

Why Cockayne syndrome patients do not get cancer despite their DNA repair deficiency

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Cockayne syndrome (CS) and xeroderma pigmentosum (XP) are human photosensitive diseases with mutations in the nucleotide excision repair (NER) pathway, which repairs DNA damage from UV exposure. CS is mutated in the transcription-coupled repair (TCR) branch of the NER pathway and exhibits developmental and neurological pathologies. The XP-C group of XP patients have mutations in the global genome repair (GGR) branch of the NER pathway and have a very high incidence of UV-induced skin cancer. Cultured cells from both diseases have similar sensitivity to UV-induced cytotoxicity, but CS patients have never been reported to develop cancer, although they often exhibit photosensitivity. Because cancers are associated with increased mutations, especially when initiated by DNA damage, we examined UV-induced mutagenesis in both XP-C and CS cells, using duplex sequencing for high-sensitivity mutation detection. Duplex sequencing detects rare mutagenic events, independent of selection and in multiple loci, enabling examination of all mutations rather than just those that confer major changes to a specific protein. We found telomerase-positive normal and CS-B cells had increased background mutation frequencies that decreased upon irradiation, purging the population of subclonal variants. Primary XP-C cells had increased UV-induced mutation frequencies compared with normal cells, consistent with their GGR deficiency. CS cells, in contrast, had normal levels of mutagenesis despite their TCR deficiency. The lack of elevated UV-induced mutagenesis in CS cells reveals that their TCR deficiency, although increasing cytotoxicity, is not mutagenic. Therefore the absence of cancer in CS patients results from the absence of UV-induced mutagenesis rather than from enhanced lethality.

mutagenesis | dipyrimidines | transcription arrest | apoptosis | RNA pol II

The nucleotide excision repair (NER) syndromes xeroderma pigmentosum (XP) and Cockayne syndrome (CS) lie at the extremes of increased cancer and neurodegeneration, respectively (1). The XP-C group of XP patients has mutations in the DNA damage-recognition protein XPC involved in global nucleotide excision repair (GGR). They are characterized by UV hypersensitivity, sun-induced cutaneous features such as hypopigmentation and hyperpigmentation, and a greatly (>1,000-fold) increased incidence of cancer (1–3). In contrast, CS patients have mutations in the RNA polymerase II cofactors CSA and CSB, which recognize damage in transcribed regions through transcription arrest (transcription-coupled repair, TCR). CS patients are characterized by neurological and developmental symptoms such as early cessation of growth, microcephaly, mental retardation with dysmyelination, cachexia, and a greatly reduced life expectancy (1). The reported average life expectancy of patients with CS is only 12 y (4). CS patients are also highly photosensitive, burning and blistering after only minutes of sun exposure (5). However, in stark contrast to the dramatic increase in skin cancer incidence in XP-C patients, no CS patient has ever been reported to develop cancer, skin or otherwise (1, 4–8). Because cancer, especially in skin, is associated with mutagenesis (9, 10), we hypothesized that, unlike defects in GGR, which are associated with enhanced UV-induced mutagenesis in XP-C cells (11), the TCR defects in CS cells may not lead to increased mutagenicity.

Most sensitive mutagenesis studies in XP and CS cells have been confined to a few selectable genes, mutation of which confers drug resistance. Maher showed that UV-induced mutations in the hypoxanthine phosphoribosyl transferase (*HPRT*) gene that conferred resistance to 6-thioguanine (6-TG) were greatly enhanced in XP cells from both excision-defective and polymerase-defective groups (11, 12). Similar studies in CS cells, however, failed to show an increase in UV-induced mutations in *HPRT*, T-cell receptor, or glycophorin A gene loci (13). In contrast, an episomal plasmid (pZ189), irradiated with UV and passed through CS cells, showed increased levels of mutations (14, 15). The limitations of these methodologies include the small number of potential gene loci suitable for drug selection and the possibility that episomal vectors may not fully induce the DNA-damage response of whole cells, thereby resulting in a high mutation frequency that is not representative of mutagenesis in chromosomal loci. These limitations complicate the disparate results of these previous studies, leaving unresolved the question of whether CS cells demonstrate elevated UV-induced mutagenesis.

With the development of next-generation sequencing came the potential to survey multiple genes simultaneously and independently of selection. However, standard next-generation sequencing methodologies are highly error prone and are limited to surveying mutations present at ratios greater than 1 in 20 wild-type sequences (16). To counteract the limitations of standard next-generation sequencing platforms, we used duplex sequencing, a highly accurate sequencing method that is 100,000-fold more accurate than traditional next-generation sequencing

Significance

Cockayne syndrome (CS) and xeroderma pigmentosum (XP) are photosensitive diseases with mutations in the nucleotide excision repair (NER) pathway. XP patients have a very high incidence of UV-induced skin cancer, but CS patients have never been reported to develop cancer. Cultured cells from both diseases have similar sensitivity to UV-induced cytotoxicity. We examined UV-induced mutagenesis in cells from XP patients with mutations in the global genome repair branch of the NER pathway (XP-C cells) and CS primary cells using duplex sequencing for high sensitivity without selection. XP-C cells showed increased UV-induced mutation frequencies compared with normal cells, consistent with their increased cancer incidence. CS cells, in contrast, showed no elevated mutagenesis. Therefore the absence of cancer in CS patients results from the absence of increased mutations following UV exposure.

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methodologies. Because of its ability to remove sequencing artifacts resulting from DNA damage, as well as amplification and sequencing errors, duplex sequencing enables the detection of mutations as low as 1 in 10^8 nucleotides sequenced (17, 18).

To test our hypothesis that defects in TCR may not lead to increased UV-induced mutagenesis, unlike defects in GGR, we used duplex sequencing for high-sensitivity mutation detection in primary cells derived from normal patients and from patients with XP-C and CS. We found that, although primary XP-C and CS cells have similar sensitivities to UV-induced cell killing, the surviving cells in the two groups are radically different. Surviving XP-C cells exhibit high levels of UV-induced mutations; surviving CS cells do not.

Results

UVC- and UVB-Induced Cytotoxicity in Primary Cells. Primary fibroblasts (Table S1) derived from normal adult skin (GM05659) and normal neonatal primary foreskin (NHF-D) and primary neonatal keratinocytes were exposed to UVB or UVC, cultured for 5–7 d, and harvested. Using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma-Aldrich), we calculated the surviving fraction at the time of cell harvesting relative to untreated cells of the same genotype (Fig. 1). Primary keratinocytes were more resistant than fibroblasts to killing by UVB and UVC (Fig. S1).

Increased UVC-Induced Cytotoxicity in Repair-Deficient Primary Fibroblasts. Normal (GM05659 and NHF-D), XP-C (GM02997 and XP226BA), CS-A (GM17536 and GM01856), and CS-B (GM01428 and GM01629) primary fibroblasts were exposed to UVC, cultured for 5–7 d, and harvested. Using the MTT assay, we calculated the surviving fraction at the time of cell harvesting relative to untreated cells of the same genotype. XP-C, CS-A, and CS-B primary fibroblasts were markedly more sensitive to killing by UVC than normal primary fibroblasts (Fig. 1), as is consistent with many previous studies reporting the enhanced UV sensitivity of XP

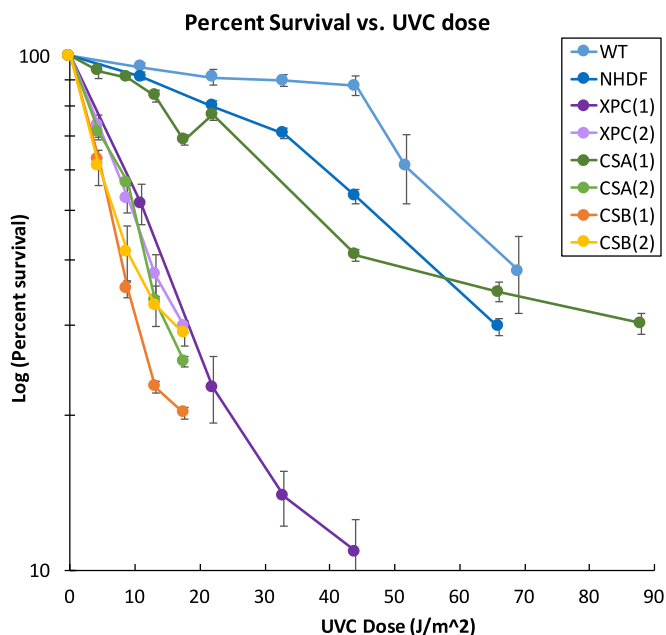


Fig. 1. Survival of normal adult (WT), normal neonatal (NHF-D), XP-C [XPC(1) and (2)], CS-A [CSA(2)], CS-B [CSB(1) and (2)], and GM17536 (originally designated "CS-A") [CSA(1)] fibroblasts. Error bars represent SD of two survival determinations.

and CS cells. One primary CS-A cell line, GM17536, appeared anomalous for CS-A and had higher survival rates than all other repair-deficient cells (Fig. 1 and Fig. S24). We therefore excluded the GM17536 cell line from our subsequent mutational analyses.

UVC and UVB Induce Subclonal Mutations in Normal Primary Cells. To validate the use of duplex sequencing to detect mutagen-induced mutations, normal fibroblasts (GM05659 and NHF-D) and keratinocytes were exposed to UVB or UVC, cultured for 5–7 d, and harvested. Genomic DNA was then isolated and subjected to a single round of duplex sequencing (18). Target genes were exonic regions of *NRAS*, *UMPS*, *PIK3CA*, *EGFR*, *BRAF*, *KRAS*, *F10*, *TP53*, and *TYMS* (Table S2), several of which were chosen for their importance in skin carcinogenesis.

The spectrum of subclonal (<20% clonal) mutations observed in normal fibroblasts and keratinocytes showed a dose-dependent increase in C:G→T:A transitions as a function of UVB dose in primary keratinocytes and as a function of UVC dose in primary fibroblasts, especially in NHF-D cells (Fig. 2A). In contrast, transversions were dose independent. Of particular interest for UV-induced mutagenesis studies are C:G→T:A mutations at dipyrimidine sites (CpT, TpC, and CpC; hereafter referred to as "Py-Py" sites), because mutations at these sites are known signatures of UV-induced mutagenesis (19, 20). Importantly, when we examined the context of the C:G→T:A transitions, the majority of UV-induced mutations occurred at Py-Py sites and showed a dose-response to UVB and UVC in all normal genotypes (Fig. 2B). These results are consistent with previous reports of UV-induced mutagenesis in reporter genes (11, 13–15) and validate our use of duplex sequencing for the detection of UV-induced mutagenesis. We chose to carry out subsequent experiments using UVC because UVB and UVC produce similar mutagenic photoproducts. In our hands the difference in cyclobutane dimer yield per joule per meter was approximately a factor of 7, measured by the cleavage of a plasmid using *Micrococcus luteus* UV endonuclease.

UVC Induces an Elevated Subclonal Mutation Frequency in XP-C Cells. Normal (GM05659 and NHF-D) and XP-C (GM2997 and XP226BA) primary fibroblasts were exposed to UVC, cultured for 5–7 d, harvested, and subjected to a single round of duplex sequencing (18). XP-C cells showed elevated subclonal mutation frequencies relative to normal fibroblasts (Fig. 3A). When we focused on C:G→T:A mutations at Py-Py sites, we found that, relative to normal cells, XP-C cells accumulated more of these UV-specific mutations with increasing UVC dose (Fig. 3B). These results are consistent with previous reports, using reporter genes, of elevated UV-induced mutagenesis in XP cells (11). Additionally, normal cells showed a smaller, shallow increase in UV-specific mutations with UVC dose, as is consistent with efficient repair that minimizes UV-induced mutations and skin cancer initiation in repair-proficient cells (10).

CS Cells Fail to Demonstrate Elevated Subclonal Frequencies of UV-Specific Mutations upon UVC Exposure. CS-A (GM01856) and CS-B (GM01428 and GM01629) primary fibroblasts were exposed to UVC, cultured for 5–7 d, harvested, and subjected to a single round of duplex sequencing (18). When we examined total subclonal mutations, all primary CS cells appeared to show an initial increase in mutation frequency, with a reduction at higher UVC doses (Fig. 3C). However, in contrast to XP-C cells, CS-A and CS-B fibroblasts showed no elevation in C:G→T:A mutations at Py-Py sites relative to normal fibroblasts (Fig. 3D). Indeed, when directly comparing XP-C and CS-A and -B cells (Fig. 3E), we found UV-specific mutations were markedly increased in XP-C fibroblasts, but there were none in CS-A/B fibroblasts despite their similar sensitivity to the cytotoxic effects of UVC (Fig. 1). When we examined the frequency of subclonal UV-specific mutations

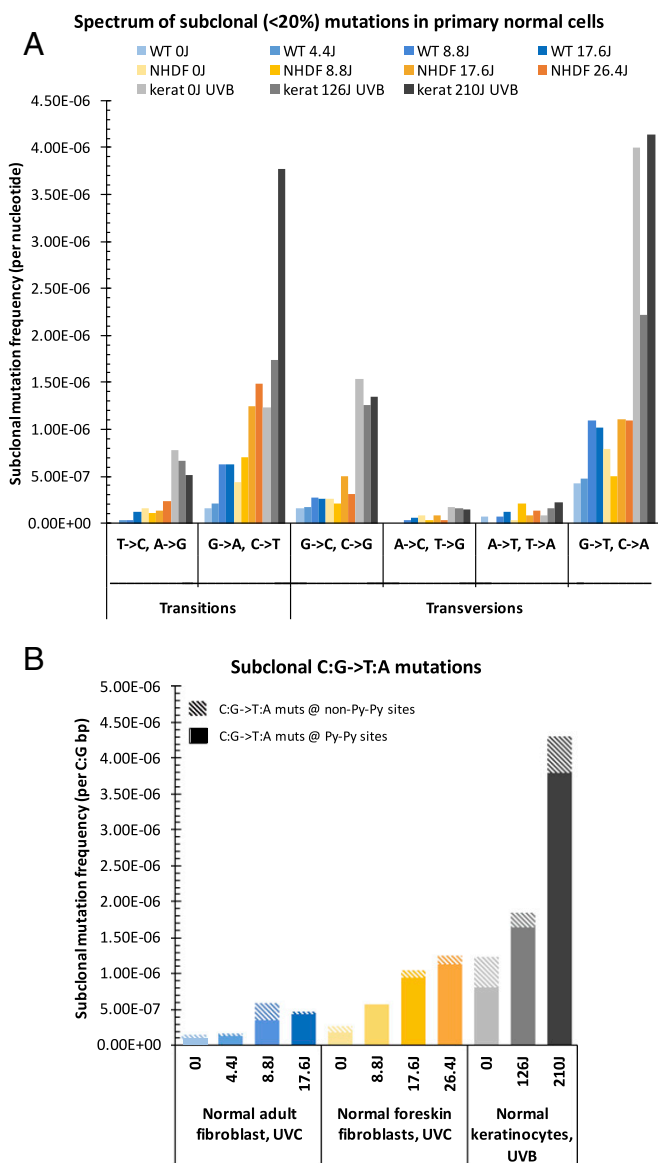


Fig. 2. UV induces unselected subclonal (<20% clonal) mutations in normal primary fibroblasts and keratinocytes. (A) Spectrum of subclonal mutations in adult (WT) and neonatal (NHDF-D) fibroblasts treated with UVC and in keratinocytes (kerat) treated with UVB. (B) Subclonal frequencies of UV-specific mutations in adult and neonatal fibroblasts treated with UVC and in keratinocytes treated with UVB. Solid bars represent UV-specific mutations (C:G→T:A mutation at Py-Py sites); hashed bars represent C:G→T:A mutations at non-Py-Py sites. Frequencies were calculated by dividing the number of mutations of each type by the number of times the wild-type base of each mutation type was sequenced.

versus cell survival, the results suggested that CS cells might have an even lower mutation frequency than normal cells at equivalent survival levels (Fig. S3).

Because UV-specific mutations did not account for the initial increase in total subclonal mutations seen in CS cells (Fig. 3C), and particularly in CS-B cells, we sought to determine if the UV-induced mutations in CS cells had an oxidative-damage signature, because CS-B has been implicated in oxidative DNA-damage repair (21–23). Indeed, we found that the majority of UV-induced mutations in CS-B cells were G:C→T:A mutations, a signature of 8-oxo-dG-induced mutagenesis (Fig. 3F), which can be caused by direct oxidation of DNA by UV and, more indirectly, by singlet

oxygen formation in cells following UV irradiation (24–26). This result is consistent with increased mutagenesis resulting from deficient oxidative DNA-damage repair in CS cells.

Duplex Sequencing Enables In-Depth Analyses of the Mutagenic Consequences of UVC. Because duplex sequencing allows us to study rare mutational events, we examined the distribution of UVC-induced mutations by combining all cells exposed to UV doses into a pool, designated “UVC,” and comparing the mutations in these cells with those in untreated cells, designated “control” (Table 1 and Fig. S4). We determined the distribution of UVC-induced mutations in active and inactive genes based on the gene-expression status of each gene in skin (GeneCards; www.genecards.org/) and the distribution of mutations in the template (transcribed) and coding (nontranscribed) strands of active genes (Table 1, Fig. S4, and Table S2). In XP-C cells, there was an increased ratio of C:G→T:A mutations in inactive genes, relative to active genes; there also was an elevated ratio of C→T mutations in the coding strand of active genes, relative to the template strand. These biases are consistent with the GGR deficiency of XP-C cells. In CS-B cells, there was little difference in the ratios of C:G→T:A mutations as a function of gene activity; there was, however, an elevated ratio of C→T mutations in the template strand, relative to the coding strand, as is consistent with the TCR deficiency of CS-B cells. Thus, although TCR deficiency influences the strand distribution of mutations, it does not increase the overall yield.

During our analysis we observed 39 instances of multiple mutations within the same read (multiplets). Because the “classic” UV-induced mutation signature is the CC→TT mutation, we were intrigued by the presence of multiple other types of multiplet mutations (Table S3). Although CC→TT mutations are the most frequent type of multiplet mutation observed, we encountered many other types, all of which occurred in UV-treated cells. Of 39 multiplet mutations, all but one occurred at or directly adjacent to a Py-Py (CpC, CpT, TpC, TpT) dinucleotide, as is consistent with the mutations resulting from error-prone bypass of UV-induced damage. In addition to mutations that occurred within a doublet (i.e., CC→TT) or triplet (i.e., CTC→TTT), six of the multiplet mutations were two single mutations occurring 3–7 nt apart.

Discussion

Both XP and CS patients have defects in NER. The XP-C patients display extreme UV sensitivity and are highly prone to develop skin, corneal, and eyelid cancers because of their defects in GGR. CS patients, defective in the TCR branch of NER (1, 27), present a very different clinical picture, one of developmental defects and neurodegeneration; many but not all patients are also photosensitive, some developing blistering sunburns (5, 28). In contrast to XP-C patients, no known CS patient has ever developed cancer (4, 5, 8). Early studies of CS presented a discordant picture as to whether CS cells show a higher frequency of UV-induced mutation relative to normal cells and differed depending on the method used. Therefore, to determine definitively whether CS cells show an elevated frequency of UV-induced mutations, we used duplex sequencing, a highly accurate next-generation sequencing methodology that enables the detection of rare mutagenic effects (18), to study UV-induced mutagenesis in primary cells derived from normal persons and from XP-C, and CS-A and -B patients. In contrast to previous methods, our use of duplex sequencing (18) enabled us to study the mutagenic consequences of UV damage independent of selective pressures and in far greater detail than previously possible.

Our study of normal fibroblasts and keratinocytes validated our use of UVC to induce subclonal UV-specific mutations (C:G→T:A at Py-Py sites); we also validated our application of duplex sequencing to analyze the mutagenic consequences of UV in primary cultured cells absent selective pressures. Our analysis of

Table 1. Ratio of pooled C:G→T:A and C→T mutations after UV irradiation, relative to controls

Gene expression and transcription	Genotype		
	Normal	CS-B	XPC
Gene-expression status*			
Expressed	2.9	6.0	3.4
Not expressed	2.1	5.5	7.4
Strand of active genes, relative to transcription [†]			
Nontranscribed (coding)	5.8	4.2	5.3
Transcribed (template)	2.5	7.7	2.9

Because these ratios are calculated relative to controls of the same genotype, the absolute numbers are cell-type dependent. The ratios should be compared according to gene activity or strand specificity for each cell type independently.

*C:G→T:A mutations at C:G base pairs.

[†]C→T mutations in active genes at cytosines.

cells. This elevated UV-induced mutagenesis occurred primarily in inactive genes, as evident from the greater than twofold increase in C:G→T:A mutations in inactive genes versus active genes, as is consistent with defective GGR (Table 1 and Fig. S4C). The bias between the template (transcribed) and coding (nontranscribed) strands in XP-C cells was similar to that of the normal cells (Table 1 and Fig. S4F), indicating that, despite the GGR deficiency, TCR of the template strand is unaffected.

In contrast to XP-C primary cells, CS-B primary cells showed no increase in UV-specific mutations following UVC irradiation, relative to normal primary cells (Fig. 3D), despite having a survival profile akin to that of XP-C cells (Fig. 1C). Similar to normal cells, CS-B fibroblasts showed no bias in the accumulation of C:G→T:A mutations between active and inactive genes (Table 1 and Fig. S4A and B), as is consistent with proficient GGR. However, in contrast to both normal and XP-C primary fibroblasts, both of which had reduced C→T mutations in the template strand of active genes relative to the coding strand, CS-B cells had increased C→T mutations in the template strand (Table 1 and Fig. S4D–F). This bias is consistent with defective TCR in CS-B primary cells.

An interesting observation in our in-depth spectrum studies is that, in contrast to normal and XP-C cells, CS-B primary fibroblasts accumulated more G:C→T:A mutations than C:G→T:A mutations upon UVC irradiation (Fig. 3F and Fig. S4B). Because G:C→T:A mutations are a signature of mutagenesis induced by 8-oxo-dG, the most common oxidative lesion in cells (37), this observation alludes to CSB's additional role in oxidative DNA-damage repair (21–23), loss of which could result in increased oxidative damage-induced mutagenesis. We previously reported that CSB interacts with complex I of the mitochondria to quench surplus reactive oxygen species (38). Given the neurological involvement in CS, further studies on the mutagenic consequences of oxidative DNA damage may be worthwhile for understanding the pathologies seen in CS.

In addition to the gene- and strand-specific analyses afforded by our duplex sequencing approach, we gained greater insight into the mutational consequences of UVC-induced damage, beyond that of C:G→T:A mutations at Py–Py sites. Specifically, we observed numerous types of multiplet mutations (Table S3). These included the classic signature of UV-induced mutagenesis, CC→TT, and also extended to triplets, such as CTC→TTT, and doublet mutations spaced 3- to 7-nt apart. These multiplet mutations are likely the consequence of error-prone bypass polymerization during translesion synthesis and are consistent

with the processivity of bypass polymerases persisting for several nucleotides after the bypass-requiring blocking lesion (39–41).

Our results reveal that UV-induced mutagenesis is no higher in CS cells than in normal cells (Fig. 3D and Fig. S3). In normal individuals, the average age of skin cancer incidence is 55 y (2), 33 y beyond the average lifespan of a CS patient and, indeed, 24 y longer than the lifespan of the longest-lived CS patient on record (31 y) (42). Although increased exposure to sunlight or use of tanning beds can result in much earlier diagnosis of skin cancers (early in the third decade of life) in normal individuals (43, 44), this age of onset is still a decade beyond the average lifespan of a CS patient. Thus, if CS cells accumulate mutations in response to UVC at the same rate as normal cells, CS patients simply do not live long enough to develop cancer. However, when analyzing UV-specific mutations plotted relative to survival, it appears that UV-induced mutagenesis might even be lower in CS cells than in normal cells (Fig. S3). This possibility suggests that, even if CS patients could attain normal lifespans, they might never get cancer; TCR deficiency may even be protective against UVC-induced mutagenesis. Further experiments examining normal versus CS cells would be necessary to determine if mutation frequencies are indeed lower in CS cells than in normal cells.

In conclusion, we have determined that, in human cells, defects in TCR fail to increase UV-induced mutagenesis as defects in GGR do. Thus, CS patients, defective in TCR, fail to develop cancer because they do not accumulate mutations more quickly than repair-proficient individuals.

Methods

Work with human cells was approved by the University of California, San Francisco Committee on Human Research, inclusive of informed consent (IRB11-05993 to J.E.C.). Normal, XP-C, and CS-A and -B human fibroblasts were obtained from the Coriell Institute (Table S1). One XP-C culture (XP226BA) was derived from discarded tissue after cancer surgery of patients in Guatemala (45). The fibroblast culture NHF-D was a gift from D. Oh, University of California, San Francisco. A culture of pooled neonatal keratinocytes was developed in house. One normal (GM05659T) and one CS-B (GM01428T) culture was transfected with lentivirus expressing hTERT and was grown continuously for at least 2 y.

To measure survival, cells were grown for 48 h in 96-well plates, drained of medium, and then exposed to a range of doses of UVC (254 nm) or UVB (280–320 nm) using a battery of five fluorescent tubes for each wavelength. The UVB lamps were filtered to remove UVC. The plates were opaque to UVC, but additional shielding was used for UVB. Cells then were allowed to grow for 5–7 d and were harvested. Survival was measured colorimetrically with MTT (Sigma-Aldrich) at 570 nm. Relative survival was calculated from the ratios of exposed to unexposed wells, based on the average 570-nm absorbance in four to six wells per exposure condition. We chose to measure the survival at 5–7 d, which corresponded to the time of harvest for our mutagenesis analysis. The surviving cell numbers represent a combination of cell lysis, growth delays, and rates of regrowth.

To measure UV-induced mutagenesis, cultures of ~10⁷ cells were washed in PBS, irradiated, and grown for 5–7 d. Cells then were harvested by trypsin, washed in PBS, and rapidly frozen in dry ice/methanol. DNA was isolated, and mutations were measured by one round of duplex sequencing (18), as described more fully in the *SI Methods*. Target genes were exonic regions of *NRAS*, *UMPS*, *PIK3CA*, *EGFR*, *BRAF*, *KRAS*, *F10*, *TP53*, and *TYMS*, several of which were chosen for their importance in skin carcinogenesis (Table S2). We required a minimum depth of 100 duplex molecules to call a position, either mutant or not; all samples had a midexon peak depth of 1,000–4,000 duplex molecules across all captured exons.

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