# A new sub-pathway of long-patch base excision repair involving 5' gap formation

Jordan Woodrick[*1], Suhani Gupta[*1], Sharon Camacho[1], Swetha Parvathaneni[2], Sujata Choudhury[1], Amrita Cheema[1], Yi Bai[1], Pooja Khatkar[1], Hayriye Verda Erkizan[1], Furqan Sami[2], Yan Su[3], Orlando D. Schärer[3], Sudha Sharma[2§], and Rabindra Roy[1§]

[1]Department of Oncology, Lombardi Comprehensive Cancer Center

Georgetown University, Washington, DC

[2]Department of Biochemistry and Molecular Biology, College of Medicine, Howard University, Washington, DC[3]Department of Pharmacological Sciences & Department of Chemistry, Stony Brook University, Stony Brook, NY

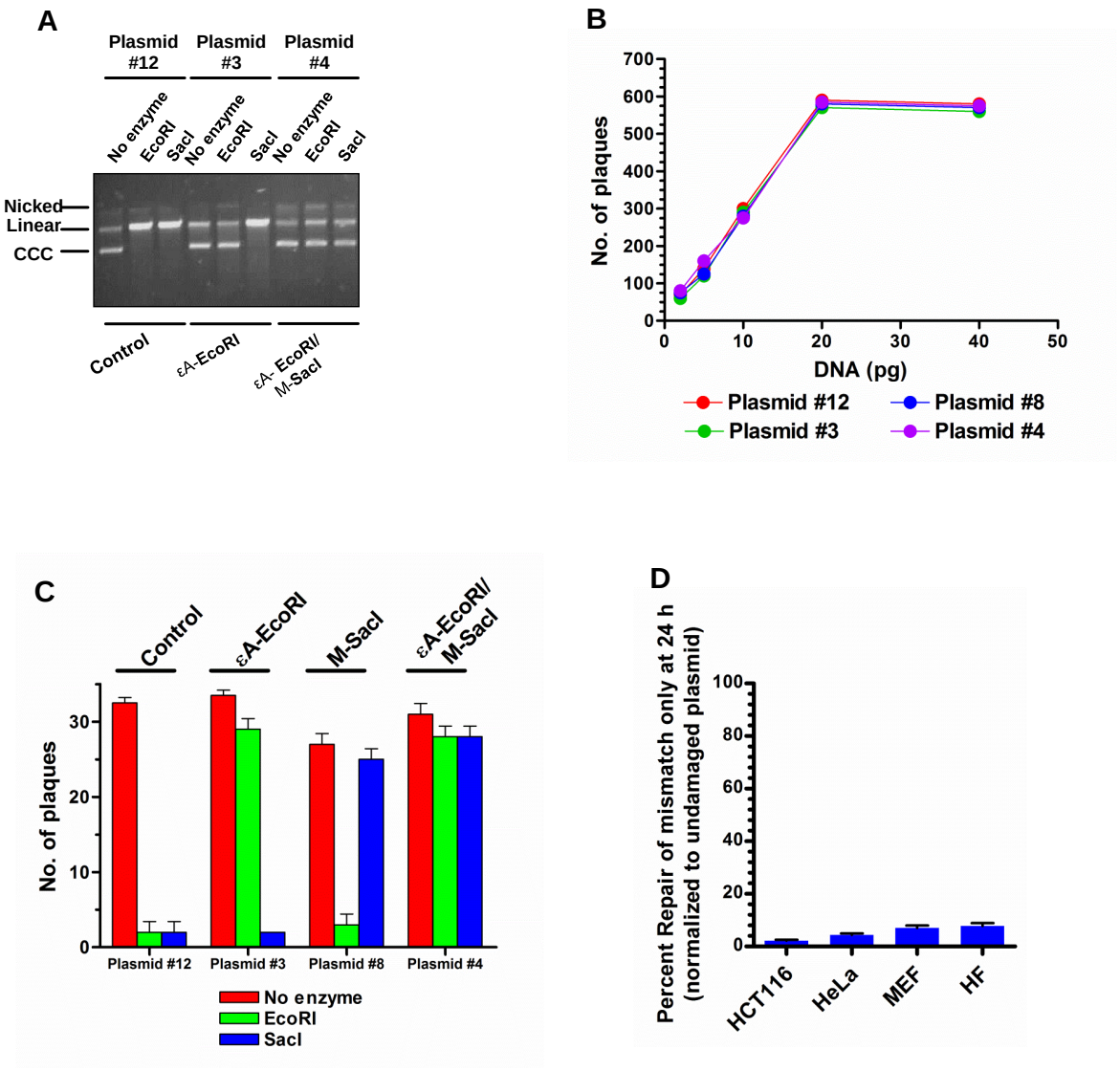
[*]Both authors contributed equally to this study.

[§]Address correspondence to Rabindra Roy: rr228@georgetown.edu or Sudha Sharma: sudha.sharma@howard.edu

**Appendix Table of Contents**

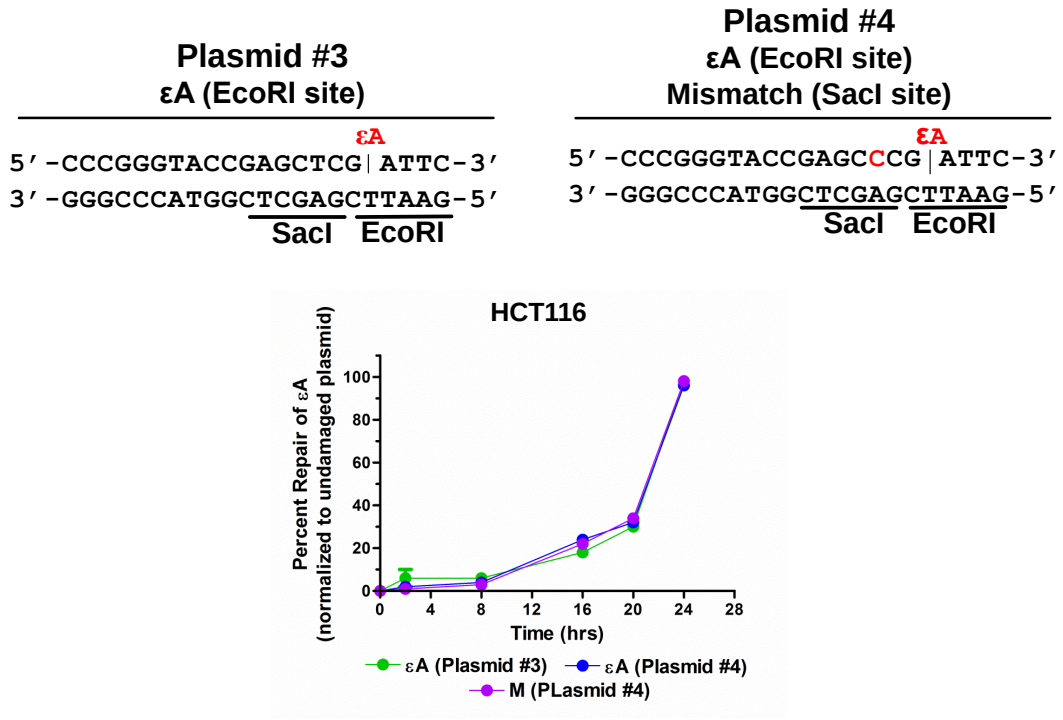
| Plasmid no. | Description of the construct | Position of adduct | Position of mismatch | Orientation to adduct |
|---|---|---|---|---|
| 1 | eA (+1 PstI)<br>5'-GCATGCCTGC**X**GGTCGACTCTAG-3' | PstI | N/A | N/A |
| 2 | eA (+1 PstI), M (+4 SalI)<br>5'-GCATGCCTGC**X**GG**C**CGACTCTAG-3' | PstI | SalI | 3' (+4) |
| 3 | eA (+1 EcoRI)<br>5'-CCGAGCTCG**X**ATTCGTAATC-3' | EcoRI | N/A | N/A |
| 4 | eA (+1 EcoRI), M (-4 SacI)<br>5'-CCGAGC**C**CG**X**ATTCGTAATC-3' | EcoRI | SacI | 5' (-4) |
| 5 | eA (+1 EcoRI), M (-12 KpnI)<br>5'-GGATCCCCGGG**C**ACCGAGCTCG**X**ATTCGTAATC-3' | EcoRI | KpnI | 5' (-12) |
| 6 | eA (+1 BamH1), M (-7 XbaI)<br>5'-GTCGACTC**C**AGAGG**X**TCCCCGG-3' | BamH1 | XbaI | 5' (-7) |
| 7 | eA (+1 BamH1) M (-9 XbaI)<br>5'-GTCGAC**C**CTAGAGG**X**TCCCCGG-3' | BamH1 | XbaI | 5' (-9) |
| 8 | M (+4 SalI)<br>5'-GCATGCCTGCAG**C**CGACTCTAG-3' | N/A | SalI | N/A |
| 9 | eA (+1 PstI), M (+11 XbaI)<br>5'-GCATGCCTGC**X**GGTCGACTC**C**AG-3' | PstI | XbaI | 3' (+11) |
| 10 | eA(+1 PstI), M (+12 XbaI)<br>5'- GCATGCCTGC**X**GGTCGACTCT**C**GAGGATCCC -3' | PstI | XbaI | 3' (+12) |
| 11 | eA (+1 PstI); M (+13 XbaI)<br>5'-GCATGCCTGC**X**GGTCGACTCTA**A**AGGATCCC-3' | PstI | XbaI | 3' (+13) |
| 12 | Control (no damage)<br>5'-CCGAGCTCGAATTCGTAATC-3' | N/A | N/A | N/A |
| 13 | 8oxoG (+1 EcoRI), M (-3 SacI)<br>5'-CCGAGC**C**C**X**AATTCGTAATC-3' | EcoRI | SacI | 5' (-3) |
| 14 | 5-OHU(+1 EcoRI), M (-8 SacI)<br>5'-CCGAGC**C**CGAATT**X**GTAATC-3' | EcoRI | SacI | 5' (-8) |

**Appendix Table S1.** Plasmid DNA used in this study was generated as described in the Appendix Supplementary Methods section using oligonucleotides indicated in the table. Red "X" indicates position of the adduct, and green "C" indicates position of the mismatch.

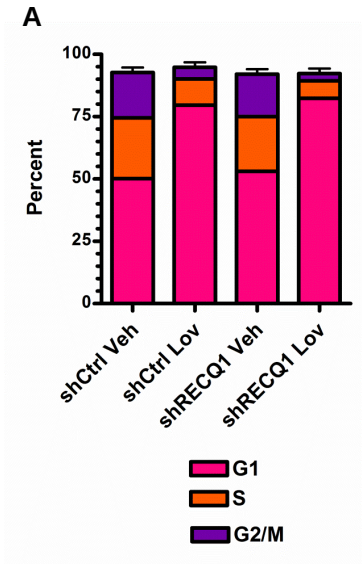


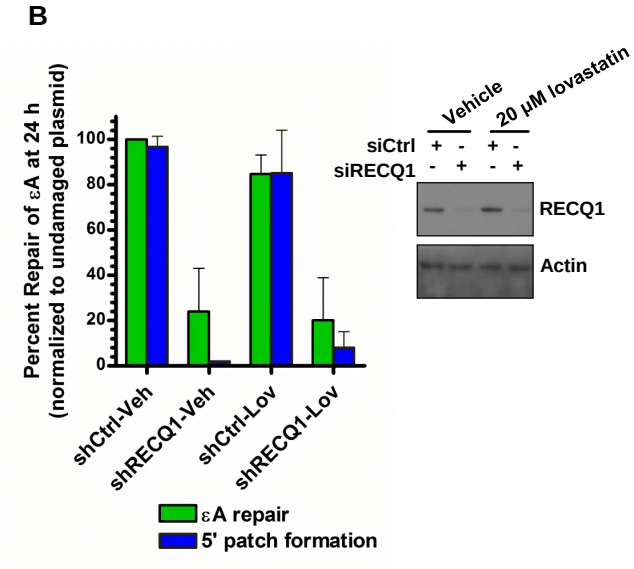

**Appendix Figure S1. Plaque assay validation *in vitro* and in-cell.** For monitoring patch size during BER in live cells, we modified a plaque-based in-cell repair assay previously developed by our laboratory (Choudhury et al, 2008). (A) Validation of plasmid. Indicated plasmids were digested with EcoRI and SacI and resolved on a 1% agarose gel. εA and/or a mismatch at the EcoRI and SacI sites, respectively, render the plasmid resistant to both restriction enzymes. (B) Plaque formation with increasing

concentrations of the indicated plasmid DNA (see Appendix Table S1 for plasmid details) demonstrates that plaque formation is not altered by modifications in the plasmid DNA and is DNA concentration-dependent. (C) The indicated plasmids were digested with EcoRI and SacI. The reaction products were subjected to plaque assay and plaques quantified, which demonstrated that linearization of the plasmid by restriction digestion yields little to no plaques after transformation. Modification of the restriction sites by εA and/or a mismatch consequently rendered the plasmid resistant to digestion and plaque number was unchanged compared to undigested control. (D) Plasmid containing only a C:A mismatch at the SalI site (plasmid #8) was assayed for repair of the mismatch in the cell lines used in this study 24 h post-plasmid transfection, which demonstrated that the mismatch was not repaired independent of BER in cells.

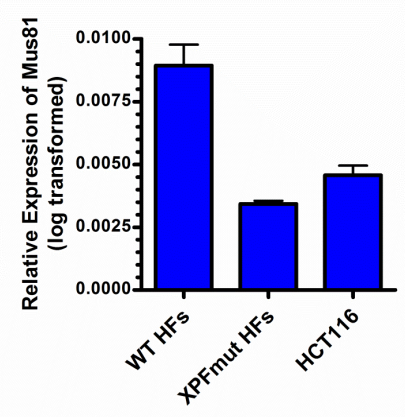



**Appendix Figure S2. 5' patch formation step during BER in live cells.** The indicated plasmids (#3 and #4, Appendix Table S1 for plasmid details) were transfected into HCT116 cells and harvested at the indicated time points post-transfection. Repair of εA or the C:A mismatch (M) in the harvested plasmid DNA was assessed by the plaque assay. Diagrams depict relevant portions of the M13mp18 MCS region with modifications indicated in red.

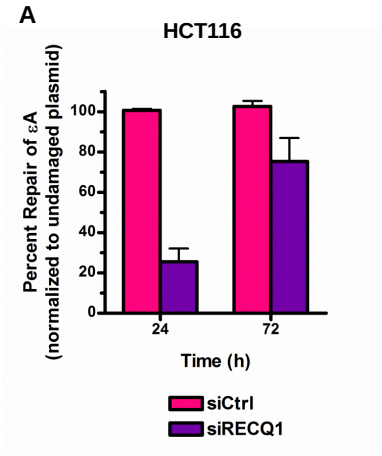


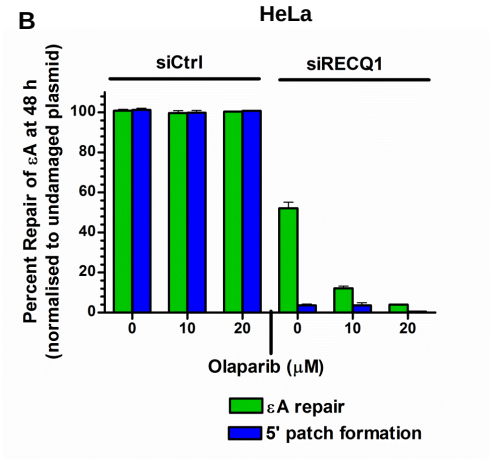




**Appendix Figure S3. 5' patch-mediated LP-BER is operational in G1-arrested cells.** (A) Cell cycle distribution measured by DNA content analysis (propidium iodide staining) using flow cytometry was monitored in stable control (shCtrl) and RECQ1 KD (shRECQ1) HeLa cells after 33 h of treatment with vehicle (0.14% ethanol) or 20 μM lovastatin. (B) After 33 h of treatment with vehicle or 20 μM lovastatin, εA repair and 5' patch formation were monitored by real time PCR-based repair assay 24 h post-plasmid transfection (plasmid #4) in stable control and RECQ1 KD HeLa cells.

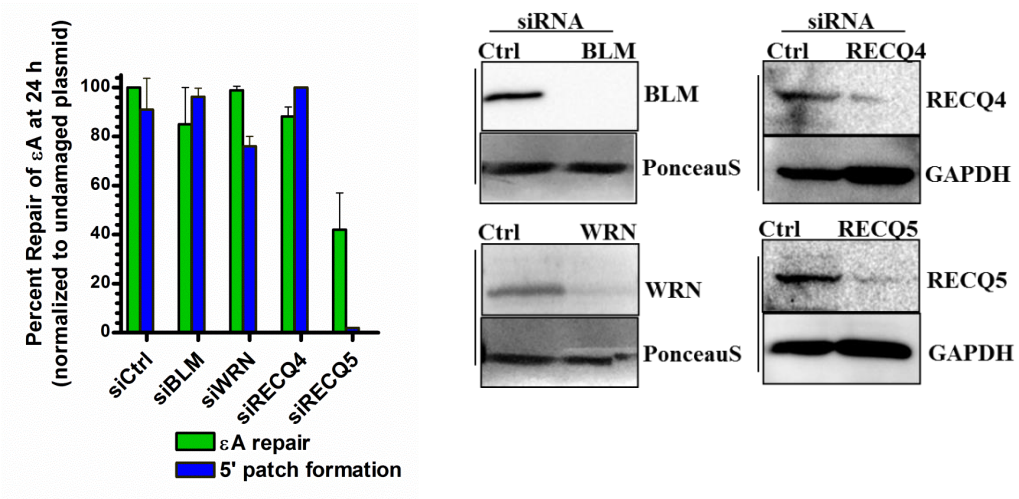



**Appendix Figure S4. Expression of Mus81 in experimental cell lines.** The expression of Mus81 was assessed by real time PCR using mRNA from WT and XPF mutant HFs and HCT116 cells. Bar graph indicates relative expression of Mus81 normalized to GAPDH expression.

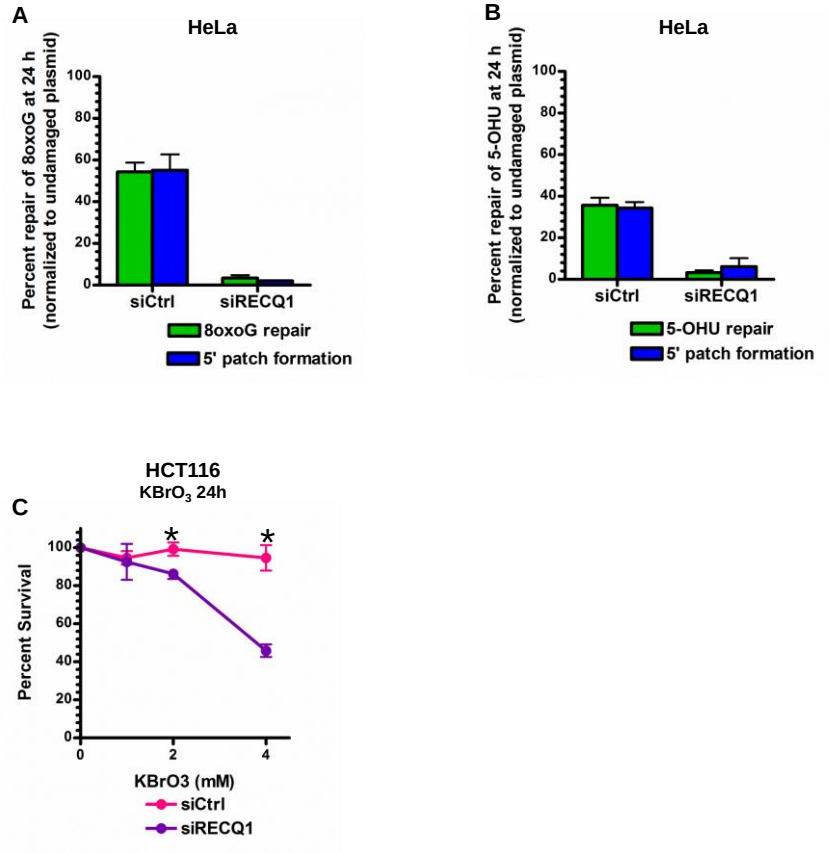



**Appendix Figure S5. RECQ1-PARP1 regulation of BER sub-pathway preference.** (A) εA repair on a plasmid #3 (no mismatch at SacI) was monitored by real time PCR-based repair assay in control and RECQ1 KD HCT116 cells at 24 and 72 h post-transfection. (B) εA repair and 5' patch formation were monitored by plaque assay post-plasmid transfection (plasmid #4) in olaparib-treated control and RECQ1 KD HeLa cells at 48 h in the same manner as described in Figure 4B.




**Appendix Figure S6. Involvement of RecQ family helicases in BER.** εA repair and 5' patch formation were monitored by plaque assay 24 h post-plasmid transfection (plasmid #4) in the same manner as described in Figure 1D in control and BLM KD, WRN KD, RECQ4 KD, or RECQ5 KD HeLa cells. Inset shows Western blot analysis of KD of each gene. Ponceau S staining and GAPDH Western blotting were used for loading controls.




**Appendix Figure S7. RECQ1 is required for BER of oxidative DNA damage in HeLa cells.** (A) 8-oxoG repair and 5' patch formation and (B) 5-OHU repair and 5' patch formation were monitored by real time PCR-based repair assay in control and RECQ1 KD HeLa cells 24 h post-plasmid transfection (plasmids #13 and 14, respectively) in the same manner as described in Figure 1D. (C) Survival of control and RECQ1 KD HCT116 cells treated for 24 h with indicated doses of $KBrO_3$ was measured by cell counting. Asterisk (*) denotes p-value of student's t-test < 0.05.

## Appendix Supplemental Methods

*Chemicals and enzymes*

Restriction enzymes (EcoRI, SacI, PstI, SalI, BspHI, BamHI, XbaI, HindIII, and NarI) were purchased from New England Biolabs, and all digestions were performed according to manufacturer's protocol. [$\gamma$-$^{32}$P]-$NAD^+$ and [$\gamma$-$^{32}$P]-ATP were purchased from Perkin Elmer. Recombinant MPG, APE1, XPF-ERCC1, and mutant RECQ1 were expressed and purified as previously described (Adhikari et al, 2010; Adhikari et al, 2007; Enzlin & Scharer, 2002; Sharma et al, 2005). *In vitro* autopoly(ADP-ribosyl)ated PARP1 was prepared as previously described (Berti et al, 2013). Purified RPA was a gift from Dr. Altaf Sarkar of Lawrence Berkeley National Laboratory. MMS and $KBrO_3$ were purchased from Sigma-Aldrich. Olaparib was purchased from Selleckchem. Aphidicolin was purchased from Calbiochem. Lovastatin was purchased from TCI America.

*Cell Culture*

All media were supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine and 1% penicillin/streptomycin. HCT116, parental HeLa, stable RECQ1 KD HeLa (Li et al, 2014), and *Recq1*$^{+/+}$ and *Recq1*$^{-/-}$ MEFs (Sharma et al, 2007) were cultured in supplemented DMEM. *Parp*$^{+/+}$ and *Parp*$^{-/-}$ MEFs (gift from Dr. Mitchell Jung of Georgetown University) were cultured in supplemented RPMI 1640 media. Normal, XPF mutant, and XPA mutant human fibroblasts (GM00637, GM04313, GM04429 respectively; Coriell) and XPF mutant fibroblasts lentivirally transduced with wild-type XPF (Staresincic et al, 2009) were cultured in supplemented MEM media.

*In vitro M13mp18 plasmid BER substrate preparation for repair assays*

The preparation of the control and εA/8-oxoG/5-OHU-containing plasmid DNA was performed *in vitro* as previously described with some modification (Choudhury et al, 2008). The *in vitro* construct preparation included three main steps: phoshorylation of the damage-containing primer oligonucleotide, annealing of the oligonucleotide to the ssDNA, and the primer extension reaction. Typically, for each preparation of a double stranded construct, six individual reactions were performed simultaneously according to the following protocol. Phosphorylation of the primers was performed by incubating 2 μg of damage- and/or mismatch-containing oligonucleotide (2 μL) (See Table S1) with 1X PNK buffer, 400 nM ATP, 50 mM DTT, and 10 U of T4 polynucleotide kinase (New England Biolabs) in a 30 μL reaction volume at 37°C for 45 min. The phosphorylated oligonucleotide was purified through a G-25 column (GE Healthcare Life Sciences) according to the manufacturer's protocol. Then 6 μL of this purified oligonucleotide was incubated with 2 μg of M13mp18 ssDNA in an annealing buffer containing 10 mM Tris-HCl, pH 7.5, and 50 mM NaCl in a 20 μL reaction volume. This annealing reaction was incubated at 80°C for 5 min followed by room temperature for 1 h and on ice for 30 min. Then the annealing reaction was incubated with an extension reaction mixture containing 1X T7 DNA polymerase buffer, 1.5 mM ATP, 1.5 mM of each dNTP, 10 mM DTT, and 160 μg/mL BSA, 10 U of T7 DNA polymerase (New England Biolabs), and 400 U of T4 DNA ligase (New England Biolabs) in a final reaction volume of 30 μL for 5 min on ice followed by 5 min at room temperature. The extension reaction was subsequently incubated at 37°C for 1 h. After an hour, 50 nmol of ATP and 200 U of T4 DNA ligase were added to the extension reaction and incubated at 14°C overnight for efficient

ligation to occur. The six individual reactions were then pooled together and incubated with 1X Supercoil-It buffer (Bayou Biolabs) and 2 μL of Supercoil-It enzyme mixture at 37°C for 3 h. Plasmid DNA was recovered after the incubation by purification using Qiaquick PCR Purification kit (Qiagen, Gaithersburg, MD). The DNA was eluted from the column using 50 μL of molecular grade water. Concentration of the eluted DNA was measured using a Nanodrop spectrophotometer, and the DNA was stored at -20°C for use in the *in vivo* repair assays.

*In-cell repair assay*

**εA/8-oxoG/5-OHU-M13mp18 plasmid transfection and RNA interference with/without complementation:** For monitoring in-cell repair of εA/8-oxoG/5-OHU and BER patch formation, typically 300,000 cells were seeded in a 6-well plate for overnight at 37°C. Transfections of both siRNA and phagemids were performed using 8 μL Lipofectamine 2000 (Life Technologies) in antibiotic-free media for the time periods indicated. For experiments requiring gene-specific RNA interference, cells were transfected with 25 nM RECQ1 siRNA (Santa Cruz Biotechnology), 50 nM APE1 siRNA (Saha et al, 2010), 25 nM FEN1 siRNA (5'- GCAGCACAAUGAUGAGUGCTT-3';(Sampathi et al, 2009)), 25 nM RPA32 siRNA #1 (5'-GGCUCCAACCAACAUUGUUTT-3';(Grudic et al, 2007)), 50 nM RPA32 siRNA #2 (Dharmacon ON-TARGETplus Human RPA2 SMARTpool), 10 nM XPF siRNA (Dharmacon ON-TARGETplus Human ERCC4 SMARTpool), 25 nM BLM siRNA (Dharmacon ON-TARGETplus Human BLM SMARTpool), 25 nM WRN siRNA (Dharmacon ON-TARGETplus Human WRN SMARTpool), 100 nM RECQ4 siRNA

(Dharmacon ON-TARGETplus Human RECQ4 SMARTpool) or 100 nM RECQ5 siRNA (Dharmacon ON-TARGETplus Human RECQ5 SMARTpool). After 24 h of siRNA transfection, 1.5 μg of εA- or control-M13mp18 phagemid was co-transfected into cells with a second dose of gene-specific siRNA. Cells were harvested at different time points post-phagemid transfection (24, 36, 48, 72 h) as indicated in the text, and the phagemid DNA was recovered using Mini Prep Kit (Qiagen) according to manufacturer's protocol for isolation of plasmid DNA from E. coli (Choudhury et al, 2008).

For RECQ1 complementation in stable RECQ1 KD HeLa cells, cells were seeded as described above then transfected with 2.5 μg empty vector or piRES-RECQ1 (expressing shRNA-resistant RECQ1) (a gift from Dr. Alessandro Vindigni of St. Louis University) (Berti et al, 2013) with 7.5 μL Lipofectamine 2000 for 6 h in Opti-MEM (Life Technologies) after which cells were supplemented with 2 mL Opti-MEM with 20% FBS for 48 h. After 48 h damage-containing plasmid was transfected and harvested as described above for siRNA experiments.

For RPA complementation in HCT116 cells, cells were seeded and transfected with 25 nM RPA32 siRNA #1 as described above. After 24 h of siRNA transfection, cells were transfected with 500 ng CMV-driven siRNA-resistant RPA32 expression plasmid (Sino Biologicals). The RPA32 open reading frame sequence was mutated at the siRNA target site by site-directed mutagenesis (5'-GGCTCCAACCAACATTGTT-3' mutated to 5'-GGCTCCAACAAATATCGTT-3') make the exogenous RPA32 siRNA-resistant. After 48 h of siRNA-resistant RPA32 expression plasmid transfection, damage-

containing plasmid was transfected and harvested as described above for siRNA experiments.

Experiments in XPF mutant human fibroblasts (lentivirally transduced with empty vector or WT XPF) were performed by seeding cells as above followed by transfecting and harvesting damage-containing plasmid as described for siRNA experiments above.

**Restriction digestion and plaque formation assay:** After retrieving the plasmid DNA, four aliquots of each sample were prepared. Two aliquots of the recovered plasmid DNA were digested individually with restriction enzymes according to the restriction sites where εA and the mismatch were placed within the construct (see Appendix Table S1 for construct information). Another aliquot was digested with a restriction enzyme whose recognition site was unmodified, serving as an internal control. Finally, one aliquot was mock-digested, serving as an undigested control. The digested plasmid DNA was transformed into electrocompetent XL1-Blue MRF' cells by electroporation, and the transformed cells were subjected to the plaque assay as described previously (Choudhury et al, 2008). The total number of plaques was scored on restriction enzyme digested vs undigested plates and the repair efficiency was calculated as follows:

$$Total\ repair\ = 100\ X\ \left[\frac{(\#\ \text{plaques from undigested DNA}) - (\#\ \text{plaques from digested DNA})}{\#\ \text{plaques from undigested DNA}}\right]$$

**Real time PCR approach:** For 8-oxoG and 5-OHU repair assays, we utilized a real time PCR-based approach for detecting repair used previously in our laboratory, which uses

the same restriction digestion products as the plaque-based assay (Woodrick et al, 2015). Instead of plaque number, the $C_t$ values are used to calculate repair.

*Cell cycle analysis*

After 24 h of siRNA transfection or damaging agent treatment, cells were fixed by ethanol fixation and DNA was stained with propidium iodide. DNA content analysis was then performed by flow cytometry to determine the percentage of each cell pool in each phase of the cell cycle. For G1 arrest with lovastatin treatment, fixation and DNA content analysis was performed after 33 h of treatment with 20 μM lovastatin.

*Western blotting*

Western blotting was performed using standard protocols with the following antibodies: mouse monoclonal anti-APE1 antibody (1:1000, Novus), rabbit monoclonal FEN1 antibody (1:1000; Abcam), rabbit monoclonal RPA32 antibody (1:1000; Bethyl Labs), rabbit polyclonal RECQ1 antibody (1:1000; Bethyl Labs), rabbit polyclonal PARP1 antibody (1:500; Cell Signaling), mouse monocolonal XPF antibody (1:800; Thermo Scientific), goat polyclonal BLM antibody (1:1000; Bethyl Labs), rabbit polyclonal WRN antibody (1;500; Santa Cruz), rabbit polyclonal RECQ4 antibody (1:500, Cell Signaling), rabbit polyclonal RECQ5 antibody (1:1000; Bethyl Labs), rabbit monoclonal OGG1 antibody (1:5000; Abcam). Mouse monoclonal β-actin antibody (1:200; Sigma-Aldrich) or GAPDH antibody (1:1000; Novus Biologicals) was used to detect β-actin or GAPDH as loading controls, respectively.

*Expression of Mus81 by quantitative PCR (qPCR)*

Total RNA was prepared from WT and XPF mut HFs (Coriell GM00637 and GM04313, respectively) and HCT116 cells using the RNAspin Mini kit (GE Healthcare) according to manufacturer's instructions. 400 ng RNA was subsequently reverse transcribed to cDNA using qScript cDNA Supermix (Quanta Biosciences) according to manufacterer's instructions. The qPCR analysis was performed as previously (Wu et al, 2008), using 3 μL of cDNA synthesis product as template in a 20 μL reaction volume containing 1X Maxima Sybr Green PCR mix (ThermoFisher Scientific) and 500 nM of forward (5'-TGTGGACATTGGCGAGAC-3') and reverse (5'-GCTGAGGTTGTGGACGGA-3') primers. Thermal cycling conditions were as follows: 95°C for 10 min followed by 40 cycles of 95°C for 10 s, 55°C for 30 s, and 72°C for 30 s.

*Nuclear extract preparation*

Nuclear extract was prepared using buffers and protocol as previously described with some modification (Matthias et al, 1989). Briefly, $10^6$ HCT116 cells were scraped from a 10 cm plate using 400 μL of Buffer A. The cells were collected in a microcentrifuge tube and incubated on ice for 15 min. Then 25 μL of 10% NP-40 was added to the cells, and the samples were vortexed followed by centrifugation at 1500 rpm for 1 min at 4°C to pellet the nuclei. The supernatant was discarded, and the pellet was resuspended in approximately 60-70 μL of Buffer C. The samples were rocked vigorously at 4°C for 20 min and subsequently centrifuged at 14,000 rpm for 10 min at 4°C. The supernatant was aliquoted and stored at -80°C.

*In vitro gap formation and mapping assays*

*In vitro* gap formation and mapping assays were performed with 24 fmol εA- or control-M13mp18 phagemid DNA and 60 µg aphidicolin-treated (5 µM for 15 min on ice) HCT116 nuclear extract (or purified proteins as described below) in a reaction buffer containing 50 mM Tris HCl (pH 7.5), 5 mM $MgCl_2$, 0.4 mM $MnCl_2$, 25 mM KCl, 5 mM ATP, 10% glycerol, 1 mM DTT, and 100 µg/ml BSA at 37°C for 1 h in a 50 µL reaction volume. The reactions were terminated by addition of 4 mg/ml proteinase K, 25 mM EDTA and 2% SDS followed by incubation at 37°C for 15 min. DNA samples recovered following phenol-chloroform extraction and ethanol precipitation.

Reactions using purified proteins were performed similarly, using 80 nM MPG, 25 nM APE1, 25 nM RECQ1 (or mutant RECQ1, where indicated), 25nM PARP1 (or PARylated PARP1, where indicated), and 25 nM XPF-ERCC1 and 16 nM of RPA32.

***In vitro* gap formation assay:** Figure 2A is a schematic depiction of the restriction digestions used to detect gap formation during repair of εA *in vitro*, which is a slightly modified version of a previously described method (Zhang et al, 2005). After ethanol precipitation, resuspended DNA was divided into two aliquots and digested with restriction enzymes according to the placement of εA in the construct in order to detect dsDNA structure 5' or 3' to the lesion. Each digestion also included the restriction enzyme BspHI, which linearized the phagemid at a region distant from the MCS (See Figure 2A). Digested samples were resolved on a 1% agarose gel.

Experiments with $NAD^+$ were performed as described above with the addition of 5-400 µM $NAD^+$ (Figure 3C).

***In vitro* gap mapping assay:** After treatment with the indicated purified proteins (Figure 3B), resuspended phagemid DNA was digested with HindIII and NarI, and digested samples were desalted using G-25 columns (GE Healthcare). Desalted samples were subsequently radiolabeled at 5' termini by T4 PNK (ThermoScientific)-mediated exchange reaction with [$\gamma$-$^{32}$P]-ATP according to the manufacturer's protocol. Radiolabeled products were purified from unbound [$\gamma$-$^{32}$P]-ATP using G-25 columns and resolved on 10% polyacrylamide sequencing gel containing 8M urea.

*Single-nucleotide gap recruitment assay*

**Preparation of nicked biotinylated M13mp18 plasmid DNA:** Ethenoadenine-containing M13mp18 plasmid DNA was prepared as described under heading "*In vitro* εA-M13mp18 construct preparation for repair assays." During the primer extension reaction, 70 μM biotinylated dCTP was added to each reaction, resulting in the production of biotinylated εA-M13mp18. The biotinylated phagemid DNA (600 fmol) was treated with 300 nM MPG in a reaction buffer containing 25 mM HEPES, pH 7.9, 150 mM NaCl, 100 μg/mL BSA, 0.5 mM DTT, and 10% glycerol in a 50 μL reaction volume at 37°C for 1.5 h. After 45 min, an additional 300 nM MPG was added for the remaining 45 min. After the completion of MPG treatment, 200 nM APE1 and 5 μM $MgCl_2$ were added to the reaction and incubated for 15 min at 37°C. After treatment the plasmid DNA was purifed by phenol extraction and ethanol precipitation as described under heading "*In vitro* gap formation and mapping assays" and utilized in the recruitment assay (see below).

**Recruitment assay:** In a 50 µL reaction volume, 300 fmol purified biotinylated nick-containing M13mp18 DNA was incubated with 25 nM PARP1, 25 nM RECQ1, and 16 nM RPA in a binding buffer containing 50 mM Tris HCl (pH 7.5), 25 mM KCl, 10% glycerol, 1 mM DTT, and 100 µg/ml BSA at room temperature for 20 min. Then 20 µL of pre-washed strepatividin-coated magnetic Dynabeads (Life Technologies) was added to each reaction and incubated at room temperature for 20 min, rolling. Using a magnetic separator, the beads were washed twice in 200 µL of 1X binding buffer. An aliquot of washed beads was retained for DNA estimation while the remaining aliquot was analyzed for protein recruitment by Western blotting analysis. The recruited proteins were eluted from the beads by resuspension in SDS loading buffer and boiling at 95°C for 5 min, followed by magnetic separation. The supernatant was resolved by SDS-PAGE on a 4-12% Bis-Tris protein gel (Life Technologies) and transferred to a nitrocellulose membrane for Western blotting as described under the heading "Western blotting."

An aliquot of the washed beads was analyzed for DNA content using the PicoGreen dsDNA assay kit (Life Technologies) according to manufacturer's protocol. Control experiments were also performed as described above but with non-biotinylated nick-containing M13mp18 DNA or beads only.

*PARP1 PARylation assay*

Ethenoadenine-containing M13mp18 plasmid DNA was treated as described under heading "*In vitro* gap formation and mapping assays" with proteins indicated in Figure 3D. Each reaction contained 5 or 400 µM $NAD^+$ supplemented with 10% [$\gamma$-$^{32}$P]-$NAD^+$ by volume. After 1 h of incubation at 37°C, reactions were resolved by SDS-

PAGE on a 4-12% Bis-Tris protein gel (Life Technologies). The gel was subsequently dried on DE81 anion exchange paper and exposed to X-ray film. Parylation of PARP1 was detected by autoradiography.

*RECQ1 expression and purification*

Full length RECQ1 was expressed and purified as described previously for RECQ4 with some modification (Xu & Liu, 2009). The full length RECQ1 open reading frame (ORF) with N-terminal His-tag was cloned into pET28a vector (Novagen) between Nhe1 (at 5' having ATG codon for expression controlled by T7 promotor) and Xho1 (in frame with 3' 6His and stop codon). *E. coli* BL21DE3 cells carrying the construct with RECQ1-His was grown in Magnificent Broth (MacConnell Research) at 37°C until the absorbance at 600 nm reached 0.4. The culture was subsequently induced overnight at 16°C with 0.1 mM IPTG. The cells were harvested by centrifugation and lysed in lysis buffer A (50 mM Tris-HCl, pH 8.0, 400 mM NaCl, 0.1% Tween 20, 1 mM DTT, 10% glycerol) followed by sonication (10 X 45 sec) on ice at 50% power using a Braun-Sonic U until lysis was complete. Lysate was centrifuged at 21,000 rpm for 30 min in an ultracentrifuge (Beckman XL-90 Ultracentrifuge) at 4°C. Supernatant was applied to His Trap HP column (GE Healthcare) equilibrated with lysis buffer A at a flow rate of 1 mL/min. The column was washed with wash buffer (Buffer A with 12.5 mM imidiazole) at a flow rate of 1 mL/min. His-tagged protein was subsequently eluted by a gradient of buffer B (Buffer A with 500 mM imidazole) by fast protein liquid chromatography (FPLC). The eluate was dialyzed against Buffer C (50 mM Tris-HCl, pH-7.2, 10% glycerol, 1 mM EDTA, 1 mM DTT, 0.2% Triton X-100, 70 mM KCl) and applied to a

SP-Sepharose ion exchange column (GE Healthcare). The SP column was washed with Buffer D (Buffer C with additional 30 mM KCl), and the protein was eluted by a gradient of buffer E (50 mM Tris-HCl, pH 8.0, 10% glycerol, 1 mM EDTA, 1 mM DTT, 500 mM KCl) by FPLC. The purified RECQ1 was stored at -80°C in aliquots.

*PARP1 expression and purification*

Recombinant PARP1 protein was expressed using the Baculovirus expression system (Kim et al, 2004). Baculovirus stock was generated by PARP1 bacmid vector in adherent SF9 cells (Invitrogen). Rapid Titer kit (Clontech) was used to determine the multiplicity of infection (MOI) of virus stock. One liter of $2.5x10^6$ cells/ml serum-independent suspension Sf9 cells (Invitrogen) was infected with 2.5 MOI of virus stock. Cells were harvested after 42–44 h of infection and all purification steps were carried out at 4°C. Whole cell extract was prepared by homogenizing cells in ice cold hypotonic lysis buffer (25 mM Tris pH 8, 10 mM NaCl, 5 mM $MgCl_2$, 0.1% Tween-20, 7 mM BME, 1 mM PMSF and 1X protease inhibitor (Roche)) followed by the addition of equal volume of hypertonic buffer (25 mM Tris pH 8, 1 M NaCI, 5 mM $MgCl_2$, 0.1 % Tween-20, 20 % glycerol, 40 mM imidazole, 7 mM BME, 1 mM PMSF and 1X protease inhibitor (Roche)). Lysate was incubated on ice for 30 min then centrifuged for 10 min at 9,500 rpm. To remove the nucleic acids, lysate was treated with 2 mg/ml of protamine sulfate (Sigma) and spun at 9500 rpm for 15 min. Immobilized Metal Affinity Chromatography (IMAC) was performed with Nickel charged Hi-Trap chelating columns (GE Healthcare) by fast protein liquid chromatography (FPLC). Protein fractions were eluted by continuous gradient of the binding buffer (25 mM Tris pH 8, 250 mM NaCl,

10% glycerol, 5 mM $MgCl_2$, 0.1% Tween-20, 20 mM imidazole and 7 mM BME) and the elution buffer (25 mM Tris pH 8, 250 mM NaCl, 10 % glycerol, 5 mM $MgCl_2$, 0.1 % Tween 20, 700 mM imidazole and 7 mM BME).

The second step of purification was carried out on a Heparin column (GE Healthcare) after IMAC eluted fraction was dialyzed to decrease NaCl concentration to 50 mM. The binding buffer was composed of 25 mM Tris pH 8, 50 mM NaCl, 10% glycerol, 5 mM $MgCl_2$, and 1 mM tris(2-carboxyethyl)phosphine (TCEP). Fractions were eluted with a continuous gradient of NaCl (0-1 M). SDS-PAGE, silver staining (BioRad) and Coomassie staining (Pierce) were performed to assess the protein purity.

*Cell survival and fast micromethod DNA SSB assays*

Cells were seeded in a 6 well plate (300,000 cells per well) for SSB assays or in a 24 well plate (20,000 cells per well) for survival assays. The following day cells (HCT116 or HeLa) were transfected with control, 25 nM RECQ1 (Santa Cruz Biotechnology), and/or 50 nM OGG1 (Dharmacon siGENOME human OGG1 SMARTpool) siRNA as described under "In-cell repair assay" in the Materials and Methods section. After 24 h of transfection, cells were treated for 1 h (SSB assay) or 24 h (survival assay) with the indicated doses of MMS or $KBrO_3$, in triplicate, with or without olaparib. For 1 h treatment experiments, treatment was removed and replaced with growth media containing olaparib for the remaining 24 h. Following 24 h incubation, cell survival was assessed either by cell counting or tetrazolium salt, 3-4,5 dimethylthiazol-2,4 diphenyl tetrazolium bromide (MTT) assay. For MTT assay the media was aspirated, and 500 μL of a tetrazolium salt, 3-4,5 dimethylthiazol-2,4 diphenyl

tetrazolium bromide (MTT) (Sigma-Aldrich) solution (0.5 mg/mL in DMEM) was added to the cells and incubated for 4 h at 37°C. The MTT solution was subsequently aspirated and cells were incubated for 30 min shaking at room temperature with 1 mL DMSO for solubilization. Absorbance was measured at 570 nm and 650 nm (background). Absorbance values at 570 nm were background subtracted, and percent survival was calculated according to the untreated control wells.

To detect single strand breaks, cells were treated with olaparib and nonlethal dose of damaging agent as stated above, and the fast micromethod DNA SSB assay was performed 24 h later following a published method (Elmendorff-Dreikorn et al, 1999). Briefly, treated cells were lysed and incubated with PicoGreen dsDNA reagent (Life Technologies), a fluorescent nucleic acid stain for dsDNA in solution, for 1 h in the dark in a 96-well black plate. Fluorescence was subsequently monitored every 2 min for 30 min in the presence of 0.1 M NaOH (pH 12.4) with excitation at 480 nm and emission detected at 520/13.5 nm in a microplate reader for fluorescence detection (BioTek Synergy H4 Hybrid Reader). The rate of unwinding was calculated using the amount of fluorescence at 0 min as reference, and the results are calculated as the $\log_{10}$ of the ratio of the percentage of dsDNA from treated and control samples, respectively (Batel et al, 1999; Schroder et al, 2006).

*Modified SSB assay for detecting Fpg- and MPG-sensitive sites*

After treatment with MMS or $KBrO_3$ and olaparib, genomic DNA was isolated following an established method (Besaratinia et al, 2007). However, for MMS-treated cells, lysis was performed overnight at room temperature rather than 37°C to prevent spontaneous

loss of alkylated purines. Isolated genomic DNA was then digested with either *E. coli* Fpg or recombinant human MPG and APE1 for detecting oxidative DNA damage and alkylation DNA damage, respectively. Fpg treatment was performed with 600 ng genomic DNA in a 1X REC buffer (Trevigen) and 3.3 U of Fpg (Trevigen) or no enzyme in a reaction volume of 20 μL for 30 min at 30°C. MPG/APE1 treatment was performed with 600 ng genomic DNA and 100 nM MPG (or no enzyme) in 25 mM HEPES, pH 7.9, 150 mM NaCl, 100 ug/mL BSA, 0.5 mM DTT, and 10% glycerol in a reaction volume of 20 uL for 30 min at 37°C. Reaction with or without MPG were both then supplemented with 500 nM APE1 and 5 μM $MgCl_2$ and incubated for 15 min at 37°C. Reaction products (no enzyme, Fpg, or MPG/APE1-treated) were subsequently diluted with 220 μL of suspension buffer (250 mM myo-inositol, 10 mM sodium phosphate dibasic, 1 mM $MgCl_2$, pH 7.4). SSBs were quantified by a SSB assay modified for naked DNA as described previously (Muller et al, 2013) with some modification. Solution volumes were reduced by half and added manually (instead of automated) on ice and Picogreen (final dilution 1:200 in each well) was used to detect dsDNA instead of Sybr Green. Using the no enzyme (no Fpg or MPG/APE1) reactions as control, results are calculated as the $\log_{10}$ of the ratio of the percentage of loss of fluorescence from enzyme-treated and control samples, respectively.

## **Appendix Supplementary** References


Adhikari S, Manthena PV, Sajwan K, Kota KK, Roy R (2010) A unified method for purification of basic proteins. *Anal Biochem* **400:** 203-206

Adhikari S, Uren A, Roy R (2007) N-terminal extension of N-methylpurine DNA glycosylase is required for turnover in hypoxanthine excision reaction. *J Biol Chem* **282:** 30078-30084

Batel R, Jaksic Z, Bihari N, Hamer B, Fafandel M, Chauvin C, Schroder HC, Muller WE, Zahn RK (1999) A microplate assay for DNA damage determination (fast micromethod). *Anal Biochem* **270:** 195-200

Berti M, Ray Chaudhuri A, Thangavel S, Gomathinayagam S, Kenig S, Vujanovic M, Odreman F, Glatter T, Graziano S, Mendoza-Maldonado R, Marino F, Lucic B, Biasin V, Gstaiger M, Aebersold R, Sidorova JM, Monnat RJ, Jr., Lopes M, Vindigni A (2013) Human RECQ1 promotes restart of replication forks reversed by DNA topoisomerase I inhibition. *Nat Struct Mol Biol* **20:** 347-354

Besaratinia A, Kim SI, Bates SE, Pfeifer GP (2007) Riboflavin activated by ultraviolet A1 irradiation induces oxidative DNA damage-mediated mutations inhibited by vitamin C. *Proc Natl Acad Sci U S A* **104:** 5953-5958

Choudhury S, Adhikari S, Cheema A, Roy R (2008) Evidence of complete cellular repair of 1,N6-ethenoadenine, a mutagenic and potential damage for human cancer, revealed by a novel method. *Mol Cell Biochem* **313:** 19-28

Elmendorff-Dreikorn K, Chauvin C, Slor H, Kutzner J, Batel R, Muller WE, Schroder HC (1999) Assessment of DNA damage and repair in human peripheral blood mononuclear cells using a novel DNA unwinding technique. *Cell Mol Biol (Noisy-le-grand)* **45:** 211-218

Enzlin JH, Scharer OD (2002) The active site of the DNA repair endonuclease XPF-ERCC1 forms a highly conserved nuclease motif. *EMBO J* **21:** 2045-2053

Grudic A, Jul-Larsen A, Haring SJ, Wold MS, Lonning PE, Bjerkvig R, Boe SO (2007) Replication protein A prevents accumulation of single-stranded telomeric DNA in cells that use alternative lengthening of telomeres. *Nucleic Acids Res* **35:** 7267-7278

Kim MY, Mauro S, Gevry N, Lis JT, Kraus WL (2004) NAD+-dependent modulation of chromatin structure and transcription by nucleosome binding properties of PARP-1. *Cell* **119:** 803-814

Li XL, Lu X, Parvathaneni S, Bilke S, Zhang H, Thangavel S, Vindigni A, Hara T, Zhu Y, Meltzer PS, Lal A, Sharma S (2014) Identification of RECQ1-regulated transcriptome uncovers a role of RECQ1 in regulation of cancer cell migration and invasion. *Cell Cycle* **13:** 2431-2445

Matthias P, Muller MM, Schreiber E, Rusconi S, Schaffner W (1989) Eukaryotic expression vectors for the analysis of mutant proteins. *Nucleic Acids Res* **17:** 6418

Muller N, Moreno-Villanueva M, Fischbach A, Kienhofer J, Martello R, Dedon PC, Ullrich V, Burkle A, Mangerich A (2013) An automated Fpg-based FADU method for the detection of oxidative DNA lesions and screening of antioxidants. *Toxicology* **310:** 15-21

Saha T, Rih JK, Roy R, Ballal R, Rosen EM (2010) Transcriptional regulation of the base excision repair pathway by BRCA1. *J Biol Chem* **285:** 19092-19105

Sampathi S, Bhusari A, Shen B, Chai W (2009) Human flap endonuclease I is in complex with telomerase and is required for telomerase-mediated telomere maintenance. *J Biol Chem* **284:** 3682-3690

Schroder HC, Batel R, Schwertner H, Boreiko O, Muller WE (2006) Fast micromethod DNA single-strand-break assay. *Methods Mol Biol* **314:** 287-305

Sharma S, Sommers JA, Choudhary S, Faulkner JK, Cui S, Andreoli L, Muzzolini L, Vindigni A, Brosh RM, Jr. (2005) Biochemical analysis of the DNA unwinding and strand annealing activities catalyzed by human RECQ1. *J Biol Chem* **280:** 28072-28084

Sharma S, Stumpo DJ, Balajee AS, Bock CB, Lansdorp PM, Brosh RM, Jr., Blackshear PJ (2007) RECQL, a member of the RecQ family of DNA helicases, suppresses chromosomal instability. *Mol Cell Biol* **27:** 1784-1794

Staresincic L, Fagbemi AF, Enzlin JH, Gourdin AM, Wijgers N, Dunand-Sauthier I, Giglia-Mari G, Clarkson SG, Vermeulen W, Scharer OD (2009) Coordination of dual incision and repair synthesis in human nucleotide excision repair. *EMBO J* **28:** 1111-1120

Woodrick J, Gupta S, Khatkar P, Dave K, Levashova D, Choudhury S, Elias H, Saha T, Mueller S, Roy R (2015) A novel method for monitoring functional lesion-specific recruitment of repair proteins in live cells. *Mutat Res* **775:** 48-58

Wu F, Liu SY, Tao YM, Ou DP, Fang F, Yang LY (2008) Decreased expression of methyl methansulfonate and ultraviolet-sensitive gene clone 81 (Mus81) is correlated with a poor prognosis in patients with hepatocellular carcinoma. *Cancer* **112:** 2002-2010

Xu X, Liu Y (2009) Dual DNA unwinding activities of the Rothmund-Thomson syndrome protein, RECQ4. *EMBO J* **28:** 568-577

Zhang Y, Yuan F, Presnell SR, Tian K, Gao Y, Tomkinson AE, Gu L, Li GM (2005) Reconstitution of 5'-directed human mismatch repair in a purified system. *Cell* **122:** 693-705