformed into *Escherichia coli* (strain BL21 DE3). Bacteria were cultured in Luria–Bertani media in the presence of 50 μg/ml carbenicillin to midlog phase at 37°C and GST fusion protein purification was performed as described previously (Romano *et al.*, 2006). The production and purification of the His-tagged p63 proteins from bacteria were performed according to the standard protocols. Briefly, bacteria were induced at 15°C by addition of IPTG (isopropyl-beta-D-thiogalactopyranoside). Cells were centrifuged and lysed by sonication following which the lysed extracts were centrifuged at 8,500 × g for 30 minutes. The supernatant was mixed with Ni-charged His-Bind resin (Novagen, Madison, WI) and the mixture agitated for 30 minutes at room temperature. The His-bind resin was washed thoroughly first with binding buffer (0.5 M NaCl, 20 mM Tris-HCl, 5 mM imidizole, pH 7.9) followed by wash buffer (0.5 M NaCl, 60 mM imidizole, and 20 mM Tris-HCl, pH 7.9). The recombinant proteins were eluted in buffer containing 1 M imidazole, 0.5 M NaCl,

and 20 mM Tris-HCl, pH 7.9. Purified His-ΔNp63α and GST-ΔNp63α proteins were run on a 12% SDS-PAGE gel, which was then stained with Coomassie Brilliant Blue to ascertain protein purity and quantity.

Cell culture

HeLa (human cervical adenocarcinoma), ME-180 (human cervical squamous cell carcinoma), NIH3T3 (mouse fibroblasts), HEK293 (human embryonic kidney epithelial), and HaCaT (human epidermal keratinocytes) cells were grown in DMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. A spontaneously immortalized MK cell line, was grown as described before (Romano *et al.*, 2006). Ptk2 (rat kangaroo kidney epithelial) cells were grown in Modified Eagle's Medium with 10% fetal bovine serum, 1% Modified Eagle's Medium non-essential amino acid solution, 100 U/ml penicillin, and 100 μg/ml streptomycin. MCF-7 (human breast adenocarcinoma) cells were grown in RPMI (Rosewell Park Memorial Institute) media supplemented with 10% fetal bovine serum and antibiotics.

RT-PCR

Total RNA from HeLa, HEK293, MCF-7, ME-180, HaCaT, and transfected Ptk2 cells was isolated and purified by TRIzol (Invitrogen, Carlsbad, CA) according to established protocols. Two μg of total RNA was reverse transcribed with the iScript complementary DNA synthesis kit (Bio-Rad, Hercules, CA). All primers were designed to span at least one intron. Primers specific for ΔNp63α consist of a forward primer 5′-GTACCTGGAAAACAATGCCCAG-3′ and a reverse primer 5′-GAGGAATGTGATCGTGTCTGG-3′. A fragment of human K14 was PCR amplified from the first strand complementary DNA using the forward primer 5′-CCAGTTCTCCTCTGGATCGCAG-3′ and the reverse primer 5′-GGATCTTCCAGTGGGATCTGTGTC CA-3′. β-Actin was used as a control and a 425 bp fragment was amplified with specific primers 5′-GCTCACCATGGATGATGA TATCGC-3′ and 5′-GATAGCATAGCCTGGATAGCAACG-3′. PCR amplifications were carried out using Hot Start Taq Polymerase (Sigma-Aldrich, St Louis, MO). Reactions were performed for 35 cycles of denaturation at 94°C for 20 seconds, annealing at 56–62°C for 20 seconds, and extension at 72°C for 60 seconds.

Preparation of nuclear extracts and EMSAs

Nuclear extracts from most cell types were prepared, and EMSAs were performed as described (Romano *et al.*, 2006). Nuclear extracts from B16 (mouse melanoma), PC12 (rat pheochromocytoma), and K-562 (human chronic myeloid) cells were obtained from commercial source (Active Motif, Carlsbad, CA). Competition assays were performed by incubating the nuclear extracts with 20- or 100-fold excess cold oligonucleotides before addition of radiolabeled probe; 1 μg of poly(dA-dT)·poly(dA-dT) was added to each reaction as nonspecific DNA. For supershift assays, nuclear extracts were incubated with 1 μl of relevant antibodies for 15 minutes before radiolabeled oligonucleotides were added to the reaction. Antibodies used in EMSAs were anti-HA antibody (3F10) (Roche, Indianapolis, IN), anti-p63 (RR-14), Poly6190 (Biolegend, San Diego, CA) and H-129 (Santa Cruz Biotechnology, Santa Cruz, CA)

Western blot

Ptk2 cells were plated at 125,000 cells/well in a six-well culture plate 1 day before transfection. Cells were transfected using FUGENE 6 transfection reagent (Roche) with 1 μg each of expression plasmid constructs encoding the various HA-tagged p63 isoforms. Cells were harvested 48 hours post-transfection, washed in phosphate-buffered saline (PBS), and centrifuged at 2,000 r.p.m. for 5 minutes. Cell extracts were prepared by resuspending the pellets in Laemmli Sample Buffer (Bio-Rad). Approximately equivalent amounts of each sample was denatured at 96°C for 10 minutes, separated by SDS-PAGE, and transferred to a polyvinylidene fluoride membrane. The membrane was blocked with 5% non-fat dry milk diluted in 150 mM NaCl, 10 mM Tris pH 7.5, and 0.1% Tween 20. Primary antibodies used (1:2,000 dil) include rat anti-HA, rabbit anti-K14 (AF64) (Covance, Berkeley, CA), mouse anti-Histone H1 AE4 (Santa-Cruz), mouse anti-tubulin (Chemicon, Germany), and rabbit anti-p63 (RR-14). Secondary antibodies (1:5,000 dil) used were ImmunoPure goat anti-rat, goat anti-rabbit, and goat anti-mouse, horseradish peroxidase-conjugated IgG (Pierce, Rockford, IL). Chemiluminescence detection of horseradish peroxidase-conjugated secondary antibodies was performed using the horseradish peroxidase stabilizer kit from KPL (Gaithesburg, MD).

Immunofluorescence

Ptk2 cells were plated at 75,000 cells/well in 12-well cell culture plates lined with micro-cover glasses. Cells were transfected at 30% confluency with 1 μg of expression plasmid containing HA-tagged mouse TAp63α, ΔNp63α, ΔNp63β, ΔNp63γ, and ΔNp63αMT. Forty-eight hour post-transfection, cells were fixed on cover glasses with 4% paraformaldehyde for 10 minutes and washed in PBS for 30 minutes. Cells were then permeabilized with 0.1% Triton X-100 (diluted in PBS) for 3 minutes, rinsed briefly in PBS, and blocked with 5% BSA for 2 hours. Samples were then incubated for 1 hour at room temperature with rat anti-HA (Roche) and rabbit anti-Keratin 14 (Covance) at (1:500 dil) in 5% BSA. Primary incubations were followed by several washes in PBS for 30 minutes. Samples were further incubated at room temperature for 45 minutes with 4′,6-diamidino-2-phenylindole (Molecular Probes Inc. Eugene, OR) along with secondary antibodies, anti-rat IgG Alexa 568 (Molecular Probes), and anti-rabbit IgG FITC (BD Biosciences) (1:500 dil). Cover glasses were then mounted on microscope slides and viewed with a Nikon FXA fluorescence microscope. Images were captured using a Nikon digital camera and analyzed using ImageJ, Adobe Photoshop, and Adobe Illustrator software.

Transient transfections and reporter assays

MK cells were plated in six-well plates the day before transfection. Transfections were performed using Fugene 6 reagent (Roche) according to the manufacturer's instructions. One microgram of each luciferase reporter construct was transfected per well along with 0.25 μg of *CMVLacZ* plasmid DNA to serve as an internal control for transfection efficiency. Reporter assays were performed as described previously (Romano *et al.*, 2006). Means and SD were calculated based on data from three independent transfection experiments.

ChIP assays

ChIP experiments with anti-p63 antibodies from cells in culture have been described previously (Romano *et al.*, 2006). For *in vivo* ChIP, newborn mouse skin samples were isolated and treated with Dispase II (Roche) overnight at 4°C. The epidermis was separated, washed

five times with PBS and crosslinked with 1% formaldehyde in PBS at room temperature for 15 minutes. The crosslinking process was stopped by adding 0.125 M glycine for 3–5 minutes followed by five washes with PBS. The epidermis was finely chopped and standard ChIP procedures were followed. ChIPed DNA was subjected to routine PCR as well as real-time PCR. Real-time PCR was performed using an icycler IQ real-time detection System (Bio-Rad) according to manufacturers recommendation with SYBR green for detection. Cycling parameters were: 95°C for 8 minutes, followed by 35 cycles of 95°C for 15 seconds, and 60° C for 1 minute. Primers used to amplify the 5′ region of the human *K14* gene were P1 (5′-GCTCCTAGGCCACAGTAGTGG-3′), P2 (5′-GAGGAATGT GATCGTGTCTGG-3′), P3 (5′-GATGTGAGATCCTCACCATAGG-3′) and P4 (5′-CTGTGCTGAGAAGTCTGTCC-3′). Human glyceraldehyde-3-phosphate dehydrogenase control primers were forward 5′-GAGTACGCTGCAGGGCCTCACTCCTTTTGC-3′ and reverse 5′-CATGCCAGTGAGCTTCCCGTTCAGCTCAG-3′. The following primers were used to amplify the 5′ region of the mouse *K14* gene: P5 (5′-TCAGCCTCAAGAGTACATCTTAGC-3′), P6 (5′-AACCCA GCTAATTCTGGTTCAGAG-3′), P7 (5′-TGTGGGCTTCTACACCAA GGC-3′), P8 (5′-TGTGGTTAGTGAGTCTCCTAC-3′). Mouse glyceraldehyde-3-phosphate dehydrogenase control primers were forward 5′-GCTAGGACTGGATAAGCAGG-3′ and reverse 5′-GG TCCGGCTTGCACACTTC-3′. All experiments involving animals were performed according to SUNY at Buffalo IACUC protocols.

Knockdown of p63 by small interfering RNA

HaCaT cells were seeded in 100-mm dishes. Transfections were performed when cells were 30–40% confluent with SiGenome SMART pool Human TP73L NM-003722 (Dharmacon, Lafayette, CO) using Lipofectamine 2000 (Invitrogen, San Diego, CA). Cells were collected ~60 hours after transfection and analyzed for knockdown of p63 by real-time PCR and Western blot analysis. RNA was extracted from small interfering RNA-transfected and control HaCaT cells by using TRIzol (Invitrogen, San Diego, CA). Total RNA (2 μg) was reverse transcribed with the iScript complementary DNA synthesis kit (Bio-Rad). Real-time PCR was performed as described above. Two separate RNA preparations from transfected cells were evaluated and PCR reactions were performed at least twice in triplicates. The relative transcript level of p63 and K14 in comparison with the reference house keeping gene β_2-microglobulin was determined by using Pfaffl method. β_2-Microglobulin was amplified with forward primer 5′-CTTGTCTTTCAGCAAGGACTG G-3′ and reverse primer 5′-CATGATGCTGCTTACATGTCTC-3′. Primers for p63 and K14 were the same as described for RT-PCR. Western blot analysis was performed with rabbit anti-K14 (AF64) (Covance), mouse anti-tubulin (Chemicon), and rabbit anti-p63 (RR-14).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Figure S1. The sequences of the wild-type (WT) and MT oligonucleotides used in EMSA.

Figure S2. Knockdown of p63 in HaCaT cells leads to a reduction in K14 messenger RNA levels.

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