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

Graphical Abstract

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

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.
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,3,4 Hong Yan,6 Max E. Gottesman,7 Tatsuro S. Takahashi,8 and Jean Gautier1,9,*

1Institute of Cancer Genetics, Columbia University, New York, NY 10032, USA
2Graduate School of Science, Osaka University, Toyonaka, Japan
3Department of Chemistry and Department of Pharmacological Sciences, Stony Brook University, Stony Brook, NY 11794, USA
4Department of Biochemistry, Duke University Medical Center, Durham, NC 27710, USA
5Institute for Basic Science Center for Genomic Integrity and School of Life Sciences, Ulsan National Institute of Science and Technology, Ulsan, South Korea
6Fox Chase Cancer Center, Philadelphia, PA 19111, USA
7Institute of Cancer Research, Columbia University, New York, NY 10032, USA
8Department of Biology, Kyushu University, Fukuoka, Japan
9Lead 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 (Enou 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

This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).
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 MutS\(x\) complex (MSH2–MSH6 heterodimer), which recognizes and binds single base mismatches and 1–2 base insertion-deletion loops (IDLS). Binding of MutS\(x\) to mispaired bases leads to the recruitment of downstream DNA repair proteins including the MutL\(x\) 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 MutS\(x\) (MSH2 and MSH6) account for 50%–60% of germline mutation-activated (Lynch syndrome) and 15%–20% of sporadic colorectal cancers (Reyes et al., 2015).

Importantly, MutS\(x\) has multiple roles outside of replication. MutS\(x\) 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 (O\(^{6}\)meG) are processed in a MutS\(x\)-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 O\(^{6}\)meG, MutS\(x\) specifically recognizes bulky lesions such as O\(^{4}\)-methylthymine adducts and cisplatin intranuclear crosslinks in vitro. Crystal structures of human MutS\(x\) 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 MutS\(x\) to DNA lesions leads to a variety of cellular outcomes including repair, checkpoint activation, and apoptosis, underscoring the versatile and critical role that MutS\(x\) 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 MutS\(x\) binds to plasmids bearing a single site-specific ICL and harnesses the entire MMR machinery, including the MutL\(x\) and EXO1 nucleaseases, 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**

**MutS\(x\) (MSH2–MSH6) Senses DNA ICLs**

The MutS\(x\) complex recognizes a diverse array of aberrant DNA structures including base-base mispairs and chemically modified DNA adducts. We hypothesized that the ability of MutS\(x\) to recognize distortions within DNA might also extend to ICLs, and that sensing of ICLs by MutS\(x\) could initiate ICL repair by a set of reactions analogous to MMR. To test this hypothesis, we first asked whether MutS\(x\) 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\(^{\prime}\) 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 MutS\(x\) 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 MutS\(x\) 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]; 2KNU) (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 MutS\(x\) (Figure 1C, left) but did not reduce the cytotoxic levels of other MMR proteins including MLH1 and EXO1 (Figure 1C, right). Compared to mock-depleted extracts, depletion of MutS\(x\) significantly decreased ICL repair (Figure 1D). Importantly, addition of recombinant MutS\(x\)WT to MSH2-depleted extracts restored ICL repair to the level of mock-depleted extracts.
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 (pMM1AC) 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.
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 MutS<sub>a</sub> 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 MutS<sub>a</sub> to mismatches via histone methylation has also been described (Li et al., 2013).

We used plasmid pull-down assays to ask whether MutS<sub>a</sub> was recruited to ICLs in a PCNA-independent manner. As shown in Figure 1B, the MutS<sub>a</sub> complex and PCNA were recruited to SJG-treated plasmids in HSS extracts. MutS<sub>a</sub> recruitment was not dependent on PCNA; MSH2 and MSH6 were efficiently recruited to crosslinked plasmids in PCNA-depleted extracts. Furthermore, the extent of MutS<sub>a</sub> recruitment was unaffected by supplementation of extracts with recombinant PCNA protein (Figure 2C). This experiment strongly suggests that MutS<sub>a</sub> 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 MutS<sub>a</sub> 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 MutS<sub>a</sub> in vitro (Figure S2G). We tested the ability of this MutS<sub>a</sub>PIP to support ICL repair in MutS<sub>a</sub>-depleted extracts. We find that MutS<sub>a</sub>PIP is unable to support ICL repair (Figure 2B). This indicates that, whereas MSH6-PCNA interaction is not required for MutS<sub>a</sub> recruitment to ICLs, and presumably for ICL sensing, the interaction between the two is essential for coordinating downstream repair events.

**ICLs Are Processed by MutL<sub>a</sub> 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
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 plasmids 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 DQHAX2E(X)4E. 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-PMS2E674K) (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...
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 MutLa, EXO1 recruitment was dependent on MutSa: 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 EXO1WT, but not catalytically inactive EXO1D173A (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 (EXO1352) from *E. coli* as previously described (Shi et al., 2017). EXO1352 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 EXO1352 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 EXO1352 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 EXO1352 was incubated with similar ds-DNA oligonucleotides containing a single SJG-136 ICL lesion, EXO1352 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). EXO1352 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.
(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 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 (Räsänen 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.
repair efficiency (Figure 1E), further suggesting MutSξ senses ICLs, and that such sensing is a rate-limiting step in repair.

We observe that MutSξ-dependent ICL repair has slower kinetics than the repair of mismatches in Xenopus extracts (Kawase 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 detectable 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 MutSξ during RIR may also be influenced by sequence context, as has been proposed for MMR (Mazurek et al., 2009).

Of note, MutSξ (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 MutSξ 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 MutSξ, which fully rescues ICL repair, helps assign the specificity of ICL recognition to MutSξ (Figure 1D). In mammalian cells, MutSξ 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 MutSξ 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 MutSξ (Figure 2B) strongly suggest that MutSξ binding to DNA is critical for ICL repair, we cannot formally rule out that additional proteins participate in recruiting MutSξ to ICLs.

The Role of PCNA during RIR

Previously, we reported that PCNA is an essential RIR factor. We found 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 MutSξ recruitment to ICLs (Figure 2C). However, we find a requirement for interaction between MutSξ and PCNA for ICL repair (Figure 2B). This suggests that PCNA may help retain MutSξ at damage sites, or perhaps enhance MutSξ’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 PCNA126A,128A (Figures S2E and S2F). However, we were not able to determine the contribution of the PCNA-MutSξ interface to ICL repair using this mutant, since binding between MutSξWT and PCNA126A,128A was enhanced, as measured by co-immunoprecipitation, compared to binding between MutSξWT and PCNAWT (Figure S2G). Accordingly, both PCNAWT and PCNA126A,128A were able to rescue ICL repair defects in PCNA-depleted extracts (Figures S2H and S2I).

Insights into Nucleolytic Processing of ICLs by MutLξ 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 Douvel 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 MutLξ strongly suggest that MutLξ promotes one or both of these incisions. However, because it is generally thought that MutLξ 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 MutSξ and MutLξ 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
complex, chemically modified DNA substrates, including cross-linked bases. However, our in vitro experiments in which EXO1 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 MutS are processed by MutL 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/Sprigen 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 described previously (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 ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001; ****p ≤ 0.0001.

Protein Expression and Purification
Recombinant MutSΔs, MutLΔs, 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 EXO1Δexo. 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. Apárico and G. Sidi 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 ST22 CA009503-27 to N.K.), the NIH (ST32 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


Pizzolato, J., Mukherjee, S., Schärer, O.D., and Jiricny, J. (2015). FANCD2-associated nuclelease 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.


