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A novel assay revealed that ribonucleotide reductase is functionally important for interstrand DNA crosslink repair

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Abstract

Cells have evolved complex biochemical pathways for DNA interstrand crosslink (ICL) removal. Despite the chemotherapeutic importance of ICL repair, there have been few attempts to identify which mechanistic DNA repair inhibitor actually inhibits ICL repair. To identify such compounds, a new and robust ICL repair assay was developed using a novel plasmid that contains synthetic ICLs between a CMV promoter region that drives transcription and a luciferase reporter gene, and an SV40 origin of replication and the large T antigen (*LgT*) gene that enables self-replication in mammalian cells. In a screen against compounds that are classified as inhibitors of DNA repair or synthesis, the reporter generation was exquisitely sensitive to ribonucleotide reductase (RNR) inhibitors such as gemcitabine and clofarabine, but not to inhibitors of PARP, ATR, ATM, Chk1, and others. The effect was observed also by siRNA downregulation of RNR. Moreover, the reporter generation was also particularly sensitive to 3-AP, a non-nucleoside RNR inhibitor, but not significantly sensitive to DNA replication stressors, suggesting that the involvement of RNR in ICL repair is independent of incorporation of a nucleotide RNR inhibitor into DNA to induce replication stress. The reporter generation from a modified version of the plasmid that lacks the *LgT*-SV40ori motif was also adversely affected by RNR inhibitors, further indicating a role for RNR in ICL repair that is independent of DNA replication. Intriguingly, unhooking of cisplatin-ICL from nuclear DNA was significantly inhibited by low doses of gemcitabine, suggesting an unidentified functional role for RNR in the process of ICL unhooking. The assay approach could identify other molecules essential for ICLR in quantitative and flexible manner.

Graphical Abstract

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Author Contributions

NF designed the research and conducted the project, generated the pGL(*LgT*-SV40ori)-ICL and pRL(*LgT*-SV40ori) plasmids, performed the ICLR assays, and wrote the paper. BJE performed the comet ICL unhooking assay and coimmunoprecipitation assay for SLX4. MLA produced aoNao. AI designed the research, maintained the cell cultures, generated the pGL-ICL plasmid and FLAG-XPF expression plasmid, and performed siRNA experiments, immunoblotting, the ICLR assays of RRM2-depleted cells, and coimmunoprecipitation assay for XPF. All authors have approved the final version of the manuscript.

Notes

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Keywords

ribonucleotide reductase; interstrand DNA crosslink; DNA repair; chemical inhibitor; assay development

1. INTRODUCTION

Interstrand DNA crosslinks (ICL) are the most powerful drivers of cell death induced by bifunctional chemotherapeutic drugs such as cisplatin. Mammalian cells have evolved sophisticated mechanisms for ICL repair (ICLR) in the form of networks of many DNA damage response/repair (DDR) processes, including nucleotide excision repair (NER), mismatch repair (MMR), translesion DNA synthesis (TLS), and homologous recombination (HR). (1,2) The selection of these DDRs is influenced by several factors, such as the cell-cycle phase at which the cell detects the ICL (1) and the chemical structure and steric size of the ICL moiety (3). These factors define ICLR pathways as either replication-dependent or -independent mechanisms. For instance, if a cell in S phase detects an ICL during DNA replication, then it activates a replication-dependent ICLR mechanism using HR as one of the DDR steps. This correction is made possible by the presence of a replicated sister chromatid that serves as a template to prevent mutagenesis during the repair. However, if a cell in G0/G1-phase detects an ICL, then it activates a replication-independent ICLR mechanism because the absence of a sister chromatid precludes HR. (1) Because cancer cells co-opt these networks to promote their survival when challenged by ICL-inducing agents, each of these pathways provides an attractive target for cancer therapeutic intervention and chemosensitization. Genetic strategies have shown the importance of several DDR molecules, including XPF, in ICLR (4,5). Unfortunately, chemical inhibitors of those DDR molecules are not available, preventing their validation as targets of sensitization for ICL-inducing chemotherapies.

There are several chemical compounds that are broadly classified as DNA repair inhibitors, some of which have been determined to inhibit specific DDR molecules. These agents have been used to study functions of DDR molecules and cancer treatments (6), and thus are an attractive alternative strategy for studying ICLR pathways that have been delineated exclusively by genetic approaches. However, such a strategy has not yet been applied to ICLR pathway studies because functional ICLR assays robust enough for a chemical genetic approach have not been developed. To enable such an approach, a new ICLR assay was developed by using newly constructed ICL-containing plasmids (4) to improve the scalability and robustness of host-cell reactivation assays. This approach has identified inhibitors of ribonucleotide reductase (RNR) as being particularly effective ICLR inhibitors.

2. MATERIALS AND METHODS

2.1. Materials

Unless otherwise stated, all chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) or obtained from our in-house chemical library collection. *N, N*-bis(aminooxyacetyl)-1,5-diaminonaphthalene (aoNao) was synthesized as described previously (7). All chemical compounds were prepared as 10 mM solutions in DMSO, except aoNao (100 mM in DMSO), cisplatin (5 mM in 0.9% aqueous sodium chloride), and gemcitabine (10 mM in water). All oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA). All enzymes for DNA molecular biology including restriction enzymes, nucleases, glycosylase, and DNA ligase, were purchased from New England BioLabs (Ipswich, MA). Other sources of materials are indicated where needed.

2.2. Cell culture

HT1080 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). GM04312 (8), GM15876, and GM08437 (XP2YO[SV] (9)) cells were obtained from Coriell Institute Biorepository (Camden, NJ). All cells were and maintained at subconfluent levels in Dulbecco's modified Eagle's medium (Mediatech, Inc., Manassas, VA) supplemented with 10% fetal bovine serum (Thermo Scientific, Waltham, MD) at 37°C in a humidified 5% CO₂ atmosphere. Unless otherwise stated, cells were seeded into 6-well cluster plates at 1.0×10^5 cells per well and allowed to attach overnight prior to treatment the following day. Each specific treatment schedule is defined in the relevant section.

2.3. Generation of reporter plasmids for ICLR assay

The SV40 LgT sequence was PCR-amplified from the pSP189 SV40 shuttle vector (10) by using 5'-ATATCCTAGGCTTTTGCAAAAAGCTCGATTCTGGTAAATATAAAATTTTAAAGTG TATAATGTGTT AAACCTAC-3' as a forward primer, 5'-ATATGTCGACCAGACATGATAAGATACATTGATGAGTTTGGACA-3' as a reverse primer, and *Pfu* Turbo Cx Hotstart DNA polymerase (Stratagene, La Jolla, CA) according to the manufacturer's recommendation. The PCR product and the pGL4.50 plasmid (Promega, Fitchburg, WI) were each double-digested by using *Avr*II and *Sal*I-HF, ligated by using Quick Ligation kit (New England BioLabs) according to the manufacturer's recommendations, and cloned by using a standard procedure to transform *E. coli* DH5α strain. This generated a modified pGL4.50 plasmid (pGL(LgT-SV40ori)) in which the hygromycin-resistance gene under the control of an SV40 origin/promoter was replaced by the LgT gene. The SV40 origin/promoter-LgT sequence of pGL(LgT-SV40ori) was excised and inserted into pGL4.75 (Promega) by using *Bam*HI and *Sal*I-HF for cassette-out/in and the Quick Ligation kit. This generated a modified pGL4.75 plasmid (pRL(LgT-SV40ori)) having a sequence that is almost identical to that of pGL(LgT-SV40ori), except that it encodes a Renilla *Rluc* reporter is encoded instead of the firefly *luc2* reporter.

To insert two ICLs into the pGL(LgT-SV40ori) in a site-specific manner, two duplex oligonucleotides were produced, each containing an ICL and a unique *Eco*RI site. The sequences of these oligonucleotides were 5'-phospho-

GGTTTAGTGAACCGTCAGATCAdUCTGAGAATTCTCCGATTA-3' and 5'-CGGAGAATTCTCAGdUTGATCTGACGGTTCATAAACCAGCT-3' (dU= deoxyuridine). A mixture of these oligonucleotides (4 nmol of each) were treated with uracil-DNA glycosylase (UDG, 200 U) in UDG buffer (1000 µL) at 37°C for 8 h. After the products were re-annealed at 4°C for 0.5 h, aoNao (20 µL, 20 µmol) was added. The mixture was incubated at room temperature with rotation for 16 h and then treated with phenol-chloroform, desalted, and concentrated by centrifugation in a Microcon 3K microconcentrator (Millipore, Billerica, MA). The sample was denatured by heating with urea loading buffer and purified by TBE-urea PAGE (15%, Invitrogen, Waltham, MA) to isolate the aoNao-crosslinked duplex, which is henceforth referred to as ICL-duplex 1. Another ICL-duplex was produced from the oligonucleotides 5'-phospho-GGCCCTTCTTAATGTTTTTGGCATCTTCCATGGTGGCTTTdUCCGGATTGCCAAGCTTGACCGAATT CGCCT-3' and 5'-ATTAGGCGAATTCGGTCAAGCTTGGCAATCCGGdUAAAGCCACCATGGAAGATGCCAAAAACATT AAGAAG-3' using a similar procedure, except that alkaline agarose gel electrophoresis (2.5%, 30 mM NaOH, 1 mM EDTA, 5V/cm) was used instead of TBE-urea PAGE. This second duplex is henceforth referred to as ICL-duplex 2.

The plasmid pGL(LgT-SV40ori) was double-digested by using *SacI* and *PspOMI* and purified by agarose gel electrophoresis. The cut pGL(LgT-SV40ori) (40 µg) was ligated to ICL-duplex 1 (10.7 µg, 50 mol eq) and ICL-duplex 2 (18.7 µg, 50 mol eq) by T4 DNA ligase (40000 U) in T4 DNA ligase buffer (4000 µL) at 16°C for 2 days. The mixture was processed for phenol-chloroform treatment, desalted, and concentrated by centrifugation in a Microcon 3K microconcentrator. This procedure generated a linear product in which the 3'-end (*SacI* site) is ligated to ICL-duplex 1, and the 5'-end (*PspOMI* site) is ligated to ICL-duplex 2. To confirm completion of these two ligations, a small portion of the product was double-digested with *SnaBI* and *SgrAI*, generating fragments containing ICL-duplex 1 and ICL-duplex 2, respectively, which are longer than each original ICL-duplex, as confirmed by TBE-urea PAGE (6%, Invitrogen). Next, to generate the pGL(LgT-SV40ori)-ICL plasmid, *EcoRI* sites in the two ICL-duplex capping portions were digested and self-ligated. The ligated linear product containing ICL-duplexes (21.3 µg) was treated with *EcoRI* (1600 u) and purified by agarose gel electrophoresis to isolate the digested product (13.7 µg). This product (2.0 µg) was treated with T4 DNA ligase (8000U) in T4 DNA ligase buffer (800 µL) at 16°C for 2 days. The mixture was processed for phenol-chloroform treatment, desalted, and concentrated in a Microcon 3K microconcentrator. Unfortunately, the self-ligation at the *EcoRI* site was incomplete, leaving ~30% uncyclized linear product. To remove the uncyclized product, the entire product was again digested with *SalI*-HF (100 U) and purified by agarose gel electrophoresis to remove two smaller fragments generated from the uncyclized contaminant and isolate the linear pGL(LgT-SV40ori)-ICL (0.39 µg). This isolated plasmid was then re-cyclized by T4 DNA ligase (160 U) in T4 DNA ligase buffer (135 µL) at 16°C for 1 day to generate pGL(LgT-SV40ori)-ICL. The mixture was processed for phenol-chloroform treatment, desalted, and concentrated in a Microcon 3K microconcentrator. A small portion of the product was digested with *BamHI* and analyzed by agarose gel electrophoresis to confirm the presence of a single band of the

expected size (7.6 kb) and absence of fragments generated from uncyclized product. Overall strategy is outlined in Figure 1D.

To produce pGL-ICL (Figure 3A), pGL4.50 was double-digested with *Bam*HI and *Sal*I-HF, blunted by using mung bean nuclease, self-ligated by T4 DNA ligase, and cloned by a standard procedure. This process generated a modified pGL4.50 plasmid from which the hygromycin-resistance gene and SV40 origin/promoter-LgT sequence were deleted. Introduction of the two ICLs into this plasmid product was carried out in a manner similar to that described for production of pGL(LgT-SV40ori)-ICL.

2.4. Screening of chemical compounds for ICLR using the ICLR reporter plasmids

HT1080 cells (1.8×10^5 /well) were cultured in DMEM (500 μ L/well) in a 24-well cell culture plate overnight. The transfection mixture [pGL(LgT-SV40ori)-ICL: 9 ng, pRL(LgT-SV40ori): 15 ng, pET15b: 1.2 μ g (carrier DNA), FuGene HD transfection reagent (Promega): 4.5 μ L, and Opti-MEM (Thermo Fisher Scientific, Waltham, MA): 141 μ L] was prepared per the manufacturer's recommendation, and one-third of the volume was added to each of 3 wells of the cells. After 3.5 h incubation, the transfected cells were rinsed with PBS, lifted with trypsin, and resuspended in fresh DMEM. They were then combined and counted to determine the number of cells. The cells (1.5×10^3 in 12 μ L DMEM) were then transferred to a white opaque 384-well plate (Corning, #8804BC). Serial dilutions of each compound solution or DMSO (3X concentrations of the final dose in DMEM) were prepared in a 96-well plate and added to the cells (6 μ L each) in triplicate. After the cells were cultured for 20 h, luciferase activity was measured on an EnVision plate reader (PerkinElmer Life Sciences, Waltham, MA) by using Dual-Glo reagent (Promega) per the manufacturer's recommendation. ICLR signals were calculated as firefly luciferase signal divided by *Renilla* luciferase signal and normalized to the average of that in DMSO-treated cells, which was defined as 1 for each assay plate.

2.5. ICLR assays in GM04312 and GM15876 cells

GM04312 cells (2.0×10^5 /well) and GM15876 cells (1.6×10^5 /well) were cultured in DMEM (500 μ L/well) in a 24-well cell culture plate overnight. Due to different activities of CMV and SV40 promoters of these cell lines, amount of each reporter plasmids were re-optimized and different from those used for HT1080 described above. Two transfection mixtures [(pGL(LgT-SV40ori)-ICL: 20 ng, pRL(LgT-SV40ori): 40 ng, pET15b: 800 μ g, FuGene HD reagent: 6.0 μ L, and Opti-MEM: 70 μ L) and (pGL-ICL: 40 ng, pGL4.75: 40 ng, pET15b: 800 μ g, FuGene HD reagent: 6.0 μ L (Promega), and Opti-MEM: 70 μ L)] were prepared per the respective manufacturer's recommendation, and half volumes of each were added to 1 well each of the two cell lines (i.e., 2 transfection mixtures $\times$ 2 cell lines = 4 combinations). After incubating for 3.5 h, the transfected cells were rinsed with PBS, lifted by trypsin (100 μ L), and resuspended in fresh DMEM (330 μ L). The cell suspensions (60 μ L each) were transferred to a white opaque 96-well plate (Corning, #3917) and then gemcitabine (3 μ M in DMEM) or DMEM was added to the cells (30 μ L each) in triplicate. After the cells were cultured for 20 h, luciferase activity was measured on an EnVision plate reader by using the Dual-Luciferase Reporter Assay System (Promega) per the

manufacturer's instructions. ICLR signals were calculated as firefly luciferase signal divided by *Renilla* luciferase signal.

2.6. Analysis of the ubiquitination of FANCD2

HT1080 cells treated as indicated were washed twice with ice-cold PBS, collected in a centrifugation tube, and lysed on ice for 0.5 h with RIPA buffer supplemented with Halt Protease Inhibitor Cocktail and Halt Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific, Waltham, MA). The protein concentration was determined by performing a BCA assay (Thermo Fisher Scientific) according to the manufacturer's recommendation. Normalized amounts of samples were separated in NuPAGE 3–8% Tris-Acetate Gel (Thermo Fisher Scientific) and electrotransferred to a nitrocellulose membrane by using an iBlot apparatus (Thermo Fisher Scientific). The membrane was blocked with Odyssey Blocker (PBS) (Li-Cor, Lincoln, NE) and incubated with anti-FANCD2 antibody (Fl-17, sc-20022, Santa Cruz Biotechnology, Dallas, TX) at a dilution of 1:200 in the same blocking buffer at 4°C overnight, rinsed with TBS-0.05% (v/v) Tween 20 three times for 10 min each, incubated with goat anti-rabbit IRDye 800CW (Li-Cor, 10:000) at room temperature for 1 h, and then rinsed with TBS-0.05% Tween 20 three times for 10 min each. Protein bands were detected by fluorescence using an Odyssey Imager (Li-Cor).

2.7. ICLR assay in RRM2-depleted cells

RNAi of RRM2 in HT1080 cells was carried out by transfecting the cells with an siRNA oligonucleotide. The sequences of the RNA oligonucleotides targeting RRM2 were 5'-GGAGCGAUUUAGCCAAGAAGU-3' and 5'-UUCUUGGCUAAAUCGCUCCAC-3' (11). Oligonucleotide sequences targeting GFP were 5'-GCUGACCCUGAAGUUCAUCtt-3' and 5'-GAUGAACUUCAGGGUCAGCtt-3' (12), and were used as a negative control. Cells (2.5×10^5) were seeded in a 24-well plate and transfected with 40 pmol of the siRNA oligonucleotide by using Lipofectamine RNAiMAX Reagent (Thermo Fisher Scientific) according to the manufacturer's recommendation. Eighteen hours after transfection, 1.6×10^5 cells were re-propagated into new wells. Twenty-four hours after re-propagation, control and RRM2-depleted cells were transfected with 9.3 ng pGL(LgT-SV40ori)-ICL, 9.0 ng pRL(LgT-SV40ori), and 400 ng pBluescript (carrier DNA) by using FuGene HD Transfection Reagent (Promega). Cells in nontransfected wells were used to confirm RRM2 protein downregulation by immunoblotting, in a manner similar to that described for FANCD2. Three hours after the second transfection, the cells in each well were trypsinized and transferred to 4 wells in a 96-well plate. Twenty-one hours after re-propagation, ICLR assays were performed in a manner similar to that used for GM04312 and GM15876.

2.8. ICL unhooking assayed by using the modified alkaline comet assay

A modified version of the alkaline comet assay (Figure 4B) was used to measure the amount of cisplatin ICLs within cellular DNA (13). HT1080 cells (1.0×10^5 /well) were initially seeded in 6-well cluster plates and allowed to attach overnight. Cells were treated with 20 μ M cisplatin or vehicle for 2 h, washed thoroughly with PBS, and subsequently released into drug-free medium for 4 h. Each sample was then exposed to 0, 3.3, or 10 nM gemcitabine continuously for 42 h. At designated time points throughout this schedule, cells were treated

with 200 μ M hydrogen peroxide or PBS vehicle for 15 min to induce random breaks in cellular DNA. After this incubation, samples were immediately harvested and stored in freezing solution (10% DMSO, 90% FBS) at -80°C until the day of the assay. Upon thawing, each sample was initially resuspended in 1% molten low-gelling temperature agarose (Type VII) and was subsequently spread onto glass slides pre-coated with agarose and allowed to set. Cells were then lysed in gel by submerging samples in ice-cold lysis buffer (100 mM EDTA, 2.5 M NaCl, 10 mM Tris-HCl, pH 10.5, and 1% Triton X-100) for 60 min and then washed with ice-cold Milli-Q water 4 times for 15 min each. Each sample was then bathed in chilled alkali electrophoresis buffer (300 mM NaOH, 1 mM EDTA) for 60 min and then electrophoresed at 4°C for 30 min at 25 V. Samples were then neutralized by the addition of neutralization buffer (0.5 M Tris-HCl, pH 8.0), washed with PBS twice for 10 min, and allowed to dry overnight. DNA was stained twice with $1 \times$ SYBR Green for 5 min, and the samples were destained using 3 washes with Milli-Q water for 10 min each. Samples were then visualized by epi-fluorescence microscopy, and the extent of DNA damage was quantified by using the scoring system described by Collins (14). At least 100 comets were scored per slide. Data plot and statistical analysis were performed using Prism 6.01 (GraphPad Software, La Jolla, CA) as indicated.

2.9. Generation of expression plasmid for FLAG-XPF

The plasmid expressing FLAG-tagged human XPF protein was constructed by modifying pcDNA3-XPF (15). Briefly, the sequence GCCATG around the start codon was replaced with GCTAGC (i.e., generating a *NheI* site) by site-directed mutagenesis using GENEART Site-Directed Mutagenesis System (Life Technologies). This procedure replaced the methionine with a serine. The sequences of the oligonucleotides used for the mutagenesis were 5'-CCGGCTCGACGGATTGCATGCGCGCCGCTGCTGGAG-3' and 5'-CTCCAGCAGCGGCGCGCTAGCAATCCGTCGAGCCGG-3'. A double-stranded oligonucleotide (5'-CGCCGCCATGGACTACAAGGACGACGATGACAAGTCTGACTACAAGGATGACGATGACAAAACA GACTACAAGGACGATGATGACAAAACCGGTG-3' and 5'-CATGGCGGCGGTACCTGATGTTCTCTGCTGCTSCTGTTTCAGACTGATGTTCTTCTGCTACTGTTTTG TCTGATGTTCTGCTACTACTGTTTTGGCCACGATC-3') that encodes 3 repeats of FLAG-tag sequence (MDYKDDDDKSDYKDDDDKTDYKDDDDKTGAS) was inserted into *KpnI* and *NheI* sites of the mutated plasmid. This provided the plasmid expressing $3 \times$ FLAG sequences in frame to the coding region for XPF (pcDNA3-FLAG-XPF), which was verified by sequencing. To verify the $3 \times$ FLAG-XPF protein expression, GM08437 (XP2YO[SV] (9)) cells that lack XPF protein expression were un-transfected or transfected with pcDNA3-FLAG-XPF, lysed and analyzed for $3 \times$ FLAG-XPF expression by immunoblotting using mouse anti-FLAG M2 antibody (1:2000, Sigma-Aldrich) and rabbit anti-XPF antibody (1:1000, Bethyl Laboratories, Montgomery, TX). Un-transfected cell lysate did not show any bands except a low molecular weight nonspecific band. Meanwhile, the transfected cell lysate additionally showed a clear band at ~ 110 kDa for both antibodies (data not shown); indicating specific expression of $3 \times$ FLAG-XPF protein (108 kDa).

2.10. Co-immunoprecipitation for XPF

HEK293T cells were cultured in two 10-cm dishes (2.5×10^6 /dish) overnight and transfected with pcDNA3-FLAG-XPF (2 μ g) by using FuGene6 Transfection Reagent (Promega) according to the manufacturer's recommendation. The cells were lifted by trypsin and re-propagated into four 6-cm dishes 7 h after transfection and cultured for additional 24 h. The cells were treated with 20 μ M cisplatin for 1 h, rinsed once with PBS, and cultured for 47 h in the drug-free medium. The cells were recovered and cell pellets were lysed in LT200 buffer (50 mM Tris-HCl pH 7.4, 200 mM NaCl, 5 mM EDTA, 0.1% NP-40, 10% glycerol, 1 mM DTT, and Halt Protease Inhibitor Cocktail (Thermo Fisher Scientific)). The protein concentration was determined by performing a BCA assay (Thermo Fisher Scientific) according to the manufacturer's recommendation. Protein lysates (1000 μ g) were incubated with 10 μ L (bead volume) of anti-FLAG M2 beads (Sigma-Aldrich) at 4°C overnight. The beads were washed 3 times with LT200 buffer for 10 min. Recovered proteins on the beads were eluted by boiling the samples in a loading buffer and were detected by immunoblotting with mouse anti-FLAG M2 antibody (1:2000, Sigma-Aldrich), goat anti-RRM2 antibody (1:500, sc-10846, Santa Cruz), and rabbit anti-RRM1 antibody (1:1,000, D12F12, Cell Signaling, Danvers, MA) in a manner similar to that described for FANCD2.

2.11. Co-immunoprecipitation for SLX4

HEK293 cells (6×10^6) were initially seeded into a 10 cm dish and allowed to attach overnight. Cells were subsequently transfected with pCR3.1-FLAG-SLX4 plasmid (20 μ g) (16) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. The cells were detached and then re-propagated in 10 cm dishes (2×10^6 /dish) 24 h following transfection. Samples were subsequently treated with 20 μ M cisplatin for 1 h, rinsed once with PBS and then cultured for 47 h in drug-free medium. Following treatment, samples were harvested and lysed in ice-cold NETN buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.5% NP-40 and Complete Mini protease inhibitor cocktail tablets [Roche]). The protein concentration of each lysate was determined using a BCA assay (Thermo Fisher Scientific) according to the manufacturer's instructions. Protein lysates (185 μ g) were incubated with 15 μ L (bed volume) anti-FLAG M2 beads (Sigma-Aldrich) in the presence or absence of 20 μ g/mL 3 $\times$ FLAG peptide overnight at 4°C. Beads were then washed 3 times with ice-cold NETN buffer for 10 min each. Proteins on the beads were recovered by boiling samples in loading buffer and were subsequently detected by immunoblotting with mouse anti-FLAG M2 antibody (1:1000, Sigma-Aldrich), goat anti-RRM2 antibody (1:800, sc-10846, Santa Cruz), rabbit anti-RRM1 antibody (1:1000, D12F12, Cell Signaling, Danvers, MA), and rabbit anti-XPF antibody (A301–315A, Bethyl Laboratories, Montgomery, TX), as described for XPF immunoprecipitation.

3. RESULTS

3.1. A new assay enabled robust readout in a scalable protocol for measuring both replication-dependent and -independent ICLR activity

By using a host-cell reactivation assay with a plasmid incorporating one large ICL generated by click chemistry bridging of a base pair, we previously showed that T2AA, an inhibitor of

K164-monoubiquitinated PCNA, inhibits ICLR (17). However, a fundamental problem in this protocol prevented its use for chemical library screening: The ICL is repaired too rapidly, making the compound's effect undetectable unless it was added concurrently with reporter transfection. Indeed, a single ICL in a plasmid is repaired within 2–4 h *in vitro* (18). This scenario prohibits large-scale screening in a multiwell-plate format and compromises the effect of the compound on transfection and ICLR, resulting in large deviations. To solve these problems, we entirely redesigned the assay system to make it robust enough for chemical library screening.

First, the assay uses a newly designed pair of luciferase reporter plasmids that share the same backbone. The reporter plasmid pGL(LgT-SV40ori)-ICL (Figure 1A) contains ICLs between the CMV promoter and the firefly luciferase gene, generating a signal in ICLR, plasmid replication, and reporter generation. The control plasmid pRL(LgT-SV40ori) (Figure 1B) contains no ICL and encodes the Renilla luciferase gene, generating a signal during replication of the plasmid backbone (which is identical to that of the reporter plasmid, except for the reporter) and reporter generation. Therefore, ICLR activities are represented simply as firefly luciferase signal divided by Renilla luciferase signal, eliminating deviations caused by multi-step normalizations with other controls. Second, the new plasmids encode the SV40 large T antigen (LgT) driven by an SV40 promoter/origin (Figures 1A, 1B). These plasmids generate LgT protein in mammalian cells via the SV40 promoter, and the LgT replicates the plasmid from the SV40 origin. Therefore, ICLs are repaired by both replication-dependent ICLR that is driven by the LgT-promoted self-replication and by replication-independent ICLR that is driven by transcription of the CMV promoter. Thus, the assay identifies ICLR inhibitors for both mechanisms. Third, the ICL reporter plasmid incorporates two ICLs (Figure 1A), preventing them from being repaired too rapidly, and thus, allowing sufficient time for plating of gross-transfected cells prior to adding the test compounds. This feature makes the assay much more adaptable for large-scale screening than any existing ICLR assay methods. Fourth, the ICL is an aromatic ring unit (aoNao, (7)) that is chemically stable and easily produced in a site-specific manner on a large scale, enabling production of enough ICLR reporter plasmids for chemical library screening. Fifth, the screening is performed in a non-SV40-transformed HT1080 fibrosarcoma cell line. HT1080 cells have very high CMV and SV40 promoter activities (19) that will not be deprived by the promoter sequences shared by the two plasmids. Therefore, it produces very high signals with little promoter interference among the two reporters. Moreover, this cell line was established from a patient who had never received radiation or chemotherapies (20), therefore its DNA repair pathways should not be mutated. The production of pGL(LgT-SV40ori)-ICL is outlined in Figure 1D.

To verify the promoter interference, HT1080 cells were transfected with various amounts of parental non-ICL pGL plasmid pGL(LgT-SV40ori) and a constant amount of pRL(LgT-SV40ori). The ratio of firefly luciferase to Renilla luciferase was almost completely linear over a >2-log scale range of the pGL(LgT-SV40ori) titration (Figure 1E), showing an absence of promoter interference between the two reporter plasmids in this cell line. This allows direct data normalization among these two (i.e., ICLR = firefly luciferase expression/Renilla luciferase expression) over a >2-log scale of ICLR activity range. To determine the

optimal time for plasmid transfection prior to addition of a compound to be assayed, the cells were transfected with the ICL assay plasmids for various amounts of time. The cells were then replated in triplicate in a 96-well plate and cultured in fresh medium, and luciferase signals were read 24 h after transfection. ICLR signals (firefly luciferase) increased until 3 h after transfection and then remained unchanged to 4 h (Figure 1F), indicating that cells should be transfected with ICL assay plasmids for 3–4 h before adding the assay compound for optimal efficiency. Next, this optimized assay condition was verified for its ability to assay a compound on the 384-well scale. T2AA, an inhibitor of the K164-monoubiquitinated PCNA was used as a test compound because it had been previously verified for ICLR inhibition (17). HT1080 cells were gross-transfected with the ICL assay plasmids for 3.5 h and then replated in a 384-well plate before the addition of various concentrations of T2AA. The luciferase signals were reduced by T2AA in a dose-dependent manner (Figure 1G), with the IC_{50} being reasonably consistent with the compound's biochemical potency as a PCNA inhibitor (21). Finally, the performance of the assay protocol was verified by using T2AA and DMSO as positive and negative controls, respectively (Figure 1H). The Z' score was acceptable for a cell-based reporter assay (22), indicating that the assay is suitable for chemical library screening. The protocol required only ~300 pg of each plasmid per data point; therefore, limited plasmid supply is not an obstacle to large-scale screening.

3.2. ICLR was impaired by inhibitors of K164-monoubiquitinated PCNA and ribonucleotide reductase

Numerous chemical compounds are classified as mechanistic small-molecule inhibitors of DNA repair (23). We hypothesized that components of the DNA repair process that are critical for ICLR could be identified by screening DNA repair inhibitors in our ICLR plasmid reactivation assay. We assayed compounds that are frequently used to inhibit specific components of DNA repair pathways. Among those examined, reactivation of the reporter was particularly sensitive to T2AA and gemcitabine (24), an inhibitor of ribonucleotide reductase (RNR) (25). Meanwhile, inhibitors of other DDR mediators such as ATR, ATM, Chk1, PARP, Rad51, and BLM showed no or modest ICLR inhibition at 10–40 μ M, the concentration on which they are inhibitory to their target (Figure 2A).

Dose-dependent effects of RNR inhibition on ICLR inhibition were further validated for three different mechanistic RNR inhibitors (25): all had potent activity in which the nanomolar IC_{50} was consistent with the compound's potency reported for RNR inhibition (Figures 2B–D). Dose-response on Renilla luciferase signal by the RNR inhibitors was much less significant (data not shown), suggesting that the ICLR inhibition is not due to cytotoxicity. A caveat with the chemical approach is the possibility of nonspecific off-target effects. However, such effects are highly unlikely to have occurred in this case because the structurally different three RNR inhibitors are all potent inhibitors of ICLR. Furthermore, in the siRNA approach, even ~50% suppression of RRM2 RNR subunit expression impaired ~90% of the ICLR activity in the same cell line (Figures 2E–F). These results strongly suggest that RNR is functionally indispensable for ICLR.

3.3. RNR inhibitors inhibited both replication-dependent and -independent ICLR equally

Given that inhibition of DNA replication is not solely responsible for ICLR inhibition by RNR inhibitors, we hypothesized that RNR inhibitors inhibit both replication-dependent and -independent ICLR. To verify this, the ICLR assay system was modified to distinguish between these two ICLR mechanisms. To read signals specific to replication-dependent ICLR, we used GM04312 cells, which lack the functional XPA that is an essential component for replication-independent ICLR (4). To read signals specific to replication-independent ICLR, an ICL plasmid lacking the LgT-SV40 promoter/ori sequence (pGL-ICL) was generated, thereby preventing plasmid replication (Figure 3A). The ICLR assay was performed with four possible combinations of plasmids (pGL[LgT-SV40ori]-ICL or pGL-ICL) and cells (GM04312 or GM15876 [an XPA-intact isogenic clone derived from GM04312]). As expected, no ICLR signal was generated from pGL-ICL in GM04312 cells because of the deficiency of both replication-dependent and -independent ICLR.

Gemcitabine significantly inhibited ICLR in the other three combinations of reporter and cells (Figures 3B–C), indicating that gemcitabine inhibits both ICLR mechanisms. The potencies of signal-generation inhibition by the two ICLR plasmids were almost identical for both gemcitabine and 3-AP (Figures 3D–E). These observations strongly indicate that RNR supports processes common in replication-dependent and -independent ICLR.

3.4. Gemcitabine inhibited ICL unhooking but not Fanconi anemia pathway activation

Given that RNR inhibitors inhibited both replication-dependent and -independent ICLR (Figure 3), we hypothesized that the RNR inhibitor could inhibit early steps common in these two ICLR mechanisms. When cells detect an ICL, the Fanconi anemia (FA) pathway is activated, triggering recruitment of components for incision of the ICL site to unhook it. (26) Monoubiquitination of FANCD2 was induced in HT1080 cells by cisplatin treatment, but it was not influenced by gemcitabine (Figure 4A), indicating that gemcitabine does not inhibit FA pathway activation.

To determine the functional significance of RNR in the ICL incision process, an ICL unhooking assay was carried out by using a previously established method (Figure 4B) (13). Most DNA damage caused by cisplatin consists of base monoalkylation and intrastrand crosslinks, which are repaired more quickly than are ICLs. (27) An ICL is formed by 2-step dialkylation via a monoalkylation intermediate (28), and thus appears at a later stage of the crosslinking reaction. Therefore, to enrich the proportion of ICL species, HT1080 cells were cultured in cisplatin-free medium for several hours after being sufficiently treated with cisplatin. The cells were then cultured in various concentrations of gemcitabine and processed for the alkaline comet ICL unhooking assay after shredding the DNA by using hydrogen peroxide. ICLs prevent strand separation of the shredded DNA in alkaline conditions; thus, comet tailing is inversely proportional to the number of ICLs. Cisplatin significantly reduced the amount of comet tail formation, representing ICL formation. After cells were cultured in drug-free medium for 2 days, amount of comet tail of cisplatin-treated cells significantly increased, representing ICL unhooking. In this assay, although gemcitabine itself showed little effect, it significantly suppressed tail formation from the cisplatin-treated cells in a dose-dependent manner (Figure 4C), indicating that gemcitabine inhibits unhooking of the cisplatin-ICL. This is consistent with previous studies observing

unhooking inhibition of carboplatin ICL by gemcitabine co-administered to human patients with ovarian cancer. (29)

3.5 RNR did not interact to SLX4/XPF

XPF is a DNA endonuclease that is essential for unhooking of ICL to initiate the ICL repair process. To secure successful ICL unhooking, XPF interacts with SLX4 scaffolding protein to coordinate with other endonucleases including SLX1 and MUS81 (16,30,31). Given that RNR inhibitors inhibited ICL unhooking (Figure 4C), we asked whether RNR interacts with SLX4/XPF. To investigate this scenario, we performed a co-immunoprecipitation assay in cells after inducing ICL. The HEK293T or HEK293 cells were transfected with an expression plasmid for FLAG-XPF (Methods) or FLAG-SLX4 (16) and pulse-treated with cisplatin to induce ICL. The FLAG-XPF or FLAG-SLX4 expressed in the cell lysate was isolated by co-immunoprecipitation using anti-FLAG antibody-conjugated beads, and proteins bound on the precipitate were analyzed for RNR (see Methods for detail). Although we found strong bands in anti-FLAG immunoblotting of the immunoprecipitates, which are matched to full-length XPF and SLX4 respectively and were disappeared when incubating the beads with a FLAG peptide, neither of the immunoprecipitates showed bands in anti-RRM1 nor -RRM2 immunoblotting (data not shown). Meanwhile, anti-XPF immunoblotting of the anti-FLAG immunoprecipitate from lysate of cells transfected with FLAG-SLX4 showed strong band at the size of XPF, indicating that SLX4 and XPF are forming a complex (data not shown). These observations suggest that RNR does not interact to SLX4/XPF in this experimental condition.

4. DISCUSSION

ICL is the most powerful chemotherapeutic driver for cancer cell elimination. ICL disables cells engaging in either gene transcription or DNA replication, which is catastrophic. Many chemotherapies use this principal to selectively eliminate cancer cells because ICL is more toxic in actively replicating cells. Thus, establishing a strategy for preventing repair of ICL has important clinical implications, because ICLR reduces chemotherapy efficacy. Even today, traditional DNA-damaging chemotherapy remains absolutely essential in clinics even when an oncogene-targeting therapy is administrated. Chemical strategies targeting ICLR are rarely studied, regardless of its importance for chemotherapy, because reliable tools and methodology are not developed yet. Thus, the key question: which DDR inhibitors inhibit ICLR, has almost never been addressed, due to lack of robust and scalable ICLR assays.

We provided scalable screening protocols for directly determining ICLR activity. In theory, our assay can identify chemical inhibitors of any steps in the complex and diverse ICLR pathways virtually in any mammalian cells regardless of the phase of cell cycle, and thus, offers the most global readout of ICLR activity. The assays offer several possible unique opportunities for studying ICLR, such as: 1) applying a chemical biology approach to identify biomolecules previously uncharacterized for ICLR pathways, like RNR; 2) determining ICLR activities in each cell-cycle by assaying in synchronized cells; 3) comparison of ICLR activities among any mammalian cell cultures that could address which cancers upregulate ICLR, and interpreting in the context of genomic signatures to identify responsible genes; and 4) determining the kinetics for ICLR in cells. Few studies have

accomplished this, which hinders determining the significance of ICL level for chemotherapeutic efficacy. This situation limits our strategy for ICL-based chemotherapy to empirical measurement (i.e., only the relationship of toxicity and chemotherapy dose is determined but not actual ICL level) and disallows rational interpretation based on ICL levels. Our approach can be used to assess the quantitative significance of residual ICL for chemotherapeutic efficacy, which has rarely been accomplished. Limitations of the ICLR assay are: 1) the ICL substrate (Figure 1C) may be repaired by different mechanisms from those for ICLs generated by chemotherapeutic drugs (e.g., cisplatin); and 2) ICLR driven by SV40-promoted replication and CMV-promoted transcription may not be used in real cancer growth.

ICLR has been identified as a combination of DDR events: detection and incision of an ICL from one strand, TLS on the strand with the ICL remnant, NER removal of the ICL remnant, and further repair on the strand from which the ICL remnant was removed. (1,2) The final step may include HR, depending on the availability of a sister chromatid template (i.e., during S phase or later). By using genetic strategies such as RNAi, several DDR molecules have been identified as being functionally important mediators of ICLR, including XPF (5,32) and DNA polymerase ζ (18,33). This target-oriented rational approach is straightforward but limited to studying only a specific molecule for addressing whether it is required for ICLR. The target-independent nature of chemical approach can complement this knowledge by identifying and functionally characterizing uncovered ICLR molecules. In this study, we used a chemical approach and found that RNR is exceptionally indispensable for ICLR.

The ineffectiveness of inhibitors of some established DDR molecules such as PARP and ATR was rather unexpected. Nanomolar chemical inhibitors of ATM and Chk1 induced dose-responsive but only weak ICLR inhibition at $>20 \mu\text{M}$ (Figure 2A). This observation may suggest that these kinases are not absolutely essential for ICLR, or their function for ICLR is redundantly shared with other kinases; and thus, inhibition of each single kinase does not effectively inhibit ICLR. Weak ICLR inhibition activity could be due to weak activity to off-target kinases that may be responsible to ICLR, which could be addressed by panel kinome screening data of those compounds. In contrast, effectiveness of inhibitors of RNR appears as solid in our assay that is consistent to their on-target potency of RNR inhibition (Figure 2B). Given that synergistic utility of gemcitabine for carboplatin chemotherapy ('GemCarbo') established in clinics since the 1990s (34) that has been validated in human specimens (29), our observation added mechanistic evidence of the clinical utility of gemcitabine.

RNR is tightly coupled to DDR at the transcriptional and translational levels (35). RNR is thought to be essential for DDR because of its function as a dNTP supplier for DNA replication (25). This explanation is reasonable but has received little validation because it is technically challenging to quantify dNTP production by RNR recruited to the DNA damage site. In this study, we found that RNR inhibitors inhibit ICLR whether it is replication-dependent or -independent. Therefore, RNR could be functional at the stage of the ICLR pathway, which is independent of DNA replication. Our observation that RNR inhibitors inhibited the incision of an ICL also is consistent with being inhibitory for both ICLR

mechanisms. This scenario is puzzling if RNR is merely a dNTP supplier. Why does RNR affect the DNA incision step that requires DNA endonuclease such as XPF but not dNTP? How does RNR support the incision without affecting FA pathway activation (Figure 4A)? One possible scenario is that RNR is being recruited before the incision to ensure availability of the dNTP supply. ICL incision may be allowed only after RNR is recruited, thereby preventing incisions where dNTPs are not supplied. This could make the ICLR process safe by ensuring timely coordination of the incision and DNA synthesis steps. Indeed, RNR is reportedly recruited to the DNA damage site by binding to Tip60 (36). Such sequential assembly of protein components of DDR machineries is well described (37).

This study was intended to identify DDR molecules essential for ICLR by using a chemical approach. There is little doubt that RNR is much more functionally important for ICLR than other DDR molecules examined in this study. However, the observation that RNR promotes ICLR unhooking (29) remains a mystery. Elucidation of the biochemical mechanism of RNR-mediated ICLR, especially in the ICL incision process, requires further investigation. An unexplored scenario is that recruitment of RNR to Tip60 (36) on the DNA lesion may trigger ERCC1 upregulation via E2F1 (38), resulting activation of ICL unhooking by ERCC1. However, further mechanistic investigation is beyond of scope of this study.

5. CONCLUSION

This study is an example of an unbiased chemical genetics approach for identifying the “Achilles’ heel” of a cellular function. To our knowledge, this is the first study of characterizing so-called DNA repair inhibitors in bona-fide ICL repair inhibition assay. Such an approach has successfully identified tankyrase as a mediator of the Wnt/ β -catenin pathway (39). This same strategy could be used to identify other potent ICLR inhibitors having novel mechanisms of action and/or novel molecular targets for ICLR inhibition. Although we have focused on ICL, theoretically our approach can be applied to any type of DNA damage such as DNA-histone crosslinking. The combination of small molecule library and chemically engineered reporter plasmid are powerful tools to identify novel DDR mechanism *and* chemical inhibitors of the DDR pathway concurrently. Hit compounds found by using this approach could ultimately be used as novel chemosensitizers for cancer treatments.

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ABBREVIATIONS

ATM	Ataxia telangiectasia mutated
ATR	ATM and rad3-related
BCA	bicinchoninic acid
Chk1	checkpoint kinase 1
DDR	DNA damage response/repair
DMSO	dimethyl sulfoxide
EDTA	ethylenediaminetetraacetic acid
FBS	fetal bovine serum
HEPES	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
HR	homologous recombination
ICL	interstrand DNA crosslink
ICLR	ICL repair
MMR	mismatch repair
NER	nucleotide excision repair
PBS	phosphate-buffered saline
PAGE	polyacrylamide gel electrophoresis
PCNA	proliferating cell nuclear antigen
RNR	ribonucleotide reductase
RRM1	RNR M1 subunit
RRM2	RNR M2 subunit
SDS-	sodium dodecyl sulfate
TLS	trans-lesion DNA synthesis
T2AA	T2 amino alcohol
TBE	Tris-borate, EDTA
TBS	Tris-buffered saline

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Figure 1.

A plasmid-reactivation assay of ICLR activity, optimized to increase robustness and enable screening of chemical libraries. **A**) The ICLR reporter plasmid. Two small ICLs (black bars, structure shown in **C**) are site-specifically incorporated between the CMV promoter and firefly luciferase region. **B**) The control plasmid's backbone is identical to that of the ICLR reporter plasmid (**A**) but lacks the ICLs and encodes *Renilla* luciferase as the reporter. **C**) The chemical structure of the ICL (aoNao (7) in bold) in the reporter plasmid (**A**). The ICL is replacing two nucleotide bases at the interstrand counter positions and, thus, is not projected from the helix. **D**) Outline of the production of pGL(LgT-SV40ori)-ICL. See Materials and Methods for detail. **E**) There is no promoter interference among the two assay plasmids. HT1080 cells were transfected with the indicated amount of non-ICL parent pGL(LgT-SV40ori) plasmid, 5 ng of pRL(LgT-SV40ori) (**B**), and 400 ng pET15b carrier DNA for 4 h. The cells were then replated in triplicate in a 96-well plate and cultured in fresh medium for 20 h. **F**) Optimal time duration of plasmid transfection for ICLR signal generation. Experimental conditions were the same as those in **E**, except that pGL(LgT-SV40ori)-ICL was transfected instead of the non-ICL pGL(LgT-SV40ori) plasmid. Error bars represent standard deviation (n=3). **G**) Dose-response of T2AA (17) for ICLR inhibition. Experimental conditions were the same as those in **F**, but with replating to a 384-well plate in the presence of the indicated final concentration of T2AA. Error bars represent standard deviation (n=3). **H**) The assay is robust and scalable for screening chemical compounds. Experimental conditions were the same as those in **G** except that the cells were

treated as indicated. Data are shown as boxplots of ICLR signals in DMSO- or T2AA- (20 μ M) treated cells (n=27 for each). Z' score = 0.43.

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Figure 2.

RNR is essential for ICLR. **A**) Of the compounds tested, gemcitabine is the only DNA repair inhibitor that inhibits ICLR effectively. Indicated compounds were assayed for ICLR inhibition (Figure 1F). The targets of each inhibitors are as follows: K164-monoubiquitinated PCNA (T2AA (17)), PARP (olaparib (40)), ATR (VE821 (41)), ATM (KU60019 (42)), Chk1 (SCH900776 (43)), Rad51 (RI-1 (44)), BLM (ML216 (45)), RNR (gemcitabine (24)), DNA methyltransferase (5-azacytidine (46)), thymidylate synthase (5-fluorouracil, 5-FU (47)), and DNA Pol α (aphidicolin (48)). Note that KU60019 and SCH900776 are nanomolar inhibitors of their targets but inhibit ICLR only weakly at >20 μ M. Error bars represent standard deviation ($n=3$). **B–D**) The different mechanistic RNR inhibitors inhibit ICLR with nanomolar potency. Shown are the chemical structures and dose-response of **B**) gemcitabine (irreversible RRM1 inhibitor), **C**) clofarabine (reversible RRM1 inhibitor), and **D**) 3-AP (RRM2 inhibitor). Error bars represent standard deviation ($n=3$). **E–F**) Even partial downregulation of RNR severely dampens ICLR. HT1080 cells were transfected with siRNA targeting RRM2 or GFP (negative control). After 42 h of siRNA transfection, cells were transfected for the ICLR assay as described in Figure 1F except that no compounds were added. The cells were analyzed by immunoblotting for RRM2 expression (**E**, densitometric quantification is indicated beneath each lane) and ICLR (**F**, Error bar represents standard deviation [$n=4$]).

Figure 3.

RNR inhibitors inhibit both replication-dependent and -independent ICLR. **A)** The reporter plasmid (pGL-ICL) containing no LgT-SV40ori to specifically measure replication-independent ICLR activity. ICLR activity in GM04312 (XPA (-)) cells (**B**) and in GM15876 (XPA (+)) cells (**C**), measured using pGL(LgT-SV40ori)-ICL (Figure 1A)/ pRL(LgT-SV40ori) (Figure 1B) or pGL-ICL (Figure 3A)/ pGL4.75 (identical to pRL containing no LgT-SV40ori, control for inhibition of reporter generation by the CMV promoter, see Methods) in the absence or presence of gemcitabine (1 μ M). Absence of XPA prevents replication-independent ICLR. Accordingly, no ICLR activity was observed in GM04312 for pGL-ICL. Both gemcitabine (**D**) and 3-AP (**E**) showed ICLR inhibition equipotent for pGL(LgT-SV40ori)-ICL and pGL-ICL in HT1080 cells. Error bars represent standard deviation (n=3).

Figure 4.

Mechanistic characterization of gemcitabine in the ICLR pathway. **A)** Gemcitabine did not affect cisplatin-induced FANCD2 monoubiquitination in HT1080 cells. **B)** Overview of the ICL unhooking assay developed by modifying a method reported by Hartley et al (13). Red stars represent ICLs and blue stars represent ICL remnants that were unhooked but not removed yet. ICLs prohibit strand separation and the ICL unhooking allows it. Therefore, ICL formation decreases electrophoresis mobility of nuclear DNA, and unhooking of ICLs increases comet tail score regardless of the presence of unhooked ICL remnants. **C)** Gemcitabine inhibited unhooking of cisplatin-ICL in HT1080 cells. The schedule of treatments for each group of cells is outlined above each data group. Numbers of score counting (n) were as indicated. The comet tail of each counted cells was scored as 0, 0.5, 1.0, 2.0, 3.0, 4.0, and summarized as a box for 25–75% range with a whisker for 5–95% range. A two-tailed unpaired *t*-test (parametric) was performed for statistical analyses.