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A half-site of the p53-binding site on the keratin 14 promoter is specifically activated by p63

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Three members of p53 family, p53, p63 and p73, can transactivate their specific target genes through a p53 consensus sequence-binding motif which consists with direct repeats of PuPuPuC(T/A)(T/A)GPyPyPy as a whole-site of p53-binding site. p63, an epidermal stem cells marker, can regulate epidermal development and differentiation, but p53 has no similar biological activity. One isoform of p63, TAp63α, can active an epidermal basal cell marker, keratin 14. However, the p53-binding site does not exist as a whole-site in the K14 promoter region, although it contains three putative p53 half-binding sites at −269 to −1 of the K14 promoter. Two of three putative half-sites of the p53-binding site can be bound by p63α by electrophoresis mobility shift assay and DNA affinity purification assay. Only mutation of the p53 half-binding site at −140 to −131, the TAp63α induced K14 promoter activity can be abolished. This half-site was specifically activated by p63, but not by p53. Once we extend this p53 half-site to a whole p53-binding site in K14 promoter, both p53 and p63 expression vectors can activate its activity. Therefore, we propose that the different length of p53-binding site would determinate the gene regulated by different p53 family proteins.

Keywords: epidermal differentiation/half-site/K14/p53/p63.

Abbreviations: AP2, activator protein 2; ATCC, American Type Culture Collection; ChIP, chromatin immunoprecipitation; DAPA, DNA affinity purification assay; DIG, digoxygenin; DTT, dithiothreitol; ECL, enhanced chemiluminescence; EMSA, electrophoresis mobility shift assay; HOTC, human ovary teratocarcinoma cell; IgG, immunoglobulin G; K14, keratin 14; K5, keratin 5; NCBI, National Center for Biotechnology Information; PARP, poly-ADP ribose polymerase; PMSF, phenylmethylsulphonyl fluoride; PVDF, polyvinylidene difluoride; RT–PCR, reverse transcription–polymerase chain reaction; SCC, chloride-sodium citrate; SDS–PAGE, sodium dodecylsulphate–polyacrylamide gel electrophoresis; TA, transactivation; TPA, tetradecanoyl phorbol acetate.

The p53 family of genes comprises three members: p53, p63 and p73 (*1*). The protein structure of p63, like that of p53, comprises three functional domains: a transactivation (TA) domain, a DNA-binding domain and an oligomerization domain. Different isoforms have been identified for each of the p53 family gene products through multiple promoters or alternative splicing processing. The three members of the p53 family genes all contain more than one promoter and alternative splicing sites, and therefore, they all have different isoforms of their gene products (*2*, *3*). The gene products of p63 exist in six isoforms. One group has a TA domain with three alternative splicing isoforms: TAp63α, TAp63β and TAp63γ (Fig. 1). The TA domain of the other groups is truncated and has three alternative splicing isoforms: ΔNp63α, ΔNp63β and ΔNp63γ, which are considered dominant-negative isoforms (*4*). The DNA-binding domain is more highly conserved than the other functional domains in the p53 family gene (*5*). Therefore, all members of the p53 family isoforms bind to the same DNA sequences: PuPuPuC(T/A)(T/A)GPyPyPy-*N*(0–13)–PuPuPuC(T/A)(T/A)GPyPyPy, where *N*(0–13) indicates a spacer of 0–13 bases (*6*). We reasoned that if p53 family isoforms bind to the same sequences, they should regulate the same downstream genes. However, knockout experiments have shown that each different p53 family gene is present in different phenotypes. For example, p53 null mice develop normally, but have an increased incidence of cancer growth (*7*). p63 null mice demonstrate limb, craniofacial and epithelial defects (*8*, *9*), and p73 null mice present with neurological, pheromonal and inflammatory defects (*10*). These findings indicate that the different isoforms of the p53 family transcription factors may regulate different target genes even when they contain similar DNA-binding domains. A minor change of the p53 DNA-binding sequence PuPuPuCGTGPyPyPy direct repeats can be activated only by p63 and not by p53 or p73 (*11*). In addition to a change of sequence, differences in the length of the p53-binding site may cause specific activation by different members of p53 family. Tetrameric p53 can bind to a whole-site of the p53-binding site

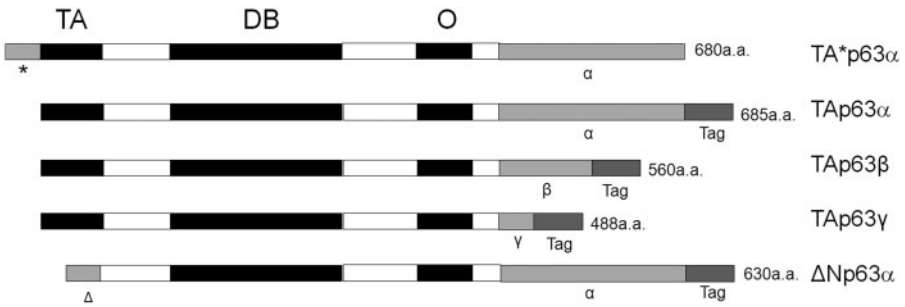

Fig. 1 The diagram of p63 isoforms. TA*p63α: this isoform contains 39-amino acid N-terminal extension in transactivation (TA*) domain and α alternative splicing in C-terminal region. DB: DNA-binding domain. O: oligomerization domain. TAp63α: this isoform contains N-terminal TA domain and α alternative splicing C-terminal extension with a his-myc fusion tag.TAp63β: this isoform contains the N-terminal TA domain and β alternative splicing C-terminal extension with a his-myc fusion tag.TAp63γ: this isoform contains the N-terminal TA domain and γ alternative splicing C-terminal extension with a his-myc fusion tag.ΔNp63α: this isoform contains the N-terminal truncated TA domain (ΔN) and α alternative splicing C-terminal extension with a his-myc fusion tag.

while maintaining transcriptional activity and can bind to a half-site of the p53-binding site PuPuPuC(T/A)(T/A)GPyPyPy, but without any transcriptional activity (*12*, *13*). In contrast, four p53 half-binding sites in the first intron of integrin α_3 can be activated by p63 only as an enhancer (*14*).

p63 has been identified in basal cells of human epidermis and is considered an epidermal stem cell marker (*15*). During basal cells differentiation into transactivated basal cells and subsequent differentiation into spinous cells, p63 is down-regulated and the epidermal basal cell markers, keratin 5 (K5) and keratin 14 (K14), are concomitantly down-regulated (*16*). During embryogenesis in the foetal epidermis, TAp63 isoforms are expressed earlier than are ΔNp63 isoforms and their expression correlates with K14 expression (*17*, *18*). Epidermal basal cells of p63 knockout mice do not express K14 (*8*). Using antisense oligonucleotides to knock down the TAp63 isoforms promotes down-regulation of K14 in limbal keratinocytes *in vivo*, but knocking down the ΔNp63 isoforms does not influence the K14 expression (*19*). K14 expression can be induced by TAp63 isoform expression vectors, not ΔNp63 isoforms, transiently transfected to simple squamous carcinoma cell lines that do not express p63 and K14 (*17*).

Sequence analysis shows no p53 whole-binding site in the K14 promoter, although it contains three putative p53 half-sites at the −269 region. It is possible that p63 activation of the K14 promoter mediates the p53 half-sites or that the region −233 to −224 contains an activator protein 2 (AP2)-binding site. AP2α is a strong activator for K14 expression (*20*), and AP2α can interact with p53 to regulate their target genes (*21*). It is possible that AP2α can also interact with p63 to regulate the K14 promoter.

We used the human ovary teratocarcinoma cell (HOTC)-C9 cell as a model system to study keratinocyte differentiation (*22*). Our previous studies showed that tetradecanoyl phorbol acetate (TPA) inhibits K14 expression in C9 cells and promotes their differentiation. To investigate the regulation by TAp63 of K14 expression and resistance to the TPA response, we used C9 cells (which express ΔNp63α), HeLa cells (which do not express ΔNp63α), HepG2 cells (AP2-null) and H1299 cells (p53-null) to clarify the mechanisms by which p63 regulates the K14 promoter expression.

Materials and Methods

Cell culture

C9 and H1299 (American Type Culture Collection (ATCC), Cat. No. CRL-5803) cells were maintained in RPMI 1640 medium. HeLa (ATCC, Cat. No. CCL-2) cells were maintained in minimal essential medium. HepG2 (ATCC, Cat. No. B-8065) cells were maintained in Dulbecco's modified Eagle's medium. All media were supplemented with 10% bovine serum product and 1% penicillin G-streptomycin sulphate at 37°C in a humidified 5% CO_2 incubator.

Plasmids

pCMV-SPORT6-TA*p63α (IMAGE id: 5552611) and pCMV-SPORT6-AP2α (IMAGE id: 4432023) clones were purchased from the ATCC's IMAGE bank. TAp63α was amplified from TA*p63α by polymerase chain reaction (PCR) and introduced into HindIII and NotI. The PCR product was cloned into the HindIII and NotI sites of pCMV-cDNA 3.1-myc-his ver.C (Invitrogen). ΔNp63α was created from TAp63α as the template by site-directed mutagenesis method. pCMV–CEP4–p53 was a gift from Dr L.-J. Juan (*23*). K14 promoter 2 kb-firefly luciferase and 269 bp-firefly luciferase were cloned in our laboratory. p21 promoter 2.4 kb-firefly luciferase was a gift from Dr B. Vogelstein (*24*).

Transient transfection, drug treatment and luciferase assay

Cells were grown in six-well tissue culture dishes to 80% confluence and then transfected with 1 μg of pCMV-cDNA 3.1, pCMV-cDNA 3.1-TAp63α, pCMV-SPORT6-TA*p63α, pCMV-CEP4-p53 or pCMV-SPORT6-AP2α in the presence of 1 μg of pGL3 firefly luciferase (Promega), 1 μg of K14 promoter 2 kb-firefly luciferase, K14 promoter 269 bp-firefly luciferase, or P21 promoter 2.4 kb-firefly luciferase and 10 ng of pCMV-renilla luciferase plasmids (Promega). Twenty-four hours post-transfection, cells were treated with 100 ng/ml TPA, and 48 h post-transfection, cells were harvested in 1 ml reporter lysis buffer and subjected to a dual-luciferase assay according to the manufacturer's protocol (Dual-Luciferase Reporter Assay System, Promega). Firefly luciferase activity was normalized to renilla luciferase activity and is presented as the mean ± standard deviation of three independent experiments, each performed in triplicate.

Reverse transcription–PCR

Total RNA was extracted using Trizol reagent, and 1 μg of RNA was synthesized to cDNA using a first-strand cDNA synthesis kit for reverse transcription (RT) according to the manufacturer's instructions (Advantage RT-for-PCR kit, Clontech). Five individual primers were used in pairs to amplify each of the six major isoforms

in separate reactions (*25*). Specific paired primers amplification of K14, K5, β-actin and AP2γ were measured. PCR was performed with recombinant *Taq* polymerase as follows: 30 cycles (K14, K5 and β-actin) or 35 cycles (p63 isoforms and AP2γ) at 94°C for 50 s, annealing for 45 s and 72°C for 2 min. Amplified products were analysed on 1% agarose gels. The specific primers were as follows: TAp63 5′-TTAGCATGGACTGTATCCGC-3′; ΔNp63 5′-CCAGACTCAATTTAGTGAGC-3′; p63α 5′-ACTTGCCAGATCATCCATGG-3′; p63β 5′-TCAGACTTGCCAGATCCTG-3′; p63γ 5′-AAGCTCATTCCTGAAGCAGG-3′; K14 forward 5′-GACCATTGAGGACCTGAGGA-3′; K14 reverse 5′-GGCTCTCAATCTGCATCTCC-3′; K5 forward 5′-TGGACCTGGACAGCATCATCG-3′; K5 reverse 5′-TCTCAGCCTCTGGATCATCCG3′; β-actin forward 5′-ACCATGGATGATGATATCGC-3′; β-actin reverse 5′-TTGCTGATCCACATCTGCTG-3′; AP2γ forward 5′-GAAATGAGATGGCAGCTAGG-3′; and AP2γ reverse 5′-CAGGGTTCATGTAGGATTTG-3′. The product sizes were as follows: TAp63α, 1,487 bp; TAp63β, 1,397 bp; TAp63γ, 1,109 bp; ΔNp63α, 1,453 bp; ΔNp63β, 1,363 bp; ΔNp63γ, 1,075 bp; K14, 224 bp; K5, 197 bp; β-actin, 1,079 bp and AP2γ, 269 bp. The annealing temperatures were 62°C for K14; 60°C for all p63 isoforms, β-actin and K5; and 53°C for AP2γ.

Western blot analysis

Ten micrograms of total protein extracts or nuclear extracts from cells was run on 10% sodium dodecylsulphate (SDS)–polyacrylamide gel electrophoresis (PAGE). Proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane and blocked with 3% skim milk–phosphate-buffered saline–Tween 20. Antibodies were used specific for all isoforms of p63 (mouse polyclonal, Sigma), K14 (goat polyclonal, Santa Cruz), AP2α (rabbit polyclonal, Santa Cruz), β-actin (rabbit polyclonal, Lab Vision) and poly-ADP ribose polymerase (PARP) (rabbit polyclonal, Lab Vision) were used as the primary antibody. Goat anti-mouse, goat anti-rabbit or donkey anti-goat immunoglobulin G (IgG) conjugated with horseradish peroxidase were used as the secondary antibody (Santa Cruz), and chemiluminescent signals were detected using an enhanced chemiluminescence (ECL) kit (Amersham).

Electrophoresis mobility shift assay

Ten micrograms of nuclear extracts was prepared from C9 and HepG2 cells. Nuclear extracts were prepared as follows. Briefly, cells were collected and suspended in homogenization buffer [10 mM HEPES-KOH, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulphonyl fluoride (PMSF)], kept on ice for 10 min and centrifuged at 300 g for 10 min at 4°C. The pellets were suspended in homogenization buffer plus 0.05% (v/v) NP-40 and homogenized using a homogenizer for 5 min, and then centrifuged at 1,200 *g* for 10 min at 4°C. The pellets were suspended in re-suspension buffer (40 mM HEPES-KOH, 20 mM KCl, 1 mM DTT, 0.1 mM PMSF and 10% glycerol), 6% (v/v) of 5 M NaCl was added and the suspended pellets were kept on ice for 30 min and then centrifuged at 20,000 g for 30 min at 4°C. The supernatant was collected and added to salt-free resuspension buffer (40 mM HEPES-KOH, 1 mM DTT, 0.1 mM PMSF, 10% glycerol) to dilute the salt to <150 mM. The paired DNA probes for annealing were as follows: K14p1 (−211 to −178) (+)CTGTGAATCACGCCTGGCGGGACAAGAAAGCCCA, (−)TGGGCTTTCTTGTCCCGCCAGGCGTGATTCACAG; K14p2 (−145 to −126) (+)ATGACAGACATGATGAGGCG, (−)CGCCTCATCATGTCTGTCAT; K14p3 (−101 to −82) (+)AGTTGGCGCTAGCCTGTGGG and (−)CCCACAGGCTAGCGCCAACT. A digoxygenin (DIG) labelling and electrophoresis mobility shift assay (EMSA) reaction were performed according to the manufacturer's protocol (DIG gel shift kit, Roche). The competition assay was performed by adding a 50-fold excess of an unlabeled competitor oligonucleotide. The binding mixture was run on a 6% acrylamide:bis-acrylamide gel (29:1) in 0.5 × Tris-borate–EDTA buffer at room temperature. DNA was transferred onto a positively charged nylon membrane that had been presoaked with 2× chloride-sodium citrate (SCC) buffer and cross-linked at 120 mJ in a transilluminator for 3 min. The DIG signal was detected according to the manufacturer's protocol.

DNA affinity purification assay

One hundred micrograms of nuclear extract protein was prepared from H1299 cells transfected with TAp63α. The nuclear extracts were pre-cleared by incubation with 60 µl streptavidin–agarose beads (4%) in a 50% slurry at 4°C for 1 h with rotation and then centrifuged at 5,000 *g* for 10 min at 4°C. The supernatant was collected and prepared for the binding reaction. The positive strand biotin-labelling probes for annealing were as follows: p53 half-binding site (+) biotin–AGCTTAGACATGCCTATCGA, (−)TCGATAGGCATGTCTAAGCT; p53 half-binding site point mutation (+) biotin–AGCTTA GACAT**C**CCTATCGA, (−)TCGATAG**G**CACGTCTAAGC; K14p1 (−211 to −192) (+) biotin–CTGTGAATCACGCCTGGCGG, (−)CCGCCAGGCGTGATTCACAG; K14p2(−145 to −126) (+) biotin–ATGACAGACATGATG A GGCG, (−)CGCCTCATCATGTCTGTCAT; K14p3 (−101 to −82) (+) biotin–AGTTGGCGCTAGCCTGTGGG, (−)CCCACAGGCTAGCGCCAACT. The annealing probe (0.3 nmol) was mixed with 60 µl streptavidin–agarose beads (4%) in a 50% slurry, and the mixture was incubated at 4°C for 3 h with rotation. The DNA-conjugated beads were washed three times with cold washing buffer (20 mM Tris–HCl 8.1, 0.1% NP-40, 1 mM DTT, 0.1 mM PMSF, 150 mM KCl, 1 mM EDTA and 10% glycerol). The binding reaction was performed by mixing the pre-cleared nuclear extract proteins with the DNA-conjugated beads and incubated at 4°C overnight with rotation. The beads were washed three times with cold washing buffer. The binding proteins were eluted with the loading buffer and separated by SDS–PAGE followed by western blot analysis and probed with p63 antibody.

Site-directed mutagenesis

The following point mutation clones were created by site-directed mutagenesis according to the manufacturer's instructions (QuikChange II XL Site-Directed Mutagenesis kit, Stratagene). The K14 promoter 269 bp mtAP2 (−233 to −224)-firefly luciferase was created using the following primers (boldface font indicates the mutant bases): GGTCCGATGGGAAAGTGTAG**AGGATCC**GCCCACACCTCCCCCTGTG and CACAGGGGGAGGTGTGGGC**GGATCCT**CTACACTTTCCCATCGGACC. The K14 promoter 269 bp mtP1 (−206 to −197)-firefly luciferase was created using the following primers: CTCCCCCTGTGAAT**G**AC**C**CCTGGCGGGACAAG and CTTGTCCCGCCAGG**G**GT**C**ATTCACAGGGGGAG. The K14 promoter 269 and 2,000 bp mtP2 (−140 to −131)-firefly luciferase was created using the following primers: CCAGTAAAATATGACAGA**G**AT**C**ATGAGGCGGATGAG and CTCATCCGCCTCAT**G**AT**C**TCTGTCATATTTTACTGG. The K14 promoter 269 bp mtP2L (−158 to −149)-firefly luciferase was created using the following primers: CACTCCAAACAATGAGTTT**G**CA**C**TAAAATATGACAGACATG and CATGTCTGTCATATTTTA**G**TG**C**AAACTCATTGTTTGGAGTG. The K14 promoter 269 bp mtP2R (−114 to −105)-firefly luciferase was created using the following primers: GATGAGAGGAGGGA**G**CT**C**CCTGGGAGTTGGCG and CGCCAACTCCCAGG**G**AG**C**TCCCTCCTCTCATC. The following point addition or deletion clones were created by site-directed mutagenesis according to the manufacturer's instructions (Phusion Site-Directed Mutagenesis kit, Finnzymes). The K14 promoter 1.5X-P2-firefly luciferase was created using the following primers: GACAGACATGATGAGACAAGGCGGATGAGAGG and CCTCTCATCCGCCTTGTCTCATCATGTCTGTC. The K14 promoter 2X-P2-firefly luciferase was created using the following primers: CAGACATGATGAGACATGATGAGGCGGATGAGAGG and CCTCTCATCCGCCTCATCATGTCTCATCATGTCTG. ΔNp63α was created from TAp63α as the template using the following primers: TTGTTTTCCAGGTACAACATGGTGGAAAGCTTAACTAGCCAGCTTG and TGCCCAGACTCAATTTAGTGAGCCACAGTACACGAACCTGGGGCTC. The K14 promoter 269 bp P2 half-site-deletion–firefly luciferase and K14 promoter 2,000 bs P2 half-site-deletion–firefly luciferase were created using the following primers: GTCATATTTTACTGGAAACTCATTGTTTGGAGTG and AGGCGGATGAGAGGAGGGACC. K14 promoter 2,000 bp enhancer half-site (−1421 to −1412)-deletion–firefly luciferase was created using the following primers: CAACAGCCCCTGCTGGAGGC and GAGGAGATAGGATGCGTCAGGCAGC. All constructs were checked by DNA sequencing.

Chromatin Immunoprecipitation

The C9 cells were grown to 90% confluence in a 10-cm culture dish and then performed chromatin immunoprecipitation (ChIP) according to the manufacturer's instructions (EZ ChIP kit, Upstate) using

IgG (rabbit, Sigma) or isoforms of p63 antibody (rabbit polyclonal, Santa Cruz) antibodies. DNA eluted from precipitated complexes was amplified the fragment of K14 promoter by PCR using the following primers: K14 promoter (−1569 to −1384): forward (5′-GCT CCTAGGCCACAGTAGTGG-3′) and reverse (5′-GAGGAATGT GATCGTGTCTGG-3′); K14 promoter (−689 to −551): forward (5′-GATGTGAGATCCTCACCATAGG-3′) and reverse (5′-CTGT GCTGAGAAGTCTGTCC-3′); K14 promoter (−220 to −52) forward (5′-CACCTCCCCCTGTGAATCAC-3′) and reverse (5′-GGC ACTTTCCATTCCCCTTG-3′). PCR was performed as follows: 35 cycles at 95°C for 40 s, annealing at 60°C for 40 s and 72°C for 40 s. PCR products were loaded into the 2% agarose gel.

Results

Characterization of C9 cells

We established the C9 cells from HOTC, which are characterized as epidermal keratinocytes. These cells can differentiate both *in vitro* and *in vivo* (*22*), and they express K14 when incubated in normal media with serum, but can be induced to differentiate by the addition of differentiating agents. Hypodermic injection of C9 cells into nude mice produces tumor growth and keratin pearl formation. Because the keratinocyte differentiation agent, TPA, can induce C9 cell differentiation and K14 down-regulation (*22*), we used C9 cells as a model system to study keratinocyte differentiation. Screening of p63 isoforms in C9 cells by RT–PCR identified mRNA of all ΔNp63 isoforms, but no expression of TAp63 isoforms (Fig. 2). However, only ΔNp63α was detected by western blot analysis (Fig. 3, lane 1). This expression profile of p63 in C9 cells is similar to that observed in primary cultures of human oral keratinocytes (*26*).

The effect of TAp63α on C9 cell expression of K14

We wished to clarify whether the TAp63 isoforms can regulate K14 expression in C9 cells. TAp63α or TA*p63α [TA* is a 39-amino acid amino-terminal extension encoded in *TP63* transcripts (*4*)] was transfected into C9 cells. The endogenous ΔNp63α and transfected TAp63α or TA*p63α were identified by western blot analysis (Fig. 3A). Overexpression of TAp63α or TA*p63α up-regulated K14 expression, but not AP2α expression (Fig. 3A). TPA decreased expression of endogenous ΔNp63α, but not of transfected TAp63α, and K14 expression did not appear to change after TPA treatment in transfected TAp63α cells compared with non-treated cells (Fig. 3B).

TAp63α and AP2α enhance K14 expression independently of each other

ΔNp63α is a dominant-negative isoform of p63 that can inhibit the TA function of TAp63 isoforms (*4*). However, ΔNp63α is expressed endogenously and K14 expression remains normal in the absence of TAp63 expression in C9 cells (Fig. 3A, lane 1). To clarify this discrepancy, we selected various cells, such as HeLa cells lacking ΔNp63α and K14 expression; HepG2 cells lacking ΔNp63α, AP2α and K14 expression and H1299 cells lacking p53, ΔNp63α, AP2α and K14 expression (Figs. 4A and 7A). We first co-transfected K14-2000 and K14-269 promoters with the expression vector TAp63α into C9 and HeLa cells and found that TAp63α up-regulated both K14 promoters in both cell types (Fig. 4B). In TPA-treated cells, the K14 promoters were down-regulated in C9 cells, but up-regulated in HeLa cells (Fig 4B). These results suggest that ΔNp63α does not act as a dominant-negative isoform for the K14 promoter in C9 cells. In HepaG2 cells, co-transfection of the K14-269 promoter with the expression vector TAp63α or AP2α activated the K14 promoter activity in a dose-dependent manner (Fig. 4C). Co-immunoprecipitation data showed no detectable protein–protein interaction between TAp63α and AP2α (data not shown). Because HepG2 is an AP2 null cell, p63 can also up-regulate the K14 expression. So these data suggest that TAp63α enhances the K14 promoter response directly and independently of the effect of AP2α.

TAp63α-enhanced K14 expression is mediated through a p53 half-site of the K14 promoter

Both the 2-kb and 269-bp regions of the K14 promoter can be activated by p63 (Fig. 4B). The sites responding to p63 were predicted as follows. Three putative half-sites of the p53-binding sites (P1–P3) are located at −269 to −1 of the K14 promoter (Fig. 5). The core

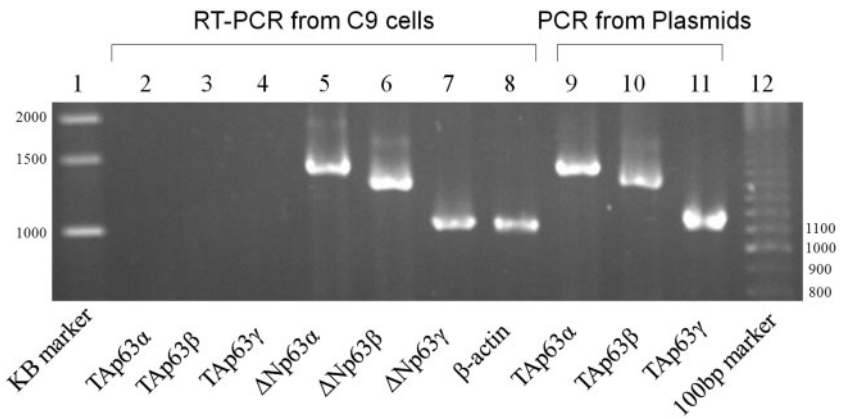

Fig. 2 The expression profile of p63 at the mRNA level in C9 cells. The expression of p63 isoforms in C9 cells determined by RT–PCR. Line 1: KB marker. The expression of ΔNp63α, ΔNp63β and ΔNp63γ were identified (lines 5–7), but not expression of TAp63 isoforms in C9 cells (lines 2–4). β-Actin expression was the internal control (line 8). Lines 9, 10 and 11 are the PCR product of the TAp63α, TAp63β and TAp63γ vectors, separately. Line 12: 100 bp marker.

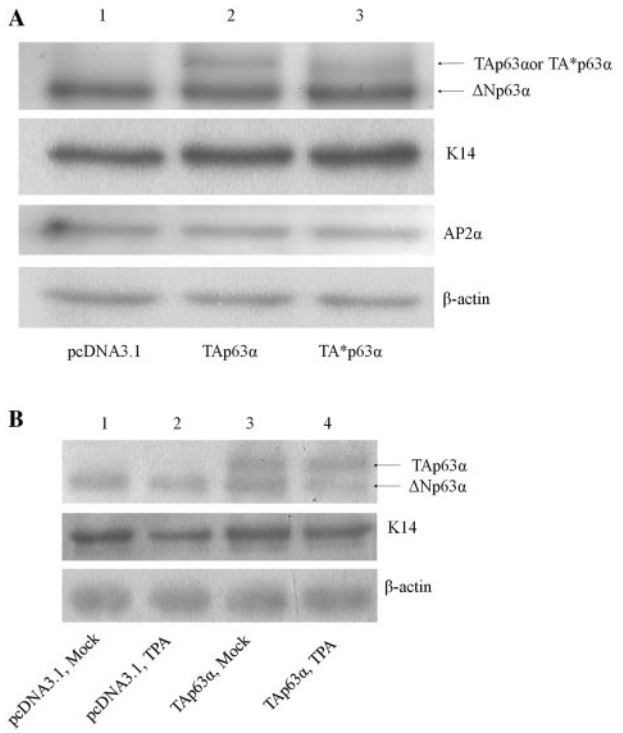

Fig. 3 Western blot analysis showed that K14 is up-regulated by TAp63α at the protein level in C9 cells. (A) C9 cells expressed only ΔNp63α (lane 1). After transfection with TAp63α or TA*p63α expression vectors in C9 cells, exogenous expression of TAp63α or TA*p63α was detected (lanes 2 and 3). Expression of K14, but not AP2α, was enhanced by TAp63α or TA*p63α (lanes 2 and 3). β-Actin expression was the internal control. Each well was loaded with 10 μg of total protein. (B) TPA treatment reduced the expression of K14 and endogenous ΔNp63α (lanes 1 and 2). β-Actin expression was the internal control. Overexpression of TAp63α enhanced K14 expression (lanes 1 and 3), and this enhancement was also observed under TPA treatment (lanes 2 and 4). Each well was loaded with 10 μg of total protein.

sequence of P1 (CACG) does not match the core sequence C(T/A)(T/A)G of the p53-binding site, but the TA function of p63 is higher in the CACG sequence than in the CAAG and CTTG sequences (*11*). There is a Sp1-binding site besides P1, and Sp1 is a p53 co-factor in the TA of the *BAX* gene (*27*). P1 is a half-site plus a quarter-site of the p53-binding site. The 1.5X p53 half-binding site can be transactivated by p53 (*12*, *13*). The complete P1 is a 1.5X p53 half-binding site plus a Sp1 site. Its binding affinity for p63 may be higher than for the other putative half-sites P2 and P3. EMSA was used to confirm the binding affinity of P1, P2 and P3 to C9 nuclear protein. The P1 and P2 sites were more tightly bound to the specific nuclear protein than was the P3 site (Fig. 6A). To identify whether p63 specifically binds to the P1 and P2 sites, we transfected H1299 cells with the TAp63α expression vector and then extracted the nuclear protein. We used DNA affinity purification assay (DAPA) to analyse the oligomers of the K14 promoter region using −211 to −192 (the p53 half-binding site of P1) and −145 to −126 (P2) as probes to purify nuclear proteins and western blot analysis using p63 antibody. DAPA identified the nuclear extract protein bound to the P1, P2 and consensus sequence of p53 half-site with p63 (Fig. 6B). An experiment using a p53 half-binding site point-mutation probe demonstrated that the binding of p63 was depleted (Fig. 6B, lane 3). These data indicate that the half-site of the p53-binding site on K14 promoter, P1 and P2, is bound specifically by p63.

To evaluate the transcriptional activity of p63 action at the sites of the K14-269 promoter, the sites −233 to −224 (an AP2 site), −206 to −197 (a p53 half-binding site of P1) and −140 to −131 (a p53 half-binding site, P2) were mutated (Fig. 7B). The mutation construct plasmids with reporter genes were transfected to H1299 cells and the luciferase activity was measured (Fig. 7C). Only the −140 to −131 region mutant abolished the p63 TA activity, indicating that p63 activates K14 expression only through the p53 half-site P2 (−140 to −131).

In order to prove that only a p53 half-site sequence can be activated by p63, one special case must be considered. A p53 whole-site on the promoter of GPX2, a member of the glutathione peroxidase 2 family, is located at −131 GGA**CCTG**TTTttgctaagtCAT**CCTG** GGG −101 (*28*). This previous study claimed that the core sequence **CCTG** can be specifically activated by p63. A nine-base spacer is between the two half-sites. The first half-site has perfect 5′ purine and 3′ pyrimidine sequences, but the second half-site has five of six errors in the 5′ purine and 3′ pyrimidine sequences. Comparison with the p53 half-site sequence within the K14-269 promoter, eight bases upstream of the P2 (−158 TTT**CCAG**TAA −149, marked as K14P2L) is another p53 half-site candidate, which also has five of the six errors in 5′ purine and 3′ pyrimidine sequences (Figs. 5 and 7B). The K14P2L mutant cannot influence the K14 promoter TA function by p63 (Fig. 7C). In this case, K14P2L is unlikely to be another p53 half-site.

Candi *et al.* (29) identified a putative p53 whole-site specifically activated by ΔNp63α on the K14 promoter using the ChIP assay and a large-range deletion-promoter functional assay. They listed the functional p53 whole-site at −134 GGG**ACCTG**CCT GGGAGTTGGC−114, but the exact location is at −115 to −95 on the transcriptional initiation site of the National Center for Biotechnology Information (NCBI) reference sequence of K14 gene [GenBank: NM000526] (Fig. 5, shown in grey). This sequence contains only one core sequence, **CCTG**, which may be another p53 half-site candidate (−114 GGA**CC TG**CCT−105) (Fig. 5). This half-site is located just 16 bases downstream of K14P2 and has been named K14P2R. The 16-base spacer is too long to fit the p53 whole-site sequence, and the spacer between the two half-sites must be shorter than 13 bases (*6*). However, there is an exception because 15 bases of the spacer retain p53 transcriptional activity (*12*). The K14P2R mutant cannot influence the K14 promoter TA function by p63 (Fig. 7C). In this case, K14P2R is unlikely to be another p53 half-site. The K14P2 mutant of the K14-2000 promoter also lost its TA activity of p63 (Fig. 7D). These results indicate that the K14 promoter possesses only one functional p53 half-site, P2 site, specific activated by TAp63α.

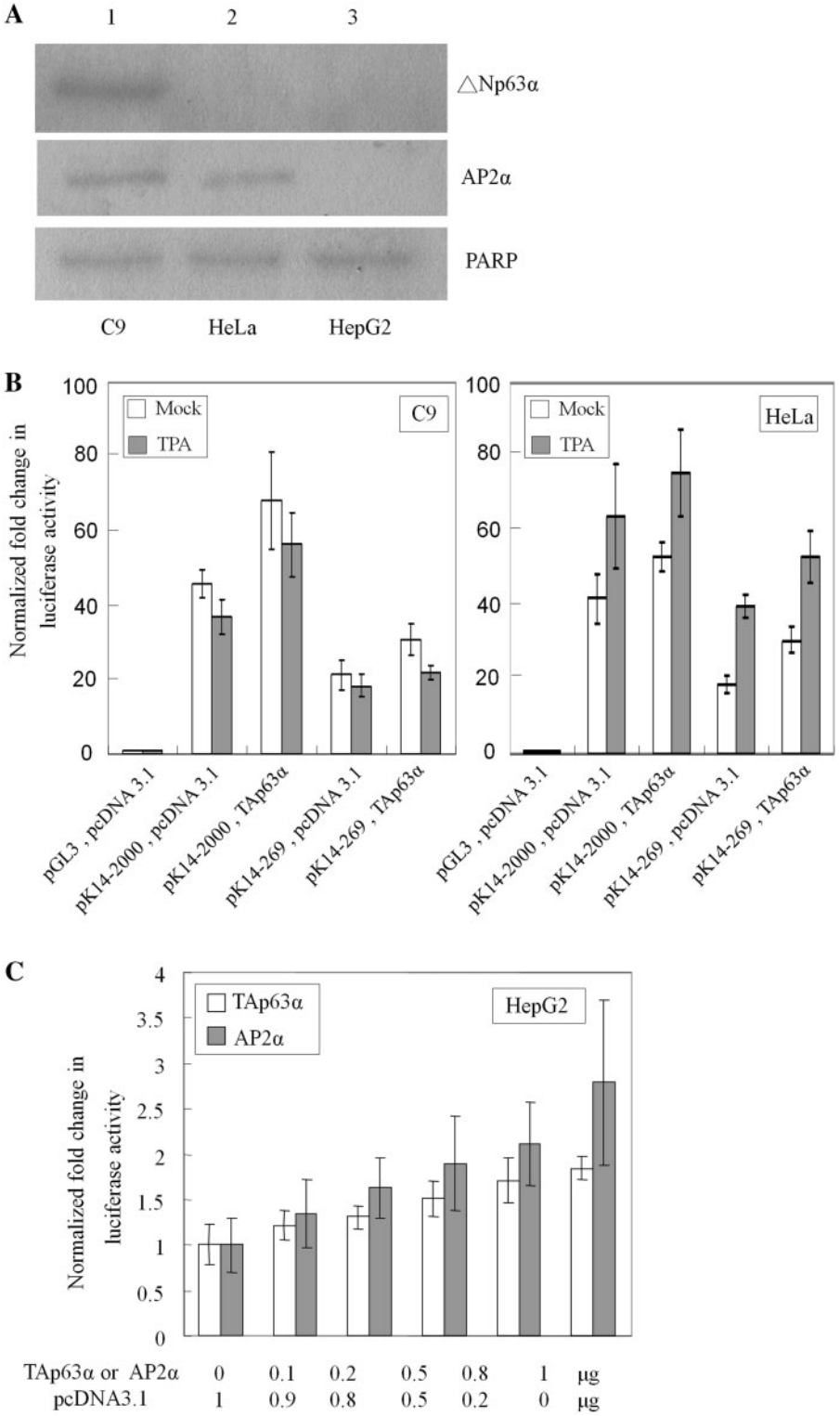

Fig. 4 Overexpression of TAp63α and AP2α directly enhances the K14 promoter response. (A) Western blot analysis of the expression profiles of p63 and AP2α in nuclear extraction proteins of different cell lines. ΔNp63α and AP2α were both expressed in C9 cells, but only AP2α, and not ΔNp63α, was expressed in HeLa cells. Neither ΔNp63α nor AP2α was expressed in HepG2 cells. PARP was the internal control. Each well was loaded with 10 μg of nuclear protein. (B) Luciferase activity of pK14-2000 and pK14-269 in C9 cells. The activity of both K14 promoters was up-regulated by TAp63α, and TPA treatment down-regulated the activity of both K14 promoters. In HeLa cells, the activity of both K14 promoters was up-regulated by TAp63α and TPA treatment. (C) Luciferase activity of pK14-269 in HepG2 cells. Transfection with either TAp63α or AP2α expression vectors enhanced pK14-269 activity in a dose-dependent manner.

Fig. 5 Three putative half-sites of p53-binding sites (P1–P3) in the K14 promoter (–269 to –1). P1 is located at –206 to –197 (–206 to –188 is considered a 1.5X half-site); P2 is located at –140 to –131; P3 is located at –96 to –87. The other two candidates for the half-site are P2L (–158 to –149) and P2R (–114 to –105), neighbours of P2. The rule of p53 conservative sequences is underlined as follows. The whole p53-binding site:PuPuPuC(T/A)(T/A)GPyPyPy-N(*013*)-PuPuPuC(T/A)(T/A)GPyPyPy, N(0–13) indicates a spacer of 0–13 bases. The half-binding site is indicated by RRRC(T/A)(T/A)GYYY. Candi *et al.* (*29*) predicted a p53 whole-site, which is marked in grey. This map of the promoter sequence refers to a K14 promoter sequence form GenBank database [GenBank: HSU11076], and the transcriptional initiation site refers to the NCBI reference sequence of human K14 gene [GenBank: NM000526].

p63, but not p53, can up-regulate K14 expression

After co-transfection with TAp63α or p53 expression vectors and K14 or p21 promoters separately into H1299 cells, the results indicated that p53 can activate p21 promoter activity but not at K14 promoter in H1299 cells (Fig. 8A and B). TAp63α activated both the K14 and p21 promoters (Fig. 8A and B). These results clearly demonstrate that p53 can activate only the p21 promoter with whole p53-binding sites (*24*), but cannot activate the K14 promoter at a p53 half-site. To further clarify that p53 can only up-regulate a whole p53-binding site on the promoter region, we constructed various length of P2 site as 0, 1, 1.5 and 2X p53 half-binding site from K14-269 promoter (Fig. 9A). According to the promoter activity results, the TAp63α can up-regulate depending on the length of p53-binding site (Fig. 9B). Normally, the p53 can suppress the K14 promoter activity (Fig. 8A), but in this case we found that p53 can up-regulate the 2X P2 site as a whole p53-binding site (Fig. 9B). Therefore, a half p53-binding site can only be activated by p63 but not by p53 on K14 promoter.

p63 TA of K14 expression is independent of AP2γ activation

Koster *et al.* (30) demonstrated that p63-regulated K14 expression is mediated by activation of AP2γ in Ptk2, HR9 and F9 cells. However, because p63 directly activates the K14 promoter in our cell model systems, we wanted to clarify whether the p63-activated K14 is independent of AP2γ activation. Transfection with the TAp63α expression vector in C9, HeLa, H1299 and HepG2 cells showed no induction of AP2γ expression and reduced expression in some cells, as well as induction of both K5 and K14 expression (Fig. 10). These

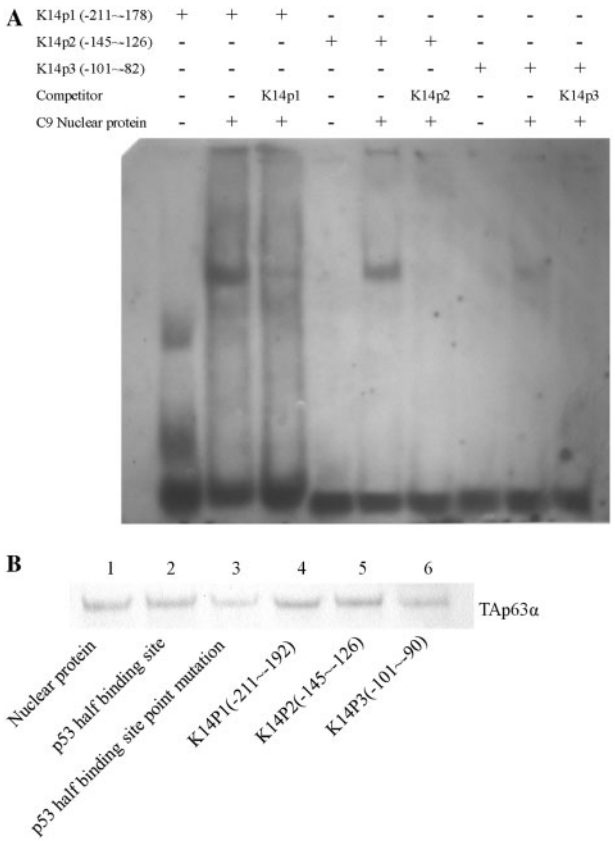

Fig. 6 Confirmation of K14P1 and K14P2 as the p53 half-binding sites by EMSA and the binding to p63 by DAPA. (A) EMSA was used to verify that K14P1, K14P2 and K14P3 bind to nuclear proteins. The oligonucleotides of P1, P2 and P3 were synthesized as probes and then incubated with nuclear protein extracts from C9 cells. P1, P2 and P3 all bound to the nuclear proteins, but the binding affinities of P1 and P2 were much higher than that of P3. All their unlabeled self-competitors can compete their binding to demonstrate its specific binding. (B) DAPA was used to purify the nuclear protein extract from H1299 cells transfected with the TAp63α expression vector. The nuclear proteins were then incubated with the designed oligonucleotide probes of the p53 whole-binding site, p53 half-binding site, p53 half-site with point mutation, K14P1, K14P2 and K14P3, and blotted with p63 antibody. The grading of binding affinity of p63 was as follows: p53 half-binding site (lane 2) = K14P1 (lane 4) = K14P2 (lane 5) > point mutation of the p53 half-binding site (lane 3) = K14P3 (lane 6). Lane 1, 10 μg of nuclear protein only.

data confirm that K14 activation by p63 in our cell model was independent of AP2γ activation.

p63 can bind to the K14 enhancer region with no obvious transactivation function

Romano *et al.* (*31*) demonstrated that there is another half p53-binding site (–1421 to –1412) at the K14 enhancer region which can bind by p63. According to their enhancer activity assay in TK promoter system, the p63 can enhance the TK promoter activity with two or three copies of this half-site in keratinocytes. We also found that p63 could bind to both –220 to –52 and –1569 to –1384 of K14 promoter by ChIP–PCR analysis in C9 cells (Fig. 11). However, in K14-2000 promoter functional assay, deletion of the p53 half-binding site at enhancer region had no obvious effect to influence K14 promoter

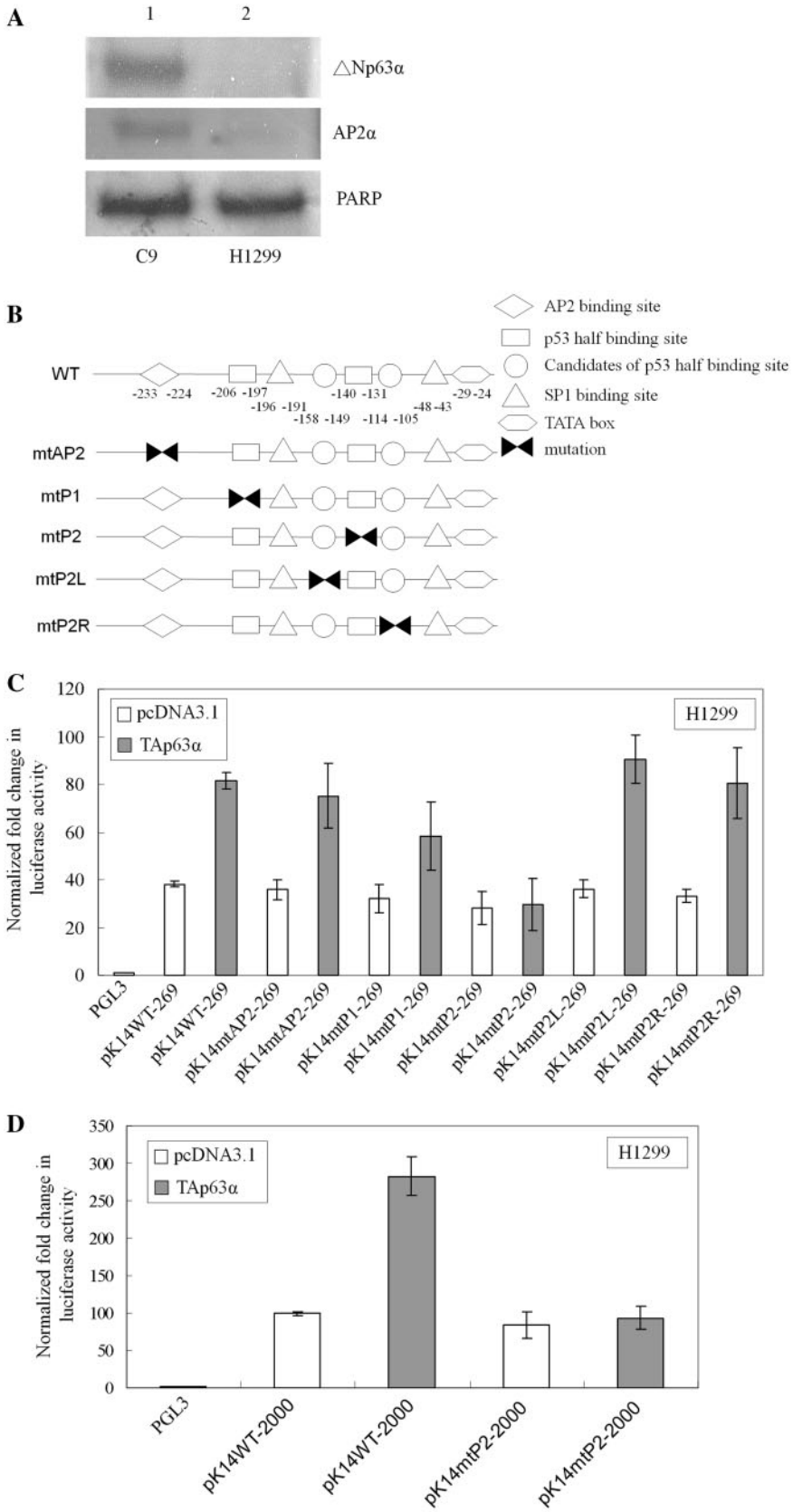

Fig. 7 TAp63α enhances K14 promoter expression mediated by a p53 half-site K14P2 (−140 to −131) in H1299 cells. (A) Nuclear proteins of C9 and H1299 cells were blotted with antibodies to p63, AP2α and PARP. No p63 or AP2α was expressed in H1299 cells. C9 nuclear proteins acted as a positive control and PARP as an internal control. Each well was loaded with 10 μg of nuclear protein. (B) pK14-269 mutant constructs: WT, wild-type; mtAP2, AP2 site mutant; mtP1, K14P1 site mutant; mtP2, K14P2 site mutant; K14P2L, a candidate of the p53 half-site mutant to the left of K14P2; K14P2R, a candidate of the p53 half-site mutant to the right of K14P2. (C) The luciferase activity of the construct mutants shows that only the K14P2 mutant abolished the TAp63α TA function in the K14 promoter in the transfection assay. (D) TAp63α activates the wild-type K14 promoter (pK14WT-2000), but not the P2 mutant promoter (pK14mtP2-2000) in H1299 cells.

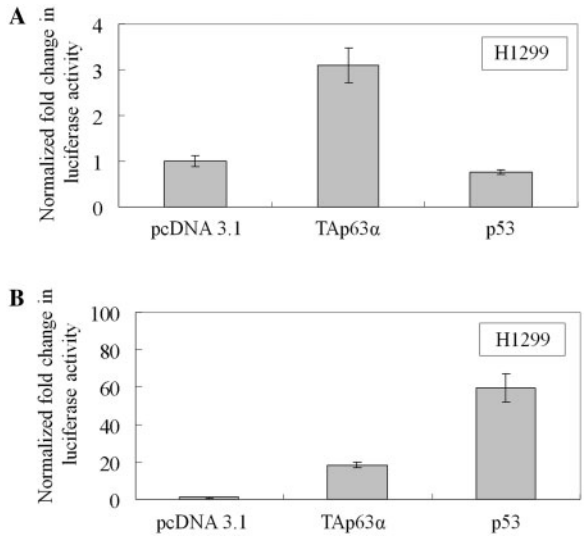

Fig. 8 p63, but not p53, activates the K14 promoter specifically. (A) H1299 cells were co-transfected with pK14WT-2000 and TAp63α or p53 expression vectors and the luciferase activity measured. TAp63α activated pK14WT-2000 promoter activity, but p53 did not. (B) H1299 cells were co-transfected with the p21-2.4 kb promoter and the TAp63α or p53 expression vectors. Both TAp63α and p53 enhanced the p21-2.4 kb promoter activity.

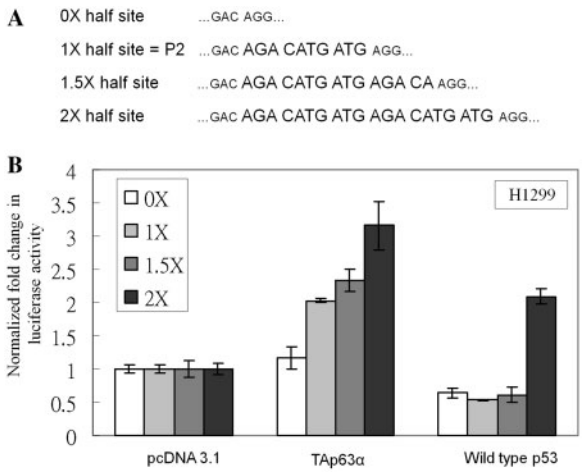

Fig. 9 p53 and p63 have different transactivation function which mediated the different length of p53-binding sites. (A) Using the site-directed mutagenesis methods, the K14 promoter clones with different length of p53-binding sites can be prepared from 1X P2 half-site as the pK14WT-269. (B) p63 presents much more TA activity mediated by the lengthening of p53-binding site, but p53 can only up-regulate the p53 whole-binding site.

activated by the different isoforms of p63 expression (Fig. 12A and B). This result is similar to that K14 promoter with −1558 to −570 deletion is also activated by both TA and ΔNp63 such as the wild-type K14 promoter (*29*).

Discussion

We identified two p53 half-binding sites on the K14 promoter region (Fig. 13). One is located at −206 to −197 (K14P1) and the other is located at −140 to −131 (K14P2). p63 directly activates the K14 promoter, but this is not mediated through AP2α and AP2γ (Figs. 4C and 10). DAPA showed that p63 can bind both p53 half-sites (Fig. 6B). However, the K14 promoter functional assay showed that only the P2 site, and not the P1 site, can be activated by p63 (Fig. 7C). This is the first study to demonstrate that the p63 transactivates a downstream gene through a p53 half-binding site on the promoter region but not on enhancer region. It is generally acknowledged that the activation of their target genes by p53 family members is mediated at the whole-site of the p53-binding site (*32*). Although all members of the p53 family can bind to a p53 half-site, the TA activity of different members varies. p53 can bind to a p53 half-site without exhibiting TA activity (*12*, *13*). In contrast, p63 binds a p53 half-site of the K14 promoter and can transactivate the K14 promoter (Figs. 6B and 7C). According to addition mutation of K14 promoter assay with different length of p53-binding site, both p53 and p63 could up-regulate the mutant keratin 14 promoter with whole-site of p53-binding site (Fig. 9B). Therefore, different length of p53-binding site could determinate the gene regulated by different p53 family members. Based on this phenomenon, we were able to identify the p63-related developmental and differentiated target genes with a p53 half-site specifically activated by p63.

The protein structure of p63 contains three functional domains: N-terminal TA domain, middle DNA-binding domain and C-terminal oligomerization domain. TA*p63α contains an extra 39 amino acids in the N-terminal more than TAp63α and can inhibit the TA domain function (*4*, *33*). ΔNp63α, with a truncated TA domain, acts as dominant-negative regulator (*4*, *34*). p63 also can bind to the whole p53-binding site (*26*), but that the TA activity varies in different p63 isoforms. TAp63α can bind to the p53 whole-site and transactivate downstream genes such as the p21 promoter (Fig. 8B). The other two isoforms, TA*p63α and ΔNp63α, are not capable of transactivating the p21 promoter with the p53 whole-site (*35*). Thus, N-terminal TA domain is necessary for p63 TA of the p53 whole-site. Our results showed that the TA*p63α and TAp63α can both activate K14 expression (Figs. 3A and 12B), and ΔNp63α also can up-regulate the K14 promoter (Fig. 12B) (*29*). Therefore, the N-terminal TA domain seems unnecessary for p63 activation of the p53 half-site in the K14 promoter. The other TA domain (TA2) has been identified and located in the C-terminal of p63 (*35*), and this domain is required for TA function of ΔNp63 isoforms (*5*, *35*, *36*). On the other hand, it had been known that p53 did not have the C-terminal TA2 domain (*5*, *36*, *37*). In our study, TA*p63α, TAp63α and ΔNp63α were all capable of transactivating the K14 promoter through a p53 half-binding site (Fig. 12B), but p53 could not activate the K14 promoter (Fig. 8A). Therefore, TA2 domain may be necessary for p63 TA of the p53 half-site in the K14 promoter. Further study is needed to clarify the

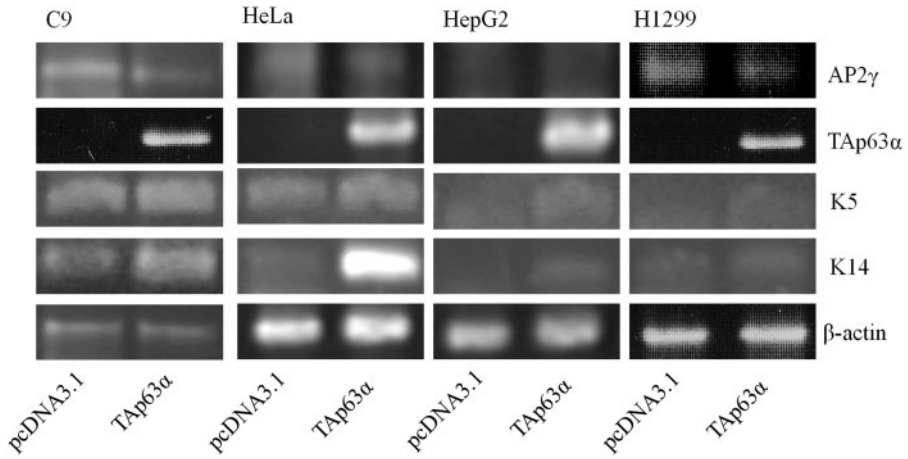

Fig. 10 TAp63α in C9, HeLa, HepG2 and H1299 cells cannot induce AP2γ expression. Forty-eight hours after transfection with the TAp63α expression vector, mRNA was isolated from each cell type and the expressions of AP2γ, Tap63α, K5, K14 and β-actin were examined by RT–PCR. AP2γ expression decreased in C9, HeLa and H1299 cells after transfection with TAp63α, and K5 and K14 expression was up-regulated by TAp63α in all cells. β-Actin expression was the internal control.

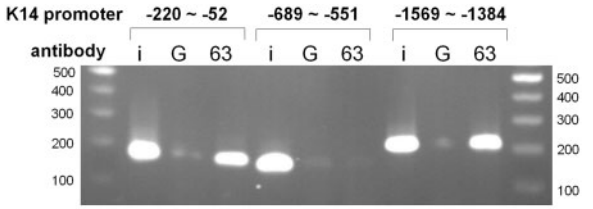

Fig. 11 ChIP assay showed p63 could bind to both the K14 promoter and enhancer regions. After ChIP assay with the specific antibody for α isoforms of p63 in C9 cells, specific PCR products could be amplified in −220 to −52 and −1569 to −1384 but not −689 to −551 regions on K14 promoter (symbol 'I': 10% input; 'G': IgG; '63': p63).

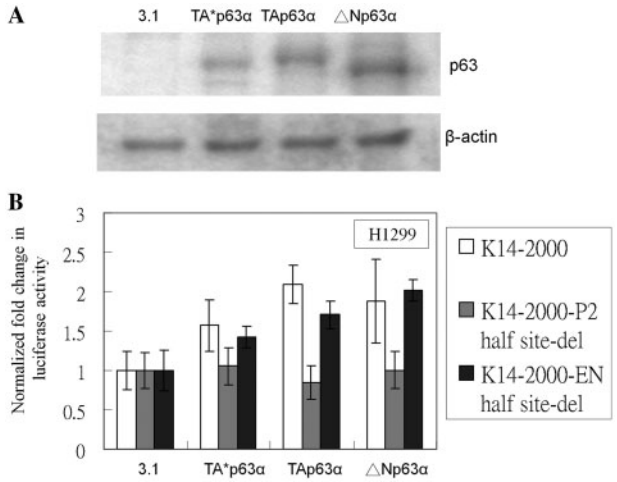

Fig. 12 All the α isoforms of p63 can up-regulate at P2 site of K14 promoter. (A) TA*p63α, TAp63α and ΔNp63α expression vectors can express in H1299 cells by western blot assay. The molecular weight of TA*p63α is smaller than TAp63α due to lack of C-terminal tag (Fig. 1). (B) TA*p63α, TAp63α and ΔNp63α can up-regulate the pK14-2000 or pK14-2000 enhancer half-site (−1421∼−1412) deletion 1421∼1412, but all p63 isoforms cannot up-regulate pK14-2000 p2 half-site (−140∼−131) deletion.

molecular mechanism responsible for p63 TA of different lengths of the p53-binding site medicated through different TA domains.

Our results showed that TPA treatment down-regulated K14 expression in C9 cells, but up-regulated K14 expression in HeLa cells (Fig. 4B). In HeLa cells, TPA-induced AP2α phosphorylation by protein kinase C might enhance its transcriptional activity (*38*). However, in human epidermal keratinocytes, TPA also induces AP2α phosphorylation by an unidentified cytosolic kinase that inhibits its transcriptional activity (*39*). Therefore, the different responses of K14 expression in C9 and HeLa cells caused by TPA treatment may indicate that TPA activates different kinases to phosphorylate different sites of AP2α in C9 and HeLa cells. However, the AP2α-phosphorylation site was identified only at Ser239 by protein kinase A activation (*40*). To identify the other AP2α phosphorylation sites by different kinases is important to understanding the signal pathways induced by TPA treatment in different cells.

In conclusion, we identified that p63 can regulate K14 expression mediated by a p53 half-site (−140 to −131) in the K14 promoter (Fig. 13). However, there are three p53 half-binding sites on K14-2000 promoter, only P2 site with functional activity even other two p53 half binding sites also can be bound by p63. In the related study of p53 regulated target genes, p53 needs SP1 as the co-factor to regulate the p53 whole-binding sites on p21 and BAX promoters (*27*). p63, like p53, also needs SP1 as co-factor to activate the p53 whole-binding site on p21 promoter (*41*). Dose p63 regulated the p53 half-binding site also need SP1 or other co-factors to activate P2 site on K14 promoter which must be further investigated.

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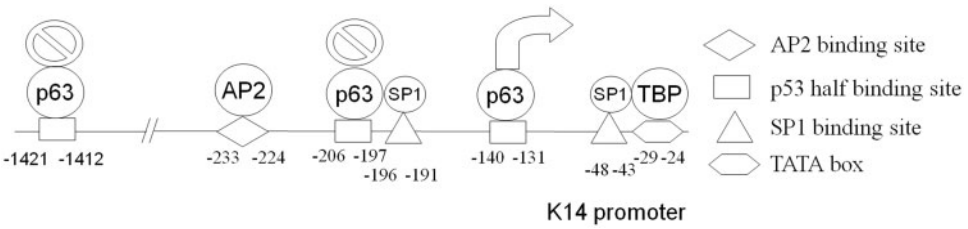

Fig. 13 The diaphragm for indicating p63-binding sites in regulation of K14 promoter. We proposed that there are three independent p53 half-binding sites in K14 promoter. But p63 can only regulate one of the p53 half-binding sites at −140 to −131. This map was modified from Tomic-Canic *et al.* (*42*).

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Conflict of interest

None declared.

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