

Original Paper

The transcriptional regulatory function of p53 is essential for suppression of mouse skin carcinogenesis and can be dissociated from effects on TGF- β -mediated growth regulation

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Abstract

Transcriptional regulation by p53 is critical for p53-mediated tumour suppression; however, p53-mediated transactivation has been dissociated from p53-mediated biological processes including apoptosis, DNA repair, and differentiation. We compared the effects of a mutant allele, *p53^{QS-val135}*, containing a double mutation in the amino-terminus abrogating transactivation activity and a modification at amino acid 135 partially affecting DNA binding, to complete loss of p53. We applied *in vitro* endpoints correlated with epithelial tumorigenesis and an *in vivo* assay of tumour phenotype to assess whether loss of p53-mediated transcriptional regulation underlies the malignant phenotype of *p53^{-/-}/v-ras^{Ha}*-overexpressing keratinocytes. Transactivation deficiency of *p53^{QS-val135}* was confirmed by reporter gene assays in fibroblasts and differentiating keratinocytes. *Ras* oncogene-induced senescence was lost in both *p53^{QS-val135}/QS-val135* and *p53^{-/-}* keratinocytes. Similarly, *p53^{QS-val135}/QS-val135*, like *p53^{-/-}*, cooperated with *v-ras^{Ha}* to enhance malignant conversion. The tumours arising in *p53^{QS-val135}/QS-val135* keratinocytes displayed strong nuclear p53 expression; thus, the *p53^{QS-val135}* allele was maintained and the deficient transactivation function of the expressed p53^{QS} mutant protein was supported by absence of p21^{waf1} in these tumours. The *p53^{QS-val135}* allele did not confer a dominant-negative phenotype, as *p53^{+ / QS-val135}* keratinocytes senesced normally in response to *v-ras^{Ha}* expression and formed benign tumours. While *p53^{-/-}* keratinocytes displayed diminished response to TGF- β , *p53^{QS-val135}/QS-val135* and *p53^{+ / +}* keratinocytes responded equivalently, indicating that the requirement for p53 in maximizing TGF- β -mediated growth regulation is independent of its transactivation domain and that the ability of keratinocytes to respond to TGF- β is insufficient to suppress the malignant phenotype in this model. Furthermore, TGF- β enhances p53^{QS}-induced activation of a dual p53-TGF- β responsive reporter in a keratinocyte cell line. These findings support an essential role for p53-mediated transcriptional regulation in suppressing malignancies arising from *ras*-induced skin tumours, consistent with previous findings in spontaneous carcinogenesis in other organs, and highlight the potential importance of senescence for tumour suppression *in vivo*.

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Introduction

The frequency with which the p53 pathway is inactivated in human cancers suggests a critical role for the wild-type protein in maintaining normal tissue homeostasis and points to reconstituting the pathway as an

attractive strategy for cancer therapies [1]. Approaches designed to this end include overexpressing wild-type p53, reactivating mutant p53 to restore wild-type function, and inhibiting negative regulators of the endogenous wild-type protein [2–4]. Key to the success of these therapies is an understanding of the

mechanism(s) of tumour suppression by p53 within each given context, and the structural requirements for this activity.

The most frequently observed mutations in the *p53* gene occur within the core DNA binding domain, suggesting that the ability to bind DNA in a sequence-specific manner, as imparted by this domain, is a critical component of p53-mediated tumour suppression. This binding is a prerequisite for the transactivation function of p53, which is mediated through the amino-terminal acidic transactivation domain [5]. While the ability of p53 to bind DNA and regulate gene transcription has been widely considered central to its function as a tumour suppressor, several studies suggesting that p53-mediated biological processes including apoptosis, DNA repair, and differentiation can occur *in vitro* independently of p53-mediated transactivation have called this requirement into question [6–9]. Notably, transactivation-independent functions of p53 in DNA repair and apoptosis have been linked to its ability to participate in protein–protein interactions mapped to the carboxyl terminus and DNA binding domains, respectively [9,10]. A proline-rich domain in the amino-terminus of p53 has also been implicated in the p53-mediated apoptotic response [11].

We and others have previously shown that a null mutation in *p53* cooperates with oncogenic *ras* to enhance the malignant progression of mouse skin tumours [12,13]. This effect of p53 deficiency could reflect loss of the transcriptional regulatory activity of p53 or of transactivation-independent functions. To distinguish between these possibilities, we have utilized previously described knock-in mice that express a *p53*^{W25Q/L26S} double mutation under the endogenous p53 promoter [14]. A W25QL26S mutant retains the ability to bind DNA but renders p53 transcriptionally inactive. In this mouse line, the *p53*^{QS} mutation was introduced into a *p53* allele harbouring an additional genetic change which resulted in an alanine-to-valine substitution at amino acid 135, strongly and differentially compromising sequence-specific DNA binding to p53-responsive gene promoters [15,16]. Previous studies of cells derived from *p53*^{QS-val135/QS-val135} mice demonstrated that the homozygous *p53*^{QS-val135/QS-val135} mutation was indistinguishable from a homozygous *p53*^{-/-} mutation, with regard to loss of p53-dependent stress responses such as PALA-induced growth arrest of murine embryonic fibroblasts, DNA damage-induced apoptosis of thymocytes and ES cells, and the ability to suppress oncogene-driven transformation [14–17]. This is demonstrated even more so by the similar spectrum of spontaneous tumours observed in the *p53*^{QS-val135/QS-val135} and *p53*^{-/-} mice [15], indicating that the *in vivo* tumour suppression activity of p53 depends on its transactivation function.

Further elucidation of the function of the *p53*^{QS-val135} allele as a tumour suppressor in relation to its effects on different cellular functions could help

to define the essential mechanisms of p53-mediated tumour suppression *in vivo*. As the p53 response is largely context-dependent, we explored the ability of the *p53*^{QS-val135} allele to substitute for *p53*^{+/+} in a mouse grafting model of squamous cell carcinogenesis, correlating this *in vivo* assay with *in vitro* endpoints that have been linked to p53 activity and epithelial cell transformation.

Materials and methods

Genotyping

Genotypes were determined by the amplification of *p53* exon 2 from genomic DNA, followed by MscI restriction enzyme digestion of the resultant PCR product to identify an MscI site introduced by the *QS* mutation as described previously [15]. The sequences of the primers were 5'- AGT GGA TCC TTT ATT CTA CCC TTT CCT ATA AGC CAT A-3' and 5'- AGT GGT ACC TTA GTT CCT GAT TTC CTT CCA TTT TTT G-3' (Integrated DNA Technologies, Coralville, IA, USA). Target sequences were amplified in a Touchdown Thermocycler (Hybaid, Ashford, Middlesex, UK) in a 60 µl reaction mixture containing 1× PCR Buffer II, 1.5 mM MgCl₂, 1 mM dNTPs, 3 U of AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA, USA), 1 µM of each primer, and 1 µl of DNA. Following a 2 min hot start at 92.5 °C, the reaction profile was as follows: 35 s at 92.5 °C; 1 min at 56 °C; and 30 s at 72 °C for 35 cycles, followed by 5 min at 72 °C. PCR products were then digested with MscI (New England Biolabs, Beverly, MA, USA) to reveal the digestion of the 450 base pair (bp) PCR product in samples containing the *p53*^{QS} allele [14].

Primary cell preparation and culture

Mice expressing the *p53*^{QS-val135} allele were generated by homologous recombination [14]. *p53*^{+/-} breeder pairs [18] were purchased from Taconic Farms (Germantown, NY, USA). Primary fibroblasts were isolated from newborn *p53*^{+/+}, *p53*^{+/QS-val135}, and *p53*^{QS-val135/QS-val135} mice, and keratinocytes were isolated from newborn *p53*^{+/+}, *p53*^{+/QS-val135}, *p53*^{QS-val135/QS-val135}, *p53*^{+/-}, and *p53*^{-/-} mice following established methods [12]. Keratinocytes were maintained in standard keratinocyte growth medium composed of SMEM (Invitrogen, Grand Island, NY, USA) containing 8% chelexed fetal bovine serum (Gemini BioProducts, Calabasas, CA, USA) adjusted to a final calcium concentration of 0.05 mM, under incubator conditions of 36 °C and 7% CO₂. Keratinocyte differentiation was induced by elevating extracellular Ca²⁺ levels to more than 0.1 mM [19].

Assays for transcriptional activity

Transfections were performed in six-well culture dishes using Lipofectamine Plus reagent according to

the manufacturer's protocol (Invitrogen, Grand Island, NY, USA). Transactivation activity was measured using the Luciferase Assay System (Promega, Madison, WI, USA) and quantified using a Perkin Elmer Microplate Luminometer LB 96V (EG & G Berthold GmbH and Co, Bad Wildbad, Germany).

The p53-responsive plasmid, PG₁₃luc, and its corresponding control, MG₁₅luc [20], were provided by Dr Burt Vogelstein. Primary keratinocytes were maintained for 1 week in standard keratinocyte growth medium prior to transfection and cultures were overlaid with fresh growth medium immediately following transfection. After 24 h, cultures were fed fresh growth medium or shifted to 0.12 mM Ca²⁺-containing medium to induce differentiation. Cultures were harvested 24 h later (48 h post-transfection).

The Mix.2 reporter plasmid contains the promoter of the *Xenopus* Mix.2 homeobox gene, which is under joint control of TGF- β and p53 upstream of the gene encoding luciferase [21]. The Mix.2 plasmid and control MutMix.2 plasmid with a mutation in the p53 binding site [21] were generously provided by Dr Stefano Piccolo. The p53-null Ak1b murine keratinocyte cell line was cultured as previously described [22] and transfected with a 1:1 ratio of Mix.2 in combination with CMV-p53^{QS}-ala135 or CMV-vector control. The following day, cultures were treated with TGF- β 2 (1 ng/ml) and harvested for luciferase activity 22–24 h later.

In vitro senescence assay

Primary keratinocytes were isolated from newborn p53^{+/+}, p53^{+/QS-*val135*}, or p53^{QS-*val135*/QS-*val135*} mice and cultured in standard keratinocyte growth medium containing 0.05 mM Ca²⁺. On day 3, cultures were infected with a retrovirus encoding the *v-ras*^{Ha} oncogene. Eleven days later, cells were fixed with 0.25% glutaraldehyde and senescent cells were detected by staining for β -galactosidase activity at pH 6.0, as described previously [23,24]. Each experiment was performed using a minimum of triplicate samples for each condition and was performed four times with consistent results. An additional experiment was performed using keratinocytes from p53^{+/+} or p53^{-/-} mice for comparison.

BrdU incorporation assay for detection of TGF- β inhibitory effects

TGF- β 2 was purchased from R&D Systems (Minneapolis, MN, USA). Primary keratinocyte cultures of each genotype were treated in parallel with TGF- β 2 (1 ng/ml) for 24 h and pulsed with BrdU for the final 4 h prior to harvesting. BrdU incorporation was determined by FACS analysis, following previously described procedures [25]. A minimum of three replicates were assayed for each condition. Results are presented as the per cent of cells in S-phase in treated cells relative to the control cell population, for each of three

p53^{QS-*val135*} experiments. An additional experiment was performed with p53^{+/+}, p53^{+/-}, and p53^{-/-} keratinocytes for comparison and to confirm previous results [12].

Grafting assay

Primary keratinocytes were isolated, pooled by genotype (p53^{+/+}, p53^{+/QS-*val135*}, p53^{QS-*val135*/QS-*val135*}), and plated in standard keratinocyte growth medium. After 4 days, keratinocyte cultures were infected with a retrovirus encoding the *v-ras*^{Ha} oncogene [26] and grafted 5 days later onto the dorsal surfaces of nude mice, using established methods [12]. Each graft site received a suspension of 3.5 \times 10⁶ keratinocytes derived from p53^{+/+}, p53^{+/QS-*val135*}, or p53^{QS-*val135*/QS-*val135*} mice in combination with 6.0 \times 10⁶ fibroblasts that had been isolated from wild-type C57Bl6/NCr mice and cultured for up to 1 week [12]. Tumours were observed for up to 33–35 days, with weekly measurements over this time (length \times width \times height) using calipers, and then harvested for histological analysis. Classification of the tumour phenotype was performed by two independent blinded observers, with 100% correlation. Parallel samples of cells for grafting were subjected to western analysis to confirm p53 status and uniform expression levels of *ras*^{Ha} across cells of the different genotypes. Additional experiments were performed with p53^{+/+}, p53^{+/-}, and p53^{-/-} keratinocytes for comparison and to confirm previous results [12]. All animal work was performed in accordance with NIH established guidelines.

Immunohistochemistry

Formalin- or ethanol-fixed, paraffin-embedded sections from graft sites containing p53^{QS-*val135*/QS-*val135*}, p53^{+/+}, p53^{+/QS-*val135*} or p53^{-/-} keratinocytes were deparaffinized and hydrated by standard methods. For cytokeratin analysis, sections were pre-incubated in 3% H₂O₂ in phosphate-buffered saline (PBS) at room temperature for 30 min to quench endogenous peroxidase activity, followed by an additional 30 min in normal goat serum (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA) to block non-specific binding. Rabbit monoclonal antibodies to keratin 1 and keratin 14 (Covance Laboratories, Berkeley, CA, USA) were diluted 1:500 in PBS containing 12% bovine serum albumin (BSA) and applied to the tissue sections for overnight incubation at 4°C. Sections were then washed with PBS, incubated for 30 min at room temperature with biotinylated secondary goat anti-rabbit antibody (Kirkegaard & Perry Laboratories), and then washed and incubated an additional 30 min with avidin-biotin peroxidase complex (Novo Castra Laboratories, Newcastle upon Tyne, UK). Antibody binding was visualized as peroxidase activity using diaminobenzidine (DAB) as a substrate (Novo Castra Laboratories).

For p53 immunostaining, sections were pretreated with 0.3% peroxide, followed by 2% BSA, and then reacted with rabbit polyclonal IgG directed to p53 (FL-393, 6243; Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:50 in PBS/12% BSA in an overnight incubation at 4 °C. Incubation with biotinylated secondary antibody (E0353; Dako Cytomation, Glostrup, Denmark) followed by antigen-antibody complex (Vectastain ABC kit PK6100, Vector Laboratories, Burlingame, CA, USA) and visualization using DAB (K3465; Dako Cytomation) was essentially as above.

Ki67 and p21^{waf1} immunostaining was performed using the Ventana Discovery Automated Stainer (Ventana Medical Systems, Tucson, Arizona) following the manufacturer's suggestions. Deparaffinization was done in the Ventana machine and sections were heat-treated in Tris-borate EDTA buffer, pH 8.0, for epitope retrieval and blocked with 2% BSA in PBS. Sections were treated with primary antibodies at the dilutions indicated for 1 h at 37 °C. The primary antibodies used were rabbit monoclonal (SP6) to Ki67 (Neomarkers), 1:200; and polyclonal affinity-purified rabbit anti-p21 (C-19: sc-397; Santa Cruz Biotechnology, Inc), 1:50. Primary antibodies were diluted in 1% BSA, 0.1% Tween-20 in PBS. The secondary antibody was a polyclonal biotinylated swine anti-rabbit IgG (Dako E0353), diluted 1:200 in Ab-diluent (Ventana Medical Systems). A streptavidin-biotin horseradish peroxidase-based DAB kit (Ventana) was used for the detection of antibody reactivity. Sections were counterstained with haematoxylin, rehydrated in graded ethanol rinses, cleared in Xylene, and mounted in Per-tex (Histolab, Gothenburg, Sweden).

Results

The p53^{QS-val135} mutant is well characterized with regard to its lack of transcriptional regulatory activity [14,15]. As shown in Figure 1A, endogenous levels of wild-type p53 in fibroblasts isolated from p53^{+/+} mice transactivate the p53-responsive PG₁₃luc reporter construct, whereas this reporter gene activity is abrogated in p53^{QS-val135/QS-val135} fibroblasts, confirming the transactivation-deficient status of this homozygous mutation. It is important to note that parallel fibroblast cultures derived from p53^{+/QS-val135} mice display levels of PG₁₃luc reporter activity similar to that observed in control p53^{+/+} fibroblasts (Figure 1A, middle versus left bars), indicating that p53^{QS-val135} mutant protein, in spite of its deficient sequence-specific DNA binding, does not exert the dominant-negative effect on transcriptional regulation that is typically observed with p53 DNA binding domain mutants [5]. While p53-mediated transactivation in wild-type keratinocyte cultures increases under differentiating conditions, consistent with our previous findings (Figure 1B and ref 19), this relative increase in transcriptional activity is diminished in p53^{QS-val135/QS-val135} keratinocytes (Figure 1B).

p53^{QS-val135/QS-val135} mice develop a spectrum of spontaneous tumours essentially identical to those arising in p53^{-/-} mice [15], and cells derived from mice homozygous for each genotype display similar properties in a variety of *in vitro* assays [14,15,17]. We compared keratinocyte cultures derived from p53^{QS-val135/QS-val135}, p53^{+/QS-val135}, p53^{+/+}, p53^{+/-}, and p53^{-/-} mice for their ability to undergo oncogene-induced senescence, a normal cellular

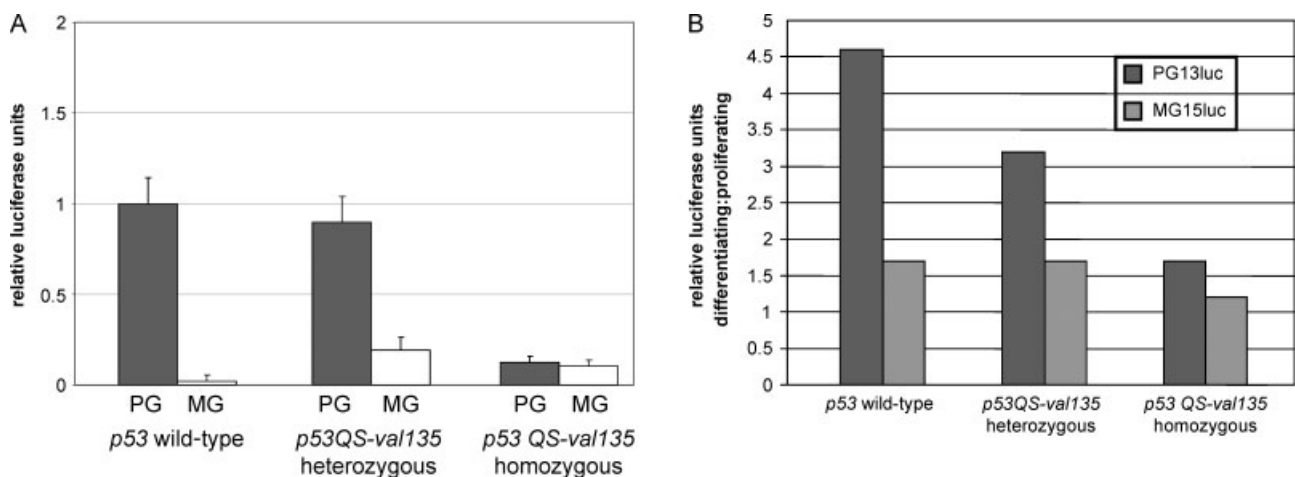


Figure 1. Cultures derived from p53^{QS-val135/QS-val135} mice are deficient in p53-mediated transcriptional activity. Primary p53^{+/+}, p53^{+/QS-val135}, and p53^{QS-val135/QS-val135} cultures were transfected with the p53-responsive reporter PG₁₃luc, or control plasmid MG₁₅luc, which contains a mutation in the p53 consensus binding sequence. (A) Fibroblast cultures established from the skin of newborn mice of each genotype were transfected with PG₁₃luc, or MG₁₅luc, and harvested after 48 h. As seen by luciferase reporter gene activity, p53^{QS-val135/QS-val135} cultures are deficient in transactivation activity, while the p53^{+/QS-val135} cultures display transactivation activity similar to that of p53^{+/+} cultures. (B) Primary epidermal keratinocytes derived from newborn p53^{+/+}, p53^{+/QS-val135}, and p53^{QS-val135/QS-val135} mice were transfected with the PG₁₃luc or MG₁₅luc plasmids and cultured under proliferating conditions or induced to differentiate for 24 h prior to harvest. As previously reported [19], p53-dependent transcriptional activity increases in p53^{+/+} keratinocytes under differentiating conditions. This enhanced transcriptional activity normally observed in conjunction with differentiation is diminished in p53^{QS-val135/QS-val135} keratinocytes

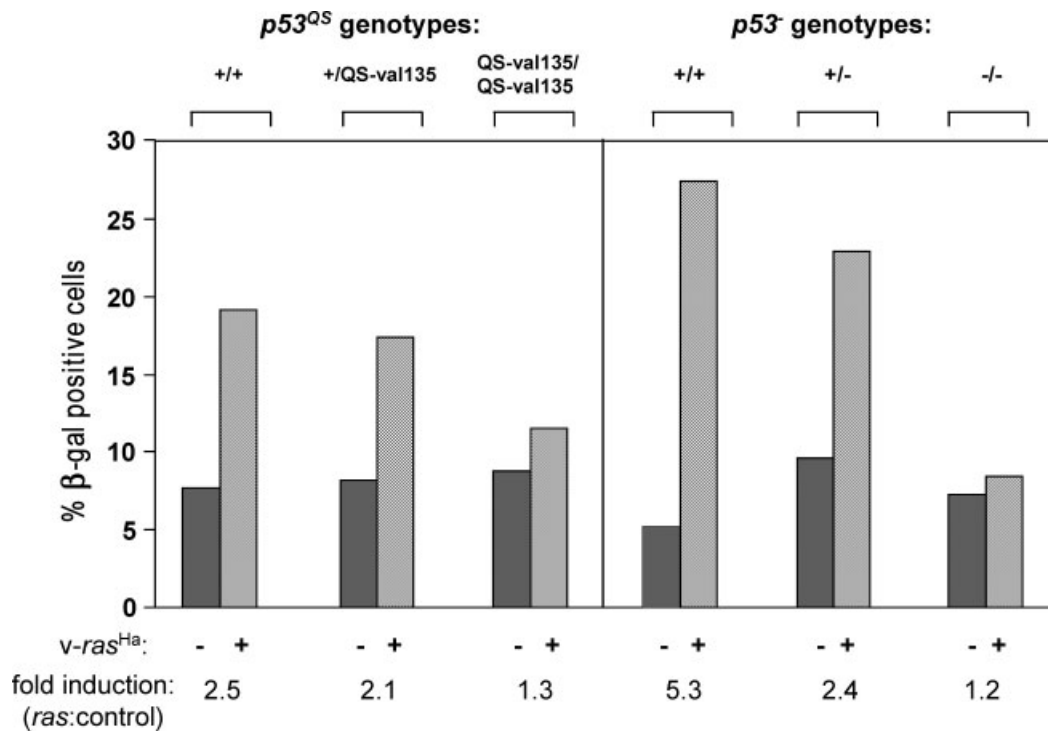


Figure 2. Both p53^{QS}-val135 and p53-null mutations overcome senescence induced by oncogenic *ras* in keratinocytes. Primary keratinocytes were isolated from newborn p53^{+/+}, p53^{+ / QS-val135}, p53^{QS-val135 / QS-val135}, p53^{+ / -} or p53^{- / -} mice and cultured in medium containing 0.05 mM Ca²⁺. On day 3, cultures were infected with a retrovirus encoding the v-ras^{Ha} oncogene. Eleven days later, cells were fixed with 0.25% glutaraldehyde and stained for β -gal activity at pH 6.0 [23,24]. The results shown are representative of four independent experiments. Both p53^{QS-val135 / QS-val135} and p53^{- / -} keratinocytes overcome senescence induced by oncogenic *ras*. p53^{+ / QS-val135} keratinocytes behave similarly to p53^{+/+} in this assay

response contributing to the maintenance of normal tissue homeostasis [27]. p53^{+/+} control cultures showed an increased number of senescing cells following the introduction of oncogenic *ras* (Figure 2), consistent with previous reports [23]. Total loss of p53 confers the ability to overcome this oncogene-mediated cellular senescence, as seen in p53^{- / -} keratinocyte cultures (Figure 2, right panel). p53^{QS-val135 / QS-val135} keratinocytes behaved identically to p53^{- / -} keratinocytes in this assay (Figure 2, left panel); thus, the integrity of the remaining domain structures outside of the transactivation and DNA binding domains is insufficient to maintain the normal cellular senescence response to oncogenic *ras*. Again, p53^{+ / QS-val135} keratinocytes behaved similarly to p53^{+/+} keratinocytes in this assay, indicating that the QS-val135 mutant p53 does not interfere with the remaining wild-type protein. These findings suggest that a functional amino-terminal transactivation domain of p53 is required for the normal senescence response to activated v-ras^{Ha}.

Loss of TGF- β responsiveness in epithelial cells is a hallmark of cancer development and has been associated with p53 loss of function [12,28,29]. We have previously shown using [³H]thymidine incorporation studies that p53-null keratinocytes partially overcome the growth arrest induced by TGF- β [12]. This was confirmed in the present study using flow cytometry analysis of BrdU incorporation as a measure of growth arrest (Figure 3A, right panel). In contrast to p53^{- / -} cultures, p53^{QS-val135 / QS-val135} keratinocytes retained

the same degree of responsiveness to TGF- β -mediated growth arrest as parallel p53^{+/+} control cultures isolated from littermates (Figure 3A, left panel).

We further explored the ability of p53^{QS} to support p53-dependent TGF- β responsiveness using transactivation assays in the p53-null keratinocyte cell line, AK1b. We first showed that this cell line was capable of responding to TGF- β with enhanced levels of phospho-Smad2 and that this response was not abrogated in the presence of p53^{QS}-ala135, unlike previous reported findings with a DNA binding mutant [30] (Figure 3B). We assessed the ability of p53^{QS}-ala135 to cooperate with TGF- β in activating the Mix.2 reporter plasmid, which contains both p53- and TGF- β -responsive elements from the *Xenopus* homeobox *Mix.2* gene [21]. By using p53^{QS}-ala135 instead of -val135 cDNA, we could focus on the regulation of p53 transactivation activity by the N-terminal amino acids 25 and 26 (human 22 and 23), as the DNA binding ability of p53^{QS}-ala135 is intact. AK1b cells were co-transfected with the Mix.2 reporter construct in combination with p53^{QS} or empty vector. As seen in Figure 3C, p53^{QS} was able to transactivate the Mix.2 reporter (Figure 3C). This was further enhanced by the addition of TGF- β , indicating that the mutations within the transactivation domain at amino acids 22 and 23 do not interfere with this biological function of p53.

We and others have previously shown that loss of p53 cooperates with expression of a mutant

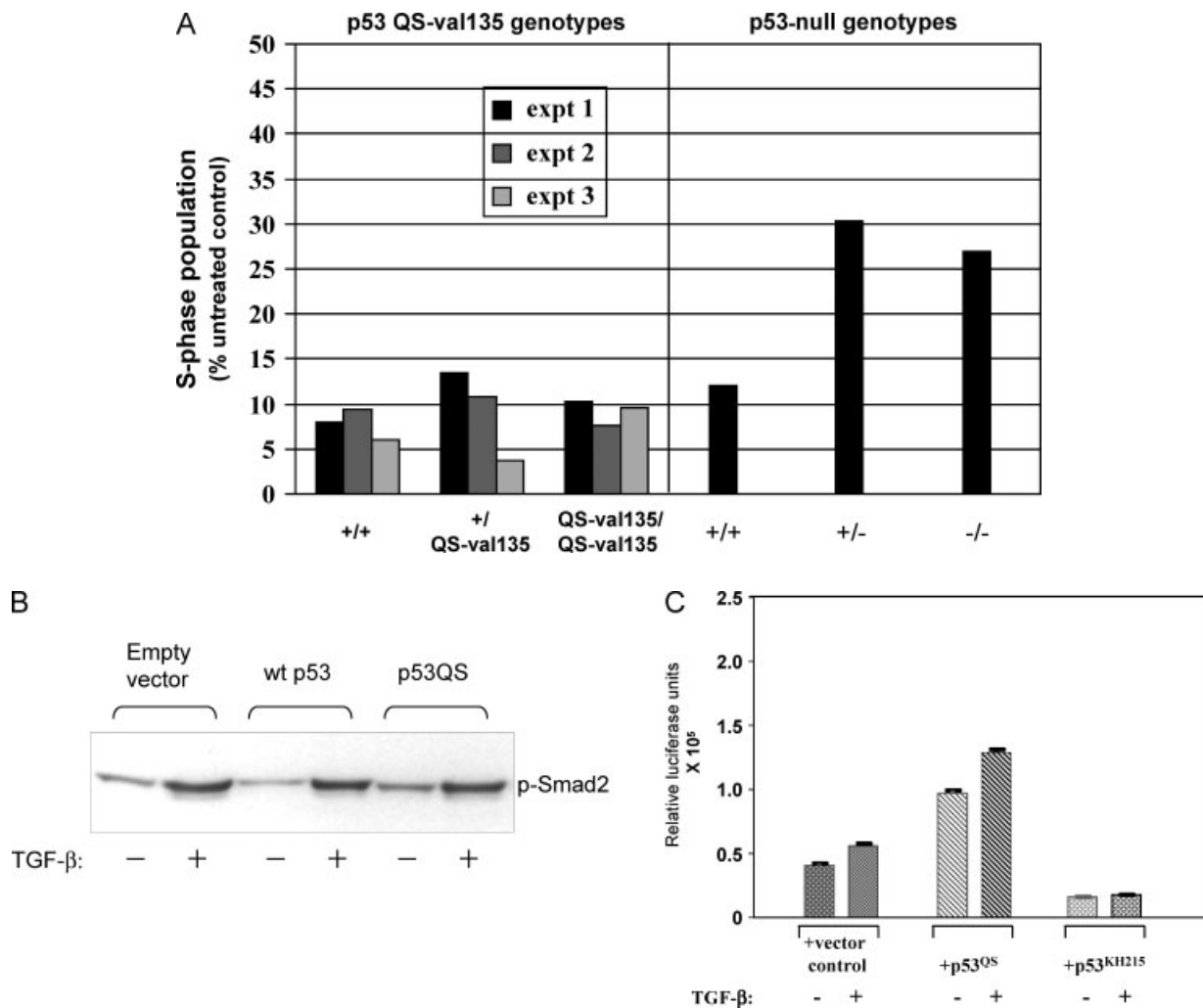


Figure 3. $p53^{QS-val135/QS-val135}$ keratinocytes retain TGF- β responsiveness. Primary keratinocyte cultures of each genotype were treated in parallel with TGF- β 2 (1.0 ng/ml) for 24 h and pulsed with BrdU for the final 4 h prior to harvesting for FACS analysis. $p53^{+/+}$ keratinocytes undergo growth arrest in response to TGF- β 2, as demonstrated by a decrease in the S-phase population in TGF- β 2-treated cultures relative to parallel control cultures. $p53$ -null keratinocytes display a partial block in TGF- β response using this assay, consistent with earlier published findings [12]. In contrast to $p53^{-/-}$ keratinocytes, $p53^{QS-val135/QS-val135}$ and $p53^{+/QS-val135}$ keratinocytes respond to TGF- β 2 by undergoing p53-dependent growth arrest to the same extent as wild-type controls. The results shown of the $p53^{QS-val135}$ experiment are representative of three experiments performed in triplicate, with three to six replicates per experiment. (B) The $p53$ -null AK1b keratinocyte cell line was transfected with empty vector cDNA or cDNA encoding wild-type or $p53^{QS}$ and treated with TGF- β 2 (1.0 ng/ml) for 1 h prior to harvest for western blot analysis. Expression of the $p53^{QS}$ mutant does not alter levels of phosphorylated Smad2 (p-Smad2) in response to TGF- β . (C) The $p53$ -null AK1b keratinocyte cell line was transfected with the Mix.2 plasmid containing both p53 and TGF- β response elements, in combination with empty vector or cDNA encoding mutant $p53^{QS-ala135}$. Increased Mix.2 reporter gene activity is observed in the presence of both $p53^{QS-ala135}$ and TGF- β . The DNA binding domain mutant KH-215 completely abrogates this activity even in the presence of TGF- β . No response to $p53^{QS}$ or TGF- β was seen using a Mix.2 plasmid with a mutation in the p53 binding site (results not shown)

ras^{Ha} oncogene to allow malignant conversion of murine epidermal keratinocytes [12,13]. To evaluate the *in vivo* ability of $p53^{QS-val135}$ to function as a tumour suppressor, primary epidermal keratinocytes isolated from $p53^{+/+}$, $p53^{+/QS-val135}$ or $p53^{QS-val135/QS-val135}$ mice were infected with a retrovirus encoding the *v-ras^{Ha}* oncogene and then grafted onto the dorsal surface of nude mice. Similar experiments were performed in parallel with $p53^{+/+}$, $p53^{+/-}$, and $p53^{-/-}$ keratinocytes, as previously described [12], and the resulting tumour phenotypes were compared (Figure 4 and Table 1) and confirmed by histological analysis of haematoxylin and

eosin (H&E)-stained sections. In studies of $p53^{+/+}$ keratinocytes, all resulting tumours were benign papillomas, as expected. $p53^{+/QS-val135}$ grafts behaved like $p53^{+/+}$ grafts and gave rise to well-differentiated papillomas, with no predisposition towards malignant conversion, while $p53^{QS-val135/QS-val135}$ and $p53^{-/-}$ keratinocytes yielded only undifferentiated carcinomas following grafting.

Tumours arising in the graft sites were further analysed histologically after H&E staining and after immunohistochemical staining for the keratinocyte differentiation markers keratin 1 (K1) and keratin 14 (K14) (Figure 5). As expected, papillomas of both

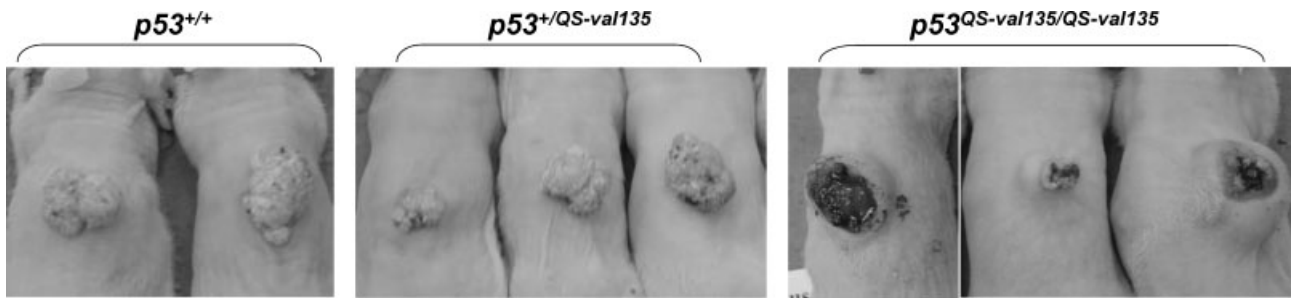


Figure 4. $p53^{Q5-val135/Q5-val135}$ behaves like a null mutation in its ability to cooperate with *ras* in a grafting model of mouse skin carcinogenesis. Primary keratinocytes from wild-type mice or mice heterozygous or homozygous for the $p53^{Q5-val135}$ mutation [14] were transduced with a retrovirus encoding the $v-ras^{Ha}$ oncogene and grafted to nude mouse hosts. Representative results are shown 34 days post-grafting. Carcinomas arising from $p53^{Q5-val135/Q5-val135}$ grafts are ulcerating and poorly differentiated and similar to those observed in p53-null keratinocytes following the same procedure (not shown)

Table 1. The conversion frequency of tumours arising in $p53^{Q5-val135/Q5-val135}/v-ras^{Ha}$ grafted keratinocytes is similar to that of $p53^{-/-}/v-ras^{Ha}$ grafted cells. Phenotypes of $p53^{Q5-val135}$ grafts were confirmed by histology 31–36 days post-grafting and reflect the results of three independent experiments. p53-null grafts were performed in two independent experiments. Their phenotypes were recorded 24–26 days post-grafting and were consistent with previously published results [12]

	p53 ^{Q5-val135} mutant*			p53-null		
	p53 ^{+/+}	p53 ^{+/Q5}	p53 ^{Q5/Q5}	p53 ^{+/+}	p53 ^{+/-}	p53 ^{-/-}
Papilloma	11	10	0	6	8	1
Carcinoma	0	0	9	0	3	7
Total	11	10	9	6	11	8
% converted	0	0	100	0	28	88

* χ^2 analysis of conversion frequency, $p < 0.0001$.

$p53^{+/+}$ and $p53^{+/Q5-val135}$ genotypes stained positive for K1 (Figures 5A and 5B) and K14 (Figures 5D, 5E). Tumours derived from $p53^{Q5-val135/Q5-val135}$ keratinocytes were histologically similar to those from $p53^{-/-}$, yielding undifferentiated carcinomas (Figures 5C, 5F, and 5I) [12]. The lack of cytokeratin staining in these carcinomas (Figures 5C and 5F) further confirmed the highly malignant nature of the $p53^{Q5-val135/Q5-val135}$ tumours. Thus, keratinocytes homozygous for the $p53^{Q5-val135}$ mutation are predisposed towards malignant conversion in the presence of a $v-ras^{Ha}$ oncogene and are indistinguishable from $p53^{-/-}$ keratinocytes in this assay. In our previous study, tumours arising from $p53^{+/-}$ keratinocytes demonstrated both an increase in malignant conversion and enhanced growth relative to $p53^{+/+}$ tumours [12]. The difference in malignant conversion and growth that was previously observed between $p53^{+/+}$ and $p53^{+/-}$ tumours was not observed between $p53^{+/+}$ and $p53^{+/Q5-val135}$ tumours in the present study (Figure 4 and Table 1; data not shown).

Using immunohistochemistry, we were able to confirm the presence of the p53 protein in $p53^{Q5-val135/Q5-val135}$ tumours (Figures 6E and 6F). p53^{Q5-val135} protein was abundant, as visualized by strong nuclear staining in areas of invasive carcinoma (Figure 6E). The differentiated papillomatous parts of the same grafts displayed abundant basally located cells with nuclear p53 staining (Figure 6F). Cells with nuclear p53 accumulation were also observed in the basal

layer of $p53^{+/+}$ (Figure 6A) and $p53^{+/Q5-val135}$ (Figure 6C) squamous cell papillomas, but to a lesser degree.

Tumours were analysed for expression of p21^{waf1} and the antibody yielded distinct nuclear staining in the positive cells (Figure 7). $p53^{+/+}$ and $p53^{+/Q5-val135}$ genotypes stained strongly positive for p21^{waf1} in the basal layer, whereas tumours derived from $p53^{Q5-val135/Q5-val135}$ keratinocytes showed loss of p21^{waf1} expression in the majority of cells, with only a few scattered weakly positive cells in the tumour. The lack of p21^{waf1} expression in these $p53^{Q5-val135/Q5-val135}$ genotypes further supports the fact that p53-mediated transcriptional regulation is compromised in homozygotes and is intact in heterozygotes.

$p53^{+/+}$ and $p53^{+/Q5-val135}$ papillomas stained positive for Ki67, which was visualized as positive nuclei in the basal layer of the papillomatous epithelium (Figure 8). $p53^{Q5-val135/Q5-val135}$ carcinomas showed an abundance in Ki67 expression, indicating that these tumours are highly proliferative.

Discussion

Using a grafting model of $v-ras^{Ha}$ -transformed keratinocytes, we have previously established that p53 suppresses the malignant phenotype of tumours induced by expression of oncogenic *ras* [12] and that this activity is dissociable from induction of p21^{waf1},

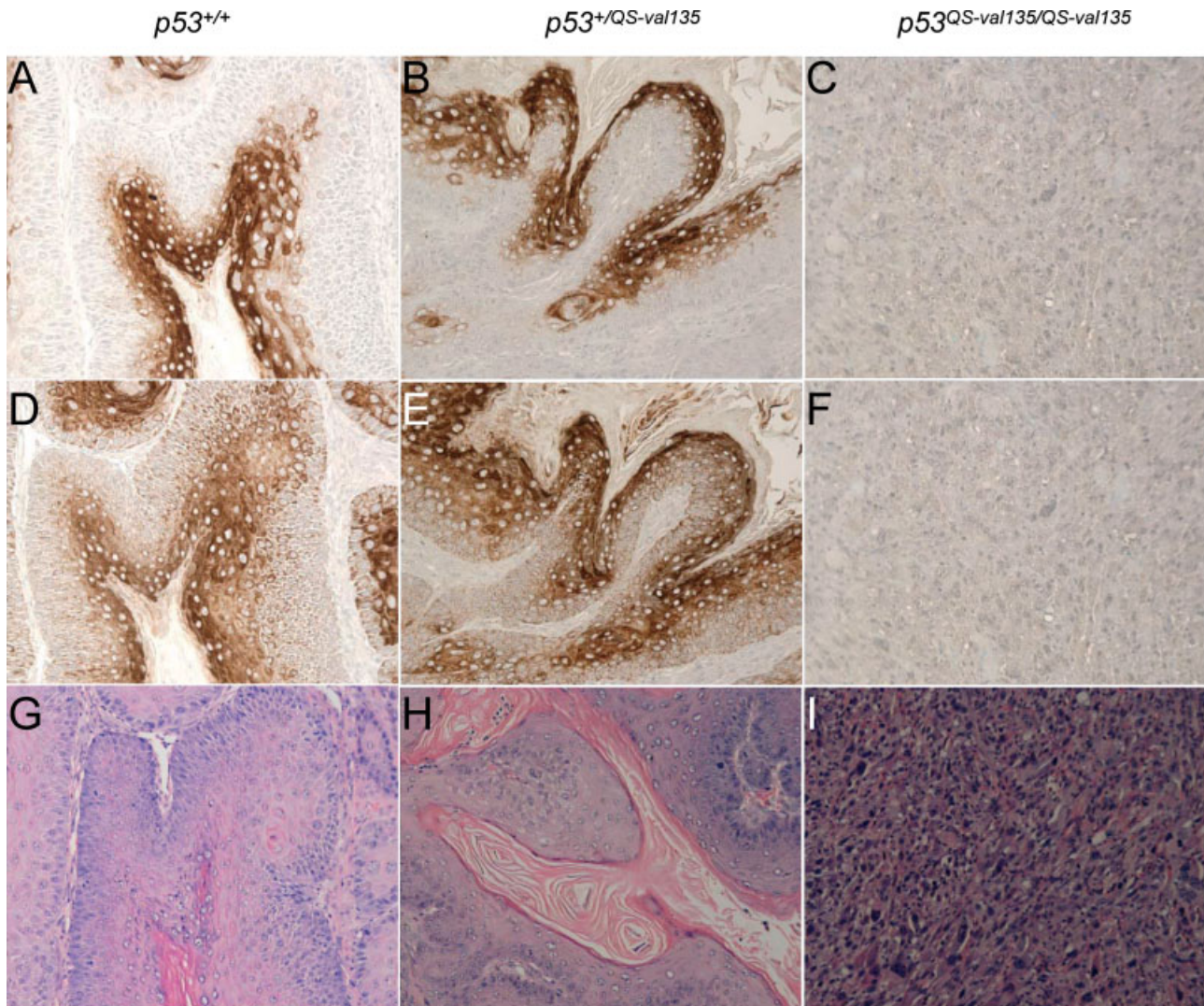


Figure 5. Histological analysis of grafted tumours. $p53^{+/+}/v-ras^{Ha}$ and $p53^{+/QS-val135}/v-ras^{Ha}$ keratinocytes give rise to well-differentiated papillomas, while $p53^{QS-val135/QS-val135}/v-ras^{Ha}$ keratinocytes yield undifferentiated carcinomas similar to those observed previously in $p53^{-}/v-ras^{Ha}$ grafts [12]. $p53^{+/+}$ papillomas (A, D, G), $p53^{+/QS-val135}$ papillomas (B, E, H), and $p53^{QS-val135/QS-val135}$ carcinomas (C, F, I) were analysed for expression of keratin I (K1) and keratin 14 (K14). (A–C) Immunohistochemical staining using K1 antibody. Positive cytoplasmic staining is prominent in the upper parts of epithelial layers and is seen in both $p53^{+/+}$ (A) and $p53^{+/QS-val135}$ (B) papillomas, while staining is negative in the $p53^{QS-val135/QS-val135}$ carcinoma (C). (D–F) Immunohistochemical staining using K14 antibody. Positive cytoplasmic staining variably distributed through the whole epithelium, including basal and suprabasal layers, is present in both $p53^{+/+}$ (D) and $p53^{+/QS-val135}$ (E) papillomas, while staining is negative in the $p53^{QS-val135/QS-val135}$ carcinoma (F). (G–I) H&E staining of parallel sections from the same tumour specimen as were used for keratin staining. Tumours derived from $p53^{+/QS-val135}$ keratinocytes (H) were papillomas, histologically similar to those from $p53^{+/+}$ (G), while tumours developing from $p53^{QS-val135/QS-val135}$ (I) were undifferentiated carcinomas. Thus, tumours derived from $p53^{QS-val135/QS-val135}$ keratinocytes were similar to those described from $p53^{-/-}$, where epithelial structure and differentiation characteristics were lost [12]

a downstream effector of p53-mediated growth regulation [31]. We applied the same strategy to assess whether disrupting the transactivation domain of p53 can reiterate the *in vivo* malignant phenotype observed in p53-null/ $v-ras^{Ha}$ -overexpressing keratinocytes. The identical *in vivo* response shared between $p53^{QS-val135/QS-val135}$ and $p53^{-/-}$ mice presented here suggests that, as previously reported for spontaneous tumours [15], p53-mediated transcriptional regulation is critical to the ability of p53 to suppress the malignant phenotype in this model. Furthermore, the $p53^{QS-val135}$ modification is equivalent to the loss of the entire protein in its ability to contribute to

$v-ras^{Ha}$ -induced senescence, while having no effect on the ability to mediate TGF- β -induced growth arrest.

After the initial description of the $p53^{W25Q/L26S}$ transactivation-deficient mice, it was determined that the p53 construct used to generate the $p53^{W25Q/L26S}$ knock-in mouse line harboured an additional genetic change yielding a protein with an alanine-to-valine substitution at amino acid 135 [15]. The $p53^{val135}$ protein arising from this DNA binding domain mutation is well characterized to be temperature-sensitive [32,33] and has been shown to act in a dominant-negative manner in the presence of wild-type p53 to enhance *in vitro* transformation induced by the

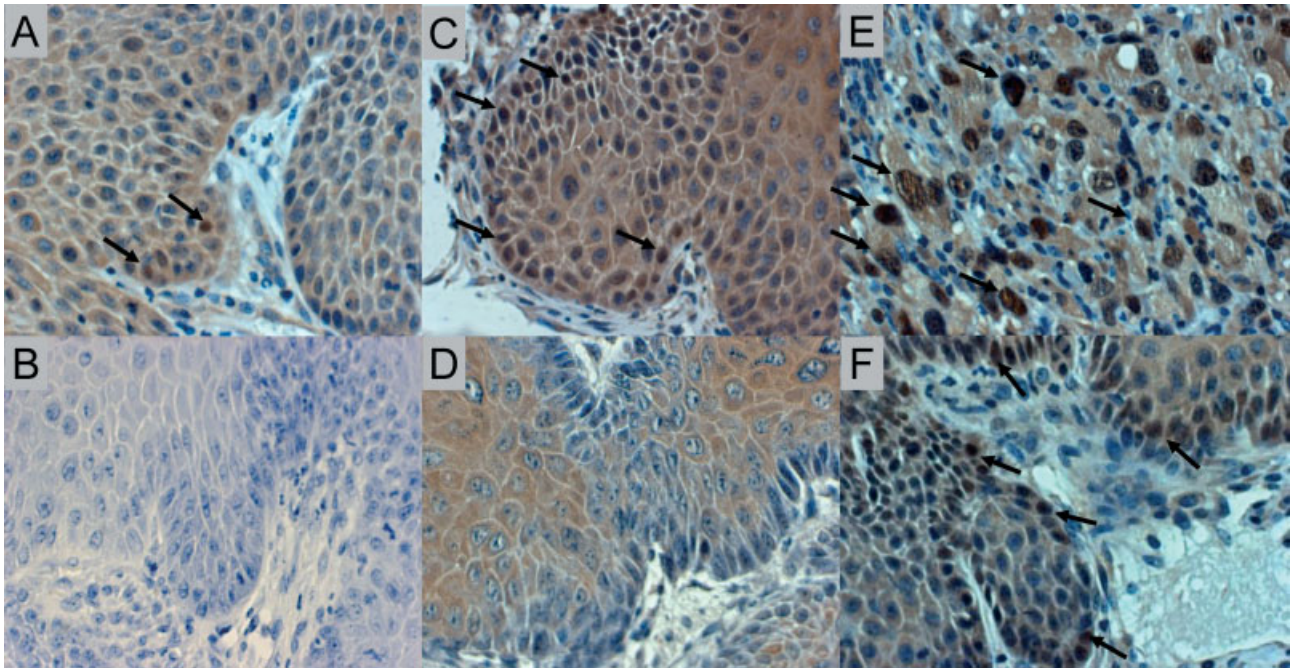


Figure 6. p53 protein expression in tumours arising in graft sites. (A, C–F) Immunohistochemical staining using anti-p53 antibody, as described in the Materials and methods section. Nuclear staining in a $p53^{QS-val135/QS-val135}$ invasive carcinoma (E), and in a superficial non-invasive part of the same tumour (F), is evident due to stabilization of the p53^{QS-val135} protein. Nuclear staining is present but less evident in a $p53^{+/QS-val135}$ papilloma (C) and in the $p53^{+/+}$ papilloma (A). (B) Negative control section processed in parallel with the above reactions but with omission of the primary antibody. (D) Papillomatous epithelium from $p53^{-/-}/v-ras^{Ha}$ grafts were used as background controls for the primary antibody. The antibody demonstrated non-specific cytoplasmic reactivity in keratinocytes of the stratum spinosum, but not in cells of the basal layer, and there was clearly no nuclear background staining

combination of oncogenic *ras* and E1A [34]. *In vivo*, p53^{val135} demonstrated dominant-negative properties, resulting in accelerated tumour development when expressed as a transgene in a $p53^{+/-}$ background, while being unable to suppress tumourigenesis in a $p53^{-/-}$ background [35]. Studies have established that the p53^{QS-val135} protein is severely compromised for DNA binding activity [15,16], a property previously shown to be reversed in the p53^{val135} mutant protein under permissive conditions [36]. We were unable to detect functionally relevant temperature sensitivity for the p53^{QS-val135} protein compared with the p53^{QS-ala135} protein [15], and the biological properties of p53^{QS-val135} appear identical to those demonstrated in an independent study of p53^{QS}-expressing ES cells that were generated using a separately derived construct [14,17]. Thus, it is unlikely that the phenotypes observed in the p53^{QS-val135} mice used in the present study were due to the additional modification of alanine to valine at amino acid 135, as has been suggested [37].

That the $p53^{val135}$ mutation does not contribute significantly to the phenotype observed in tumours arising from $p53^{QS-val135/QS-val135}$ keratinocytes in this study is supported by our findings presented here that in multiple assays, the $p53^{+/QS-val135}$ keratinocytes behave biologically like $p53^{+/+}$ keratinocytes. Consistent with this, we previously reported that the p53^{val135} protein is predominantly nuclear in keratinocytes under the cell growth conditions used here [22], in contrast to previous reports in rat embryo

fibroblasts [32]. This is also the case when $p53^{val135}$ keratinocyte cultures are maintained at 39 °C, an even more permissive temperature for a mutant phenotype [22,32]. Moreover, we found the p53^{QS-val135} mutant protein to be exclusively nuclear in the skin tumours generated in this study. *In vitro*, $p53^{+/QS-val135}$ keratinocytes maintained the ability to undergo senescence in the presence of oncogenic *ras*, and all tumours arising *in vivo* from $p53^{+/QS-val135}$ keratinocytes were benign, even past 30 days post-grafting. These findings suggest that the val135 mutation does not confer a mutant conformation in this context, and does not behave in a dominant negative manner or significantly interfere with the functioning of the wild-type allele (Figure 1), even though it could theoretically hetero-tetramerize with wild-type p53 protein. The facts that the p53 immunostaining intensity was similar in $p53^{+/+}$ and $p53^{+/QS-val135}$ tumours, but much higher in $p53^{QS-val135/QS-val135}$ tumours and that p21^{waf1} immunostaining was similar in $p53^{+/+}$ and $p53^{+/QS-val135}$ but virtually lost in $p53^{QS-val135/QS-val135}$ tumours indicate that the p53^{QS-val135} mutant protein may form dimers and be processed with the wild-type p53 protein, and furthermore support the absence of a dominant-negative effect of the double mutant protein.

Previous studies have shown that the p53^{QS-val135} protein is differentially defective in binding to p53 target gene sequences in DNA, while it is totally deficient in transcriptional activation, with the exception of a low induction of Bax [15,16]. Johnson *et al*

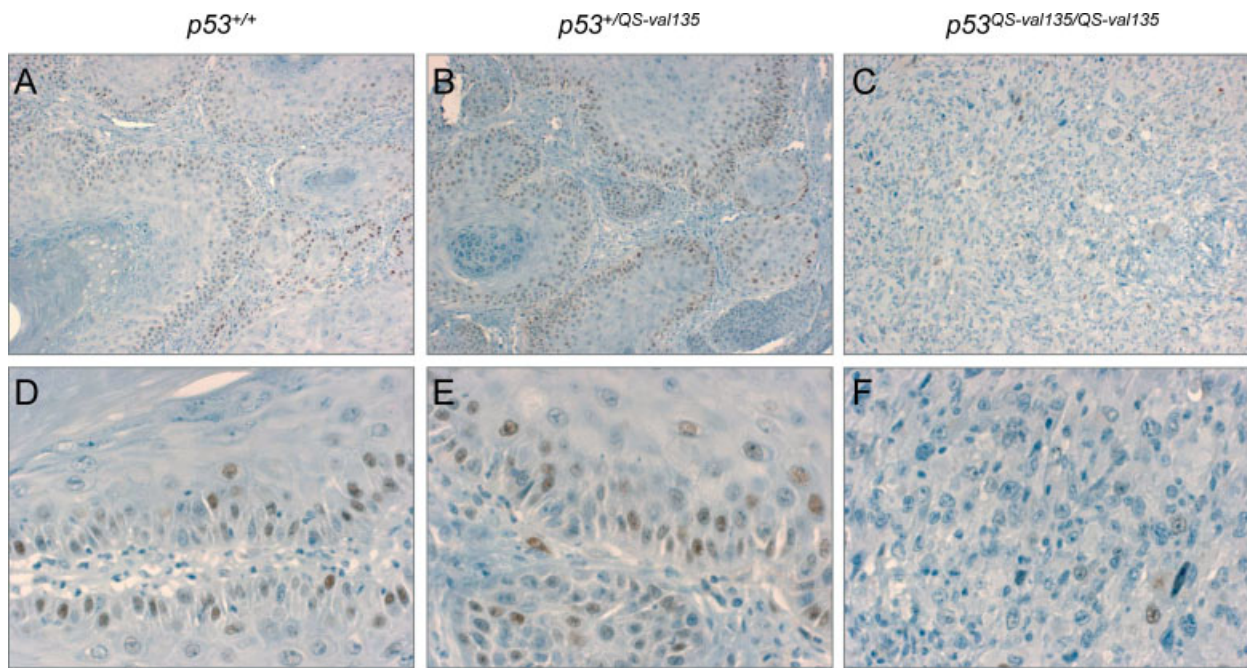


Figure 7. Expression of the p53-regulated gene $p21^{waf1}$ is lost in $p53^{QS-val135/QS-val135}$ tumours. Immunohistochemical staining using anti- $p21^{waf1}$ antibody. (A, B) Positive nuclear staining is prominent in the basal layers of both $p53^{+/+}$ and $p53^{+/QS-val135}$ papillomas, while staining is strikingly absent in the $p53^{QS-val135/QS-val135}$ carcinoma (C). Panels A–C were photographed under a 10 \times objective. (D–F) The same tumour sections under higher magnification (40 \times objective)

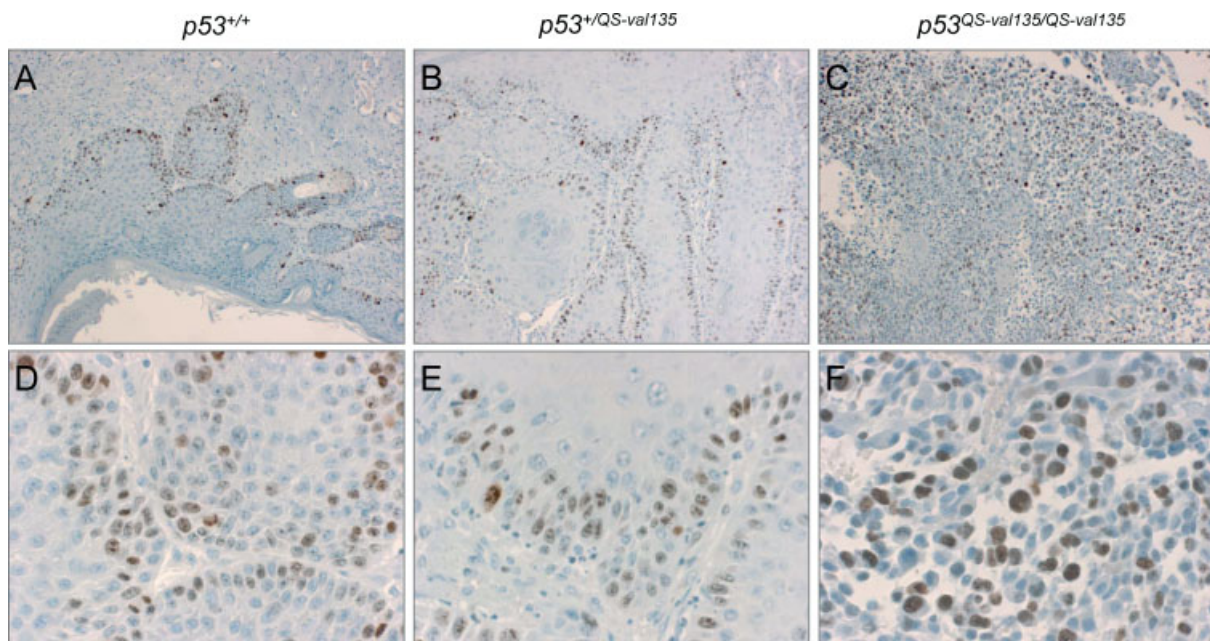


Figure 8. The proliferating cell compartment is expanded in $p53^{QS-val135/QS-val135}$ tumours. Immunohistochemical staining using anti-Ki67 antibody, as described in the Materials and methods section. (A, B) Brown nuclear staining is localized to the basal layer of the papillomatous part of both $p53^{+/+}$ and $p53^{+/QS-val135}$ tumours. (C) A distinct increase in the intensity of the staining is seen in the $p53^{QS-val135/QS-val135}$ carcinoma, indicating increased proliferation of the cells that converted to a malignant, invasive phenotype. Panels A–C were photographed under a 10 \times objective. (D–F) The same tumour sections under higher magnification (40 \times objective)

generated a conditional $p53^{QS}$ mouse mutation with intact DNA sequence-specific binding and some low-level activation of $p21^{waf1}$ and Bax genes. This *in vivo* mutation resulted in defective DNA damage-induced G1 cell cycle arrest and apoptosis, while retaining some apoptotic function induced by serum-deprivation

or hypoxia [38]. These findings indicate that distinct molecular mechanisms are invoked in the apoptotic response to different stresses. Surprisingly, heterozygosity of the conditional $p53^{QS}$ mutation resulted in embryonic lethality, suggested by the authors to be due to hypoxia-induced apoptosis in the embryo [38].

The ability of p53^{QS-val135/QS-val135} keratinocytes to respond to TGF- β to the same degree as those expressing wild-type p53 protein (Figure 3A) was unexpected and in contrast to the complete loss of G1 arrest and apoptosis reported previously in other p53^{QS-val135/QS-val135} cells following exposure to DNA-damaging agents [14,17]. This implies that the TGF- β effect on keratinocytes is largely independent of the p53 transactivation function. Surprisingly, however, p53^{QS-ala135} retained substantial activity on the p53- and TGF- β -responsive Mix.2 reporter when tested in p53-null keratinocytes, and most of the induction appeared p53-dependent, with only a minor component from TGF- β . This may be a result of the fact that the p53^{QS} mutant protein is very stable and binds to chromatin. It has also been suggested that p53 has a second transactivation domain [39], which would not be affected by the QS mutations and which may operate on this promoter.

Importantly, the retention of TGF- β responsiveness was the only functional contribution of the mutant p53^{QS-val135} protein that distinguished it from a total lack of p53 and provides insight into the mechanism by which p53 contributes to this response. It has been proposed that p53 and the SMAD proteins bind to distinct elements on promoters that are under the joint control of p53 and TGF- β , such as p21^{waf1}, PAI-1, and MMP-2 [21]. We speculate that the ability of p53^{QS-val135} to support the p53-mediated growth arrest response to TGF- β is independent of its amino-terminal transactivation domain and reflects the ability of p53^{QS-val135} protein to interact with SMAD2/3 signalling proteins on gene promoters, as has been shown for wild-type p53 [21]. It has also been shown that Ras/MAPK signalling-induced phosphorylation at p53 Ser⁶ and Ser⁹ is essential for establishing the p53-Smad cooperation required for the induction of p21^{waf1} and p15^{ink4b} by TGF- β [40]. These amino acids are intact in the p53^{QS-val135} protein and may mediate the effect observed here. It is also of interest that multiple variants of the p53 homologue, Δ Np63, which lack the p53-homologous amino-terminal transactivation domain, cooperate with the TGF- β pathway to at least the same extent as TAp63 forms, which contain this domain, in driving the p53- and TGF- β -responsive Mix.2 reporter [21]. As noted, Johnson *et al* [38] reported some remaining transcriptional functions of a p53^{QS-Ala135} allele. These effects may also be mediated by the second p53 transactivation domain [39,41], which is not affected by the QS mutations. The mechanism(s) of interaction between p53 and SMAD proteins, and the integrity of the requisite structural features in p53 for full TGF- β responsiveness in keratinocytes require further studies. However, from the results presented here, we can conclude that the biological activity of p53 as a tumour suppressor in skin appears to be independent of its role in mediating TGF- β -induced growth arrest.

Further studies strengthen the importance of senescence as an *in vivo* p53-transactivation-mediated function. Johnson *et al* reported a knock-in mouse model in which the N-terminal 80 amino acids of p53 were replaced with a heterologous transactivation domain of the Herpes Simplex Virus VP16 protein [42]. The resulting protein was transcriptionally active and maintained p53 target gene specificity, but lacked the proline-rich domain and transactivation-independent functions mapped to the amino-terminus. Despite activation of established pro-apoptotic genes, this mouse mutant was deficient in apoptosis induction, yet displayed intact DNA damage- and oncogene-induced cell cycle arrest and senescence. Thus, p53-induced senescence and apoptosis are mediated by separate functional pathways *in vivo* and while senescence induction is strictly dependent on the transactivation function, apoptosis is not [42]. Although it still remains to be tested, this further highlights the potential importance of senescence for tumour suppression *in vivo*.

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