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Replication-Independent Repair of DNA Interstrand Crosslinks

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SUMMARY

DNA interstrand crosslinks (ICLs) are cytotoxic lesions that covalently link opposite strands of the DNA helix and block DNA unwinding. ICLs are repaired during and outside S phase, and replication-independent ICL repair (RIR) is critical to maintain genomic integrity and to allow transcription in nondividing or slowly dividing cells. Here, we show that the Y family DNA polymerase kappa (Pol κ) is essential for RIR of a site-specific ICL lesion in *Xenopus* egg extracts, and that both its catalytic activity and UBZ domains are required for this function. We also demonstrate a requirement for PCNA and its modification on lysine 164. Finally, we show that Pol κ participates in ICL repair in mammalian cells, particularly in G0. Our results identify key components of the RIR pathway and begin to unravel its mechanism.

INTRODUCTION

Interstrand crosslinks (ICLs) are cytotoxic DNA lesions that arise endogenously and are also induced by several classes of drugs widely used in cancer therapy. ICLs are sensed and repaired during and outside S phase (reviewed in Deans and West, 2011). During DNA replication, ICL repair is activated when one or possibly two replication forks (Räschle et al., 2008) encounter the lesion that blocks DNA unwinding. It is thought that the FancM/FAAP24 complex acts as a molecular sensor that recognizes the stalled fork (Ciccia et al., 2007). The mechanism for the recognition of ICLs in the absence of DNA replication is not clearly defined, although the structure of the lesion influences the outcome of repair (Mu et al., 2000; Smeaton et al., 2008). There is also evidence that proteins in the nucleotide excision repair (NER) pathway may play a role in lesion recognition (reviewed in Hlavin et al., 2010). Our work has shown that checkpoint signaling is activated from a single ICL in the absence of origin firing (Ben-Yehoyada et al., 2009).

Following activation of the Fanconi anemia (FA) pathway (Deans and West, 2009), repair is initiated through the action of nucleases that make dual incisions in one DNA strand, enabling unhooking of the ICL lesion (reviewed in Sengerova et al., 2011) and generating a double-strand break (DSB). The incision step is regulated by the FANCD2/FANCI heterodimer (Knipscheer et al., 2009). Replication-dependent ICL repair requires the homologous recombination machinery (Deans and West, 2011). Indeed, broken sister chromatids generated during ICL processing are repaired via a Rad51-dependent mechanism (Long et al., 2011). Similar to replication-dependent ICL repair, repair of ICLs in G0/G1 is

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SUPPLEMENTAL INFORMATION

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likely to involve incisions in one DNA strand, inducing unhooking of the ICL lesion (Deans and West, 2011).

Following unhooking, translesion synthesis (TLS) polymerases perform synthesis past the lesion. DNA polymerase zeta (Pol ζ) is required for replication-dependent ICL repair in *Xenopus* extracts (Räschle et al., 2008) and has been specifically associated with RIR in budding yeast (Sarkar et al., 2006). Multiple other DNA polymerases and the deoxycytosine monophosphate (dCMP) transferase Rev1 have also been implicated directly or indirectly in ICL repair (reviewed in Ho and Scharer, 2010).

DNA polymerase kappa (Pol κ) is a member of the Y family of TLS polymerases homologous to the *E.coli dinB* gene (Gerlach et al., 2001; Ogi et al., 1999) and has a lower error rate (a single-base substitution rate of 7×10^{-3}) than other Y family polymerases (Ohashi et al., 2000). Pol κ plays a role in NER synthesis in human cells (Ogi and Lehmann, 2006; Ogi et al., 2010) and is able to insert nucleotides opposite various types of crosslinked lesions in vitro (Ho et al., 2011; Minko et al., 2008). Loss of Pol κ also sensitizes cells to N^2-benzo[a]pyrene dG adducts and DNA alkylating agents (Ogi et al., 2002; Takenaka et al., 2006). TLS polymerases can be recruited by PCNA and RFC complexes to perform NER in arrested mammalian cells (Ogi et al., 2010), or via the 9-1-1 complex for TLS in fission yeast (Kai and Wang, 2003). It is not known whether TLS polymerases are recruited to ICL lesions through similar mechanisms.

RESULTS

The Mechanism of RIR Is Distinct from Replication-Dependent ICL Repair

Previously, we developed an assay to monitor repair of a single site-specific ICL in *Xenopus* egg extracts (Ben-Yehoyada et al., 2009). The plasmid represented in Figure 1A harbors a helix-distorting trimethylene crosslink (Dooley et al., 2003) and is subjected to quantitative PCR (qPCR) using “X” or “C” primers that span the ICL or a control region in the undamaged backbone, respectively. The X:C ratio is a measure of ICL repair that can be taken prior to or following incubation of the plasmid in extract. We find that membrane-free cytosol (HSS extract), which is not capable of supporting plasmid DNA replication (see Figure S1A available online), promotes ICL repair (Figure 1B), albeit with slower kinetics than replication-dependent repair (Räschle et al., 2008). This is consistent with our previous data which established that replication-competent extracts (HSS + NPE [Walter and Newport, 1997]) support ICL repair following addition of inhibitors of DNA replication initiation (Ben-Yehoyada et al., 2009). The ICL is located within an AluI restriction site that can be cleaved only if both DNA strands are accurately repaired. AluI digest of the repaired products prior to qPCR eliminated the PCR signal (Figure S1B), indicating that replication-independent ICL repair (RIR) in HSS occurs on both DNA strands. In addition, the ICL lesion activated the ATR-dependent signaling branch of the DNA damage response, triggering a robust checkpoint in HSS extract (Figure S1C), consistent with our previous studies (Ben-Yehoyada et al., 2009). Activation of the ATR checkpoint suggests the generation of ssDNA-RPA repair intermediates during RIR.

The FANCD2-FANCI heterodimer is important for the initiation of replication-dependent ICL repair (Knipscheer et al., 2009). In contrast, depleting HSS extract of FANCI (Figure S1D), which is required for FANCD2 ubiquitination and foci formation (Smogorzewska et al., 2007), did not impair RIR. Likewise, curcumin, a small molecule inhibitor of the FA pathway (Chirnomas et al., 2006), had no effect on ICL repair in HSS (Figure 1C). Replication-dependent ICL repair in replicating *Xenopus* extracts also requires Rad51 (Long et al., 2011). However, depletion of Rad51 from HSS (Figure S1E) did not inhibit RIR (Figure 1D), as expected for a repair process that does not require replicated sister

chromatids. Depletion of the TLS polymerase Pol ζ from replicating *Xenopus* extracts abrogates replication-dependent ICL repair (Räschle et al., 2008). Additionally, ICL repair studies in G1 yeast cells (Sarkar et al., 2006) also demonstrated a role for Pol ζ in yeast RIR. To investigate whether Polζ was required for lesion bypass in vertebrate RIR, we depleted the Rev7 regulatory subunit of Polζ (Figure S1F). Efficient depletion of Rev7 from HSS extract did not affect ICL repair (Figure 1E). This suggests that the mechanism of TLS in vertebrate RIR past ICL lesions involves a different TLS polymerase(s). Taken together, these data demonstrate that the mechanism of RIR is independent of the FA pathway and Polζ, and is distinct from replication-dependent ICL repair.

The Y Family Polymerase Pol κ Is Essential for RIR

The Y family polymerase Pol κ is able to insert a nucleotide opposite the crosslinked guanine of various types of ICL lesion in vitro (Ho et al., 2011; Minko et al., 2008) and therefore represents a potential candidate for TLS in RIR. We generated antibodies against *Xenopus* Pol κ that efficiently deplete Pol κ from extracts (Figure S2A). Depletion of Pol κ dramatically reduced ICL repair, indicating a critical role for Pol κ in repair of ICLs outside S phase (Figure 2A). To further confirm the requirement for Pol κ in RIR, we inhibited origin firing in the replicating extract system HSS+NPE using roscovitine, a CDK2 inhibitor. Treatment of HSS+NPE with roscovitine resulted in a 40% decrease in repair, consistent with our previous results (Ben-Yehoyada et al., 2009). Depletion of Pol κ from HSS and NPE inhibited by roscovitine resulted in the abrogation of most of the remaining ICL repair (Figure S2B). Importantly, the ICL repair defect could be rescued by the addition of partially purified recombinant *Xenopus* Pol κ from reticulocyte lysates (Figure 2A and Figure S2C). In contrast, a catalytically inactive Pol κ mutant harboring D199A and E200A substitutions (Pol $\kappa^{D199A,E200A}$) (Gerlach et al., 2001) was ineffective, indicating that Pol κ catalytic activity is necessary for RIR (Figures 2A and 2B and Figure S2C). Both Pol ζ and Pol κ interact with Rev1, a deoxycytidyl transferase (D'Souza and Walker, 2006; Guo et al., 2003); thus Rev1 could act as a common platform for TLS loading during RIR and replication-dependent ICL repair. Interactions between Rev1 and Pol κ are mediated by adjacent phenylalanines (F562/F563 in *Xenopus* Pol κ). We purified Pol κ protein harboring F562A and F563A substitutions (Pol $\kappa^{F562A,F563A}$) (Figure 2B and Figure S2D) that block interactions with Rev1 (Ohashi et al., 2009). Addition of Pol $\kappa^{F562A,F563A}$ restored RIR in Pol κ-depleted extracts (Figure 2C), despite the fact that the interaction between Pol $\kappa^{F562A,F563A}$ and Rev1 is impaired, as shown by in vitro pull-down assays with the C terminus of *Xenopus* Rev1 (Figure S2E), indicating that the Pol κ-Rev1 interaction is not required for RIR. Taken together, our data suggest that DNA synthesis in RIR and replication-dependent ICL repair utilize different TLS polymerases and that these pathways are thus mechanistically distinct.

We reasoned that if Pol κ is involved in RIR, it should bind specifically to crosslinked DNA. To monitor the interaction of Pol κ with the ICL plasmid, we performed chromatin immunoprecipitation (ChIP) using a single primer pair adjacent to the ICL site. ICL or control plasmids (prepared using the same method as ICL plasmids, Figure S2F) were incubated in Pol κ-depleted extract supplemented with Pol κ or Pol $\kappa^{D199A, E200A}$. As predicted, both mutant and wild-type Pol κ were enriched on the ICL plasmid. Enrichment was greater with Pol $\kappa^{D199A, E200A}$ than with wild-type enzyme (Figure 2D), suggesting that Pol κ associates transiently with the ICL plasmid and that the interaction is stabilized when the enzyme is unable to promote DNA synthesis.

Mechanism of Pol κ Recruitment

Next, we explored the mechanism of Pol κ recruitment to ICL lesions. The *S. pombe* 9-1-1 complex has been implicated in loading the Pol κ homolog, DinB, during DNA replication

following treatment with MMS (Kai and Wang, 2003). To determine whether 9-1-1 is involved in Pol κ loading at ICL lesions in *Xenopus* extracts, we depleted the complex using an antibody against the Rad1 subunit of 9-1-1 (Lupardus et al., 2002). Efficient depletion of Rad1 (Figure S3A) did not alter ICL repair levels (Figure 3A), suggesting that 9-1-1 is not needed to load Pol κ onto DNA carrying an ICL.

PCNA is also known to be responsible for recruiting TLS polymerases to damaged DNA (reviewed in Lehmann, 2006), and we therefore speculated that it might be required for RIR. We compared ICL repair in mock- or PCNA-depleted HSS (Figure S3B) (Mattock et al., 2001). Depletion of PCNA abolished ICL repair (Figure 3B). However, repair was fully restored by adding recombinant wild-type *Xenopus* PCNA protein to the depleted extract. Ubiquitylation and sumoylation of PCNA on the highly conserved lysine 164 enhances DNA damage tolerance by TLS (Ulrich, 2005). Addition of PCNA harboring a lysine-to-arginine mutation at amino acid 164 ($PCNA^{K164R}$) did not support ICL repair (Figure 3B), supporting the idea that PCNA modification is required for RIR. Next, we reasoned that if PCNA is required for RIR, it should be specifically recruited to ICL-containing DNA. To monitor PCNA binding to the ICL plasmid, we used biotinylated Lac repressor protein (Bio-LacR) to immobilize plasmid DNA on streptavidin magnetic beads. We pulled down ICL or control plasmids incubated in HSS extract for 40 min and identified plasmid-bound proteins by western blotting. Consistent with its role in RIR, PCNA preferentially bound to the ICL plasmid (Figure 3C).

Monoubiquitylation of PCNA at lysine 164 increases its affinity for various TLS polymerases, including Pol κ (Bi et al., 2006). Pol κ contains two ubiquitin-binding UBZ domains that mediate enhanced interaction with ubiquitylated PCNA and are required for the formation of Pol κ nuclear foci following UV irradiation (Guo et al., 2008). Binding to PCNA can also be mediated by a PCNA interaction protein box (PIP box) motif, QXX(I, L, or M) XXF(F or Y) (Kelman and Hurwitz, 1998), found in many proteins that participate in DNA replication and repair. Mutation of the two C-terminal hydrophobic residues of the PIP box in the Y family polymerases Pol eta (Pol η) and Pol iota (Pol ι) abrogates PCNA binding (Haracska et al., 2001; Vidal et al., 2004). Pol κ contains a noncanonical PIP box motif (Ogi et al., 2005), which displays a weaker binding affinity for PCNA compared to the PIP boxes of the other Y family polymerases (Hishiki et al., 2009). To assess the relative contribution of the Pol κ UBZ domains and PIP box in RIR, we generated two Pol κ mutants. The first contains amino acid substitutions D634A and D789A (Pol $\kappa^{D634A,D789A}$) within the UBZ domains, which abrogate enhanced interaction with ubiquitylated PCNA (Guo et al., 2008). The second harbors amino acid substitutions I857A, F860A, and F861A (Pol $\kappa^{I857A,\ F860A,F861A}$), which are the conserved hydrophobic residues in the PIP box of *Xenopus* Pol κ (Figure 2B and Figure S3C). Addition of Pol $\kappa^{D634A,D789A}$ did not support RIR in Pol κ-depleted extracts, whereas Pol $\kappa^{I857A,\ F860A,\ F861A}$ fully restored RIR to wild-type levels (Figure 3D). Our data thus support a model in which PCNA, ubiquitylated on lysine 164, recruits Pol κ to ICL lesions.

Pol $\kappa^{-/-}$ MEFs' Sensitivity to Crosslinking Agents Correlates with a Defect in ICL Repair

To determine directly whether Pol κ could process our ICL in vivo, we developed a GFP-based assay to monitor repair of single ICL molecules in mammalian cells, similar to cell-reactivation assays for ICL repair (Smeaton et al., 2009). The plasmid pEGFP-N3 was modified by deleting the SV40 origin to prevent plasmid replication in cells expressing SV40 T-antigen. Oligonucleotides harboring an ICL or control oligonucleotides of the same sequence were synthesized and ligated into the modified form of pEGFP-N3 in frame with the GFP gene. Cells successfully transfected with control plasmid express GFP, whereas ICL repair is required for GFP expression in cells transfected with the ICL plasmid (Figure 4A). The GFP-ICL repair assay was carried out in $PolK^{-/-}$ MEFs which have no obvious

documented cellular phenotype (Schenten et al., 2002; Shimizu et al., 2005). *Pol* $\kappa^{-/-}$ and *Pol* $\kappa^{+/+}$ mouse embryonic fibroblasts (MEFs) were transfected with control-or ICL-containing plasmids, and the number of GFP-positive cells was measured by fluorescence-activated cell sorting 24 hr posttransfection. ICL repair efficiency, as determined by the ratio of GFP cells in the two populations, was reduced by ~40% in *Pol* $\kappa^{-/-}$ cells compared to *Pol* $\kappa^{+/+}$ cells (Figure 4B). The remaining repair is likely accomplished by a distinct pathway.

We previously demonstrated that repair of the ICL plasmid used in this study is a faithful readout for the sensitivity of a cell to DNA crosslinking agents, since cells derived from FA patients, which are sensitive to crosslinking drugs, harbor a defect in its repair (Ben-Yehoyada et al., 2009). We reasoned that if Pol κ is indeed required for ICL repair, Pol κ-deficient cells should be more sensitive to crosslinking agents than normal cells. We carried out clonogenic survival assays using *Pol* $\kappa^{+/+}$ and *Pol* $\kappa^{-/-}$ MEFs. Cells were treated acutely for 3 hr with the crosslinking drug mitomycin C (MMC), either during exponential growth or during release into G1 following G0 arrest by serum starvation. *Pol* $\kappa^{-/-}$ cells were more sensitive to MMC in either phase than their wild-type counterparts; however, *Pol* $\kappa^{-/-}$ MEFs treated during G1 were significantly more sensitive than *Pol* $\kappa^{-/-}$ cells treated during exponential growth (Figure 4C). In contrast, *Pol* $\kappa^{+/+}$ cells treated in G1 were only marginally more sensitive than cells treated while growing exponentially. This suggests that Pol κ functions in ICL repair in mammalian cells, especially during the G0/G1 phases of the cell cycle. Next, we tested the sensitivity of *Pol* $\kappa^{-/-}$ MEFs to another crosslinking agent, cisplatin. As was the case with MMC, *Pol* $\kappa^{-/-}$ MEFs were more sensitive to cisplatin than *Pol* $\kappa^{+/+}$ cells (Figure 4D), demonstrating that Pol κ participates in the repair of multiple types of crosslinked lesions. The sensitivity of *Pol* $\kappa^{-/-}$ cells to MMC and cisplatin correlates with a reduced ability to repair a single site-specific ICL in the GFP-based assay. We conclude that the sensitivity of *Pol* $\kappa^{-/-}$ MEFs to crosslinking drugs is due primarily to a defect in ICL repair pathways, rather than pathways that remove other lesions, e.g., intrastrand crosslinks, generated by these drugs.

DISCUSSION

In this report we define components of a pathway to repair ICLs outside of S phase. Our data show that the Y family polymerase Pol κ is recruited to DNA containing a site-specific ICL and that its catalytic activity is essential for RIR, indicating that Pol κ carries out a nonredundant DNA synthesis step in this pathway. Interestingly, interaction between Pol κ and Rev1 is not required for Pol κ function in RIR, whereas this interaction is necessary for Pol κ-dependent repair of DNA damaged with benzo(a)pyrene or UV (Ohashi et al., 2009). This sets RIR apart from NER with respect to Pol κ function. We show that Pol κ UBZ domains are essential for RIR but that the PIP box, a potential PCNA-interacting motif carried by Pol κ, can be mutated without affecting RIR. We also establish that RIR requires PCNA modification on lysine 164, and that PCNA is preferentially loaded onto DNA harboring an ICL. Since the UBZ domains of Pol κ are necessary for enhanced interaction with ubiquitylated PCNA and for localization of Pol κ to UV-induced foci (Guo et al., 2008), we propose that monoubiquitylated PCNA facilitates Pol κ recruitment to ICL-containing DNA via the Pol κ UBZ domains. The absence of a role for the PIP box is consistent with surface plasmon resonance studies that have shown that a Pol κ PIP box peptide shows a very weak affinity for PCNA compared to the PIP boxes of other Y family polymerases such as Pol ι and Pol η (Hishiki et al., 2009). Interestingly, the Polη PIP box is required for binding to PCNA, whereas its UBZ domain is dispensable for its function in TLS (Acharya et al., 2010). This indicates that recruitment of each member of the Y family of TLS polymerases to sites of DNA damage may be differentially regulated.

It is likely that more than one DNA polymerase operates in the RIR pathway. The requirement for PCNA modification suggests a polymerase switch during repair (Moldovan et al., 2007). For example, Pol κ might add a nucleotide across the unhooked ICL, whereas an additional polymerase(s) would synthesize DNA up to and beyond the ICL. We sequenced a 245 bp region spanning the ICL-containing region of the plasmid from 56 independent clones derived from repair in HSS using a high-fidelity polymerase (error rate of 2.3×10^{-6}). A total of four substitutions were observed, three of which were within 15 bp on either side of the ICL lesion. Assuming the size of the newly synthesized region is similar to that of NER (approximately 30 bp), an error rate of 1.7×10^{-3} can be calculated, which is similar to the error rate for Pol κ of 7×10^{-3} (Ohashi et al., 2000). Notably, we do not observe any mutations opposite the adducted G. However, since the size of the region synthesized by Pol κ, and the role of other TLS polymerases, is not precisely known, it is not possible to determine the exact error rate of DNA synthesis in RIR that can be specifically attributed to Pol κ.

Our data demonstrate that distinct translesion polymerases are required for RIR and replication-coupled ICL repair. Notably, Pol ζ is required in replication-coupled ICL repair (Räschle et al., 2008) but is dispensable for RIR in vertebrates. The fact that Pol ζ is required for RIR in yeast but not vertebrates might be explained by the fact that Pol κ is absent in *S. cerevisiae* (Waters et al., 2009). In addition, RIR, unlike replication-coupled ICL repair, does not require the FA pathway or Rad51. Together, these data establish that RIR and replication-dependent ICL repair operate by distinct mechanisms.

RIR is the sole repair mechanism by which nondividing or slowly dividing cells can maintain genomic integrity and perform transcription after generation of ICLs. The physiological relevance of this pathway is emphasized by the fact that mammalian *Pol* $\kappa^{-/-}$ cells are exquisitely sensitive to crosslinking drugs in G0/G1. There is a direct link between the ability of cells to repair a single site-specific ICL and their sensitivity to multiple types of crosslinking drug, implying that Pol κ participates in repair of different crosslinked lesions. Studies of synthetic ICL lesions in *Xenopus* cell-free extracts are entirely consistent with results from the cell studies, indicating that the extracts represent a faithful model for understanding RIR.

The distinction between RIR and replication-dependent ICL repair is a means by which drugs can be rationally designed to target quiescent or slowly cycling cells. Such drugs could complement conventional chemotherapies, which target rapidly proliferating cells. We note that tumors show significant heterogeneity in proliferation rate, and that slowly proliferating cells are largely resistant to standard chemotherapy (reviewed in Moore and Lyle, 2011). In particular, a fraction of these quiescent cells, speculated to be cancer stem cells, survive therapies that kill bulk tumor cells. The development of strategies that sensitize these cells to crosslinking therapies can be informed by an understanding of the mechanism of ICL repair in the absence of DNA replication.

EXPERIMENTAL PROCEDURES

Plasmid Construction

ICL and control plasmids used in Figure 1, Figure 2, and Figure 3 were prepared as described in Ben-Yehoyada et al. (2009) except that linear DNA backbone was purified by electroelution from agarose gels and an additional cesium chloride density-gradient centrifugation purification step was performed. For GFP-ICL plasmids, the SV40 origin of pEGFP-N3 was deleted to make pEGFP-N3-ΔSV40 as described in the Supplemental Information. Crosslinked or control oligonucleotides (a generous gift from C. Rizzo) were

ligated into pEGFP-N3-ΔSV40, and closed circular monomeric molecules were purified by electroelution.

Xenopus Egg Extract Preparation and Immunodepletions

Xenopus leavis were handled in accordance with guidelines provided by the institutional Animal Care and Use Committee at Columbia University, protocol AA5192. Cell-free extracts were prepared from unfertilized *Xenopus* eggs as described (Lebofsky et al., 2009; Shechter et al., 2004). Immunodepletions were performed using anti-Rad51, -FANCI, -Pol κ, -Rev7, and -Rad1 antibodies coupled to protein A Sepharose CL-4B beads (Amersham Biosciences; Pittsburgh, PA). Mock depletions were performed with preimmune serum. For PCNA depletions, a p21 peptide with the sequence CKRRQTS MTDFYHSKRRLIFS (Mattock et al., 2001) was immobilized on SulfoLink columns (Pierce Biotechnology; Rockford, IL). Uncoupled SulfoLink resin was used for mock depletions. Two to four rounds of depletion were performed by incubating extracts with antibody-bound beads or peptide-bound resin at 4°C for 25 min. Rev7 depletions were carried out by one round at 4°C and two rounds at room temperature.

Replication Assay, Checkpoint Activation Assay, and ICL Repair Assays

Replication assays were performed as described in Ben-Yehoyada et al. (2009). For checkpoint activation assays, ICL or control plasmids were incubated in HSS at 20 ng/μl in the presence or absence of ATR inhibitor ETP-46464 (gift from O. Fernandez-Capetillo) (Toledo et al., 2011) for 1 hr at 21°C, and extract was analyzed by western blotting. For ICL repair assays, ICL plasmid was incubated in extract at 5–10 ng/μl for 2–4 hr at 21°C, and DNA samples were processed as described in Ben-Yehoyada et al. (2009). In experiments in which replicating extracts were used (Figure 1B and Figure S2C), the recovered ICL-DNA was digested with the restriction enzymes PvuI and PvuII prior to qPCR analysis. Where shown, roscovitine (Sigma #R7772 in DMSO) or curcumin (Sigma X458-37-7 in EtOH) was added to repair reactions at the indicated concentrations. qPCR was carried out using an Applied Biosystems 7500 fast thermocycler and Absolute Blue QPCR SYBR Green low ROX PCR mix (Abgene; Surry, UK; Cat #AB-4322B) using the primers and cycling conditions described in the Supplemental Information.

Expression and Purification of Recombinant PCNA

pET28aPCNAwt and pET28aPCNAK164R vectors were a gift from Vincenzo Costanzo (CRUK, London, UK). 6× His-tagged wild-type or mutant PCNA was induced with 1 mM IPTG and expressed in BL21 Star(DE3)pLysS cells at 25°C for 5 hr. Cells were lysed by sonication in 20 mM sodium phosphate, 500 mM NaCl, and purified with Ni-NTA agarose (QIAGEN) using standard methods. PCNA-depleted extracts were supplemented with amounts of recombinant protein similar to that present in extract based on western blot analysis.

Expression of Pol κ in Rabbit Reticulocyte Lysates

Xenopus leavis Pol κ was expressed by incubating pCMV-Sport.ccdb-Pol κ (Open Biosystems, accession number BC076794) in TnT SP6 Quick Coupled Transcription/ Translation System (Promega) and partially purified by ammonium sulfate precipitation (for further information, see the Supplemental Information). Mutants of Pol κ were generated directly in pCMVsport.ccdb-Pol κ using QuikChange XL Site-Directed Mutagenesis Kit (Agilent Technologies; La Jolla, CA; catalog number 200517) and the primers described in the Supplemental Information. The numbering of mutant residues refers to the amino acid number of the *Xenopus* protein. Partially purified Pol κ was used to supplement Pol κ-depleted extracts at approximately 5-fold higher concentrations than those present in extract.

In Vitro GST Pull-Down Assay

The C terminus of *Xenopus* Rev1 (amino acids 815–1230), the equivalent region to that shown in human Rev1 to contain the Pol κ interaction domain (Ohashi et al., 2004), was cloned and purified as described in the Supplemental Information. GST-Rev1 (815–1230) was bound to glutathione Sepharose (GE Healthcare) for 30 min at room temperature. Beads (20 μl) containing approximately 5 μg GST-Rev1 (815–1230) were incubated with 2.2 μl of partially purified Pol κ proteins (as described above) in 250 μl binding buffer (50 mM Tris-Cl [pH 8], 150 mM NaCl, 5 mM EDTA, 10% glycerol, 0.1% Tween, 0.75 mg/ml BSA, 5 μg/μl leupeptin) for 2 hr at 4°C. Beads were washed three times in binding buffer supplemented with 4 mM glutathione and three times in binding buffer. Proteins were eluted from beads by boiling for 5 min at 95°C in Laemmli buffer and analyzed by western blotting.

Lac Repressor Pull-Down Assay

Purified Bio-LacR (gift from K. Marians) was prebound to M-280 streptavidin dynabeads (Invitrogen catalog number 112.05D). ICL or control plasmids were incubated in HSS at 21°C for 40 min before the addition of Bio-LacR-coated beads and further incubation for 20 min at room temperature. Beads were washed with 10 mM HEPES (pH 7.7), 4 mM $MgCl_2$, 50 mM KCl, 1 mM DTT, 250 mM sucrose, 50 mM NaCl, and 0.015% Triton X-100. Proteins were eluted from dynabeads by boiling for 5 min at 95°C in Laemmli buffer and analyzed by western blotting.

Chromatin Immunoprecipitation

ICL or control plasmids were incubated in extract at 5–10 ng/μl, and ChIP was carried out as described in Long et al. (2011) using either Pol κ antibodies or preimmune serum. Immunoprecipitated DNA was quantified using qPCR as described above, using the primers 5′-CTCATTTTTTAACCAATAGGC-3′ and 5′- CCTGATAGACGGTTTTTCGCC-3′.

GFP-ICL Repair Assay and Clonogenic Survival Assays

Pol $\kappa^{+/+}$ and *Pol* $\kappa^{-/-}$ MEFs (a gift from Drs. H. Ohmori and M. Moriya) were grown in DMEM + 10% FBS + 1 mM sodium pyruvate at 37°C in 5% CO_2. For GFP-ICL repair assays, 12-well plates were seeded with 4×10^4 cells per well and transfected using Nanojuice transfection kit (EMD Chemicals; catalog number 71902-3) with 500 ng of carrier plasmid, and the amount of ICL and control plasmids indicated. GFP expression was analyzed using a FACSCalibur Analyzer and CellQuest software. For clonogenic survival assays, *Pol* $\kappa^{+/+}$ and *Pol* $\kappa^{-/-}$ cells were plated at a density of 750 cells per 100 mm plate. Growth media was supplemented with MMC or cisplatin at the concentrations stated for 3 hr, then replaced with fresh untreated growth media for 10 days. Colonies were fixed with methanol and stained with 1% crystal violet. Colonies were counted and cell survival was calculated as a percentage of colonies counted in the untreated sample.

Additional experimental procedures are described in the Supplemental Information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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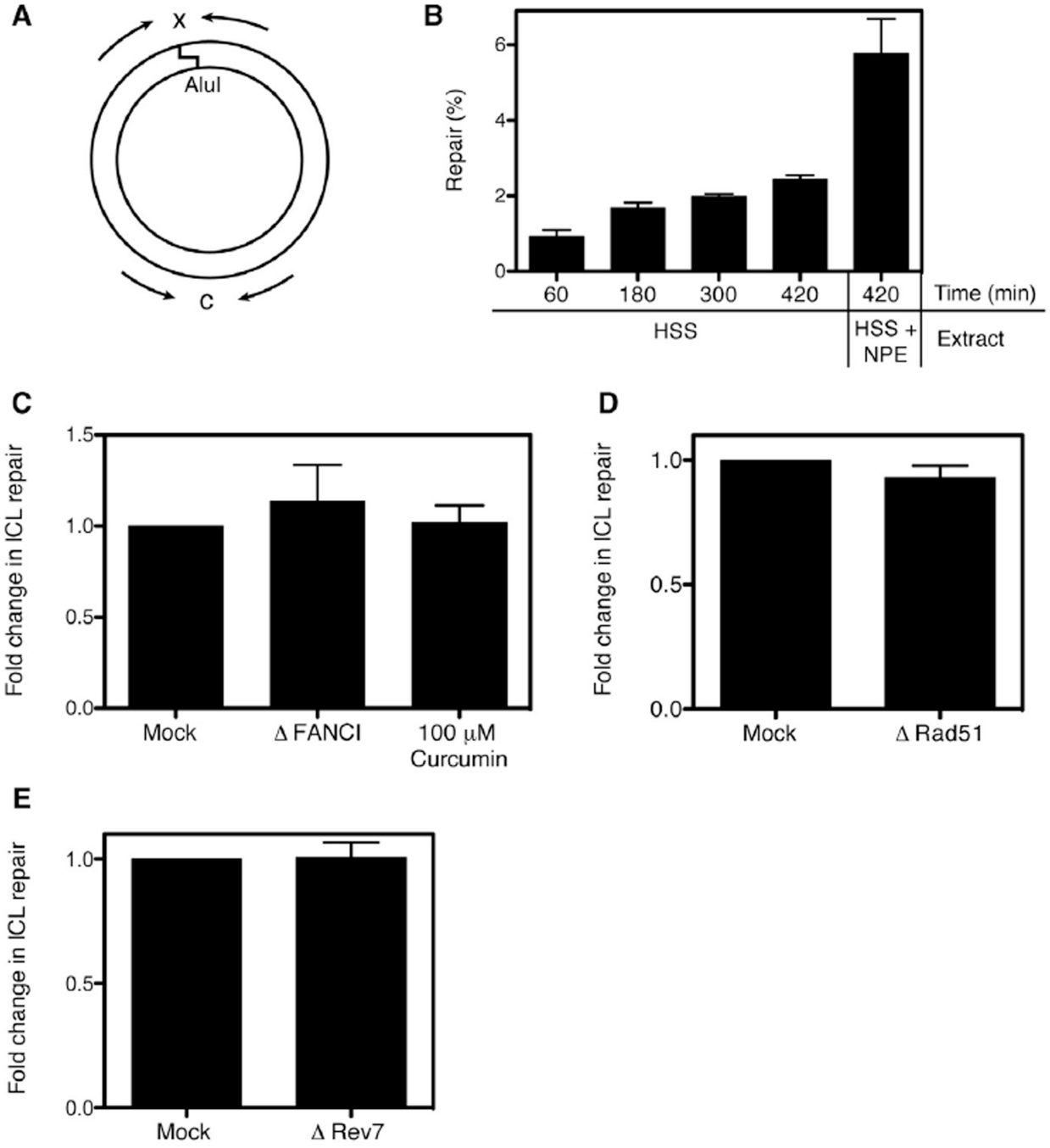


Figure 1. Replication-Independent ICL Repair Occurs via a Mechanism that Is Distinct from Replication-Dependent ICL Repair

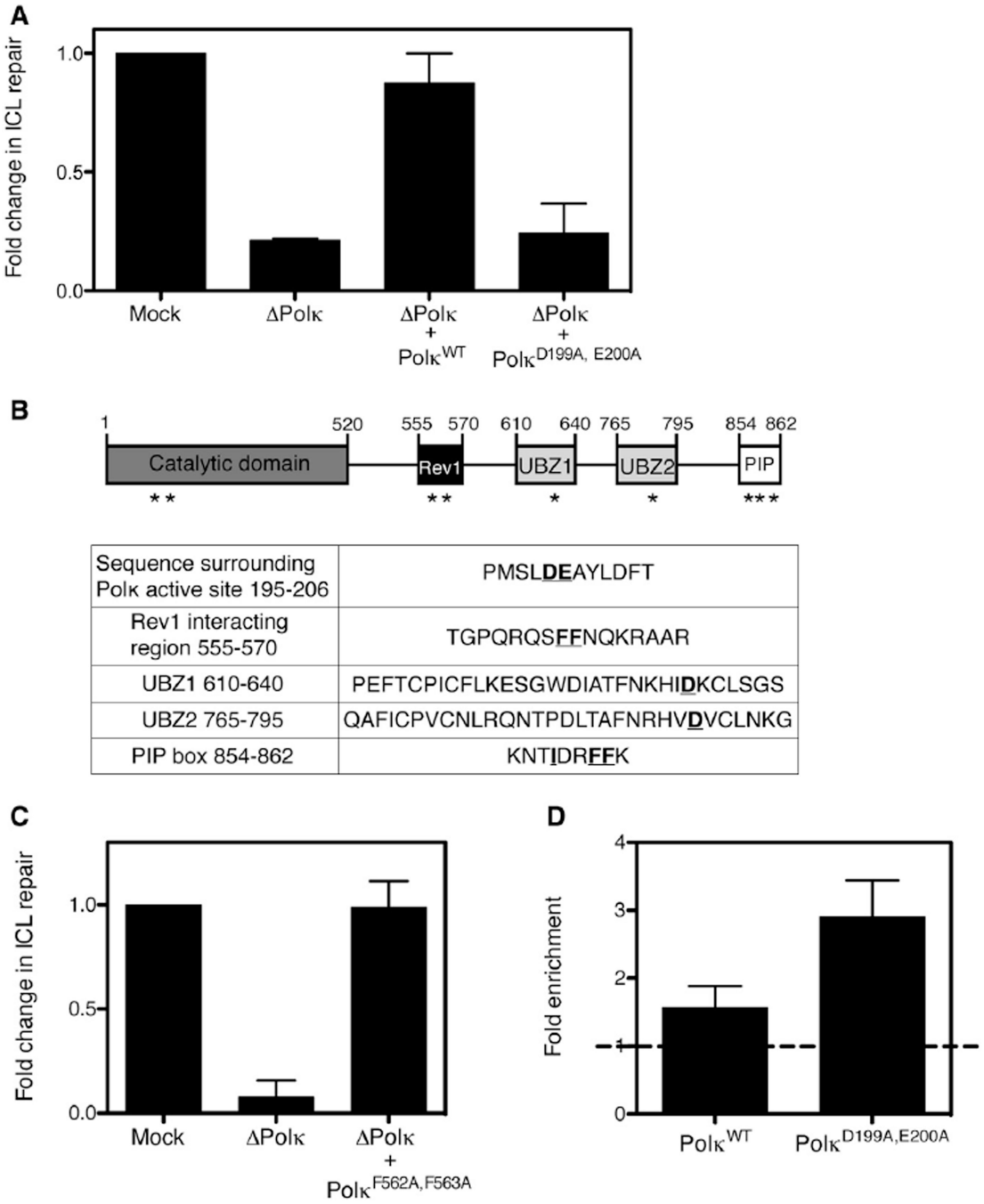
(A) Schematic representation of the ICL plasmid. Regions amplified by the X and C primers are illustrated.

(B) ICL plasmid was incubated in HSS (non-replicating) or HSS + NPE (replication-competent) extract for the times indicated. Repair (%) is measured as the fraction of the X:C ratio of the ICL plasmid over the X:C ratio of the control plasmid. See also Figure S1.

(C) Quantification of ICL repair in mock- and FANCI-depleted HSS or in HSS treated with 100 μM curcumin.

(D) Quantification of ICL repair in mock- and Rad51-depleted HSS.

(E) Quantification of ICL repair in mock- and Rev7-depleted HSS. Results represent the mean and SEM from at least three independent experiments.



Sequence surrounding Polκ active site 195-206	PMSL**DE**AYLDFT
Rev1 interacting region 555-570	TGPQRQS**FF**NQKRAAR
UBZ1 610-640	PEFTCPICFLKESGWDIATFNKHI**D**KCLSGS
UBZ2 765-795	QAFICPVCNLRQNTPDLTAFNRHV**D**VCLNKG
PIP box 854-862	KNT**I**DR**FF**K

Figure 2. Pol κ Is Required for RIR

(A) ICL repair in mock-, Pol κ-, and Pol κ-depleted HSS supplemented with recombinant Pol κ^{WT} or Pol $\kappa^{D199A, E200A}$. See also Figure S2.

(B) Schematic showing the domain structure of Pol κ and the location of mutations used in this study. Asterisks mark the location of the mutations shown in bold and underlined in the table below.

(C) Quantification of ICL repair in mock-depleted HSS, Pol κ-depleted HSS, and Pol κ-depleted HSS supplemented with recombinant Pol $\kappa^{F562A, F563A}$.

(D) ICL and control plasmids were incubated in Pol κ-depleted HSS, supplemented with either Pol κ^{WT} or Pol $\kappa^{D199A, E200A}$ and anaylzed by Pol κ ChIP. The data are expressed as

the fold enrichment of DNA pulled down from ICL plasmid compared to control plasmid. A value of 1 (dashed line) indicates no enrichment. Results represent the mean and SEM from at least three independent experiments.

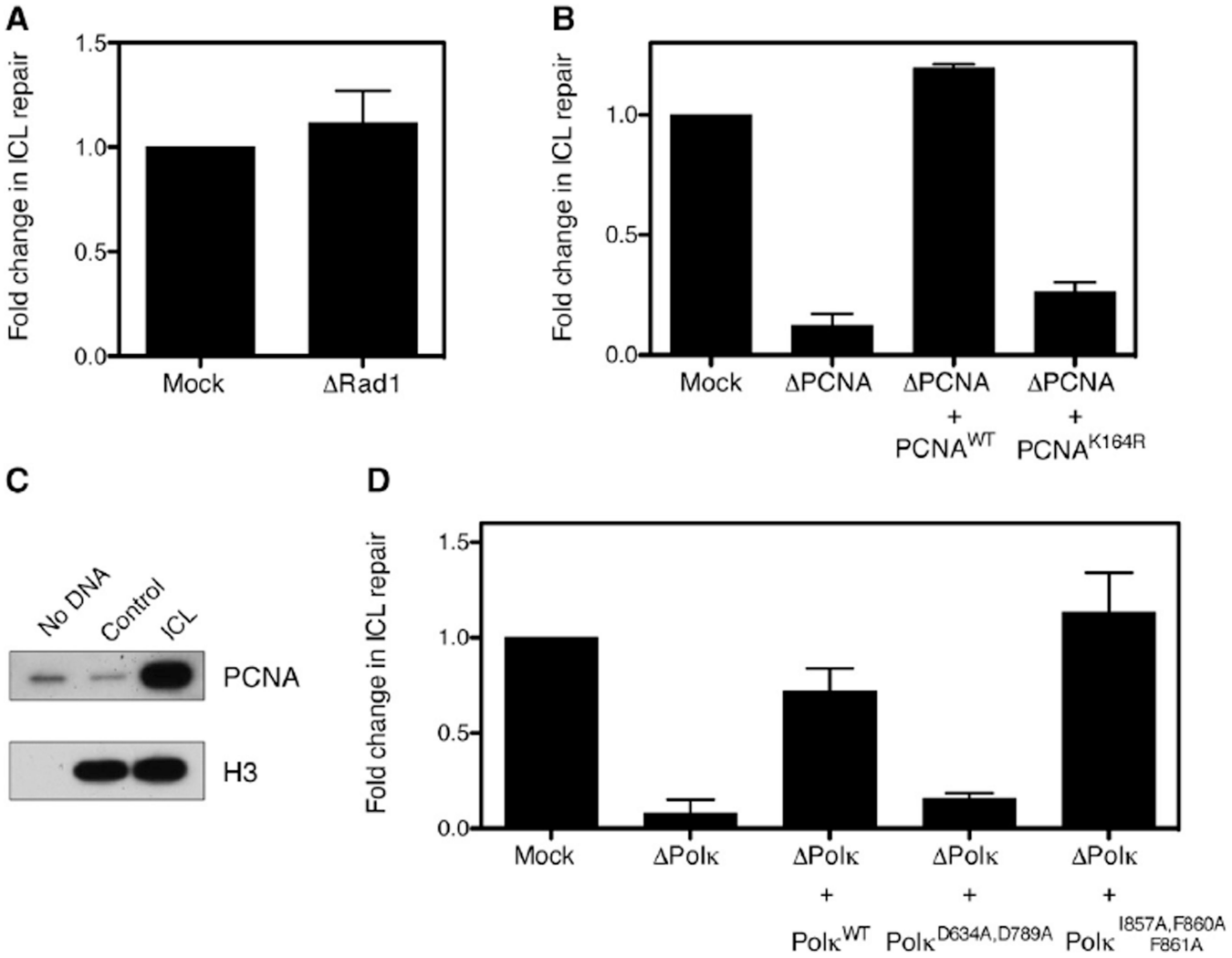


Figure 3. PCNA Modification on Lysine 164 and Pol κ UBZ Domains Are Required for RIR

(A) Quantification of ICL repair in mock- and Rad1- depleted HSS. See also Figure S3.

(B) Quantification of ICL repair in mock-depleted HSS, PCNA-depleted HSS, and PCNA-depleted HSS supplemented with $PCNA^{WT}$ or $PCNA^{K164R}$.

(C) Control or ICL plasmids were incubated in extract for 40 min and immobilized using biotinylated LacR protein. DNA-bound proteins were analyzed by western blotting.

(D) Quantification of ICL repair in mock-, Pol κ-, or Pol κ-depleted HSS supplemented with Pol κ^{WT}, Pol $\kappa^{D634A,D789A}$, or Pol $\kappa^{I857A,F860A,F861A}$, as indicated. Results represent the mean and SEM from at least three independent experiments.

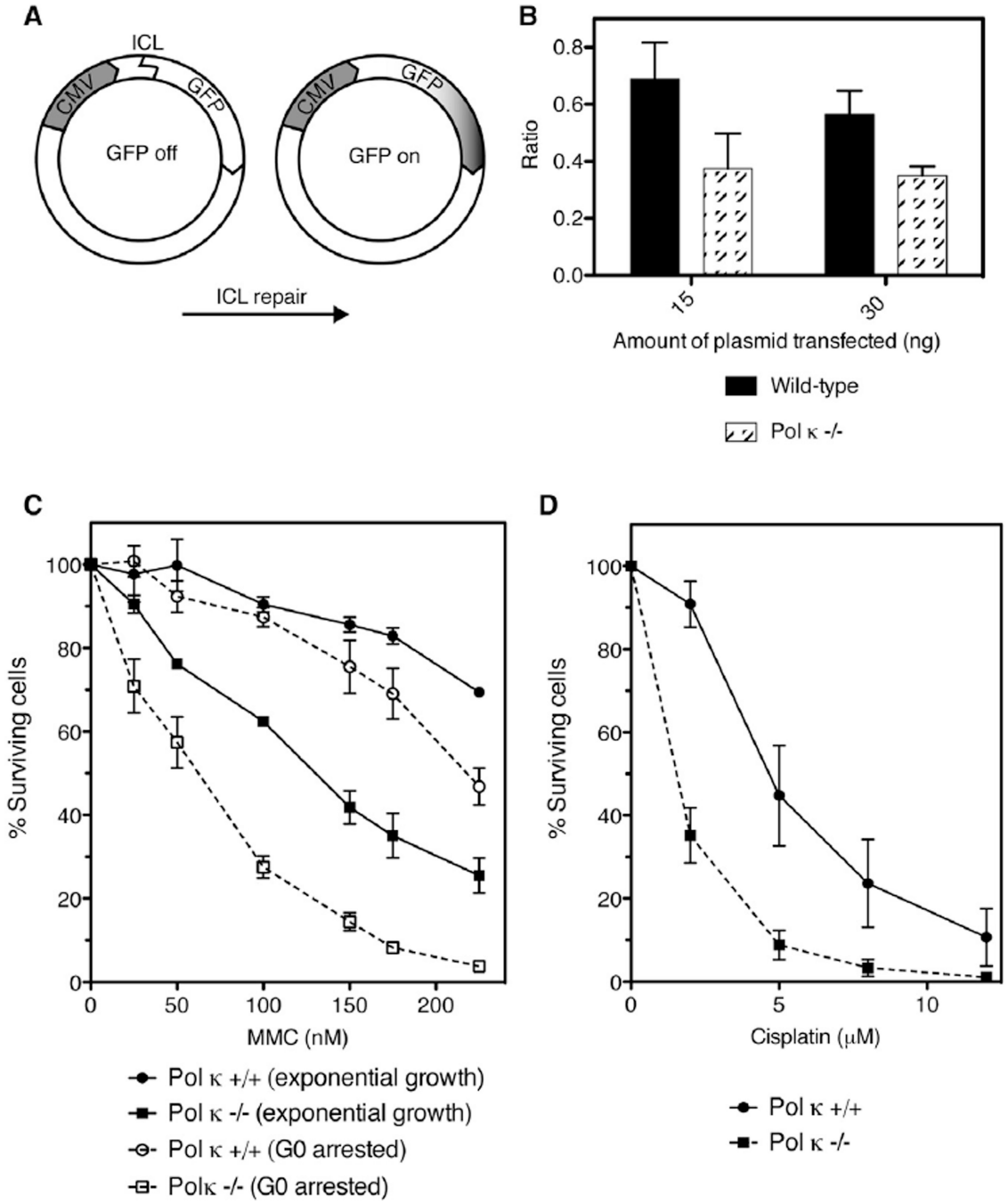


Figure 4. *Pol* k$^{-/-}$ MEF Sensitivity to Crosslinking Agents Correlates with a Defect in ICL Repair

(A) Schematic of GFP-based ICL assay.

(B) Quantification of the repair of a single site-specific ICL in either *Pol* $\kappa^{-/-}$ or *Pol* $\kappa^{+/+}$ MEFs using the GFP-based assay shown in (A). Repair is expressed as the fraction of GFP-positive cells transfected with the ICL plasmid over GFP-positive cells transfected with the control plasmid.

(C) Clonogenic survival assays with *Pol* $\kappa^{-/-}$ or *Pol* $\kappa^{+/+}$ MEFs in the presence of the indicated concentration of MMC. Cells were treated during exponential growth or during G0 arrest.

(D) Clonogenic survival assay with *Pol* $\kappa^{-/-}$ or *Pol* $\kappa^{+/+}$ MEFs in the presence of the indicated concentration of cisplatin during exponential growth. Results represent the mean and SEM from at least three independent experiments.