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A novel role for transcription-coupled nucleotide excision repair for the in vivo repair of 3,\(N^4\)-ethenocytosine

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ABSTRACT

Etheno (\(\varepsilon\)) DNA base adducts are highly mutagenic lesions produced endogenously via reactions with lipid peroxidation (LPO) products. Cancer-promoting conditions, such as inflammation, can induce persistent oxidative stress and increased LPO, resulting in the accumulation of \(\varepsilon\)-adducts in different tissues. Using a recently described fluorescence multiplexed host cell reactivation assay, we show that a plasmid reporter bearing a site-specific 3,\(N^4\)-ethenocytosine (\(\varepsilon\)C) causes transcriptional blockage. Notably, this blockage is exacerbated in Cockayne Syndrome and xeroderma pigmentosum patient-derived lymphoblastoid and fibroblast cells. Parallel RNA-Seq expression analysis of the plasmid reporter identifies novel transcriptional mutagenesis properties of \(\varepsilon\)C. Our studies reveal that beyond the known pathways, such as base excision repair, the process of transcription-coupled nucleotide excision repair plays a role in the removal of \(\varepsilon\)C from the genome, and thus in the protection of cells and tissues from collateral damage induced by inflammatory responses.

INTRODUCTION

Etheno (\(\varepsilon\)) DNA adducts are highly mutagenic base lesions characterized by an exocyclic (imidazole) ring. They are produced endogenously through reactions with lipid peroxidation (LPO) products generated under oxidative stress conditions (1–4). \(\varepsilon\)-Adducts were first identified as products resulting from exposure of DNA bases to chloroaacetalddehyde (CAA) (5). The role of these lesions in carcinogenesis became evident after occupational exposure to vinyl chloride (VC), a precursor of chloroethylene oxide and CAA (6), which was correlated with the development of a rare tumor, angiosarcoma of the liver (7–9). Studies in rodents have shown comparable carcinogenic effects of both VC (10) and urethane (11) exposure. The toxicity and mutagenicity of \(\varepsilon\)-adducts have also been demonstrated in both bacteria (12–16) and mammalian cells (13,17,18).

With the development of more sensitive techniques to detect \(\varepsilon\)-adducts, background levels of 1,\(N^6\)-ethenoadenine (\(\varepsilon\)A) and 3,\(N^4\)-ethenocytosine (\(\varepsilon\)C) were first measured in rodents (19,20). Interestingly, a range of adduct levels was detected for different tissues, suggesting differential exposure to relevant DNA-damaging agents and/or variation in tissue DNA repair capacity (19). It was later shown that LPO products represent a major endogenous source of \(\varepsilon\)-adducts (1–3). LPO is triggered by the reaction of reactive oxygen and nitrogen species with polyunsaturated fatty acid residues of phospholipids. Conditions such as inflammation, impaired metal transport or dietary imbalance can induce persistent oxidative stress and LPO excess, which in turn can cause an accumulation of \(\varepsilon\)-adducts in different tissues. Notably, many of these conditions are characteristic of cancer-prone diseases (21).

Two DNA repair pathways have been shown to repair \(\varepsilon\)-adducts, namely base excision repair (BER) and direct reversal (DR). BER is a finely tuned process that begins with the excision of a damaged base by a DNA glycosylase, generating an abasic (AP) site. For responses involv-
ing a monofunctional DNA glycosylase, the AP site is processed by apurinic/apyrimidinic endonuclease 1 (APE1), which produces a single strand break with a 3'-OH and 5'-deoxyribosephosphate (5'-dRP). DNA polymerase β removes the 5'-dRP moiety and fills in the gap by inserting a nucleotide with the complementary base to the undamaged strand. Repair is completed by the action of a DNA ligase to seal the nick (22). DR by the AlkB homolog (ALKBH) enzymes does not require any other proteins or DNA processing per se, but instead involves direct reversal of the lesion via removal of the etheno ring. Specifically, the repair reaction begins with epoxidation of the etheno ring after which the addition of water opens the ring to form a glycol intermediate that is spontaneously released as glyoxal, leaving behind a normal base (23).

In mammalian cells, εA is excised by the alkyladenine DNA glycosylase (AAG, a.k.a. MPG) (24,25) or reversed transcriptional blockage and, when bypassed, can promote the incorporation of an inappropriate ribonucleotide into the transcript opposite the lesion site, in a process known as transcriptional mutagenesis (41). By taking advantage of our recently described fluorescence multiplexed host cell re-activation (FM-HCR) assays for measuring DNA repair capacity, we further demonstrate that εC is repaired by TC-NER.

**MATERIALS AND METHODS**

**Plasmids**

As described previously (42), EGFP, mOrange and tag-BFP reporter genes subcloned into the pmaxCloning Vector (Lonza) between the KpnI and SacI restriction sites in the multiple cloning site were employed. Plasmids were amplified in *Escherichia coli* DH5α (Invitrogen) and purified using Qiagen endotoxin-free Maxi and Giga kits.

**UV treatment.** Plasmids were irradiated in TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 7.0) at a DNA concentration of 50 ng/μl in a volume of 1.5 ml in 10-cm polystyrene Petri dishes (without lids) with 800 J/m² UV-C light generated by a Stratalinker 2000 box.

**Site-specific thymine dimer reporter.** pMax:GFP 615AA plasmid (described previously (42)) was prepared using a Giga Prep kit according to manufacturer instructions (Qiagen). The plasmid was nicked with Nb.BtsI (New England Biolabs) to generate a single-strand break in the transcribed strand. The nicked strand was then digested with ExoIII (New England Biolabs), and the remaining single-stranded circular DNA (ssDNA) was purified using a ⅓ agarose gel. The ssDNA was extracted from the agarose gel using a Gel Extraction Kit (Qiagen). Twenty micrograms of the ssDNA were combined with 9 μl of a 10 mM oligonucleotide sequence containing a thymine dimer localized at bases 614 and 615 of the plasmid [5’TACAGGCGAXGTTGC3’, where X denotes a thymine dimer] (Trilink Biotech) and 1× Pfu Polymerase Buffer (Thermo Scientific). The mixture was heated to 85°C in a thermocycler for 6 min, and then allowed to anneal by cooling to 37°C at 1°C/min. Following annealing, 12 μl of 10 mM dNTPs and 30 U Pfu Polymerase AD were added to the mixture. The oligo was extended at 61°C for 1.5 h. Following extension, the plasmid was purified using a PCR Purification kit following manufacturer instructions (Qiagen). After elution in 105.4 μl EB Buffer (10 mM Tris–Cl, pH 8.5), 5 μl T4 DNA Ligase buffer (New England Biolabs), 2 μl 10 mM dNTPs, 2 μl 25 mM ATP, 0.5 μl 10 mg/ml BSA, 1.5 U T4 DNA Polymerase (New England Biolabs) and 8 U T4 DNA Ligase (New England Biolabs) were added, and the solution was incubated at 16°C for 1 h. The solution was subjected to electrophoresis in a ⅓ agarose gel to purify the ligated band. Ligated plasmid was extracted from the gel using a Gel Extraction kit (Qiagen). Gel extraction was performed according to manufacturer instructions except that plasmids were eluted in TE buffer (10 mM Tris–Cl, pH 8.0, 1 mM EDTA) (Invitrogen).

**Site-specific εA (GFP-616-εA) and εC (GFP-615-εC) reporter.** pMax:GFP WT plasmid was prepared using
a Giga Prep kit according to manufacturer instructions. Single stranded plasmid DNA was prepared as described above. Annealing of a site-specific oligo was performed as described above, using an oligonucleotide containing εA [5’GTCAGGGCGGXCCTGGGTGCTCAG GTAGTG, where X denotes εA or εC [GTCAGGG CCGAXTGGGTGCAGTTAGTG, where X denotes εC] (Life Technologies). Following annealing, 12 μl of 10 mM dNTPs and 30 U of Pfu Polymerase AD were added to the mixture, and the oligo was extended at 68°C for 1.5 h. After extension, the plasmid was purified using phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation. The plasmid DNA was resuspended in a mixture containing 105.4 μl EB Buffer (10 mM Tris–Cl, pH 8.5), 5 μl T4 DNA Ligase buffer (New England Biolabs), 2 μl 10 mM dNTPs, 2 μl 25 mM ATP and 0.5 μl 100X BSA. Once the DNA was resuspended, 1.5 U T4 DNA Polymerase and 8 U T4 DNA Ligase (New England Biolabs) were added, and the solution was incubated at 16°C for 1 h. In order to remove excess salts from the solution, the buffer was changed following ligation using 30,000 NMWL Filtration columns (Millipore). The plasmid-containing solution was concentrated three times and finally resuspended in 150 μl TE for purification by gel electrophoresis. In order to recover the plasmid, a 1.5–2% NuSieve GTG low melting point agarose gel (Lonza) was used. The plasmid DNA was extracted from the gel according to the manufacturer’s instructions. Briefly, the gel was melted and a series of phenol/chloroform extractions were performed, followed by ethanol precipitation and resuspension of the plasmid in TE buffer.

Primary cells and cell lines

Animals. Aag−/− and Triple Knockout (TKO, Aag−/−, Alkbh2−/−Alkbh3−/−) mice were described previously (43). All mice used for experiments were on a mixed B6/129s background. Age- and gender-matched mice were 6–10 weeks old at the time of euthanasia. Mice were euthanized by CO2 asphyxiation and their spleen was subsequently removed. Single-cell suspensions of splenocytes were prepared by pushing tissue pieces through a 70 μm pore size nylon mesh screen. Erythrocytes were lysed in ACK lysis buffer.

Mice were housed in an Association of Assessment and Accreditation of Laboratory Animal Care–accredited facility. All procedures were approved by the Massachusetts Institute of Technology Committee on Animal Care.

Human cells. B-lymphoblastoid cells (GM01712, CSB; GM01857, CS; GM01953, healthy; GM02246, XPC; GM02345, XPA; GM02253, XPD; GM07752, healthy and GM21148, XBP) were maintained in RPMI 1640 medium (Gibco) supplemented with 15% FBS, 2 mM L-glutamine and 1% penicillin and streptomycin. SV40-transformed CSA-deficient CS3BE and CSB-deficient CS1AN cell lines (Coriell Institute) transfected with pcDNA-CSA or pcDNA-CSB, respectively (previously described (44)) were maintained in DMEM medium (Gibco) supplemented with 10% HI-FBS, 2 mM L-glutamine, and 1% penicillin and streptomycin. Adherent cell lines were sub-cultured by trypsinization. Cells were maintained at 37°C and 5% CO2.

Rodent cells. Mouse embryonic fibroblasts (MEFs) were prepared from wild-type C57BL/6J and previously described Aag−/− mouse models (45). Primary MEFs were previously immortalized by transfecting MEFs with a pSV3-neo plasmid expressing large T-antigen. Immortalized MEFs were cultured in DMEM medium supplemented with 10% HI-FBS, 2 mM L-glutamine and 1% penicillin and streptomycin, and were subcultured by trypsinization. Cells were maintained at 37°C and 5% CO2.

Splenic lymphocytes were cultured in supplemented RPMI Glutamax: 20% heat-inactivated FBS, 1% penicillin/streptomycin. B-lymphocytes were stimulated with 10 μg/ml Kdo2-Lipid A (Avanti Polar Lipids) for 48 h before transfection.

DNA repair assays transfections

Electroporation. Lymphoblastoid cell lines were transfected by electroporation. 2 × 10⁶ lymphoblastoid cells in 100 μl of complete medium were combined with a reporter plasmid mixture (Table 1). Cells were electroporated using a 96-well electroporation plate and gene pulser (Bio-Rad) with an exponential waveform at 260 V and 950 μF. Following electroporation, 100 μl of complete medium were added to each well in the plate, and the 200 μl volume was transferred to a 12-well plate containing 800 μl of equilibrated medium.

Lipofection. MEFs and human fibroblasts were transfected by lipofection using Lipofectamine LTX (LifeTechnologies), following manufacturer’s instructions. Briefly, plasmid reporter DNA mixture (Table 1) combined with Opti-MEM, Lipofectamine and Plus reagents, and 200 μl of the total mixture were added to each well of the 6-well plate.

Flow cytometry analysis of lesions

Eighteen hours after transfection, cells suspended in culture media were analyzed for fluorescence on a BD LSR II cytometer running FACSDiva software. Cell debris, doublets and aggregates were excluded based on their side- and forward-scatter properties. TO-PRO-3 was added to cells 5–10 min before analysis and was used to exclude dead cells from the analysis. The following fluorophores and their corresponding detectors (in parentheses) were used: tagBFP (Pacific Blue), EGFP (FITC), mOrange (phycoerythrin; PE) and TO-PRO-3 (allophycocyanin; APC). Compensation was set by using single-color controls. Regions corresponding to cells positive for each of the five fluorescent proteins were established by using single-color dropout controls. For reporters that required compensation in more than one detector channel, fluorescence in the reporter channel was plotted separately against each of the channels requiring compensation. Using these plots, both single-color controls and the dropout control (in which the reporter of interest was excluded from the transfection) were used to establish regions corresponding to positive cells.
Table 1. Combinations of reporter plasmids and types of DNA damage used in each experiment

<table>
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<tr>
<th>Comb.</th>
<th>BFP</th>
<th>GFP</th>
<th>Carrier</th>
<th>mOrange</th>
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<tr>
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<td>WT</td>
<td>90 ng</td>
<td>30 ng</td>
</tr>
<tr>
<td>2</td>
<td>WT</td>
<td>616-eA</td>
<td>90 ng</td>
<td>30 ng</td>
</tr>
<tr>
<td>3</td>
<td>WT</td>
<td>615-eC</td>
<td>90 ng</td>
<td>30 ng</td>
</tr>
<tr>
<td>4</td>
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<td>WT</td>
<td>400 ng</td>
<td>100 ng</td>
</tr>
<tr>
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<td>WT</td>
<td>616-eA</td>
<td>400 ng</td>
<td>100 ng</td>
</tr>
<tr>
<td>6</td>
<td>WT</td>
<td>615-eC</td>
<td>400 ng</td>
<td>100 ng</td>
</tr>
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</tr>
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<td>450 ng</td>
</tr>
<tr>
<td>16</td>
<td>WT</td>
<td>615-eC</td>
<td>25 ng</td>
<td>450 ng</td>
</tr>
<tr>
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<td>WT</td>
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<td>25 ng</td>
</tr>
<tr>
<td>18</td>
<td>WT</td>
<td>615-eC</td>
<td>25 ng</td>
<td>450 ng</td>
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<td></td>
<td></td>
<td></td>
<td>25 ng</td>
<td>375 ng</td>
</tr>
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Each column corresponds to a different plasmid reporter expressing a fluorescent protein, with the exception of ‘carrier’ which corresponds to an empty vector. WT = wild-type; eA = 1,N6-ethenoadenine; eC = 3,N4-ethenocytosine; TˆT = thymine dimer. Numbers before eA, eC and TˆT correspond to the site of the lesion in the plasmid. UV corresponds to plasmids treated with ultraviolet light at 800 J/m².

Calculation of percent fluorescent reporter expression. Every experimental setup consisted of two sets of transfections; a control transfection (CT) and a sample transfection (ST) containing one or more reporters with DNA lesions. Both transfections included the same color combination with the same undamaged reporter to normalize each set for transfection efficiency.

Fluorescence index (FI) for a given reporter within one transfection was calculated as follows:

\[
FI = \frac{C_F \times MFI}{C_L}
\]

where \(C_F\) is the number of positive fluorescent cells for that given fluorophore, \(MFI\) is the mean fluorescence intensity of the \(C_F\), and \(C_L\) is the total number of live cells.

The normalized fluorescence index for a given reporter \(FIO\) was calculated as follows:

\[
FIO = \frac{FI^n}{FI^E}
\]

where \(F^n\) corresponds to the \(FI\) of a reporter normalized to the \(FI\) of the transfection efficiency normalization plasmid, \(FI^E\).

Normalized reporter expression from a sample transfection, \(FIO_{ST}\), and that from the same reporter plasmid in control transfection, \(FIO_{CT}\), were used to compute the percent reporter expression (\%R.E.) as follows:

\[
\%R.E. = \frac{FIO_{ST}}{FIO_{CT}} \times 100
\]

RNA isolation for RNA-Seq

At 18 h, a fraction of transfected cells were harvested by centrifugation, washed twice with PBS, and resuspended in 1 ml TRIzol reagent. The suspension was extracted with 200 μl chloroform. The aqueous phase was removed, combined with one volume of absolute ethanol, and applied to a Qiagen RNeasy miniprep spin column. The column was washed two times with 500 μl Buffer RPE (Qiagen) and finally eluted in 40 μl diethylpyrocarbonate (DEPC)-treated water. The quality of the RNA preparation was determined by using a bioanalyzer to confirm an RNA integrity number (RIN) of at least 9.0. At least 190 ng of total RNA were stored in TE buffer at −80°C until submission for mRNA sequencing (RNA-Seq).

Total RNA samples were submitted to the Massachusetts Institute of Technology BioMicroCenter for preparation and sequencing. Briefly, total RNA was poly-A purified, fragmented, and converted to cDNA by using the Clontech SMARTseq protocol. Library construction from cDNA was performed using the Beckman Coulter SPRIworks system. During library amplification, a unique bar code was introduced for each of the 12 samples corresponding to the four transfections performed in triplicate (Supplementary Table S1) and from which total RNA was generated. All samples were combined and ran on a NextSeq instrument.

Next generation sequencing data analysis

RSEM (46) (version 1.2.15) was used to estimate gene expression based on hg19 UCSC known gene annotations. The count table was imported into DESeq2 (47) (version 1.10.1) for differential gene expression analysis. BWA (48) (version 0.7.10) was used to map the raw reads to the plasmids. Then all properly paired, uniquely and perfectly mapped reads were counted to estimate expression of the reporters. Relative expression levels of the reporters were computed by normalizing their counts to BFP expression in the same sample. SAMTools (49) (version 1.3) mpileup (options: -d1000000 -B) was used to process the bam alignments, followed by VarScan (50) (version 2.3.6) to call mutations on the reporters.
RESULTS

εA is repaired in vivo by AAG, but not ALKBH2 or ALKBH3; εC is not repaired by any of them

In order to test the in vivo role of known DNA repair proteins in the repair of εA and εC adducts, we performed an HCR assay by transfecting reporter constructs harboring site-specific lesions into repair-proficient WT B6 and Aag−/− cell lines derived from C57BL/6 background mice whereas WTB6/129s and TKO (triple knockout, Aag−/−/Alkbh2−/−/Alkbh3−/−) cell lines are derived from mixed background mice (C57BL/6 and 129s). Error bars show standard deviation of at least three biological replicates. Two-tailed unpaired t test ***P ≤ 0.001; ns, not significant.

TC-NER and downstream NER components are involved in the repair of εC

Given the strong decrease in %R.E. observed for site-specific εC lesions, we sought to determine if this decrease was a result of a transcription block that could be repaired by the NER pathway. To this end, we used a panel of B-lymphoblastoid cell lines derived from individuals with CS or XP, diseases characterized by deficiencies in the different sub-pathways of NER and sensitivity to UV light. Previously, we established a reporter assay system to evaluate DNA repair capacity for UV-induced DNA damage using plasmids treated with 800 J/m^2 UV that were subsequently transfected into cell lines deficient in NER (XPA, XPB, XPC or XPD) (42). In this study, deficiencies in XPA, XPB, XPC and XPD resulted in decreased reactivation of UV-treated plasmids compared to reactivation in healthy controls (Figure 3A, plasmid combinations #7 and 8 in Table 1). We also determined DNA repair capacity of UV damage in CS cell lines defective in TC-NER. Cells lacking CSA displayed significantly decreased reactivation; while plasmid reactivation appeared lower in CB-deficient cells in comparison to cells from the healthy control, the difference did not quite reach significance (P = 0.056).

We had previously shown that XPA-deficient lymphoblastoid cells could not efficiently repair plasmids containing a site-specific thymine dimer (T*T), the most common lesion caused by UV light (42). Here, we tested whether the NER deficiencies of the other XP and CS cell lines could be phenotyped using a site-specific thymine dimer, a lesion known to block transcription. Following transfection of a GFP reporter bearing a site-specific T*T, we reproduced our previous XPA mutant results. Moreover, we observed similar repair deficits in cells defective for TC-NER (CSA and CSB) as well as the other downstream NER components that operate after the damage recognition step (XPB and XPD). Interestingly, deficiencies in the GG-NER sub-pathway (cells deficient in XPC) did not appear to affect the repair of this site-specific lesion (Figure 3B, plasmid com-
Figure 3. Repair of UV-irradiated and site-specific thymine dimer, εA and εC plasmids by a panel of B-lymphoblastoid cell lines with varying NER deficiencies. Cell lines were transfected with GFP repair reporters irradiated with 800 J/m² UV-C (A) or with GFP reporters containing a site-specific thymine dimer (T^-T^-) (B). Black boxes correspond to NER proficient cells; dark gray, TC-NER deficient; light grey, GG-NER deficient; white, downstream-NER (TC-NER and GG-NER) deficient. Dashed lines represent a threshold between repair assumed to occur through TC-NER (above line) and GG-NER (below line) based on the lowest repairing TC-NER deficient cell line tested. Error bars show standard deviation of at least three biological replicates. Two-tailed unpaired t test between healthy control and each deficient cell line: *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001; ns, not significant.

Figure 3A: Repair of UV (800 J/m²) Reporter

Figure 3B: T^-T^- Reporter

Figure 3C: εA Reporter

Figure 3D: εC Reporter

Figure 3. Repair of UV-irradiated and site-specific thymine dimer, εA and εC plasmids by a panel of B-lymphoblastoid cell lines with varying NER deficiencies. Cell lines were transfected with GFP repair reporters irradiated with 800 J/m² UV-C (A) or with GFP reporters containing a site-specific thymine dimer (T^-T^-) (B). Black boxes correspond to NER proficient cells; dark gray, TC-NER deficient; light grey, GG-NER deficient; white, downstream-NER (TC-NER and GG-NER) deficient. Dashed lines represent a threshold between repair assumed to occur through TC-NER (above line) and GG-NER (below line) based on the lowest repairing TC-NER deficient cell line tested. Error bars show standard deviation of at least three biological replicates. Two-tailed unpaired t test between healthy control and each deficient cell line: *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001; ns, not significant.

After validating that NER-deficient cell lines were deficient in the repair of a site-specific transcription blocking base lesion, we transfected the site-specific εA or εC plasmid into CS or XP cells. None of the cells displayed a deficiency in the repair of εA; curiously, the XPC-deficient cell line actually displayed higher εA repair capacity than the healthy control (Figure 3C, plasmid combinations #7 and 10 in Table 1). In contrast, repair of εC was deficient in five out of six NER-deficient cell lines, closely resembling the repair phenotypes observed for the site-specific T^-T^- lesion (Figure 3D, plasmid combinations #7 and 11 in Table 1). Deficiencies in TC-NER, as well as the downstream NER factors, resulted in significantly diminished εC repair capacity. Again, the GG-NER-deficient XPC line appeared more repair-proficient than the healthy control. It should be noted that the ‘healthy’ cell line GM07752 happens to have a particularly low NER capacity compared with other ‘healthy’ lymphoblastoid cell lines (42).

To further investigate the role of TC-NER in the repair of εC, we utilized human SV40-transformed fibroblasts derived from CS patients deficient in CSA (CS3BE) or CSB (CS1AN) plus their respective isogenic repair-proficient cDNA-complemented counterparts. We determined the repair capacity of these four cell lines for UV treated plasmids and for plasmids with a site-specific T^-T^- and εC lesion. Consistent with our results in the non-isogenic lymphoblastoid cells, both CSA- and CSB-deficient cells showed decreased repair of UV treated plasmids as well as site-specific T^-T^- and εC plasmids, in comparison to their complemented equivalents. Furthermore, no significant difference in the repair of εA was detected in either CSA- or CSB-deficient cells relative to the complemented controls (Figure 4A and...
in vivo determinewhetherthislesioncouldsometimesbebypassed by RNAPII resulting in transcriptional mutagenesis of thymine dimers caused by UV irradiation of AA to AG in CSA-deficient cells (Supplementary Figure S1). As expected, the repair-proficient CSA-complemented cells showed a lower percentage of transcriptional mutagenesis events given that the lesion is being actively repaired more efficiently by TC-NER. Finally, sequence level analysis of the UV irradiated mOrange reporter revealed transcriptional mutagenesis of thymine dimers caused by UV irradiation of AA to AG in CSA-deficient cells (Supplementary Figure S1).

**DISCUSSION**

Using a multiplexed HCR assay, we were able to reveal a novel role for the NER pathway, specifically TC-NER, in the repair of the highly mutagenic and cytotoxic DNA lesion, εC. Given that this lesion is a product of endogenous LPO products, characteristic of cancer-prone inflammatory diseases, we propose that TC-NER may play an important role in protection against inflammation-associated disease. That both humans and mice deficient in TC-NER undergo premature aging could in part be due to a deficiency in the repair of inflammation associated DNA damage (51,52).

It is noteworthy that we did not observe a major contribution by Alkbh2 and Alkbh3 in the repair of εA, in either MEFs or mouse splenic-B-lymphocytes (Figures 1 and 2). Both enzymes have been shown in vitro to repair εA DNA lesions (26–28). Deletion of both enzymes together with Aug, in chronic-inflammation mouse models, has been
Figure 5. HCR readout by flow cytometry and RNA-Seq. DNA repair capacity of patient derived fibroblasts deficient in CSA (CS3BE) and their CSA complemented counterpart (CS3BE + CSA). (A) Cells were transfected with BFP as a transfection control reporter, GFP bearing a site-specific εC and mOrange irradiated with 800 J/m² UV-C. (B) %R.E. for flow cytometry data (filled bars) was calculated as explained in Materials and Methods. %R.E. RNA-Seq data (empty bars) was calculated directly from transcript counts and normalized to efficiency normalization plasmid counts and the respective control transfection. (C) Representative gene expression profile for undamaged versus damaged transfections of both cell lines. Endogenous transcripts are shown in black and reporter plasmids are colored in blue, green and orange. (D) RNA-Seq sequence variants detected in transcripts at position 615 in a GFP reporter containing either a C or an εC on the transcribed strand. Frequencies are reported for the expected variant (G) as well as for the remaining 3 possible variants (C, G, T/U). Error bars show standard deviation for three biological replicates. Two-tailed unpaired t test: *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001; ns, not significant.

shown to lead to increased accumulation of ε-adducts in colon epithelium (specifically 1,N²-εG, while εA did not reach significance) compared to Aag deletion alone (43). It is possible that the contribution of these enzymes in εA repair is cell-type specific and not expressed or active in MEFs or splenic-B-lymphocytes; although they have been shown to be expressed in the spleen of C57BL/6 mice (53). Alternatively, in vivo, they may simply not play a prominent role in the repair of εA.

Interestingly, εC can be bound by AAG (25), yet it is not excised by this DNA glycosylase (Figures 1 and 2). In vitro, the interaction between AAG and εC blocks the repair of this lesion by ALKBH2 (29). Surprisingly, Aag⁻/⁻ mice show increased accumulation of εC (in the absence of Aag) in the colonic mucosa of inflammatory bowel disease mouse models (54). We hypothesized that if Aag binds to εC and recruits downstream repair enzymes, we would observe decreased εC plasmid reactivation in Aag⁻/⁻ cells. However, one could also argue that there would be increased εC repair in Aag⁻/⁻ cells, if the absence of Aag allowed Alkbh2 to access the lesion. In fact, neither turned out to be the case in MEFs or primary splenic-B-lymphocytes as no difference in the repair of εC was observed in the absence or presence of Aag. Given (i) the lack of a clear role for Aag and the Alkbh enzymes in εC repair and (ii) the strong decrease in reporter expression observed in the presence of this lesion, we propose that εC causes a transcription blockage similar to that seen for the site-specific T'T reporter (42). We further propose that εC is repaired by the TC-NER machinery. Nevertheless, we cannot rule out the possibility that ‘naked’ εC and the Aag-εC complex have the same effect on transcription, namely transcriptional blockage. Finally, it is striking that the closely related lesions εA and εC can have such diverse effects on transcription. Through crystallographic analysis of the Aag-εC complex we have previously shown that the inability to protonate εC at C5
in comparison to the N7 position of εA renders the lesion incapable of excision by Aag (55). While this does not directly explain why εC is repaired by TC-NER and εA is not, it does provide a paradigm for why seemingly closely related DNA base lesions may be perceived very differently by DNA polymerases, RNA polymerases and DNA repair proteins.

It is notable that XPC-deficient cells exhibited reduced repair capacity for plasmids containing high levels of UV-damage located randomly in the reporter plasmid, indicating that this HCR-based assay is capable of monitoring GG-NER (Figure 3). However, it is also notable that GG-NER plays no significant role in repairing a single transcription-blocking lesion located in an actively transcribed region, probably because RNAPII lesion detection outcompetes XPC-mediated GG-NER lesion detection. As such, our method cannot differentiate between cells being deficient in GG-NER for the repair of site-specific transcription-blocking lesions or cells simply not utilizing GG-NER for the repair of a single DNA lesion located in a highly transcribed gene. Given that the plasmid used is non-replicative and that transcription from the CMV promoter is vigorous, it is possible that the observed roles for TC- and GG-NER would change if the plasmid were either replicative or transcribed less efficiently. Another indication that repair of site-specific transcription-blocking lesions is occurring through TC-NER and not GG-NER is that TC-NER deficient cells showed similar repair phenotypes for site-specific T’T repair as cells deficient in downstream-NER steps, even though this was not the case with UV-irradiated plasmids.

The fact that the repair phenotype of a site-specific εC by human lymphoblastoid cell lines deficient in NER so closely recapitulated that of a site-specific T’T strongly supports our conclusion that the repair of εC occurs via the TC-NER pathway (Figure 3). This observation was further validated in a different cell type, namely human fibroblasts, deficient or complemented for either CSA or CSB (Figure 4). Importantly, each pair of cell lines was isogenic, minimizing the contribution of any inter-individual variations in NER capacity.

TC-NER proteins have been postulated to play a role in the repair of BER substrates. In particular, the contribution of CSB to the repair of 8oxoG and uracil has been shown to be DNA glycosylase dependent (OGG1 and UNG, respectively) in yeast (56), mouse cells (57,58) and human fibroblasts (59). Similarly, CSB has been implicated in the repair of single-strand breaks (SSBs) in actively transcribed genes (60). These SSBs could also be produced through the BER pathway. Nevertheless, we did not observe at any point the participation of Aag in TC-NER of εA adducts. We cannot rule out the possibility that εC repair by TC-NER involves, to some degree, formation of BER intermediates created by the action of other DNA glycosylases (such as TDG, MBD4 or SMUG1) on εC. Nevertheless, Aag does not seem to play a role in modulating TC-NER for the repair of εC under our experimental conditions.

RNA-Seq analysis of our reporter transcripts allowed us to corroborate that, in fact, fewer transcripts are produced in the presence of εC, validating the interpretation of our flow cytometry results as indicating transcription blockage by εC (Figure 5). Moreover, we were able to describe the transcriptional mutagenesis properties of εC in vivo. The only studies on the effect of εC on transcription were done in vitro and date back to the 1980s; CAA treated poly(dC) oligos were shown to block transcription and induce ribonucleotide misincorporation by calf thymus RNAPII (61). To our knowledge, the transcription blockage and mutagenic properties of εC described here represent novel in vivo features for this lesion. RNAPII transcriptional mutagenesis tends to resemble the misincorporation profile exhibited by mammalian replicative DNA polymerases during synthesis opposite DNA lesions (41). In the case of εC, misincorporation during DNA replication has the preference T->A->C (17). We observed a similar trend with A and T(U) misincorporation being favored and incorporation of C being the least likely event.

In the broader context of LPO-induced DNA damage in mammals, while bulky HNE–DNA and malondialdehyde-derived adducts are widely known to block transcription and known to be repaired by TC-NER (4,35,36), for the non-bulky ε-adducts, only 1,N2-εG had been reported to block transcription in vitro (62). Altogether, our in vivo results showing transcriptional blockage and transcriptional mutagenesis by εC complement the notion that DNA damage and repair play a pivotal role in the etiology of disease; in particular, cancer-prone inflammatory diseases, degenerative diseases and other diseases of old age, which are characterized by increased oxidative stress that can lead to the formation of LPO products. In these situations, repair of εC becomes highly relevant in both a replicative and non-replicative context. On the one hand, it can contribute to the accumulation of potentially deleterious mutations during replication; and on the other hand, it can perturb cellular homeostasis via RNAPII blockage or production of mutated transcripts via transcriptional mutagenesis.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.

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