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Powerful Skin Cancer Protection by a CPD-Photolyase Transgene

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Summary

Background: The high and steadily increasing incidence of ultraviolet-B (UV-B)-induced skin cancer is a problem recognized worldwide. UV introduces different types of damage into the DNA, notably cyclobutane pyrimidine dimers (CPDs) and (6-4) photoproducts (6-4PPs). If unrepaired, these photolesions can give rise to cell death, mutation induction, and onset of carcinogenic events, but the relative contribution of CPDs and 6-4PPs to these biological consequences of UV exposure is hardly known. Because placental mammals have undergone an evolutionary loss of photolyases, repair enzymes that directly split CPDs and 6-4PPs into the respective monomers in a light-dependent and lesion-specific manner, they can only repair UV-induced DNA damage by the elaborate nucleotide excision repair pathway.
Results: To assess the relative contribution of CPDs and 6-4PPs to the detrimental effects of UV light, we generated transgenic mice that ubiquitously express CPD-photolyase, 6-4PP-photolyase, or both, thereby allowing rapid light-dependent repair of CPDs and/or 6-4PPs in the skin. We show that the vast majority of (semi)acute responses in the UV-exposed skin (i.e., sunburn, apoptosis, hyperplasia, and mutation induction) can be ascribed to CPDs. Moreover, CPD-photolyase mice, in contrast to 6-4PP-photolyase mice, exhibit superior resistance to sunlight-induced tumorigenesis.
Conclusions: Our data unequivocally identify CPDs as the principal cause of nonmelanoma skin cancer and provide genetic evidence that CPD-photolyase enzymes can be employed as effective tools to combat skin cancer.

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Introduction

The integrity of the genome is continuously threatened by a variety of endogenous and environmental agents. Exposure of the skin to ultraviolet (UV) light results in the formation of two main types of DNA damage: cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6-4) pyrimidone photoproducts (6-4PPs) [1]. These photolesions interfere with the vital cellular processes of transcription and replication, resulting in reduction of RNA synthesis, arrest of cell cycle progression, and apoptosis. Acute skin effects of UV exposure involve erythema (commonly known as sunburn), edema, and hyperplasia. Importantly, replication of UV-damaged DNA can induce mutations that ultimately may lead to the formation of skin cancer [2]. Indeed, UV-B light is a well-known universal carcinogen with increasing impact, as illustrated by the growing incidence of skin cancer in Western society, a change which may be attributed to altered lifestyle (i.e., natural and artificial sun tanning) and depletion of the protecting ozone layer [3, 4].

In mammals, the deleterious effects of the main photolesions are kept within bounds by nucleotide excision repair (NER), a repair system that continuously guards the genome and removes lesions from the DNA [2, 5]. The relevance of this repair mechanism for human health is highlighted by the inherited disorders xeroderma pigmentosum, Cockayne syndrome, and trichothiodystrophy, in which mutations in NER genes cause increased sensitivity of the skin to UV and, in the case of xeroderma pigmentosum, an over 1000-fold-increased susceptibility to sunlight-induced skin cancer [6].

NER proceeds via a complex multistep "cut and patch" reaction, involving the concerted action of ~30 proteins [2, 5, 7, 8], and consists of two subpathways: global genome NER (GG-NER) and transcription-coupled NER (TC-NER). GG-NER repairs helix-distorting lesions throughout the entire genome but is hampered by the fact that certain types of damage are less well recognized by the main damage-sensing XPC-HR23B complex and are accordingly less efficiently repaired. To prevent that such lesions, when present in the transcribed strand of active genes, block transcription elongation for too long, TC-NER preferentially removes transcription-blocking DNA injury, presumably with stalled RNA polymerase II (RNApolII) as the damage sensor [9–11]. Thus, TC-NER acts as an efficient backup system

for lesions that are slowly or not at all repaired by GG-NER.

Because of the distinct helix-deforming characteristics of CPDs and 6-4PPs, considerable differences exist in the efficiency at which these photoproducts are processed by NER. UV-exposed human and rodent cells efficiently remove 6-4PPs from their genome (>75% within 4 hr), mainly by GG-NER [1, 11]. On the other hand, GG-NER-mediated repair of the less helix-distorting CPD lesions proceeds considerably slower in human fibroblasts (>60% repair in 24 hr) and is virtually absent in rodent cells [1, 9].

Apart from the evolutionary highly conserved NER pathway, nature has evolved a very powerful repair mechanism for removal of UV-induced DNA lesions: photoreactivation. This is accomplished by photolyases, enzymes that bind CPDs or 6-4PPs in a lesion-specific manner and rapidly revert the damage by splitting the dimer back to undamaged bases with the energy of visible light [12]. Photolyases are highly conserved and likely represent an ancient repair system that has existed from very early on in evolution. Intriguingly, placental mammals do not possess photolyases [13, 14]. As a result, man and mice rely solely on the complex NER for removal of UV-induced damage.

Because the number of CPDs induced by UV is approximately 3-fold higher than the number of 6-4PPs, much of the mutagenic and carcinogenic potential of sunlight is expected to arise from CPDs [15–18]. However, studies with plasmids containing site-specific lesions have shown that 6-4PPs are potentially much more mutagenic [1]. Because 6-4PPs and CPDs are induced simultaneously, and NER eliminates both photolesions, it has been impossible to determine in an unequivocal fashion the relative contribution of CPDs and 6-4PPs to cytotoxic, mutagenic, and carcinogenic events at the level of the intact organism. With the substrate-specificity and light-dependent action of photolyases and the absence of this repair mechanism in placental mammals, we recently started to explore the biological effects of CPDs in the skin of transgenic mice that ubiquitously express a marsupial CPD-photolyase. We have shown that accelerated repair of CPD lesions by photoreactivation dramatically reduces the acute UV-mediated skin effects [19].

To assess the contribution of 6-4PP independently, we report here the generation of mice expressing the *Arabidopsis thaliana* 6-4PP-photolyase from the ubiquitous β-actin promoter. This mouse, together with the CPD-photolyase mouse and transgenic animals that express both photolyases, offers the unique opportunity to dissect the individual effects of CPDs versus 6-4PPs in UV-induced responses such as mutagenesis and carcinogenesis.

Results

Generation and Characterization of 6-4PP-Photolyase Transgenic Mouse and Cell Lines

To obtain mice ubiquitously expressing a 6-4PP-specific photolyase transgene (β-act-6-4PP-PL mice, hereafter referred to as "6-4PP-PL" mice), we have made a construct containing the *Arabidopsis thaliana* 6-4PP photolyase cDNA [20], preceded by the chicken β-actin promoter and CMV enhancer, and completed with the 3′ part of the human β-globin gene (Figure 1A). We generated several independent 6-4PP-PL transgenic founder lines. The transgene was transmitted in a Mendelian fashion, whereas heterozygous offspring were fertile and did not show any overt phenotype. We further investigated a 6-4PP-PL mouse line containing three copies of the transgene at chromosome 7F3-4 (data not shown).

RT-PCR analysis on total skin RNA showed the presence of a 300 bp PCR fragment, indicating proper transcription and splicing of the transgene (Figure 1B). Immunoblot analysis of protein extracts from wild-type and 6-4PP-PL transgenic MDFs with anti-6-4PP-PL antibodies yielded a band of the expected size (60 kDa) in transgenic cells only (Figure 1C). Immunocytochemical analysis of these cells revealed a nuclear localization (Figure 1D).

6-4PP-PL MDFs were subjected to an immunocytochemical repair assay to investigate whether the 6-4PP-PL gene product was biologically active [21]. As shown in Figure 1E, immediately after exposure of cells to 20 J/m^2 UV-C, bright immunofluorescent signals indicate the induction of 6-4PPs. When cells are subsequently kept in the dark (thus withholding the photolyase from its source of energy), 6-4PPs are still present, indicating that NER-mediated removal of 6-4PP lesions is relatively slow (75% repair in 4 hr [9, 22]) when compared to photoreactivation because it leaves most of the lesions unrepaired after 1 hr. In marked contrast, exposure of UV-irradiated 6-4PP-PL MDFs to 1 hr of photoreactivating light resulted in a strong reduction of 6-4PP levels (Figure 1E), whereas levels of CPDs remained unchanged (data not shown).

Taken together, these data demonstrate that we have successfully generated a transgenic mouse model that expresses a biologically active *A. thaliana* 6-4PP photolyase and allows light-dependent and lesion-specific repair of 6-4PPs.

Effects of CPD and/or 6-4PP Photoreactivation on Survival of UV-Exposed Fibroblasts

To investigate the contribution of 6-4PPs to UV-induced cell death, we first completed our panel of CPD-PL and 6-4PP-PL transgenic MDF lines with the isolation of cell lines expressing both photolyases. To this end, 6-4PP-PL mice were bred with β-act-CPD-PL mice (hereafter referred to as "CPD-PL" mice) to obtain double-transgenic animals. Like the parent mouse lines, CPD-PL/6-4PP-PL mice were born in a Mendelian ratio and are phenotypically normal. As expected on the basis of results obtained with single-transgenic cell lines, UV-irradiated MDFs isolated from these mice show light-dependent removal of both CPDs and 6-4PPs (data not shown).

The UV sensitivity of the various MDF lines was determined with a ^{3}H-thymidine incorporation-based survival assay [23]. Figure 2A shows the fraction of surviving cells upon exposure to increasing doses of UV-C. In line with our previous observations, CPD-PL MDFs display wild-type UV sensitivity when kept in the dark, whereas

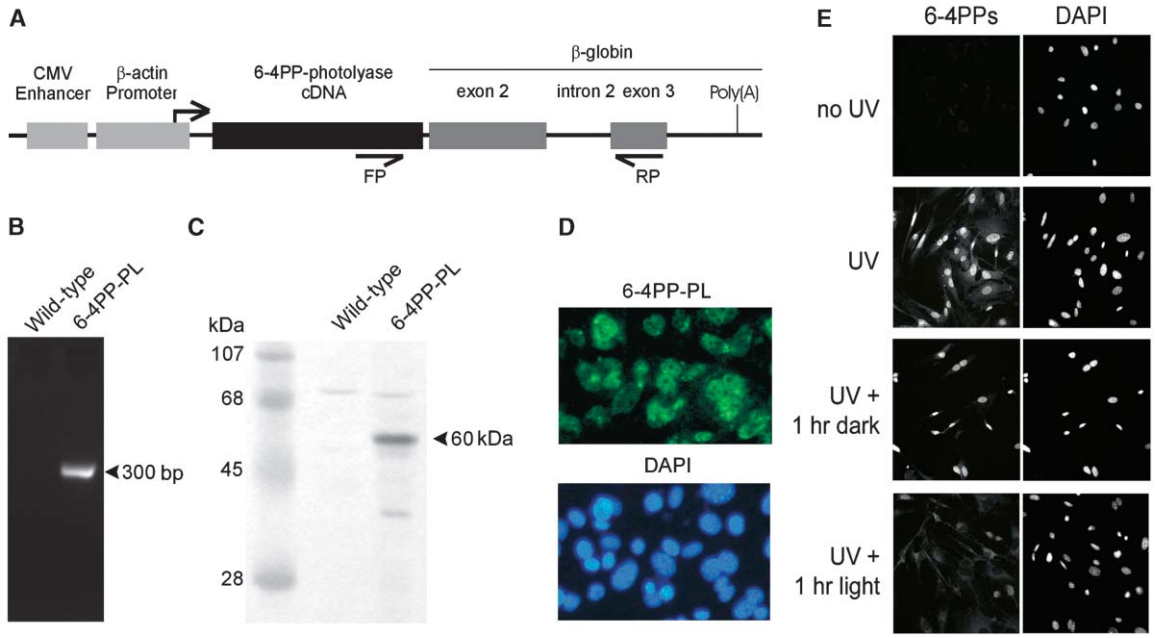

Figure 1. Expression of *Arabidopsis thaliana* 6-4PP Photolyase in Transgenic Mice

(A) Expression construct for the generation of transgenic mice expressing *Arabidopsis thaliana* 6-4PP photolyase. Arrows indicate primers used for the RT-PCR. FP denotes forward primer, and RP denotes reverse primer.

(B) RT-PCR on skin extracts of a transgenic mouse line shows the presence of 6-4PP photolyase mRNA.

(C) Immunoblot analysis of protein extracts from cultured wild-type and 6-4PP-PL mouse dermal fibroblasts (MDFs) shows the presence of the 60 kDa 6-4PP photolyase.

(D) Immunocytochemical detection of 6-4PP photolyase in cultured 6-4PP-PL MDFs. Nuclei are visualized by DAPI staining.

(E) Photoreactivation 6-4PP lesions in cultured 6-4PP-PL MDFs. Cells were exposed to 20 J/m^2 of UV-C light and were subsequently exposed to photoreactivating light for 1 hr or kept in the dark. Photolesions were detected by immunofluorescent labeling with 6-4PP-specific antibodies and FITC-conjugated secondary antibodies. Nuclei are visualized by DAPI staining.

fast repair of CPDs by exposure of cells to photoreactivating light dramatically reduces the level of cell death, as evident from the enhanced cellular survival [19]. Similarly, 6-4PP-PL and CPD-PL/6-4PP-PL MDFs demonstrate a UV sensitivity in the wild-type range when kept in the dark. Importantly, photoreactivation of 6-4PPs did not significantly enhance cellular survival. Photoreactivation of both lesions in UV-exposed CPD-PL/6-4PP-PL MDFs reveals a UV sensitivity comparable to that observed in CPD-PL cells. Thus, no additive effect of photoreactivation of 6-4PPs could be observed, pointing toward CPDs as the major trigger for UV-induced cell death.

UV-exposed fibroblasts undergo a transient reduction in RNA synthesis. To investigate which class of lesions is responsible for this response, we have measured the relative incorporation of ^{3}H-uridine (indicative for the level of RNA synthesis) in UV-treated photolyase cells. As expected, when kept in the dark, all MDF lines showed a reduced level of RNA synthesis 15 hr after exposure to 10 J/m^2 of UV-C (Figure 2B). However, photoreactivation of CPDs in UV-exposed CPD-PL cells largely reduced the drop in RNA synthesis. In marked contrast, exposure of 6-4PP-PL cells to photoreactivating light did not significantly affect the inhibition of RNA synthesis. When UV-exposed CPD-PL/6-4PP-PL MDFs were treated with photoreactivating light, we noticed an attenuation of the inhibiting effect of UV on RNA synthesis; the attenuation was comparable to that observed when only CPDs were photoreactivated (Figure 2B).

Taken together, these findings suggest that UV-induced transcription inhibition and cell death can be mainly ascribed to CPD lesions in the DNA, whereas 6-4PPs appear of minor importance in these processes.

Contribution of 6-4 Photoproducts to Acute Skin Effects

Before studying the consequence of rapid removal of 6-4PPs on acute UV-mediated skin effects, we tested whether 6-4PP-PL mice are able to remove 6-4PPs from the skin. To this end, we employed an immunocytochemical assay for detection of 6-4PPs. When the depilated back of 6-4PP-PL mice was exposed to 1 MED (minimal erythemal dose) of UV-B and animals were subsequently kept in the dark for 3 hr, we observed bright nuclear signals in epidermal and (upper) dermal cells, showing the presence of 6-4PPs (Figure 3, middle). Despite a functional NER mechanism in these animals, 6-4PPs were still present. This finding suggests that repair of 6-4PPs in the intact skin apparently may progress slower than in cultured cells. Importantly, exposure of UV-treated animals to photoreactivating light appeared sufficient to cause a strong reduction in 6-4PP levels in dermis and epidermis (Figure 3, bottom). Immunohistochemical staining with CPD antibodies revealed

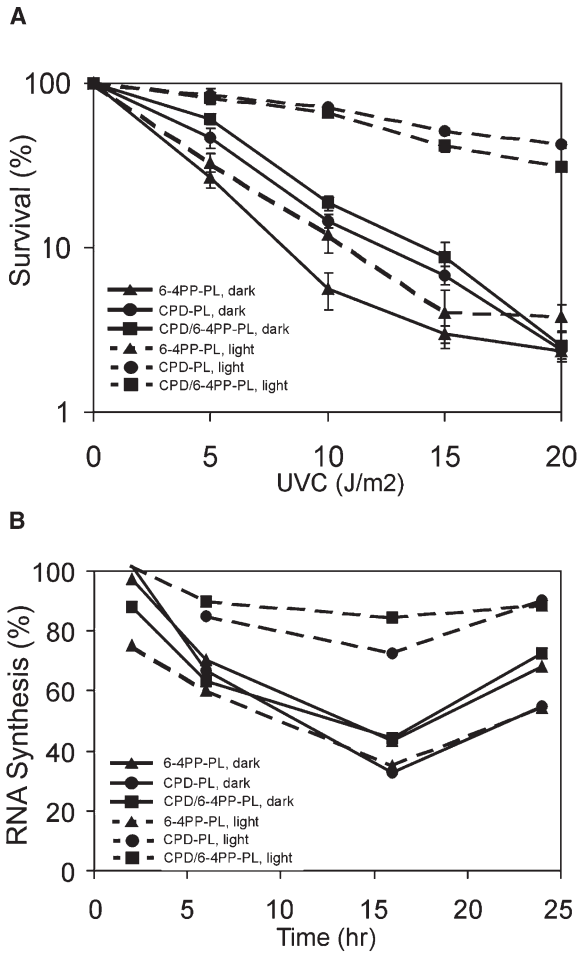

Figure 2. Effect of Photoreactivation of Photoproducts on Cellular Survival and RNA Synthesis

(A) UV survival of CPD-PL, 6-4PP-PL, and double-transgenic CPD-PL/6-4PP-PL MDFs upon exposure to increasing doses of UV-C light, with or without subsequent treatment with photoreactivating light for 1 hr.

(B) RNA synthesis of CPD-PL, 6-4PP-PL, and double-transgenic CPD-PL/6-4PP-PL MDFs followed in time after exposure to a single dose of 10 J/m^2 of UV-C light with or without subsequent treatment with photoreactivating light.

no alterations in the level of CPDs in UV-exposed 6-4PP-PL mice (data not shown). In conclusion, the *A. thaliana* 6-4PP-PL is active in epidermal as well as dermal cells and can specifically photoreactivate the majority of 6-4PPs within 3 hr, leaving the CPDs unaffected.

Exposure of the skin to UV light results in the formation of so-called sunburn cells because of an apoptotic response of keratinocytes. To investigate whether rapid removal of 6-4PPs by photoreactivation suppresses induction of apoptosis, we exposed 6-4PP-PL mice to 1 MED UV-B. Subsequently, animals were either kept in the dark or exposed to photoreactivating light for 3 hr. Forty hours after UV exposure, mice were sacrificed, and skin biopsies were processed for TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling) analysis. As expected, apoptotic nuclei were visible in the UV-exposed epidermis of 6-4PP-PL animals that had not been exposed to photoreactivating light (Figure 4A). However, in contrast to photoreactivation of CPDs [19], rapid removal of 6-4PPs by photolyase did not result in a detectably decreased apoptotic response. Treatment of UV-exposed CPD-PL/6-4PP-PL double-transgenic mice with photoreactivating light reduced the apoptotic response to a level comparable to that observed when only CPDs were removed.

Repeated exposure to UV results in epidermis thickening, known as hyperplasia. Photolyase transgenic mice were exposed to 1 MED UV-B light or 1 MED UV-B light and photoreactivating light for 4 consecutive days to investigate whether 6-4PPs play a role in the induction of hyperplasia. One week after the start of the experiment, animals were sacrificed. As shown in Figure 4B, haematoxylin/eosin-stained skin sections of UV-exposed 6-4PP-PL mice that had not received photoreactivating light revealed a clear induction of epidermal hyperplasia. Whereas light-mediated CPD removal in UV-exposed CPD-PL mice prevents the induction of hyperplasia [19], treatment of UV-exposed 6-4PP-PL mice with photoreactivating light did not abolish hyperplasia. On the other hand, photoreactivation of both CPDs and 6-4PPs in double-transgenic animals resulted in a strong inhibition of hyperplasia, as observed when only CPDs have been removed by photolyase. These findings point toward CPDs as the major trigger for UV-induced apoptosis and hyperplasia in NER-proficient mice.

Contribution of CPDs and 6-4PPs to Mutagenic Events

Photolyase mice were bred with *lacZ* reporter mice containing a repeat of 20 copies of a *lacZ* reporter plasmid to investigate whether rapid removal of CPDs and/or 6-4PPs affects the UV-induced mutation frequency [24]. Animals were exposed to a single dose of 1 MED UVB, with or without subsequent exposure to photoreactivating light, and after 2 weeks (the time required to allow fixation of mutations) genomic DNA was extracted from the epidermis. Rescued *lacZ* plasmids were used to transform a *lacZ*$^-$/*galE*$^-$ *E. coli* strain and determine mutation frequencies by growing the cells on selective plates. Typically, mutation frequencies of 150.10^{-5} were observed for UV-exposed photolyase mice that subsequently had been kept in the dark. Analysis of epidermal DNA of nonirradiated animals yielded a background mutation frequency of 15.10^{-5}. Figure 5A shows the mutation frequencies, expressed as a percentage of the mutation frequency observed in animals that have not been exposed to photoreactivating light, in UV-exposed wild-type, CPD-PL, 6-4PP-PL, and CPD-PL/6-4PP-PL mice. Nearly complete photoreactivation of CPDs (as determined by immunohistochemistry) in CPD-PL mice reduced the UV-induced mutation frequency in the epidermis by 40%. In contrast, removal of 6-4PPs did not significantly affect the mutation frequency. When both 6-4PPs and CPDs were photoreactivated in UV-exposed CPD-PL/6-4PP-PL mice, we observed a mutation frequency similar to that found in CPD-PL mice. Thus, rapid removal of CPDs rather than 6-4PPs reduces the UV-induced mutation frequency.

Mutations in the *p53* tumor suppressor gene play a crucial role in the early development of squamous cell carcinomas. Notably, mutant p53 protein can be immu-

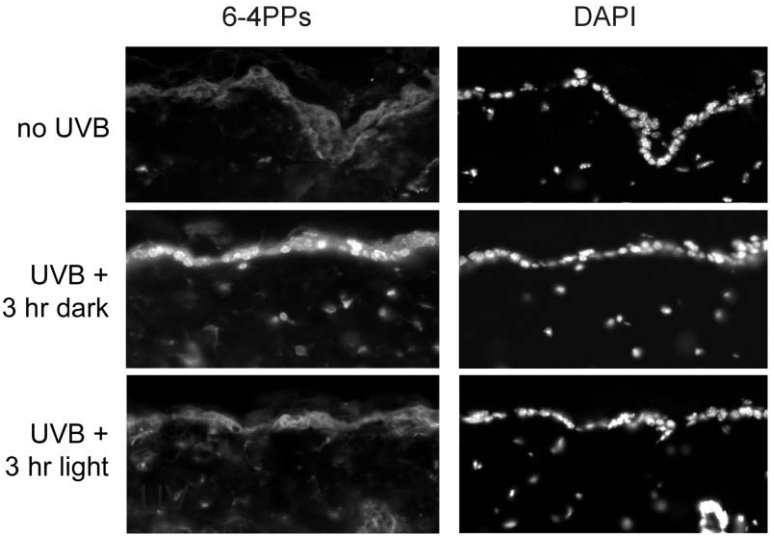

Figure 3. Photoreactivation of 6-4PPs in the Skin of 6-4PP Photolyase Transgenic Mice

Induction of 6-4PPs in the depilated dorsal skin of 6-4PP-PL mice by 1 MED of UV-B light and the effect of subsequent exposure to photoreactivating light for 3 hr. Photolesions were detected by immunofluorescent labeling with 6-4PP-specific antibodies and FITC-conjugated secondary antibodies. Nuclei are visualized by DAPI staining.

nohistochemically detected in clusters of preneoplastic epidermal cells induced upon chronic treatment with UV, and the number of these "p53 patches" appears a direct measure for UV-induced tumor risk [25, 26]. To investigate the protective effect of photoreactivation on the process of carcinogenesis, we gave hairless photolyase mice a daily dose of UV-B (1 MED) with or without subsequent treatment with photoreactivating light. Twenty-one days after the first exposure, mice were sacrificed, and epidermal sheets were analyzed for the presence of p53 patches. Figure 5B shows the number of p53 patches observed in a fixed area on the back of photolyase mice. Whereas p53 patches were absent in nonexposed animals (data not shown), UV irradiation resulted in a clear induction of p53 patches in both CPD-PL and 6-4PP-PL mice. Interestingly, photoreactivation of CPDs resulted in a dramatic reduction in the number of p53 patches and reached levels close to that of unirradiated control animals. Removal of 6-4PPs, however, did not significantly reduce the number of p53 patches. These data suggest a substantial role for unrepaired CPDs in the early stages of skin cancer.

Contribution of CPDs and 6-4PPs to Skin Cancer

Photolyase mice and wild-type littermates were subjected to daily treatments with 500 J/m^2 UV-B and then exposed to photoreactivating light for 3 hr to determine whether enhanced removal of photolesions also affects tumor formation. As shown in Figure 6A, such a chronic UV exposure experiment results in a continuous increase in the number of tumor-bearing wild-type animals, the increase starting 6 weeks after the first exposure to UV and reaching a maximum after 17 weeks, when all the animals have developed one or more tumors. An exponential increase in tumor yield was observed in wild-type animals, resulting in an average of seven tumors per animal after 20 weeks of treatment (Figure 6B). Histopathological analysis identified the tumors as skin carcinomas (data not shown). Strikingly, photoreactivation of CPDs results in a tremendous reduction in carcinogenic events. As shown in Figure 6, both the fraction of tumor-bearing CPD-PL mice (Figure 6A) and the tumor yield (Figure 6B) remain remarkably low. These data demonstrate a clear protective effect of photoreactivation of CPD lesions against skin tumor formation in mice. In marked contrast, photoreactivation of 6-4PPs (either in a wild-type or in a CPD photolyase transgenic background) seems to result in a slight, but statistically not significant, reduction in tumor yield and incidence. Thus, in line with the mutagenesis and p53 patch data, CPD lesions appear the major cause of UV-induced carcinogenesis in the skin.

Discussion

Photolyase Transgenic Mice

Previously, with CPD photolyase transgenic mouse models, we have shown that photoreactivation of CPDs prevents the onset of apoptosis, epidermal hyperplasia, and erythema (sunburn), pointing to CPDs as a major trigger for acute skin effects [19]. However, these studies neglected long-term effects and the contribution of the even more helix-distorting 6-4PPs on these endpoints. To define the biological consequences of unrepaired 6-4PPs in DNA, we have generated transgenic mice carrying the *Arabidopsis thaliana* 6-4PP photolyase gene under control of a ubiquitous promoter. Although more related 6-4PP photolyase cDNAs are available (such as the *Drosophila melanogaster* 6-4PP photolyase), we have chosen to use the plant enzyme because it was previously shown to be active in transiently transfected mammalian cells [27]. 6-4PP-PL mice properly express the transgene, and the heterologous photolyase is biologically active, as shown by the ability of transgenic mice and corresponding fibroblast lines to efficiently and specifically remove 6-4PPs in a light-dependent manner.

CPDs, Rather Than 6-4PPs, Form the Main Trigger for UV-Induced Cell Death and Transcription Inhibition

DNA lesions that block elongating RNApolII have been shown to cause a (temporary) overall inhibition of transcription, preventing cells from transcribing damaged

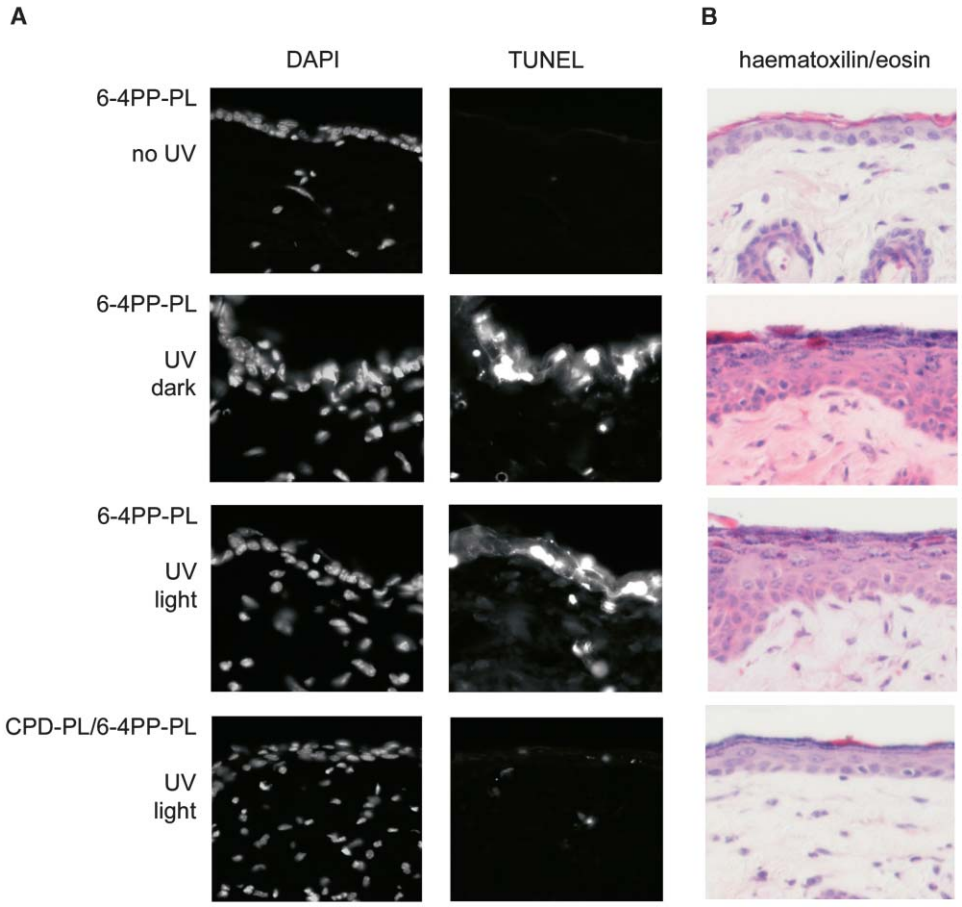

Figure 4. Effect of Photoreactivation of 6-4PPs and CPDs on UV-B-Induced Acute Responses

(A) Apoptotic response in the skin of photolyase mice. Animals were exposed to 1 MED of UV-B light and then exposed to either photoreactivating light for 3 hr or darkness. Apoptosis was measured 40 hr after UV exposure by a TUNEL assay.

(B) Hyperplasia in the epidermis of photolyase mice. Animals were exposed to 1 MED UV-B light for 4 subsequent days and then exposed to photoreactivating light (3 hr) or darkness. Four days after the last exposure, mice were sacrificed, and skin sections were stained with haematoxylin and eosin.

genes [28]. Moreover, transcription-blocking DNA lesions in the transcribed strand of active genes trigger an apoptotic response [29–31]. Because both CPDs and 6-4PPs potentially block RNApolII [2], photoreactivation of either class of photolesions is expected to ablate UV-induced cell killing and transcription inhibition. However, whereas photoreactivation of CPD lesions results in a markedly improved survival of UV-exposed cells, photoreactivation of 6-4PPs apparently does not noticeably affect UV-resistance, even in a CPD-PL transgenic background. Similarly, rapid CPD removal by photolyase reduces UV-mediated transcription inhibition, whereas photoreactivation of 6-4PPs had no marked effect. The different response of UV-exposed cells to photoreactivation of CPDs or 6-4PPs might be explained to some extent by the fact that the number of CPDs induced by UV is approximately 3-fold higher than the number of 6-4PPs. More likely, however, because RNApolII blocking lesions form the major trigger of apoptosis and overall suppression of transcription initiation, the difference reflects the efficiency at which NER can remove CPDs and 6-4PPs from the template strand of active genes. Whereas rodent cells relatively rapidly remove 6-4PPs by both GG-NER and TC-NER, CPDs are only removed by TC-NER [9]. Providing NER-proficient mouse cells with a CPD photolyase may therefore be significantly more effective than introduction of a 6-4PP photolyase. In conclusion, our data provide evidence that in an NER-proficient background, CPDs rather than 6-4PPs are the main trigger of cell death and inhibition of transcription initiation. These findings confirm previously performed transfection studies and marsupial system studies in which CPDs were reported to be the main intermediate in UV-induced apoptosis [16, 32].

Similar experiments need to be performed in totally NER-deficient (XPA) cells supplemented with either CPD-PL, 6-4PP-PL, or both photolyases to unequivocally establish the relative intrinsic effects of unrepaired CPDs and 6-4PPs. Because repair of UV lesions in these cells is completely dependent on photoreactivation, the UV dose can be adapted in such a way that, after exposure of cells to photoreactivating light, equal amounts of unrepaired CPDs or 6-4PPs remain present in the genome, thereby enabling a true comparison of cyto-

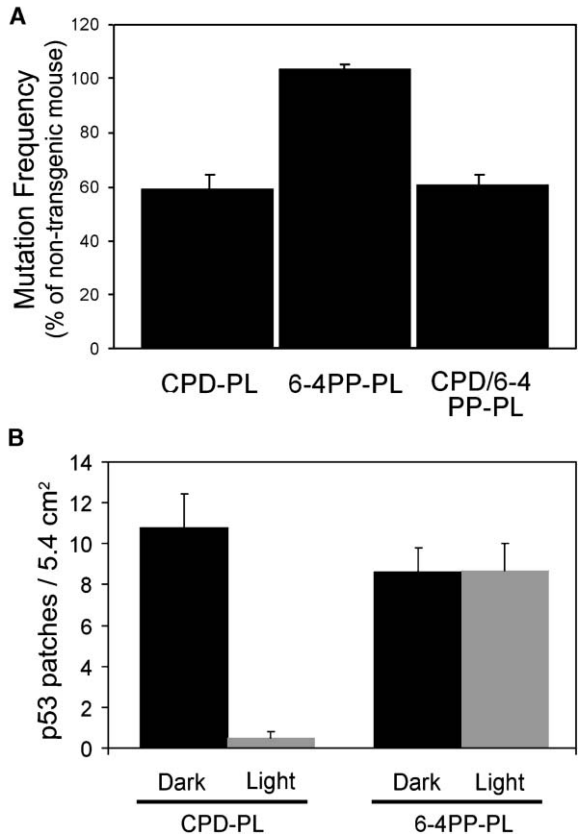

Figure 5. Effect of Photoreactivation of 6-4PPs and CPDs on UV-B-Induced Mutation Frequency and Induction of p53 Patches

(A) Photolyase transgenic mice, carrying the IM30 *lacZ* mutation frequency reporter gene, received a single dose of UV-B light (1 MED) and were either exposed to photoreactivating light (3 hr) or kept in the dark. Subsequently, animals were kept in the dark for 2 weeks to allow mutation fixation. The epidermal mutation frequency in the *lacZ* reporter gene of mice exposed to photoreactivating light was determined and expressed as a percentage of the frequency in mice that were kept in the dark.

(B) Photolyase mice were exposed to a daily dose of 1 MED UV-B light and subsequently received photoreactivating light (3 hr) or were kept in the dark. After animals were housed for 21 days in darkness, animals were sacrificed and epidermal sheets were isolated. The epidermis was further processed for immunocytochemical staining of mutant p53 and mounted on glass slides. Clusters of more than 10 immunoreactive cells were marked as p53 patches, and the number of p53 patches per 5.4 cm^2 was counted. Error bars indicate the standard error of the mean.

toxic effects of these lesions. Indeed, with human XPA cells supplemented with photolyases, Nakajima and coworkers [33] showed that in the absence of NER, a single 6-4PP is more toxic than a CPD.

Photoreactivation of CPD and/or 6-4PPs in UV-Exposed Skin

Exposure of the skin to UV light causes basal keratinocytes to undergo apoptosis, as shown by the dose-dependent occurrence of sunburn cells in the epidermis. Upon repeated exposure, however, keratinocytes proliferate, resulting in epidermal hyperplasia. Previously, we have shown a considerable contribution of CPDs to the induction of both UV-induced epidermal apoptosis and hyperplasia [19]. Here we provide evidence that photoreactivation of 6-4PPs does not detectably decrease the magnitude of these processes, which further points toward CPDs as the major trigger for apoptosis and hyperplasia in the epidermis of the murine skin. Analogous to the different UV sensitivity of cultured CPD and 6-4PP photolyase cells, the lack of a reduction of acute skin effects in UV-exposed 6-4PP-PL animals can be explained by the fact that 6-4PPs, in contrast to CPDs, are properly repaired by NER.

Mutagenic and Carcinogenic Properties of CPDs and 6-4PPs

With a *lacZ* mutation reporter mouse model, we showed that photoreactivation of CPDs reduces the mutation frequency in the epidermis of UV-exposed animals, whereas photolyase-mediated removal of 6-4PPs (even in combination with photoreactivation of CPDs) hardly affected the UV mutation frequency. This may find its origin in the ability of NER to efficiently remove 6-4PPs (thus prohibiting fixation of mutations), whereas CPDs are not repaired by NER in the transcriptionally silent *lacZ* repeats. These data fit well with a study by You and coworkers [27], showing that photoreactivation of CPDs rather than 6-4PPs reduces the mutation frequency in cultured mouse embryonic fibroblasts carrying the BigBlue mutation reporter system.

Despite the fact that most of the CPDs in UV-exposed CPD-PL mice are removed by photoreactivation [19], the reduction in mutation frequency appears limited to some 40%. Evidently, not all CPD lesions have been removed from the heterochromatin of the transcriptionally silent *lacZ* gene, which in part may be explained by the fact that photoreactivation of CPDs (at least in yeast) is modulated by chromatin structure [34]. The relatively small reduction in the *lacZ* mutation frequency likely finds its origin in the fact that photoreactivation of CPDs not only reduces the number of mutagenic DNA lesions, but concomitantly eliminates the apoptotic response, thus permitting survival of keratinocytes with a relatively low number of mutations.

Induction of mutations is of crucial importance in the multistep process of carcinogenesis. The frequency of clusters of cells containing mutated p53 protein has been shown to correlate well with rates of carcinoma induction in murine skin [25, 26]. We observed an over 20-fold reduction in the formation of p53 patches upon photoreactivation of CPDs. This finding at first sight contrasts the only 2-fold reduction in mutation frequency in the *lacZ* gene in the UV-exposed skin of CPD-PL mice. However, consistent with the multihit hypothesis, p53 patches are likely to arise not only from mutations in the p53 gene, but also from mutations in other genes in order to confer the growth advantage that allows clonal expansion of a mutated keratinocyte. Thus, if p53 patch formation would require mutations in, for example, four genes, a 2-fold reduction in mutation frequency by photoreactivation of CPDs (as determined for the *lacZ* gene) will already translate into a 16-fold reduction in the number of p53 patches.

Last but not least, in addition to a strong inhibition of

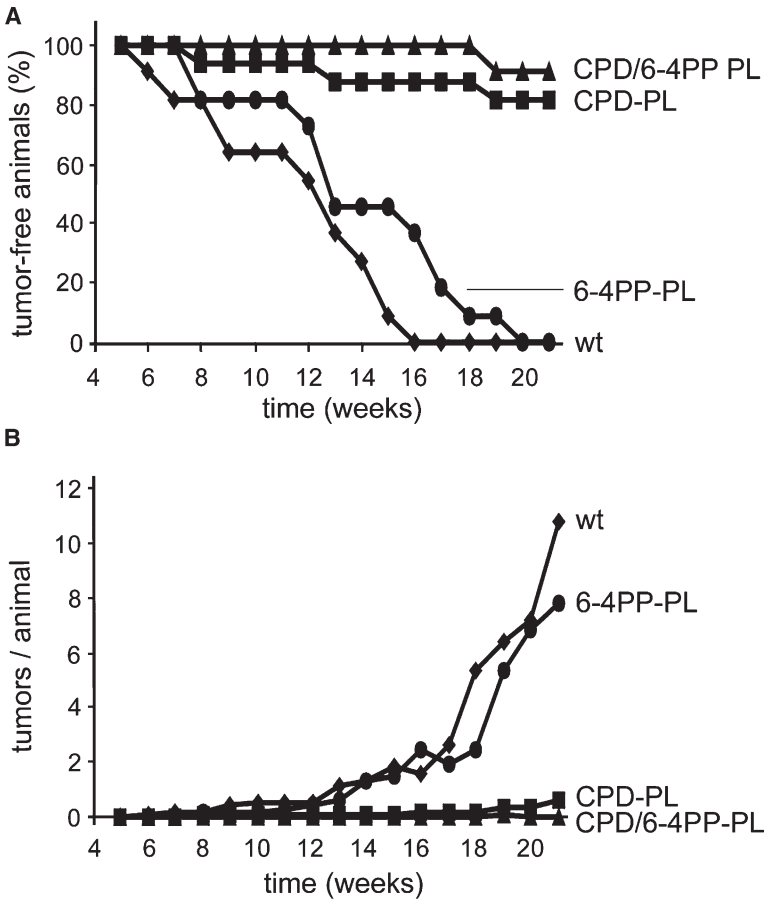

Figure 6. Effect of Photoreactivation of 6-4PPs and CPDs on Skin Carcinomas

CPD-PL (n = 11; squares), 6-4PP-PL (n = 16; circles), and CPD/6-4PP-PL (n = 11; triangles) mice, as well as their wild-type littermates (n = 11; diamonds), received daily treatments of UV-B light (500 J/m^2/day) and then exposure to photoreactivating light for 3 hr.

(A) The fraction of tumor-bearing mice in time after the first UV treatment.

(B) The average yield of squamous-cell carcinomas per mouse in time after the first UV treatment.

the early stages of skin cancer, we show a tremendous profit of photoreactivation of CPDs in terms of actual tumor formation. Apparently, the speed at which mutations accumulate in chronically UV-exposed CPD-PL mice is sufficiently low to retard the appearance of tumors. Interestingly, 6-4PP-PL transgenic mice seem to show a tendency to develop less squamous cell carcinomas than wild-type mice, which may point to mild carcinogenic properties of this lesion compared to CPDs. However, as also holds for other parameters, a direct comparison of the carcinogenic potential of 6-4PPs versus CPDs should await a semiquantitative carcinogenesis study of photolyase transgenic mice in an *Xpa*-deficient background, allowing induction of comparable levels of unrepaired 6-4PPs and CPDs.

Although crucial, the process of carcinogenesis takes more than the induction of mutations; it also requires the proper environment for precancerous cells to develop into tumors. A prerequisite for the occurrence of carcinomas is suppression of the immune system; without UV-induced immunosuppression, tumors will be rejected [35]. DNA damage has been implicated to play an important role in UV-mediated immunosuppression. Therefore, the observed skin cancer protection may arise through a reduced mutation load in combination with an altered immune response. Whether the immune response is indeed affected by photoreactivation of CPDs or 6-4PPs is currently under investigation.

Concluding Remarks

We have generated a comprehensive set of photolyase transgenic mouse models that enable thorough analysis of the contribution of individual classes of photolesions to UV-induced responses in the prime target of UV light, the skin. The overall picture emerging from this work is that CPD lesions are the major intermediate in all UV-induced processes—including mutagenesis and, importantly, carcinogenesis—studied thus far in the epidermis of these mouse models. To reveal the true relative potential of CPDs and 6-4PPs to mediate these processes, we are currently breeding photolyase mice with totally NER-deficient *Xpa* animals [36].

When extrapolating these data to man, one should take into account that humans, in contrast to rodents, have some repair of CPDs residing in nontranscribed DNA [1, 4]. Therefore, the protective effect of CPD photolyase may be somewhat less pronounced in man. Yet previous work on the opossum *Monodelphis* suggests that CPD photolyase improves protection against UV-induced skin cancer even in the presence of a functional NER system [37]. Recently, topically applied liposome encapsulated photolyase enzymes were found to effectively repair CPD lesions in the human skin [38]. Whereas the best remedy against skin cancer remains protection from UV exposure (i.e., prevention from exposure and sunscreens), our results predict that photoreactivation of CPDs in the skin by photolyase introduced via lipo-

somes may alleviate the incidence of skin cancer. A major drawback of liposome usage, apart from the temporary mode of action, is the variable efficiency of transfer and the need for careful and timely application. As the skin is one of the most accessible organs for gene therapy, this method, once absolutely safe, may provide the additional defense required to counteract the tremendous increase in skin cancer. Particularly, it may alleviate the extreme sun sensitivity and proneness to skin cancer of patients suffering from xeroderma pigmentosum.

Experimental Procedures

Generation of β-act-6-4PP Photolyase Transgenic Mice

The construct for the generation of 6-4PP-PL transgenic mice was cloned in vector pSP72 (Promega) and contained the chicken β-actin promoter with the CMV enhancer (from pCY4B), followed by the *Arabidopsis thaliana* 6-4PP-PL cDNA [20]. At the 3′ end, exon 2 (the last 22 bp), intron 2, exon 3, and the 3′ untranslated region (including the polyadenylation signal) of the human β-globin gene were inserted. The construct was excised from the plasmid and injected in the pronucleus of fertilized eggs derived from FVB/N intercrosses, as described previously [39].

Transgenic animals were identified by Southern blot analysis of genomic tail DNA, with the 6-4PP-PL cDNA as a probe. The number of integrated transgenes was determined as described by Schul et al. [19]. The site of chromosomal integration was determined by fluorescent in situ hybridization (FISH) analysis of metaphase chromosome spreads [40] with mouse dermal fibroblasts and the 6-4PP-PL cDNA probe. Routine genotyping of mice was performed by PCR analysis with primer set 5′-GCA CGA TTC AGC AAG CAA GG-3′ and 5′-CGG TAC CTC TAC CTA TTT GAG TTT TG-3′.

DNA Repair Assays

UV Survival

UV radiation sensitivity was determined as described previously [23], except that a photoreactivation step was incorporated in the assay. MDFs were washed with PBS, exposed to UV-C light (Philips TUV germicidal lamp) and subsequently kept in Hank's buffer. Next, the cells were either exposed to photoreactivating light or kept in the dark for 1 hr, as described above, after which cell culturing was continued in the dark. After 2 days, the number of proliferating cells was determined from the amount of radioactivity incorporated during a 3 hr pulse with [^{3}H]thymidine. Cell survival is expressed as the percentage of radioactivity in exposed cells in relation to that in untreated cells.

RNA Synthesis Recovery

The recovery of RNA synthesis after UV was measured by labeling cells with [2-^{14}C]thymidine (0.05 μCi/ml, specific activity 56 Ci/mmole) for 24 hr and then exposing them to 10 J/m^2 of UV-C light. Cells were subsequently photoreactivated or kept in the dark, as described above. At different time points after UV, cells were labeled with [5,6-^{3}H]uridine (10 μCi/ml, specific activity 47 Ci/mmole) for 1 hr and processed for scintillation counting. The relative rate of RNA synthesis is expressed as the ratio of activity of UV-irradiated over nonirradiated cells.

Photoreactivation in Mouse Skin

Mice were anesthetized, and hairs were removed from a small area on the back of the animal by plucking. One-third of the hairless area was covered with black nonadhesive tape, and the remaining area was exposed to the light of two Philips TL-12 (40W) tubes emitting UV-B. Subsequently, half of the UV-exposed area was covered with tape, and mice were exposed for 3 hr to the light, filtered through 5 mm of glass, of four white fluorescent tubes (General Electric Lightning Polylux XL F36W/840). Mice were sacrificed, and skin samples were taken from the unexposed area, the UV-irradiated area that was covered, and the UV-irradiated area that was exposed to photoreactivating light. The skin samples were fixed for 1 hr in a mixture of 75% methanol and 25% acetic acid, incubated in 70% ethanol for 1 hr and then 5% sucrose (1 hr), and snapfrozen in TissueTek (Sakura). Eight micrometer cryosections were incubated in 70% ethanol containing 0.07 N NaOH for 4 min and were subsequently washed. Immunofluorescence was performed as described above.

Acute UV Responses

Apoptosis

For detection of apoptotic cells in the skin, we used a TUNEL assay (Fluorescein Apoptosis Detection System, Promega). Mice were exposed to UV and photoreactivating light, as described above, and were subsequently kept in the dark. Skin samples, taken 40 hr after UV exposure, were fixed overnight in 4% paraformaldehyde, washed in PBS, and embedded in paraffin. Skin sections (5 μm) were deparaffinized and incubated as described by the manufacturer.

Hyperplasia

Mice were anesthetized, and an area on the back was depilated by plucking. Mice were exposed to 1 MED UV-B and photoreactivating light for 4 consecutive days. One week after the start of the experiment, mice were sacrificed, and 8 μm skin sections were obtained. Sections were further processed and stained with haematoxylin and eosin.

Mutation Frequency

Photolyase mice were mated with IM1 transgenic mice [24] harboring approximately 20 copies of the *lacZ* gene in an SKH1 hairless background. Mice were treated with 1 MED UV-B and then photoreactivation or darkness, as described above. Two weeks after UV exposure, mice were sacrificed and skin was removed. Epidermal sheets were isolated by placing the skin floating with the dermis facing 0.25% trypsin/0.04% EDTA in PBS. DNA was isolated from the epidermis, and mutation frequencies were determined after rescuing *lacZ*-containing plasmids from genomic DNA as described previously. In brief, *lacZ* plasmids were recovered from genomic DNA by HindIII digestion in the presence of magnetic beads that had been precoated with a LacZ/LacI fusion protein. Plasmids were eluted with isopropylthio-β-galactoside (IPTG), circularized with T4 DNA ligase, and electroporated into *E. coli C* (*lacZ*$^-$/*galE*$^-$). Plasmids with a mutation in the *lacZ* gene were positively selected on plates containing the lactose analog phenyl β-D-galactoside (*p*-gal; Sigma). Mutant frequencies were calculated as the number of mutant colonies on the selective plate versus the number of total transformants obtained on a nonselective plate.

Carcinogenesis

P53 Patches

Hairless mice were exposed to UV-B for 21 consecutive days, followed by 3 hr of photoreactivation. The UV dose was gradually increased, starting with 0.7 MED at day 1, 0.8 MED (day 2), 0.9 MED (day 3), and then 1 MED daily from day 4 to the end of the experiment. At day 21, mice were sacrificed, and rectangular parts of the skin were isolated and placed floating on a 200 μg/ml thermolysin solution (Sigma) in PBS containing 2 mM $CaCl_2$. After overnight incubation at 4°C, the epidermis was isolated, fixed, permeabilized, and stained for p53 protein in mutant conformation with Pab240 antibodies (Novocastra) as described previously [25, 26].

Formation of Skin Carcinomas

Hairless photolyase mice and their wild-type littermates (8–12 weeks old) were exposed daily to 500 J/m^2 UV-B (Philips TL-12 tubes) followed by 3 hr of photoreactivating light (General Electric Lightning Polylux XL F36W/840 lamps). Animals were screened weekly for the occurrence of skin abnormalities. Typically, carcinomas were expected to occur after 3 months of treatment. Mice were sacrificed when tumors >4 mm occurred, and tumor biopsies were taken for routine haematoxylin/eosin staining.

Supplemental Data

Detailed Experimental Procedures are available at http://www.current-biology.com/cgi/content/full/15/2/105/DC1/.

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