p51/p63 controls subunit \( \alpha_3 \) of the major epidermis integrin anchoring the stem cells to the niche

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Running Title: p51/p63 induces integrin \( \alpha_3 \)
Summary

*p51*/p63, a member of the tumor suppressor *p53* gene family, is crucial for skin development. We describe here identification of *itga3* encoding integrin α3 as a target of its *trans*-activating function, proposing that *p51*/p63 allows epidermal stem cells to express laminin receptor α3β1 for anchorage to the basement membrane. When activated by genotoxic stress or overexpressed ectopically in non-adherent cells, p51/p63 transduced a phenotype to attach to extracellular matrices, which was accompanied by expression of *itga3*. Motifs matching the p53-binding consensus sequence were located in a scattered form in intron 1 of human *itga3*, and served as p51/p63-responsive elements in reporter assays. In addition to the *trans*-activating ability of the TA isoform, we detected a positive effect of the ΔN isoform on *itga3*. The high level α3 production in human keratinocyte stem cells diminished upon elimination of p51/p63 by siRNA or by Ca²⁺-induced differentiation. Furthermore, a chromatin immunoprecipitation experiment indicated a physical interaction of p51/p63 with intron 1 of *itga3*. This study provides a molecular basis for the standing hypothesis that *p51*/p63 is essential for epidermal-mesenchymal interactions.
Introduction

The p51/p63 gene (1,2) codes for tumor suppressor p53-like nuclear proteins whose structures, tissue localization and biological significance are well conserved among diverse organisms from humans to zebrafish (2-6) as reported to date. Studies showed enhanced expression of p51/p63 in squamous cell carcinomas of head, neck (7,8) and lung (9), but detected less tight association of p51/p63 with other cancers (10). The gene knockout mice exhibited severe defects in skin, limb and maxillofacial tissues (3,6), reflecting the normal embryonic p51/p63 expression localized in epidermis, apical ectodermal ridge (AER) of the limb buds and surface ectoderm at the branchial arches. Germ line p51/p63 mutations in humans are associated with the EEC (ectroductyly, ectodermal dysplasia and cleft palate) syndrome (11) and other malformation syndromes (12). Immunohistochemical analyses with skin tissues revealed nuclear expression of the p51/p63 protein confined to the basal layer of epidermis (2). By a clonal analysis of human keratinocytes for proliferative potential and protein composition, it was determined that p51/p63 is specifically expressed in keratinocyte stem cells and transit amplifying cells, predominantly and less predominantly, respectively (13). Consistently, p51/p63-null mice exhibited striking epidermal defects: absence of keratinocyte stratification and differentiation, lack of normal basal cells expressing keratin 14, and exposed dermis (3,6). Thus, true biological activities of p51/p63
essential for skin development should be identified in keratinocyte stem cells.

More than 6 isoforms arise from p51/p63 by using the TA- and ΔN-type transcriptional initiation sites and by RNA splicing to form the C-terminal A/γ, B/α, and C/γ variants (2). Reflecting the structural similarity to p53 in the DNA binding domain, many of the genes inducible by p53 are also responsive to the p51/p63 proteins (1,2,14). Each of the TA isoforms acts more or less as a trans-activation factor, whereas the ΔN isoforms lacking the TA domain exhibit dominant-negative activities against p53 and the p51/p63 TA isoforms in reporter assays (2). More recently, however, a report revealed that a ΔN isoform of p73, another p53 homolog involved in neurogenesis, acts both as a positive and negative regulator of transcription (15). Furthermore, the TA isoforms of p51/p63 are unstable due to degradation by proteasome under normal conditions, but can accumulate in response to DNA damage to induce gene expression (16-18).

p51/p63 has been implicated in transcriptional events related to cell growth and differentiation. Those include down-regulation of the EGF-receptor expression (19), trans-activation of Jagged-1 encoding a Notch ligand (20), induction of β-globin expression indicative of erythroleukemic cell differentiation (16,17), and activation of REDD-1 implicated in redox stress responses (21). With all these findings, however, essential molecular and cellular mechanisms that are assigned to p51/p63 for epidermal stem cell
regulation still remain obscure.

We report here that \( p51/p63 \) trans-activates the \( itga3 \) gene coding for integrin subunit \( \alpha3 \), also referred to as CD49c and VLA3-\( \alpha \). The \( \alpha3 \) subunit pairs with \( \beta1 \) to form the \( \alpha3\beta1 \) (VLA3) complex which falls into the category of major epidermis integrins with other members, \( \alpha2\beta1 \), and \( \alpha6\beta4 \) (22-24). Integrin \( \alpha3\beta1 \) is expressed predominantly in keratinocyte stem cells (23), and functions as a receptor for laminin, a major extracellular matrix (ECM) protein in the basement membrane (also referred to as basal lamina). The critical interactions between the epidermal stem cells and their niche (25) may be facilitated by \( p51/p63 \) through its ability to induce \( itga3 \).
Experimental Procedures

Cell culture U937 cell culture has been described (26). ECM-binding assays were performed using culture dishes coated with laminin, fibronectin, collagen or poly-lysine (BioCoat, BD Biosciences). Sixteen hours after incubation with actinomycin D, dishes were once washed with phosphate-buffered saline, and adherent and non-adherent cells counted. Neonatal human keratinocyte (NHK) preparations from foreskin were from Cambrex. The basal medium was supplemented with human recombinant epidermal growth factor (0.2 ng/ml), insulin (10 µg/ml), hydrocortisone (1 µg/ml), gentamicin (0.1 mg/ml), amphotericin-B (100 ng/ml) and bovine pituitary extract (Keratinocyte Growth Medium BulletKit, Cambrex). Experiments with NHK were accomplished within three sub-culturing cycles, during which cells maintained a high proliferating potential: more than 80% of the cells grew to form 16-cell to 32-cell colonies on day 3 after attachment to the basement (day 0).

RNA purification and RT-PCR Total RNA was purified with RNAwiz (Ambion) in combination with DNase I (Invitrogen)-treatment. One-step RT-PCR was performed with the SuperScript One-Step RT-PCR system (Invitrogen). After the RT reaction with 0.2-0.5 µg of RNA and PCR primers for 30 min at 50-54°C, synthesized DNA was directly amplified by 28-34 cycles of PCR consisting of annealing at 55-60°C, elongation at 72°C and DNA
strand dissociation at 94°C. 18S rRNA was amplified for 16 cycles. The following primer pairs were used: p21\textsuperscript{waf1}, 5'-GTTCCCTTGGAGCCCGAGC-3' (forward) and 5'-GGTACAAGACGTAGCAGGT-3' (reverse); glyceraldehydes-3-phosphate dehydrogenase (GAPDH), 5'-GAAGGTGAAGGTCGGAGTC-3' and 5'-GAAGATGGTGATGGGATTTC-3'; integrin α3 (#1), 5'-TCCATGAACTACTCTTTACCTTTGCGGATGC-3' and 5'-TGCCAGACTCACCATCAGTCGTC-3'; for integrin α3 (#2), 5'-AAGCCAAGTCTTGAGAGA-3' and 5'-GTAGATTTGGTCCCAGGAGCT-3'(27); integrin α5, 5'-CATTTCGGAGTCTGGGCCAA-3' and 5'-TGGAGGCTGAGCTGAGCTT-3'; integrin α6, 5'-TGTGACCTCGGAATCTTTT-3' and 5'-CAACTCCGAGACCGATAAA-3'; integrin αM, 5'-TCCCACCGTGCTCTGTG-3' and 5'-CCCAATGGACTCGGAAAT-3'; integrin β1 (#2), 5'-ACACGTCTCTCTCTCTCG-3', 5'-CAGTGTACGGCAGCCT-3' (28); integrin β1 (#1), 5'-AATGGTGAACGTGCAAGC-3' and 5'-CAACCAAATGGATCTCTCAGTT-3' (29); involucrin, 5'-TGCTCTCCTGGCCAGACGTG-3' and 5'-ATTCTCTACCTGTTCCAGGATGC-3'; ΔN(p51/p63), 5'-GGAAAAATAGGCCAGACTC-3' and 5'-GAAGGACACGTCGAAAATG-3'; TA(p51/p63), 5'-ATGCCAGACACACAG-3' and 5'-AGCTCATGTTGGGAC-3'; p53, 5'-AGTGGATCCAGACTGCTTCC-3' and 5'-AGCTCATGTTGGGAC-3'.
5'-TAGGGCACCACCACACTAT-3'; 18S RNA, 5'-GTAACCCGTTGAACCCCATT-3' and 5'-CCATCCAATCGGTAGTAGCG-3' (31).

Western blotting We described Western blotting previously (17). An anti-integrin α3 antibody (Biogenesis, 5355-1305), an anti-p51/p63 antibody (4A4, Santa Cruz), an anti-p21\textsuperscript{waf1} (H164, Santa Cruz), a rabbit anti-human p53 antibody (FL393, Santa Cruz) and an anti-β-actin antibody (AC-15, Sigma) were purchased. Proteins separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis with 7%, 8.5% or 10% gel were transferred to polyvinyl difluoride (PVDF, Millipore) membrane, pore-size 0.45 μm. For detection of p21\textsuperscript{waf1}, 12% polyacrylamide gel and PVDF membrane with 0.2 μm pores were used.

Transfection Plasmids were transfected into HeLa and Saos-2 cells with Effectene transfection reagent (Qiagen). Luciferase assay was performed with a Steady-Glo Luciferase Assay System and Glo Lysis Buffer (Promega). DMRIE-C transfection reagent (Invitrogen) was used for transient and permanent transfection of U937 cells. Small interfering RNAs (siRNAs) with sequences 113-130 (p51/p63-siRNA1) and 282-300 (p51/p63-siRNA2) were synthesized (Greiner Japan), in which nucleotide number 1 corresponded to the A residue of the translation start codon of the ΔN cDNAs. siRNAs were transfected into NHK by Amaxa Nucleofector apparatus (Amaxa Biosystems) with Human
Keratinocyte Nucleofector Solution.

**Inducible p51/p63 expression in HEK293 cells** We transfected HEK293 cells with pFRT/LacZeo and selected Zeocin resistant clones. The pFRT-positive cells were next transfected with tetracyclin repressor expression plasmid pcDNA6/TR, and Blasticidin S resistant clones obtained. The pcDNA5/FRT/TO-vector with each of the p51/p63 isoform cDNAs was co-transfected with pOG44 into the HEK293 clones having pFRT and pcDNA5/TR. Stable hygromycin-resistant clones were maintained as cell lines that induce p51/p63 expression in response to tetracyclin (2 μg/ml)

**Plasmids** Overexpression of p51/p63 with the pRcCMV vector was described, as were luciferase expression constructs, pRGC-luc (18), and pGL-ES (32). To construct p(1+2)luc, a synthetic double-stand DNA having sequences 594-612 and 877-901 derived from intron 1 of human *itga3* was inserted into the multiple cloning site of a luc expression vector, pGL3-promoter. p(3+4)luc was constructed similarly, so that nucleotides 2465-2490 and 6437-6461 were inserted in tandem between the Nhe I and Xho I sites located 5’ to the SV40 promoter. The (3+4) segment was inserted at the Nhe I/Xho I sites of p(1+2) to create p(1+2+3+4)luc. Human genomic DNA was purified from peripheral blood cells obtained from a healthy volunteer donor (Y.T.) A DNA region encompassing intron 1 of *itga3* was amplified by PCR with primers targeting the 3’ end of exon 1 and 5’ end of exon 2:
5'-CTCCGCCTTTCAACCTGGATACCCGATTCT-3' and 5'-GGTACACAGCACCAGTCCGTGTGGTGAGC-3', respectively. The 7.6 kb segment containing the full intron 1 sequences was inserted into pGEM-T Easy. By Bgl II-digestion, the 1.4 kb 5' terminal region was excised to be cloned in pGL3-promoter at the Bam HI site (Promega). Nucleotide mutations were introduced with a GeneTailor Site-Directed Mutagenesis System (Invitrogen). PCR primers used were 5'-GAGAGAGGAGGACTTTTCCCAACTCT-3' and 5'-AAGTCCTCCTCTCTCTCTTTTCACC-C-3' for the M1 mutation, and 5'-ATGCCCAAGAGGCTT-3' and 5'-ATGCTCCTGGGCATGAGCGGTCT-3' for the M2 mutation. Nucleotide sequences were confirmed with ABI 377 sequencer. Plasmids were purified from *Escherichia coli* DH5α by a GenElute Endotoxin-free Plasmid Kit (Sigma-Aldrich).

**Indirect immunofluorescence microscopy** Mouse skin sections were fixed in 4% paraformaldehyde (PFA), embedded with paraffin, and sectioned at 8 μm. Keratinocytes cultured on slide glass were also fixed with 4% PFA. Deparaffinized tissue sections and cultured samples were heated at 95° C for 40 minutes in a buffer (10 mM sodium citrate buffer, pH 6.0; or with 50 mM glycine-HCl buffer, pH 3.5, with 0.01% (w/v) EDTA) for epitope retrieval. Samples were permeabilized with 0.1% Triton X-100, blocked in PBS containing 5% nonfat dry milk and 5% goat serum (30 min, RT), and incubated with a
primary antibody (for 16 h, 4 °C). Samples probed with FITC- or rhodamin-labeled secondary antibody, for 1 h at 22 °C, were washed and mounted in an anti-fade medium (Dako, Japan). Photomicrographs were obtained with a Leica DMRXA microscope equipped with an air-cooled camera (Synsys) controlled by QFISH software (Leica).

Chromatin immunoprecipitation (ChIP) HEK293 cells seeded on 15-cm plates were transfected with a plasmid for expression of human influenza virus hemagglutinin epitope (HA)-tagged p51A/TAp63γ (HAp51A) (1). After 48 hr, cells were fixed with formaldehyde, and the nuclei purified. Sheared chromatin was immunoprecipitated with non-immune rabbit IgG, an anti-TFIIB antibogy (Active Motif), or an anti-HA rabbit antibody (Zymed, 71-5500). Protein G-agarose blocked with salmon sperm DNA, RNase A, proteinase K, a proteinase inhibitor cocktail, mini-columns for DNA purification and buffers were supplied in the ChIP-IT kit by Active Motif. Adsorption of the antibody/protein/DNA complexes to protein G-agarose, washing, reversal of cross-links, removal of RNA, protein digestion, and DNA purification were performed following the manufacturer’s protocol. A 166 base-pair (bp) segment of the GAPDH gene promoter locus was amplified by PCR with primers: 5’-TACTAGCGGTTTACGGCG-3’ and 5’-TCGAACAGGAGGACAGAGCGA-3’. For detection of the itga3 intronic sequences encompassing the first and second p53(p51/p63)-binding consensus sequences, a 390 bp segment was amplified with
primers: 5'-CTCTCGTAATGGAAGACC-3' and 5'-GCATTATGAGACATCCAC-3'.
Results

Induction of an ECM-binding phenotype and integrin α3 expression

As we reported earlier, p51A/TAp63γ, a potent trans-activator isoform of p51/p63, accumulated in response to DNA damage to induce p21waf1 in a mouse cell line (16,26). In this study, we propagated U/p51A and U/p53 cell lines derived from p53-deficient U937 human lymphoid cells after transfection with an RSV promoter-driven, low-level expression vector for p51A/TAp63γ and p53, respectively (16). In U/p51A cells, p51A/TAp63γ accumulated 8-24 h after exposure to 5 nM of actinomycin D without an alteration in the mRNA level (Figure 1A). The RT-PCR and Western blot analyses also showed activation of the p21waf1 and GADD45 expression with an increase in the p21waf1 protein amount. U/p53 cells incubated with actinomycin D also stabilized p53, inducing p21waf1. Control U937 cells caused a slight up-regulation of p21waf at 16 h, probably by a p53-independent mechanism (33).

We examined those cells for attachment to basements coated with laminin, fibronectin or collagen (Figure 1B). More than 90% of the U/p51A cells incubated with actinomycin D attached to laminin-coated dishes within 16 h. Approximately 55% and 16% of the cells bound to fibronectin and collagen, respectively, with little evidence of their binding to poly-lysine. In contrast, only a 9% fraction of the drug-treated U/p53 cells bound to laminin,
and a yet smaller fraction to fibronectin. Control U937 cells adhered to neither of the extracellular matrix proteins under the conditions used.

To find the molecular basis of the p51A-caused cell attachment to ECM, cellular RNA was analyzed for expression of integrin genes by RT-PCR (Figure 1C). With two different primer pairs, \( \alpha 3(#1) \), and \( \alpha 3(#2) \), an increase in \( \text{itga3} \) mRNA was evident at 8-24 h after exposure to actinomycin D. By contrast, \( \alpha 4, \alpha 5, \alpha 6 \) and \( \alpha M \) remained constant through the time course. Although the \( \beta 1 \) gene expression slightly increased, reaching maximum at 8 h, the response was not specific to U/p51A. Consistent with the RT-PCR analysis, the 145 kDa \( \alpha 3 \) (ref. (34)) content increased in U/p51A cells during the period (Figure 1C, bottom).

Since the \( \alpha 3 \) subunit associates with \( \beta 1 \) to form the \( \alpha 3\beta 1 \) heterodimer whose major and minor ligands are laminin and fibronectin/collagen (35), respectively, we hypothesized that p51/p63 promotes \( \text{itga3} \) expression directly or indirectly to cause the cell-ECM attachment.

**Transient or drug-controlled p51/p63 expression also activates \( \text{itga3} \)**

We performed transient overexpression of p51/p63 in U937 cells using a cytomegalovirus promoter vector with a liposome-based transfection carrier. The p51A/TAp63\( \gamma \) expression vector, but not the vector-only plasmid, caused a laminin-binding activity (Figure 2A, B). Reflecting the transfection efficiency, 10\%, determined by a G418-resistant colony formation assay, an approximately 10\% fraction of the cells bound to
laminin (Figure 2B). Although the apparent increases in the mRNA and protein of α3 were not so great (2 to 3-fold) when the entire culture was analyzed (Figure 2A), we could speculate that there was a greater increase in the α3 expression in the attached cell population (10%). Furthermore, ΔNp51A/ΔNp63γ lacking the trans-activation domain also generated cells adherent to laminin (Figure 2B), which was accompanied by itga3 activation as detected by the mRNA and protein analyses (Figure 2A). In contrast to the poor retention of 57 kDa p51A/TAp63γ having the N-terminal sequences that determine its fate for degradation by proteasome (16,18), ΔNp51A/ΔNp63γ was stable enough to form an intense band at 52 kDa in Western blotting (Figure 2A). Thus, neither the laminin-adherent phenotype nor the α3 induction shown in Figure 2 required a cellular signaling event caused by actinomycin D. When expressed to a certain level, the TA and ΔN proteins seemed to induce itga3.

Human embryonic kidney (HEK) 293 cells were genetically engineered so that established cell lines, 293-1, -2, and -5 expressed cDNAs of p51A/TAp63γ, p51B/TAp63α and ΔNp51A/ΔNp63γ, respectively, from the same position on the chromosome upon induction with tetracycline. Twelve hours after induction, each cell line achieved a high level production of p51A/TAp63γ (57 kDa) (16), p51B/TAp63α (85 kDa) (17) or ΔNp51A/ΔNp63γ (52 kDa) (36) (Figure 2C, left). Expectedly, the itga3 mRNA increased approximately
10-fold in p51A/TAp63γ-expressing 293-1 cells (Figure 3C, right). p51B/TAp63α- or ΔNp51A/ΔNp63γ- expressing cells also caused a 3- or 2-fold activation of itga3, respectively, whereas control HEK293 cells showed no detectable change. Consistently, the 145 kDa integrin α3 protein increased in tetracycline-induced 293-1, 293-2 and 293-5 cells, reflecting the relative amounts of the itga3 mRNA (Figure 2C, left). Processed integrin α3 light chain (30 kDa) was not detectable. Supporting the experiments with U937 cells (Figure 1 and 2A,B), these results indicated that itga3 is inducible by the TA and ΔN isoforms of p51/p63.

**p51/p63-responsive elements in the first intron of itga3**

We found sequences matching RRRC(T/A)(T/A)GYYY at position 597, 881, 2475 and 6422 in intron 1 of itga3 on human chromosome 17 (at 17q21.33), in which position numbers are relative to nucleotide 1 at the 5’ end of intron 1 (Figure 3). Another motif was present 2594 bp upstream of the transcription start site lacking a nearby TATA box. The decanucleotide motif corresponded to a half-site of the full consensus sequence occupied by a p53 tetramer (a dimer of a dimer), RRRC(T/A)(T/A)GYYY-N(0-13)-RRRC(T/A)(T/A)GYYY, where N(0-13) indicates a spacer of 0 to 13 bases in the classical definition (37). Because a half-site is bound by a p53 dimer (38,39) to cause moderate transcriptional activation (40), the four motifs scattered in intron 1 were speculated to serve
as the cis-acting elements for the regulation by p51/p63.

We constructed luc expression plasmids, p(1+2)luc, p(3+4)luc, and p(1+2+3+4)luc, by placing the 1st (597), 2nd (881), 3rd (2475) and 4th (6422) motifs upstream of the SV40 promoter in different combinations (Figure 3A). Trans-activation assays were performed by co-transfection of the luc plasmids with a p53, p51A/TAp63γ, ΔNp51A/ΔNp63γ or control expression vector (pRcCMV) in HeLa cells derived from human cervical epithelia (Figure 3B). p51A/TAp63γ activated the (1+2), (3+4) and (1+2+3+4) promoters by 4.5-, 1.8- and 3.4-fold, respectively. p53 also caused 3.4, 1.4, and 3.8-fold increase with those plasmids. The alignment of the 1st and 2nd motifs was more effective than that of the 3rd and 4th motifs in this assay. ΔNp51A/ΔNp63γ did positively affected transcription from either of the plasmids, although the ΔN isoform certainly exerted a dominant-negative type activity in our assay system with the ribosomal gene cluster promoter as reported previously (36). The activation profile detected with p(1+2)luc was consistent with the endogenous itga3 responses to p51/p63 observed in U937 and HEK293 cells (Figure 1,2).

We next performed reporter assays with the (α3-1)luc plasmid which had the 1.4 kb 5′ terminal intron segment containing the 1st and 2nd motifs immediately downstream of the luc-coding region in the 3′ to 5′ orientation (Figure 3C,D). (α3-1)luc increased its luciferase expression by 1.2-, 3.2- and 1.9-fold in the presence of p53, p51A/TAp63γ and
ΔNp51A/ΔNp63γ, respectively. Combined expression of p51A/TAp63γ and ΔNp51A/ΔNp63γ caused a 2.5-fold activation of (α3-1)luc, implying an interaction between the TA and ΔN isoforms. When a single mutation, G603T, and a double mutation, G603T/G887T, were introduced into (α3-1)luc to render M1(α3-1)luc and M1M2(α3-1)luc, respectively, the efficiency of trans-activation by p51A/TAp63γ dropped to 1.4-fold and 1.2-fold. These results indicated that the 1st and 2nd half-site motifs cooperatively play an essential role to respond to p51A/TAp63γ. The ΔN isoform seemed to act on the same sites to cause the moderate activation. On the other hand, (α3-4)luc, in which the 1.4 kb segment was placed in the 5’ to 3’ orientation, produced a high background of the luciferase activity, and less sensitive to p53 and p51/p63 (Figure 3D), implying that G/C-enriched sequences in the 5’ terminal region of the insert affected the heterologous viral promoter activity in the vector.

**Concurrent expression of p51/p63 with α3 in epidermis development**

Immunostaining of skin sections from mouse embryos on day 14 (E14) showed p51/p63 protein localization in the inner layer of the double-layered surface ectoderm or the periderm (Figure 4A, far left). The p51/p63 nuclear stain intensified in the basal layer of epidermis on E16 when epidermis stratification was in progress. In newborn mice, however, the overall p51/p63 stain significantly decreased, leaving p51/p63-positive cells in clusters
that corresponded to the patches of keratinocyte stem cells (13). The double
immunofluorescence analysis showed that nuclear p51/p63 stain (FITC) coincided with
peripheral α3 (Rhodamin) stain in the basal cells of the E14, E16 and newborn tissues
(Figure 4A, three right panels). Furthermore, the α3 label was also markedly weakened at
birth. In their temporal and spatial expression profiles, p51/p63 and α3 were closely related
to each other in mouse skin development.

The α3 expression is associated with p51/p63 in keratinocyte stem cells

We cultured neonatal human keratinocytes (NHK) to analyze p51/p63 and integrin α3
expressions. More than 90% of the cells in the NHK culture were able to replicate to form a
colony of 16 or 32 descendant cells within 3 days after plating with medium containing 0.1
mM of Ca²⁺ (low Ca²⁺). As judged by the high growth potential, epidermal stem cells were
predominant in the culture. By changing the extracellular calcium concentration, we could
control growth and differentiation of the epidermal cells (41-43). When the Ca²⁺
concentration was raised to 1 mM (high Ca²⁺), those cells ceased replicating and
underwent differentiation as described. Seven days after incubation with high Ca²⁺, we
detected expression of involucrin, a keratinocyte differentiation marker (Figure 4B).

Both the TA and ΔN type transcription occurred in NHK (Figure 4B, day 0). Western
blotting also detected the p51/p63 proteins, p51A/TAp63γ (57 kDa), p51B/TAp63α(β) (85
kDa), ΔNp51A/ΔNp63γ (52 kDa), and ΔNp51B/ΔNp63α(β) (80 kDa), as identified by experimental expression in U937, HEK293 (Figure 2) and HeLa cells (36). The ΔN-type transcripts gradually decreased on day 4 and 7, whereas the TA-type mRNA did not change significantly up to day 7. However, Western blotting revealed that both the TA and ΔN proteins gradually diminished during the period (Figure 4B). The protein degradation system controlling p51/p63 may be enhanced in the cells incubated with high Ca^{2+}. Thus, the depletion of p51/p63 during the keratinocyte differentiation (13) seemed to involve transcriptional and post-transcriptional mechanisms.

In NHK (day 0), 145 kDa full-length α3 appeared predominant in the Western blot with the antibody reactive with the cytoplasmic peptide. On day 4 of the Ca^{2+}-induction, the 30 kDa light chain of α3 (44), instead of the 145 kDa protein, formed an intense band, indicating that the α3 maturation process had became active (Figure 4B). We did not detect a decrease in the α3 gene expression by RT-PCR for at least 4 days after the Ca^{2+}-input. On day 7, when the p51/p63 protein content had markedly decreased, the α3 mRNA and protein levels had declined markedly. In contrast, p21^{waf1} gene expression was up-regulated on day 4 and 7 compared with day 0, possibly due to the decline in the ΔN isoforms that negatively regulate p21^{waf1}. The level of Integrin β1 mRNA did not change significantly up to day 7. Thus, p51/p63 protein decrease preceded suppression of itga3
during the keratinocyte differentiation \textit{in vitro}.

By immunocytostaining, more than 99% of the cells were positive in p51/p63 (Figure 5A, upper panels), assuring that the NHK culture was enriched in keratinocyte stem cells (13). Double staining indicated the presence of $\alpha_3$ in perinuclear regions as observed in $\alpha_3$-transfected CHO and NIH3T3 cells (45,46). Seven days after incubation with high Ca$^{2+}$, we detected morphological features of \textit{in vitro} keratinocyte differentiation: cell flattening and formation of cell-to-cell contacts (Figure 5A, lower panels). The nuclear p51/p63 label was significantly weakened, supporting the Western blot analysis (Figure 4B). The $\alpha_3$ label had not only faded, but had also changed its localization to the cell-cell borders as found in MDCK cells (45), probably by forming a complex with proHB-EGF and DRAP27/CD9 proteins (47).

Furthermore, we introduced siRNA-1 and -2 targeting the DNA binding domain-encoding sequences of $p51/p63$ into NHK using an electrical transfection system (Figure 5B). Twenty-four hours later, approximately 40% of the cells in the culture transfected with siRNA1 or siRNA2 displayed a decrease in p51/p63. Obviously, $\alpha_3$ also decreased in the cells that underwent suppression of p51/p63. Unlike the differentiation process with high Ca$^{2+}$ (Figure 5A), the siRNA experiment showed no evidence of the $\alpha_3$ protein shift from the perinuclear regions to the cell-cell borders. Control double labeling
with an anti-pan-keratin antibody indicated that the siRNA did not affect the total level of cytokeratins. Thus, the integrin α3-inducing ability of p51/p63 seemed vital in keratinocyte stem cells.

Detection of an interaction of p51/p63 with intron 1 of itga3.

To assess whether p51/p63 can directly interact with the 1st intron of itga3 on the chromosome, a ChIP experiment (48) was carried out with HEK293 cells transfected with an HAp51A-expression vector and control untransfected cells. Expression of HAp51A was confirmed by Western blotting with an anti-HA antibody and 4A4 anti-p51/p63 antibody (Figure 6A). An increase in 145 kDa integrin α3 was also detectable in the HAp51A-expressing cells.

For immunoprecipitation, we used three different antibodies: control non-immune IgG, an anti-HA antibody and an antibody against TFIIB, a general transcription factor. 4A4 was not reactive with either of the p51/p63 isoforms unless proteins were fully denatured, and was not useful for ChIP. DNA fractions recovered from the immunoprecipitates were examined by PCR for a GAPDH promoter locus and an itga3 intron 1 segment (553-942) encompassing the 1st and 2nd half-sites. Both from the HAp51A-transfected and untransfected cells, the GAPDH promoter segment was more abundantly precipitated by the anti-TFIIB antibody than by the control and anti-HA antibodies (Figure 6B, left panels).
Constant transcription of the house-keeping gene was thus detectable by this ChIP experiment. On the other hand, the \textit{itga3} segment was more enriched in the anti-TFIIB and anti-HA precipitates from the HAp51A-expressing cells than in the control non-immune IgG precipitates. None of the three immunoprecipitates from untransfected cells was enriched for the \textit{itga3} sequences (Figure 6B, right panels). These results not only indicated a physical interaction of p51/p63 with intron 1 of \textit{itga3}, but also verified that \textit{itga3} transcription is induced by p51/p63.
Discussion

We have shown (i) induction of integrin $\alpha 3$ by DNA damage-caused p51/p63 protein activation and by transient and controlled p51/p63 overexpression, (ii) transduction of an ECM-binding phenotype relevant to integrin $\alpha 3\beta 1$ in a non-adherent leukemic cell line, (iii) presence of p51/p63-responsive sites in the 1st intron of human $itga3$, (iv) suppression of $\alpha 3$ by p51/p63 knockdown with siRNAs and by differentiation in the NHK culture, and (v) association of p51/p53 with intron 1 of $itga3$ on the chromatin. Transcriptional activation of $itga3$ may be one of the pivotal roles of p51/p63 in epidermis development.

Integrin $\alpha 3\beta 1$ is a major epidermis laminin receptor whose expression is confined to the stem cells (23). Although $\alpha 3$-null mice caused occasional skin blisters where the epidermis separated from the dermis (49), conditional $\beta 1$ knock-out mice displayed extreme skin blistering, hair defects and basement membrane disassembly, indicating more critical roles for the partners of $\beta 1$ in skin development (50). The terminal differentiation program was, however, preserved even in the absence of $\beta 1$ (50). A double mutation in $\alpha 3$ and $\alpha 6$ impaired basement membrane assembly and epidermal cell compaction in AER, and thereby caused abnormal limb patterning (51). Defects in the urogenital tracts and other organs were also observed in the $\alpha 3^{+/}\alpha 6^{+/}$ mice. Thus, most of the abnormalities in the integrin $\alpha$ or $\beta$ knock-out embryos are closely related to the
phenotypes of the EEC syndrome (11,12) and p51/p63-deletion (3,6). Our p51/p63-knockdown experiment with siRNA provided evidence that p51/p63 is a dominant factor inducing the \( \alpha_3 \) expression in keratinocyte stem cells. Due to the localized, abundant expression in undifferentiated keratinocytes (23), the p51/p63 proteins may control \( \alpha_3 \) efficiently in those cells. However, the more striking skin phenotypes observed in the p51/p63-null mice imply cooperation of \( \alpha_3 \) with other signaling and/or structuring molecules under the control of p51/p63.

Most of the known p53-responsive promoters have a full-site that forms a stable complex with a p53 tetramer (38). However, a half-site can accommodate a p53 dimer (39), and serves as a minimal p53-responsive element when placed adjacent to a Sp1 site (40). Furthermore a full p53-binding site with a 33-base spacer has recently been identified (52). The half-sites at 597 and 881 were active not only in the full-site context at the enhancer position in p(1+2)luc, but also in the original half-site context placed downstream of luc in (\( \alpha_3 \)-1)luc. It remains to be determined whether the p51/p63 proteins also form functional tetramers, although the peptide-peptide interaction in the oligomerization domain was detectable by an yeast two-hybrid assay, indicating dimer formation (53). The mechanism of \( \alpha_3 \) regulation by p51/p63 may involve topological arrangement of the separate half-sites in intron 1, interactions of p51/p63 with other transcriptional regulatory factors,
and association between different p51/p63 isoforms.

The mouse α3 gene also contains three half-site motifs in intron 1, in which the 1st and 2nd motifs are located in the 5' terminus, suggesting similar regulatory mechanism in intron 1 by p51/p63. The promoter/enhancer region extending to -4 kb has been extensively studied (32,54) to determine that the Ets binding site at –133 is critical for the gene expression in MKN1 gastric carcinoma cells. However, the 4 kb promoter/enhancer region lacked a putative p51/p63-binding site, and did not respond to p51/p63 in our assay (data not shown).

When assayed with the promoters of p53 target genes, the ΔN isoforms suppressed the trans-activating ability of p51A/TAp63γ (2), by which the ΔN proteins were originally determined to be dominant-negative type isoforms. A recent study demonstrated that a ΔN isoform of p73 is able to both positively and negatively regulate p53 target genes (15). p53 activates the human BAX promoter using Sp1 as a cofactor (40), whereas p51A/TAp63γ suppresses EGF-receptor gene expression by an interaction with Sp1 (19). Gene regulation by the TA and ΔN forms of p51/p63 now appears more elusive than originally characterized. To exert the positive effect, the ΔN proteins may interact with an activation factor whose expression could be cell-type specific.

α3β1 is essential for cell spreading on the basement membrane, ECM assembly,
hemidesmosome stability, establishment/maintenance of the cytoskeletal organization, epidermal proliferation and the control of cell migration (24,25,50,55-57). It may be through the α3β1-caused epidermal-mesenchymal interactions that p51/p63 facilitates generation of an undifferentiated basal cell population. In addition to the epidermis, p51/p63 expression occurs in the basal cells of epithelia of the mammary gland (58), oral tissues (59), uterocervix (60) and bladder (30), where the α3-inducing role of p51/p63 may also function. Furthermore, the overexpression of p51/p63 in squamous cell carcinomas of head, neck and lung (7,9) might contribute to the high-level α3 expression which determines cellular capability of invasion and metastasis (61-63). It is interesting to envisage that p51/p63, a hypothetical ancestor gene to tumor suppressor p53 (64), had evolved as an inducer of the critical interaction between growing epithelial cells and their niche.
References


Footnote

Abbreviations: ECM, extracellular matrix; NHK, neonatal human keratinocytes; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; siRNA, small interfering RNA; ChIP, chromatin immunoprecipitation; HA, human influenza virus hemagglutinin epitope; HAp51A, HA-tagged p51A/TAp63γ.

Acknowledgements

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Figure Legends

Figure 1. Induction of laminin-binding activity and integrin α3 expression in U/p51A cells in response to DNA damage.

(A) Accumulation of p51A and p53 in response to actinomycin D. RT-PCR and Western blot analyses of U/p51A, U/p53 and U937 cells incubated with the drug are shown. Time course (in h), mRNAs, and proteins are indicated. Western blotting with an anti-p51/p63 antibody (4A4) is shown for U/p51A cells, as are results with an anti-p53 antibody (FL432) for U/p53 and U937 cells. GAPDH mRNA and β-actin served as standards. (B) ECM-adhering activity detected in U/p51A cells cultured with actinomycin D. Cells were tested for affinity to ECM by plating on culture dishes coated with laminin (LN), fibronectin (FN), collagen (CL) or poly-lysine (poly-L). Microscopic observation at 16 h after the input of actinomycin D and the ratios (%) of (adherent cell count) / (total cell count) are shown in the left and right panels, respectively. (C) Induction of integrin α3 in p51A-expressing cells. Upper panels show various integrin subunit mRNAs analyzed by RT-PCR. We used two different primer pairs, α3(#1), and α3(#2) to detect the itga3 mRNA. The β1 gene expression in U937 cells was examined with the β1(#2) primer pair. Lower panels show Western blot analyses of the U/p51A cell samples for integrin α3 and β-actin. Dots indicate positions of the size markers (kDa).
**Figure 2.** Integrin α3 induction by transient or induced expression of p51/p63.

(A) RT-PCR and Western blot analyses for U937 cells transiently transfected with a p51A, ΔNp51A or control expression vector. *itga3* (with the α3#1 primers), the TA and ΔN isoforms of p51/p63 and control 18S rRNA were amplified. p51A/TAp63γ (57 kDa), ΔNp51A/ΔNp63γ (52 kDa) and a non-specific band (NS) at 47 kDa (36) reactive with monoclonal antibody 4A4 are indicated. (B) p51/p63-transfected U937 cell attachment to laminin. Transfected cells were plated on dishes coated with laminin or poly-lysine (poly-L). Forty-eight hours after transfection, plates were observed microscopically (upper panel) and quantified for cell attachment (lower panel). (C) *itga3* expression in a tetracycline-controlled p51/p63 expression system of HEK293 cells. Western blots (left panels) show detection of the p51/p63 isoforms, p51A, p51B, and ΔNp51A with monoclonal antibody 4A4 in 293-1, -2 and -5 cells cultured in the presence (+) or absence (-) of tetracycline. Integrin α3 (145 kDa) is indicated by arrow. RT-PCR (right panels) show induction of *itga3* and *p21waf-1* expression.

**Figure 3.** Finding of p51/p63-responsive sequences in intron 1 of *itga3*.

(A) Schematic presentation of the 5’ end of human *itga3*. The promoter-enhancer region, exon 1 (279 bp), intron 1 (7431 bp) and the 5’ part of exon 2 are shown. Closed triangles at positions 597, 881, 2475 and 6422 (relative to the 1st nucleotide of intron 1) indicate
putative p53(p51/p63)-responsive sites. The (1+2) and (3+4) segments inserted 5' to the promoter of the luc vector are depicted in coordination with their original locations in intron 1. Nucleotide sequences matching the p53(p51/p63)-binding consensus sequences are underlined. (B) Luc-expression assays with p(1+2)luc, p(3+4) and p(1+2+3+4)luc. The core structure comprising the enhancer-type insert, the promoter (P) and luc coding region (luc) is schematically shown for each reporter construct. Co-expressed activators, p53, p51A/TAp63γ, and ΔNp63γ, are indicated below the columns. Basal level luciferase activities were obtained by co-transfection with the vector (pRcMCV). Luminescence intensity is presented in relative light units (RLU). (C) Cloning of the 5' terminal region of intron 1 and in vitro mutagenesis. The 1434 bp segment cloned into the vector are indicated by bold line. The half-site consensus sequences, the motifs at 597 and 881, and the M1 and M1 mutations (open triangles) are shown. Nucleotide substitutions in M1 and M2 are in bold letters. (D) Luciferase assays with (α3-1)luc, its mutants and (α3-4)luc. Relevant components and their layout are illustrated for each vector.

Figure 4. Changes in p51/p63 and integrin α3 during keratinocyte differentiation.

(A) p51/p63 immunostain developed with 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt (far left panels) and double immunofluorescent staining for p51/p63 with FITC and integrin α3 with Rhodamin (three right panels). Skin sections from E14 and E16 embryos
and newborn were stained simultaneously on the same slide glass. Bars indicate 50 μm.

(B) Analyses for mRNA and protein compositions in NHK. An NHK culture that had been maintained with 0.1 mM Ca^{2+} (day 0) was incubated with 1 mM Ca^{2+} (high Ca^{2+}) in the medium to day 4 and day 7. mRNAs examined by RT-PCR are indicated (left panels). Western blots (right panels) show p51/p63 isoforms, immature (145 kDa) and mature (30 kDa) forms of integrin α3 and β-actin (41 kDa). Dots indicate protein standards (sizes in kDa) in lane M.

Figure 5. Suppression of α3 coincides with p51/p63 depletion in human keratinocytes.

(A) NHK and the Ca^{2+}-incubated culture on day 7 were doubly stained for p51/p63 and α3.

(B) NHK cultures transfected with p51/p63 siRNA1 or 2 were analyzed for alterations in p51/p63 and α3 (upper panels). Large and small arrows indicate apparently complete and substantial suppression of p51/p63, respectively. Also shown is a double stain for p51/p63 (FITC) and pan-keratins (Rhodamin) in a siRNA2-transfected culture.

Figure 6. Association of p51/p63 with the target region in itga3 intron 1.

(A) HAp51A-transfected HEK293 cells were tested for expression of HAp51A and induction of integrin α3 by Western blotting. Vector- or HAp51A-transfected cell lysates were analyzed with antibodies indicated. Arrow shows the position of HAp51A (two upper panels) or 145 kDa integrin α3 (third panel).
(B) ChIP was performed with sheared chromatin preparations from HAp51A-transfected (HEK293/HAp51A) and untransfected cells. Antibodies used in immunoprecipitation (control, anti-TFIIB and anti-HA) are indicated. Panels show PCR analyses for a 166-bp segment of the GAPDH promoter and a 390-bp segment in itga3 intron 1.
### Figure 1A

#### A

**RT-PCR**

<table>
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<th>U/p51A</th>
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<tr>
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<td>GAPDH</td>
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**Western Blot**

- p51A or p53
- p21waf1
- β-actin

**Actinomycin D (5 nM)**

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</table>
Figure 1B,C
Figure 2

Panel A: RT-PCR

- itga3
- TA
- ΔN
- 18S rRNA

Panel B: Western blot

- Integrin α3
- p51A
- ΔNp51A
- NS
- β-actin

Panel C: HEK293 cells

- Western Blot
- Control, p51A, p51B, ΔNp51A
- Tet:
  - -
  - +

- p51/p63
- Integrin α3
- 18S rRNA
- β-actin

- RT-PCR
- Control, p51A, p51B, ΔNp51A
- Tet:
  - -
  - +

- itga3
- p21waf1
- 18S rRNA
Figure 3

A

Intron 1 (7431 bp)

Exon 1

Exon 2

-GAGGACATGTCCCAACTCCAGGAGCATGTCCAGG-

-AGGGGGCTAGCCCACCCGCCTAGGGCTAGGCTTCCTC-

(1+2)

(3+4)

B

Luc activity (RLU, x10^4)

Vector p53 p51A Np51A

Luc activity (RLU, x10^4)

Vector p53 p51A Np51A

Luc activity (RLU, x10^4)

Vector p53 p51A Np51A

Luc activity (RLU, x10^4)

Vector p53 p51A Np51A

C

-2594

Exon 1

Exon 2

1434 bp

HEK293 cells

consensus: RRRRC   GYYY

TT

597: GGACTTGTCC

881: GAGCATGTCC

M1: GGACTTTCC

M2: GAGCATTTC

D

Luc activity (RLU, x10^5)

vector p53 p51A Np51A

Luc activity (RLU, x10^5)

vector p53 p51A Np51A

Luc activity (RLU, x10^5)

vector p53 p51A Np51A

Luc activity (RLU, x10^5)

vector p53 p51A Np51A

95

95

95

95

Figure 3
Figure 4
Figure 5

A

NHK

Ca^{2+}

B

siRNA1

siRNA2

siRNA2

p51/p63
Integrin α3
Merged

p51/p63
Integrin α3
Merged

p51/p63
Cytokeratins
Merged
Figure 6
p51/p63 controls subunit alpha3 of the major epidermis integrin anchoring the stem cells to the niche

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