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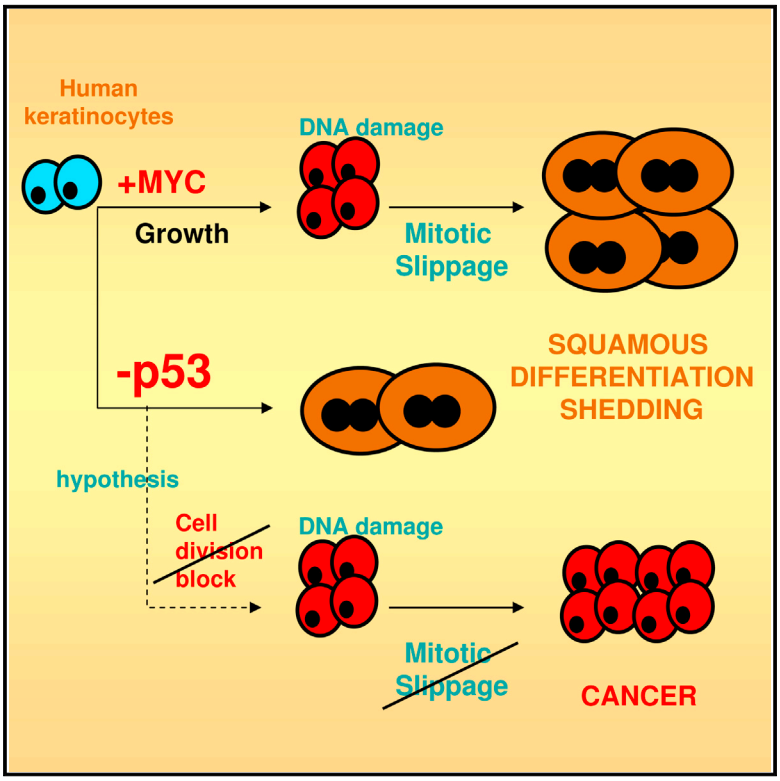
Inactivation of p53 in Human Keratinocytes Leads to Squamous Differentiation and Shedding via Replication Stress and Mitotic Slippage

Graphical Abstract



Highlights

p53 limits the power of the proto-oncogene MYC to drive epidermal differentiation

Loss of p53 causes replication stress and mitotic slippage in human keratinocytes

p53 protects the proliferative potential of the keratinocyte stem cell compartment

Loss or mutation of p53 promotes human squamous differentiation and shedding

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In Brief

The p53 tumor suppressor is frequently inactivated in squamous cell carcinoma, yet loss of p53 does not initiate nonmelanoma skin cancer, suggesting that epithelial skin cells have self-protective mechanisms. Freije et al. show that p53 enhances proliferation and inhibits differentiation in keratinocytes by preventing endoreplication. p53 loss leads to squamous differentiation and expulsion of mutant cells, which may confer the epidermis with a molecular protective response.

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Inactivation of p53 in Human Keratinocytes Leads to Squamous Differentiation and Shedding via Replication Stress and Mitotic Slippage

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SUMMARY

Tumor suppressor p53 is a major cellular guardian of genome integrity, and its inactivation is the most frequent genetic alteration in cancer, rising up to 80% in squamous cell carcinoma (SCC). By adapting the small hairpin RNA (shRNA) technology, we inactivated endogenous p53 in primary epithelial cells from the epidermis of human skin. We show that either loss of endogenous p53 or overexpression of a temperature-sensitive dominant-negative conformation triggers a self-protective differentiation response, resulting in cell stratification and expulsion. These effects follow DNA damage and exit from mitosis without cell division. p53 preserves the proliferative potential of the stem cell compartment and limits the power of proto-oncogene MYC to drive cell cycle stress and differentiation. The results provide insight into the role of p53 in self-renewal homeostasis and help explain why p53 mutations do not initiate skin cancer but increase the likelihood that cancer cells will appear.

INTRODUCTION

Mutations of tumor suppressor *p53* are detected in 50% of human cancer and are associated with poor prognosis (http://p53.free.fr; Petitjean et al., 2007). p53 is a transcription factor with a major role in halting the cell cycle upon DNA damage, allowing DNA repair and chromosomal integrity, its loss of function often causing genomic instability (Di Leonardo et al., 1997; Taylor and Stark, 2001; Aylon and Oren, 2011; Carvajal and Manfredi, 2013). For these reasons, it has been referred to as the "guardian of the genome" (Lane, 1992). p53 upregulates diverse cell cycle inhibitors such as p21CIP1 or the 14-3-3 family. Concomitantly, it induces MDM2, which by a negative loop promotes p53 degradation. Most p53 mutations render it inactive, avoiding degradation and resulting in protein accumulation (Lane, 1992; Aylon and Oren, 2011). Insufficient DNA repair in the presence of p53 can result in cell death via apoptosis (Yonish-Rouach et al., 1991; Roos and Kaina, 2006; Aylon and Oren, 2011).

Squamous cell carcinoma (SCC) is the most frequent human malignancy. SCC of the skin is rapidly increasing worldwide, especially in fair individuals (Rogers et al., 2010; http://seer.cancer.gov). SCC tends to be aggressive and develop metastasis, also in the skin (4%–5%; Karia et al., 2013), and due to its high incidence, the associated mortality is reaching significant numbers. In the United States, SCC of the skin caused 4,000 to 8,000 deaths in 2012 (Karia et al., 2013). The frequency of p53 inactivation is strikingly high in skin SCC (over 80%; Shea et al., 1992; Brash, 2006; http://p53.free.fr). This is mostly due to the mutagenic effects of UV light (Jonason et al., 1996). Understanding the function of p53 in human epidermis provides insight into its role in malignancy.

p53 has been reported to accumulate and mediate apoptosis in the epidermis in response to sunburn (Hall et al., 1993; Ziegler et al., 1994). However, the role of p53 in epidermal homeostasis is not well understood, although it is detected in proliferative cells and downregulated during differentiation (Woodworth et al., 1993; Kallassy et al., 1998; Dazard et al., 2000). Studying this issue has in part been hampered because the skin of *p53* knockout mice appears unaffected (Donehower et al., 1992). These mice develop spontaneous tumors and die mainly of thymic lymphoma by 4–6 months of age. Intriguingly, during this time, they do not develop skin carcinomas. The skin must have powerful self-protective mechanisms, since patches or "columns" of cells containing mutated p53 have been frequently reported in otherwise asymptomatic skin (Jonason et al., 1996; Ren et al., 1997; le Pelletier et al., 2001). These mutations are UV associated and do not lead to SCC, further indicating that disruption of p53 in mouse skin alone is insufficient to drive

skin epithelial cancer. Consistently, the absence of p53 does not increase the number or growth rate of chemically induced benign tumors but accelerates their progression to carcinoma (Kemp et al., 1993). Accordingly, the rare human Li-Fraumeni inherited syndrome, caused by heterozygous autosomal *p53* mutation, involves spontaneous cancer excluding cutaneous carcinomas (Malkin et al., 1990; Srivastava et al., 1990). Similarly, mice overexpressing mutant p53 do not display higher rates of spontaneous skin carcinomas (Lavigueur et al., 1989; Harvey et al., 1995). The mechanisms protecting the skin from loss of p53 remain intriguing (Kemp et al., 1993).

We have previously identified an epidermal checkpoint that responds to deregulated S phase of the cell cycle and DNA damage by triggering terminal squamous differentiation (Gandarillas, 2012). This response involves mitotic bypass or slippage (failure to maintain G2/M arrest, leading to defective exit of mitosis and re-replication in the absence of cell division; Andreassen and Margolis, 1994; Blagosklonny, 2007; Gandarillas, 2012). During mitotic slippage, the nuclei can divide and return to G1 or not, but cells stay in a special G2/M, unable to achieve cytokinesis (Mantel et al., 2008; Zanet et al., 2010; Freije et al., 2012). Overexpression of MYC or cyclin E triggers this mitosis-differentiation checkpoint in human keratinocytes as an oncogene-induced differentiation response (OID; Freije et al., 2012; Gandarillas, 2012).

It has been shown that overfunction of MYC and other oncogenes can cause DNA damage (DNA strand breaks), and this in turn induces p53-mediated apoptosis (Pusapati et al., 2006; Halazonetis et al., 2008). Ectopic cyclin E in human keratinocytes mediates MYC-induced differentiation and causes accumulation of DNA damage (Freije et al., 2012). In addition, MYC induces p53 in keratinocytes as they commit to differentiation (Dazard et al., 2000). Altogether, these results raise the possibility that MYC might promote epidermal differentiation by causing DNA damage, and this response might be mediated by p53. We aimed to investigate this issue by suppressing p53 function in human primary keratinocytes from different individuals, with or without overactivation of MYC, by making use of three different well-characterized small hairpin RNA (shRNA) constructs, a temperature-sensitive mutant, and stratifying organotypic cultures. The results reveal a human squamous differentiation mechanism protecting self-renewal homeostasis against genetic damage.

RESULTS

Replication Stress Initiates MYC-Induced Differentiation of Human Epidermal Keratinocytes

In order to determine whether MYC-induced keratinocyte differentiation is mediated by DNA damage, we generated primary keratinocytes isolated from human skin expressing a conditional MYCER (HKMYCER). This fusion protein renders MYC activatable by 4-OH-tamoxifen (OHT; Littlewood et al., 1995). Activation of MYC by OHT in keratinocytes for 2 days induces the cell cycle (Freije et al., 2012) and for 5 days promotes terminal differentiation and polyploidy (Gandarillas and Watt, 1997; Gandarillas et al., 2000).

We compared the dynamics of DNA replication, p53 induction, and DNA damage after activation of MYCER by OHT. We studied this by measure of bromodeoxyuridine (BrdU) incorporation (DNA synthesis) and phosphorylation on serine 139 of histone H2AX (γH2AX, a marker of DNA strand breaks; Rogakou et al., 1998). Activation of MYC rapidly caused an increase of the proportion of cells undergoing DNA replication (Figure 1A, BrdU 12h). This induced the p53/p21 pathway (Figures 1B, 1C, and S1A), as reported previously (Dazard et al., 2000; Pusapati et al., 2006), accumulation of cells in the G2/M phase of the cell cycle (Figure 1A), and expression of cyclin A and metaphasic phosphohistone H3 (P-H3, strong at condensed chromosomes; Figures 1C and S1B). The rapid activation of the cell cycle shortly preceded an increase of the DNA damage marker γH2AX peaking by 24 hr (Figures 1A and S1A). These changes are consistent with cell cycle deregulation and replication stress leading to DNA strand breaks (Bartkova et al., 2006). By 2 days, a striking increase of nuclear and nucleolar size and binucleation followed (Gandarillas et al., 2000; Figures 1B and S1A). These results show that MYC causes DNA damage prior to increased differentiation in human keratinocytes.

In keratinocytes, a mitosis block by genotoxic agents triggers squamous differentiation (Gandarillas, 2012). To test whether MYC at least in part might enhance differentiation by causing irreparable DNA damage, we treated HKMYCER cells with KU55933 (KU), a specific inhibitor of the DNA-break repair ataxia telangiectasia mutated (ATM) pathway (Hickson et al., 2004). Interestingly, treatment with KU augmented the power of MYC to induce differentiation by 6 days, as measured by involucrin expression (Figure 1D), suggesting that unrepaired DNA damage contributes to MYC-induced keratinocyte differentiation. In order to further test whether accumulation of DNA damage and failure to complete DNA repair triggers a differentiation response, we treated human keratinocytes with NU1025 (NU) a specific inhibitor of the DNA repair poly(ADP-ribose)polymerase (PARP; Griffin et al., 1995). Interestingly, treating keratinocytes with NU caused accumulation of DNA damage and subsequently induced terminal differentiation as monitored by involucrin expression and cell stratification (Figures 1E and S1C). These results provide evidence suggesting that a DNA damage-induced differentiation response might be part of the normal program of keratinocytes. Consistently, keratinocytes heavily labeled for γH2AX were found stratifying into the differentiated layer (Figure S1D; Movie S1).

Inactivation of p53 Potentiates MYC-Induced Differentiation

We aimed to investigate whether p53 mediates MYC-induced keratinocyte differentiation as a response to replication stress. To this end, we adapted shRNA technology for primary keratinocytes and knocked down endogenous p53 in the presence of conditional MYC (MYCER). We have made use of three well-characterized lentiviral constructs bearing p53 shRNA (see Experimental Procedures; Kim et al., 2007; Rodriguez et al., 2011; Sandler et al., 2011; from hereon shP53), one of which (as its vector control) carries a gene for GFP. Figures 2A–2C show the inhibition of endogenous p53 by immunofluorescence, western blotting (protein), and RT-PCR (mRNA). Immunofluorescence analyses showed undetectable levels of p53 in individual GFP-expressing cells shortly after infections (Figure 2A and not shown). shP53 suppressed MYC induction of p53 and its

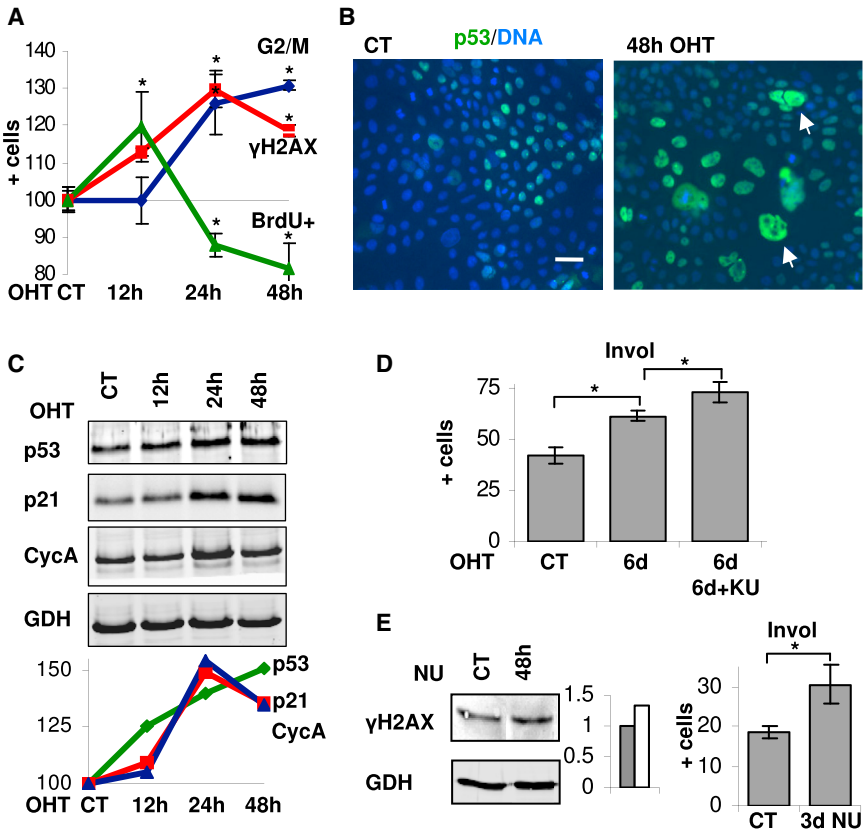

Figure 1. DNA Damage Initiates MYC-Induced Differentiation of Human Epidermal Keratinocytes

(A) Flow-cytometry quantitation of human keratinocytes (HK) expressing conditional MYCER after activation of MYC by OHT for the periods of time indicated with respect to controls with no OHT (CT): percent of cells undergoing DNA synthesis (BrdU+, green), in G2/M (4N, DNA content, blue), or expressing the DNA damage marker γH2AX (red).

(B) Immunostaining for p53 (green) in CT or after activation of MYC for 48 hr. Nuclear DNA by DAPI (blue). Arrows: large nuclei positive for p53. Scale bar, 50 μM. See also Figure S1A.

(C) Expression of the cell cycle regulators indicated in MYCER cells after activation of MYC as indicated. Plots: quantitation of the western blots normalized to the loading control housekeeping protein GAPDH (GDH); CycA, cyclin A. See also Figure S1B.

(D) Flow-cytometry quantitation of the epidermal differentiation marker involucrin (Invol) in MYCER cells untreated or treated with the ATM inhibitor KU55933 (KU) for 6 days, as indicated.

(E) Effects of the inhibition of PARP repair polymerase in HK by treatment with NU1025 (NU) as analyzed by western blot for γH2AX (left) or by flow cytometry for Invol (large bar histogram, right), for the periods indicated. Small bar histogram is a quantitation of the γH2AX western blots normalized to GDH. The Invol positive region was determined by a negative isotype antibody control (CD8). See also Figure S1C.

Error bars are SEM. Data are average of duplicate samples of representative experiments. *$p < 0.05$.

transcriptional target protein, p21CIP1 (p21; Figures 2A–2C). Unexpectedly, the induction of differentiation 5 days after activation of MYC was not attenuated but augmented in the presence of shP53, as this provoked a higher rate of stratification, morphological changes, and expression of the terminal differentiation markers involucrin and keratin K1 (Figures 2D–2G). Activation of MYC and inactivation of p53 therefore cooperated in driving cells toward differentiation. This indicates that in keratinocytes, endogenous p53 does not mediate MYC-induced differentiation but rather restrains it.

Inactivation of p53 in Human Normal Keratinocytes Causes Replication Stress

Enhanced differentiation in HKMYCER after inhibition of p53 suggested that endogenous p53 opposes the action of MYC. To explore the cell cycle mechanisms by which p53 functions, we infected normal primary keratinocytes freshly isolated from human skin with the three shP53 constructs. The shP53 constructs efficiently downregulated p53 even upon treatment with the genotoxic drug doxorubicin (DOXO), which causes acute DNA damage and strongly induces p53 (Figures 3A–3C and S2A). In the absence of DOXO, western blotting and immunofluorescence staining or RT-PCR showed barely detectable levels of the endogenous protein or mRNA, respectively, 2 or 3 days after delivery of the shP53s (Figures 3B–3D and S2A). p53 target p21CIP was also downregulated (Figures 3A, 3B, and S2A), indicating that both p53 protein and function were suppressed. We first tested whether the loss of p53/p21 in human keratinocytes might cause cell cycle deregulation and failure to repair DNA breaks. Inhibition of p53 did not alter significantly the expression of its related factor, p63 (Figure 3A), but provoked a rapid induction of cell cycle markers phospho-Rb, premitotic cyclin A, and mitotic phosphohistone pH3 (Figures 3B, S3A, and S3B). Given the reported capacity of p63 to induce cell cycle and epidermal differentiation molecules (Koster, 2010), it might contribute to the observed effects.

Interestingly, the cell cycle changes caused by shP53 resulted in increased DNA damage as measured by γH2AX foci (Figure 3B, 3D, and 3E). The induction of phospho-Rb and γH2AX after inactivation of p53 was transient (Figures 3B and S5B). Inactivation of p53 in keratinocytes therefore caused cell cycle and replication stress as identified by S phase deregulation and accumulation of DNA strand breaks.

Loss of p53 Provokes Mitotic Slippage and Loss of Proliferative Potential

The early cell cycle activation upon suppression of p53 led to a transient increase of DNA replication (BrdU incorporation; Figures 4A and S2B, 48 hr). Subsequently, the peak of BrdU-positive cells shifted from the diploid to the polyploid range (Figures 4A and S2B), thus indicating that some replicating cells lost the capacity to divide. As a result, the cell cycle profile of the whole

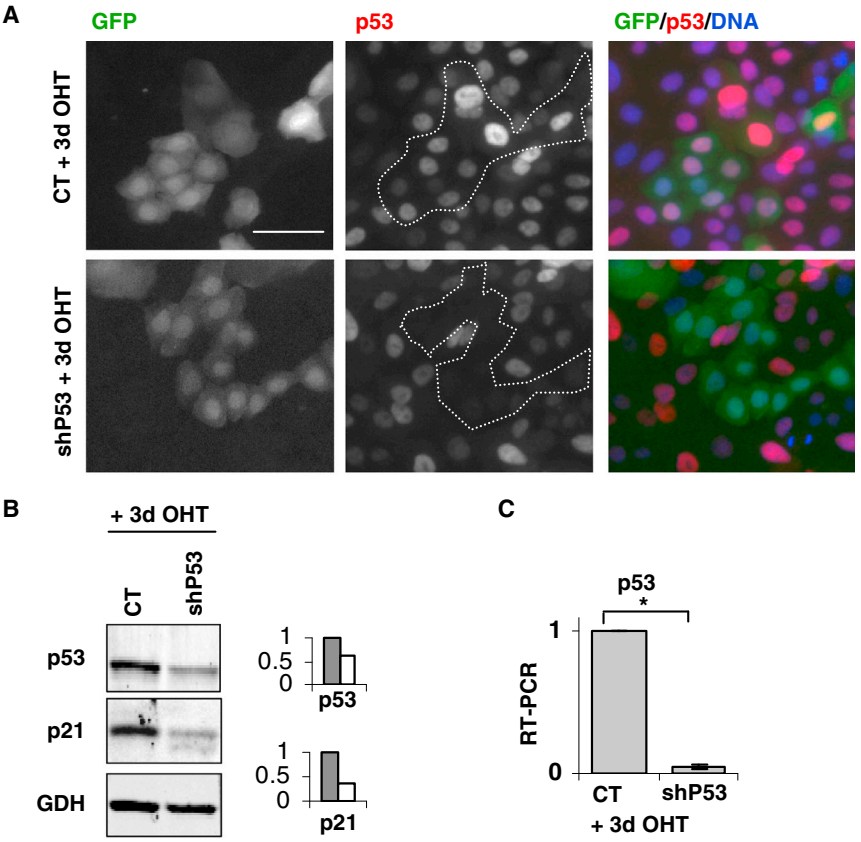

Figure 2. p53 Restrains MYC-Induced Epidermal Differentiation

(A) Expression of GFP (green) and p53 (red) by immunofluorescence of HKMYCER cells infected with control vector CTGFP (CT) or with shP53 as indicated after activation of MYC by addition of OHT for 3 days. Note that within the mixed cultures, p53 is undetected in cells expressing shP53 (green cells, broken line).

(B) Detection of p53 and its target p21 by western blot in CT or shP53 cells treated with OHT for 3 days as indicated. Bar histograms represent quantitation of the western blots normalized to GAPDH (GDH).

(C) Expression of *p53* as measured by RT-PCR in CT or shP53 cells after activation of MYC by OHT for 3 days.

(D) Typical phase contrast and green fluorescence (GFP) of live shP53 cells 4 days postinfection with or without OHT as indicated.

(E) Percent of CT or shP53 GFP cells expressing the terminal differentiation marker keratin K1 after addition of OHT for 5 days, as determined by immunofluorescence.

(F) Immunofluorescence for involucrin (green) of CT or shP53 cells, 5 days after addition of OHT. Nuclear DNA by DAPI (blue).

(G) Percent of CT or shP53 cells expressing involucrin (Invol) after addition of OHT for 5 days, as determined by flow-cytometry.

Error bars are SEM of duplicate samples of representative experiments. *$p < 0.05$. Scale bar, 50 μm.

population decreased in G1/diploidy and increased in polyploidy (with frequent multinucleation), while the G2/M fraction remained basically unchanged (Figures 4B, S2B, and S2C). All in all, we found no great increase of S phase or proliferative mitosis 3 days after inhibition of p53, but a cell cycle shift toward polyploidy as a result of mitotic bypass or mitotic slippage shP53 (re-replication or endoreplication in the absence of cell division; Andreassen and Margolis, 1994; red arrow in Figure 4B and Figure S2B). Consistently, there was a transient accumulation of mitotic markers cyclin A and cyclin B and the metaphase marker P-H3 (Figures 3B, 4C, S3A, and S3B; Movies S2, S4, S5, and S6), an increase of cells expressing postmitotic keratin K1 (Figure 4C), and an accumulation of cyclin E that typically associates with keratinocyte mitotic slippage (Figures 3B and S3C; Zanet et al., 2010; Freije et al., 2012). Concomitantly, a high frequency of cells displayed striking accumulation of cytoplasmic cyclin A or B (Figures 4C and S3A; Movie S2). Inhibition of shP53 induced a shorter form of cyclin A (Figure 3B), likely inactive (Kaufmann et al., 2001; Wang et al., 2012), even more upon overactivation of MYC (Figure S3D). Timely degradation of cyclins A and B is required for exit from mitosis, and their cytoplasmic accumulation is likely to associate with defective mitosis (Murray, 2004; Zanet et al., 2010; Rosa-Garrido et al., 2012).

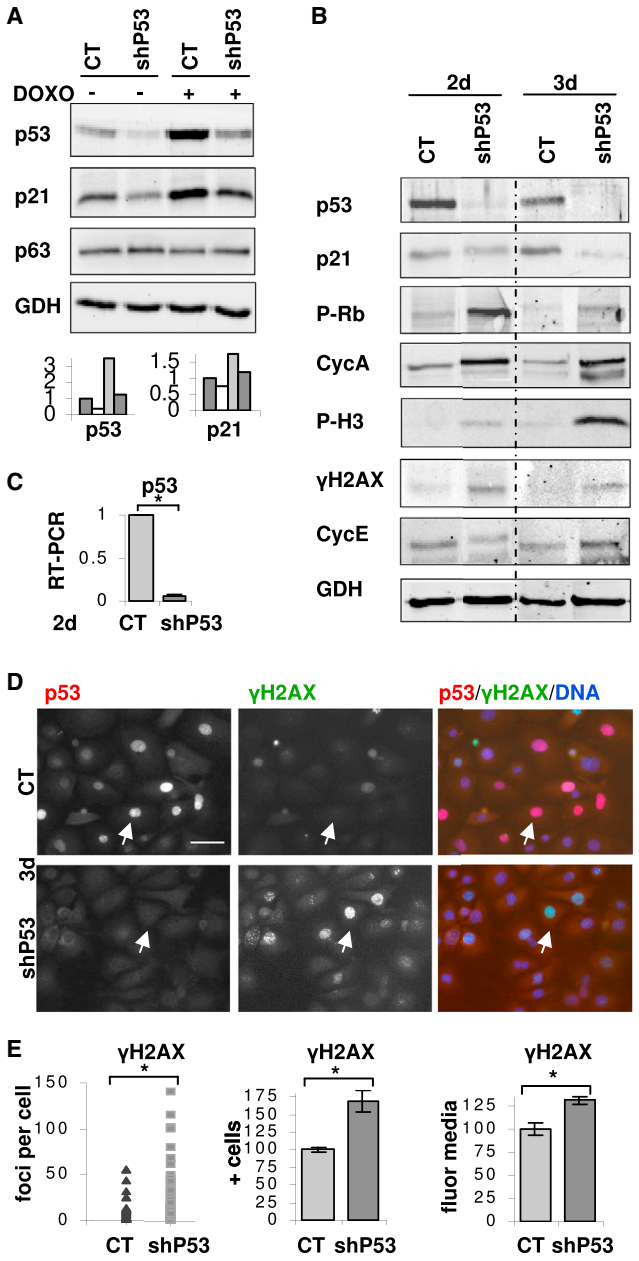

Figure 3. Disruption of Endogenous p53 in Human Keratinocytes Causes Replication Stress

(A) Expression of p53, its target p21, and p63 in HK infected with CTGFP (CT) or with shP53 as indicated, untreated or treated with the genotoxic drug doxorubicin (DOXO) for 24 hr. Bar histogram represents quantitation of western blots normalized to GAPDH (GDH).

(B) Western blot for the cell cycle regulators indicated in CT or shP53 cells, 2 or 3 days postinfection; Cyc, cyclin. See also Figure S2A.

(C) Bar histogram shows expression of *p53* in CT or shP53 cells as measured by RT-PCR 2 days postinfection. See also Figure S2A.

(D) Double immunostaining for p53 (red) and γH2AX (green) 3 days postinfection. Note that cells with low p53 tend to accumulate DNA damage (arrows) and vice versa. Nuclear DNA by DAPI (blue). See also Figure S5. Scale bar, 50 μm.

(E) Distribution histogram shows number of foci of γH2AX per nucleus, as scored by immunofluorescence as in Figure 3D and image analyses. Left bar histogram shows percentage of positive γH2AX cells as quantitated by flow cytometry. Right bar histogram shows the mean γH2AX intensity per cell as measured by flow cytometry in the whole population.

Error bars are SEM of duplicate samples of representative experiments. *$p < 0.05$.

As a consequence of failure to exit cytokinetic mitosis, loss of p53 did not result in increased proliferation but did result in a significant loss of clonogenic potential (Figure 4D). Cells expressing shP53 were impaired especially in the generation of large colonies that are founded by the great potential of stem cells (Jones and Watt, 1993). The loss of clonogenic potential by inactivating p53 did not involve detectable apoptosis (absence of a sub-G1 DNA peak; Figure S2B). Accumulation of mitotic markers and metaphase figures, loss of the capacity to divide, polyploidy, BrdU incorporation by polyploid cells, and multinucleation altogether indicate that loss of p53 in keratinocytes leads to mitotic slippage, by which cells enter mitosis but fail to divide (see also below).

Loss of p53 Promotes Squamous Differentiation

Mitosis failure in keratinocytes triggers terminal differentiation, and cell cycle stress triggers this mitosis-differentiation checkpoint (Zanet et al., 2010; Freije et al., 2012; Gandarillas, 2012). We explored whether loss of p53 triggered this response, thus explaining the observed loss of clonogenic potential. As shown in Figures 5A–5C and S4, inhibition of p53 strikingly induced the squamous differentiation markers involucrin, keratin K1 (see also Figure 4C), keratin K10, and filaggrin as well as cell-size increase and morphological changes typical of terminal differentiation. As a consequence, cells with low levels of p53 were found to stratify and detach at a higher rate than control cells in confocal analyses (Figure S5A; Movie S3). The time-lapse videos also showed that HKshP53 stressed and accumulated in mitosis attempting to divide (Movies S4, S5, and S6). To better study the effect on differentiation, we placed cells in a high-calcium medium that allows stratification closely resembling the process in human epidermis (Rheinwald, 1989). Cells expressing the GFP marker displayed very low levels of p53, stratified, and detached (Movies S3 and S7). Shed cells were counted in the culture medium showing that inactivation of p53 produced a higher rate of desquamation (Figure 5D). Consistently, within unselected mixed cultures, 5–6 days after delivery of shP53, cells with detectable levels of p53 and low levels of γH2AX had repopulated the basal proliferative layer (Figure S5B). This further indicates that cells expressing p53 are more proliferative. Maintaining the proliferative potential might be a keratinocyte stem cell p53 function, since in parallel experiments, inhibition of p53 led to increased proliferation and impaired senescence in pluripotent mesenchymal stem cells (Figure S6).

Inactivation of a Temperature-Sensitive p53 Mutant Protein Promotes Differentiation, whereas Its Activation Attenuates It

Often, in skin tumors and in patients with Li-Fraumeni syndrome, mutated *p53* coexists with the normal allele. In this context, inactive p53 mutants can have a dominant-negative effect by disrupting the function of the endogenous protein (Hann and

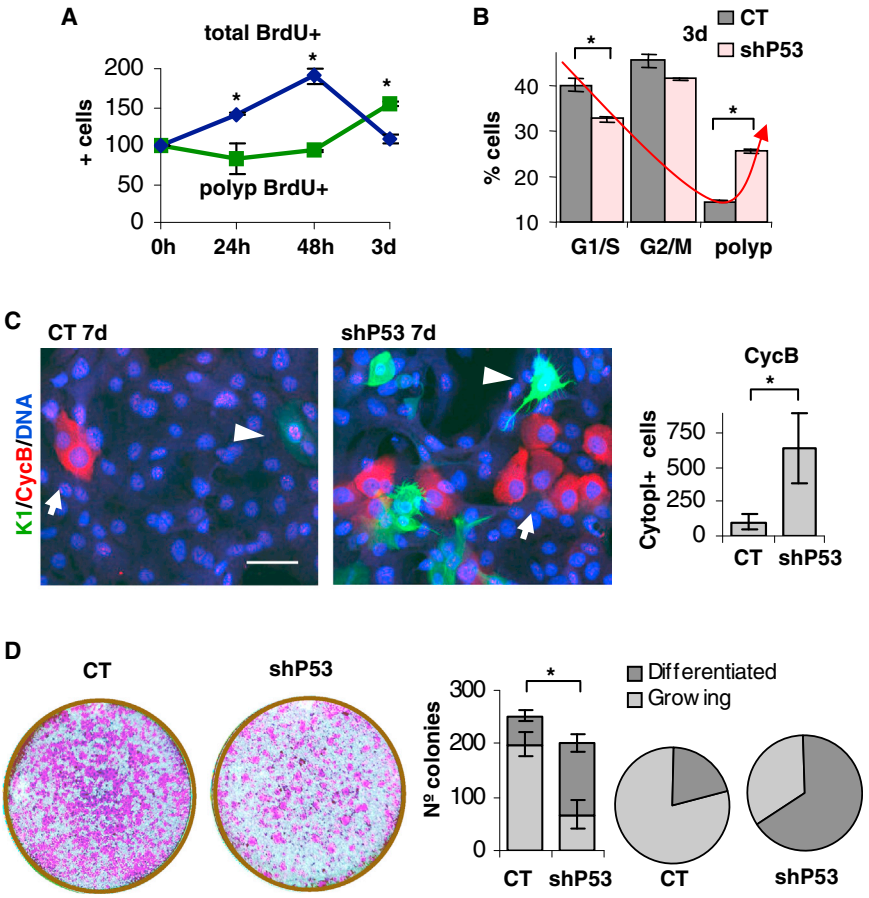

Figure 4. Disruption of Endogenous p53 in Human Keratinocytes Causes Mitotic Slippage and Loss of Clonogenic Potential

(A) Percent of total or polyploid (polyp) HK infected with shP53 undergoing DNA synthesis (BrdU+) for the periods of time postinfection indicated, as quantitated by flow cytometry and normalized to HK infected with CTGFP (CT). See also Figure S2B.

(B) Percent of cells in the G1/S (2N), G2/M (4N), or polyp phases of the cell cycle in CT (gray) or shP53 (light red) cells 3 days postinfection, as determined by flow cytometry. Red arrow shows the shift of shP53 cells toward polyploidy, a consequence of mitotic slippage. See also Figures S2B and S2C.

(C) Double immunostaining for K1 (green, arrow heads) and mitotic cyclin B (CycB, red; arrows). Nuclear DNA by DAPI (blue). shP53 cells displayed frequent accumulation of cytoplasmic cyclin B (cytopl+), and scoring is shown in the bar histogram. See also Movie S2.

(D) Clonogenic capacity of CT or shP53 cells (10,000 cells plated 3 days postinfection). Bar and circle histograms show the number (bars) and percent (circles) of actively shP53 growing colonies (light gray) versus small abortive, differentiated colonies (dark gray).

Error bars are SEM of duplicate (A–C) or triplicate (D) samples of representative experiments. *p < 0.05.

Lane, 1995; Blagosklonny, 2000; Sigal and Rotter, 2000; Lee et al., 2012). To mimic this condition, we have overexpressed in epidermal keratinocytes (HKp53ts) a well-characterized mutant form of p53 (p53ts), whose conformation state can be regulated by temperature (Michalovitz et al., 1990). At 32°C, p53ts displays the wild-type conformation, whereas at 39°C, it adopts an inactive conformation and can thus behave as a dominant-negative mutant. It is well established that active p53 is rapidly degraded due to an autoregulatory feedback loop with MDM2, whereas the inactive protein stabilizes and accumulates (Haupt et al., 1997). Consistently, HKp53ts expressed higher levels of p53 at 32°C than control cells, but the protein accumulated further at 39°C as it adopted the inactive conformation (antigen pab240 positive; Figures 6A, 6B, and S7A; Gannon et al., 1990; Michalovitz et al., 1990). In addition, while p53 was absent from suprabasal HKp53ts at 32°C, as the endogenous protein, it accumulated in suprabasal involucrin-positive cells at 39°C (Figure 6B and S7A). This further indicated that the protein was inactivated at 39°C.

Inactivation of p53ts in human keratinocytes by the 39°C temperature switch resulted in increased differentiation monitored as high light scatter and expression of the suprabasal markers involucrin and keratin K16 (Figures 6B, 6C, and S7B, and S7C). Conversely, inducing the active conformation of p53ts at 32°C retained keratinocytes in the proliferative compartment as determined by an increased proportion of cells with basal-like low-scatter parameters and decreased expression of the suprabasal markers (Figures 6B, 6C, S7B, and S7C). Loss or gain of cell cycle control by inactivation or activation of p53 was reflected in the increase or decrease of hyperproliferative suprabasal K16 at 39°C or 32°C, respectively. As observed after suppressing endogenous p53 by shRNA, the inactive form of p53ts caused binucleation, increased nuclear size and γH2AX-associated DNA damage (Figures 6B and S7A, S7D, and S7E; arrows), and reduced the clonogenic potential of the stem cell compartment once cells were returned to 37°C (Figure 6D). No additional gains of function were observed.

DISCUSSION

Role of p53 in Human Keratinocytes

Altogether, our results demonstrate a human epidermal cell response to loss of p53 function consistent with an oncogene-induced differentiation checkpoint (OID). Keratinocytes lacking p53 differentiated, as determined by the expression of various differentiation markers, morphological changes, and loss of proliferative capacity. This process was preceded by mitosis defects, suggesting that endogenous p53 interferes with the initiation of epidermal differentiation by retaining cells within the proliferative compartment. p53 might cause this by two mechanisms (Figure 7): (1) by holding cells in the G1 and G2/M phases of the cell cycle and ensuring efficient DNA repair, or (2) by opposing cyclin E function. Interestingly, p53 has been proposed

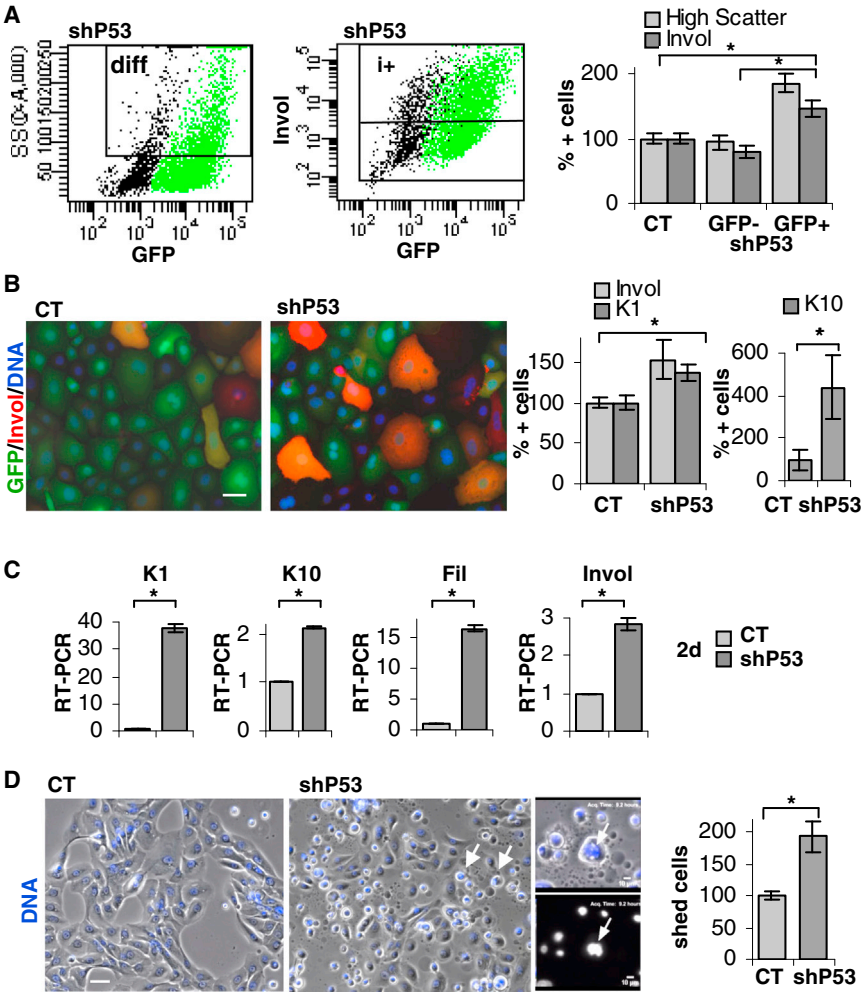

Figure 5. Loss of Endogenous p53 in Human Keratinocytes Triggers Terminal Differentiation, Stratification, and Shedding

(A) Dot plots show flow-cytometry analyses for GFP (green) versus light scatter (SSC-A; left) or involucrin (invol, right) of HK infected with shP53. Bar histograms show percent of differentiating (high light scatter; diff. in dot plot) or Invol positive (i+ in dot plot) cells in the GFP (+) or non-GFP (–) populations, as indicated. CT, cells infected with control vector. See also Figure S4A.

(B) Double immunostaining for GFP (green) and Invol (red) 5 days postinfection of HK with CTGFP (CT) or shP53 as indicated; nuclear DNA by DAPI (blue). Bar histograms show percentage of cells positive for Invol and K1 (flow cytometry), or K10 (immunofluorescence) in shP53 cells relative to CT cells. Scale bar, 50 μm. See also Figure S4.

(C) Expression of differentiation markers *K1*, *keratin K10*, *filaggrin* (Fil), and *Invol*, as determined by RT-PCR in shP53 cells relative to CT cells 2 days postinfections.

(D) Snap frames from Movies S4 and S6 showing the accumulation of shP53 cells in mitosis (arrows) 5 days postinfection. Nuclear DNA by NucBlue (blue). Amplified insets show a cell blocked in metaphase (bottom panel: DNA only). See also Movies S4, S5, and S6. Bar histogram shows percent of shP53 cells shed into the medium, relative to control cells.

Error bars are SEM of duplicate samples of representative experiments. *$p < 0.05$. Scale bars represent 50 μm (B and D) or 10 μm (small insets in D).

to constitute a barrier against hyperactivation of cyclin E/cdk2 (Minella et al., 2002), and cyclin E mediates MYC-induced keratinocyte cell cycle stress and differentiation (Freije et al., 2012). Loss of p53 may thus allow cyclin E/cdk2 (ergo MYC) full power to trigger the differentiation response. This would mitigate the cell cycle stress/OID response and the cell division block that triggers squamous differentiation (Zanet et al., 2010; Freije et al., 2012; Gandarillas, 2012).

The results provide insight into the enigmatic role of p53 in epidermal homeostasis with implications in stem cell regulation. First, they show that p53 is dispensable for the epidermal differentiation signal. Second, by promoting the correct timing of the cell cycle checkpoints and avoiding mitotic slippage, p53 appears to protect the proliferative capacity of epidermal stem cells (Figure 7). Consistent with this model, p53 is downregulated as epidermal differentiation initiates (see Dazard et al., 2000 and references therein), and its overexpression inhibited growth and squamous differentiation markers in cell-reconstituted epidermis (Woodworth et al., 1993). p53 was also dispensable for the differentiation signal caused by overactivation of MYC. This signal might be triggered in cells accumulating irreparable DNA damage (strand breaks) and escaping the protective role of p53 (Figure 7), since it was enhanced by inhibition of the DNA repair ATM pathway. Interestingly, the differentiation signal was also enhanced in normal keratinocytes by inhibition of the DNA repair poly-(ADP-ribose)-polymerase.

Protecting the proliferative potential of stem cells from differentiation might be a keratinocyte function of p53, since in other cell types, it promotes apoptosis, senescence, or even differentiation. In this study, shP53 prolonged the lifespan of human pluripotent mesenchymal stem cells. As mentioned above, we have shown that MYC in keratinocytes promotes differentiation, while in other cells it promotes proliferation. As we have suggested for MYC (Gandarillas, 2012; Muñoz-Alonso et al., 2012), the cell-growth deregulation that follows p53 loss might trigger differentiation in cells where this process is associated with cellular growth. Paradoxical inhibition of senescence by p53 has also been reported (Demidenko et al., 2010). Conversely, p53 has been shown to induce apoptosis in keratinocytes after acute damage caused by sunburn (Ziegler et al., 1994). p53 might thus play a dual role in epidermal survival by keeping proliferative cells "healthy" and inducing cell death of severely damaged cells.

Implications into Skin Homeostasis and Cancer

Our fluorescent confocal and video analyses on mixed (GFP and no-GFP cells) stratifying cultures showed that

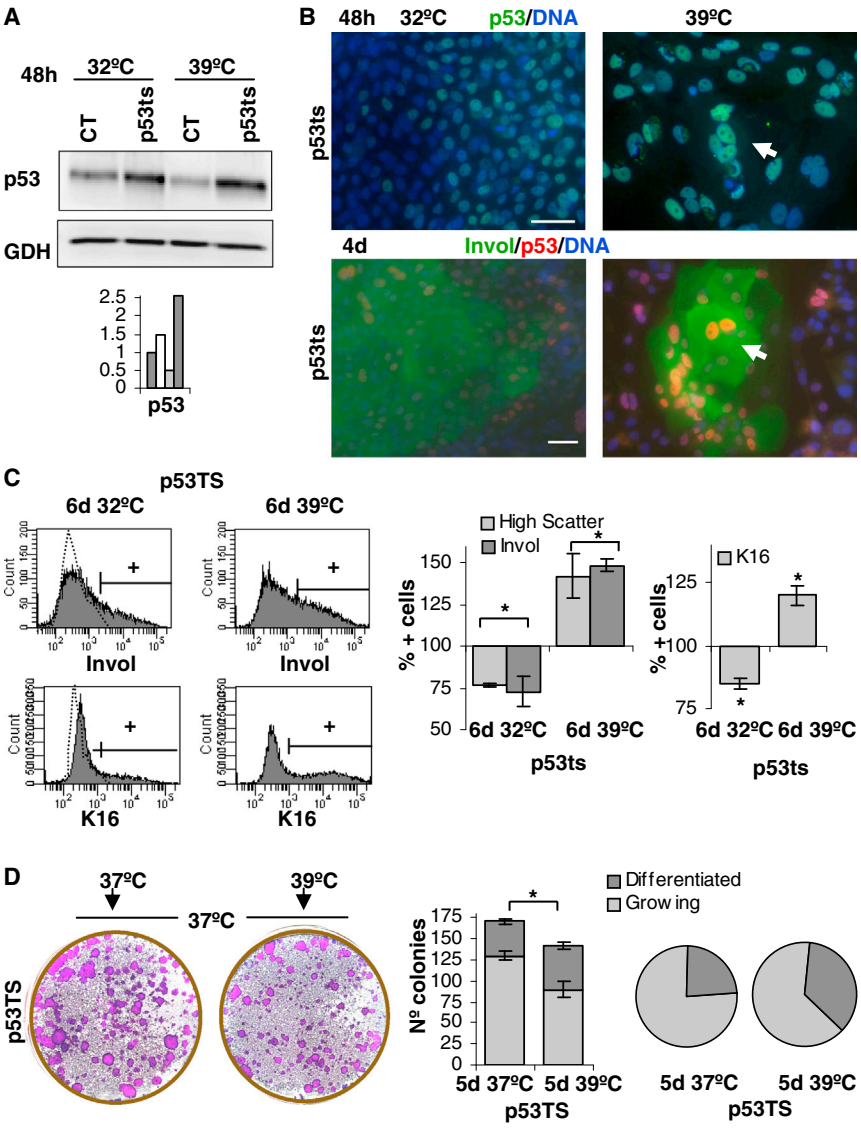

Figure 6. A Mutant Inactive Conformation of p53 Induces Keratinocyte Differentiation and Reduces the Great Proliferative Potential Cell Compartment

(A) Detection of p53 by western blotting in keratinocytes expressing control vector (CT) or the temperature-sensitive mutant p53 (p53ts) 48 hr after the temperature switch to 32°C or 39°C as indicated. Bar histogram show quantitation of p53 western blots normalized to GAPDH (GDH). Note that p53 accumulates at 39°C (inactive conformation).

(B) Top: immunofluorescence for p53 (green) on p53ts cells as in (A); Bottom: double immunofluorescence for involucrin (Invol, green) and p53 (red) on p53ts cells 4 days after the temperature switch. Note the binucleate differentiating p53ts cells that accumulate p53 at 39°C (arrows). Nuclear DNA by DAPI (blue). See also Figure S7A. Scale bar, 50 μm.

(C) Representative flow cytometry for the expression of Invol (top) or the hyperproliferative marker keratin K16 (bottom) in p53ts cells 6 days after the temperature switch, as indicated. Bar histograms show percent of morphologically differentiated (high scatter) cells or cells positive for Invol or K16 as indicated, normalized to CT cells; quantitation of positive cells by the gates indicated on the histograms (+), as determined by a negative isotype antibody control (dotted line; CD8). See also Figure S7B.

(D) Clonogenic capacity of p53ts cells plated 5 days after the 39°C temperature switch. A total of 2,500 proliferative cells were plated. Bar and circle histogram show number (bars) or percent (circles) of actively shP53 growing colonies (light gray) versus small abortive, differentiated colonies (dark gray).

Error bars are SEM of duplicate (C) or triplicate (D) samples of representative experiments. *p < 0.05.

p53-defective keratinocytes had a higher tendency to stratify and detach than the normal cells. It is intriguing that the absence of p53 did not cause apparent physiological defects in mouse skin in vivo (Donehower et al., 1992; Martínez-Cruz et al., 2008). This, in part, might be due to compensatory mechanisms. Also, the in vivo niche might rescue the proliferative capacity of stem cells. For instance, lack of MYC renders mouse keratinocytes unable to proliferate in vitro, but they can form an epidermis in vivo (Zanet et al., 2005). Interestingly, however, the specific ablation of p53 in mouse epidermis caused mitotic defects (Martínez-Cruz et al., 2009). In addition, our model for mitosis-induced differentiation is that keratinocytes undergoing a prolonged arrest in mitosis are pushed to stratify and differentiate by other more proliferative and more adherent neighbor cells (Gandarillas, 2012). This might thus involve competition phenomena. Within these lines, it is interesting that in mouse epidermis, p53 mutant clones are growth limited by normal adjacent clones unless the skin is UV irradiated (Zhang et al., 2001).

Altogether, our results have implications into the mechanisms leading to epithelial skin cancer (mainly SCC), in which *p53* is frequently mutated. Disruption of p53, however, does not seem to be an initiating event in this malignancy, since mice lacking p53 die around 4–6 months, mainly of thymic lymphoma, and do not develop early skin tumors (Donehower et al., 1992). Consistently, epidermal-specific *p53* knockout mice develop spontaneous tumors from 5 months onward (Martínez-Cruz et al., 2008). This is also consistent with humans usually developing skin carcinomas at an old age. In addition, *p53* knockout mice are not more susceptible to chemically induced skin carcinogenesis (Donehower et al., 1992; Kemp et al., 1993). Within similar lines, Li-Fraumeni patients having one mutant *p53* allele do not display a higher rate of skin carcinomas (Malkin et al., 1990; Srivastava et al., 1990). Altogether, these observations suggest that the epidermis has mechanisms to be protected from *p53* mutations. This is further supported by the well-documented presence of frequent clones or "columns" of p53 mutant cells in

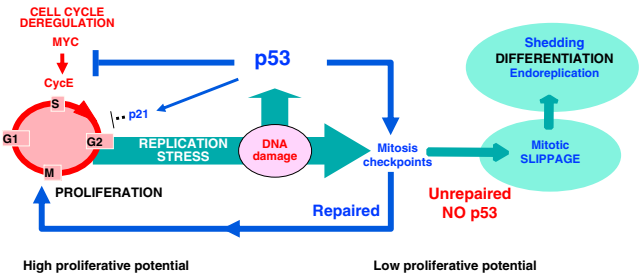

Figure 7. Model for the Role of p53 in Proliferation and Differentiation of Human Epidermal Keratinocytes

p53 would hold cells, allowing DNA repair. Cells with successful repair would re-enter the proliferative compartment, and cells with irreparable levels of DNA damage would undergo mitosis slippage, terminal differentiation, stratification, and shedding. p53 would protect the proliferative potential of stem cells (left), and its loss of function would lead to loss of proliferative potential (right).

sun-exposed asymptomatic skin that do not seem to lead to cancer (Jonason et al., 1996; Ren et al., 1997; le Pelletier et al., 2001). Our results herein provide a mechanism explaining these observations. Loss of cells with inactive p53, by stratification and shedding due to unrepaired DNA damage, may explain how the skin protects itself from mutations hitting this tumor suppressor. Precancerous keratinocytes would be removed via squamous differentiation, therefore avoiding clinical impact.

Although loss of p53 does not sensitize the skin to early tumorigenesis, it accelerates cancer progression (Kemp et al., 1993; Bornachea et al., 2012). Interestingly, genomic instability appears to contribute to the spontaneous tumors developed in *p53* knockout mouse epidermis (Martínez-Cruz et al., 2009). In addition, mouse skin deficient in p53 or overexpressing a mutant form develops tumors more frequently when UV irradiated (Li et al., 1995; Jiang et al., 1999). UV causes keratinocytes to arrest in G2 (Pavey et al., 2001). Our results support a model that might reconcile these observations. In our experiments, inhibition of p53 impaired efficient G2 arrest, resulting in mitotic slippage and terminal differentiation, thereby suppressing cell division (Figure 7). Additional alterations cooperating with inactivation of p53 might hit the control of mitosis and bypass the proliferative block. This would allow p53-mutated cells to escape terminal differentiation and expulsion and divide in spite of a high genomic instability context caused by genetic damage, thus promoting malignant clones to appear.

EXPERIMENTAL PROCEDURES

Cell Culture, Plasmids, Viral Infections, and Treatments

Ethical permission for this study was requested, approved, and obtained from the Ethical Committee for Clinical Research of Cantabria Council, Spain. In all cases, human tissue material discarded after surgery was obtained with written consent presented by clinicians to the patients, and it was treated anonymously.

Primary keratinocytes were isolated from neonatal human foreskin and cultured in the presence of a mouse fibroblast feeder layer (inactivated by mitomycin C) in Rheinwald FAD medium as described previously (10% serum and 1.2 mM Ca^{+2}; Rheinwald, 1989; Gandarillas and Watt, 1997). Low passages (one to five) of keratinocytes from four different individuals were utilized.

For gene delivery in primary keratinocytes the following viral constructs driven by viral constitutive promoters were used. Retroviral constructs were pBabe empty vector, pBabe-MYCER (Littlewood et al., 1995; Gandarillas and Watt, 1997; MYC fusion protein with the ligand binding domain of a mutant estrogen receptor that responds to 4-OH-hydroxytamoxifen; OHT), or pBabe-p53Val135 (p53ts; kindly provided by M. Oren, Weizmann Institute, Revohot, Israel) carrying a mutant p53 whose conformation can be regulated by a temperature shift (wild-type at 32°C, inactive at 39°C; Michalovitz et al., 1990). All pBabe constructs carried puromycin resistance and were delivered by cell infection, and cells were selected as described previously (Freije et al., 2012). Lentiviral constructs were control pLKO1 (CT; Sigma-Aldrich), control GFP pLVTHM (CTGFP), and three constructs expressing shRNA specific against p53: the GFP expressing vector pLVUH-shp53 (shP53) and two non-GFP constructs pLKO1-p53-shRNA-427 (shP53-427; Addgene; Kim et al., 2007) and pLKO1-p53-shRNA-941 (shP53-941; Addgene; Kim et al., 2007). More details can be found in Supplemental Experimental Procedures.

For delivery of shRNA expressing constructs in primary human keratinocytes, two alternative optimized methods of infection were used giving "low" (25%–35%) or "high" (80%–90%) efficiency. Cells were then placed on low calcium or high calcium media depending on the experiment. See Supplemental Experimental Procedures for method details.

MYCER was activated by addition of 100 nM OHT (Sigma-Aldrich) to the culture medium for the periods of time indicated. Keratinocytes were grown at 37°C and 5% CO_2 except for induction of active or inactive conformations of p53ts, when the temperature was shifted to 32°C and 39°C, respectively, for the periods of time indicated.

Primary keratinocytes were treated for 6 days with the ATM inhibitor KU55933 (10 μM; Tocris Bioscience) or with the PARP inhibitor NU1025 (200 μM; Millipore) for 24 or 72 hr. Parallel control cultures were always subjected to the DMSO vehicle only.

For clonogenicity assays, 15,000 keratinocytes grown in low-calcium medium or 2,500 grown in high-calcium FAD medium were plated per T6 well triplicates and cultured in FAD medium. About 10 days later, the cultures were stained with rhodanile blue as described previously (Jones and Watt, 1993). This dye colors keratinocytes pink (darker the more differentiated) and feeder fibroblasts purple.

Cell Shedding

Quantitation of cell shedding was measured by counting cells detached into the culture medium. Data were obtained from duplicate samples and normalized to controls.

Antibodies

Primary and secondary antibodies utilized in this study are listed in Supplemental Experimental Procedures.

Flow Cytometry

Keratinocytes were harvested, fixed, and stained for BrdU and DNA (propidium iodide [PI]) or for involucrin and analyzed by flow cytometry as described in Supplemental Experimental Procedures. All antibody stainings were controlled by the use of a similar concentration of isotype-negative immunoglobulins (mouse anti-CD8 or rabbit serum). A total of 10,000 events were gated and acquired in list mode for every sample except for some GFP-positive analyses, when 50,000 cells were acquired. In order to gate out cell aggregates, the area of the fluorescent pulse of PI (DNA content; PE-A) was plotted versus the width of the fluorescent pulse (PE-W).

Immunofluorescence and Confocal Microscopy

Keratinocytes were grown on glass coverslips, fixed and stained as previously described (Freije et al., 2012). After washing with PBS, coverslips were stained with 0.1 μg/ml DAPI, mounted with ProLong Gold Antifade Reagent (Life Technologies), and visualized and photographed under AxioVision Zeiss fluorescent microscopy. z stack 3D digital images were reconstructed after frame collection by confocal microscopy (Nikon A1R, 20× numerical aperture [NA] 0.75) and processed by NIS Elements software (AR, 3.2 64 bits; Nikon) as indicated in the supplemental movie legends.

Whole-Cell Extracts and Western Blotting

Cells were washed twice with PBS and incubated for 30 min on ice in lysis buffer as described previously (Freije et al., 2012). Supernatant concentrations were determined using the Qubit protein assay kit (Life Technologies). For western blot analysis, 80 μg of cell extracts was separated by SDS-PAGE (10% or 12%) and transferred to nitrocellulose membranes. After blocking and incubating with the primary antibodies, the membranes were subjected to secondary antibodies and subjected to enhanced chemiluminescence substrate (SuperSignalTM West Femto, Pierce), following the supplier's protocol, and analyzed with an Odyssey scanner (Li-Cor) or C-Digit scanner (Li-Cor).

RT-PCR

Total RNA was isolated using NucleoSpin RNA (Macherey-Nagel) according to the manufacturer's instructions. A total of 1 μg of total RNA was reverse transcribed with the iScript cDNA synthesis kit (Bio-Rad) in a 20 μl reaction for 30 min at 42°C. The cDNAs (1 μl) were amplified by real-time PCR (Bio-Rad iQ SYBR green supermix) and normalized to β-actin mRNA levels. Primers utilized in this study are listed in Supplemental Experimental Procedures.

Time-Lapse Videos

For Movies S4, S5, and S6, keratinocytes 4 days postinfection with a control (CT) or non-GFP-expressing shP53 construct (shP53-427) were treated with NucBlue Live ReadyProbes Reagent (Life Technologies) and filmed by time-lapse imaging for 24 hr, photographed every 7 min. For Movie S7, keratinocytes 5 days postinfection with shP53-GFP construct were filmed by time-lapse imaging for 11 hr, photographed every 10 min. In all cases, phase-contrast and green- or blue-fluorescence images were obtained by an epifluorescence microscope (NIKON Ti; 10× NA 0.30) by an ORCA R2 camera.

Statistical Analyses

Data sets were compared using an unpaired Student's t test. A p value of less than 0.05 was considered statistically significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and seven movies and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.10.012.

AUTHOR CONTRIBUTIONS

A.G. conceived the project. A.G. and A.F. designed the study and wrote the paper. A.F., R.M., L.C., M.C., and P.A. performed assays. P.M., R.R., D.A., and E.D.D. provided materials and technical input and made critical suggestions to the paper.

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