Error-prone translesion replication of damaged DNA suppresses skin carcinogenesis by controlling inflammatory hyperplasia

Anastasia Tsaalbi-Shtylika, Johan W. A. Verspuy, Jacob G. Jansen, Heggert Rebel, Leone M. Carlé, Martin A. van der Valk, Jos Jonkers, Frank R. de Gruijl, and Niels de Wind

Departments of Toxicogenetics and Dermatology, Leiden University Medical Center, 2300 RC Leiden, The Netherlands; and Divisions of Experimental Pathology and Molecular Biology, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands

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The induction of skin cancer involves both mutagenic and proliferative responses of the epidermis to ultraviolet (UV) light. It is believed that tumor initiation requires the mutagenic replication of damaged DNA by translesion synthesis (TLS) pathways. The mechanistic basis for the induction of proliferation, providing tumor promotion, is poorly understood. Here, we have investigated the role of TLS in the initiation and promotion of skin carcinogenesis, using a sensitive nucleotide excision repair-deficient mouse model that carries a hypomorphic allele of the error-prone TLS gene Rev1. Despite a defect in UV-induced mutagenesis, skin carcinogenesis was accelerated in these mice. This paradoxical phenotype was caused by the induction of inflammatory hyperplasia of the mutant skin that provides strong tumor promotion. The induction of hyperplasia was associated with mild and transient replicational stress of the UV-damaged genome, triggering DNA damage signaling and senescence. The concomitant expression of Interleukin-6 (IL-6) is in agreement with an executive role for IL-6 and possibly other cytokines in the autocrine induction of senescence and the paracrine induction of inflammatory hyperplasia. In conclusion, error-prone TLS suppresses tumor-promoting activities of UV light, thereby controlling skin carcinogenesis.

DNA translesion synthesis | Interleukin-6 | skin cancer | tumor initiation | tumor promotion

Tumor initiation by mutagenic agents and tumor promotion by inflammatory agents are critical determinants of carcinogenesis. Ultraviolet light (UV) is considered a complete carcinogen, as it induces not only mutations but also a mild inflammatory and proliferative response of the skin, mediated by growth factors and inflammatory cytokines (1–3).

Mutagenesis induced by DNA-damaging agents depends on specialized TLS DNA polymerases that replicate damaged nucleotides, such as UV-induced photodimers, in an error-prone fashion. Thereby, TLS safeguards the perpetuation of replication on damaged templates at the expense of mutagenesis (4). Rev1 is a key actor in error-prone TLS (5). Although the protein can incorporate deoxycytosines opposite abasic nucleotides and some damaged guanines, Rev1 plays a regulatory, rather than a catalytic, role in error-prone TLS of other damages, including photodimers (5–7). The role of Rev1 in a particularly error-prone subpathway of TLS of photodimers is mediated by its N-terminal BRCT domain (6, 7). In agreement, mouse embryonic stem cells with a disruption of this domain (Rev1B/B cells) lack all UV-induced nucleotide transversion mutations and part of the transitions (8). Nevertheless, this Rev1 allele is hypomorphic as in Rev1B/B cells photodimers ultimately are replicated, in contrast to completely Rev1-deficient cells (7). Rev1B/B mice display no spontaneous phenotypes, unlike completely Rev1-deficient mice, supporting the hypomorphic nature of the Rev1B allele, also at endogenous DNA damages (8, 9).

Here, we have investigated the premise that tumor initiation by error-prone TLS of photodimers is a rate-limiting step in UV-induced skin cancer, using Rev1B/B mice as a model. In addition to the hypomorphic Rev1 allele, the mice carried a defect in nucleotide excision repair (NER) of photodimers, predisposing them to skin cancer. Surprisingly, despite the defect in error-prone TLS, UV-induced skin carcinogenesis was further accelerated by the Rev1B/B deficiency. We found that exposure of the skin of these mice to UV did induce severe inflammatory hyperplasia, providing strong tumor promotion. This result demonstrates that, paradoxically, error-prone TLS controls carcinogenesis by suppressing tumor-promoting activities of UV light, at the expense of the induction of mutations.

Results

Hypomorphic Rev1 Defect Accelerates Skin Carcinogenesis. We used NER-deficient (Xpc−/−) hairless albino mice to investigate the effect of the Rev1B/B allele on UV-induced skin carcinogenesis. In this sensitive model (i) UV dosimetry is not affected by melanin, and the absence of fur excludes shaving-induced artifacts (10); (ii) a very low, subtoxic, UV dose suffices to efficiently induce skin cancer with short latency (10, 11, reviewed in ref. 12); and (iii) the NER deficiency further increases the dependence on error-prone TLS of photodimers (7). The Rev1B/B mutation only slightly increased the UV sensitivity of the Xpc−/− skin as the minimal erythema/edema (toxic) dose (MED; 13) was reduced from 500 J/m2 to 350 J/m2. To induce skin cancer, mice were exposed to a very low (40 J/m2) daily UV dose and tumors were counted and measured weekly. Unexpectedly, squamous cell carcinomas (SCC) appeared significantly earlier in the Rev1−/− mutant mice than in the controls (Fig. 1), although the total tumor load, growth, and histology were independent of the genotype.

Rev1B/B Xpc−/− SCC Display a Defect in Mutagenesis but Normal Genome Stability. Mutational inactivation of p53 precedes skin cancer (3, 11, 14). The Rev1 defect resulted in only a slight reduction of the number of p53-mutant patches in premalignant skin (Table 1) in apparent contrast with the phenotype of cultured Rev1B/B cells. To provide better evidence for a defect in error-prone TLS in vivo we determined the p53 mutation...
UV Induces Inflammatory Acanthosis in the Rev1\textsuperscript{B/B}:Xpc\textsuperscript{-/-} Epidermis.

To analyze responses of the Rev1-mutant epidermis to UV, we measured cell cycle progression in vivo. Thus, mice were irradiated with a single subtoxic UV dose. One hour before killing, replicating cells were pulse-labeled in vivo with Bromodeoxyuridine (BrdU) and epidermal cell suspensions were analyzed by bivariate flow cytometry (Fig. 2 A and B). In both genotypes an S phase delay was induced early after UV exposure, evidenced by reduced BrdU incorporation per cell (population 3). At later time points BrdU incorporation in many S-phase cells ceased completely (population 4), as shown before (16). Populations 3 + 4 were transiently enlarged in the Rev1-mutant epidermis indicating a slightly increased S phase delay. Strikingly, in the Rev1 mutant a population of very actively replicating cells emerged beyond 48 h after exposure (population 5). Such an excessive mitogenic response was also induced by the UV regimen of the carcinogenesis experiment since, after 8 days of very low-dose exposure, the Rev1-mutant skins displayed severe acanthosis (hyperplasia of the skin) and obvious signs of inflammation (Fig. 2C and Figs. S3 and S4). We then investigated responses of skin patches to a single high UV dose, equitoxic for each genotype. As expected, initial levels of erythema and edema were very similar. However, after 3 weeks, in the Rev1-mutant mice the exposed patches were strongly elevated, erythematous and keratotic, resembling psoriatic plaques in humans (Fig. 2D). In conclusion, uniquely in the Rev1-mutant skin, UV induces mitogenic activity followed by inflammatory acanthosis, and this phenotype is independent of the UV dose and toxicity.

### Table 1. Mutant p53 patches in premalignant skin, and p53 mutations and gross genomic alterations in SCC

<table>
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<tr>
<th></th>
<th>Xpc\textsuperscript{-/-}</th>
<th>Xpc\textsuperscript{B/B}</th>
<th>Xpc\textsuperscript{-/-}</th>
<th>Xpc\textsuperscript{B/B}</th>
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<td>57 ± 22\textsuperscript{1}</td>
<td>91 ± 35\textsuperscript{1}</td>
<td>57 ± 22\textsuperscript{1}</td>
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<td>53</td>
<td>61</td>
<td>53</td>
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<tr>
<td>No PCR product</td>
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<td>1 (2%)</td>
<td>2 (3%)</td>
<td>1 (2%)</td>
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<tr>
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<td>12 (20%)</td>
<td>12 (23%)</td>
<td>12 (20%)</td>
<td>12 (23%)</td>
</tr>
<tr>
<td>SCC with ambiguous p53 mutations</td>
<td>3 (5%)</td>
<td>4 (8%)</td>
<td>3 (5%)</td>
<td>4 (8%)</td>
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<tr>
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<td>44 (72%)</td>
<td>36 (68%)</td>
<td>44 (72%)</td>
<td>36 (68%)</td>
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<td>54</td>
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<td>0</td>
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<td>44</td>
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<td>28 (63%)\textsuperscript{3}</td>
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<td>3\textsuperscript{11}</td>
<td>2.7\textsuperscript{11}</td>
<td>3\textsuperscript{11}</td>
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\textsuperscript{a}Number of patches ± SEM per isolated epidermal sheet after chronic exposure, as detected using a mutant-p53-specific antibody.

\textsuperscript{1}No significant difference (t test, \(P = 0.28\)).

\textsuperscript{2}p53 mutations in cDNA from individual SCC.

\textsuperscript{3}Percent of transitions at YCpG sites of the total number of transitions at dipyrimidines. Significantly different (\(\chi^2, P = 0.0018\)).

\textsuperscript{4}See Fig. S2.

\textsuperscript{5}Only tumors with amplifications/losses are taken into account.

\textsuperscript{**}No significant difference (\(\chi^2, P = 0.50\)).

\textsuperscript{11}No significant difference (t test, \(P = 0.76\)).
and apoptosis. At later stages, however, mitogenic signaling induces excessive proliferation of basal-layer keratinocytes that contain mutagenic DNA damage, and inhibitory consequences of DNA damage signaling are negated. The expression and secretion of IL-6, shortly after UV exposure and at later stages, is in agreement with a causal role in both the establishment of senescence and in the mitogenic signaling.

**DNA Damage Signaling in Rev1B;Xpc−/− Cells Is Caused by Transient Perturbation of Replication.** We used mouse embryonic fibroblast (MEF) lines to study whether the enhanced DNA damage signaling in the Rev1 mutant is caused by stressed replication. Rev1B;Xpc−/− MEFs showed only slightly increased UV sensitivity (Fig. S5A) but phosphorylation of the Atr substrate Chk1 was markedly enhanced in the mutant (Fig. S5B). Thus, the MEF lines react in a similar fashion to UV exposure as the epidermis.

We measured whether TLS of photolesions and the global progression of replication were affected by the Rev1 defect using the alkaline denaturation assay (Fig. 4C) and a sensitive alkaline sucrose gradient-based assay (Fig. 4D), respectively. Both assays revealed a delay of approximately 2 h in Rev1B;Xpc−/− MEFs (Fig. 4C and D). Therefore, the enhanced DNA damage responses in the Rev1 mutant are caused by a mild TLS defect and the consequent transient perturbation of replication. Given that replication is completed normally, albeit with some delay, the extent of the DNA damage signaling in the Rev1B;Xpc−/− MEFs and skin may be inappropriate.

**Discussion**

UV light induces nucleotide substitutions, as well as proliferation, required for the establishment of transformed clones (3). Tumor initiation and promotion determine the incidence and
latency of carcinogenesis. Our data demonstrate that a defect in error-prone TLS, while probably leading to a reduction in skin cancer initiation, increases tumor promotion, which ultimately results in the acceleration of tumorigenesis. Of note, our mice carried a defect in NER that itself strongly accelerates skin carcinogenesis. It is of interest to investigate whether the Rev1B allele accelerates skin carcinogenesis in NER-proficient mice, displaying long tumor latency.

In contrast to the significantly reduced mutability of Rev1B/B cells (8), UV-induced mutagenesis at p53 appears only marginally decreased in the Rev1B/B; Xpc−/− epidermis. However, because the hyperproliferation increases the number of mutable cells in the Rev1-mutant skin, the actual reduction in the mutation rate may be stronger than the apparent one. In Rev1-mutant SCC, the distribution of C to T transitions at p53 was shifted toward YCpG sites, presumably reflecting Rev1-independent mutagenic TLS. Spontaneous deamination converts 5-methylcytosines within YCpG-CPDs to thymidines that can be replicated by TLS polymerase, incorporating adenosines and resulting in C to T transitions (26, 27; pathway 1 in Fig. S6). Frequencies of gross genomic alterations were unaltered in the Rev1B/B; Xpc−/− mice indicating that skin carcinogenesis is not driven by genomic deletions, caused by collapsed replication forks. For these reasons, we hypothesize that poly-

Fig. 3. DNA damage responses of the epidermis, 24 h after acute subtoxic UV exposure. (Scale bar, 100 μm.) Error bars: SEM. (A) IL-6 in the epidermis and its quantification. Cytoplasmic granular IL-6 expression throughout the basal layer. Nuclei are stained blue (DAPI). Rev1B/B; Xpc−/−: p_UV < 0.001, p_Xpc < 0.001. (B) Activation of DNA damage signaling and its quantification. SB: suprabasal layer; Xpc−/−: p_UV < 0.001. Rev1B/B; Xpc−/−: p_UV < 0.001, p_Xpc = 0.03. B: basal layer; Xpc−/−: p_UV < 0.001. Rev1B/B; Xpc−/−: p_UV < 0.001, p_Xpc < 0.001. (C) Senescence and its quantification. Rev1B/B; Xpc−/−: p_Xpc < 0.001. (D) Proliferation and its quantification. Rev1B/B; Xpc−/−: p_Xpc = 0.012. (E) Apoptosis and its quantification.

Fig. 4. Perturbation of replication in Rev1B/B; Xpc−/− MEFs. (A) Measurement of replication fork progression by alkaline denaturation. Top: following UV treatment, nascent forks are pulse labeled with [3H]thymidine (interrupted lines). The persistence of label at DNA ends indicates fork stalling. (B) Measurement of global replication. Top: the template is uniformly labeled with [14C]thymidine (uninterrupted lines), followed by UV treatment. Middle: nascent DNA is labeled with [3H]thymidine (dashed line). Bottom: DNA is cleaved at CPDs and [14C]-labeled fragments serve as internal standards. Fragments are fractionated on alkaline sucrose gradients and a size increase of [3H]-labeled fragments indicates progress of replication at damaged templates. (C, Top) Fork progression at undamaged templates. Bottom: delayed TLS in Rev1B/B; Xpc−/− MEFs. (D) Transient perturbation of replication in Rev1B/B; Xpc−/− MEFs is illustrated by the double arrows that have the same length in both Top and Bottom. Open symbols: 14C-labeled internal standard.
merase \( \eta \)-dependent TLS initiates carcinogenesis in the Rev1-mutant skin. In cultured Rev1\(^{B/B}\);Xpc\(^{-/-}\) MEFs, replication of the UV-damaged genome was delayed only transiently, possibly owing to the activity of the compensatory TLS pathway. Nevertheless, UV-induced DNA damage signaling was enhanced in these cells and in the skin. In the epidermis, this was initially associated with increased levels of senescence and apoptosis, and with the expression of IL-6 throughout the basal layer of the skin. In the epidermis, this was initially associated with induction of senescence may be partially dependent on Rev1-independent mutagenic TLS, underlies the acceleration of skin tumorigenesis. Thus, a defect in error-prone TLS shifts the activity of UV light from a tumor initiator toward a tumor promoter.

Replication stress-induced acanthosis may play a role in skin carcinogenesis in humans and mice defective for polymerase \( \eta \) (35, 36). It may also explain the finding that the additional disruption of error-prone TLS polymerase \( \eta \) in polymerase \( \eta \)-deficient mice further accelerates skin carcinogenesis (37, 38). Apparently, in skin carcinogenesis, mutagenesis by error-prone TLS is the price to pay for preventing the tumor-promoting consequences of inappropriate DNA damage responses.

**Materials and Methods**

**Generation of \( \text{Rev1}^{B/B};\text{Xpc}^{-/-} \) Mice.** Rev1\(^{B/B}\) 129 Sv/Ola mice (8) were crossed with Xpc\(^{-/-}\) mice (12) in the SKH albino hairless background for two generations and then intercrossed to obtain albino hairless Rev1\(^{B/B};\text{Xpc}^{-/-}\) and littermate control Xpc\(^{-/-}\) mice. Genotyping was performed as described (8, 13). All required permissions for the mouse experiments were obtained.

**Determination of the MED of UV and Skin Tumor Induction.** Patches of the lateral-dorsal skin were exposed to a Hanovia Kromayer lamp, as described in ref. 13. All exposures were given in duplicate on separate mice in two independent experiments. The mice were checked daily for erythema and/or edema and the MED was determined after 7 days.

To induce skin tumors, 11 Rev1\(^{B/B};\text{Xpc}^{-/-}\) and 14 Xpc\(^{-/-}\) mice (the ratios of males and females were similar in both groups) were placed in individual cages and chemically irradiated with a daily UV dose of 40 \( J/m^2 \), using Philips TL-12/40W lamps, as described before (13). This dose equals 0.08 \( x \) MED for Xpc\(^{-/-}\) mice. The dorsal skins were inspected weekly and the moment of appearance, number, size, morphology, and location of the tumors were recorded. When the largest tumors reached 4 mm in diameter, the animals were killed. The dissected tumors were split in two: one part was frozen in liquid nitrogen, while the other part was fixed in 4% buffered formaldehyde.

**Histology and Immunohistochemistry.** For investigating responses to acute UV exposure, four Rev1\(^{B/B};\text{Xpc}^{-/-}\) and four Xpc\(^{-/-}\) mice were treated with 350 \( J/m^2 \) at 46 days. Then, epidermal sheets were isolated from tumors and from untreated skin of the same mouse. Comparative genomic hybridization using a mouse BAC microarray containing 2,803 unique BAC clones from mouse genomic libraries at 1-Mb intervals was performed as described in ref. 39.

**Analysis of p53 Mutations.** Seven Rev1\(^{B/B};\text{Xpc}^{-/-}\) and 7 Xpc\(^{-/-}\) mice were treated with 40 \( J/m^2 \) for 46 days. Then, epidermal sheets were isolated and immunostained for mutant p53 patches as described in ref. 40.

**Cell Cycle Analysis ex Vivo.** Rev1\(^{B/B};\text{Xpc}^{-/-}\) (n = 20) and Xpc\(^{-/-}\) (n = 20) mice were treated with 250 \( J/m^2 \) UV using Philips TL-12/40W lamps, or mock-treated. At each time point, four mice per genotype received an intraperitoneal injection of BrdU (5 mg in 300 \( \mu L \) of PBS), 1 h before killing and dissection of the mid dorsal skin. An epidermal cell suspension was obtained and fixed in 7% ethanol. The DNA was stained for BrdU and with propidium iodide.

**Generation and Analysis of MEF Lines.** MEFs were isolated from 13.5-day-old embryos and immortalized with shRNA against p53 (42).

**UV-C exposure (Philips TL-UV lamp, peak 284 nm) was performed on growing MEF lines, washed with PBS. At a similar dose, UV-C induces an approximately 100-fold higher frequency of photolesions compared with longer wavelength UV, used in the in vivo experiments. Measurement of TLS...
by alkaline denaturation, after treatment with 10 J/m² UV-C, was performed as described (Fig. 4A) (7). Perturbation of replication in UV-exposed MEFs (5 J/m²) was determined as described (Fig. 4B) (7).

**Western Blot Analysis.** Proteins were separated on 10% polyacrylamide-SDS gels followed by transfer to Hybond-P membranes (Amersham Biosciences). Antibodies are described in Table S1.

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Supporting Information

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Fig. S1. C to T transitions at p53 in UV-induced SCC. (A) Distribution along p53 of the C to T transitions at dipyrimidines within YCpG sites. (B) Distribution along p53 of the C to T transitions at dipyrimidines outside of YCpG sites.
Fig. S2. Comparative Genomic Hybridization of DNA from UV-induced SCC. (A) Ten different SCC from Xpc−/− mice. Horizontal axis, chromosome number. Vertical axis, copy number alterations. Amplifications are in red; deletions in green. (B) Nine different SCC from Rev1B;Xpc−/− mice.
Fig. S2. Continued.

Rev1<sup>B/B</sup>, Xpc<sup>−/−</sup>
Fig. S3. Acanthosis and inflammatory responses of the Rev1B/B;Xpc−/− skin after 8 days of chronic UV treatment (40 J/m²/day). Hematoxylin and eosin (H&E) staining of frozen sections. The skin of the Rev1B/B;Xpc−/− mice is characterized by severe acanthosis, thickened stratum corneum, dermal edema, increased vascularization, and local infiltration of immune cells, including eosinophiles. E. epidermis; D, dermis; SC, stratum corneum; HF, hair follicle; white arrows, local infiltration of immune cells; black arrows, neo-vascularization.
Fig. S4. Different physiology of the Xpc−/− and Rev1B/Xpc−/− skins after 8 days of chronic UV treatment (40 J/m²/day). Proliferation (Ki-67), DNA damage signaling (SQ/TQ), senescence (Dec1), IL-6 and secreted growth factors (exemplified by amphiregulin). (Scale bar, 100 μm.) See the main text for details.
Fig. S5. Responses of Rev1<sup>fl</sup>; Xpc<sup>−/−</sup> MEFs to UV exposure. (A) Clonal survival to UV-C light was determined as described in ref. 1. (B) Phosphorylation at Chk1<sup>S317</sup> was assayed by Western blotting at different time points after exposure to 5 J/m<sup>2</sup> UV-C light. Hybridization with an Msh2 antibody serves as loading control.

Fig. S6. Putative mechanisms for the induction of C to T transitions at CPDs. (1) The efficient spontaneous deamination of a 5-methylcytosine (dotted C), embedded within a CPD (Top), generates a thymidine that can be bypassed by Polymerase η. Although TLS itself is “error-free,” bypass results in a C to T transition. (2) Inefficient deamination of an unmethylated cytosine embedded within a CPD to uracil (Top) triggers subsequent error-free TLS by Polymerase η. (3) Direct, Rev1-dependent “error-prone” TLS of a cytosine within a CPD.
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<th>Cellular process/ antigen class</th>
<th>Primary antibody</th>
<th>Visualization</th>
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*Nuclear counterstaining, DAPI. **To visualize UV-damaged cells, the sections stained for proliferation marker Ki-67 were treated with 0.1% of pepsin in 2N HCl during 30 min at room temperature, prior to the anti-CPD staining.