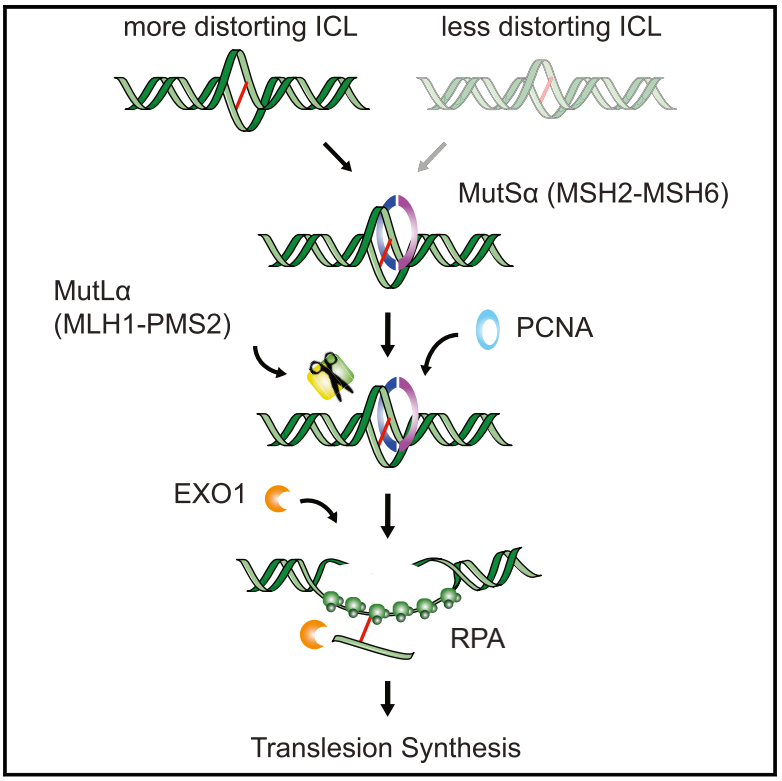
Article

Cell Reports

Sensing and Processing of DNA Interstrand Crosslinks by the Mismatch Repair Pathway

Graphical Abstract



Authors

Niyo Kato, Yoshitaka Kawasoe, Hannah Williams, ..., Max E. Gottesman, Tatsuro S. Takahashi, Jean Gautier

Correspondence

jg130@cumc.columbia.edu

In Brief

Kato et al. identify a mechanism of ICL recognition that operates independently of DNA replication and transcription. In the absence of these processes, ICLs are recognized and repaired by the MMR machinery. MutSα is critical for ICL recognition, while MutLα and EXO1 contribute to key downstream nucleolytic steps during ICL repair.

Highlights

- ICLs are sensed and repaired independently of replication and transcription
- MutSα (MSH2–MSH6) binds to ICLs and initiates repair
- ICL structure influences recognition and repair efficiency
- MMR-dependent ICL repair requires the nuclease activities of MutLα and EXO1

Kato et al., 2017, Cell Reports *21*, 1375–1385
October 31, 2017
https://doi.org/10.1016/j.celrep.2017.10.032

Cell Reports
Article

Sensing and Processing of DNA Interstrand Crosslinks by the Mismatch Repair Pathway

Niyo Kato,[1] Yoshitaka Kawasoe,[2] Hannah Williams,[1] Elena Coates,[1] Upasana Roy,[3] Yuqian Shi,[4] Lorena S. Beese,[4] Orlando D. Schärer,[3,5] Hong Yan,[6] Max E. Gottesman,[7] Tatsuro S. Takahashi,[8] and Jean Gautier[1,9,*]
[1]Institute of Cancer Genetics, Columbia University, New York, NY 10032, USA
[2]Graduate School of Science, Osaka University, Toyonaka, Japan
[3]Department of Chemistry and Department of Pharmacological Sciences, Stony Brook University, Stony Brook, NY 11794, USA
[4]Department of Biochemistry, Duke University Medical Center, Durham, NC 27710, USA
[5]Institute for Basic Science Center for Genomic Integrity and School of Life Sciences, Ulsan National Institute of Science and Technology, Ulsan, South Korea
[6]Fox Chase Cancer Center, Philadelphia, PA 19111, USA
[7]Institute of Cancer Research, Columbia University, New York, NY 10032, USA
[8]Department of Biology, Kyushu University, Fukuoka, Japan
[9]Lead Contact
*Correspondence: jg130@cumc.columbia.edu
https://doi.org/10.1016/j.celrep.2017.10.032

SUMMARY

DNA interstrand crosslinks (ICLs) that are repaired in non-dividing cells must be recognized independently of replication-associated DNA unwinding. Using cell-free extracts from *Xenopus* eggs that support neither replication nor transcription, we establish that ICLs are recognized and processed by the mismatch repair (MMR) machinery. We find that ICL repair requires MutSα (MSH2–MSH6) and the mismatch recognition FXE motif in MSH6, strongly suggesting that MutSα functions as an ICL sensor. MutSα recruits MutLα and EXO1 to ICL lesions, and the catalytic activity of both these nucleases is essential for ICL repair. As anticipated for a DNA unwinding-independent recognition process, we demonstrate that least distorting ICLs fail to be recognized and repaired by the MMR machinery. This establishes that ICL structure is a critical determinant of repair efficiency outside of DNA replication.

INTRODUCTION

DNA interstrand crosslinks (ICLs) are lesions that covalently link opposing strands of the double helix. ICLs physically block cellular processes that require the unwinding of the DNA molecule, such as replication, recombination, and transcription. ICLs can also interfere with essential protein-DNA binding events such as transcription factor binding. Chemicals that induce ICLs, such as nitrogen mustards, platinum drugs, and mitomycin C are therefore extremely cytotoxic and are routinely used in the clinic as anti-cancer chemotherapies (Deans and West, 2011). Importantly, ICLs also arise as a consequence of cellular metabolism, for instance, through production of reactive aldehydes such as malondialdehyde, acetaldehyde, and formaldehyde (Duxin and Walter, 2015) or by chemical rearrangements at abasic DNA sites (Price et al., 2014).

ICLs vary greatly in structure and the degree to which they distort DNA. These structural differences can influence the manner in which cells process the ICLs. Indeed, in yeast, the repair pathway that responds to crosslinking damage differs according to the crosslinking drug (Beljanski et al., 2004). In mammalian cells, the efficiency of ICL unhooking and repair is greater for a distorting ICL than for a non-distorting ICL (Hlavin et al., 2010; Smeaton et al., 2008). Furthermore, at least two replication-coupled ICL repair mechanisms have been described in *Xenopus* extracts for which the primary determinant of repair pathway choice is based on ICL structure (Semlow et al., 2016).

In proliferating cells, most repair occurs during S-phase, when ICL sensing is a direct consequence of replication. Active replisomes stall at ICLs and trigger a complex reaction that requires the Fanconi anemia (FA) proteins (Ben-Yehoyada et al., 2009; Klein Douwel et al., 2014; Knipscheer et al., 2009; Räschle et al., 2008) or the NEIL3 glycosylase (Semlow et al., 2016). Non-dividing yeast cells grown to stationary phase (Sarkar et al., 2006) and G1-arrested mammalian cells (Hlavin et al., 2010; Muniandy et al., 2009; Shen et al., 2006) harness an alternative modality of repair termed replication independent repair (RIR) (Williams et al., 2012). To date, the molecular components that contribute to RIR have not been fully defined. A fundamental question that remains unanswered is how ICLs are recognized without replisome/ICL clashes. Collision between the transcription machinery and ICLs could activate repair. Indeed, ICLs are more efficiently processed when placed in transcribed regions (Islas et al., 1991), and repair of ICLs placed in constitutively transcribing plasmids shows dependence for transcription-coupled and global-genome nucleotide excision repair proteins (Enoiu et al., 2012). However, RIR is initiated in *Xenopus* extracts in the absence of both replication and transcription (Ben-Yehoyada et al., 2009; Williams et al., 2012), suggesting ICLs can be directly funneled into a repair pathway by DNA damage sensor proteins. But no ICL sensor has yet been unambiguously identified, and it

is not clear how structurally distinct ICL lesions are recognized and/or subsequently repaired during RIR.

The mismatch repair (MMR) pathway is a highly conserved DNA repair mechanism that primarily functions to correct replication errors that escape proofreading. MMR is initiated by the MutSα complex (MSH2–MSH6 heterodimer), which recognizes and binds single base mismatches and 1–2 base insertion-deletion loops (IDLs). Binding of MutSα to mispaired bases leads to the recruitment of downstream DNA repair proteins including the MutLα endonuclease (MLH1-PMS2 heterodimer), replication protein A (RPA), EXO1, and proliferating cell nuclear antigen (PCNA). Together, these proteins catalyze an excision-repair reaction to restore proper Watson-Crick base-pairing and can effectively increase the fidelity of replication by up to three orders of magnitude. In humans, mutations in either subunit of MutSα (MSH2 and MSH6) account for 50%–60% of germline mutation-based (Lynch syndrome) and 15%–20% of sporadic colorectal cancers (Reyes et al., 2015).

Importantly, MutSα has multiple roles outside of replication. MutSα is active in quiescent (post-mitotic) mammalian cells, such as terminally differentiated neurons, where the complex participates in genome maintenance mechanisms by recognizing not only canonical DNA mismatches, but also responding to chemically modified DNA bases (Iyama and Wilson, 2013; Schroering et al., 2007). For example, plasmids containing single O^6-methyl-guanine adducts (O6meG) are processed in a MutSα-dependent fashion in non-replicating mammalian (York and Modrich, 2006) and *Xenopus* extracts (Olivera Harris et al., 2015), although leading sometimes to futile repair cycles (Fu et al., 2012). In addition to O6meG, MutSα specifically recognizes bulky lesions such as O4-methylthymine adducts and cisplatin intrastrand crosslinks in vitro. Crystal structures of human MutSα bound to these DNA substrates reveal a common mechanism of damage recognition that requires an N-terminal FXE motif found in the MSH6 subunit (Warren et al., 2007). Binding of MutSα to DNA lesions leads to a variety of cellular outcomes including repair, checkpoint activation, and apoptosis, underscoring the versatile and critical role that MutSα plays in genome maintenance.

In this study, we demonstrate a role for the MMR machinery in ICL repair. Using non-replicating and transcriptionally silent *Xenopus* extracts, we show that MutSα binds to plasmids bearing a single site-specific ICL and harnesses the entire MMR machinery, including the MutLα and EXO1 nucleases, to perform ICL repair. We also compare the repair of structurally different ICLs, including two analogs of the malondialdehyde ICL and a nitrogen mustard-like ICL (Guainazzi et al., 2010). We conclude that ICL structure influences lesion recognition and repair efficiency during RIR.

RESULTS

MutSα (MSH2–MSH6) Senses DNA ICLs

The MutSα complex recognizes a diverse array of aberrant DNA structures including base-base mispairs and chemically modified DNA adducts. We hypothesized that the ability of MutSα to recognize distortions within DNA might also extend to ICLs, and that sensing of ICLs by MutSα could initiate ICL repair by a set of reactions analogous to MMR. To test this hypothesis, we first asked whether MutSα binds preferentially to ICLs in non-replicating *Xenopus* extracts (high-speed supernatant [HSS]) using a plasmid pull-down assay. Notably, these cell-free extracts support ICL repair and restore the integrity of both DNA strands (Williams et al., 2012).

We used SJG-136, a rationally designed crosslinking drug, to generate plasmids with a single site-specific ICL lesion. SJG-136 preferentially forms ICLs between guanine residues at 5′-purine-GATC-pyrimidine-3′ sequences (Figure S1A) (Gregson et al., 2001). We treated oligonucleotide duplexes containing a single SJG-136 reaction site (Figure S1B) and purified crosslinked duplexes by denaturing PAGE. These oligos were ligated into a small plasmid (pBS: pBlueScript) and further purified by cesium chloride density ultracentrifugation.

Following incubation in HSS, we isolated plasmid and plasmid-bound proteins from the extract using biotinylated lac repressor protein and streptavidin beads, as previously described (Williams et al., 2012). Western blot analysis revealed that the crosslinked plasmid was enriched in bound MSH2 and MSH6 when compared to identical undamaged control plasmids (Figure 1A).

In order to study the dose-dependent response of MutSα to crosslinks, we conducted a similar experiment with plasmids containing multiple ICLs by treating double-stranded pBS plasmids in vitro with increasing doses of SJG-136 (Figures S1C–S1E). We observed dose-dependent increase of MSH2 and MSH6 binding to damaged plasmids (Figure 1B), after normalizing for plasmid recovery using qPCR (Figure S1F). We also observe the recruitment of PCNA and RPA to these plasmids in a dose-dependent manner, suggesting that the crosslinked plasmids were undergoing processing and repair. Consistent with the accumulation of RPA and with our previous studies (Ben-Yehoyada et al., 2009; Williams et al., 2012), incubation of the SJG-136-treated plasmids in HSS triggered phosphorylation of cytosolic Chk1 (Figure S1G), a marker for ataxia telangiectasia and Rad3-related kinase (ATR) checkpoint activation.

Next, we asked whether MutSα is required for ICL repair. ICL plasmids harboring a single, site-specific trimethylene ICL lesion (5′GpC-ICL), for which a nuclear magnetic resonance (NMR) structure has been described were used as substrates (Protein Data Bank [PDB]: 2KNL) (Dooley et al., 2003). In contrast to the temperature-sensitive ICLs generated by SJG-136, the trimethylene crosslink is extremely stable at high temperature. We can therefore monitor ICL repair by quantitative PCR by comparing the increase in amplification of the damaged "X" region to that of an undamaged "C" region on the plasmid backbone over time (Ben-Yehoyada et al., 2009; Williams et al., 2012) (Figure S1B, right).

Incubation of cytosol with MSH2 antibodies raised against *Xenopus* MSH2 protein depleted both MSH2 and MSH6 subunits of MutSα (Figure 1C, left) but did not reduce the cytosolic levels of other MMR proteins including MLH1 and EXO1 (Figure 1C, right). Compared to mock-depleted extracts, depletion of MutSα significantly decreased ICL repair (Figure 1D). Importantly, addition of recombinant $MutS\alpha^{WT}$ to MSH2-depleted extracts restored ICL repair to the level of mock-depleted extracts.

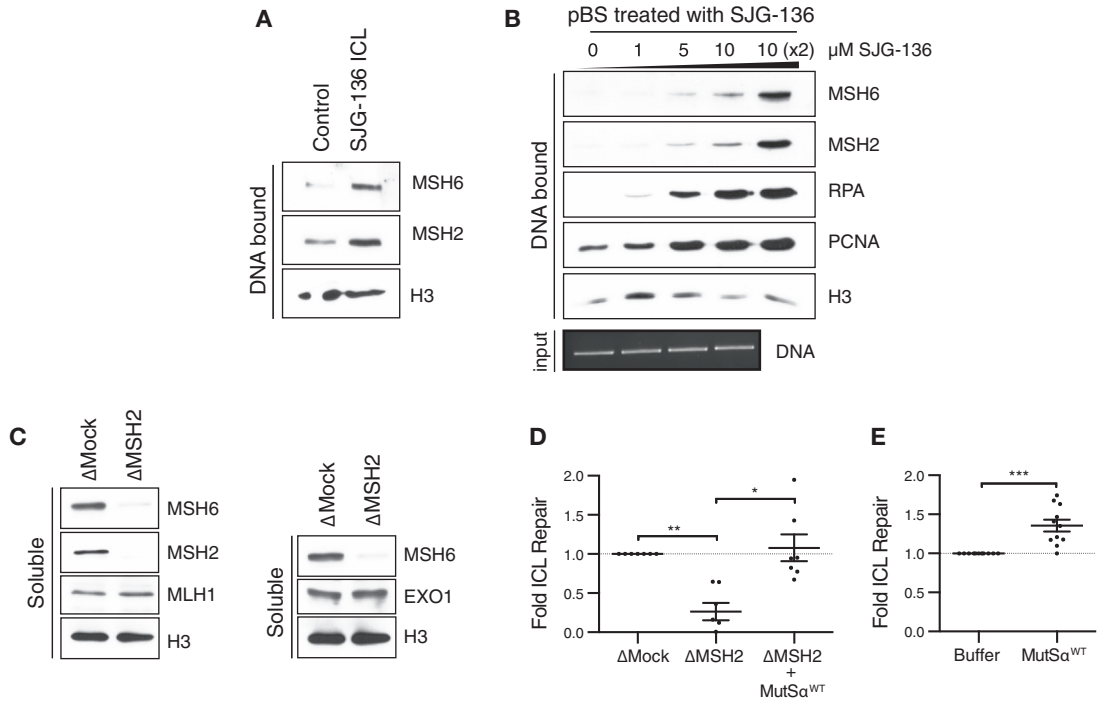


Figure 1. ICL Sensing by MutSα (MSH2–MSH6)

(A) Plasmid pull-down assay of control plasmids or plasmids containing a single site-specific SJG-136 ICL lesion. Plasmids were incubated for 40 min in HSS and then purified from extracts using recombinant Bio-LacR protein coupled to streptavidin beads. Plasmid-bound proteins were analyzed by western blot (WB) using the indicated antibodies.

(B) Plasmid pull-down assay and WB of SJG-136-treated plasmids. In the last lane, labeled (10×2), twice the amount of 10 μM-treated plasmids was incubated in HSS. Input was plasmid DNA run on an agarose gel.

(C) MSH2–MSH6 immunodepletion. Mock- and MSH2-depleted HSS was analyzed by WB.

(D) Quantification of ICL repair in mock- and MSH2-depleted HSS at 3 hr. Results represent mean ± SEM from n = 7 independent experiments.

(E) Quantification of ICL repair in HSS and HSS supplemented with MutSα^{WT} at 3 hr. Results represent mean ± SEM from n = 11 independent experiments.

See also Figure S1.

Moreover, overexpression of MutSα^{WT} in undepleted HSS increased the ICL repair efficiency (Figure 1E), suggesting that lesion recognition by MutSα is a rate-limiting step during RIR. These data establish that MutSα is required for ICL repair in the absence of DNA replication and transcription.

Mechanism of ICL Recognition by MutSα

MutSα discriminates aberrant DNA substrates amid a vast excess of normal DNA bases. The MSH6 subunit contributes critically to this process in at least two ways. First, the MSH6 subunit contains a highly conserved FXE motif (Figures S2A and S2B) that functions during damage recognition (Lamers et al., 2000; Malkov et al., 1997): the aromatic ring of the phenylalanine residue stacks with the mispaired base, whereas the carboxyl group of the glutamic acid residue hydrogen bonds with the mispaired base (Warren et al., 2007). A single amino acid substitution of this phenylalanine residue prevents efficient recognition of mispaired bases in yeast (Bowers et al., 1999) and results in defective repair in human cell extracts (Dufner et al., 2000).

To test the possible role of the FXE motif in MutSα-dependent repair of ICL in *Xenopus* extracts, we cloned and purified *Xenopus* mismatch binding-deficient MutSα^{FXE} complex (Figures S2A, S2B, and S2D). First, we asked whether the F411A substitution affected MMR using a plasmid-based MMR assay in HSS extracts. We used plasmids containing a single A:C mismatch and a 15-nt gap on the 3′ side of the A-strand (pMM1^{AC}) to trigger gap-directed strand-specific MMR (Figure 2A), as previously described (Kawasoe et al., 2016). Repair of this plasmid occurs preferentially on the A-strand, and the correction of the A:C site to a G:C pair generates a BamHI restriction site (Figure 2A, top). Background levels of repair on the C-strand are monitored by digestion with XhoI (Figure 2A, bottom). 60 min after incubation in mock-depleted extracts, 37% of the A:C mismatches were repaired to G:C. As anticipated, MMR was abolished in MSH2-depleted extracts. Addition of recombinant MutSα^{WT} but not MutSα^{FXE} restored repair (Figure 2A, top). The requirement for the FXE motif for MMR is therefore conserved in *Xenopus*.

Next, we determined whether the F411A substitution ablated ICL repair. As shown in Figure 2B, recombinant MutSα^{FXE} was unable to support repair of ICLs in MSH2-depleted extracts. The FXE domain is, therefore, critical for ICL damage recognition.

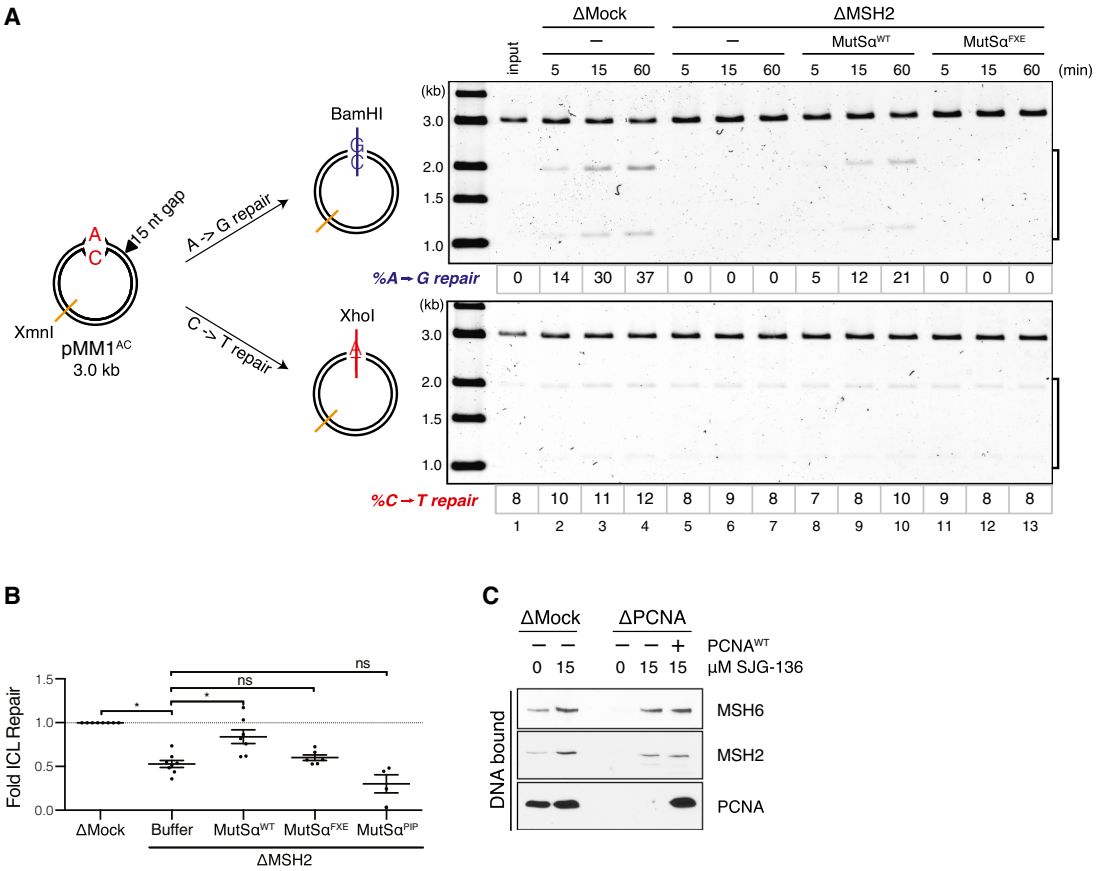


Figure 2. Mechanism of ICL Recognition by MutSα (MSH2–MSH6)

(A) Plasmids containing a single site-specific A:C mismatch were incubated in mock-depleted, MSH2-depleted, or MSH2-depleted HSS supplemented with recombinant MutSα^{WT} or MutSα^{FXE} for the indicated amount of time. Mismatch repair efficiency was measured by digestion with XmnI with BamHI or XhoI restriction enzymes, for A→G repair (top) and C→T repair (bottom), respectively. Data are representative of three independent experiments.

(B) Quantification of ICL repair in mock-depleted, MSH2-depleted, or MSH2-depleted HSS supplemented with recombinant MutSα^{WT} (n = 7), MutSα^{FXE} (n = 6), or MutSα^{PIP} (n = 4) at 3 hr. Results represent mean ± SEM of independent experiments.

(C) Plasmid pull-down assay and WB of SJG-136-treated plasmids in mock- or PCNA-depleted extract.

See also Figure S2.

In addition to recognizing a mismatched base, MSH6 also interacts with proliferating cell nuclear antigen (PCNA). Studies using fluorescently tagged proteins show that the interaction between MSH6 and PCNA is important for the colocalization of the MutSα complex to replication factories in *S. cerevisiae* (Hombauer et al., 2011) and in human cells undergoing MMR (Kleczkowska et al., 2001). Yet, disruption of the PCNA-MSH6 interface results in a modest MMR phenotype (Shell et al., 2007), and other PCNA-independent recruitment of MutSα to mismatches via histone methylation has also been described (Li et al., 2013).

We used plasmid pull-down assays to ask whether MutSα was recruited to ICLs in a PCNA-independent manner. As shown in Figure 1B, the MutSα complex and PCNA were recruited to SJG-treated plasmids in HSS extracts. MutSα recruitment was not dependent on PCNA; MSH2 and MSH6 were efficiently recruited to crosslinked plasmids in PCNA-depleted extracts. Furthermore, the extent of MutSα recruitment was unaffected by supplementation of extracts with recombinant PCNA protein (Figure 2C). This experiment strongly suggests that MutSα can be recruited to ICLs independently of PCNA.

The interaction between MSH6 and PCNA is mediated by a PCNA interaction motif (PIP box) in MSH6 (Clark et al., 2000; Flores-Rozas et al., 2000). We constructed recombinant MutSα complex with a triple amino acid substitution in its conserved PIP box motif: $Q(X)_2LI(X)_2FF$ (Figures S2A, S2C, and S2D) that disrupts PCNA interaction with MutSα in vitro (Figure S2G). We tested the ability of this MutSα^{PIP} to support ICL repair in MutSα-depleted extracts. We find that MutSα^{PIP} is unable to support ICL repair (Figure 2B). This indicates that, whereas MSH6-PCNA interaction is not required for MutSα recruitment to ICLs, and presumably for ICL sensing, the interaction between the two is essential for coordinating downstream repair events.

ICLs Are Processed by MutLα and EXO1 Nucleases

The earliest catalytic events during ICL repair are thought to be dual endonucleolytic incisions flanking the ICL lesion in a process referred to as "unhooking." However, neither the exact

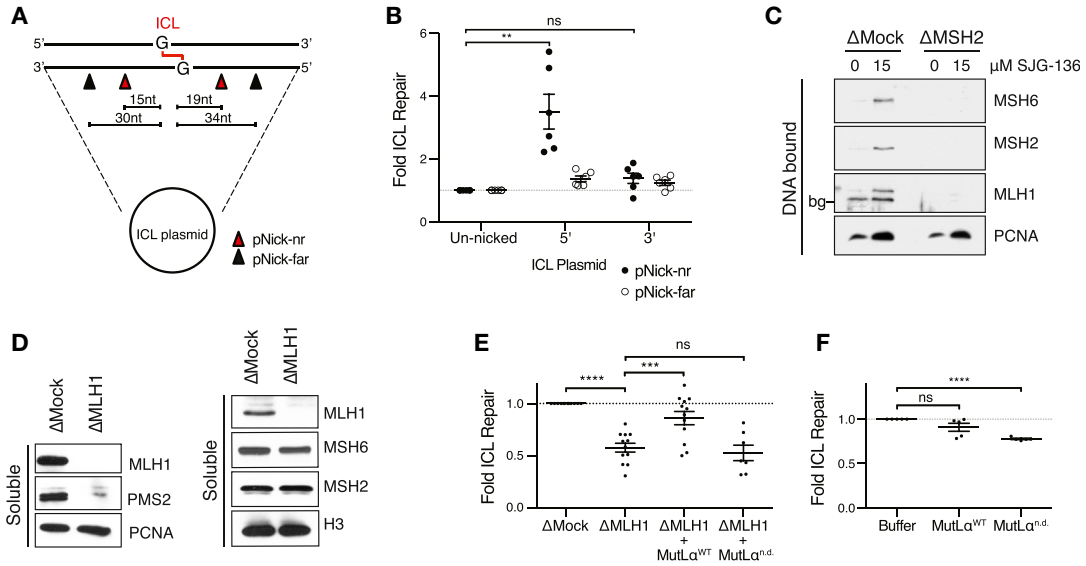


Figure 3. Nucleolytic Incision of ICL Lesions by MutLα (MLH1-PMS2)

(A) Schematic of nicking sites engineered into ICL pBS plasmids (see also Figure S3A).

(B) Quantification of ICL repair in HSS of unnicked, 5′ or 3′ nicked plasmids at 90 min. Results represent mean ± SEM from n = 6 independent experiments.

(C) Plasmid pull-down assay and WB of SJG-136-treated plasmids in mock- or MSH2-depleted extract.

(D) MLH1-PMS2 immunodepletion. Mock- and MLH1-depleted HSS was analyzed by WB.

(E) Quantification of ICL repair in mock-depleted, MLH1-depleted, or MLH1-depleted HSS supplemented with recombinant MutLα^{WT} (n = 12) or MutLα$^{n.d}$ (n = 7) at 90 min. Results represent mean ± SEM independent experiments.

(F) Quantification of ICL repair in HSS with overexpression of buffer, MutLα^{WT}, or MutLα$^{n.d.}$ at 90 min. Results represent mean ± SEM from n = 5 independent experiments.

See also Figure S3.

mechanism nor the nuclease(s) involved in the process are known for RIR.

We engineered the ICL-containing plasmids with 4 unique nicking sites flanking the ICL (Figures 3A and S3A). We hypothesized that presenting the extract with a pre-nicked plasmid might stimulate ICL repair if the substrate resembled a physiologically relevant ICL repair intermediate. Indeed, introduction of a single nick 19 bp 5′ to the ICL lesion stimulated repair by 4-fold. In contrast, nicks 15 or 30 bp 3′ to the ICL or 34 bp 5′ to the ICL failed to stimulate repair (Figure 3B). We conclude that incision 5′ to the ICL is a rate-limiting step during the repair process. It further suggests that a 5′ incision close to the ICL (19 versus 34 bp) is optimal for repair and could reflect the physiological site of incision. Finally, the asymmetry between 3′ and 5′ incisions could reflect the action of 2 distinct nucleases.

Our evidence that MutSα is recruited to ICLs and is required for their repair prompted us to investigate the possible role of the mismatch-associated MutLα (MLH1-PMS2 heterodimer) endonuclease in ICL repair. Recruitment of MutLα to SJG-136-treated plasmids was observed following incubation of SJG-136-treated plasmid in extracts (Figure 3C). Notably, MLH1 recruitment was critically dependent upon MutSα, since MLH1 binding to the ICL-containing plasmid was abrogated in MSH2-depleted extracts (Figure 3C).

Specific antibodies against *Xenopus* MLH1 quantitatively depleted both MLH1 and PMS2 from extracts (Figure 3D, left). Importantly, MLH1 depletion did not affect the levels of MSH2 and MSH6 in the cytosol (Figure 3D, right), and MSH2 and MSH6 were still enriched on SJG-136-treated plasmids in MLH1-depleted extracts (Figure S3E, left). Removal of MLH1/PMS2 reduced ICL repair by 50%, and addition of recombinant MutLα^{WT} to MLH1-depleted extract restored repair (Figure 3E). Therefore, MutLα is not only recruited by MutSα to ICL plasmids, it is also necessary for efficient ICL repair.

The PMS2 subunit of MutLα complex contains a conserved endonuclease motif DQHA(X)$_2$E(X)$_4$E. Substitution of E707 to K abolishes the endonucleolytic activity associated with PMS2 and yields a strong mutator phenotype in *S. cerevisiae* (Smith et al., 2013). Mutation of the corresponding residue (PMS2-E702K) in mice increases genomic mutation rates and cancer predisposition (van Oers et al., 2010). We generated the equivalent mutation in *Xenopus* MutLα (MLH1-PMS2^{E674K}) (Figures S3B–S3D). Recombinant MutLα lacking endonuclease activity did not support ICL repair in MLH1-depleted extract (Figure 3E). Furthermore, overexpression of nuclease deficient MutLα complex significantly reduced ICL repair, possibly by acting as a dominant-negative (Figure 3F). Overexpression of recombinant MutLα complexes did not interfere with MutSα recruitment to SJG-136-treated plasmids (Figure S3E, right). These results indicate that PMS2-associated nuclease activity is required for ICL repair.

The dual incision flanking the ICL lesion during "unhooking" produces an oligonucleotide that remains covalently attached to DNA by the ICL adduct and is base-paired on each side of

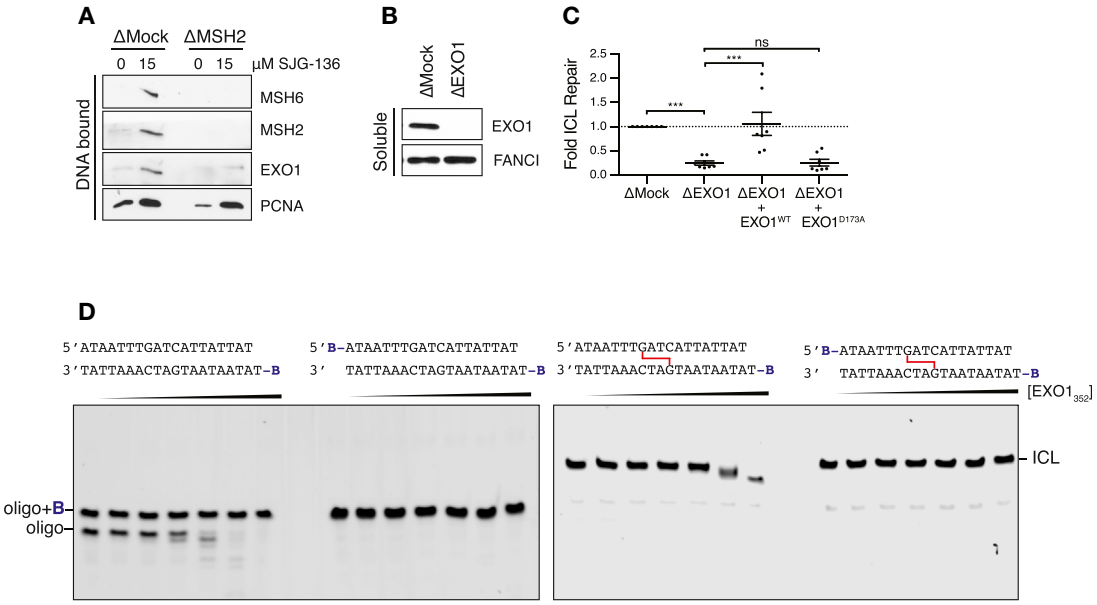


Figure 4. Nucleolytic Processing of ICL Lesions by EXO1

(A) Plasmid pull-down assay and WB of SJG-136-treated plasmids in mock- or MSH2-depleted extract.

(B) EXO1 immunodepletion. Mock- and EXO1-depleted HSS was analyzed by WB.

(C) Quantification of ICL repair in mock-depleted, EXO1-depleted, or EXO1-depleted HSS supplemented with recombinant $EXO1^{WT}$ or $EXO1^{D173A}$ at 3 hr. Results represent mean ± SEM from n = 7 independent experiments.

(D) $Exo1_{352}$ nuclease assay with control or SJG-treated oligonucleotides. Reaction products were run on a denaturing polyacrylamide gel and stained with SYBR Gold.

See also Figure S4.

the ICL. During replication-coupled ICL repair, this DNA fragment is degraded with 5′→ 3′ polarity by the SNM1A exonuclease, presumably allowing DNA polymerases to be loaded (Wang et al., 2011), and for other downstream repair reactions to take place.

We speculated that the 5′→ 3′ EXO1 exonuclease could fulfill a similar function in RIR. In an in vitro reconstituted vertebrate MMR system, EXO1 degrades the single-strand oligonucleotide harboring the mismatch (Genschel et al., 2002). We found that EXO1 was recruited to SJG-136-treated plasmids. As is the case for MutLα, EXO1 recruitment was dependent on MutSα: EXO1 binding to the ICL-containing plasmid was reduced in MSH2-depleted extracts (Figure 4A). A specific antibody generated against *Xenopus* EXO1 quantitatively depleted EXO1 from extracts (Figure 4B). ICL repair was significantly reduced in EXO1-depleted HSS. This defect could be rescued by addition of $EXO1^{WT}$, but not catalytically inactive $EXO1^{D173A}$ (Figure 4C) (Liao et al., 2011). This demonstrates that EXO1 and its nuclease activity are required for ICL repair in the absence of DNA replication and transcription.

To evaluate the specific contribution of EXO1 during ICL repair, we conducted in vitro nuclease assays (Wang et al., 2011). First, we purified the catalytic domain of human EXO1 ($EXO1_{352}$) from *E. coli* as previously described (Shi et al., 2017). $EXO1_{352}$ harbors robust nuclease activity in vitro but lacks its C-terminal region (Figure S4A), important for making connections with various protein binding partners including MSH2, MLH1, PCNA, and RPA (Shi et al., 2017).

Duplex oligonucleotides containing a single SJG-136 ICL lesion are easily distinguished by size from uncrosslinked duplex on a denaturing polyacrylamide gel (Figure S4B). Similarly, the reaction products upon incubation with $EXO1_{352}$ can be visualized and identified. We generated control duplex DNA with only one free 5′ end available for processing (the 5′ end of the other strand blocked by addition of a biotin moiety). This template is efficiently processed by $EXO1_{352}$ from the biotin-free 5′ end in the 5′ to 3′ direction as anticipated (Figure 4D, left). Exonucleolytic processing was abolished when both ends of the duplex are blocked with biotin, confirming that under these experimental conditions, only the exonuclease activity contributes to the processing of the DNA substrates. When $EXO1_{352}$ was incubated with similar ds-DNA oligonucleotides containing a single SJG-136 ICL lesion, $EXO1_{352}$ was able to initiate processing of the substrate from an available 5′ end. However, EXO1 processing was blocked by the ICL lesion (Figure 4D, right). $EXO1_{352}$ is therefore unable to bypass an ICL under these conditions.

Incubation of plasmids harboring ICL lesions activates the RPA-ATR-pChk1 branch of the DNA damage response (Figure S1G). Next, we assessed the role of RPA in ICL repair. Trimeric RPA protein complex was depleted using an antibody generated against RPA1. Depletion of RPA from HSS extracts completely abolished ICL repair. ICL repair was restored by addition of recombinant *Xenopus* trimeric RPA complex (Figures S4C–S4E). This is consistent with our previous studies in which we reported a requirement for RPA in ICL repair in LSS extracts

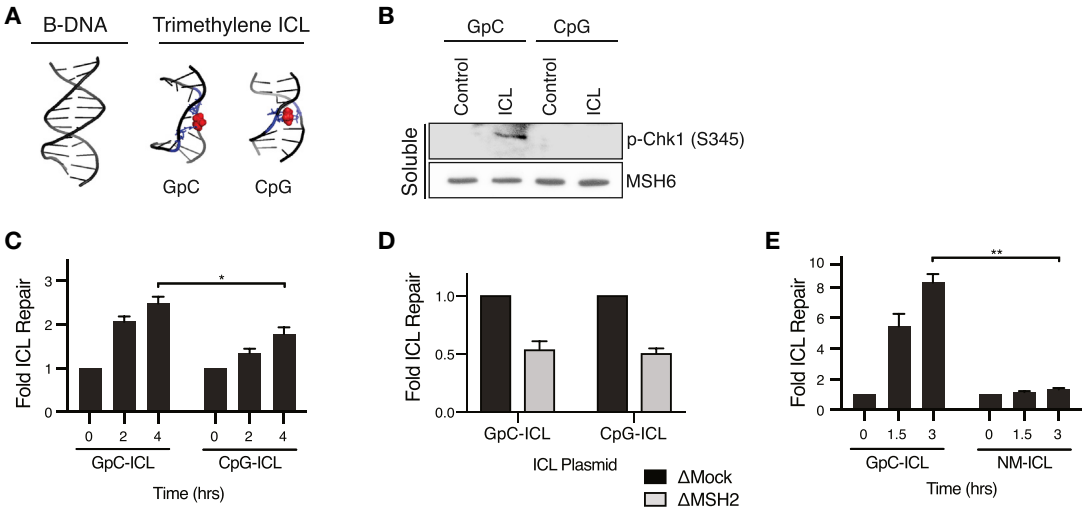


Figure 5. ICL Structure and Repair Efficiency

(A) Structures of normal B-form DNA (PDB: 1BNA) and DNA duplexes containing trimethylene 5′ GpC-ICL (PDB: 1LUH) and 5′ CpG-ICL (PDB: 2KNK) lesions.

(B) Plasmids containing single trimethylene GpC or CpG-ICL were incubated in HSS, and soluble extracts were analyzed by WB.

(C) Quantification of ICL repair of trimethylene GpC and CpG plasmids at the indicated time points. Results represent mean ± SEM from n = 4 independent experiments.

(D) Quantification of ICL repair of trimethylene GpC and CpG plasmids in mock- and MSH2-depleted HSS at 3 hr. Results represent mean ± SEM from n = 4 independent experiments.

(E) Quantification of ICL repair of trimethylene GpC and NM-like ICL plasmid using TaqMan qPCR reagent at the indicated time points. Results represent mean ± SEM from n = 3 independent experiments.

See also Figure S5.

(low-speed supernatant) treated with geminin and roscovitine to inhibit replication (Ben-Yehoyada et al., 2009).

We predicted that RPA binding to crosslinked plasmids during ICL repair would occur primarily after ICL recognition and initiation of ICL unhooking events. We found that MSH2, MSH6, and MLH1 were loaded to similar levels to SJG-136-treated plasmids following incubation in mock- and RPA-depleted extracts (Figure S4F). This result supports a model in which MutSα and MutLα play early roles during ICL repair, preceding nucleolytic events during the repair.

The Efficiency of RIR Is Influenced by ICL Structure

In replicating extracts, ICLs are sensed by a translocating replisome that stalls at the ICL. In RIR, we suggest ICL sensing is dependent on distortion of the DNA helix at the lesion. To test this idea, we compared the kinetics of repair between chemically related trimethylene ICLs in two distinct structural conformations: the more distorting 5′-GpC-ICL (Dooley et al., 2003) and the less distorting 5′-CpG-ICL (Dooley et al., 2001) (Figures 5A and S5A). Both ICLs were ligated into a pEGFP vector backbone (Figure S5B). We found that the more distorting ICL induced a stronger ATR checkpoint activation (Figure 5B). Furthermore, repair of the more distorting lesion was 40% more efficient than the less distorting lesion (Figure 5C). We next asked whether repair of these ICLs was dependent upon MutSα. As shown in Figure 5D, repair of both trimethylene crosslinks displayed a similar requirement for MSH2. Thus, MutSα is able to recognize structurally distinct ICL lesions.

Finally, we monitored repair of a chemically distinct nitrogen mustard-like ICL lesion (Figures 5E and S5A). This NM-ICL is generated within the major groove with minimal distortion of the DNA helix, allowing the DNA to maintain B-DNA conformation (Guainazzi et al., 2010; Mukherjee et al., 2014). Interestingly, even after 3 hr of incubation there was no detectable repair of this NM-ICL (Figure 5E). This supports our notion that ICL recognition in RIR depends on DNA distortion and is consistent with a previous report, which indicates that the repair of NM-ICLs is entirely dependent on replication (Räschle et al., 2008).

DISCUSSION

MutSα Is a Bona Fide ICL Sensor

The mechanism(s) by which ICLs are sensed and repaired in non-dividing cells has been difficult to elucidate. In this study, we used *Xenopus* HSS extracts, which do not replicate or transcribe, to identify eukaryotic MMR complex, MutSα (MSH2–MSH6), as a sensor of ICL lesions. Our conclusion is supported by the following observations. First, MutSα was recruited to plasmids containing ICL lesions (Figures 1A and 1B). Second, MutSα carrying its mismatch binding FXE motif was required for repair of trimethylene ICLs (Figure 2B). Third, binding of MutSα to ICL was required for recruitment of downstream repair proteins including MutLα (Figure 3C) and EXO1 (Figure 4A).

We conclude that binding of MutSα to ICLs precedes nucleolytic processing and thus acts at an early step in ICL repair. Moreover, overexpression of MutSα in extracts enhanced ICL

repair efficiency (Figure 1E), further suggesting MutSα senses ICLs, and that such sensing is a rate-limiting step in repair.

We observe that MutSα-dependent ICL repair has slower kinetics than the repair of mismatches in *Xenopus* extracts (Kawasoe et al., 2016; Radman, 2016). We surmise that the complexity of the ICL lesion, which requires two rounds of repair synthesis during RIR, could account for this difference.

Importantly, we find that structurally distinct ICLs were repaired with varying efficiencies. Trimethylene-ICLs, which significantly distort DNA, were repaired with robust efficiency, in contrast to non-distorting NM-like ICLs, which had no detectible repair (Figures 5C and 5E). This indicates that ICL repair by RIR is critically dependent on ability of the ICL lesion to be recognized by DNA damage sensor proteins. Thus, repair of ICLs in G0/G1 utilizes a fundamentally different mechanism than ICL repair during S-phase, when lesion sensing occurs by replisomes stalled at ICLs irrespective of crosslink structure. Moreover, ICL recognition by MutSα during RIR may also be influenced by sequence context, as has been proposed for MMR (Mazurek et al., 2009).

Of note, MutSα (MSH2–MSH6) is one of two MMR sensors that operate during MMR in vertebrates. The other is MutSβ (MSH2–MSH3), which is about 10 times less abundant than MutSα in mammalian cells and has higher specificity for recognizing larger IDLs and branched DNA substrates (Genschel et al., 1998). Depletion of MSH2 from extracts quantitatively depleted MSH6 and MSH3. Our depletion-rescue experiments with recombinant MutSα, which fully rescues ICL repair, helps assign the specificity of ICL recognition to MutSα (Figure 1D). In mammalian cells, MutSβ along with the global genome nucleotide excision repair sensor, XPC (xeroderma pigmentosum complementation group C) have been linked to psoralen ICL repair (Muniandy et al., 2009; Thoma et al., 2005; Zhang et al., 2002; Zhao et al., 2009). However, how these complexes contribute to the repair of ICLs remains unclear.

We were unable to detect direct binding of MutSα to SJG-136-treated oligonucleotides using electrophoretic mobility shift assay (EMSA) experiments. This could be due to the design of the oligo duplex itself, which is restricted in length (~20 bases) to ensure SJG-136 generates a single ICL and to avoid the formation of secondary structures, since the duplex must be almost exclusively be composed of A and T nucleotides. While our data using the mismatch binding deficient (FXE) mutant MutSα (Figure 2B) strongly suggest that MutSα binding to DNA is critical for ICL repair, we cannot formally rule out that additional proteins participate in recruiting MutSα to ICLs.

The Role of PCNA during RIR

Previously, we reported that PCNA is an essential RIR factor. We showed that PCNA ubiquitinylated at lysine (K164) is required to recruit Polκ to ICL sites for repair synthesis (Williams et al., 2012). The experiments described here indicate that PCNA is not essential for MutSα recruitment to ICLs (Figure 2C). However, we find a requirement for interaction between MutSα and PCNA for ICL repair (Figure 2B). This suggests that PCNA may help retain MutSα at damage sites, or perhaps enhance MutSα's damage recognition specificity, as has been suggested during MMR (Clark et al., 2000; Flores-Rozas et al., 2000; Kleczkowska et al., 2001). Our data show that PCNA plays an upstream role during ICL repair, in addition to supporting Polκ recruitment for repair synthesis.

We also probed the requirement for binding between PCNA and components of the MMR machinery by generating mutations in the interdomain connector loop (IDCL) of PCNA. Mutations in this region abrogate interactions with MSH6 and PMS2 in vitro, and cause MMR defects in yeast (Lee and Alani, 2006). We made the equivalent mutations in *Xenopus* $PCNA^{L126A,I128A}$ (Figures S2E and S2F). However, we were not able to determine the contribution of the PCNA:MutSα interface to ICL repair using the this mutant, since binding between $MutS\alpha^{WT}$ and $PCNA^{L126A,I128A}$ was enhanced, as measured by co-immunoprecipitation, compared to binding between $MutS\alpha^{WT}$ and $PCNA^{WT}$ (Figure S2G). Accordingly, both $PCNA^{WT}$ and $PCNA^{L126A,I128A}$ were able to rescue ICL repair defects in PCNA-depleted extracts (Figures S2H and S2I).

Insights into Nucleolytic Processing of ICLs by MutLα and EXO1

Following sensing, an ICL is thought to be processed in two consecutive nucleolytic steps. First, dual incisions surrounding the ICL are made to produce an "unhooked" oligonucleotide, which is covalently tethered to the DNA by the ICL adduct. The unhooked lesion is then resected in a "trimming" reaction that facilitates synthesis past the adduct by translesion synthesis polymerases by eliminating the need for displacement synthesis (Roy et al., 2016). In replication-coupled ICL repair the XPF-ERCC1 endonuclease and SLX4 promote unhooking of ICLs (Klein Douwel et al., 2014), and the SNM1A exonuclease has been proposed to process unhooked ICL lesions (Wang et al., 2011).

We investigated the role of these nucleolytic reactions in RIR. We used nicked plasmids to identify that a 5′ incision 19 base pairs away from an ICL lesion was able to stimulate repair by 4-fold. This is in contrast to nicks placed 15 or 30 bp 3′ to the ICL or 34 bp 5′ to the ICL, all of which failed to stimulate repair (Figure 3B). This experiment demonstrates a striking mechanistic difference between RIR and MMR. MMR is stimulated symmetrically by either a 5′ or a 3′ nick in vitro (Constantin et al., 2005; Varlet et al., 1996). Our results also suggest that 5′ incision is a rate-limiting step during RIR, and that 2 distinct nucleases are required during ICL repair. Depletion-rescue experiments with MutLα strongly suggest that MutLα promotes one or both of these incisions. However, because it is generally thought that MutLα requires a pre-existing nick and interaction with PCNA to stimulate its otherwise latent endonuclease activity, it is possible that another, as yet unidentified nuclease, is responsible for the initial incision.

Our discovery that MutSα and MutLα participate in ICL repair highlights the versatile roles DNA repair proteins play in genome maintenance. This idea is further exemplified by our finding that EXO1 is required during RIR. EXO1 is a 5′ → 3′ exonuclease that is not only involved in MMR, but also plays critical roles in double-strand break repair and telomere maintenance (Tran et al., 2004). In all of these roles, EXO1 catalyzes digestion of DNA by hydrolyzing phosphodiester bonds between adjoining normal nucleotides. The requirement for EXO1's nuclease activity during ICL repair (Figure 4C) suggests that EXO1 could process

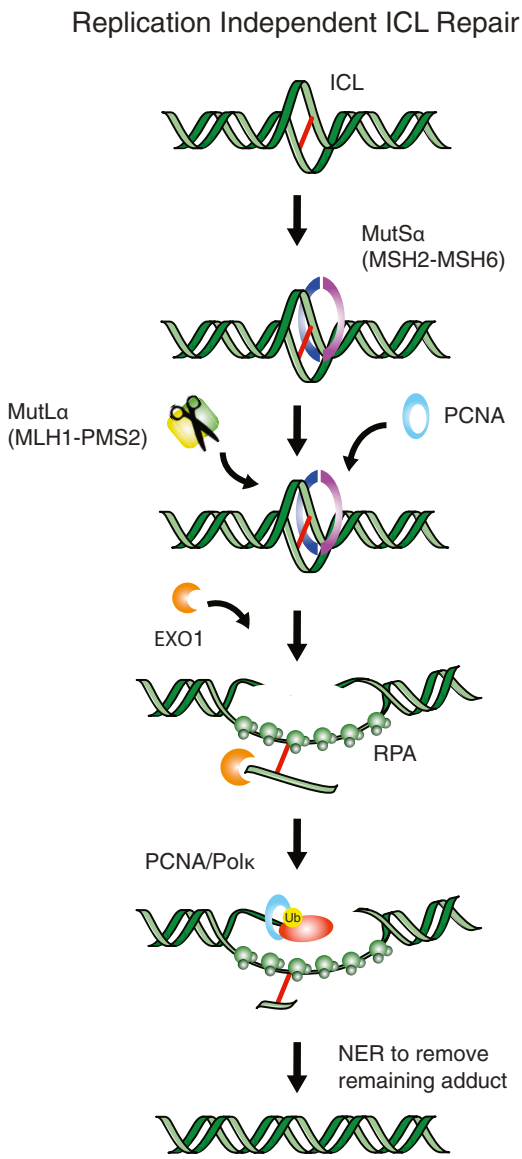


Figure 6. Model for MMR-Mediated ICL Repair

ICLs can be recognized and repaired in the absence of replication. The MutSα complex senses and binds ICL lesions and recruits downstream repair proteins including the MutLα endonuclease, PCNA, and EXO1 exonuclease. The ICL lesion is repaired through a multi-step excision resynthesis reaction. The latter requires Polκ and monoubiquitinated PCNA.

complex, chemically modified DNA substrates, including crosslinked bases. However, our in vitro experiments in which $EXO1_{352}$ was unable to bypass an ICL lesion strongly suggests that EXO1 alone cannot efficiently process ICLs (Figure 4D). However, it is conceivable that ICL processing by EXO1 requires association with other proteins through its C terminus.

During replication-coupled ICL repair, SNM1A has been proposed to operate similarly to EXO1 in processing unhooked ICL lesions (Wang et al., 2011). In addition, there is evidence that the FAN1 nuclease can digest past ICL-containing oligonucleotides in vitro (Pizzolato et al., 2015; Wang et al., 2014). However, we find that FAN1 depletion in *Xenopus* HSS extracts has no effect on ICL repair (unpublished data).

Conclusions

DNA crosslinking agents are among the most widely prescribed anti-cancer drugs used in the clinic. Yet, acquired resistance remains is a significant limitation of these drugs (Deans and West, 2011). It seems likely that alterations to pathways that contribute to the repair of ICLs, the principle cytotoxic lesion generated by these drugs, may underlie acquired resistance. It is therefore critical to fully understand how cells respond to and repair ICLs.

We provide compelling evidence that the MMR pathway contributes to ICL repair. This is in agreement with a recent study that used an unbiased proteomic approach to study protein recruitment to psoralen-ICLs using quantitative mass spectrometry. This study revealed that MSH2, MSH6, PMS2, and EXO1 associate with crosslinked chromatin (Räschle et al., 2015). Importantly, recruitment of these proteins was resistant to geminin, suggesting that MMR proteins may play a role in ICL repair both in and beyond G0/G1 phase. Indeed, requirement for MSH2 and EXO1 has been reported in repair of nitrogen mustard ICLs in S-phase yeast (Barber et al., 2005), and in S-phase mammalian cells defective in the FA pathway (Huang et al., 2011).

In this study, we provide mechanistic insights into how MMR proteins cooperate to accomplish ICL repair (Figure 6). We propose that ICLs that are recognized by MutSα are processed by MutLα and EXO1 in an incision-excision reaction, followed by DNA synthesis process that requires PCNA and Polκ. Further, our work stresses the importance of distinguishing between structurally distinct ICLs, which may be repaired in overlapping and/or divergent ways. Future experiments should address whether the sequence context of ICLs influences ICL recognition and repair.

EXPERIMENTAL PROCEDURES

Xenopus Cell-free Extract and Depletions

Xenopus laevis frogs were handled in accordance with guidelines provided by the Institutional Animal Care and Use Committee at Columbia University, protocol AAAK0551. For details regarding the preparation of cell-free extracts and depleted extracts, see Supplemental Experimental Procedures.

Preparation ICL Plasmids

SJG-136-treated plasmids were prepared by incubating pBS with indicated concentrations of SJG-136 (NCI/Spriogen LTD) in 50 mM triethanolamine and 2 mM EDTA, overnight at 37°C. Plasmids were ethanol precipitated and resuspended in water. The quality and quantity of plasmids recovered were analyzed by agarose gel electrophoresis with ethidium bromide, Nanodrop measurement, and qPCR. The amount of plasmid used in plasmid pull-down experiments was normalized accordingly. The preparation of plasmids containing a single SJG-136, trimethylene, or NM-like ICL lesion is described in detail in Supplemental Experimental Procedures.

ICL Repair and lac Repressor Plasmid Pull-Down Experiments in *Xenopus* Extracts

Lac repressor plasmid pull-down assays in undepleted and depleted HSS extracts were performed as described previously (Williams et al., 2012). ICL repair assays in HSS were performed essentially as describe previously (Ben-Yehoyada et al., 2009; Williams et al., 2012). Each of these assays, as well as the MMR assay of a site-specific A:C mispair is described in detail in Supplemental Experimental Procedures.

Statistical Analysis

All statistical analyses were performed using GraphPad Prism 7 software using paired Student's t test or ANOVA. Data are annotated as ns = $p > 0.05$; *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$.

Protein Expression and Purification

Recombinant MutSα, MutLα, RPA, PCNA, and EXO1 were purified as described in Supplemental Experimental Procedures.

Reagents

All antibodies and reagents are listed in Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and five figures and can be found with this article online at https://doi.org/10.1016/j.celrep.2017.10.032.

AUTHOR CONTRIBUTIONS

N.K. performed all experiments except Figure 2A, which was prepared by Y.K., and Figures 3B and 4C, which were generated by H.W.; E.C. contributed to Figure 5E; U.R. prepared the NM-like ICL oligonucleotide; Y.S. purified $EXO1_{352}$. N.K. and J.G. prepared the manuscript with input from all other authors: L.S.B., O.D.S., H.Y., M.E.G., and T.S.T.

ACKNOWLEDGMENTS

We thank the National Cancer Institute and Spirogen LTD for providing SJG-136, Dr. C. J. Rizzo for trimethylene-ICL crosslinked oligonucleotides, Dr. K. Marians for Bio-LacR protein, Dr. Karlene Cimprich for the RPA construct, and J. Jiricny for MSH2–MSH6 and EXO1 antibodies. We also thank T. Apáricio and G. Sidhu for RPA protein purification. This work was supported by the National Cancer Institute (1R35 CA197606 and 1P01 CA174653 to J.G., R01 CA165911 to O.D.S., and 5T32 CA009503-27 to N.K.), the NIH (5T32 GM008798-15 to N.K. and P01 CA092584 to L.S.B.), and the Korean Institute for Basic Science (IBS-R022-A1-2017 to O.D.S.).

Received: May 19, 2017
Revised: September 21, 2017
Accepted: October 8, 2017
Published: October 31, 2017

REFERENCES

Barber, L.J., Ward, T.A., Hartley, J.A., and McHugh, P.J. (2005). DNA interstrand cross-link repair in the Saccharomyces cerevisiae cell cycle: Overlapping roles for PSO2 (SNM1) with MutS factors and EXO1 during S phase. Mol. Cell. Biol. *25*, 2297–2309.

Beljanski, V., Marzilli, L.G., and Doetsch, P.W. (2004). DNA damage-processing pathways involved in the eukaryotic cellular response to anticancer DNA cross-linking drugs. Mol. Pharmacol. *65*, 1496–1506.

Ben-Yehoyada, M., Wang, L.C., Kozekov, I.D., Rizzo, C.J., Gottesman, M.E., and Gautier, J. (2009). Checkpoint signaling from a single DNA interstrand crosslink. Mol. Cell *35*, 704–715.

Bowers, J., Sokolsky, T., Quach, T., and Alani, E. (1999). A mutation in the MSH6 subunit of the Saccharomyces cerevisiae MSH2-MSH6 complex disrupts mismatch recognition. J. Biol. Chem. *274*, 16115–16125.

Clark, A.B., Valle, F., Drotschmann, K., Gary, R.K., and Kunkel, T.A. (2000). Functional interaction of proliferating cell nuclear antigen with MSH2-MSH6 and MSH2-MSH3 complexes. J. Biol. Chem. *275*, 36498–36501.

Constantin, N., Dzantiev, L., Kadyrov, F.A., and Modrich, P. (2005). Human mismatch repair: Reconstitution of a nick-directed bidirectional reaction. J. Biol. Chem. *280*, 39752–39761.

Deans, A.J., and West, S.C. (2011). DNA interstrand crosslink repair and cancer. Nat. Rev. Cancer *11*, 467–480.

Dooley, P.A., Tsarouhtsis, D., Korbel, G.A., Nechev, L.V., Shearer, J., Zegar, I.S., Harris, C.M., Stone, M.P., and Harris, T.M. (2001). Structural studies of an oligodeoxynucleotide containing a trimethylene interstrand cross-link in a 5′-(CpG) motif: Model of a malondialdehyde cross-link. J. Am. Chem. Soc. *123*, 1730–1739.

Dooley, P.A., Zhang, M., Korbel, G.A., Nechev, L.V., Harris, C.M., Stone, M.P., and Harris, T.M. (2003). NMR determination of the conformation of a trimethylene interstrand cross-link in an oligodeoxynucleotide duplex containing a 5′-d(GpC) motif. J. Am. Chem. Soc. *125*, 62–72.

Dufner, P., Marra, G., Räschle, M., and Jiricny, J. (2000). Mismatch recognition and DNA-dependent stimulation of the ATPase activity of hMutSalpha is abolished by a single mutation in the hMSH6 subunit. J. Biol. Chem. *275*, 36550–36555.

Duxin, J.P., and Walter, J.C. (2015). What is the DNA repair defect underlying Fanconi anemia? Curr. Opin. Cell Biol. *37*, 49–60.

Enoiu, M., Jiricny, J., and Schärer, O.D. (2012). Repair of cisplatin-induced DNA interstrand crosslinks by a replication-independent pathway involving transcription-coupled repair and translesion synthesis. Nucleic Acids Res. *40*, 8953–8964.

Flores-Rozas, H., Clark, D., and Kolodner, R.D. (2000). Proliferating cell nuclear antigen and Msh2p-Msh6p interact to form an active mispair recognition complex. Nat. Genet. *26*, 375–378.

Fu, D., Calvo, J.A., and Samson, L.D. (2012). Balancing repair and tolerance of DNA damage caused by alkylating agents. Nat. Rev. Cancer *12*, 104–120.

Genschel, J., Littman, S.J., Drummond, J.T., and Modrich, P. (1998). Isolation of MutSbeta from human cells and comparison of the mismatch repair specificities of MutSbeta and MutSalpha. J. Biol. Chem. *273*, 19895–19901.

Genschel, J., Bazemore, L.R., and Modrich, P. (2002). Human exonuclease I is required for 5′ and 3′ mismatch repair. J. Biol. Chem. *277*, 13302–13311.

Gregson, S.J., Howard, P.W., Hartley, J.A., Brooks, N.A., Adams, L.J., Jenkins, T.C., Kelland, L.R., and Thurston, D.E. (2001). Design, synthesis, and evaluation of a novel pyrrolobenzodiazepine DNA-interactive agent with highly efficient cross-linking ability and potent cytotoxicity. J. Med. Chem. *44*, 737–748.

Guainazzi, A., Campbell, A.J., Angelov, T., Simmerling, C., and Schärer, O.D. (2010). Synthesis and molecular modeling of a nitrogen mustard DNA interstrand crosslink. Chemistry *16*, 12100–12103.

Hlavin, E.M., Smeaton, M.B., Noronha, A.M., Wilds, C.J., and Miller, P.S. (2010). Cross-link structure affects replication-independent DNA interstrand cross-link repair in mammalian cells. Biochemistry *49*, 3977–3988.

Hombauer, H., Campbell, C.S., Smith, C.E., Desai, A., and Kolodner, R.D. (2011). Visualization of eukaryotic DNA mismatch repair reveals distinct recognition and repair intermediates. Cell *147*, 1040–1053.

Huang, M., Kennedy, R., Ali, A.M., Moreau, L.A., Meetei, A.R., D'Andrea, A.D., and Chen, C.C. (2011). Human MutS and FANCM complexes function as redundant DNA damage sensors in the Fanconi Anemia pathway. DNA Repair (Amst.) *10*, 1203–1212.

Islas, Á.L., Vos, J.-M.H., and Hanawalt, P.C. (1991). Differential introduction and repair of psoralen photoadducts to DNA in specific human genes. Cancer Res. *51*, 2867–2873.

Iyama, T., and Wilson, D.M., 3rd. (2013). DNA repair mechanisms in dividing and non-dividing cells. DNA Repair (Amst.) *12*, 620–636.

Kawasoe, Y., Tsurimoto, T., Nakagawa, T., Masukata, H., and Takahashi, T.S. (2016). MutSα maintains the mismatch repair capability by inhibiting PCNA unloading. eLife *5*, Published online July 12, 2016. https://doi.org/10.7554/eLife.15155.

Kleczkowska, H.E., Marra, G., Lettieri, T., and Jiricny, J. (2001). hMSH3 and hMSH6 interact with PCNA and colocalize with it to replication foci. Genes Dev. *15*, 724–736.

Klein Douwel, D., Boonen, R.A.C.M., Long, D.T., Szypowska, A.A., Räschle, M., Walter, J.C., and Knipscheer, P. (2014). XPF-ERCC1 acts in unhooking

DNA interstrand crosslinks in cooperation with FANCD2 and FANCP/SLX4. Mol. Cell. *54*, 460–471.

Knipscheer, P., Räschle, M., Smogorzewska, A., Enoiu, M., Ho, T.V., Schärer, O.D., Elledge, S.J., and Walter, J.C. (2009). The Fanconi anemia pathway promotes replication-dependent DNA interstrand cross-link repair. Science *326*, 1698–1701.

Lamers, M.H., Perrakis, A., Enzlin, J.H., Winterwerp, H.H., de Wind, N., and Sixma, T.K. (2000). The crystal structure of DNA mismatch repair protein MutS binding to a G x T mismatch. Nature *407*, 711–717.

Lee, S.D., and Alani, E. (2006). Analysis of interactions between mismatch repair initiation factors and the replication processivity factor PCNA. J. Mol. Biol. *355*, 175–184.

Li, F., Mao, G., Tong, D., Huang, J., Gu, L., Yang, W., and Li, G.-M. (2013). The histone mark H3K36me3 regulates human DNA mismatch repair through its interaction with MutSα. Cell *153*, 590–600.

Liao, S., Toczylowski, T., and Yan, H. (2011). Mechanistic analysis of Xenopus EXO1's function in 5'-strand resection at DNA double-strand breaks. Nucleic Acids Res. *39*, 5967–5977.

Malkov, V.A., Biswas, I., Camerini-Otero, R.D., and Hsieh, P. (1997). Photocross-linking of the NH2-terminal region of Taq MutS protein to the major groove of a heteroduplex DNA. J. Biol. Chem. *272*, 23811–23817.

Mazurek, A., Johnson, C.N., Germann, M.W., and Fishel, R. (2009). Sequence context effect for hMSH2-hMSH6 mismatch-dependent activation. Proc. Natl. Acad. Sci. USA *106*, 4177–4182.

Mukherjee, S., Guainazzi, A., and Schärer, O.D. (2014). Synthesis of structurally diverse major groove DNA interstrand crosslinks using three different aldehyde precursors. Nucleic Acids Res. *42*, 7429–7435.

Muniandy, P.A., Thapa, D., Thazhathveetil, A.K., Liu, S.T., and Seidman, M.M. (2009). Repair of laser-localized DNA interstrand cross-links in G_1 phase mammalian cells. J. Biol. Chem. *284*, 27908–27917.

Olivera Harris, M., Kallenberger, L., Artola Borán, M., Enoiu, M., Costanzo, V., and Jiricny, J. (2015). Mismatch repair-dependent metabolism of O6-methylguanine-containing DNA in Xenopus laevis egg extracts. DNA Repair (Amst.) *28*, 1–7.

Pizzolato, J., Mukherjee, S., Schärer, O.D., and Jiricny, J. (2015). FANCD2-associated nuclease 1, but not exonuclease 1 or flap endonuclease 1, is able to unhook DNA interstrand cross-links *in vitro*. J. Biol. Chem. *290*, 22602–22611.

Price, N.E., Johnson, K.M., Wang, J., Fekry, M.I., Wang, Y., and Gates, K.S. (2014). Interstrand DNA-DNA cross-link formation between adenine residues and abasic sites in duplex DNA. J. Am. Chem. Soc. *136*, 3483–3490.

Radman, M. (2016). Mismatch repair earns Nobel Prize in Chemistry 2015 to Paul Modrich for a biochemical tour de force. DNA Repair (Amst.) *37*, A22–A28.

Räschle, M., Knipscheer, P., Enoiu, M., Angelov, T., Sun, J., Griffith, J.D., Ellenberger, T.E., Schärer, O.D., and Walter, J.C. (2008). Mechanism of replication-coupled DNA interstrand crosslink repair. Cell *134*, 969–980.

Räschle, M., Smeenk, G., Hansen, R.K., Temu, T., Oka, Y., Hein, M.Y., Nagaraj, N., Long, D.T., Walter, J.C., Hofmann, K., et al. (2015). DNA repair. Proteomics reveals dynamic assembly of repair complexes during bypass of DNA cross-links. Science *348*, 1253671.

Reyes, G.X., Schmidt, T.T., Kolodner, R.D., and Hombauer, H. (2015). New insights into the mechanism of DNA mismatch repair. Chromosoma *124*, 443–462.

Roy, U., Mukherjee, S., Sharma, A., Frank, E.G., and Schärer, O.D. (2016). The structure and duplex context of DNA interstrand crosslinks affects the activity of DNA polymerase η. Nucleic Acids Res. *44*, 7281–7291.

Sarkar, S., Davies, A.A., Ulrich, H.D., and McHugh, P.J. (2006). DNA interstrand crosslink repair during G1 involves nucleotide excision repair and DNA polymerase ζ. EMBO J. *25*, 1285–1294.

Schroering, A.G., Edelbrock, M.A., Richards, T.J., and Williams, K.J. (2007). The cell cycle and DNA mismatch repair. Exp. Cell Res. *313*, 292–304.

Semlow, D.R., Zhang, J., Budzowska, M., Drohat, A.C., and Walter, J.C. (2016). Replication-dependent unhooking of DNA interstrand cross-links by the NEIL3 glycosylase. Cell *167*, 498–511.

Shell, S.S., Putnam, C.D., and Kolodner, R.D. (2007). The N terminus of Saccharomyces cerevisiae Msh6 is an unstructured tether to PCNA. Mol. Cell *26*, 565–578.

Shen, X., Jun, S., O'Neal, L.E., Sonoda, E., Bemark, M., Sale, J.E., and Li, L. (2006). REV3 and REV1 play major roles in recombination-independent repair of DNA interstrand cross-links mediated by monoubiquitinated proliferating cell nuclear antigen (PCNA). J. Biol. Chem. *281*, 13869–13872.

Shi, Y., Hellinga, H.W., and Beese, L.S. (2017). Interplay of catalysis, fidelity, threading, and processivity in the exo- and endonucleolytic reactions of human exonuclease I. Proc. Natl. Acad. Sci. USA *114*, 6010–6015.

Smeaton, M.B., Hlavin, E.M., McGregor Mason, T., Noronha, A.M., Wilds, C.J., and Miller, P.S. (2008). Distortion-dependent unhooking of interstrand cross-links in mammalian cell extracts. Biochemistry *47*, 9920–9930.

Smith, C.E., Mendillo, M.L., Bowen, N., Hombauer, H., Campbell, C.S., Desai, A., Putnam, C.D., and Kolodner, R.D. (2013). Dominant mutations in S. cerevisiae PMS1 identify the Mlh1-Pms1 endonuclease active site and an exonuclease 1-independent mismatch repair pathway. PLoS Genet. *9*, e1003869.

Thoma, B.S., Wakasugi, M., Christensen, J., Reddy, M.C., and Vasquez, K.M. (2005). Human XPC-hHR23B interacts with XPA-RPA in the recognition of triplex-directed psoralen DNA interstrand crosslinks. Nucleic Acids Res. *33*, 2993–3001.

Tran, P.T., Erdeniz, N., Symington, L.S., and Liskay, R.M. (2004). EXO1-A multi-tasking eukaryotic nuclease. DNA Repair (Amst.) *3*, 1549–1559.

van Oers, J.M.M., Roa, S., Werling, U., Liu, Y., Genschel, J., Hou, H., Jr., Sellers, R.S., Modrich, P., Scharff, M.D., and Edelmann, W. (2010). PMS2 endonuclease activity has distinct biological functions and is essential for genome maintenance. Proc. Natl. Acad. Sci. USA *107*, 13384–13389.

Varlet, I., Canard, B., Brooks, P., Cerovic, G., and Radman, M. (1996). Mismatch repair in Xenopus egg extracts: DNA strand breaks act as signals rather than excision points. Proc. Natl. Acad. Sci. USA *93*, 10156–10161.

Wang, A.T., Sengerová, B., Cattell, E., Inagawa, T., Hartley, J.M., Kiakos, K., Burgess-Brown, N.A., Swift, L.P., Enzlin, J.H., Schofield, C.J., et al. (2011). Human SNM1A and XPF-ERCC1 collaborate to initiate DNA interstrand cross-link repair. Genes Dev. *25*, 1859–1870.

Wang, R., Persky, N.S., Yoo, B., Ouerfelli, O., Smogorzewska, A., Elledge, S.J., and Pavletich, N.P. (2014). DNA repair. Mechanism of DNA interstrand cross-link processing by repair nuclease FAN1. Science *346*, 1127–1130.

Warren, J.J., Pohlhaus, T.J., Changela, A., Iyer, R.R., Modrich, P.L., and Beese, L.S. (2007). Structure of the human MutSalpha DNA lesion recognition complex. Mol. Cell *26*, 579–592.

Williams, H.L., Gottesman, M.E., and Gautier, J. (2012). Replication-independent repair of DNA interstrand crosslinks. Mol. Cell *47*, 140–147.

York, S.J., and Modrich, P. (2006). Mismatch repair-dependent iterative excision at irreparable O6-methylguanine lesions in human nuclear extracts. J. Biol. Chem. *281*, 22674–22683.

Zhang, N., Lu, X., Zhang, X., Peterson, C.A., and Legerski, R.J. (2002). hMutSbeta is required for the recognition and uncoupling of psoralen interstrand cross-links in vitro. Mol. Cell. Biol. *22*, 2388–2397.

Zhao, J., Jain, A., Iyer, R.R., Modrich, P.L., and Vasquez, K.M. (2009). Mismatch repair and nucleotide excision repair proteins cooperate in the recognition of DNA interstrand crosslinks. Nucleic Acids Res. *37*, 4420–4429.