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ARTICLE

p21CIP1 controls the squamous differentiation response to replication stress

Isabel de Pedro[1] · Jesús Galán-Vidal[1] · Ana Freije[1] · Ernesto de Diego[1,2] · Alberto Gandarillas[1,3]

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Abstract

The control of cell fate is critical to homeostasis and cancer. Cell cycle cdk inhibitor p21CIP1 has a central and paradoxical role in the regulatory crossroads leading to senescence, apoptosis, or differentiation. p21 is an essential target of tumor suppressor p53, but it also is regulated independently. In squamous self-renewal epithelia continuously exposed to mutagenesis, p21 controls cell fate by mechanisms still intriguing. We previously identified a novel epidermoid DNA damage-differentiation response. We here show that p21 intervenes in the mitosis block that is required for the squamous differentiation response to cell cycle deregulation and replication stress. The inactivation of endogenous p21 in human primary keratinocytes alleviated the differentiation response to oncogenic loss of p53 or overexpression of the DNA replication major regulator Cyclin E. The bypass of p21-induced mitotic block involving upregulation of Cyclin B allowed DNA damaged cells to escape differentiation and continue to proliferate. In addition, loss of p21 drove keratinocytes from differentiation to apoptosis upon moderate UV irradiation. The results show that p21 is required to drive keratinocytes towards differentiation in response to genomic stress and shed light into its dual and paradoxical role in carcinogenesis.

Introduction

p21CIP1 (p21) is a central regulator of cell growth. It is a subject of great attention because its function lies at a crossroads between cell cycle arrest, senescence, differentiation, and cancer [1, 2]. Consistently, p21 is deregulated in a large variety of neoplasms. However, a dual positive or negative role has been proposed in carcinogenesis in part due to its proven capacity to inhibit growth, to protect cells from apoptosis and to cause genomic instability [3–5]. p21 is a transcriptional target of tumor suppressor p53, the guardian of the genome that induces cell cycle arrest or apoptosis upon DNA damage [6]. In turn, p21 is a key regulator of cyclin dependent kinases (cdks) [2]. By inhibiting S phase Cdk2, p21 is able to impose cell cycle arrest in the G1/S transition that initiates DNA replication. By inhibiting mitotic Cdk1, it delays the G2 phase to allow DNA repair prior to cell division [5, 7]. However, p21 is known to be regulated also independently of p53 and to elicit cell cycle independent functions, notably by binding to DNA replication or DNA repair proteins [8–10].

p21 has been involved in regulating differentiation in a variety of systems where it is usually upregulated post mitotically. A pivotal homeostatic role has been proposed for p21 in the control of cell fate and cell renewal in the epidermis [11]. However, the role of p21 both in normal keratinocytes and in squamous carcinomas is paradoxical. While p21 is induced during squamous terminal differentiation in epidermis and oral mucosa [12–14], its overexpression inhibited mouse keratinocyte differentiation [15]. Conversely, although silencing of the p21 gene in mouse enhanced keratinocyte proliferation, it also induced terminal differentiation [16]. Also paradoxically, there is not clear correlation between loss of p21 and aggressiveness of epidermoid carcinomas and in some studies it is upregulated

These authors contributed equally: Isabel de Pedro, Jesús Galán-Vidal

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✉ Alberto Gandarillas
agandarillas@idival.org

[1] Cell Cycle, Stem Cell Fate and Cancer Laboratory, Institute for Research Marqués de Valdecilla (IDIVAL), 39011 Santander, Spain

[2] Paediatric Surgery, Hospital Universitario Marqués de Valdecilla, 39008 Santander, Spain

[3] INSERM, Languedoc-Roussillon, 34394 Montpellier, France

in aggressive lesions [1, 17]. The loss of p21 is reported as a marker of either good or poor prognosis [4]. Therefore, although there is consensus that p21 plays an important role in squamous homeostasis and carcinogenesis, its function is intriguing.

Epidermis is continuously self-renewed and continuously exposed to the genotoxic effects of ultraviolet light (UV). In addition, replication stress is a result of cell cycle deregulation and a source of DNA damage [18]. This causes an accumulation of errors in the DNA replication phase that eventually cells cannot readily repair. p53 is induced in response to this damage and triggers pathways leading to cell cycle arrest in G1 or G2 in order to allow the cell more time to DNA repair [6]. p21 as a main target of p53 elicits this function by inhibiting Cdk2 or Cdk1 [1, 5]. Defects in the cellular response to replication stress cause genomic instability and can lead to cancer.

To note, we have previously found that keratinocytes respond to replication stress and irreparable DNA damage by undergoing terminal squamous differentiation, and polyploidisation [19–21]. This response is triggered for instance upon inhibition of p53, overexpression of proto-oncogene MYC or the major DNA replication driver Cyclin E. We have named this response as oncogene-induced differentiation (OID) [20]. The squamous OID is mediated by G2 and mitotic checkpoints that determine keratinocyte fate towards differentiation or apoptosis [22, 23]. In all cases, the OID involves transient induction of p21. Keratinocytes undergo endoreplication in the onset of differentiation. p21 can lead to endoreplication and polyploidy due to its ability to inhibit mitotic Cdk1 yet allow DNA replication [7]. p21 is also transiently induced at the initiation of spontaneous keratinocyte terminal differentiation [24, 25]. Given that p21 is involved in mitosis control via Cdk1, we investigated whether it might be intervening in the mitosis block that leads to differentiation. To this aim, we have knocked down p21 in human primary keratinocytes undergoing replication stress caused upon loss of p53 or deregulation of Cyclin E. Interestingly, the loss of p21 alleviated the differentiation response and allowed p53 and Cyclin E precancerous alterations to drive cell multiplication. The downregulation of p21 eased the cell division block in response to replication stress. Consistently, cells driven to proliferate by suppression of p21 and loss of p53 or gain of Cyclin E had a high level of DNA damage. The results provide understanding to the role of p21 in squamous homeostasis and its paradoxical effects on differentiation and carcinogenesis.

Results

In human epidermis, p21 is expressed in individual cells within the basal layer, more frequently in peribasal cells and sporadically in more superficial layers (Supplementary Fig. 1a) [12–14]. To assess the role of p21 in the differentiation response of human keratinocytes to DNA damage, we made use of four different specific shRNA against p21 (shp21 I–IV) and lentiviral infection. We chose the most efficient constructs (I and III) for subsequent studies (Supplementary Fig. 1b; arrows). The inhibition of p21 by these constructs was very significant even upon treatment with doxorubicin (DOXO), an antitumor drug that causes acute DNA damage and strikingly induces the expression of p21 (Fig. 1A). Knockdown of p21 in human keratinocytes strikingly enhanced the proliferative capacity as observed by morphology and growing number of cells (Supplementary Fig. 1c). This is consistent with previous observations in mouse cells [16]. p21 inhibits cell cycle cdks and its knockdown produced an activation of the cell cycle as measured by the fraction of cells in S and G2/M phases and polyploidy (Fig. 1B and Supplementary Fig. 1d). Consistently, loss of p21 induced the expression of active cell cycle molecules phospho-Rb (p-Rb) and mitotic Cyclins A and B (Fig. 1C). Knocking down p21 caused a significant upregulation of mitotic Cyclin B. It is interesting that p21 promotes the degradation of Cyclin B [26]. The accumulation of Cyclin B in absence of p21 might account for a higher mitotic capacity.

Enhanced proliferation was concomitant with a significant inhibition of terminal differentiation in exponentially growing cells 3 days after infection with shp21, as monitored by analyses of cellular morphology by flow cytometry (light scattering) and decreased expression of the squamous differentiation markers keratin K1 and keratin K16 (Fig. 1D and Supplementary Fig. 1e). Consistently, the production of squamous cornified envelopes, the final product of squamous differentiation, was also impaired (Fig. 1D). However, differentiation was not abolished at confluence, as shown by increased expression of keratin K1 6 days after infection with shp21 (Supplementary Fig. 1f). This was likely a result of enhanced proliferation, resulting in more rapid acquisition of cell confluence, what in turn leads to stratification and differentiation [27, 28].

Loss of p53 causes cell cycle deregulation, increased DNA replication, DNA damage, and differentiation in normal human keratinocytes [21]. The absence of p53 also downregulates its transcriptional target p21. However, a significant amount of p21 remains in shp53 cells (Fig. 2A) [21] and this is likely due to p53-independent p21 expression pathways [8–10]. The question therefore was whether the remaining p21 protein might be responsible for the differentiation mitotic response to replication stress in the absence of p53. To address this question, we inhibited both p21 and p53 by means of shRNA in human primary keratinocytes. As shown in Fig. 2A, the inhibition of p21 observed in the double p21/p53 mutant cells was more

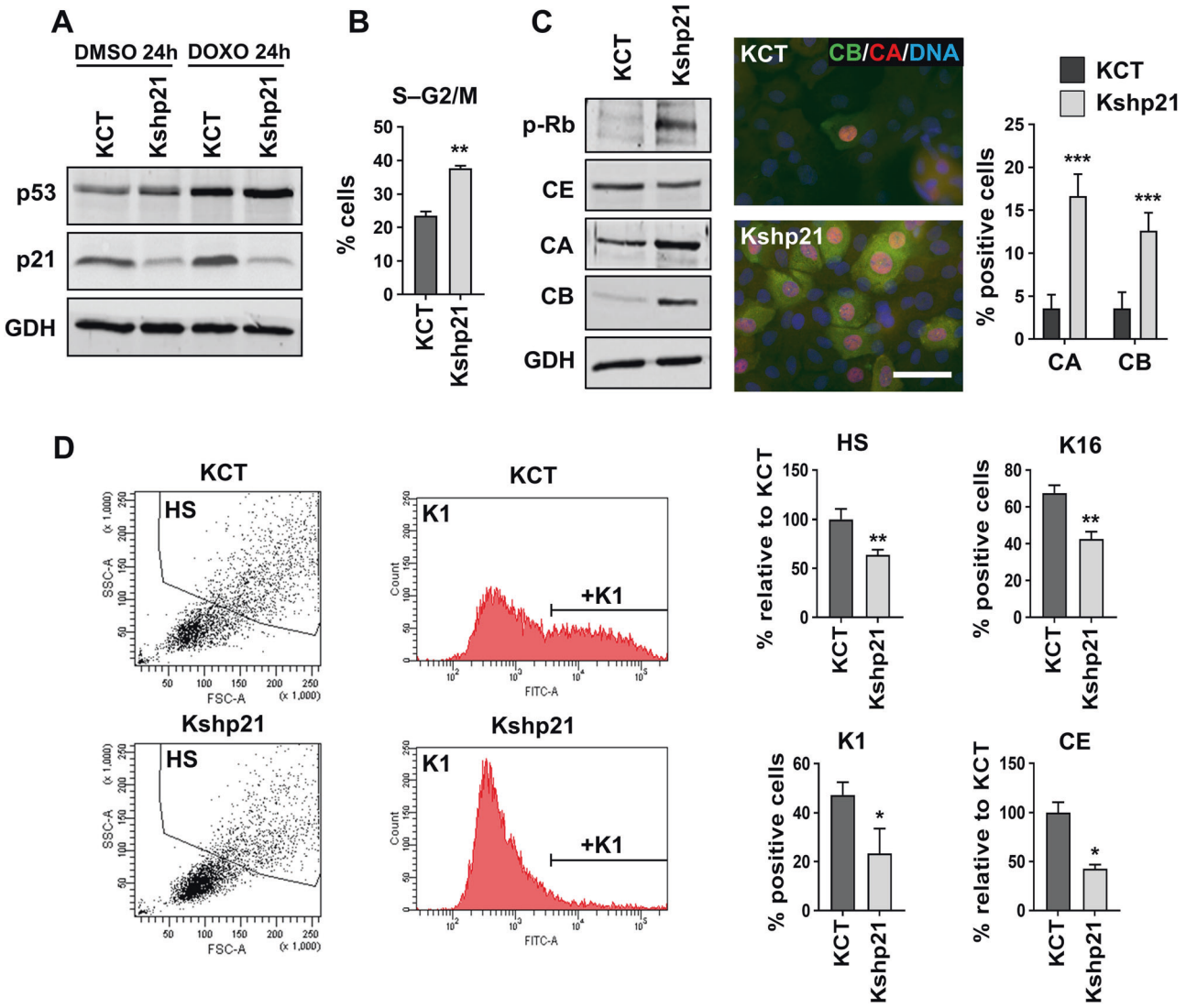

Fig. 1 p21 knockdown results in loss of squamous differentiation. **A** Detection of p21 and p53 by western blot in primary human keratinocytes 3 days after infections with shRNA to p21 (Kshp21) or the empty control vector (pLK01, KCT). Cells were treated with doxorubicin (DOXO) or the control vehicle (DMSO) for 24 h. GAPDH (GDH) as loading control. **B** Percent of cells in S-G2/M phases according to DNA content (Propidium iodide) by flow cytometry 5 days after infections ($n = 2$). **C** Left: western blotting for cell cycle markers phospho-RB (p-RB), Cyclin E (CE), Cyclin A (CA), or Cyclin B (CB). GAPDH (GDH) as loading control. Center: immunofluorescence for CB (green) and CA (red) 3 days after infection with shp21 or with CT as indicated. Blue is nuclear DNA by DAPI. Scale bar, 50 µm. Right: percent of positive CA and CB cells as quantitated by immunofluorescence ($n = 4$ randomly selected fields). **D** Left: representative flow cytometry dot plot displaying size and complexity light scatter parameters of KCT or Kshp21, as indicated. HS: the fraction of cells with high scatter values, typical of terminal differentiation. Center: representative flow cytometry histogram for differentiation marker keratin K1, 5 days after infections with shp21 or CT as indicated. Right: as indicated, percent of HS cells ($n = 3$), positive cells for keratins K16 or K1 ($n = 3$) as quantitated by flow cytometry, or cornified envelopes (CE) in the culture medium, relative to KCT ($n = 2$). In all cases, 5 days after infections. Data shown are representative or mean ± SEM of at least two independent experiments. p values were calculated with Student's t test: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

efficient than in the single shp53 mutant. Therefore, shp21 efficiently suppressed p53-independent expression of p21 in cells bearing the shp53 construct. shp21 also caused hyperactivation of Cdk1 (Fig. 2A), consistent with the known capacity of p21 to inhibit the mitotic kinase.

Interestingly, additional inhibition of p21 in Kshp53 cells allowed higher proliferative capacity and diminished the proportion of cells with high size and complexity (high light scatter, HS) typical of terminal differentiation (Fig. 2B). As for cells bearing single shp21 and unlike cells bearing single shp53, double mutant cells reached confluence faster and had a more proliferative morphology (Supplementary Fig. 2a), indicating that endogenous p21 was involved in the mitotic block induced in response to the loss of p53 [21]. As a consequence of enhanced proliferative potential and accelerated confluence, the double knockdown induced the

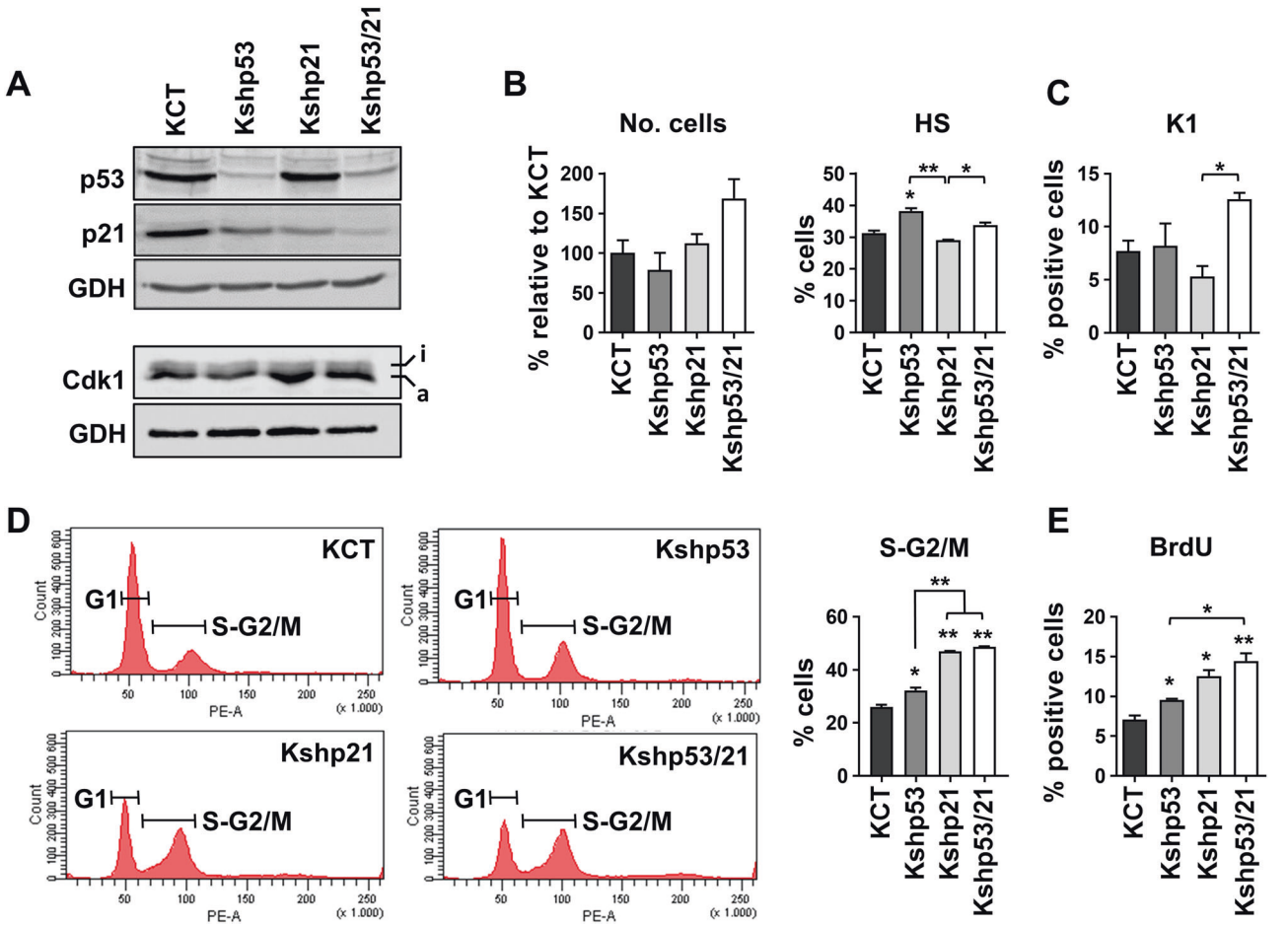

Fig. 2 p21 is required for the antiproliferative differentiation response to inactivation of p53. A Western blotting for the expression of p53 or p21 (top, 3 days post infections) and Cdk1 (bottom, 5 days post infections) in primary keratinocytes bearing empty vectors pLVTHM and pLK01 (KCT), shp53 and pLK01 (Kshp53), pLVTHM and shp21 (Kshp21), or shp53 and shp21 (Kshp53/21), as indicated. a active, i inactive. GAPDH (GDH) as loading control. **B** As indicated, 5 days after infections and relative to KCT ($n = 2$): number of harvested cells and percent of cells with high size and complexity (HS) by flow cytometry ($n = 2$) and **C** Percent of positive cells for K1 by flow cytometry. **D** Representative flow cytometry histograms of DNA content using PI, 5 days after infections. Bar histogram shows percentage of cells in S-G2/M phases ($n = 2$). **E** Percent of BrdU-positive cells measured by flow cytometry 5 days after infections ($n = 2$). Data are representative or mean ± SEM of at least two independent experiments. p values by Student's t test; $^{*}p < 0.05$, $^{**}p < 0.01$.

expression of keratin K1 5 days after infection with shp53/21 above levels of controls or those of shp21 cells (Fig. 2C).

Inhibition of p53 causes deregulation of cell cycle entry and accelerated DNA replication [6]. In keratinocytes this was observed by measure of increased S and mitotic phases (Fig. 2D). Inhibition of p21 and p53 further enhanced the DNA replication increase caused by shp53 or shp21 alone (Fig. 2E and Supplementary Fig. 2b).

As previously described, loss of p53 caused accumulation of the DNA damage marker histone γH2AX, as a result of replication stress [21, 29]. shp21 drove Kshp53 into proliferation still displaying accumulation of γH2AX (Fig. 3A–B and Supplementary Fig. 3a). 53BP foci, another marker of genetic damage, also increased in Kshp21 or Kshp53/21 cells (Fig. 3B and Supplementary Fig. 3b). Finally, cells bearing shp53 or additional shp21 contained higher levels DNA fragmentation as revealed by COMET assays (Fig. 3C). Therefore, while Kshp53 cells lost proliferative capacity in response to DNA damage, suppression of p21 allowed them to continue to proliferate bypassing the mitotic block response to unrepaired DNA damage.

Cyclin E is a major driver of DNA replication through activation of Cdk2. Variations in the levels or timing of Cyclin E expression have a significant effect on the cell cycle. When deregulated, Cyclin E is a direct promoter of replication stress and mitotic defects due to chromosome abnormalities (Supplementary Fig. 4a) [30]. In the epidermis, Cyclin E accumulates in differentiating cells [31]. In keratinocytes it strongly drives terminal differentiation when deregulated due to the OID [19]. This response involves the induction of p21. We wanted to test whether p21 was involved in the mitotic block leading to squamous

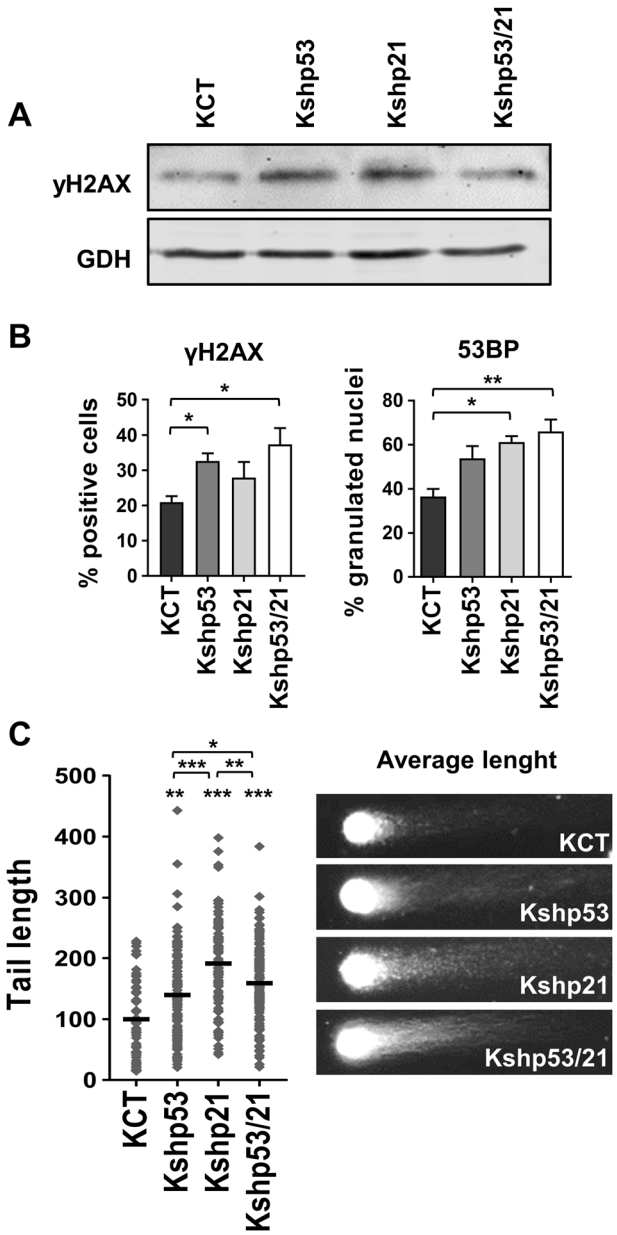

Fig. 3 Loss of p21 drives p53 knockdown cells to proliferate with high levels of DNA damage. A Western blotting for γH2AX, 5 days after infections. GAPDH (GDH) as loading control. Cells as in Fig. 2A. **B** Immunofluorescence quantitation for percent of γH2AX positive cells, 4 days after infections (left; $n = 3$ randomly chosen fields) or 53BP granulated nuclei, 5 days after infections (right; $n = 5$ randomly chosen fields), as indicated. **C** Nuclear DNA fragmentation monitored by *comet* assays, 5 days after infections. Bar histogram: quantitation of DNA fragmentation by measuring tail length, relative to control (KCT) ($n = 69$–145 cells). Horizontal small bars indicate the average value in each condition. Microphotographs show a representative average tail of each condition. Data are representative or mean ± SEM of at least two independent experiments. p values were calculated with Student's t test; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

differentiation in response to deregulation of Cyclin E. To this aim, we delivered a doxycyclin (dox) inducible Cyclin E construct in primary cells by lentiviral infection (CEdox).

Overexpression of Cyclin E strikingly induced p21 after the addition of dox, likely as a consequence of cell cycle stress (Fig. 4A and Supplementary Fig. 4b) [1]. Subsequent delivery of shp21 in cells bearing the CEdox construct abolished p21 upregulation even upon the addition of dox (Fig. 4A). As expected, overexpression of Cyclin E hyperactivated the cell cycle as monitored by a significant increase of the S and G2/M cell fractions (Fig. 4B) and led to a proliferative block due to replication stress (Supplementary Fig. 4c) [19]. Inhibition of p21 drove Cyclin E activity into enhanced proliferation and shedding due to more rapid cell confluence involving stratification and differentiation (Fig. 4C and Supplementary Fig. 4d). However, differentiation in attached cells significantly diminished (Fig. 4D).

As expected, deregulation of Cyclin E caused accumulation of the DNA damage marker γH2AX (Fig. 5A). This effect was more marked in cells that additionally expressed shp21. Consistently, KCE/shp21 cells although highly proliferative contained higher levels of nuclear DNA breaks (Fig. 5B).

Epidermal cells are continuously exposed to sublethal (non-apoptotic) UV irradiation and this is another trigger of the keratinocyte differentiation response to DNA damage [22]. We aimed to determine whether p21 is required not only for the differentiation response to replication stress but also to sublethal levels of UV irradiation. As shown in Fig. 6A, inhibition of p21 in human keratinocytes reduced the differentiation response to UV irradiation as measured by expression of terminal differentiation marker keratin K1. Acute UV irradiation induces keratinocyte apoptosis [32]. The keratinocyte choice to apoptosis or differentiation appears to be controlled by the mitotic checkpoints [22, 23]. Interestingly, the loss of p21 drove keratinocytes irradiated with sublethal UV doses from differentiation into apoptosis, as monitored by the increased fraction of cells in the sub-G1 region in the DNA content analyses (Fig. 6B) and the frequent apoptotic bodies in the cultures (Fig. 6C).

Discussion

Our results shed new insight into the still confusing mechanisms by which p21 controls cell decision making and differentiation. We show that p21 intervenes in the signal to initiate differentiation in response to replication stress. In addition to the inhibitory function on cell cycle entry through inhibition of Cdk2, p21 is known to halt mitosis by inactivating Cdk1 [5, 7]. This latter role on mitosis appears now as the limiting factor in keratinocyte differentiation. Silencing p53 would be expected to induce proliferation given the frequent mutation of this tumor suppressor in squamous carcinomas (80%) [33, 34] (www.p53.fr).

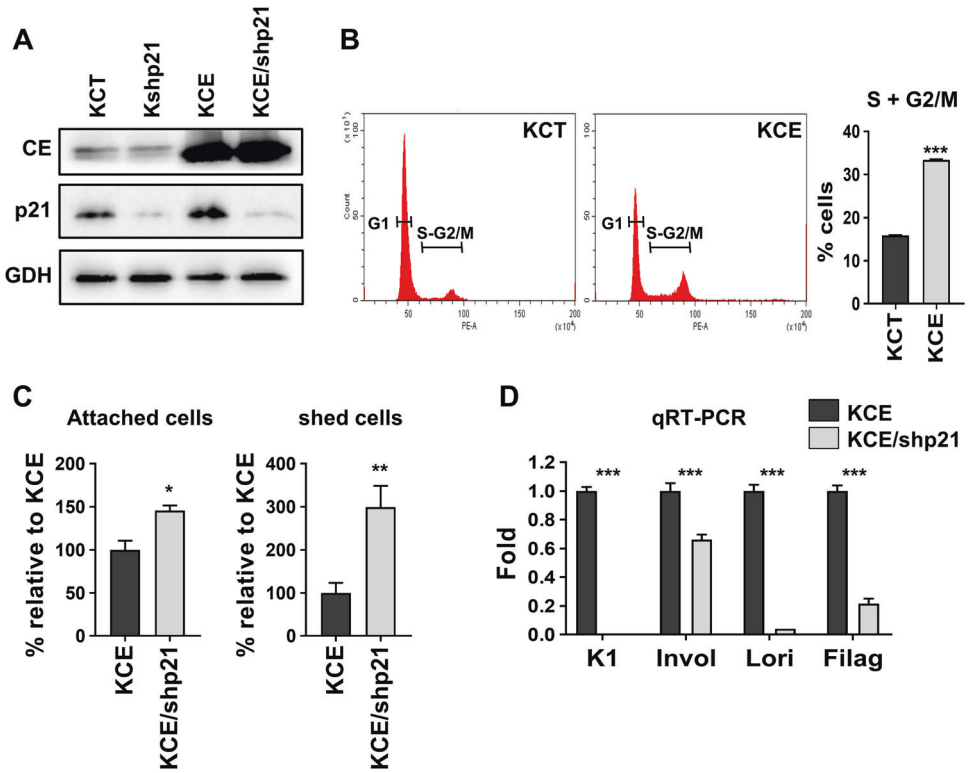

Fig. 4 p21 mediates the squamous differentiation response to deregulation of Cyclin E. A Western blotting for Cyclin E (CE) or p21 in primary keratinocytes all infected with CEdox vector (CT) and shp21 or its empty vector pLK01 3 days after infections and addition of dox. KCT are CEdox and pLK01 infected in the absence doxycycline, (dox). KCE are CEdox and pLK01 infected upon the addition of dox for 3 days. GAPDH (GDH) as loading control. **B** Cell cycle analyses by flow cytometry and DNA content by propidium iodide (PI). KCE were induced with dox for 5 days. Bar histogram shows percent of cells in S-G2/M phases ($n = 2$). **C** Number of attached cells or shed cells, relative to KCE, 5 days after infections and addition of dox ($n = 3$). **D** Expression of differentiation markers K1, involucrin (Invol), loricrin (Lori), and filaggrin (Filag), determined by qRT-PCR, 5 days after infections and addition of dox ($n = 3$). Data are representative or mean ± SEM of at least two independent experiments. p values were calculated with Student's t test; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

However, knocking p53 down results in cell cycle activation and leads to differentiation due to the mitotic checkpoints responding to replication stress [21]. In striking contrast, simultaneous loss of p53 and p21 alleviated the cell division block resulting in enhanced proliferation. The results illustrate why in keratinocytes mutation of p53 is not sufficient to drive tumorigenesis but needs additional alterations in mitosis control to be tumorigenic [35–38]. Similarly, deregulation of Cyclin E that through Cdk2 overactivation promotes replication stress, DNA damage and differentiation in normal keratinocytes [19], stimulated proliferation in absence of p21.

Although the inhibition of endogenous p21, as the inhibition of p53 or the overexpression of Cyclin E, induced cell cycle activation and DNA damage, it did not trigger the differentiation response [19, 21]. This distinct effect of shp21 must be due to the capacity of p21 to inhibit mitotic Cdk1 [5, 7]. Altogether, the results show the critical role of p21 in blocking the expansion of precancerous clones. The observation that cells with high Cyclin E or loss of p53, and loss of p21, proliferate with a high level of DNA damage shows: i—that genetically damaged cells tend to differentiate in the presence of p21 and ii—that p21 is responsible for blocking damaged cells and inducing them into stratification and differentiation, thus protecting the epithelium from genetic damage. This role, in addition to cell cycle cdk independent functions [9], might explain the importance of p21 in squamous homeostasis.

Intriguingly, suppression of p21 was not sufficient to abolish differentiation. There might be two nonexclusive explanations to this result. One is that the inhibition of endogenous p21 by the shRNAs is not complete. Second that alternative molecules inhibit Cdk1 and block mitosis in absence of p21 upon an alternative stimulus leading to differentiation, other than DNA damage. Inhibition of p21 increased proliferation when cells were exponentially growing but it allowed differentiation at confluence. It is interesting that in low-calcium conditions keratinocyte differentiation has been shown to depend more on cell confluence than on calcium concentration [27, 28]. Therefore,

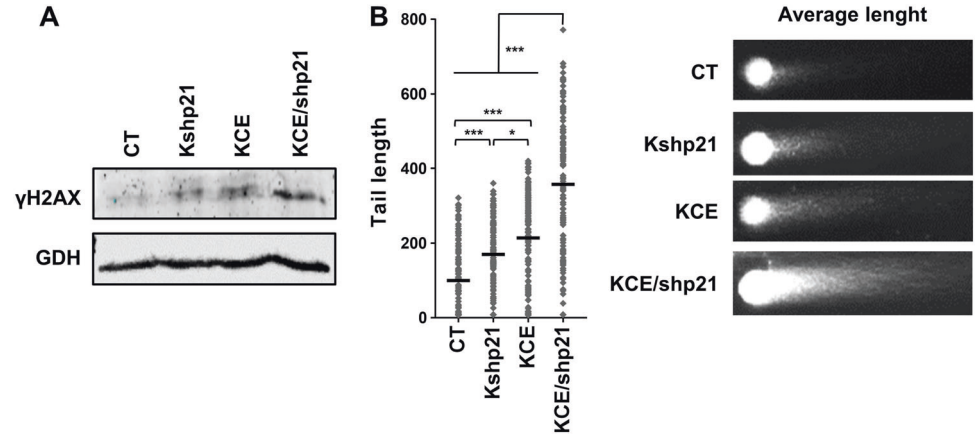

Fig. 5 Loss of p21 allows keratinocytes to proliferate with high levels of Cyclin E-induced replication stress. A Western blotting for γH2AX by 3 days after infections and 5 ng/ml addition of Doxycyclin (dox). Cells as in Fig. 4A. GAPDH (GDH) as loading control. **B** Nuclear DNA fragmentation monitored by *comet* assays 3 days after infections and addition of dox induction. Bar histogram displays quantitation of comet tail length ($n = 125–146$ cells). Micrographs show a representative sample of average length of each condition. Horizontal small bars indicate the average value in each condition. Data shown are from two independent experiments. p values were calculated with Student's t test; $^{*}p < 0.05$, $^{***}p < 0.001$.

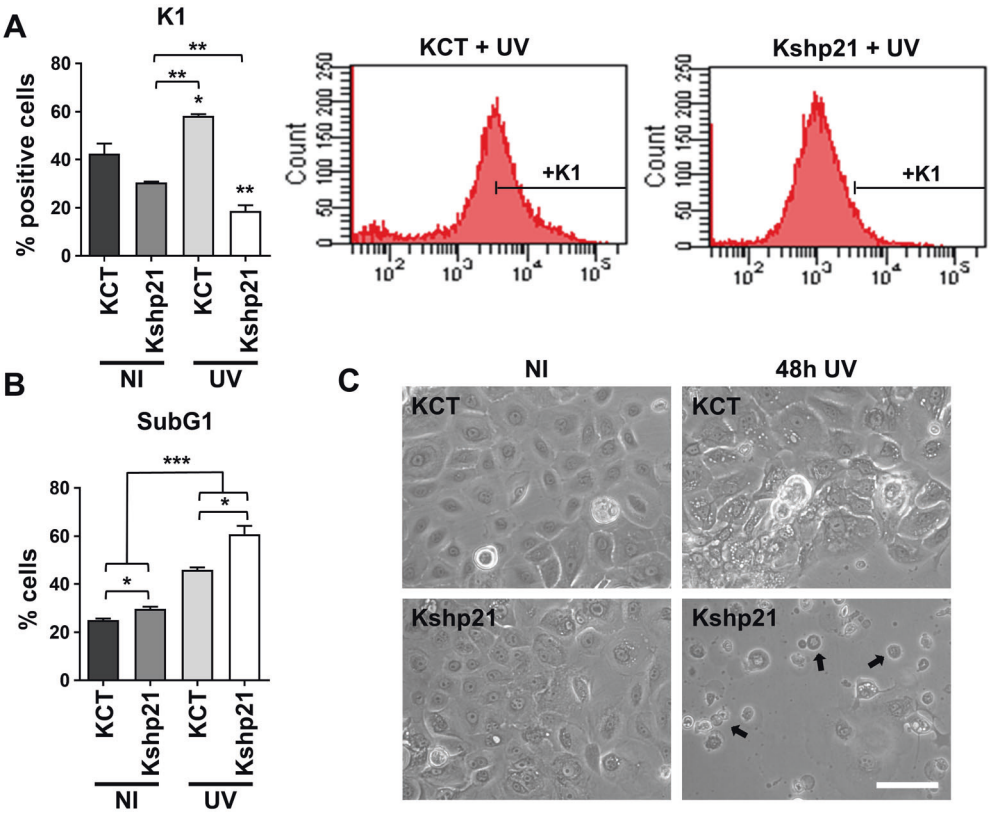

Fig. 6 Loss of p21 drives UV-induced squamous differentiation into apoptosis. A Bar histogram quantitation and representative flow cytometry histograms for the expression of squamous terminal differentiation marker K1 in primary human keratinocytes, 5 days after infections and 48 h after sublethal UV irradiation (10 mJ/cm^2). Cells as in Fig. 1A. NI non-irradiated ($n = 2$). **B** Flow cytometry DNA content analyses by Propidium iodide (PI) of detached apoptotic cells in sub-G1 phase 4 days after infections and 24 h after sublethal UV irradiation (10 mJ/cm^2) ($n = 3$). **C** Representative phase contrast micrographs of cells. Note the frequent apoptotic bodies in irradiated Kshp21 (arrows). Data are representative or mean ± SEM of two independent experiments. p values were calculated with Student's t test; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

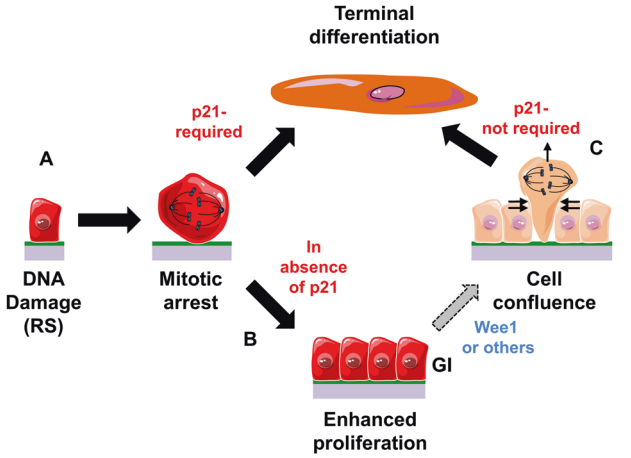

Fig. 7 Model for p21 dependent and independent triggers of squamous terminal squamous differentiation. Replication stress (RS) caused by oncogenic or spontaneous cell cycle deregulation (**A**) triggers the mitotic checkpoints which in halts mitosis. p21 by inactivating Cdk1 triggers mitotic slippage and terminal differentiation. In the absence of p21 (**B**), DNA damaged keratinocytes undergo cell division instead of mitotic slippage and proliferate, leading to genomic instability (GI). When keratinocytes are confluent (**C**), cells cannot divide for lack of space and initiate differentiation even in the absence of p21. Cell illustrations modified after templates by Servier Medical Art (https://smart.servier.com/).

keratinocytes might differentiate in response to two independent stimuli: 1—DNA damage (requiring p21) or 2—cell confluence (p21 may contribute but not essential; Fig. 7). We have previously shown that a prolonged mitosis block strikingly drives keratinocytes into differentiation [19, 23]. A prolonged mitosis arrest due to DNA damage caused by UV light induces keratinocyte differentiation [22]. Our results herein suggest that p21 plays an important role in this self-protective response but also in steady state homeostasis. Keratinocytes undergo rounds of rapid proliferation before terminal differentiation. Deregulation of the cell cycle causes replication stress and DNA damage and this in turn terminal differentiation [39]. By responding with a mitotic block, p21 would maintain the homeostatic balance between proliferation and differentiation.

Keratinocytes that are committed to differentiate undergo mitotic slippage [21, 23]. Cells slip mitosis upon inactivation of Cyclin B/Cdk1 during a G2 arrest [40–42]. In our study, inhibition of p21 caused accumulation of Cyclin B and a significant increase of the G2/M fraction of the cell cycle. High levels of Cyclin B might allow keratinocytes to stay in G2 longer thus avoiding mitotic slippage and retaining the capacity for cell division [26]. Another line of evidence supporting that p21 is involved in the mitotic control of keratinocyte cell fate comes from its protective role face to UV light-induced apoptosis. Acute UV irradiation triggers keratinocyte apoptosis whereas sublethal doses induce squamous differentiation [22]. The control of whether keratinocytes undergo apoptosis or differentiation upon UV light appears to be in the mitotic checkpoints [23]. The knockdown of p21 sensitized keratinocytes to UV-induced apoptosis, further illustrating that the regulator is involved in the mitotic control of keratinocyte fate. In the absence of p21 at cell confluence, some other molecules might inactivate Cdk1 causing mitotic slippage and differentiation. For instance, Wee1 protein responds to DNA damage and induces dephosphorilation of Cdk1 by Cdc25. It is interesting that inactivation of Wee1 lows the threshold at which keratinocytes undergo apoptosis in response to UV irradiation [22].

There is yet an intriguing paradox regarding p21 in carcinogenesis, as it is often deregulated in tumors and its expression can be marker of either good or poor cancer prognostic [4, 5]. It has been proposed that ectopic p21 can cause genomic instability by loss of DNA replication control [3]. We have suggested that squamous differentiation has a dual effect on carcinogenesis [17]. First, by suppressing proliferation, second through mitotic arrest and endoreplication, by causing mitotic defects and aneuploidy. Provoking mitotic defects in tumor cells that are able to avoid terminal differentiation, might be another mechanism to p21-induced genomic instability. The loss of p21, by allowing genetically damaged cells to proliferate, would also contribute to genomic instability.

Our results argue for a positive and essential role of p21 linking active proliferation with differentiation in epidermoid epithelia. Now the challenge is to identify the molecules that trigger squamous differentiation downstream of the p21 cell division block. These molecules ought to play an important role in squamous cancer.

Materials and methods

Cell culture, plasmids, and viral infections

Ethical permissions for this study were requested, approved, and obtained from the Ethical Committee for Clinical Research of Cantabria Council, Spain (2014.166 and 2017.259). 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, 1 ng/ml EGF (10% fetal bovine serum and 1.2 mM Ca^{+2}) [43, 44]. Low passages (1–3) keratinocytes from three different individuals were utilized. Mouse fibroblast J2 cell line used as feeder layer was cultured in Dulbecco medium 10% donor calf serum. HELA cell line was cultured in Dulbecco medium 5% fetal bovine serum.

For gene delivery in primary keratinocytes the following lentiviral constructs driven by constitutive promoters were used: control pLK01 and four different constructs expressing shRNA against p21 with different target sequences, shp21 I (TRCN0000040123), shp21 II (TRCN0000040124), shp21 III (TRCN0000287021), and shp21 IV (TRCN0000307347), all from Sigma-Aldrich (St Louis, MO, USA); control GFP pLVTHM and a construct expressing shRNA against p53, pLVUH-shp53-GFP (shp53; Addgene plasmid 11653) [45]. Otherwise, a lentiviral construct driven by an inducible promoter was used to overexpress Cyclin E1 in keratinocytes or HeLa cells: pInducer20-Cyclin E1 (CEdox, a kind gift from Jeannette G Cook (Chapel Hill, USA); Addgene plasmid 109348) [46]. To over induce the expression of Cyclin E1 cells were treated with dox (Calbiochem, Germany; rasdfef. 324385) at 5/100 ng/ml. Control cells were CEdox infected without dox.

Lentiviral production was performed by transient transfection of 293T cells. Concomitantly, keratinocytes were cultured in FAD medium until confluence and lentiviral infections were performed as in [47]. CEdox infections were made in FAD medium and cells were selected with 500 µg/ml Geneticin (Gibco, MA, USA; rasdfef. 10131035) for a week. Experiments involving lentiviral constructs bearing shRNA against p21 and/or p53 were performed in low Ca^{+2} medium due to the higher infection efficiency. shp53/21 double mutants were co-infected at the same time. Cells were transferred to low-calcium concentration (<0.1 mM) in two media: keratinocyte growth medium 2 to plate and infect (Promocell, Germany; rasdfef. C-20111) or Defined Keratinocyte-SFM to grow (serum-free, <0.1 mM Ca^{+2}; Gibco; rasdfef. 10744019), following the manufacturer's instructions. Low Ca^{+2} conditions allow differentiation of human keratinocytes but not stratification. Differentiating or apoptotic cells are shed into the medium [21, 48].

For DOXO (Sigma-Aldrich; rasdfef. 44583) treatment, 0.5 µM in dimethyl sulfoxide (DMSO) was added onto keratinocytes for 24 h. Parallel control cultures were always subjected to the DMSO vehicle.

Quantitation of cell shedding and corneocytes

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

For quantitating cornified envelopes (or corneocytes), culture supernatants were collected, cells were centrifuged, and the pellets were treated with 1 M tris-HCL (pH = 8), 2% SDS, and 2% β-mercaptoethanol solution and boiled at 100 °C for 10′. Insoluble remaining cells (corneocytes) were counted [49]. Data were obtained from triplicate samples and normalized to controls.

Antibodies

The following primary antibodies from Santa Cruz Biotechnology (Santa Cruz, CA, USA) were used: anti-GAPDH (0411, rasdfef. sc-47724; western blot, WB), anti-p53 (FL-393, rasdfef. sc-6243; WB), anti-keratin 16 (LL025, rasdfef. sc-53255; flow cytometry, FC), anti-cyclin A (H-432, rasdfef. sc-751; WB and immunofluorescence, IF), anti-cyclin E1 (HE12, rasdfef. sc-247; WB and IF) and anti-cyclin B1 (GNS1, rasdfef. sc-245; WB and IF). Other antibodies used were: anti-p21 (WAF1/Cip1, rasdfef. P1484; Sigma-Aldrich; WB), anti-pRb Ser780 (9307, rasdfef. R6275; Sigma-Aldrich; WB), anti-Cdk1 (A17, rasdfef. MAB8878; Sigma-Aldrich; WB), anti-BrdU (B44, rasdfef. 347580; BD Biosciences, San Diego, CA, USA; FC), anti-γH2AX Ser139 (JBW301, rasdfef. 05-636; Merck Millipore, Billerica, MA, USA; WB and IF), anti-keratin 1 (Poly19052, rasdfef. 905201; Biolegend, San Diego, CA, USA; FC) and anti-53BP1 (rasdfef. A300-272A; Bethyl, Montgomery, TX, USA; IF).

The following secondary antibodies from Jackson ImmunoResearch (Philadelphia, PA, USA) were used: Alexa Fluor® 488-conjugated goat anti-rabbit or anti-mouse IgG antibodies (rasdfef. 111-547-003 and 115-547-003, respectively; FC and IF); Alexa Fluor® 594-conjugated goat anti-rabbit or anti-mouse IgG antibodies (rasdfef. 111-517-003 and 115-517-003, respectively; IF). Other secondary antibodies used were: DyLight 800-conjugated goat anti-rabbit or anti-mouse IgG antibodies (rasdfef. SA5-35571 and 1 SA5-35521, respectively; ThermoFisher, Waltham, MA, USA; WB) and HRP-conjugated goat anti-rabbit or anti-mouse IgG antibodies (rasdfef. 170-6515 and 170-6516, respectively; Bio-Rad, Berkeley, CA, USA; WB).

Flow cytometry

Keratinocytes were harvested, fixed, and stained for Bromodeoxyuridine (BrdU), DNA content (Propidium Iodide, PI) and keratins K16 and K1 as previously described [50]. All antibody staining was controlled by the use of a similar concentration of isotype negative immunoglobulins (mouse or rabbit serum). After staining, cells were firmly resuspended and filtered through a 70 µM mesh to minimize the presence of aggregates and then analyzed on a Becton Dickinson FACS Canto™ and a CytoFLEX (Beckman Coulter). 10,000 events were gated and acquired in mode list. For DNA synthesis analyses, cells were cultured in the presence of 10 µM BrdU (Sigma-Aldrich; rasdfef. B5002) for 4 h and harvested by trypsin for flow cytometry. BrdU staining and DNA content analysis with PI (25 µg/ml, 12 h) were performed as described [19].

Immunodetection

For immunofluorescence, keratinocytes were grown on glass coverslips, fixed, and stained as previously described [19]. For determination of protein expression, cells were washed with PBS, lysed, and subjected to SDS-PAGE electrophoresis and western blotting as previously described [19].

UV radiation

Keratinocytes were irradiated as previously described [22] during 24 or 48 h. The light sources were fluorescent tubes of 312 nm shortwave Ultraviolet B. The effect of sublethal UV doses on squamous differentiation was assessed on primary cells from healthy skin of three different individuals, with consistent results.

Reverse transcription and polymerase chain reaction (RT-PCR)

Total RNA was isolated and reverse-transcribed using NucleoSpin® RNA (Macherey-Nagel, Germany; rasdfef. 740955.50) and the iScript™ cDNA synthesis kit (Bio-Rad; rasdfef. 4106228) according to the manufacturer's instructions. The cDNAs (50 ng) were amplified by real-time PCR using iQ™ SYBR Green supermix (Bio-Rad; rasdfef. #1708880). Primers utilized in this study for human genes were: involucrin (5′-TGCCTGAGCAAGAATGTGAG-3′ and 5′-AGCTGCTGATCCCTTTGTGT-3′), filaggrin (5′-GGCACTGAAAGGCAAAAAGG-3′ and 5′-AGCTGCCATGTCTCCAAACTA-3′), β-actin (5′-AAAATCTGGCACCACACCTTC-3′ and 5′-AGCACAGCCTGGATAGCAA-3′), keratin 1 (5′-CCAGCCAGAGTAGGACCAGT-3′ and 5′-TGCAGCAAAACAAGGAAATG-3′), loricrin (5′-TCATGATGCTACCCGAGGTTTG-3′ and 5′-CAGAACTAGATGCAGCCGGAGA-3′). RT-PCR results are presented normalized to the β-actin signal of each sample and relative to controls.

Comets assay

Comet assays or single cell gel electrophoresis were performed as described previously [51]. DNA damage was quantitated by measuring the comet tail length in pixels.

Statistical analyses

Data are presented as mean ± SD from two or three independent culture dishes conditions and at least two independent experiments as shown in each figure legend. Data sets were compared using an unpaired two-tailed Student's *t* test. *p* values considered statistically significant indicated in each figure legend. In every case sample size was chosen accordingly. Damaged samples were excluded from analyses.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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