

Telomere Length and Telomerase Activity Impact the UV Sensitivity Syndrome Xeroderma Pigmentosum C

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Abstract

Xeroderma pigmentosum (XP), a UV-sensitivity syndrome characterized by skin hyperpigmentation, premature aging, and increased skin cancer, is caused by defects in the nucleotide excision repair (NER) pathway. XP shares phenotypical characteristics with telomere-associated diseases like *Dyskeratosis congenita* and mouse models with dysfunctional telomeres, including mice deficient for telomerase (*Terc*^{-/-} mice). Thus, we investigated a hypothesized role for telomerase and telomere dysfunction in the pathobiology of XP by comparing *Xpc*^{-/-}-mutant mice and *Xpc*^{-/-}*G1-G3Terc*^{-/-} double-mutant mice and exposed them to UV radiation. Chronically UV-exposed *Xpc*^{-/-} skin displayed shorter telomeres on an average compared with wild-type skin. Strikingly, this effect was reversed by an additional deficiency in the telomerase. Moreover, aberrantly long telomeres were observed in the double-mutant mice. Telomere lengthening in the absence of telomerase suggested activation of the alternative lengthening of telomeres (ALT) in the UV-exposed skin of the double mutants. Mechanistic investigations revealed an elevated susceptibility for UV-induced p53 patches, known to represent precursor lesions of carcinomas, in *Xpc*^{-/-}*G1-G3Terc*^{-/-} mice where a high number of UV-induced skin tumors occurred that were characterized by aggressive growth. Taken together, our results establish a role for xeroderma pigmentosum, complementation group C (XPC) in telomere stability, particularly upon UV exposure. In absence of telomerase, critically short telomeres in XP mutants seem to aggravate this pathology, associated with an increased tumor incidence, by activating the ALT pathway of telomere lengthening. *Cancer Res*; 73(6): 1844-54. ©2012 AACR.

Introduction

Xeroderma pigmentosum (XP) is an autosomal recessive human skin disorder with a marked increase in skin cancer prevalence after exposure to sunlight, thereby underlining the biologic relevance of the DNA repair mechanism called nucleotide excision repair (NER; reviewed in ref. 1). NER counteracts the deleterious effects of helix distorting and transcription blocking UV-induced DNA adducts by removing them from the DNA in a "cut-and-patch"-like fashion. Two NER subpathways exist with partly distinct substrate specificity: Global genome repair (GGR) surveys the entire genome, whereas transcription-coupled repair operates exclusively on DNA damage interfering with transcription. During GGR, the recognition of the UV-induced lesion is carried out by the XPC protein,

followed by the recruitment of the other NER factors to the site of damage (reviewed in ref. 2).

Next to increased UV sensitivity and increased skin cancer, XP is characterized by premature aging of the skin, including hyperpigmentation (1, 2). XP-associated skin pathologies are remarkably similar to those of patients suffering from telomere disorders, such as *Dyskeratosis congenita*. In particular, these patients show a shortened lifespan, cutaneous pigmentation, premature hair graying, dystrophy of the nails, leukoplakia of the oral mucosa, anemia, and predisposition to cancer resulting from a mutation in telomerase (3-5).

Telomerase, an enzyme consisting of a reverse transcriptase catalytic subunit (TERT) and an RNA subunit (TERC), adds telomeric repeats *de novo* to the 3' end of chromosomes to compensate for telomeric loss during cell division, thus preventing the accumulation of critically short telomeres, which triggers replicative senescence and aging (6). Many human tumors bear short telomeres with active telomerase (7), and telomerase has also been found activated in premalignant and malignant skin tumors (8), as well as UV-exposed epidermis (9). More recently, telomeres were reported to be hypersensitive to UV radiation *in vitro* (10). *Xpc*^{-/-} mice develop similar phenotypes to patients with XPC and are prone to cancer development (1, 11). Therefore, we hypothesized that telomerase or chromosomal instability at the telomere (telomere instability) may play a

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Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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doi: 10.1158/0008-5472.CAN-12-3125

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prominent role in the pathobiology of XP. To this end, a mouse model was developed mimicking the human syndrome in the absence of telomerase ($Xpc^{-/-}G1-G3Terc^{-/-}$).

Here, we show that in absence of UV radiation, XPC is required to prevent telomeric aberrations and recombination at the telomere while dispensable for telomere length maintenance, suggesting a role for XPC in telomere stability. Chronic UV radiation resulted in aberrant skin differentiation, increased skin cancer, and was concomitant with a striking accelerated shortening of the telomere *in vivo*. Interestingly, in absence of XPC, recombination-mediated alternative lengthening of telomeres (ALT) is activated in UV-exposed skin, thereby increasing telomere aberrations and the risk for malignant transformation.

Materials and Methods

Mice

$Xpc^{-/-}Terc^{-/-}$ double-mutant mice were generated by crossbreeding Xpc (11) and $Terc$ (12) mutants and subsequently crossbreeding of the $Xpc^{+/-}Terc^{+/-}$ offspring to obtain early and late generations of telomerase knockout mice ($G1-G3Terc^{-/-}$) in $Xpc^{+/+}$ and $Xpc^{-/-}$ background. Littermates were used in all experiment.

Mice were kept with standard mouse chow and water available *ad libitum* and maintained under specific pathogen-free conditions in accordance with the recommendations of the Federation of European Laboratory Animal Science Associations. Mice were observed daily and sacrificed when signs of morbidity or tumors were found in accordance to the Guidelines for Humane Endpoints for Animals Used in Biomedical Research. All mice were exposed to day–night cycles (12 hours of light and 12 hours dark) at the Spanish National Cancer Research Centre mouse facility. The light source was white fluorescent lamps (TLD36W/840 and TLD58W/840, Phillips), which are known to emit very low doses of UV radiation between 250 and 400 nm.

Isolation of MEFs

Mouse embryonic fibroblasts (MEF) were isolated from embryos at day 13.5 postcoitum as described previously (13), maintained in Dulbecco's Modified Eagle's Medium supplemented with 10% (v/v) FBS and antibiotics/antimycotics, and grown in a 5% CO₂ humidified incubator at 37°C.

Chronic UVB carcinogenesis experiments

The dorsal skin of 6- to 7-week-old male was shaved with electric clippers and irradiated 3 times per week with 1.8 kJ/m² UVB. Scoring of tumor number and size was conducted once a week and mice were sacrificed when signs of poor health appeared (humane endpoint). Skin sections and tumors were subjected to histopathologic analysis.

Q-FISH telomere length analysis in skin

Perpendicularly cut sections of mouse skin were deparaffinized and incubated with a Cy-3-labeled telomeric probe (14). Telomere fluorescence in basal cells of the epidermis (interphase nuclei) was determined as previously described by our

group (13, 15, 16). Telomere fluorescence was represented as frequencies' histograms.

Q-FISH telomere length and cytogenetic analysis on pMEFs

Quantitative-FISH (Q-FISH) was conducted on metaphases harvested after incubation with 0.1 µg/mL colcemide (Invitrogen) for 4 hours at 37°C followed by fixation in methanol/acetic acid (3:1). Q-FISH on chromosome metaphase spreads was conducted as described previously by Samper and colleagues (17). Imaging was conducted for littermates on the same day, in parallel, and blindly. Metaphases were analyzed for chromosomal aberrations by superimposing the Q-FISH telomere image on the 4', 6—diamidino-2-phenylindole (DAPI) image using TFL-Telo software. Metaphases were scored for Robertsonian like fusions (p:p arm fusions), dicentrics (q:q arm fusions), p:q arm fusions, chromatid fusions, chromatid breaks, fragments, and MTS. Aberrations were calculated per chromosome or per metaphase (15, 18)

ImmunofISH on skin sections or MEFs

Primary MEFs were grown on p-lysine-coated coverslips and were fixed 10 to 20 minutes at room temperature in 4% paraformaldehyde (PFA), and permeabilized (19). After blocking, cells were incubated with affinity purified Rabbit anti-Mouse TRF-1 (TRF-12-A, Alpha Diagnostics), Rabbit anti-Mouse TRF-2 (TRF-21-A, Alpha Diagnostics), Rabbit anti-phospho-H2A.X (ser-139) (Millipore), and Rabbit anti-53BP1 (NB100–304, Novus) diluted 1/200 in blocking solution overnight at 4°C. After washing, cells were incubated with Alexa488-conjugated, a secondary goat anti-rabbit immunoglobulin G (IgG; 1:300; Molecular Probes, A11008), diluted 1/300 in blocking solution for 30 minutes at room temperature. After immunostaining, telomeric FISH was conducted as described previously (17) with formamide end concentration of 50%. Finally, slides were counterstained with DAPI (0.2 µg/mL) before mounting in ProLong antifade mounting medium. Images were captured in a confocal microscope (Leica TCS SP-2) and analyzed by the Leica confocal software.

Detection of ALT-associated PML bodies

Combined telomere-FISH and immunofluorescence was carried as previously described (20) with slight modifications. Briefly, deparaffinized slides were hydrated followed by antigen retrieval in citrate buffer [10 mmol/L sodium citrate, 0.05% Tween 20 (pH 6.0)] and fixation in 4% PFA. Q-FISH hybridization and subsequent washes were carried out as described above (17) followed by incubation with anti-PML (kindly provided by P. Salomoni, UCL Cancer Institute, London, United Kingdom; commercially available as no. 05-718; Upstate). Primary antibody with goat anti-mouse Alexa-Fluor 488-conjugated anti-mouse IgG (Molecular Probes A11008; 1:300), counterstained with DAPI (Sigma; 1:2000, PBS) in Vectashield. Basal cells with 3 or more telomere spots colocalizing with promyelocytic leukemia (PML), or presence of associated PML bodies (APB), were considered positive for ALT as previously described (20).

Statistical Analysis

The obtained results were subjected to statistical analysis. Statistical differences between 2 groups were assessed with a Student *t* test with Welch correction and indicated in the graphs when significance was reached.

Results

Xpc prevents telomere fragility

Telomere dysfunction and DNA damage induced by UV and/or oxidative stress have been implicated in cancer and aging (1, 11, 21). Because XPC is involved in repair of both types of DNA lesions (22, 23), we studied the impact of *Xpc* deficiency on telomere length. Q-FISH revealed that the average telomere length did not differ significantly in *Xpc*^{+/+} and *Xpc*^{-/-} primary MEFs (28.87 kb and 26.69 kb, respectively; Fig. 1A and Supplementary Fig. S1A). *G1-G3Terc*^{-/-} mice showed progressive shortening of telomeres, which was not accelerated by *Xpc* deficiency (Fig. 1A and Supplementary Fig. S1). Moreover, Q-FISH on

mouse epidermis did not reveal major changes in telomere length in aged matched mice from *G1-G3Terc*^{-/-} in *Xpc*^{+/+} and *Xpc*^{-/-} background (Supplementary Fig. S2A–S2C), although a trend to shorter telomeres is observed in the latter, both *in vivo* and *in vitro*. Together, these results suggest that XPC is dispensable for telomere length maintenance.

Because other NER proteins previously have been shown to affect telomere function (24), telomere aberrations were studied in this mouse model. Chromatid and chromosome-type fusions increased in *G2-G3Terc*^{-/-} cells concomitant with an increase in telomeric breaks and fragments (Supplementary Fig. S3A). *Xpc*^{-/-} *Terc*^{+/+} cells showed a dramatic increase in telomeres with multitelomeric signals (MTS), which was exacerbated in *Xpc*^{-/-} *G1-G2Terc*^{-/-} cells (Fig. 1B and Supplementary Fig. S3A). MTS have recently been related to DNA replication problems at telomeres and increased telomere fragility (15, 25), suggesting that *Xpc*^{-/-} cells have an increased telomere fragility even in the absence of UV-induced DNA damage. In support of this, exposure to

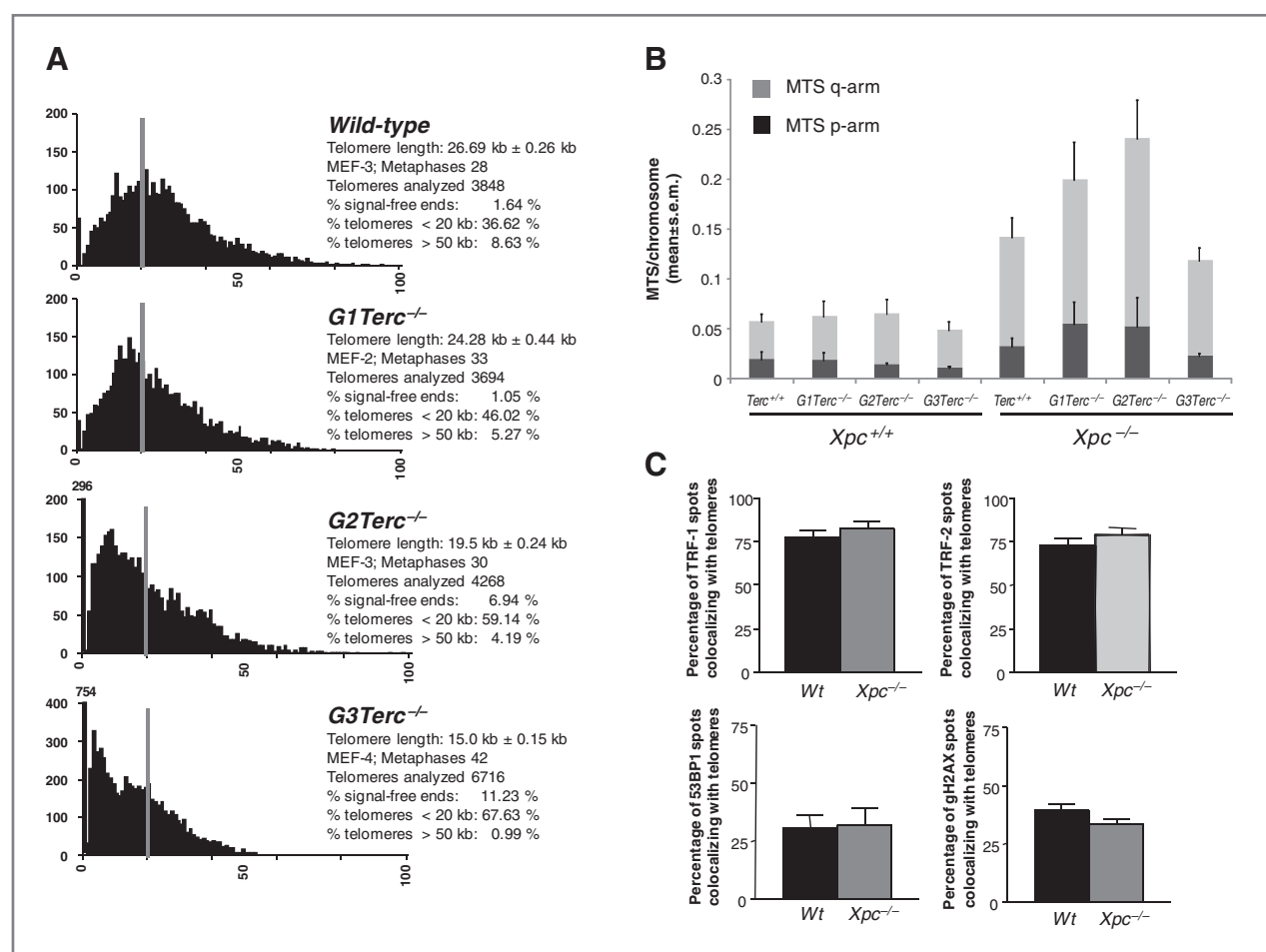


Figure 1. Analysis of telomere length and telomere aberrations. A, histogram of chromosomal telomere length in the indicated genotypes; *n* = 3. B, quantification MTS per chromosome in the indicated genotypes. C, quantification of colocalization between TRF-1, TRF-2, 53BP1, and γ -H2AX with telomeres.

Aphidicolin (0.5 $\mu\text{mol/L}$; ref. 26) further increased the frequency of MTS (Supplementary Fig. S3B). $Xpc^{-/-}$ cells exposed to hypoxia (3% O_2) showed a significant reduction of the average number of MTS per chromosome, even up to $Xpc^{+/+}$ levels ($P = 0.001$; Supplementary Fig. S3), indicating that MTS in $Xpc^{-/-}$ cells are in part caused by an increased sensitivity to oxidative damage.

To discard the possibility that the observed chromosomal instability and aberrations could be the result of displacement of telomere-capping proteins in the absence of XPC, colocalization of TRF-1 and TRF-2 with telomeres (15, 19, 27) was assessed and found to be similar in $Xpc^{+/+}$ and $Xpc^{-/-}$ fibroblasts (Fig. 1C and Supplementary Fig. S1B). In agreement with a proficient telomere-capping function, telomere-associated DNA damage signaling was unaffected by the absence of XPC as shown by the identical levels of 53BP1 and $\gamma\text{-H2AX}$ colocalization at the telomere in $Xpc^{+/+}$ and $Xpc^{-/-}$ cells (Fig. 1C). Taken together, our results show that XPC is important to prevent telomere fragility.

Longevity and aging in $XpcTerc$ mouse model

Progressive shortening of the telomeres in successive generations of $G1\text{-}G3Terc^{-/-}$ mice has been previously reported to be concomitant with a shortening of both the median and maximum life span with increasing generations (6). Here,

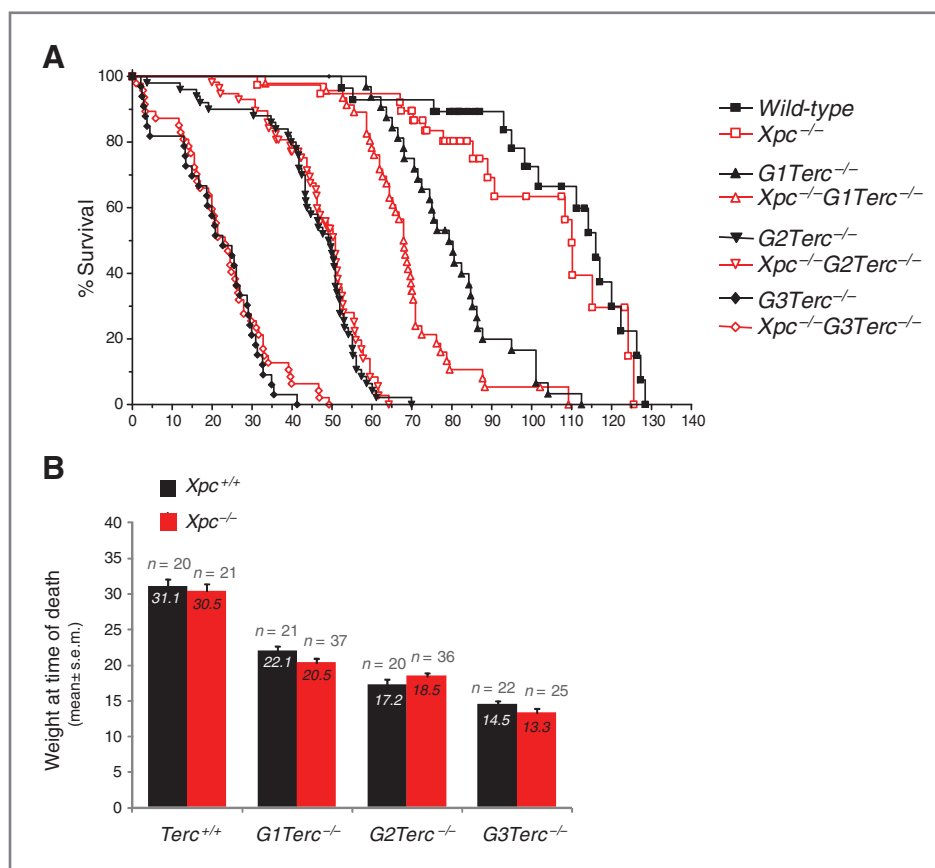
we confirmed a decreased median and maximum lifespan in $Xpc^{+/+}G1Terc^{-/-}$, $Xpc^{+/+}G2Terc^{-/-}$, and $Xpc^{+/+}G3Terc^{-/-}$ animals (Fig. 2A; ref. 28).

Interestingly, combination of XPC and TERC deficiencies did not significantly alter longevity compared with the single TERC mutant mice, in agreement with the fact that both genotypes have a similar telomere length.

Similarly, while a gradual decrease in body weight was observed in $Xpc^{+/+}G1\text{-}G3Terc^{-/-}$ mice, no significant differences were found between $Xpc^{+/+}$ and $Xpc^{-/-}$ genotypes (Fig. 2B). Concomitant with lordokyphosis, a gradual drop in bone mass density and percentage of body fat were observed in $G1\text{-}G3Terc^{-/-}$ mice, which was not affected by XPC (Supplementary Fig. S4A).

Telomerase-deficient mice display dysfunction of highly proliferative tissues such as skin, reproductive tract, and intestine as a result of telomere depletion (6, 28). Although a gradual increase in degenerative pathologies in the reproductive tract was observed in $Xpc^{-/-}$ background, especially in female mice (Supplementary Fig. S4B), XPC deficiency only marginally influenced the aging-related pathologies of $Terc^{-/-}$ mice (Supplementary Fig. S4B) including degenerative pathologies in the skin, which suggest that mutant mice suffer from premature skin aging. Of note, in unexposed single and double mutants, no benign papillomas or malignant skin tumors were reported.

Figure 2. Longevity of $Xpc^{-/-}G1\text{-}G3Terc^{-/-}$ mouse model. A, the percentage of survival $G1\text{-}G3Terc^{-/-}$ mouse cohorts in $Xpc^{+/+}$ (black) and $Xpc^{-/-}$ (red) background. B, weight at time of death in studied cohorts of mice. n represents the number of mice per genotype.



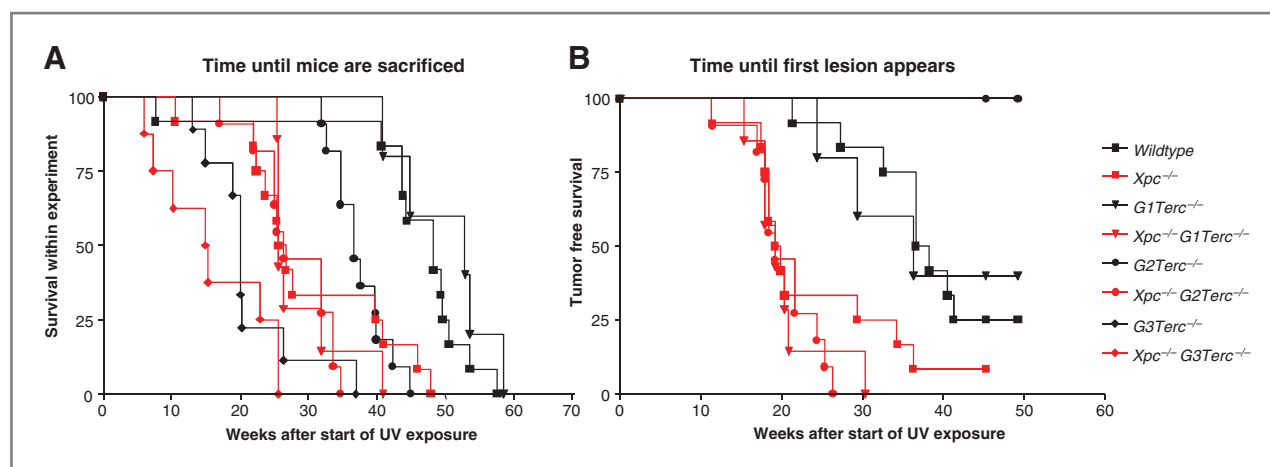


Figure 3. Telomerase deficiency accelerates UV-induced carcinogenesis in *Xpc*^{-/-} mice. Survival curves (Kaplan–Meier representation) for chronically irradiated mice (A) as well as tumor-free survival (B) of the indicated *G1*–*G3Terc*^{-/-} mouse cohorts in *Xpc*^{+/+} (black) and *Xpc*^{-/-} (red) background are presented.

UV-induced carcinogenesis is accelerated in *XpcTerc* mice

To address whether telomere length affects the rate of tumorigenesis, mice were subjected to chronic UVB irradiation and we determined survival (Fig. 3A and Supplementary Fig. S5A), tumor burden, and tumor-free survival (Fig. 3B and Supplementary Fig. S5A). From 21 weeks onwards, wild-type mice gradually developed increasing numbers of tumors that also increased in size. *Xpc*^{+/+} *G1Terc*^{-/-} mice showed a lower number of tumors compared with wild-type mice, and the first lesion appeared several weeks later than in the controls. In *Xpc*^{+/+} *G2Terc*^{-/-} and *Xpc*^{+/+} *G3Terc*^{-/-} cohorts, no tumors were observed, highlighting the previously described tumor suppressor role of short telomeres. In *Xpc*^{-/-} background, tumors appeared already at 12 weeks after the start of UV radiation and the number of tumors increased more rapidly compared with wild-type mice. Strikingly, telomerase deficiency neither delayed tumor formation nor altered the number of tumors in *Xpc*^{-/-} *G1Terc*^{-/-} and *Xpc*^{-/-} *G2Terc*^{-/-} mice (Fig. 3B and Supplementary Fig. S5A). Our results suggest that a deficiency in telomerase does not prevent UV-induced tumor formation in XPC-deficient mice. Interestingly, tumors formed in the absence of telomerase displayed more aggressive growth and development as determined by the cumulative affected dorsal skin area in *Xpc*^{-/-} and *Xpc*^{-/-} *G1-G2Terc*^{-/-} (Supplementary Fig. S5A). Of note, as a result of tumor burden, *Xpc*^{-/-} mice needed to be euthanized earlier than wild-type (humane end point). In contrast, *Xpc*^{+/+} *G2-G3Terc*^{-/-} and *Xpc*^{-/-} *G3Terc*^{-/-} animals were sacrificed as a result of age-related pathologies and without UV-related lesions (Fig. 3A and Supplementary Fig. S5A). Careful pathologic examination (not shown) of the UV-exposed skin revealed that *Xpc*^{-/-} mice presented more squamous cell carcinomas (SCC), as well as prestages, such as hyper- and dysplasia, *in situ* carcinomas, and early SCCs. Interestingly, only in the *Xpc*^{-/-} background, papillomas and multiple SCCs fused together were observed, particularly in the double-mutant background.

All together, our results indicate that Xpc deficiency is sufficient to bypass the tumor suppressor effect of *Terc* deficiency, leading to more aggressive tumors in the double mutant mice.

Chronic UV exposure leads to accelerated telomere shortening

To study the cause for the observed increased UV carcinogenesis in *Xpc*^{-/-} *Terc*^{-/-} mice, we first examined telomere length in both dorsal (UV-exposed; Fig. 4A–C and Supplementary Fig. S5B) and ventral (unirradiated) skin (Fig. 4D–F and Supplementary Fig. S5B) of the same mouse to discard inter-individual or age-related differences. Q-FISH analysis revealed a gradual drop in the average telomere length and percentage of long telomeres in ventral skin of *Xpc*^{+/+} *G1-G2Terc*^{-/-} skin, concomitant with an increase of the percentage of short telomeres. *Xpc*^{-/-} *Terc*^{+/+} ventral skin has marginally shorter telomeres, compared with *Xpc*^{+/+} *Terc*^{+/+} skin, independent of telomerase deficiency (Fig. 4D–F).

In UV-exposed skin, a gradual decrease in average telomere length and increase in the percentage of short telomeres is observed in *Xpc*^{+/+} *G1-G2Terc*^{-/-} mice, compared with *Xpc*^{+/+} *Terc*^{+/+} littermates. UV-irradiated *Xpc*^{-/-} *Terc*^{+/+} skin showed a similar response concomitant with an increase in the percentage of short telomeres. Strikingly, in UV-irradiated *Xpc*^{-/-} *G1-G2Terc*^{-/-} skin, both average telomere length, as well as the percentage of long telomeres, gradually increased compared with *Xpc*^{+/+} *Terc*^{+/+} concomitant with a drop in the percentage of short telomeres (Fig. 4A–C). This telomere lengthening suggests that alternative mechanisms involved in maintenance of telomeres are activated in UV-irradiated skin.

The overall effect of chronic UV irradiation on the average telomere length was determined by subtracting the values found in ventral skin (e.g., baseline) from those observed in the dorsal skin of the same mouse. Strikingly, chronic UV radiation provoked accelerated telomere shortening in the *Xpc*^{+/+} mice, which was further accelerated in

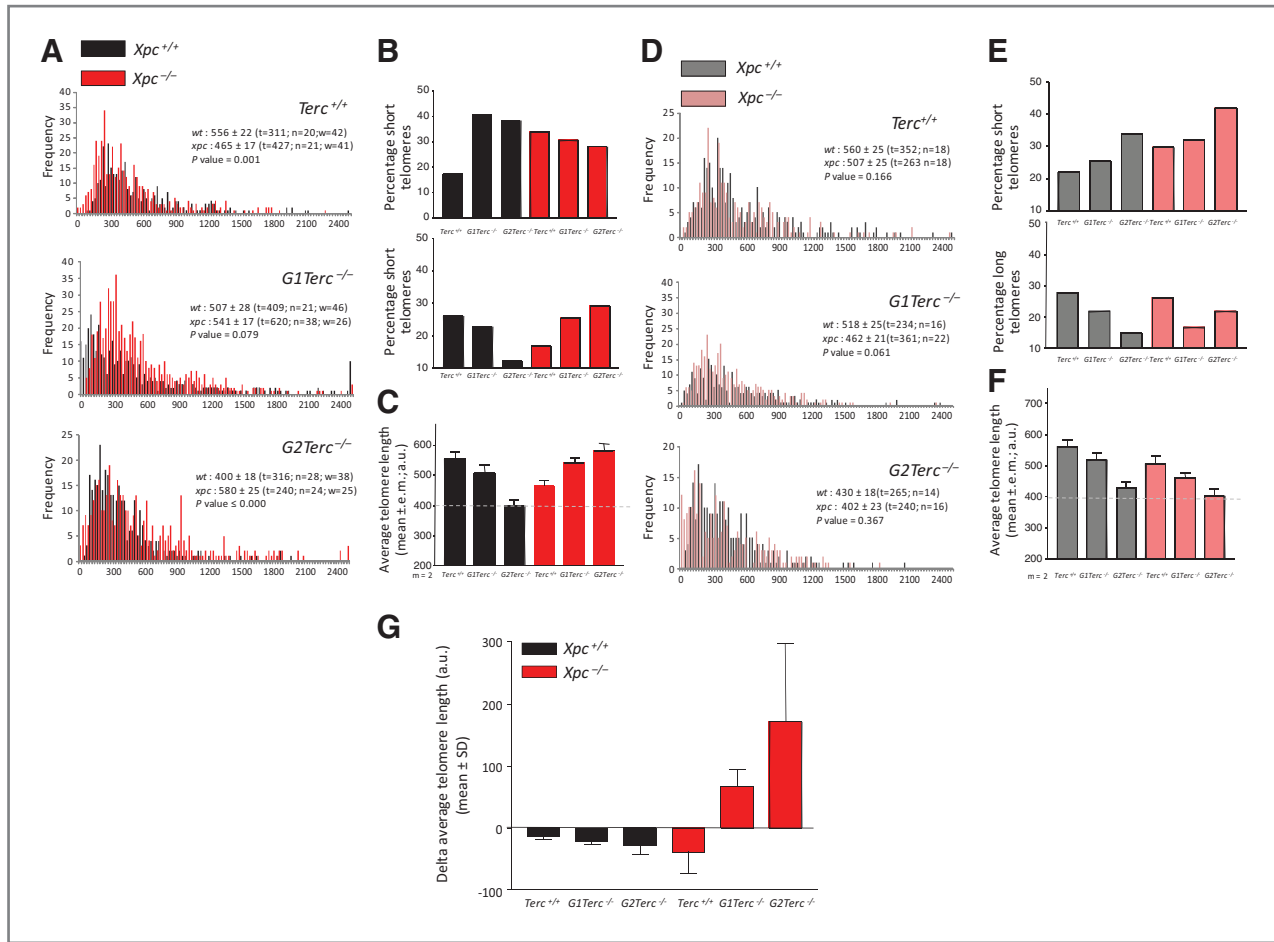


Figure 4. Chronic UV exposure leads to accelerated telomere shortening. Telomere length in basal cells chronically irradiated dorsal (A–C) and ventral (D–F) mouse skin in *Xpc*^{+/+} (black) and *Xpc*^{-/-} (red) background. Histograms (A and D) represent the frequency of telomere fluorescence in arbitrary units (a.u.) per telomere dot from at least 200 telomeres per genotype. The percentage of short telomeres (B and E—top; < 200 a.u.) and long telomeres (B and E—bottom; > 750 a.u.) and average telomere length (C and F). G, the effect of UV irradiation on telomere length is presented as delta average telomere. See Supplementary Fig S5B for more detail.

the absence of telomerase (*Xpc*^{+/+}*G1-G2Terc*^{-/-}; Fig. 4G). In *Xpc*^{-/-} skin, UV-induced telomere shortening was even more pronounced, suggesting that *Xpc* is important for telomere maintenance upon UV-induced DNA damage. In *Xpc*^{-/-}*G1-G2Terc*^{-/-} mice, we found longer telomeres in dorsal skin compared with ventral skin despite UV exposure (Fig. 4G). This observation, together with the presence of longer and more heterogeneous telomeres in *Xpc*^{-/-}*G1-G2Terc*^{-/-} skin (Fig. 4A), suggests telomere maintenance in a telomerase-independent manner. To our knowledge, this is the first report on the detrimental effect of chronic UV irradiation on telomere length *in vivo*, thereby indicating that telomeres are hypersensitive to UV radiation.

Immunohistochemistry on UV-irradiated skin

The observed accelerated telomere shortening suggests an increased rate of cell division. Indeed, a strong induction of cell division (Ki-67, Supplementary Fig. S6) and hyperplasia was observed in UV-exposed *Xpc*^{-/-}*G1-G3Terc*^{-/-} mouse skin. Although moderately increased staining for p53 and p21, as

well as γ -H2AX, was detected, apoptosis (activated caspase-3) was not elevated in *Xpc*^{+/+} or *Xpc*^{-/-} skin (independent of telomerase; Supplementary Fig. S6) indicating that cells with increased chromosomal instability and UV-induced DNA damage persist in the irradiated skin. Interestingly, UV-exposed *Xpc*^{-/-} and *Xpc*^{-/-}*G1-2Terc*^{-/-} skin displayed keratinocytes with aberrant differentiation (e.g., suprabasal cells cytokeratin 5 positive and cytokeratin 10 negative; Supplementary Fig. S6) suggesting that epidermal turnover may be responsible for removal of damaged cells (29).

Next, the number p53⁺ patches (Fig. 5A and B), groups of cells carrying a mutation in p53 (precursor lesions of neoplasias in UV-exposed skin; ref30) and hyperplasia (Fig. 5C) was quantified, which indeed confirmed hyperplasia in UV-exposed *Xpc*^{-/-} epidermis. Moreover, this revealed that p53⁺ patches are formed in all tested genotypes. Interestingly, *Xpc*^{-/-}*G1-G3Terc*^{-/-} background displayed a higher number of p53⁺ patches, particularly the large patches (Fig. 5B). Although no statistical significant difference was found, a higher total number of cells positive for p53⁺ was observed

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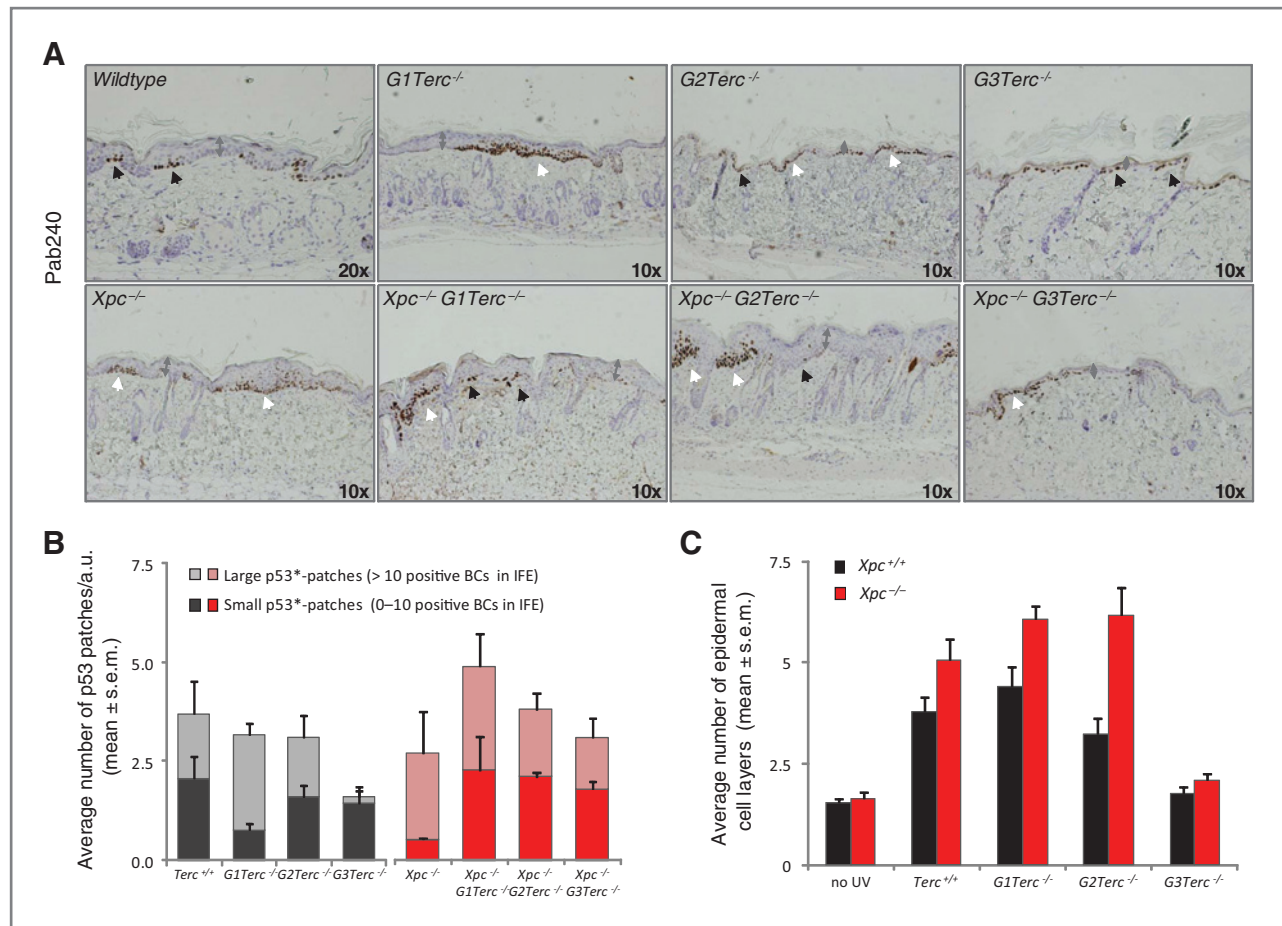


Figure 5. DNA damage response and early events in UV-induced carcinogenesis. **A**, representative low-magnification images of immunohistochemical staining for p53-Pab240 in chronically irradiated mouse epidermis of all studied genotypes. The average number of small (0–10 positive basal cells) and large (> 10 positive basal cells) p53* patches (**B**) per a.u. (1 a.u. is equivalent to 91 interfollicular basal cells) and the average number of skin layers (**C**) are determined in chronically irradiated skin of indicated. At least 10 randomly picked microscopic fields were evaluated from least 2 independently stained skin sections (*n*) from different mice.

in *Xpc*^{-/-} epidermis. Remarkably, p53* patches are observed in *Xpc*^{-/-} *G3Terc*^{-/-} skin (Fig. 5B) confirming the onset of carcinogenesis.

Activation of ALT in UV-irradiated skin of *Xpc*^{-/-} *Terc*^{-/-} skin

Q-FISH analysis on UV-exposed skin revealed telomeres with very heterogeneous lengths, and foci of very intense fluorescent signal corresponding to telomere repeats were observed in 30% and 23% of basal cells of *Xpc*^{-/-} *G1Terc*^{-/-} and *Xpc*^{-/-} *G2Terc*^{-/-} skin, respectively (Fig. 6A and B). Together, with the increase in average telomere length and the increased percentage of long telomeres (Fig. 4), this observation suggested that ALT was activated in the UV-irradiated *Xpc*^{-/-} *G1-2Terc*^{-/-} mouse skin. Indeed, immuno-FISH revealed that an elevated percentage of basal cells that show colocalization of PML and telomeres (APBs) in UV-irradiated *Xpc*^{-/-} *G1-2Terc*^{-/-} mouse skin (Fig. 6C and D) suggesting that in repair-compromised skin with short telomeres, ALT is activated as survival strategy to ensure cellular longevity at cost of

increased chromosomal instability. In support of this, Co-FISH revealed increased frequencies of Crossovers [sister chromatid exchange (SCE)], telomere-SCE (T-SCE), chromosome fusions, as well as MTSs in unirradiated *Xpc*^{-/-} MEF (Supplementary Fig. S7).

Discussion

The presented data show for the first time that chronic UV exposure *in vivo* results in accelerated telomere shortening indicating that telomeres are hypersensitive to UV-induced DNA damage as previously reported for *in vitro* experiments (10). Moreover, in the absence of *Xpc*, persistent DNA damage resulted in further telomere attrition indicating that XPC is involved in telomere integrity and stability. The latter is supported by our observations that *Xpc*^{-/-} cells show more MTS and T-SCE (Supplementary Fig. S7), as well as the activation of ALT upon chronic UV exposure (Fig. 6).

It is envisaged that the UV-induced accelerated telomere shortening is the result of increased hyperplasia upon UV

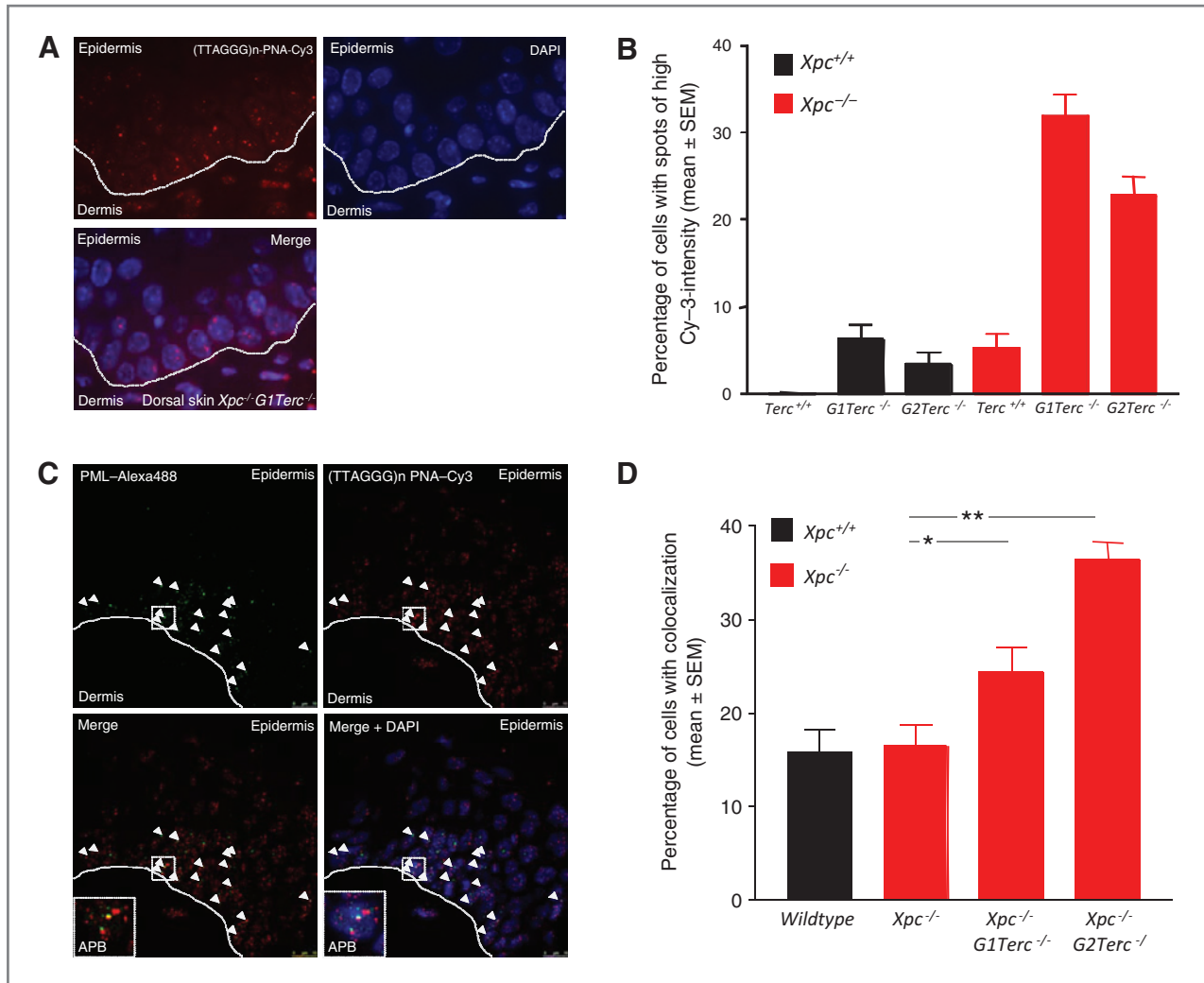


Figure 6. Activation of ALT in chronically irradiated *Xpc*^{-/-}*G1-2Terc*^{-/-} mouse epidermis. **A**, representative images of epidermal cells with high telomere fluorescence (clusters of telomeric signal) compared with normal telomeres. Magnification, $\times 100$. **B**, quantification of the percentage of basal cells displaying these intense spots of fluorescence representing telomeres. **C**, representative confocal image stack through the epidermal skin sections simultaneously stained for PML (Alexa 488, green) and telomeres (TTAGGG-PNA, Cy3, red). APBs are detected in skin section (bottom inset). Scale bar, 10 μm /L. **D**, quantification of the percentage of basal cells of mouse epidermis showing colocalization of 3 or more telomeres with PML.

exposure (Fig. 5 and Supplementary Fig. S8) in which increased proliferation together with the "end replication problem" is likely to result in accelerated telomere erosion and dysfunctional telomeres. Here, we propose that persistent DNA damage and a failure to resolve repair intermediates contribute to the accelerated telomere attrition observed in the *Xpc*^{-/-} skin.

XPC is a key component of the NER pathway, and recently has also been implicated in base excision repair (22, 31). Repair intermediates from UV damage and/or oxidative stress removal entail single-strand breaks, which have been described to be less efficiently repaired in the telomere, in turn resulting in accelerated telomere erosion upon UV damage and oxidative stress (32). Telomeres tolerate a lack of repair of UV-induced cyclobutane pyrimidine dimers (CPD) *in vitro* (10), and persistent CPDs have been reported

in wild-type, as well as *Xpc*^{-/-} epidermis. Removal of UV-induced pyrimidine (6-4) pyrimidone photoproducts (64PP) only occurred in XPC-proficient mouse skin (33). This suggests that a failure to repair 64PPs may explain the accelerated telomere shortening upon UV exposure. However, this does not exclude the possibility that UV-induced lesions are recognized via other repair pathways, such as transcription-coupled NER and/or translesion synthesis as previously suggested (10). It is envisaged that stalling of transcription or replication, unresolved repair intermediates, or even a collapse of the replication fork provokes genomic as well as telomere instability.

Failure to repair DNA breaks and telomere shortening below critical length result into replicative senescence or cell death (34). Strikingly, no apparent signs of apoptosis were found in UV-exposed *Xpc*^{-/-} skin. Moreover, a concomitant

increase in proliferation, hyperplasia, and aberrant epidermal differentiation was observed (Supplementary Fig. S6), which suggests that upon chronic UV radiation, turnover of the epidermis, and subsequent shedding of death cells, is responsible for the elimination of damaged cells thereby lowering the risk of cell cycle reentry and acquisition of mutations (29). In hyperplastic skin, an increase in the residency time of the keratinocytes is expected. This, together with a lack of apoptosis induction in cells with short telomeres and persistent DNA damage, is proposed to increase the risk of malignant transformation. In support, an increased number of cells with inactivated p53, as well as the elevated and increased carcinogenesis is observed in UV-exposed *Xpc*^{-/-}*Terc*^{-/-} mouse skin (Figs. 5 and 6 and Supplementary Fig. S5). Alternatively, the increased chromosomal instability could help keratinocytes to acquire beneficial mutations, possibly providing a growth advantage over the surrounding tissue and thereby explaining the more aggressive tumor growth observed *Xpc*^{-/-}*G1Terc*^{-/-} mice.

It was shown that in repair-compromised and telomerase-deficient skin, ALT is activated after UV exposure (Fig. 6). Indeed, DNA damage has been reported to induce formation of APBs, which can entail more than one telomere and preferentially associate with linear telomeric DNA (35–37; Supplementary Fig. S8). ALT is most efficient in S- and G₂ phase where the sister chromatids are available (34). Interestingly, *Xpc*^{-/-} keratinocytes show a cell-cycle arrest in late S-phase upon single UV exposure, both *in vivo* and *in vitro* (38, 39). It is therefore attractive to speculate that persistent DNA damage, and a possible subsequent replication fork collapse at the telomere, cause a cell-cycle arrest that may be dependent on recombination. This is further supported by our observation that more MTS are observed in *Xpc*^{-/-} primary MEFs (Fig. 1B and Supplementary Fig. S7).

The occurrence of cells with p53 mutations in otherwise unsuspecting epidermis, both in human (40) and mouse (41, 42) are considered initial steps in skin carcinogenesis (43), and are considered precursor lesions and potential indicators of skin cancer risk. More than 90% of the SCCs observed in patients with XP have been found to carry UV signature mutations in p53 (reviewed in ref. 44). Moreover, a higher frequency of UV signature mutations (e.g., CC → TT) has been observed in *Xpc*^{-/-} epidermis compared with wild-type, which contributed to the elevated carcinogenesis (33). Considering the DNA sequence of telomeres, TTAGGG (and complementary AATCCC), it is not unthinkable that telomeres carry hot-spots for UV-induced mutations, repair of which may result in irresolvable repair intermediates or collapse of a replication fork due to structural complexity of the telomere or their highly repetitive character. The subsequent rapid and drastic telomere loss, due to repeated cycles of fusion and breaks at the damaged site, simultaneously with elevated genomic instability at the telomere, may in turn provide a substrate for ALT. Indeed, dramatic telomere attrition was observed in UV-exposed *Xpc*^{-/-} skin (Fig. 4) and increased numbers of APBs observed in UV-exposed *Xpc*^{-/-}*G1Terc*^{-/-} skin (Fig. 6).

Moreover, p53* patches were observed in our mouse model and although the number between *Xpc*^{+/+} and *Xpc*^{-/-} skin are not significantly different, the onset is different since the analyzed skin is retrieved at the time of death, which is weeks earlier for *Xpc*^{-/-} animals (Fig. 3 and Supplementary Fig. S6). Strikingly, even in apparent tumor-free *Xpc*^{-/-}*G3Terc*^{-/-} mice p53* patches were found indicating that the precancerous lesions are present. However, the severely reduced lifespan of these animals censored tumor development.

Carcinogenesis studies conducted with telomerase-deficient mice in p53-deficient backgrounds have shown that p53 plays a pivotal role in response to telomere dysfunction (45). Our observation of p53* patches suggests the inactivation of the p53-dependent DNA damage response pathway and bypass of this checkpoint. In the context of telomere dysfunction, this would be catastrophic for a cell unless telomere maintenance is reestablished by ALT activation, as observed in UV-exposed *Xpc*^{-/-}*G1G2Terc*^{-/-} skin. Together these findings may explain the observed lack of tumor suppression conferred by absent telomerase activity in *Xpc*^{-/-}*G1G3Terc*^{-/-} mice (Fig. 3).

Our data and observations support a model in which persistent UV-induced DNA damage, like in *Xpc*^{-/-} skin, can result in accelerated telomere shortening and activation of UV-induced DNA damage responses, such as cell-cycle arrest or epidermal turnover. Telomerase activation, together with mutations in key tumor suppressor genes such as p53, may result in increased carcinogenesis as observed in *Xpc*^{-/-} mouse models. However, in context of short telomeres or the inability to activate telomerase chromosomal instability is envisaged to increase leading to activation of ALT, which may account for the exacerbated skin carcinogenesis observed in *Xpc*^{-/-}*Terc*^{-/-} mutants.

In conclusion, the stability of the telomeres plays an important role in cutaneous carcinogenesis, especially considering the fact that increased cell division (hyperplasia) is an intrinsic DNA damage removal strategy, resulting in telomere erosion. This implies a new complicating factor in the fight against and prevention of skin carcinogenesis, especially in patients suffering from DNA repair deficiencies like XP.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: G.J. Stout, M.A. Blasco

Development of methodology: G.J. Stout

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): G.J. Stout

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): G.J. Stout

Writing, review, and/or revision of the manuscript: G.J. Stout, M.A. Blasco

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): G.J. Stout

Study supervision: G.J. Stout, M.A. Blasco

Acknowledgments

The authors thank M.E. Varela for critically reading the manuscript and J.M. Flores for excellent work on pathologic samples. Research in the Blasco lab was funded by European Research Council (ERC) Project TEL STEM CELL

(GA#232854), European Union FP7 Projects 2007-A-20088 (MARK-AGE) and 2010-259749 (EuroBATS), Spanish Ministry of Economy and Competitiveness Projects SAF2008-05384 and CSD2007-00017, Regional of Government of Madrid Project S2010/BMD-2303 (ReCaRe), AXA Research Fund (Life Risks Project), Lilly 2010 Preclinical Biomedicine Research Award (*Fundación Lilly*, Spain), *Fundación Botín* (Spain). The authors also thank O. Aparicio, R. Serrano, M.E. Collado, S. Leceta for technical assistance, F. Mulero for the molecular imaging, and P. Salomoni who kindly provided the PML antibody.

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Received August 13, 2012; revised November 21, 2012; accepted November 28, 2012; published OnlineFirst January 3, 2013.

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