SAGE-Hindawi Access to Research
Journal of Nucleic Acids
Volume 2010, Article ID 823917, 11 pages
doi:10.4061/2010/823917

Research Article

Coincident *In Vitro* Analysis of DNA-PK-Dependent and -Independent Nonhomologous End Joining

Cynthia L. Hendrickson,[1,2] Shubhadeep Purkayastha,[1] Elzbieta Pastwa,[3] Ronald D. Neumann,[1] and Thomas A. Winters[1]

[1] *Radiology & Imaging Sciences Department, Nuclear Medicine Section, Warren G. Magnuson Clinical Center, National Institutes of Health, Bethesda, MD 20892, USA*
[2] *Applied Biosystems, Advanced Genetic Applications, 500 Cummiing Center, Suite 2450, Beverly, MA 01915, USA*
[3] *Molecular Genetics Department, Medical University of Lodz, Lodz 92-215, Poland*

Correspondence should be addressed to Thomas A. Winters, twinters@mail.nih.gov

Received 15 May 2010; Accepted 6 June 2010

Academic Editor: Ashis Basu

In mammalian cells, DNA double-strand breaks (DSBs) are primarily repaired by nonhomologous end joining (NHEJ). The current model suggests that the Ku 70/80 heterodimer binds to DSB ends and recruits DNA-PK$_{cs}$ to form the active DNA-dependent protein kinase, DNA-PK. Subsequently, XRCC4, DNA ligase IV, XLF and most likely, other unidentified components participate in the final DSB ligation step. Therefore, DNA-PK plays a key role in NHEJ due to its structural and regulatory functions that mediate DSB end joining. However, recent studies show that additional DNA-PK-independent NHEJ pathways also exist. Unfortunately, the presence of DNA-PK$_{cs}$ appears to inhibit DNA-PK-independent NHEJ, and *in vitro* analysis of DNA-PK-independent NHEJ in the presence of the DNA-PK$_{cs}$ protein remains problematic. We have developed an *in vitro* assay that is preferentially active for DNA-PK-independent DSB repair based solely on its reaction conditions, facilitating coincident differential biochemical analysis of the two pathways. The results indicate the biochemically distinct nature of the end-joining mechanisms represented by the DNA-PK-dependent and -independent NHEJ assays as well as functional differences between the two pathways.

1. Introduction

DNA double-strand breaks (DSBs) constitute the most cytotoxic form of DNA damage in the genome. DSBs are generated not only by exogenous sources, such as ionizing radiation, radiomimetic compounds, and topoisomerase inhibitors but also from endogenous cellular processes that generate reactive oxygen species [1, 2]. In mammalian cells, one of the major pathways for the repair of DSBs is nonhomologous end joining (NHEJ) [3, 4]. The principle proteins that participate in this DNA end joining pathway both *in vitro* [5–7] and *in vivo* [8–10] are the Ku 70/80 heterodimer, DNA-PK$_{cs}$, XRCC4, DNA ligase IV and XLF (XRCC4-like factor; also called Cernunnos) that has recently been identified as a binding partner of the DNA ligase IV-XRCC4 complex and as necessary for efficient ligation via NHEJ [11, 12]. Subsets of NHEJ may involve other factors such as Artemis [13]. Together with the Artemis protein, DNA-PKcs can stimulate processing of the DNA ends [14, 15]. Additional proteins, including DNA polymerases μ and λ , TDP1 (tyrosyl-DNA phosphodiesterase), PNK (polynucleotide kinase), and WRN (Werner's syndrome helicase) are also likely to play a role in DSB repair [16]. Recent reports suggest that numerous other proteins, including ATM, histones H1 and H2AX, NBS1, and Mre11 may also have some influence on the NHEJ pathway [17–19]. NHEJ is a complex, multistep process initiated by the binding of a heterodimeric complex composed of Ku70 and Ku80 subunits (encoded by the XRCC5 and XRCC6 genes, respectively) to both ends of the broken DNA molecule with high specificity and affinity [20]. Ku binds DNA DSB ends and recruits DNA-PK$_{cs}$, which is a 460 kDa serine/threonine protein kinase, to the ends [21]. Ku then translocates inward, approximately 14 bp on the DNA, allowing DNA-PK$_{cs}$ to contact DNA [22].

The resulting DNA-PK holoenzyme (Ku/DNA-PKcs) has a serine/threonine protein kinase activity that is necessary for efficient repair [23]. A current model of NHEJ suggests that inward translocation of Ku allows DNA-PKcs molecules on opposing DSB ends to interact across the DSB and form a molecular "bridge" or synapse between the two DNA ends [24], and end-joining may then be completed by ligation of the DNA ends by DNA ligase IV/XRCC4/XLF complex [25].

Therefore, DNA-PK has important roles in NHEJ that include its DNA end-bridging activity [24], and its function in regulating DSB end processing enzymes, such as the structure-dependent nuclease Artemis [15] and its requirement for the stable recruitment of the DNA ligase IV/XRCC4 complex [26]. In support of this DNA-PK-dependent NHEJ model, previous studies have shown that DNA-PK binds XRCC4-ligase IV [26, 27], but not the other mammalian DNA ligases (I or III) *in vitro* [28]. It has also been shown that wortmannin, a chemical inhibitor of DNA-PK, [29] inhibits NHEJ [5] in a way similar to that seen in cells expressing kinase deficient DNA-PK_{cs} [23]. However, the role of DNA-PK kinase activity in NHEJ has not yet been fully understood. Although DNA-PK_{cs} binds to Ku at DNA DSB sites, disruption of these DNA-PK complexes by autophosphorylation [30, 31] is required for subsequent ligation of the DNA ends [32, 33]. It has been established that DNA-dependent protein kinase (DNA-PK) undergoes a series of autophosphorylation events that facilitate successful completion of nonhomologous end joining [32]. DNA-PK_{cs} is phosphorylated at multiple sites *in vivo* in response to DNA damage, including serine 2056 [34], a cluster of sites between residues 2609–2647 (referred to as the ABCDE or Thr-2609 cluster), and threonine 3950 [35]. Although DNA-PK_{cs} in which the ABCDE sites have been mutated to alanine has normal protein kinase activity, its ability to dissociate from the Ku-DNA complex is reduced *in vitro* [36] and *in vivo* [37], suggesting that phosphorylation of the ABCDE sites plays a major role in regulating disassembly of the initial DNA-PK complex. DNA-PK also phosphorylates the Ku subunits [38] and XRCC4 [39], but mutation of these phosphorylation sites does not inhibit NHEJ [26, 40]. It has been suggested that activation of the kinase may be required for mobilization of the DNA ligase IV/XRCC4 complex [41], but the mechanism for mobilization is unknown. It has also been suggested that phosphorylation of histone H1 by DNA-PK, which reduces the affinity of the histone for DNA is required for NHEJ [42]. Alternatively, it has also been proposed that DNA-PK_{cs} stimulates, but is not essential to, NHEJ [10, 18]. Recent studies have revealed the overall structural architecture of DNA-PK_{cs} and Ku with DNA under conditions that mimic DSBs and DNA-PK_{cs} autophosphorylation [43]. The results indicate that efficient association and dissociation of the DNA-PK_{cs} at DSBs is regulated by Ku and DNA-PK_{cs} autophosphorylation that induces dramatic conformational changes in the protein. Recently, a three-dimensional crystal structure of purified DNA-PK_{cs} in complex with C-terminal fragments of Ku80 has been determined and reveals irregular regions of repetitive structures (α-helical HEAT repeats) that might provide a flexible cradle to promote DNA DSB repair [44]. Conceivably, individual phosphorylation events have different effects on DNA-PK_{cs} structure and function, both *in vitro* and *in vivo*, which in turn influences the assembly and disassembly of the initial NHEJ complex that regulates the accessibility of the DSB to other repair factors as well as pathway progression [3, 33, 45]. The DNA-PK dependent pathway could thus be characterized as the principle NHEJ pathway that employs the products of DNA-PK_{cs}, Ku70/80, DNA ligase IV, XRCC4, XLF, and Artemis. Defects in the components of this pathway have been implicated in genomic instability and development of cancer [46, 47]. The possibility however of the presence of alternative pathways for NHEJ was suggested by early experiments in which cells deficient in DNA-PK_{cs}, Ku, DNA ligase IV, or XRCC4 showed a high potential of end joining with preferential use of microhomologies [48]. The presence of at least one alternate pathway was first indicated by the observation that DNA-PK mutant MO59J cells, which do not express DNA-PK_{cs}, retain the ability to repair DNA DSBs [8] and exhibit wild-type end-joining activity *in vitro* [49], suggesting the involvement of a DNA-PK-independent end-joining pathway in these cells. At least two NHEJ mechanisms have also been identified in cells with DNA-PK_{cs} *in vivo*: an immediate, high-fidelity end joining that occurs within two hours, followed by an error-prone DSB repair with slower kinetics [49, 50]. A study of cell lines with and without DNA-PK_{cs}, M059K, and M059J, respectively, suggests that the first, faster NHEJ pathway is DNA-PK_{cs}-dependent and the second, slower NHEJ pathway is DNA-PK_{cs}-independent [51, 52]. DNA-PK-dependent and -independent repair has also been indicated *in vivo* as a function of cell cycle [53]. Recent studies have confirmed the operation of alternative pathways of NHEJ in the absence of the DNA-PK/LigIV/XRCC4 complex, in which another ligase partially substitutes for DNA ligase IV [54, 55]. Although Polβ, XRCC1, PARP-1, and DNA ligase III contribute predominantly to base excision repair (BER) and SSB repair [56], these proteins are also considered to be candidate components for backup pathways for NHEJ in which ligase III provides the major ligation activity [57, 58]. Indeed, PARP-1 has been shown to compete with Ku for repair of DNA double-strand breaks but apparently through distinct NHEJ pathways [59]. These backup pathways are not typically detectable in the presence of DNA-PK_{cs}, suggesting that the binding of the protein to the DNA inhibits DNA-PK-independent NHEJ [49, 54]. A more recent work has identified histone H1 as an additional putative factor that operates preferentially within these backup pathways [60]. Although there is a significant evidence *in vivo* and *in vitro* of a DNA-PK_{cs}-independent NHEJ pathway, this DNA end-joining mechanism has only been reported *in vitro* in the absence of the kinase subunit due to the apparent inhibition of alternate pathways by DNA-PK_{cs} [54]. In this study, we have identified *in vitro* reaction conditions that optimize the repair of DNA DSBs via a DNA-PK-independent pathway in the presence of functional DNA-PK_{cs}. We also evaluated DSB end-joining efficiency and DNA-PK activity in extracts treated with wortmannin, which is a potent and selective inhibitor of phosphatidylinositol 3-kinases (PI3K) as well as the PI3K-like DNA-PK and has a pronounced effect on DNA DSB repair [61, 62]. Under these

same conditions, we have found that DNA-PK is active in the absence of wortmannin and inhibited in the presence of wortmannin but that inhibition of DNA-PK's kinase activity does not inhibit NHEJ. Results also confirm that under reaction conditions that favor DNA-PK-dependent NHEJ, wortmannin completely inhibits DNA end joining. We have found that the individual activities of the two NHEJ repair pathways are differentially affected by reaction conditions. Furthermore, as evidenced in earlier studies [49, 50], we have observed decreased DSB repair fidelity under reaction conditions that favor DNA-PK-independent NHEJ.

2. Materials and Methods

2.1. Materials. T4 DNA ligase (10 U/μL) was purchased from Invitrogen (Carlsbad, CA.). Wortmannin was from Sigma (St. Louis, MO). Restriction enzymes were from New England BioLabs (Beverly, MA). Vistra Green was obtained from Amersham Biosciences (Piscataway, NJ). Antibodies to Ku80, XRCC4, GAPDH, PARP-1, histone H1 (IgG_{2a}), DNA-PK$_{cs}$, and ATM were purchased from Abcam, Inc. (Cambridge, MA). Antibodies to DNA ligase I, Mre11, Rad50, and NBS1 were from GeneTex, Inc. (San Antonio, TX). Antibodies to DNA ligase III were from Novus Biologicals (Littleton, CO). All antibodies were of isotype IgG_1 except where noted. The DNA-PK peptide substrate was purchased from Promega (Madison, WI). Plasmid pSP189 was a gift from Dr. Michael Seidman (National Institute of Aging, Baltimore, MD).

2.2. Cellular Extraction. Unless otherwise indicated, cervical cancer (HeLa) cells, fibroblast (WI-38) cells, and malignant glioma (M059K) cells (8×10^7 cells/mL) were extracted by triplicate rounds of freezing and thawing in B1 lysis buffer (10 mM HEPES pH 7.9, 60 mM KCl, 1 mM EDTA pH 8.0, 1 mM DTT, and 1 mM PMSF). Lysates were cleared by centrifugation at $16,000 \times g$ for 30 min at 4°C and the supernatants constituting the WCEs were stored as aliquots at −80°C.

2.3. DSB End-Joining Assays. Reactions (50 μL; 50 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$, 1 mM DTT, 1 μg/mL aprotinin, 1 μg/mL leupeptin, 10 μg/mL bestatin, 1 mM pefabloc, 1 mM ATP, 100 ng StuI-cut pSP189 plasmid DNA unless otherwise indicated, and with or without 5% (w/v) polyethylene glycol, MW ~8 kDa, ((PEG) also where indicated) were initiated with the addition of 15 μg WCE. Reactions were incubated at 30°C for the times indicated. Experiments with wortmannin were prepared and incubated on ice for 10 min before heating to 30°C. All reactions were stopped with the addition of SDS (0.4%) and incubation at 65°C for 15 min. DNA was recovered by phenol: chloroform extraction and ethanol precipitation, separated on 1% agarose gels, and stained with Vistra Green for 1 hr. Images were digitized with a FluorImager 595 system and quantified densitometrically using GelPro v2.0 software (Media Cybernetics, Gaithersburg, MD).

DSB repair fidelity experiments employed a modification of the end-joining assay described above. Repair fidelity was measured as a function of restriction enzyme recleavage efficiency for the end-joined DNA products recovered from the assay described above. Standard end-joining reactions were run with pSP189 DNA linearized with StuI, EcoRI, PvuI, or Hin1I (producing blunt, 4 nucleotide 5′-overhang, 2 nucleotide 5′-overhang, or 2 nucleotide 3′-overhang DSB ends, respectively). DNA recovered from these reactions in the ethanol precipitation step was redissolved in 20 μL of the appropriate manufacturer's restriction enzyme reaction buffer and split into two 10 μL aliquots. One aliquot was incubated at 37°C for 2 h with 2.5 U of the restriction enzyme originally used to linearize the plasmid. Following this redigestion step, both aliquots were electrophoresed and analyzed as described above for the end-joining assay. All DSB repair fidelity reactions were run in triplicate.

2.4. Kinase Assays. Kinase activity was assayed under the same conditions as DNA end joining, except for the addition of 5 μg peptide substrate (EPPLSQEAFADLWKK) and 0.2 μCi [γ-^{32}P] ATP. Reactions were incubated at 30°C for 10 min then quenched as described for the end-joining assay. The peptide substrate was isolated on 16% Tris-Tricine SDS PAGE gels and analyzed by autoradiography.

2.5. Immunodepletions. WCE (700 μg) was incubated with antibody (70 μg) for 1 hr on ice. Antibody-bound protein was removed by adding either Sepharose A or G in binding buffer (50 mM Tris-HCl, pH 7.6; 0.1 mg/mL BSA), and rotating for 1 hr at 4°C. Unbound proteins were recovered by filtration through a 0.22 μm cellulose acetate membrane and stored at –80°C. Target protein depletion was confirmed by western blot, following 4–12% Tris-Glycine SDS gradient PAGE. Immunodepletion of DNA ligase IV was performed using antibodies to XRCC4 due to the lack of ligase IV-specific antibodies. It has been reported that only trace ligase IV is found unbound to XRCC4 due to the instability of free DNA ligase IV [63]. In our hands, immunodepletion of XRCC4 resulted in nearly complete removal of the ligase.

3. Results and Discussion

3.1. Determination of Preferential Reaction Conditions for DNA-PK-Dependent, and DNA-PK-Independent Nonhomologous End-Joining Pathways. In previous studies, both DNA-PK dependent and independent NHEJ pathways have been observed *in vivo* [5, 49–51, 58], however, no *in vitro* reaction conditions in the presence of a functional DNA-PK$_{cs}$ protein have been reported to date that favor DNA-PK-independent DNA end joining. Several studies have noted the role of polymers such as PEG in stabilizing and enhancing the binding of proteins to the DNA through a macromolecular crowding effect [64]. Earlier works had shown that in the presence of high concentrations of macromolecules such as PEG, T4 DNA ligase as well as DNA ligase preparations from rat liver nuclei or from Escherichia coli actively catalyze blunt end ligations, in contrast to the poor activity of these enzymes on such substrates under conventional assay conditions [65]. These and other studies have argued that

such macromolecular crowding or confinement may perhaps play a more essential role in cell biology and physiology than otherwise noted and could well be a more adequate model for intracellular, *in vivo* conditions [66].

We observe that, in the presence of 5% polyethylene glycol (PEG), DNA end joining is insensitive to wortmannin, a potent and selective inhibitor of DNA-PK [29] that covalently binds to the protein. Whole cell extracts (WCE) from HeLa, WI-38, and M059K cells were assayed for DNA end-joining activity with and without 5% PEG and 10 μM wortmannin. The WCEs were assayed for the ability to end-join blunt-end *Stu*I-cut plasmid DNA (which we have previously shown to be NHEJ-dependent [67]) and produce plasmid dimers and trimers that were detected on agarose gels stained with Vistra Green (Figure 1(a)). After 2 hrs at 30°C in the absence of PEG, the WCEs generated 5 to 25% product, depending on cell type (Figure 1(b)). The same reactions run with 10 μM wortmannin reduced the total amount of product to between 0 to 4%, also depending on cell type. The assay was repeated in the presence of 5% PEG, resulting in 12 to 24% product in the absence of wortmannin. For HeLa and WI-38 WCEs, product yield increased up to 2-fold with the addition of PEG, while addition of 10 μM wortmannin resulted in little or no decrease in product. However, an approximately 60% decrease in product was observed with M059K WCE in the presence of PEG and wortmannin, which though significantly less than the wortmannin inhibition observed with this extract in reactions without PEG still indicated that a fraction of the NHEJ was wortmannin-sensitive even in the presence of PEG. It could be argued that a residual amount of the end-joined product is due to DNA-PK-dependent NHEJ or that alternatively, DNA-PK_{cs} may be playing a structural and/or stimulatory role in the organization of the initial NHEJ complex [43].

The end-joining reactions were repeated with HeLa WCE, 1% DMSO, and 0.1 to 10 μM wortmannin in 1% DMSO (Figure 2(a)). Complete inhibition of DNA end joining was observed in the absence of PEG with 1.5 μM wortmannin. In contrast, in the presence of 5% PEG, only a 15 to 20% reduction in product was observed with 1.5 μM wortmannin. No further inhibition was observed with up to 10 μM wortmannin. To determine the effective concentration of PEG required to favor wortmannin-insensitive NHEJ, the DNA end-joining assay was repeated for 1 hr at 30°C with 0 to 5% PEG with and without 10 μM wortmannin (Figure 2(b)). In the absence of wortmannin, a steady increase in product formation was observed with the addition of up to 4% PEG, after which, a trend towards a small decrease in product formation was observed. Conversely, in the presence of wortmannin, a minimum of 3% PEG was required for the generation of end-joined product and at 5% PEG, little or no wortmannin inhibition was observed. Presumably, at a concentration of 5% PEG, the product is being formed by a wortmannin-insensitive, DNA-PK-independent NHEJ pathway.

It has been reported previously that blunt-ended DNA activates DNA-PK less efficiently than DNA DSBs with 3′- or 5′-overhangs [68]. Therefore, we wished to confirm DNA-PK activity under our end-joining reaction conditions in the absence of PEG and wortmannin and also determine whether the loss of wortmannin sensitivity in the presence of PEG was due to the operation of a DNA-PK-independent pathway or simply due to the inability of wortmannin to inhibit DNA-PK in the presence of PEG. DNA-PK kinase activity was assayed under the same reaction conditions as those used for the end-joining assays (Figure 2(c)). Reactions were prepared in the same manner as the DNA end-joining reactions, but with the addition of 5 μg of a peptide DNA-PK-phosphorylation substrate and [γ-^{32}P] ATP. The reactions were prepared with and without DNA, 5% PEG, and 10 μM wortmannin, and incubated at 30°C for 5 minutes. As is typical with the peptide substrate-based DNA-PK activity assay, nonspecific background kinase activity is observed for samples in the absence of added activator DNA, regardless of the presence or absence of PEG. A 2.5- to 3-fold DNA-dependent increase in kinase activity was observed following addition of the blunt-ended end-joining substrate DNA, and no difference in activity was observed between reactions with and without PEG. The addition of 10 μM wortmannin inhibited all detectable kinase activity in both reactions, indicating that the PEG did not affect the ability of wortmannin to inhibit DNA-PK kinase activity. Therefore, PEG did not inhibit DNA binding, kinase activation, or wortmannin-mediated inhibition of DNA-PK. Consequently, even though DNA-PK kinase is active in the presence of PEG, DNA end joining seems to be independent of this kinase activity, since addition of 10 μM wortmannin, which completely inhibits kinase activity, does not inhibit NHEJ in the presence of PEG. Thus, in the presence of 5% PEG, *in vitro* reaction conditions are established in which the NHEJ proceeds via a DNA-PK-independent pathway or at least one that is independent of the kinase activity of DNA-PK. This result argues for a much broader role for DNA-PK in the context of the structural architecture of the initial NHEJ complex in addition to the role it plays in catalyzing phosphorylation of several other repair proteins [43, 44].

3.2. Survey of Proteins Involved in DNA-PK-Dependent and -Independent Nonhomologous End Joining. We wished to determine the contribution of individual proteins to either of the two NHEJ pathways. To this end, a variety of proteins were individually immunodepleted from the HeLa WCE and DNA end-joining assays were conducted (with and without 5% PEG) to detect depletion-dependent reductions in end-joining activity (Figure 3). Immunodepletion of Ku80 and DNA ligase IV resulted in an almost complete loss of DNA end-joining efficiency for reactions both in the presence or absence of 5% PEG. This supports our earlier observations [69] and indicates the indispensable role that Ku plays in the initial recognition and binding of DSB ends and that of ligase IV in the final ligation step of the NHEJ process. Immunodepletion of NBS1 and histone H1 resulted in a significant loss of DNA-PK-dependent DNA end-joining activity in reactions without PEG (Figure 3(a)). In comparison, in reactions with 5% PEG, only a minimal loss of presumed DNA-PK-independent activity was observed with immunodepletion of NBS1 and histone H1

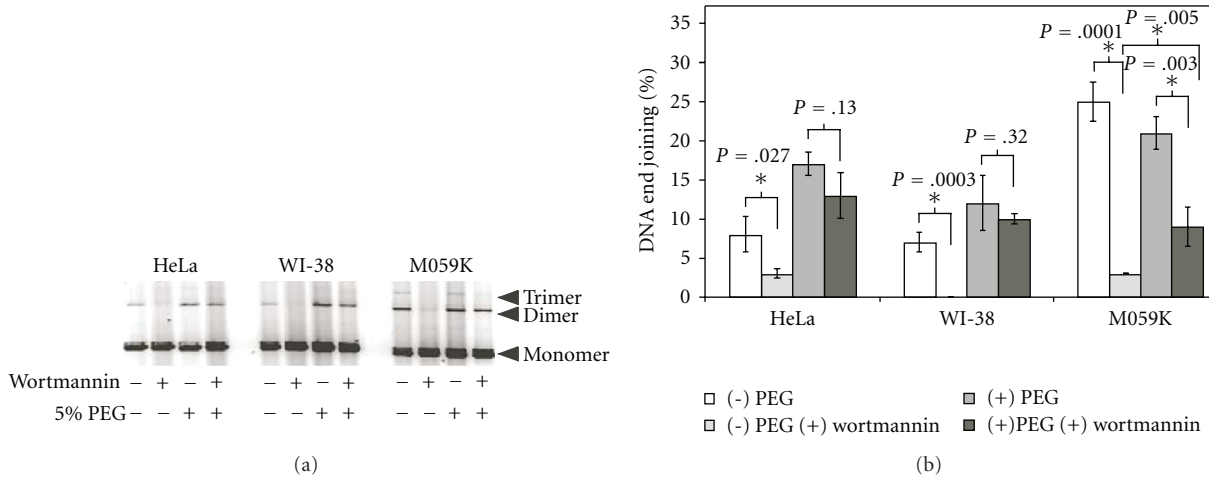

Figure 1: DNA end joining with HeLa, WI-38, and M059K WCEs with and without 5% PEG and/or 10 μM wortmannin. (a) Agarose gel image showing typical DNA end-joining reaction results. Dimer and trimer DNA end-joining product yields are calculated as a fraction of the total DNA in the lane and reported graphically as shown in panel B. (b) Graphical depiction of results for triplicate end-joining reactions run with the three cell extracts under each of the conditions indicated. Error bars indicate the standard deviation for triplicate reactions. Significant differences, as determined by the Student t-test, are indicated by an asterisk. The P-values for each pairing are given above the brackets.

(Figure 3(b)). This is an interesting result in the context of the supposed stimulatory role of NBS1 and histone H1 in the backup pathways of NHEJ, where histone H1 enhances the activity of both DNA ligase III and PARP-1 [60]. Immunodepletion of DNA-PK$_{cs}$ resulted in a 30% reduction in the efficiency of the end-joining reactions without PEG, with little reduction observed in the presence of PEG. In addition, immunodepletion of Poly (ADP-ribose) polymerase 1 (PARP) and DNA ligase III consistently resulted in a trend toward increased efficiency for both the presumed DNA-PK-dependent and -independent NHEJ over the course of multiple experiments. This observation suggests the operation of backup pathways under these conditions and supports the hypothesis that these proteins may compete for repair at the DSB ends through alternate NHEJ pathways [58, 59]. Perrault et al. reported that DNA-PK independent DNA end joining is observed after immunodepletion of DNA-PK$_{cs}$ [54]. To confirm that the DNA end joining observed after immunodepletion of DNA-PK$_{cs}$ is actually DNA-PK-independent, the immunodepleted extract was assayed for activity with and without 10 μM wortmannin (Figure 3(c)). End joining by the WCE was inhibited in the presence of 10 μM wortmannin; whereas the DNA end-joining activity of the DNA-PK$_{cs}$ immunodepleted extract was wortmannin-insensitive, indicating that a DNA-PK-independent process formed the product.

From an examination of the immunodepletion data, only Ku and ligase IV-XRCC4 complex could be specifically identified as participating in the DNA-PK-independent NHEJ in this system, while Ku, DNA-PK$_{cs}$, ligase IV-XRCC4, NBS1, and histone H1 are implicated in the DNA-PK-dependent NHEJ. These results support a previous model proposed by Riballo et al., in which one pathway consists of Ku and ligase IV-XRCC4 that can be stimulated by DNA-PK$_{cs}$, and a second, DNA-PK$_{cs}$-dependent pathway that requires NBS1 [18].

3.3. The In Vitro DNA-PK-Dependent and -Independent Nonhomologous End-Joining Pathways Exhibit Different Optimal Reaction Conditions.. Several groups have reported *in vitro* assays for DNA DSB repair but reaction conditions differ considerably. To investigate if reaction conditions could be established for the end-joining assay that favor one pathway over the other, we tested the DNA end-joining activity of HeLa WCE with and without 5% PEG under a variety of reaction conditions. Two separate buffers were used. Buffer A is composed of 50 mM Tris-HCl, pH 7.6, and 75 mM KOAc along with 5 mM $MgCl_2$, 1 mM DTT, and a protease inhibitor cocktail. Buffer B includes bis-tris-propane, pH 8.2, and 75 mM KCl with 5 mM $MgCl_2$, 1 mM DTT, and a protease inhibitor cocktail as before (see Materials and Methods for details). DSB end joining with HeLa WCE under these two conditions were measured over time with and without 5% PEG. In the absence of PEG, buffer A produced a small but consistently higher yield of DNA end-joining products over time compared to reactions in buffer B, and under both conditions, a linear increase in product was generated during the first 5 hours (data not shown). With PEG, an increase in end joining was observed with buffer B compared to buffer A (Figure 4(a)). However, under both conditions, product formation appeared to plateau after about 3 hours. Buffer A therefore, seemed to provide the most optimal reaction conditions for the DNA-PK-dependent (without PEG) end-joining whereas Buffer B seemed to provide the same for the DNA-PK-independent (with PEG) end-joining pathway in our system.

This observation was also reported by Ramsden et al., who demonstrated a modest increase in Ku-dependent ligation of DNA lacking DNA-PK$_{cs}$ on increasing the

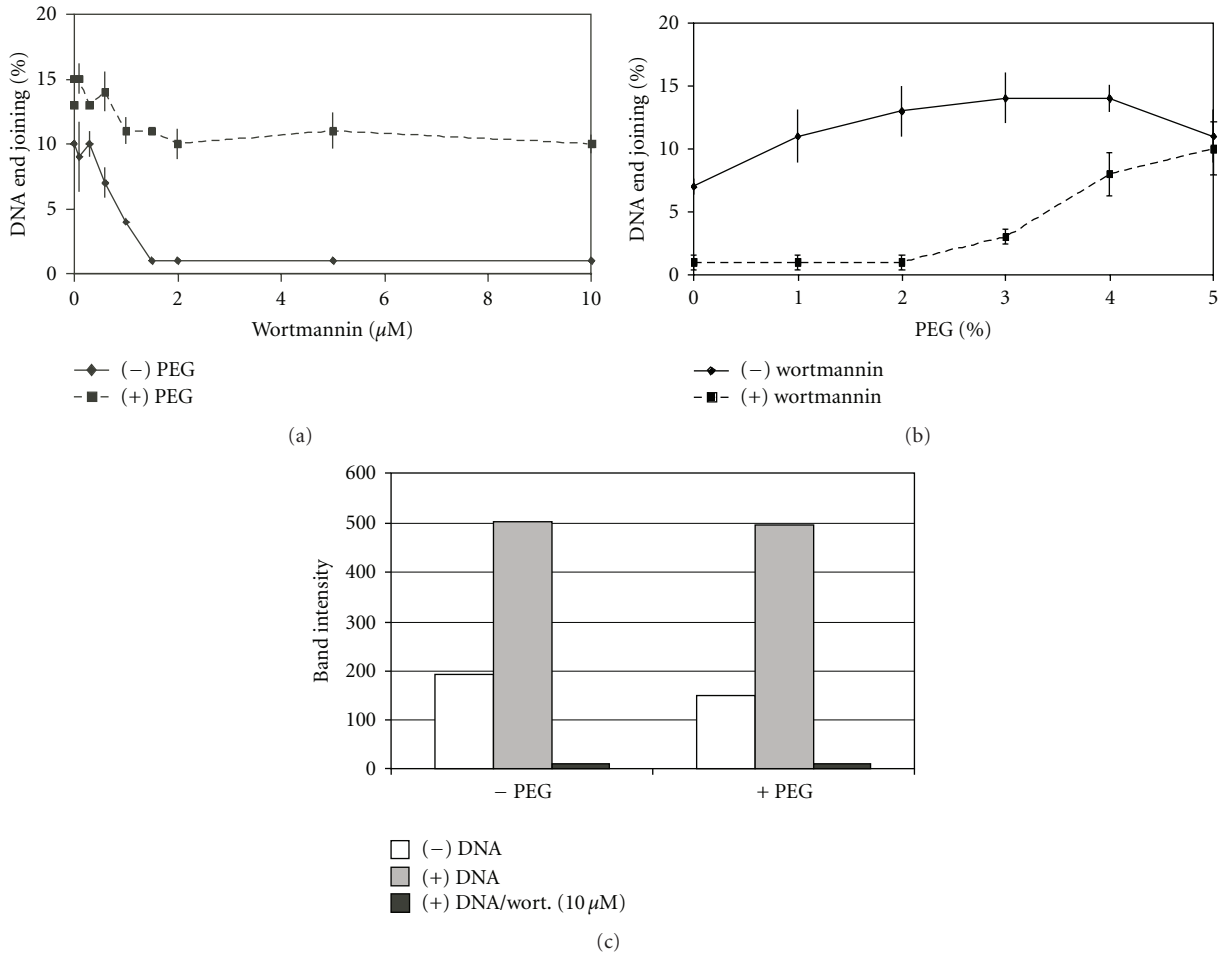

Figure 2: DNA end-joining activity and DNA-PK activity responses in PEG and/or wortmannin. (a) Wortmannin dose response. DNA end-joining reactions were run using HeLa WCE under standard conditions with or without 5% PEG and wortmannin for 1 hr at 30°C. Reactions contained 0 to 10 μM wortmannin in 1% DMSO. Error bars indicate the standard deviation for triplicate reactions. (b) PEG dependence of wortmannin sensitivity. DNA end-joining reactions were run in triplicate with and without 10 μM wortmannin at 0–5% PEG. (c) DNA-PK kinase activity under DNA end-joining reaction conditions in the presence and absence of PEG. Standard DNA end-joining reactions were run with HeLa WCE for 10 min at 30°C in the presence of a DNA-PK peptide substrate and [γ-^{32}P] ATP. The peptide was isolated on a 16% PAGE gel and the intensity of the radiolabeled band, a measure of DNA-PK kinase activity, was detected by autoradiography. The presence or absence of 5% PEG during the end-joining reaction did not affect DNA-PK kinase activity, nor did it affect the ability of wortmannin to inhibit DNA-PK kinase activity. The mean activity of duplicate reactions is plotted.

concentration of KCl from 25 mM to 120 mM [70]. High concentrations of chloride have been reported earlier to reduce protein-DNA interactions [71, 72]. Of the NHEJ proteins, only Ku can bind directly to DNA in 75 mM KCl [73]. However, since both the NHEJ-dependent and -independent pathways utilize Ku, a reduction in the number of nonspecific protein-DNA interactions competing with Ku for the DNA ends should enhance both NHEJ pathways. However, high-chloride concentrations can also inhibit protein-protein interactions and have indeed been shown to inhibit DNA-PK holoenzyme formation [72, 74].

Variable concentrations of the divalent cations Mg^{2+} and Mn^{2+} were also examined for their effect on DNA end-joining activity. Increasing $MgCl_2$ concentration from its optimum at 5 mM, up to a final concentration of 10 mM (by steps of 1, 2, and 5 mM additions of $MgCl_2$) had little effect on DNA end-joining activity with or without PEG (data not shown). Addition of up to 5 mM $MnCl_2$ in addition to the 5 mM $MgCl_2$ already in the standard reaction buffers, increased DNA-PK-dependent end-joining activity (without PEG) and reduced DNA-PK-independent end joining (with PEG) (as shown in Figure 4(b)). To further study the effect of manganese on DNA end joining, various concentrations of $MnCl_2$ were added to reactions with 5% PEG and 10 μM wortmannin (Figure 4(c)). As observed previously in the absence of $MnCl_2$ and presence of PEG, a small decrease in DNA end-joining activity occurred with the addition of 10 μM wortmannin. Without wortmannin, DNA end-joining activity was reduced at low $MnCl_2$ concentrations but partially recovered with the addition of 0.5 to 1 mM $MnCl_2$. This recovery in activity, however, was inhibited by wortmannin, indicating that even in the presence of

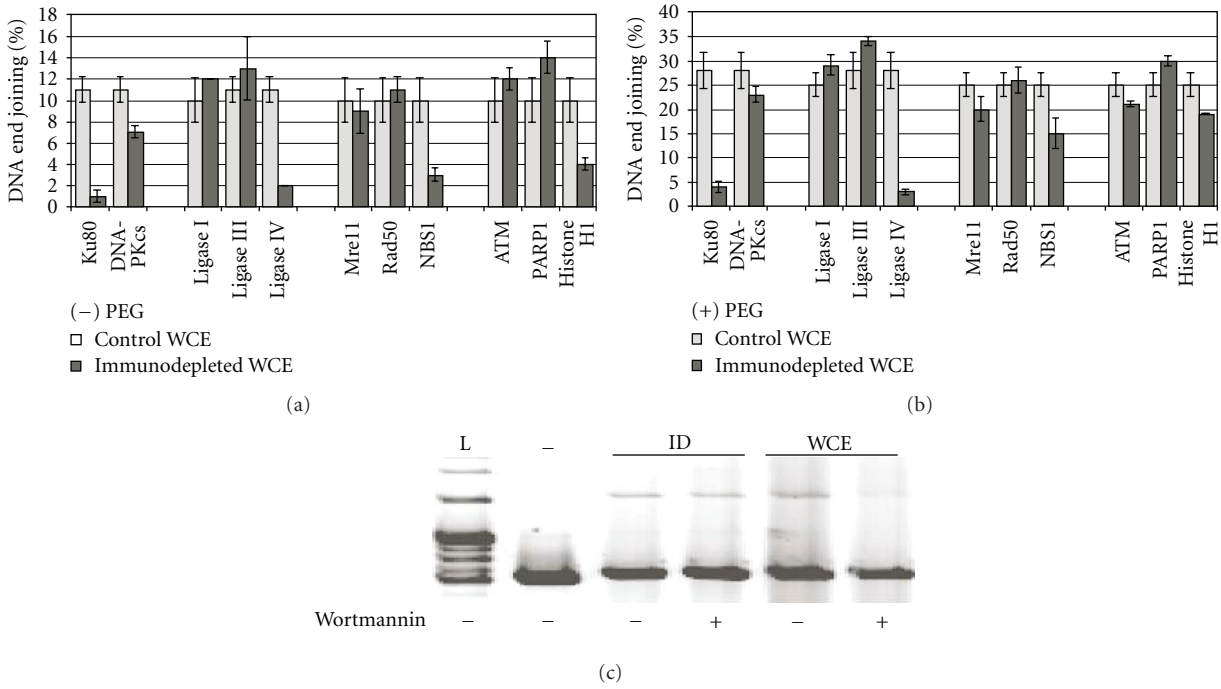

Figure 3: DNA end joining with immunodepleted extracts. HeLa WCE was immunodepleted for the various potential NHEJ proteins indicated, and immunodepletion (≥ 3-fold) of the target protein was confirmed by western blot (data not shown). The individual immunodepleted extracts were then assayed for DNA end-joining activity at 30°C for 2 hrs. (a) Results of DNA end-joining reactions performed in the absence of 5% PEG. (b) Results of DNA end-joining reactions performed in the presence of 5% PEG. All reactions were performed in triplicate and error bars indicate the standard deviation. (c) Wortmannin-insensitive DNA end joining is detectable in the DNA-PK_{cs} immunodepleted HeLa WCE in the absence of PEG. Reactions were run in the absence of 5% PEG and where indicated, in the presence of 10 μM wortmannin at 30°C for 2 hrs. (L) T4 DNA ligase positive control; (−) negative control; (ID) DNA-PK_{cs}-immunodepleted WCE.

5% PEG, a small fraction of the observed DNA end-joining activity results from the DNA-PK-dependent pathway or at least one that is dependent on the DNA-PK kinase activity. Furthermore, the addition of $MnCl_2$ increases the activity of this wortmannin-sensitive pathway. Previous studies have shown that one factor that could alter the relative influence of a particular end-joining pathway in the reaction is the concentration of divalent cations, particularly Mg^{2+} and Mn^{2+} [75]. We observe that the DNA-PK-dependent NHEJ activity present in our system is sensitive to the reaction concentration of Mg^{2+} and particularly, Mn^{2+}. Elevated concentrations of these divalent cations stimulate overall end-joining activity and mask the requirement for DNA-PK, suggesting the involvement of a DNA-PK-independent NHEJ pathway. Thus, the relative contribution of a particular pathway to the overall end-joining activity observed in WCEs seems to depend on, and in turn reflect, the *in vitro* reaction conditions used. Taken as a whole, differential reaction buffer preference and variable responses to divalent cations observed in this study emphasize the existence of distinct biochemical differences between the DNA-PK-independent and -dependent NHEJ activities observed in the presence and absence of PEG, respectively.

3.4. Functional Changes Are Associated with DNA-PK-Dependent and -Independent Reaction Conditions. Previous studies have suggested that DNA-PK-dependent and -independent repair pathways may be functionally distinct, possibly preferentially interacting with certain subclasses of DNA DSBs and/or having different DSB repair fidelity [18, 49–51, 58]. We therefore chose to investigate DSB repair fidelity under our DNA-PK-dependent and -independent reaction conditions. To test DSB repair fidelity, the ability of the HeLa WCE to accurately end-join DSBs with various DSB-end overhang configurations was determined. Standard DNA end joining assays run both with and without 5% PEG were conducted using substrate plasmid DNA that had been linearized by restriction digestion with, StuI (blunt ends), EcoRI (4 nucleotide 5′-overhang), Hin1I (2 nucleotide 5′-overhang), or PvuI (2 nucleotide 3′-overhang). The products of these end-joining reactions were then subjected to redigestion with their corresponding restriction enzyme (Figure 5). Accurate DSB end-joining restores the enzyme recognition sequence at the end-joining junction sites, resulting in product DNA that is susceptible to recutting with the restriction enzyme originally used to linearize the plasmid. DSB repair fidelity is defined as the frequency with which the DNA end-joining assays accurately join DSB ends, and is reported here as the percent of total end-joined product DNA cleaved following redigestion with the appropriate restriction enzyme. As shown in Figure 5, substantial functional differences were detected between DSB

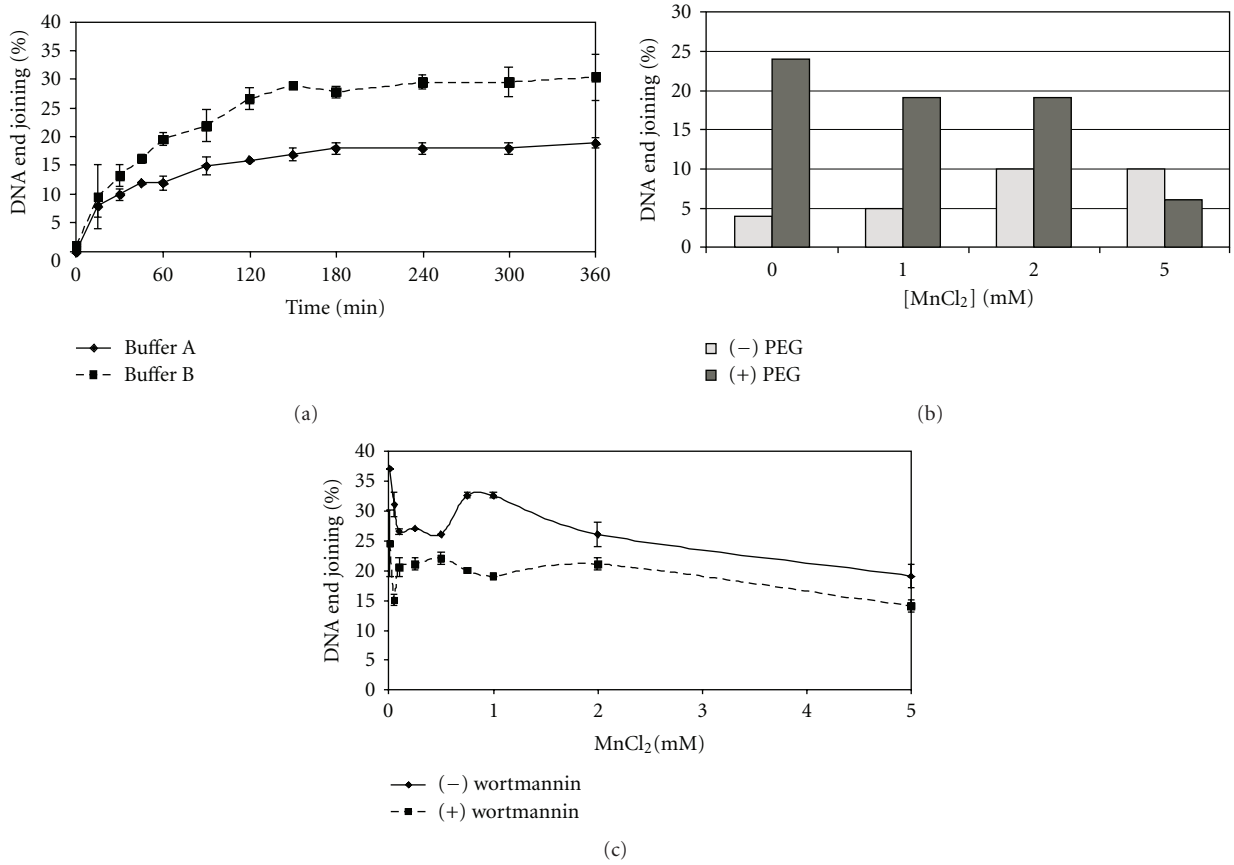

Figure 4: Effects of reaction conditions on DNA end-joining activity. (a) Reaction buffer composition effects on HeLa WCE end-joining activity time course reactions in the presence of 5% PEG. Reactions were run at 30°C for the times indicated in either of the two standard reaction buffers as described in the text. The mean values of triplicate reactions were plotted and the error bars indicate standard deviation. (b) Effects of $MnCl_2$ on HeLa WCE DNA end-joining activity. Reactions were run with the addition of 0–5 mM $MnCl_2$ to standard DNA end-joining reactions containing 5 mM $MgCl_2$ for 2 hrs at 30°C in the presence or absence of 5% PEG as indicated. (c) The DNA end-joining reactions employing $MnCl_2$ (0–5 mM) in the presence of PEG were repeated with and without 10 μM wortmannin (6 hrs at 30°C). Reactions were run in triplicate and the error bars indicate standard deviation.

repair reactions catalyzed by the DNA-PK-dependent and -independent NHEJ pathways. End joining under DNA-PK-dependent reaction conditions (without PEG) resulted in substantially higher DSB repair fidelity than reactions (with PEG) favoring the DNA-PK-independent end-joining pathway, and these results are consistent with the pathway-dependent DSB repair fidelity reported by others [51].

4. Conclusion

In summary, we have demonstrated *in vitro* assay conditions that permit coincident and differential analysis of DNA-PK-dependent and -independent NHEJ activities under conditions in which functional DNA-PK_{cs} is present. Establishing and defining these reaction conditions facilitates biochemical analysis of these important subpathways of NHEJ regardless of the cellular source of enzyme activities, and irrespective of intrinsic DNA-PK_{cs} expression status. We found that reactions containing 5% PEG favored DNA-PK-independent NHEJ while reactions lacking PEG favored DNA-PK-dependent NHEJ. The biochemically distinct nature of the pathways represented by these two reaction conditions is borne out by the differential end-joining activity observed in response to the DNA-PK inhibitor wortmannin, immunodepletion of individual proteins that may participate in NHEJ, and the pathway specific responses to divalent cations, and reaction buffer composition.

In addition to these results that indicate the biochemically distinct nature of the end-joining mechanisms represented by the DNA-PK-dependent and -independent NHEJ assays, we also observe functional differences between the two pathways. We find that DNA-PK-dependent DSB end joining is a higher fidelity process than DNA-PK-independent end joining. This latter finding is consistent with *in vivo* results reported by others using cell lines that lack expression of DNA-PK_{cs} [51, 52, 54], or following depletion of DNA-PK_{cs} from extracts of cells that do express the protein [54].

In vitro end joining of restriction enzyme-cut plasmid DNA is routinely reported as a measure of NHEJ activity, yet these reports are often conflicting with respect to what enzymes are involved in the repair of DNA DSBs. Our

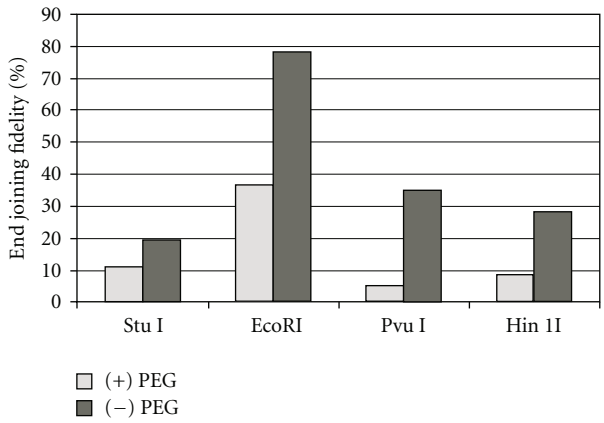

Figure 5: DNA end-joining fidelity reactions run in the presence or absence of 5% PEG. DNA DSB end joining was performed with each of the four different restriction enzyme linearized plasmid DNA substrates indicated to assess the effects of different DSB overhang configurations (see text) upon the fidelity of DSB repair occurring under DNA-PK-independent and -dependent reaction conditions. DSB repair fidelity is measured as the fraction of total DNA end-joining reaction products that are capable of being recut with the restriction enzyme that was originally used to linearize the end-joining substrate DNA. All reactions were run in triplicate. Bars represent the average restriction enzyme redigestion efficiency of the end-joining reaction products, which is reported as % fidelity.

results indicate that multiple pathways may simultaneously contribute to the production of linear plasmid multimers *in vitro*. Consequently, the ability to selectively shift the mechanism of product formation by altering reaction conditions not only suggests the need for care when evaluating data obtained by the wide variety of *in vitro* DSB repair assays currently in use, but also provides a means by which greater control may be achieved over the repair mechanism through which this end point is reached.

Application of the reaction conditions described in this report may permit concurrent investigations of the relative contributions of DNA-PK-dependent and -independent NHEJ pathways to DSB repair in any mammalian cell. This approach could be helpful in identifying proteins involved in the DNA-PK-dependent and -independent NHEJ DSB repair subpathways, and characterizing their individual roles in these multiprotein repair complexes. Information such as this is likely to be useful in identifying new and more effective approaches for manipulating cellular DSB repair activity.

Abbreviations

DSB:	Double-strand break.
DNA-PK:	DNA-protein kinase.
DNA-PK$_{cs}$:	DNA-protein kinase catalytic subunit.
NHEJ:	nonhomologous end joining.
DMSO:	dimethylsulfoxide.
PEG:	polyethylene glycol.
WCE:	whole cell extract.

Acknowledgment

This research was supported in part by the Intramural Research Program of the NIH, through the Warren Grant Magnuson Clinical Center.

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