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Embryonic Stem Cells Lacking the Epigenetic Regulator Cfp1 are Hypersensitive to DNA-Damaging Agents and Exhibit Decreased Ape1/Ref-1 Protein Expression and Endonuclease Activity

Courtney M. Tate^{1,2}, Melissa L. Fishel^{1,3}, Julianne L. Holleran⁴, Merrill J. Egorin⁴, and David G. Skalnik^{1,2,*}

¹Herman B Wells Center for Pediatric Research, Section of Pediatric Hematology/Oncology, Indiana University School of Medicine, 1044 W. Walnut St. R4-W312, Indianapolis, IN 46202

²Department of Pediatrics and Biochemistry and Molecular Biology, Indiana University School of Medicine, 1044 W. Walnut St. R4-W312, Indianapolis, IN 46202

³Department of Pharmacology and Toxicology, Indiana University School of Medicine, 1044 W. Walnut St. R4-W312, Indianapolis, IN 46202

⁴University of Pittsburgh Cancer Institute, Room G27E, Hillman Research Pavilion, 5117 Centre Avenue Pittsburgh, PA 15213-1863

Abstract

Modulation of chromatin structure plays an important role in the recruitment and function of DNA repair proteins. CXXC finger protein 1 (Cfp1), encoded by the *CXXC1* gene, is essential for mammalian development and is an important regulator of chromatin structure. Murine embryonic stem (ES) cells lacking Cfp1 (*CXXC1*^{-/-}) are viable but demonstrate a dramatic decrease in cytosine methylation, altered histone methylation, and an inability to differentiate. We find that ES cells lacking Cfp1 are hypersensitive to a variety of DNA-damaging agents. In addition, *CXXC1*^{-/-} ES cells accumulate more DNA damage and exhibit decreased protein expression and endonuclease activity of AP endonuclease (Ape1/Ref-1), an enzyme involved in DNA base excision repair. Expression in *CXXC1*^{-/-} ES cells of either the amino half of Cfp1 (amino acids 1-367) or the carboxyl half of Cfp1 (amino acids 361-656) restores normal Ape1/Ref-1 protein expression and rescues the hypersensitivity to DNA-damaging agents, demonstrating that Cfp1 contains redundant functional domains. Furthermore, retention of either the DNA-binding activity of Cfp1 or interaction with the Setd1A and Setd1B histone H3-Lys4 methyltransferase complexes is required to restore normal sensitivity of *CXXC1*^{-/-} ES cells to DNA-damaging agents. These results implicate Cfp1 as a regulator of DNA repair processes.

Keywords

Cfp1; Ape1/Ref-1; DNA damaging agents; epigenetic regulation; chromatin

*Address correspondence to: David Skalnik, Cancer Research Building, Room 327, 1044 West Walnut St., Indianapolis, IN 46202, Tel (317) 274-8977; FAX (317) 278-9298; dskalnik@iupui.edu.

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1. Introduction

Perturbations of global and gene-specific patterns of cytosine methylation are commonly observed in cancer [1,2]. Hyper-methylation and silencing of tumor suppressor genes contributes to tumorigenesis [3], and modulation of DNA (cytosine-5)-methyltransferase 1 (Dnmt1) activity alters susceptibility to cancer in otherwise normal mice [4], in mice lacking DNA mismatch repair [5], and in mice carrying mutations in the NF1 and p53 genes [6]. Hypo-methylation of DNA has also been linked to chromosome instability and increased chromosomal translocations that are frequently associated with malignancies [7]. Finally, agents, such as azacytidine that inhibit DNA methylation, and histone deacetylase (HDAC) inhibitors such as vorinostat, are being used as chemotherapeutic agents in the treatment of malignancies such as myelodysplastic syndrome, chronic myelogenous leukemia, and acute myelogenous leukemia [8-10]. Both aberrant epigenetic modifications and deficiencies in DNA repair can lead to tumorigenesis, as both processes are linked to genetic instability and play a role in the regulation of genes involved in cell growth and differentiation.

The eukaryotic cell acquires more than 10,000 DNA lesions per day. Failure to repair such lesions can lead to mutations, genomic instability, or cell death [11]. The base excision repair (BER) pathway predominantly recognizes and removes oxidative and alkylation damage, including reactive oxygen species (ROS)-induced damage and spontaneous base loss [12-15]. The damaged base is then removed by one of the DNA glycosylases resulting in a baseless or apurinic/apyrimidinic (AP) site, which is a substrate for the AP endonuclease1/redox effector factor 1 (Ape1/Ref-1). Ape1/Ref-1 is a multi-functional protein with AP endonuclease activity that hydrolyzes the phosphodiester bond immediately 5' to the AP site, after which β -polymerase and DNA ligase act to complete the repair process. Cells with reduced Ape1/Ref-1 activity are hypersensitive to numerous DNA-damaging agents such as methylmethane sulfonate (MMS), hydrogen peroxide (H_2O_2), temozolomide (TMZ), ionizing radiation (IR), cisplatin, and other alkylating and oxidative DNA-damaging agents [16-23].

The repair of DNA damage *in vivo* is complicated by the fact that genomic DNA is packaged with histone and non-histone proteins into chromatin, a highly condensed structure that hinders DNA accessibility and its subsequent repair. The efficiency of DNA repair is affected by chromatin structure, nucleosome accessibility/disruption, and transcriptional activity [24,25]. Chromatin structure is partly controlled by cytosine methylation and covalent modifications of histone proteins including methylation, acetylation, ubiquitination, and phosphorylation [26-29]. Cytosine methylation is catalyzed by Dnmt enzymes in the context of CpG dinucleotides [30] and is essential for normal mammalian development [31]. Histone variants and modifications also play a major role during DNA repair by marking the lesion, recruiting components of the repair machineries, and facilitating their action [32].

CXXC finger protein 1 (Cfp1), encoded by the *CXXC1* gene, is a protein that intersects with cytosine methylation through Dnmt1 [33] and with histone methylation through the Setd1A and Setd1B histone H3-Lys4 methyltransferase complexes [34,35]. Cfp1 contains several conserved protein domains, including a cysteine-rich CXXC DNA-binding domain that exhibits specificity for unmethylated CpG dinucleotides [36] and a cysteine-rich Set1 interaction domain (SID), which is required for interaction with the Setd1A and Setd1B histone H3-Lys4 methyltransferase complexes [33,36,37]. Targeted disruption of the *CXXC1* gene in mice results in early embryonic lethality at approximately 4.5-6.5 days postcoitus [38]. Interestingly, embryonic stem (ES) cells lacking Cfp1 (*CXXC1*^{-/-}) are viable but exhibit a variety of defects when compared to wild-type (*CXXC1*^{+/+}) ES cells. These defects include a lengthened population doubling time due to an increased rate of apoptosis, an inability to achieve *in vitro* differentiation, and a 70% decrease in global and gene-specific cytosine methylation [39]. *CXXC1*^{-/-} ES cells also exhibit perturbations in global levels of histone

methylation, including increased euchromatin-associated histone H3-Lys4 di- and tri-methylation and decreased heterochromatin-associated histone H3-Lys9 di-methylation [34]. Consequently, Cfp1 plays an important role in the regulation of cytosine and histone methylation, heterochromatin formation, and cellular differentiation.

The purpose of this study was to determine if Cfp1 plays a role in the sensitivity of ES cells to DNA-damaging agents. In these studies, an array of DNA-damaging agents were used, including MMS, TMZ, H₂O₂, IR, cisplatin, and etoposide, that cause lesions repaired by the BER pathway as well as other pathways (Table 1). Platinum-based drugs such as cisplatin, in addition to causing DNA cross-linking, generate ROS as a byproduct [40-42]. Reduced expression of Ape1/Ref-1 in neuronal cultures enhances cisplatin-induced cell killing as well as apoptosis and ROS generation, indicating that BER and Ape1/Ref-1 are important in the response to these drugs as well [20]. The results reported here demonstrate that *CXXCI*^{-/-} ES cells exhibit hypersensitivity to these DNA-damaging agents but normal sensitivity to the anti-metabolite/anti-folate agent, methotrexate (MTX), and the microtubule-stabilizing agent, paclitaxel. In addition, *CXXCI*^{-/-} ES cells express decreased levels of Ape1/Ref-1 protein and AP endonuclease activity and exhibit increased accumulation of DNA damage. Structure-function studies were utilized to determine the domains of Cfp1 that are sufficient to restore normal APE1/Ref-1 protein expression and rescue the hypersensitivity to DNA-damaging agents. These findings reveal that Cfp1 influences sensitivity to DNA-damaging agents and may regulate DNA repair processes.

2. Materials and methods

2.1. Cell culture

Generation of *CXXCI*^{-/-} murine ES cell lines was previously described [39]. *DNMT1*^{-/-} ES cells were a generous gift from En Li (Novartis Institute for Biomedical Research, Cambridge, MA). ES cell lines were cultured on 0.1% gelatin-coated tissue culture dishes in high-glucose Dulbecco's modified Eagle's medium (GIBCO, BRL, Life Technologies, Grand Island, NY) supplemented with 20% fetal bovine serum (GIBCO, BRL), 100 Units/ml penicillin/streptomycin (Invitrogen, Carlsbad, CA), 2 mM L-glutamine (Invitrogen), 1% non-essential amino acids (Invitrogen), 0.2% leukemia inhibitory factor-conditioned medium, 100 nM β-mercaptoethanol, 0.025% HEPES pH 7.5, (Invitrogen), and 1% Hank's balanced salt solution (Invitrogen).

2.2. Plasmid construction and stable transfection of ES cells

To rescue the *CXXCI*^{-/-} ES cell phenotype, murine Cfp1 cDNA [43] was subcloned into the pcDNA 3.1/Zeo mammalian expression vector (Invitrogen). The Cfp1 expression vector or the empty expression vector was electroporated into *CXXCI*^{-/-} ES cells as previously described [39]. For structure-function studies, cDNA constructs encoding full-length FLAG epitope-tagged human Cfp1 (amino acids 1-656) or various Cfp1 truncations and mutations were subcloned into the pcDNA3.1/Hygro mammalian expression vector (Invitrogen). The amino-terminal bipartite nuclear localization signal of Cfp1 (amino acids 109-121) was inserted between the FLAG epitope and Cfp1 sequence for constructs containing amino acids 361-656 and 361-656 C375A. The FLAG epitope was incorporated into each construct to permit convenient evaluation of protein expression levels. Linearized DNA (25 µg) was electroporated into *CXXCI*^{-/-} ES cells, which were grown in selection medium containing 200 µg/ml hygromycin B (Sigma-Aldrich, St. Louis, MO) for approximately two weeks before single colonies were isolated for expansion, and then maintained in medium containing 50 µg/ml hygromycin B. Expression of FLAG epitope-tagged human Cfp1 was verified by western blot analysis using anti-FLAG (Sigma-Aldrich) or anti-Cfp1 antibodies [35], as described below.

Two or three independent clones for each construct were analyzed, and representative data are shown.

2.3. Drug treatments and irradiation

Stock solutions of DNA-damaging and non-genotoxic agents were diluted with ES culture medium to the indicated concentrations, and cells were treated for various amounts of time before being harvested, counted, and plated in triplicate for colony forming assays. Cells were treated with media controls or H₂O₂ (Sigma-Aldrich) for 2 h, MMS (Sigma Aldrich) for 3 h, or TMZ (LKT Laboratories, St. Paul, MN) for 4 h. Etoposide (Sigma-Aldrich) was prepared as a 50 mM stock solution in dimethyl sulfoxide (DMSO), and cells were treated with etoposide or DMSO (vehicle control) for 3 h. Cis-diammineplatinum (II) dichloride (Cisplatin) (Sigma-Aldrich) was freshly prepared for each experiment as a 20 mM stock solution in DMSO, and cells were treated with cisplatin or DMSO for 3 h. Irradiation was carried out at room temperature using a Co-60 gamma unit (Nordion International, Kanata, Canada) that delivered various doses. After irradiation, the cells were immediately trypsinized, counted, and plated for colony forming assays. Paclitaxel (Sigma-Aldrich) was prepared in DMSO, and cells were treated with paclitaxel or DMSO for 6 h. MTX (Sigma-Aldrich) was freshly prepared in 1 M sodium hydroxide (NaOH), and cells were treated with MTX or NaOH (vehicle control) for 6 h.

2.4. Clonogenic Survival

Exponentially-growing ES cells were treated with various concentrations of each agent for the indicated times before being trypsinized and counted. For clonogenic survival assays, 400 cells were plated into gelatin-coated, 10 cm tissue culture dishes. Colonies were allowed to form for 7-9 days, and then fixed in methanol:acetic acid (3:1), stained with 0.02% crystal violet, and counted. Colony numbers from the treated samples were normalized to untreated or vehicle control-treated cells and plotted as a function of dose. Data presented represent the average of three independent experiments performed in triplicate.

2.5. Analysis of cytosine methylation

Global cytosine methylation was assessed utilizing a methyl acceptance assay as previously described [44]. This *in vitro* methylation reaction quantitates transfer of radiolabeled methyl groups from S-adenosyl-L-methionine onto unmethylated genomic CpG dinucleotide sites [45]. Briefly, 500 ng of genomic DNA was incubated with 2 μ Ci of ³H-methyl-S-adenosyl L-methionine (Amersham, GE Healthcare, Piscataway, NJ; 15 Ci/mmol) and 3 units of SssI (CpG) methylase (New England Biolabs, Ipswich, MA) in 120 mM NaCl, 10 mM Tris-HCl pH 7.9, 10 mM EDTA, and 1 mM dithiothreitol (DTT) for 1 h at 30°C in a total volume of 30 μ l. The SssI enzyme was then inactivated by heating the reaction mixture for 10 min at 65°C. *In vitro* methylated DNA was isolated by filtration through Whatman DE-81 ion-exchange filters (Fisher Scientific, Pittsburgh, PA). The filters were washed five times with 0.5 M sodium phosphate buffer (pH 7.4), air-dried, and the incorporated radioactivity was measured by scintillation counting. Background radioactivity bound to the filter from a reaction mixture lacking DNA was subtracted from the values of the reaction mixtures containing DNA. Methylation reactions were performed in duplicate and the analysis was carried out for three independent experiments.

2.6. Western blot analysis

Cfp1, Dnmt1, and Ape1/Ref-1 protein expression was assessed by harvesting whole cell extracts. Cells were lysed in cytoskeletal buffer (300 mM NaCl, 10% sucrose pH 7.2, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride [PMSF], and 3% protease inhibitor cocktail [Sigma-Aldrich]) by Dounce homogenization 30 times on ice. Soluble extracts were separated

from debris by centrifugation at $16,000 \times g$ for 10 min at 4°C. Protein concentration was quantitated by measuring absorbance at 595 nm using the Bradford Reagent (BioRad, Cambridge, UK) as previously described [46]. In order to separate proteins, 60 µg of whole cell protein was subjected to electrophoresis using PAGER-Gold pre-cast 4-12% Tris-glycine gradient gels (Lonza Group Ltd., Basel, Switzerland) and transferred to nitrocellulose membranes (Amersham, GE Healthcare). The membranes were probed with rabbit polyclonal Cfp1 [35], mouse monoclonal Ape1/Ref-1 (Novus Biologicals, Littleton, CA), or β -actin (Lab Vision) antibodies followed by the appropriate horseradish peroxidase-labeled secondary antibody. Proteins were detected with an ECL kit (Amersham, GE Healthcare) and autoradiography, and band intensities were quantitated by densitometry.

2.7. Ape1/Ref-1 endonuclease activity assay

Oligonucleotide gel-based Ape1/Ref-1 endonuclease activity assays were modified as a fluorescent assay and performed as previously described [47]. The 26 bp oligonucleotide substrate contained a single THF residue in the middle, yielding a HEX-labeled 13-mer fragment upon excision. Ape1/Ref-1 endonuclease activity was measured by incubating 0.4 pmol of HEX-labeled (2 pmol/ml) excess oligonucleotide substrate with cellular protein in a total volume of 20 µl of assay buffer (50 mM HEPES pH 7.5, 50 mM KCl, 10 mM $MgCl_2$, 1% BSA, and 0.05% Triton X-100) at 37°C for 15 min. Reactions were terminated by adding formamide loading buffer (10 µl) to each sample. The results were detected following electrophoresis and quantitated using the FMBioII fluorescence imaging system and software (Hitachi Genetic Systems, South San Francisco, CA).

2.8. H2AX phosphorylation (H2AX- γ) expression as a measure of DNA damage

Exponentially growing *CXXCI*^{+/+} and *CXXCI*^{-/-} ES cells were treated with 1 µM TMZ (LKT Laboratories) for 4 h. Whole cell protein extracts were collected after treatment, and 10 µg of each extract was utilized in western blotting as previously described [19,48] using mouse monoclonal antibody specific for phosphorylation of H2AX at Ser¹³⁹ (Upstate, Waltham, MD). Blots were also probed for β -actin (Lab Vision) as a loading control. Bands were detected using a chemiluminescence kit (Pierce, Rockford, IL), visualized using Bio-Rad Chemidoc XRS (Hercules, CA), and quantitated using Chemidoc software, Quantity One 4.6.1.

2.9. Measurement of total platinum in DNA

Atomic absorption spectroscopy was used to quantitate total platinum in DNA as previously described [49]. Exponentially-growing cells were treated with 100, 200, or 300 µM cisplatin or DMSO vehicle controls for 3 h. Genomic DNA was isolated directly after cisplatin treatment by lysing cell pellets with 600 µl of cell lysis buffer (10 mM Tris pH 8.0, 100 mM EDTA, and 0.5% SDS). Cell lysates were then incubated with 4 µl RNase A (Roche, 10 mg/ml) for 15 min at 37°C, then 200 µl of 7 M ammonium acetate was added before vortexing the sample for 30 sec and incubating on ice for an additional 5 min. Proteins and debris were pelleted by centrifugation, and the resulting supernatants were mixed with 350 µl of isopropanol to precipitate the DNA. DNA pellets were washed with 70% ethanol, air dried, and resuspended in ddH₂O. Platinum concentration was assessed with a Perkin-Elmer model 1100 flameless atomic absorption spectrometer (Perkin-Elmer, Norwalk, CT) as previously described [49, 50].

2.10. Statistical analysis

Statistical significance was assessed by two-tailed student t-tests with equal variance. A *P* value <0.05 was interpreted as statistically significant.

3. Results

3.1. Loss of Cfp1 results in hypersensitivity to a variety of DNA-damaging agents

Wild-type (*CXXCI*^{+/+}) and *CXXCI*^{-/-} ES cells were analyzed for clonogenic survival following exposure to increasing amounts of DNA-damaging agents (Table 1). *CXXCI*^{-/-} ES cells demonstrate significant decreases in cell survival after exposure to IR, MMS, H₂O₂, TMZ, etoposide, and cisplatin compared to *CXXCI*^{+/+} ES cells (Fig. 1A-F). Taken together, these results indicate that *CXXCI*^{-/-} ES cells are hypersensitive to DNA-damaging agents that cause DNA strand breaks (IR and etoposide), alkylation (MMS, TMZ), DNA cross-links (cisplatin), and oxidative damage (IR, H₂O₂, cisplatin). Because *CXXCI*^{-/-} ES cells exhibit an extended population doubling time due to an increase in apoptosis [39], longer time points for colony formation were additionally analyzed. However, extended incubation time did not alter colony forming numbers for *CXXCI*^{-/-} ES cells (data not shown).

3.2. *CXXCI*^{-/-} ES cells are not hypersensitive to non-genotoxic agents

Due to the variety of chromatin and differentiation defects observed in *CXXCI*^{-/-} ES cells [34,39], further studies were performed to determine if *CXXCI*^{-/-} ES cells are hypersensitive to any type of stress. However, there was no significant difference in clonogenic survival between *CXXCI*^{+/+} and *CXXCI*^{-/-} ES cells after treatment with the non-genotoxic agents MTX (Fig. 2A) or paclitaxel (Fig. 2B). These data indicate that the hypersensitivity of *CXXCI*^{-/-} ES cells is specific to DNA-damaging agents and is not a general sensitivity to cytotoxic agents.

3.3. Expression of full-length Cfp1 in *CXXCI*^{-/-} ES cells rescues hypersensitivity to DNA-damaging agents

Previous experiments in our laboratory demonstrated that stable expression of full-length wild-type murine Cfp1 in *CXXCI*^{-/-} ES cells (*CXXCI*^{-/-}*cDNA*) corrects the epigenetic and differentiation defects observed in *CXXCI*^{-/-} ES cells [39]. To determine if reconstitution of Cfp1 in *CXXCI*^{-/-} ES cells additionally rescues the hypersensitivity to DNA-damaging agents, *CXXCI*^{+/+}, *CXXCI*^{-/-}, and *CXXCI*^{-/-}*cDNA* ES cells were analyzed for clonogenic survival after treatment with DNA-damaging agents. For these and the following experiments, TMZ was utilized as a clinically relevant representative alkylating agent, H₂O₂ was chosen as an oxidizing agent, and cisplatin was utilized as a clinically relevant DNA cross-linking agent. These studies reveal that rescued *CXXCI*^{-/-}*cDNA* ES cells demonstrate sensitivities to TMZ, cisplatin, and H₂O₂ similar to those of *CXXCI*^{+/+} ES cells (Fig. 3). Therefore, expression of full-length Cfp1 in *CXXCI*^{-/-} ES cells restores normal sensitivity to DNA-damaging agents, indicating that the observed hypersensitivity is specifically due to loss of Cfp1.

3.4. Hypersensitivity of *CXXCI*^{-/-} ES cells to DNA-damaging agents is not solely caused by decreased cytosine methylation

Genomic DNA in *CXXCI*^{-/-} ES cells may be more accessible to genotoxic agents due to dramatically decreased levels of genomic cytosine methylation, a marker of heterochromatin [39]. To examine whether this could potentially explain the increased sensitivity to DNA-damaging agents, the sensitivity of *DNMT1*^{-/-} ES cells to DNA-damaging agents was determined. *DNMT1*^{-/-} ES cells are deficient for maintenance DNA methylation, and suffer a dramatic loss of global cytosine methylation [31]. Using an *in vitro* methyl acceptance assay, the number of radiolabeled methyl groups incorporated into a genomic DNA sample is inversely related to the native DNA methylation status. These studies reveal that genomic DNA isolated from *CXXCI*^{-/-} or *DNMT1*^{-/-} ES cells accept approximately 2.5- and 3-fold more methyl groups than does DNA derived from *CXXCI*^{+/+} ES cells, respectively, indicating a ~60% decrease of global cytosine methylation in *CXXCI*^{-/-} ES cells and a ~67% decrease in *DNMT1*^{-/-} ES cells (Fig. 4A). Consistent with a previous report [39], *CXXCI*^{-/-}*cDNA* ES cells

exhibit normal levels of cytosine methylation, indicating that the global loss of cytosine methylation observed in *CXXCI*^{-/-} ES cell DNA is specifically due to loss of Cfp1.

To determine the sensitivity of *DNMT1*^{-/-} ES cells to DNA-damaging agents, *DNMT1*^{-/-}, *CXXCI*^{+/+}, and *CXXCI*^{-/-} ES cells were treated with TMZ, cisplatin, or H₂O₂ and analyzed for clonogenic survival. These studies reveal that *DNMT1*^{-/-} ES cells are not hypersensitive to TMZ, cisplatin, or H₂O₂ (Fig. 4 B-D). In addition, *DNMT1*^{-/-} ES cells exhibit a sensitivity to the non-genotoxic agents MTX and taxol similar to that of *CXXCI*^{+/+} ES cells (data not shown). Thus, *DNMT1*^{-/-} ES cells exhibit a normal sensitivity to DNA-damaging agents, indicating that the observed hypersensitivity of *CXXCI*^{-/-} ES cells cannot be solely explained by a reduction in global cytosine methylation.

3.5. *CXXCI*^{-/-} ES cells express decreased levels of Ape1/Ref-1 protein and endonuclease activity

CXXCI^{-/-} ES cells exhibit increased sensitivity to TMZ, MMS, H₂O₂, cisplatin, and IR, agents that generate lesions that can be repaired by the BER pathway. Furthermore, reduced expression of Ape1/Ref-1 in various cell types results in hypersensitivity to these agents (20-27). Therefore, the expression level and endonuclease activity of Ape1/Ref-1 was evaluated in *CXXCI*^{+/+}, *CXXCI*^{-/-}, and *DNMT1*^{-/-} ES cells. Western blot analysis revealed a ~50% decrease in Ape1/Ref-1 protein expression in *CXXCI*^{-/-} ES cells, and a slight increase in Ape1/Ref-1 protein expression in *DNMT1*^{-/-} ES cells (Fig. 5A). In addition, expression of full-length Cfp1 in *CXXCI*^{-/-} ES cells (*CXXCI*^{-/-}*cDNA*) is sufficient to reconstitute Ape1/Ref-1 protein expression back to levels observed in *CXXCI*^{+/+} ES cells (Fig. 5A). In contrast, expression of another downstream component involved in the BER pathway, XRCC1, is unchanged in *CXXCI*^{-/-} ES cells (data not shown).

Ape1/Ref-1 endonuclease activity was measured by incubation of cellular protein extracts with a 26-mer HEX-labeled oligonucleotide substrate that contains an AP site. Consistent with Ape1/Ref-1 protein levels, Ape1/Ref-1 endonuclease activity is significantly decreased (40-50%) in *CXXCI*^{-/-} ES cells compared to *CXXCI*^{+/+} ES cells, and slightly increased in *DNMT1*^{-/-} ES cells (Fig. 5B). Western blot analysis of the protein samples analyzed in the endonuclease activity assay confirmed equal protein loading (Fig. 5B, bottom panel).

Immunoprecipitation was performed to determine if Ape1/Ref-1 physically interacts with Cfp1. Full-length FLAG epitope-tagged Cfp1 was expressed in human embryonic kidney cells (HEK-293), immunoprecipitated, and interacting proteins were detected by western blot analysis. However, Ape1/Ref-1 was not detected as a pull-down product with Cfp1 (data not shown). Co-immunoprecipitation of Cfp1 with the histone methyltransferase Setd1A was used as a positive control [34]. Ape1/Ref-1 sub-cellular localization is controlled by a strictly regulated process and is quite variable between cell types and disease states [51]. However, confocal immunofluorescence microscopy revealed no difference in the sub-cellular localization of Ape1/Ref-1 in *CXXCI*^{-/-} ES cells compared to *CXXCI*^{+/+} ES cells. Each cell line demonstrated a punctate staining of Ape1/Ref-1 distributed mostly within the nucleus with very little cytoplasmic localization (data not shown).

3.6. *CXXCI*^{-/-} ES cell DNA exhibits increased levels of DNA damage following treatment with DNA-damaging agents

To determine whether the increased sensitivity of *CXXCI*^{-/-} ES cells to cisplatin correlates with higher levels of DNA damage, atomic absorption spectroscopy was used to evaluate the total amount of platinum in DNA isolated from *CXXCI*^{+/+} or *CXXCI*^{-/-} ES cells treated with cisplatin for three hours. These studies reveal a significant increase in the amount of platinum/μg DNA in *CXXCI*^{-/-} ES cells compared to *CXXCI*^{+/+} ES cells after treatment with cisplatin (Fig. 6A).

As an independent assessment of DNA damage, the level of histone H2AX phosphorylation (H2AX- γ), a marker of double strand breaks (DSBs), was measured in *CXXC1*^{+/+} and *CXXC1*^{-/-} ES cells following TMZ treatment. Repair of DSBs is expected to be important in ES cells due to the high basal level of H2AX- γ [52] which we also observed (data not shown). Immediately after treatment with TMZ, there is a significant increase in H2AX- γ levels in *CXXC1*^{-/-} ES cells compared to *CXXC1*^{+/+} ES cells (Fig. 6B). The levels of H2AX- γ were normalized to β -actin expression, and the graph presents relative H2AX- γ expression compared to untreated controls. No increase in annexin V staining was detected in cells similarly treated with TMZ (data not shown). Thus, the increased level of H2AX- γ in Cfp1-null ES cells following TMZ exposure is not an indirect consequence of DNA fragmentation associated with apoptosis.

3.7. Redundant functional Cfp1 domains rescue hypersensitivity of *CXXC1*^{-/-} ES cells to TMZ and cisplatin

Expression of full-length Cfp1 (aa 1-656) in *CXXC1*^{-/-} ES cells rescues the hypersensitivity to cisplatin, TMZ, and H₂O₂ (Fig. 3), thereby providing a convenient method to assess structure-function relationships of Cfp1. Various FLAG epitope-tagged Cfp1 fragments were expressed in *CXXC1*^{-/-} ES cells to identify functional properties of Cfp1 that are sufficient to restore normal sensitivity to DNA-damaging agents. Stably transfected clones were selected, and clones were screened for protein expression by western blot analysis using an anti-Cfp1 antibody (Fig. 7). Multiple clones were analyzed for each construct and representative data are shown. ES cells heterozygous for the disrupted *CXXC1* allele express ~50% of Cfp1 protein compared to *CXXC1*^{+/+} ES cells but do not exhibit the epigenetic and differentiation defects observed in *CXXC1*^{-/-} ES cells [39]. In addition, *CXXC1*^{-/-} ES cells are not hypersensitive to TMZ, cisplatin, or H₂O₂ (data not shown). Consequently, clones that express at least 50% of the level of Cfp1 expression observed in *CXXC1*^{+/+} ES cells were selected for analysis.

Remarkably, *CXXC1*^{-/-} ES cells expressing either the amino half of Cfp1 (aa 1-367, containing the PHD1, CXXC, acidic, and basic domains) or the carboxyl half of Cfp1 (aa 361-656, containing the coiled coil, SID, and PHD2 domains) exhibit a sensitivity to TMZ and cisplatin similar to that of wild-type ES cells and *CXXC1*^{-/-} ES cells expressing full-length Cfp1 1-656 (Fig. 8). These data reveal that Cfp1 contains redundant functional domains capable of rescuing the hypersensitivity phenotype, and indicate that no single Cfp1 domain is required to rescue the hypersensitivity to DNA-damaging agents.

3.8. Retention of Cfp1 DNA-binding activity or interaction with the Setd1 histone H3-Lys4 methyltransferase complexes is required to restore normal Ape1/Ref-1 protein expression and rescue the hypersensitivity of *CXXC1*^{-/-} ES cells to TMZ and cisplatin

The amino half of Cfp1 (aa 1-367) contains the CXXC DNA-binding domain [37], and the carboxyl half of Cfp1 (aa 361-656) contains the SID domain that mediates interaction with the Setd1A and Setd1B histone H3-Lys4 methyltransferase complexes [33]. To define further the functional domains required for Cfp1-mediated rescue activity, Cfp1 point mutations that abolish DNA-binding activity of Cfp1 (C169A) [37] or ablate the interaction of Cfp1 with the Setd1 methyltransferase complexes (C375A) [33] were analyzed (Fig. 9A).

CXXC1^{-/-} ES cells expressing full-length Cfp1 lacking DNA-binding activity (1-656 C169A) or lacking interaction with the Setd1 histone H3-Lys4 methyltransferase complexes (1-656 C375A) exhibit sensitivity to TMZ and cisplatin similar to that of *CXXC1*^{-/-} ES cells expressing full-length Cfp1 (Fig. 9B,C). Hence, neither DNA-binding activity of Cfp1 nor association with the Setd1 histone H3-Lys4 methyltransferase complexes is required to rescue hypersensitivity to DNA-damaging agents. In contrast, abolishing DNA-binding activity ablates the rescue activity of the Cfp1 1-367 fragment (1-367 C169A), and abolishing

interaction with the Setd1 complexes ablates the rescue activity of the 361-656 Cfp1 fragment (361-656 C375A). Mutation of both DNA-binding and Setd1 interaction domains within full-length Cfp1 also ablates rescue activity (Fig. 9). Therefore, retention of either Cfp1 DNA-binding or Setd1 interaction domains is required to rescue the hypersensitivity of *CXXCI*^{-/-} ES cells to TMZ and cisplatin.

Western blot analysis was performed to determine the level of Ape1/Ref-1 protein expression in *CXXCI*^{-/-} ES cells carrying various Cfp1 mutations. Cfp1 mutations that fail to rescue the hypersensitivity to TMZ and cisplatin (Cfp1 1-367 C169A, 361-656 C375A, and 1-656 C169A, C375A) also fail to rescue Ape1/Ref-1 protein expression in *CXXCI*^{-/-} ES cells (Fig. 10). In contrast, *CXXCI*^{-/-} ES cells expressing Cfp1 mutations that rescue the hypersensitivity to TMZ and cisplatin (Cfp1 1-656, 1-656 C169A, 1-656 C375A, 1-367, and 361-656) also express normal levels of Ape1/Ref-1 protein (Fig. 10). These results reveal a strong correlation between Ape1/Ref-1 protein expression levels in *CXXCI*^{-/-} ES cells and sensitivity to DNA-damaging agents.

4. Discussion

The studies reported here demonstrate that *CXXCI*^{-/-} ES cells are hypersensitive to a variety of DNA-damaging agents, including IR, H₂O₂, TMZ, MMS, cisplatin, and etoposide. *CXXCI*^{-/-} ES cells also exhibit a ~50% decrease in expression of the DNA repair protein Ape1/Ref-1, and a concomitant decrease in Ape1/Ref-1 endonuclease activity. *CXXCI*^{-/-} ES cells exhibit increased platinum incorporation into DNA after treatment with cisplatin, and elevated levels of H2AX-γ formation after treatment with TMZ.

The structure of chromatin is a major factor affecting radio-sensitivity and DNA repair efficiency [53]. For example, mitochondrial DNA is approximately 10-fold more sensitive to DNA-damaging agents compared to nuclear DNA [54-56]. Given that mitochondrial DNA is not packaged as chromatin, it has been inferred that chromatin confers protection against DNA-damaging agents. HDAC inhibitors, such as trichostatin A (TSA), increase the acetylation of core histones, resulting in an open chromatin configuration that is more accessible to DNA-damaging agents [57]. TSA increases chromatin accessibility and sensitizes K562 cells to IR [58,59]. HDAC inhibitors also sensitize tumor cells to the induction of cell death by IR, ultraviolet radiation, and several DNA-damaging drugs [57,60-62]. In addition, H2AX-γ persists longer in melanoma cells after IR exposure in combination with sodium butyrate, another HDAC inhibitor. Sodium butyrate also causes an increase in etoposide-induced apoptosis in leukemia cells [63].

CXXCI^{-/-} ES cells exhibit a ~70% decrease in global cytosine methylation [39] and altered patterns of histone methylation that correlate with decreased amounts of heterochromatin [34]. Therefore, *CXXCI*^{-/-} ES cells may exhibit elevated levels of DNA packaged in accessible chromatin, and the increased sensitivity to DNA-damaging agents and increased accumulation of DNA damage observed in *CXXCI*^{-/-} ES cells may be due, at least in part, to epigenetic alterations that result in decreased heterochromatin. However, previous reports also suggest that the more open configuration of DNA could result in an increased accessibility for DNA repair proteins [64,65]. This does not appear to be the case in this study, as neither Dnmt1 nor Cfp1 deficiency resulted in improved survival.

Structure-function analysis reveals that expression in *CXXCI*^{-/-} ES cells of either the amino half of Cfp1 (aa 1-367) or carboxyl half of Cfp1 (aa 361-656) is sufficient to restore normal Ape1/Ref-1 protein expression and rescue the hypersensitivity to DNA-damaging agents. Thus, Cfp1 contains redundant functional domains, and no single Cfp1 protein domain is essential for rescue activity. Additional studies revealed that retention of either DNA-binding

or Setd1 interaction domains of Cfp1 is required to restore normal Ape1/Ref-1 protein expression and rescue the hypersensitivity of *CXXCI*^{-/-} ES cells to DNA-damaging agents. Interestingly, other studies in our laboratory reveal an identical requirement for the retention of either the DNA-binding domain or Setd1 complex interaction domain of Cfp1 for rescue of deficient cytosine methylation, aberrant histone methylation, and *in vitro* differentiation in *CXXCI*^{-/-} ES cells [66]. The finding of redundant functional Cfp1 domains might be explained by cross-talk between epigenetic modifications. It is thought that cytosine methylation and histone modifications are highly integrated and mutually reinforcing mechanisms that establish and maintain appropriate chromatin structure [67-73]. Thus, rescue of normal cytosine methylation might provide critical epigenetic marks to permit restoration of appropriate histone modifications, and rescue of appropriate histone methyltransferase regulation might permit subsequent restoration of appropriate patterns of cytosine methylation.

Increased sensitivity of *CXXCI*^{-/-} ES cells to DNA-damaging agents may also be due to a decrease in DNA repair activity. *CXXCI*^{-/-} ES cells exhibit a ~50% decrease in Ape1/Ref-1 protein expression and endonuclease activity. Ape1/Ref-1 is a critical component of the BER pathway, and provides >95% of the endonuclease activity in mammalian cells [74]. Consistent with the data presented here, reduction of Ape1/Ref-1 protein by specific anti-sense oligonucleotides or siRNA renders mammalian cells hypersensitive to a variety of laboratory and chemotherapeutic agents, including MMS, H₂O₂, bleomycin, TMZ, and cisplatin [20,22,23,42,75,76]. For example, suppression of Ape1/Ref-1 in rat glioma cells increases sensitivity to MMS and H₂O₂ [77], and suppression of Ape1/Ref-1 in a human glioma line and human medulloblastoma cell line results in decreased AP endonuclease activity, increased abasic site content, and increased sensitivity to 1,3-bis(2-chloroethyl)-nitrosourea (BCNU), MMS, and TMZ [75,78]. In these studies, we also observe an increase in sensitivity to the DNA crosslinking agent, cisplatin which creates crosslinks on DNA that are repaired by nucleotide excision repair and homologous recombination. However, cisplatin also generates ROS, [40-42] and Ape1/Ref-1 is well-established as a key enzyme in the repair of oxidative DNA damage [84]. To ensure genomic integrity, there is crossover, interaction and compensation within and between the various DNA repair pathways. This notion is underscored by recent data which illustrates that inhibition of the BER protein PARP (poly(ADP-ribose) polymerase) enhances agents such as cisplatin, doxorubicin, and cyclophosphamide [85,86], as well as data reported here suggesting that decreased Ape1/Ref-1 levels in *CXXCI*^{-/-} ES cells contribute to the response to a variety of DNA damaging agents. Furthermore, it is probable that BER and Ape1/Ref-1 play a role in repairing the DNA damage induced by the ROS produced secondarily by exposure to these types of agents.

In addition, *APE1/REF-1*^{+/-} mice exhibit a 40-50% reduction in Ape1/Ref-1 protein and endonuclease activity in all tissues examined [79,80], and display a 35-50% reduction in BER activity [80,81]. *APE1/REF-1*^{+/-} mice exhibit increased spontaneous mutation frequencies in somatic tissues and spermatogenic cells [81]. *APE1/REF-1*^{+/-} mice also display hypersensitivity to oxidative stress *in vivo*, and *APE1/REF-1*^{+/-} mouse embryonic fibroblasts (MEFs) exhibit diminished survival following exposure to oxidative DNA-damaging agents [79]. Taken together, this literature suggests that the ~50% decrease in Ape1/Ref-1 protein expression and endonuclease activity observed in *CXXCI*^{-/-} ES cells may contribute to the observed hypersensitivity to DNA-damaging agents. Consistent with this idea, *DNMT1*^{-/-} ES cells, which exhibit less global cytosine methylation than *CXXCI*^{-/-} ES cells, express normal levels of Ape1/Ref-1 and exhibit a normal sensitivity to TMZ, H₂O₂, and cisplatin. Therefore, a global decrease in cytosine methylation cannot solely account for the hypersensitivity to DNA-damaging agents exhibited by *CXXCI*^{-/-} ES cells. Further work is necessary to ascertain the mechanism of reduced Ape1/Ref-1 expression in *CXXCI*^{-/-} ES cells. Cfp1 functions as a transcriptional activator in co-transfection assays [36,82], and may play a role in activating transcription of Ape1/Ref-1 through its CpG island-containing promoter [77,83].

In addition to endonuclease activity, Ape1/Ref-1 possesses other activities that are both related and unrelated to its function in DNA repair including 3'phosphodiesterase activity, 3'-phosphatase activity, 3'-5' exonuclease activity, reduction of key cysteine residues located in the DNA-binding domains of transcription factors such as AP1 (Fos/Jun), p53, and HIF-1 α , as well as negatively regulating the Rac1 GTPase to prevent oxidative stress [16], all of which could contribute to the effects observed here. The causal relationship of reduced Ape1/Ref-1 expression to the observed hypersensitivity of *CXXCI*^{-/-} ES cells, and the functional domains of Ape1/Ref-1 required for rescue, can be further investigated by introducing into *CXXCI*^{-/-} ES cells expression vectors containing wild-type Ape1/Ref-1 as well as Ape1/Ref-1 mutants that are deficient in DNA repair or redox function [20].

Histone modifications are important regulators of BER. For example, *in vitro* data demonstrate that the ATP-dependent chromatin remodeler SWI/SNF stimulates the processing of 8-oxo-7,8 dihydroguanine during BER [92]. Many chromatin-associated proteins are implicated in DNA repair, and it is well established that DNA becomes more accessible during DNA repair. Nucleosomes are unpackaged, allowing the DNA repair machinery to access the DNA lesion, followed by reassembly of nucleosomes in newly replicated regions, as well as the restoration of the local epigenetic state of the chromatin [11,93,94]. *CXXCI*^{-/-} ES cells exhibit increased levels of histone H3-Lys4 tri-methylation, suggesting that Cfp1 restricts the activity of the Setd1 complexes [34]. Therefore, the hypersensitivity to DNA-damaging agents and the increased accumulation of DNA damage observed in *CXXCI*^{-/-} ES cells may be a consequence of aberrant histone methylation and potentially aberrant regulation/targeting of histone methyltransferase complexes which might lead to a reduction in DNA repair.

The studies reported here implicate Cfp1, an epigenetic regulator of cytosine methylation and histone methylation, in DNA repair processes. *CXXCI*^{-/-} ES cells provide a unique system with which to investigate how chromatin conformation impacts the effectiveness of DNA-damaging or chemotherapeutic agents. Such insights may lead to more efficacious combinations of chemotherapy in cancer patients, resulting in better overall patient outcomes.

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FIG. 1. *CXXC1*^{-/-} ES cells are hypersensitive to DNA-damaging agents. Clonogenic survival was determined for *CXXC1*^{+/+} (black diamonds) and *CXXC1*^{-/-} (gray squares) ES cells after treatment with IR (A), MMS (B), H₂O₂ (C), TMZ (D), etoposide (E), or cisplatin (F). Each data point represents the mean $\pm$ standard error from at least three experiments performed in triplicate. Asterisks denote a statistically significant ($p < 0.05$) difference compared to *CXXC1*^{+/+} ES cells using Student's t test.

FIG. 2. *CXXC1*^{-/-} ES cells are not hypersensitive to non-genotoxic agents. Clonogenic survival was determined for *CXXC1*^{+/+} (black diamonds) and *CXXC1*^{-/-} (gray squares) ES cells after treatment with MTX (A), or paclitaxel (B). Each data point represents the mean $\pm$ standard error from at least three experiments performed in triplicate.

FIG. 3.

Hypersensitivity of *CXXC1*^{-/-} ES cells to DNA-damaging agents is rescued by expression of full-length Cfp1.

Clonogenic survival was determined for *CXXC1*^{+/+} (black diamonds), *CXXC1*^{-/-} (gray squares), and rescued *CXXC1*^{-/-cDNA} (black triangles) ES cells after treatment with TMZ (A), cisplatin (B), or H₂O₂ (C). Each data point represents the mean $\pm$ standard error from at least three experiments performed in triplicate. Asterisks denote a statistically significant ($p < 0.05$) difference compared to *CXXC1*^{+/+} ES cells using Student's t test.

FIG. 4.

DNMT1^{-/-} ES cells are not hypersensitive to DNA-damaging agents.

A. Global cytosine methylation levels were determined by methyl acceptance assay for genomic DNA isolated from *CXXC1*^{+/+}, *CXXC1*^{-/-}, rescued *CXXC1*^{-/-}cDNA, and *DNMT1*^{-/-} ES cells. The graph presents the results from three independent experiments, and error bars represent standard errors. Asterisks denote a statistically significant ($p < 0.05$) difference compared to *CXXC1*^{+/+} ES cell DNA using Student's t test.

B-D. Clonogenic survival was determined for *CXXC1*^{+/+} (black diamonds), *CXXC1*^{-/-} (gray squares), and *DNMT1*^{-/-} (black triangles) ES cells after treatment with TMZ (B), cisplatin (C), or H₂O₂ (D). Each data point represents the mean $\pm$ standard error from at least three experiments performed in triplicate. Asterisks denote a statistically significant ($p < 0.05$) difference compared to *CXXC1*^{+/+} ES cells using Student's t test.

FIG. 5.

Ape1/Ref-1 protein expression and endonuclease activity is reduced in *CXXCI*^{-/-} ES cells.

A. Western blot analysis was performed on whole cell protein extracts collected from *CXXCI*^{+/+}, *CXXCI*^{-/-}, *DNMT1*^{-/-}, and rescued *CXXCI*^{-/-cDNA} ES cells using antiserum directed against Cfp1, Dnmt1, Ape1/Ref-1, or β-actin as a loading control. The graph represents results from at least three independent experiments, and error bars represent standard errors. Asterisk denotes a statistically significant ($p < 0.05$) decrease in Ape1/Ref-1 protein expression compared to *CXXCI*^{+/+} ES cells using Student's t test.

B. Ape1/Ref-1 endonuclease activity was determined in *CXXCI*^{+/+}, *CXXCI*^{-/-}, and *DNMT1*^{-/-} ES cells using an oligonucleotide gel-based Ape1/Ref-1 activity assay. Ape1/Ref-1 activity was measured by incubating HEX-labeled excess oligonucleotide substrate with 7.8 or 15.6 ng of cellular protein followed by quantitation of the level of the 13-mer (AP endonuclease cleavage product) relative to the 26-mer substrate. The bottom panel presents western blot analysis for β-actin to show relative protein concentration in the extracts utilized for the Ape1/Ref-1 endonuclease activity assay. The graph represents results from at least three independent experiments, and error bars represent standard errors. Asterisks denote a statistically significant ($p < 0.05$) decrease in Ape1/Ref-1 endonuclease activity compared to *CXXCI*^{+/+} ES cells using Student's t test.

FIG. 6.

CXXC1^{-/-} ES cells exhibit an increase in DNA damage after treatment with DNA-damaging agents.

A. *CXXC1*^{+/+} and *CXXC1*^{-/-} ES cells were treated with the indicated concentrations of cisplatin for 3 h. DNA was isolated and incorporated platinum was measured using atomic absorption spectrometry. The graph represents results from at least three independent experiments, and error bars represent standard errors. The asterisk denotes a statistically significant ($p < 0.05$) increase in platinum incorporation compared to *CXXC1*^{+/+} ES cells using Student's *t* test.

B. Effect of TMZ on DSB formation. *CXXC1*^{+/+} and *CXXC1*^{-/-} ES cells were treated with 1 μM TMZ for 4 h, and whole cell protein extracts were collected immediately following

treatment and analyzed for the presence of H2AX- γ by western blot. A representative western blot is presented, and the bar graph summarizes data from seven experiments, with β -Actin used to normalize the amount of protein per lane. The amount of H2AX- γ is expressed relative to the respective untreated controls (baseline H2AX- γ expression levels).

FIG. 7.

Protein expression of wild-type and Cfp1 mutations in *CXXC1*^{-/-} ES cells.

A. Schematic of full-length Cfp1 (1-656) and Cfp1 truncations and mutations that were stably expressed in *CXXC1*^{-/-} ES cells. The filled circle at the N-terminus of Cfp1 represents the FLAG-epitope, while NLS represents a nuclear localization signal derived from the Cfp1 protein [82]. An "X" indicates the approximate location of an amino acid point mutation made within the CXXC (C169A) or SID domain (C375A). Experiments were carried out on 2-3 independent stably expressing clones, and data is shown for representative clones.

B. Western blot analysis of clones was performed to determine protein expression level of Cfp1 truncations and mutations compared to the expression level of endogenous Cfp1 in *CXXC1*^{+/+} ES cells. Protein extracts were analyzed using antiserum raised against Cfp1 [35]. The blot was also incubated with antiserum directed against β -actin as a loading control. The graph represents results from at least three independent experiments, and error bars represent standard error.

Fig. 8.

Expression of either half of Cfp1 in *CXXC1*^{-/-} ES cells restores normal sensitivity to DNA-damaging agents.

A. Schematic of full-length human Cfp1 (1-656) and Cfp1 truncations that were stably expressed in *CXXC1*^{-/-} ES cells. The filled circle at the N-terminus of Cfp1 represents the FLAG-epitope, and NLS represents a nuclear localization signal [95]. Experiments were carried out on two or three independent clones for each expression construct, and data are shown for representative clones.

B and C. Clonogenic survival after treatment with TMZ (B) or cisplatin (C) was determined for *CXXC1*^{+/+}, *CXXC1*^{-/-}, and *CXXC1*^{-/-} ES cells expressing full-length Cfp1 (1-656), Cfp1

1-367, Cfp1 361-656, or cells carrying the empty expression vector. Each data point represents the mean $\pm$ standard error from at least three experiments performed in triplicate. Data points located below asterisks denote statistically significant ($p < 0.05$) decreases compared to full-length Cfp1 (1-656) using Student's t test.

FIG. 9.

Retention of either the DNA-binding domain or Setd1 interaction domain of Cfp1 is required to rescue hypersensitivity of *CXXCI*^{-/-} ES cells to DNA-damaging agents.

A. Schematic of full-length human Cfp1 (1-656) and Cfp1 mutations that were stably expressed in *CXXCI*^{-/-} ES cells. The filled circle at the N-terminus of Cfp1 represents the FLAG-epitope, and NLS represents a nuclear localization signal derived from the Cfp1 protein [95]. An "X" indicates the approximate location of an amino acid point mutation made within the CXXC (C169A) or SID domains (C375A). Experiments were carried out on two or three independent clones for each expression construct, and data are shown for representative clones.

B and C. Clonogenic survival after treatment with TMZ (B) or cisplatin (C) was determined for *CXXCI*^{-/-} ES cells expressing full-length Cfp1 (1-656), various Cfp1 mutations, or cells carrying the empty expression vector. Each data point represents the mean $\pm$ standard error from at least three experiments performed in triplicate. Data points below asterisks denote statistically significant ($p < 0.05$) decreases compared to full-length Cfp1 (1-656) using Student's t test.

FIG. 10.

Restoration of normal Ape1/Ref-1 protein expression levels in *CXXCI*^{-/-} ES cells is strongly correlated with rescue of hypersensitivity to DNA-damaging agents.

Western blot analysis was performed on whole cell protein extracts collected from *CXXCI*^{+/+}, *CXXCI*^{-/-}, and *CXXCI*^{-/-} ES cells expressing full-length Cfp1 (1-656) or the indicated Cfp1 mutations (or cells carrying the empty expression vector) using antisera directed against Ape1/Ref-1 and β -actin as a loading control. The graph represents results from at least three independent experiments, and error bars represent standard error. Asterisks denote statistically significant ($p < 0.05$) decreases compared to full-length Cfp1 (1-656) using Student's t test.

Table 1

Laboratory and clinical agents utilized to test the sensitivity of ES cells that lack Cfp1.

	Type of Damage / Mechanism of Action	DNA Repair Pathway	Ref.
DNA damaging agents			
Radiation	SSBs DSBs ROS	BER HR / NHEJ BER	[96]
Methyl methanesulfonate (MMS)	N7-meG N3-meA O ⁶ -meG	BER BER Direct	[97]
H ₂ O ₂	ROS	BER	[98]
Temozolomide (TMZ)	O ⁶ -meG N3-meA N7-meG	Direct BER BER	[99]
Etoposide	Topoisomerase poison SSBs DSBs	BER HR / NHEJ	[100]
Cisplatin	Inter- and intrastrand DNA crosslinks ROS	NER / MMR / HR BER	[101] [42]
Non-genotoxic Agents			
Methotrexate	Inhibitor of dihydrofolate reductase leading to inhibition of DNA replication	N.A.	[102,103]
Paclitaxel	Microtubule stabilization and block of cell cycle progression	N.A.	[104]