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The Many Roles of DNA-PKcs Phosphorylation In Facilitating DNA Double Strand Break Repair

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THE MANY ROLES OF DNA-PKcs PHOSPHORYLATION IN FACILITATING DNA DOUBLE STRAND BREAK REPAIR

By

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ABSTRACT

DNA double strand breaks (DSBs) are dangerous lesions that can have profound consequences, including genome instability, chromosomal rearrangements, or the activation of apoptotic pathways. Yet, these types of lesions are not uncommon, as cells that undergo meiotic recombination or DNA replication often encounter double strand breaks. In addition, DSBs are created as a result of oxidative reactions (radical oxygen species), ionizing radiation (x-rays, gamma rays), or chemotherapy drugs. Hence, it is critical that DSBs are repaired. Indeed, DNA DSB repair pathways are conserved in all forms of life: homologous recombination (HR) and non-homologous end joining (NHEJ).

DNA-PKcs is a central component of the NHEJ pathway in eukaryotes higher than the yeast. It is an extremely large serine and threonine protein kinase, and a member of the phosphatidylinositol 3-kinase-like kinase (PIKK) subfamily. Autophosphorylation of DNA-PKcs is essential to the NHEJ pathway. Many labs, including ours, use site-directed mutagenesis studies to elucidate the roles of DNA-PKcs autophosphorylation in facilitating DNA double strand break repair. In particular, we want to identify the autophosphorylation site that is responsible for kinase dissociation, whereby DNA-PKcs loses all of its kinase activities and releases Ku-bound DNA, presumed to be the end of the NHEJ pathway. In this dissertation, I will describe the relevance of 7 putative phosphorylation sites in facilitating kinase dissociation.

Furthermore, I will demonstrate that DNA-PKcs phosphorylation has an important role in regulating DNA repair pathway choice. Free DNA ends from DSBs are

always bound and protected by DNA-PKcs and Ku proteins, which ultimately direct repair toward the NHEJ pathway in mammalian cells. Here, I will show that the phosphorylation of T946 and S1004 of DNA-PKcs inhibits NHEJ while promoting HR.

DEDICATION

To my family, who, through their love and understanding, gave me strength and commitment to pursue my potential in science.

To the future generations, and my children, who will live longer, healthier lives.

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Above all, I thank friends and family, who supported me through the years of my pursuit in higher education, who witnessed my frustrations and would not let me give up, and who believed in all good things about me (and none of the bad). For without them, I would not have written this dissertation.

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LIST OF ABBREVIATIONS

aa	amino acids
ALA	alanine
APH	Aphidicolin
ASP	aspartic acid
ATM	Ataxia telangiectasia mutated kinase
ATR	Ataxia telangiectasia-rad3-related kinase
BRCA	Breast Cancer susceptibility protein
CHO	Chinese hamster ovarian
CtIP	CtBP-interacting protein
DNA-PK	DNA-dependent protein kinase
DNA-PKcs	DNA-dependent protein kinase catalytic subunit
dsDNA	double stranded DNA
DSB	double strand break
H2AX	Histone H2A variant
γH2AX	phosphorylated H2AX
HR	homologous recombination
IR	ionizing radiation
IRF	irradiation-induced foci
LZ	leucine zipper
MS	mass spectrometry

MRN	MRE11/RAD50/NBS1
NBS1	Nijmegen breakage syndrome protein 1
NHEJ	non-homologous end joining
OA	Okadaic acid
PI	protease inhibitor
PIKK	phosphatidylinositol 3-kinase-like kinase
PP2A	protein phosphatase 2A
RAG	recombination activating gene
RPA	Replication Protein A
RSS	recombination signal sequence
ssDNA	single-stranded DNA
XRCC	X-ray repair cross complementing (*XRCC*) gene
Zeo	Zeocin

CHAPTER 1

Literature Review

DNA double strand breaks

For all cells, a DNA double strand break (DSB) is a dangerous lesion that can have profound consequences, including genomic instability, chromosomal rearrangements, and cell death. Yet, DNA DSBs are not uncommon occurrences, as they do arise during the physiological progression of the cell cycle, such as DNA replication, meiotic exchange, and stalled or collapsed replication forks. In addition, V(D)J recombination, essential to vertebrate lymphoid cell development, is a process that relies on site-specific DSBs generated by recombinase activating gene products (RAG1/2). More clinically significant, perhaps, are DNA DSBs that result from pathological factors, such as ionizing radiation (x-rays, gamma rays), oxidizing metabolites (free radicals),

radiomimetics (bleomycin, zeocin), and topoisomerase inhibitors (etoposide). In order to prevent chromosome instability and cell death, all cells, from bacteria to man, have evolved two major repair mechanisms: non-homologous end joining (NHEJ) and the homologous recombination (HR) pathways.

In prokaryotes and yeast, HR is the primary mode of DSB repair. The NHEJ pathway in bacteria and yeast consists of homologues of Ku and DNA Ligase IV (Aravind and Koonin, 2001; Pastwa and Blasiak, 2003), but not DNA-PKcs, which developed later, and appears to specifically function in NHEJ of higher eukaryotes (Hiom, 2003). In higher eukaryotes, the HR pathway plays a minor role to NHEJ in DSB repair although the precise reason for this is not clear. HR is limited to the late S/G2 phase of the cell cycle (Rothkamm et al., 2003; Sonoda et al., 2006) whereas NHEJ is functional in all phases of the cell cycle (Hinz et al., 2005; Jeggo and Lobrich, 2006). Since most higher eukaryotic cells do not enter the cell cycle, it makes sense that the NHEJ pathway should dominate in those cells. However, a fundamental question that has remained largely unanswered is how pathway choice is regulated in higher eukaryotes. This topic will be further explored in chapter 3 of this dissertation.

Fidelity of DSB repair mechanisms

An important distinction between HR and NHEJ is the fidelity of their repaired product to the original sequences. The HR pathway is considered "error-free" because it uses a template to prime the repair synthesis, whereas the NHEJ pathway is a re-ligation mechanism independent of template, and is error-prone. Studies from murine models demonstrate that both pathways are important for maintaining genome stability (Ferguson

and Alt, 2001) and that the absence of either pathway leads to genomic instability, including potentially oncogenic translocations, and lowers the threshold for lymphomagenesis (Difilippantonio et al., 2002; Pierce et al., 2001; Roth, 2002).

The NHEJ pathway, which relies on a handful of core factors, including Ku70, Ku86, DNA-PKcs, XRCC4, XLF, and DNA Ligase IV, readily rejoin two ends of the DNA backbone with the use of short overlapping segments of 1-4 nucleotides (microhomologies) at the 3' ends of DNA (Roth and Wilson, 1986). Theoretically, this process can be faithful to the original sequence. However, DNA DSBs resulting from IR or oxidative metabolites consist of chemically modified or damaged ends that necessitate processing before they can be religated (Ma et al., 2002). During DNA processing, ends are trimmed off by nucleases and a few nucleotides can be inserted by polymerases mu and lambda independent of template to facilitate joint stability (Kovalchuk et al., 2004). Therefore, joints repaired by NHEJ are generally associated with small deletions and additions of several nucleotides (Lieber et al., 2003), thereby changing the sequence of DNA. In fact, the variability that result from end processing is an important feature of V(D)J recombination, whereby antigen receptors gain additional diversity.

In contrast, repair by HR relies on copying information from a sister chromatid or homologous chromosome, resulting in a product that is error-free. Repair by HR is crucial during stalled DNA replication fork to ensure faithful replication (Lundin et al., 2002; Saleh-Gohari et al., 2005). While precise repair would be ideal for any situation, the use of the HR for DSB repair is hardly practical in the multi-cellular organisms, which have cell populations in G0/G1 rather than in constant cycles of mitosis. NHEJ is

the predominant pathway for DSB repair in eukaryotes higher than the yeast, probably first evolved from practicality.

Cell cycle checkpoint signaling

Damaged DNA activate "sensor proteins" that relay and amplify the damage signal to various effector proteins which in turn arrest cell cycle progression and activate chromatin restructuring and DNA repair. The most important DNA DSB sensor proteins belong to a structurally unique family of protein serine-threonine kinases whose catalytic domains share a clear evolutionary relationship with those of mammalian and yeast phosphoinositide 3-kinases (PI-3K), called the phosphatidylinositol 3-kinase-like kinase (PIKK) subfamily: ataxia telangiectasia mutated (ATM), ATM and Rad3 related (ATR), and DNA-dependent protein kinase catalytic subunit (DNA-PKcs) (Abraham, 2001). While ATM is considered the main DNA damage signaling protein, it should be noted that there is significant overlapping of activities and cross-talk between ATM, ATR, and DNA-PKcs.

ATM was first identified in cells from patients with an autosomal recessive disorder characterized by progressive neuronal degeneration, immunodeficiency, sterility, and cancer predisposition (Shiloh, 2001). ATM-deficient cells (A-T) displayed significant defects in the G1, S, and G2 checkpoints. One of ATM's most important targets is the tumor suppressor protein, p53. However, ATM is known to phosphorylate many other proteins with roles in cell signaling, including Artemis, MDC1, NBS1, and Chk2 (Kobayashi et al., 2008).

A-T cells exhibit a delayed and reduced level of p53 protein accumulation following exposure to IR (Kastan et al., 1992) and a significant decrease in phosphorylation of Ser 15 on p53 protein (Canman et al., 1998; Siliciano et al., 1997). While there are reports of ATR (Tibbetts et al., 1999) and DNA-PKcs (Lees-Miller et al., 1992) phosphorylating p53 at Ser15, it is generally accepted that ATM is the major Ser15 kinase, especially during acute response to IR (Tibbetts et al., 1999). ATM has been shown to target numerous proteins, each signaling an extensive network of downstream substrates, thereby coordinating the optimal response for the cell to DNA damage, whether it be repair by NHEJ or HR, or apoptosis, as reviewed in (Kurz and Lees-Miller, 2004) and (Kastan, 2008).

Deficiencies in the non-homologous end joining pathway

The NHEJ pathway is mediated by 7 evolutionarily conserved and non-redundant core proteins: Ku70, Ku86 (also referred to as Ku80), DNA-PKcs (DNA-dependent protein kinase catalytic subunit), Artemis, XRCC4, DNA ligase IV, and XLF (XRCC4-like factor, also referred to as Cernunnos). The loss of any one of the core proteins obliterates the NHEJ pathway, and results in an individual with severe combine immunodeficiency (SCID) disease due to the absence of mature B and T cells, and ionizing radiation sensitivity.

It should be noted that while there have been human cases of Artemis and XLF deficiencies; most of our understanding of NHEJ factor deficiencies are from animal studies. In mice, the loss of DNA-PKcs or Artemis leads to the least severe phenotype, as there are generally no other defects other those that associated with NHEJ deficiency.

However, more recent reports have shown evidence of accelerated ageing phenotypes associated with DNA-PKcs deficiency (Espejel et al., 2004). In contrast, the phenotype associated with Ku deficiency in mice are qualitatively more severe than DNA-PKcs deficient mice, which included growth retardation, and dwarfism in addition to SCID syndromes due to its function in other pathways that regulate development (Gu et al., 1997; Nussenzweig et al., 1996).

Deficiencies in XRCC4 and DNA Ligase IV, essential in sealing the phosphodiester backbone of DNA, present the most severe phenotype, with embryonic lethality and massive neuronal cell death by the p53 apoptotic pathway (Frank et al., 2000; Gao et al., 2000). Neuronal cells are more sensitive to DSBs during development, and cells deficient in XRCC4, Ligase IV, XLF, and Ku, (Gu et al., 2000) present various degrees of neuronal deficiencies. The most recent factor uncovered, XLF, was initially identified in patients exhibiting severe growth retardation, dystrophy, and microcephaly (Buck et al., 2006) in addition to symptoms associated with NHEJ defects.

The current model of the NHEJ pathway

A model of the NHEJ pathway is shown in figure 1 (published in Meek, Gupta et al. 2004) and is discussed in detail below.

The initiator: Ku70/86

In the mammalian system, the NHEJ pathway is initiated upon the binding of Ku70/86 heterodimer to DNA ends. The Ku molecule, an abundant nuclear heterodimeric protein consisting of the Ku70 and Ku86 subunits, manifests as an open

ring structure (Mimori et al., 1986; Wu and Lieber, 1996). In response to ionizing radiation (IR), Ku uses its hollow center to bind broken DNA ends with high affinity (Jin and Weaver, 1997; Walker et al., 2001). DNA-bound Ku then vigorously recruits DNA-PKcs to the damaged site to form the catalytically active holoenzyme, DNA-PK (Taccioli et al., 1998).

Bringing essential factors together: DNA-PKcs

The DNA-dependent protein kinase catalytic subunit (DNA-PKcs) is a large protein of 465 kDa and a member of the serine/threonine kinase family, phosphoinositide 3 kinase related protein kinase subfamily (PIKK). Indeed, DNA-PKcs is an avid kinase protein and is known to phosphorylate many of the factors in the NHEJ pathway, including Ku70/86 (Douglas et al., 2005), XRCC4 (Calsou et al., 2003; Lee et al., 2004; Yu et al., 2003), DNA Ligase IV (Wang et al., 2004), and itself (Cui et al., 2005; Ding et al., 2003). However, with the exception of itself, phosphorylation of the said NHEJ factors does not appear to be required for NHEJ. Instead, the immense size of DNA-PKcs and its early localization to DNA double strand breaks have implicated its function as a scaffold upon which the DNA repair enzymatic machinery assembles (Meek et al., 2004).

DNA-PKcs has a leucine rich region (LRR) that has been shown to give the protein an innate ability to bind to DNA (Gupta and Meek, 2005). However, the presence of Ku bound to DNA ends significantly increases this affinity toward DSBs that is essential for DNA binding *in vivo* (West et al., 1998). Once DNA-PKcs binds to DNA-bound Ku70/86, it becomes activated and undergoes an autophosphorylation-induced

conformational change such that its contact with DNA ends are more intimate, whereby DNA ends are more deeply embedded in DNA-PKcs (DeFazio et al., 2002; Spagnolo et al., 2006). Furthermore, the binding of DNA-PKcs to Ku70/86 heterodimer also forces the Ku ring structure to translocate inward by at least one helical turn of the DNA (Yoo and Dynan, 1999). The result is the formation of a large complex, called DNA-PK, that not only actively promotes the NHEJ pathway by recruiting other NHEJ factors, but also effectively protects DNA ends from non-NHEJ factors (Smith and Jackson, 1999).

The NHEJ pathway consisting of homologues of Ku and DNA Ligase IV is evolutionarily conserved from the bacteria to man (Aravind and Koonin, 2001; Pastwa and Blasiak, 2003), but DNA-PKcs developed later, and appears to specifically function in NHEJ of higher eukaryotes (Hiom, 2003).

DNA-PKcs autophosphorylation

Autophosphorylation has been shown to be essential to many of the properties of NHEJ. When activated, DNA-PKcs becomes autophosphorylated and phosphorylated at many sites, including the 16 phosphorylation residues that have been previously described (Ma et al., 2005b; Meek et al., 2007; Olsen et al., 2006; Weterings and Chen, 2007). While the phosphorylation of not all of the sites identified have been proven essential to NHEJ, the autophosphorylation of 11 sites in two well characterized clusters, the ABCDE cluster (T2609, S2612, T2620, S2624, T2638, and T2647) and the PQR cluster (S2023, S2029, S2041, S2051, S2053, and S2056) (Chan et al., 2002; Cui et al., 2005; Ding et al., 2003) have been shown to be essential to proper DNA repair by NHEJ.

In 2003, we showed that phosphorylation of ABCDE was essential to NHEJ by facilitating proper DNA end-processing (Ding et al., 2003) and release of DNA ends (Block et al., 2004; Reddy et al., 2004). Recently, it was shown that phosphorylation of ABCDE is also essential for the 5'→3' endonuclease activities of Artemis (Goodarzi et al., 2006). On the other hand, phosphorylation of PQR appears to oppose the effects of phosphorylation at ABCDE (Cui et al., 2005). While cells expressing DNA-PKcs mutants with PQR phosphorylation sites ablated are only mildly radiosensitive, as compared to the ABCDE-ablated mutants that are severely radiosensitive, phosphorylation of PQR is important for preventing excessive nucleotide losses at DNA ends (Cui et al., 2005). Importantly, we showed for the first time that by blocking phosphorylation at PQR, cells utilized the other DNA repair pathway, HR, more readily, suggestive of DNA-PKcs as a regulator of repair pathway choice (Cui et al., 2005).

Autophosphorylation is also known to cause kinase inactivation followed by dissociation of the complex from Ku-bound DNA (Chan et al., 1996; Merkle et al., 2002). However, the mechanism leading to this event, believed to be the final step of NHEJ, remains elusive. Our lab has created a DNA-PKcs construct with 13 phosphorylation sites ablated (including ABCDE, PQR, as well as two additional sites, T3950 and S3205); yet, even this construct is able to become further phosphorylated in the presence of ATP and DNA, followed by disassembly of the complex (Douglas et al., 2007), demonstrating that the autophosphorylation site responsible for kinase dissociation are not within the 13 sites examined. There are at least 20 more possible phosphorylation sites on the DNA-PKcs (as identified by mass spectrometry) whose functions have yet to be analyzed.

DNA end trimming: nucleases

Damaged DNA ends, whether they result from pathological sources or by the controlled enzymatic activities of RAG proteins during V(D)J recombination, are often incompatible for re-ligation without processing. In the first part of DNA end processing, damaged nucleotides are removed, or trimmed, by exo- and endo- nucleases. The most well studied nuclease that participates in the NHEJ pathway is a 97 kDa protein, Artemis, a member of the metallo-β-lactamase family (Ma et al., 2002; Wang et al., 2005) which forms a tight complex with DNA-PKcs *in vivo* (Lieber et al., 2003).

Studies with oligomeric substrates revealed that Artemis possesses two distinct nuclease properties: one that is constitutive, ATP-independent, and characterized by 5' → 3' exonuclease activities toward single-stranded DNA (ssDNA); and the other, which depends on the presence of the catalytically active DNA-dependent protein kinase (DNA-PK), an endonucleolytic activity that removes 5' overhangs, shortens 3' overhangs, and opens hairpin loops at the ends of coding joints (Ma et al., 2002). Recently, it was shown that the endonuclease activities of Artemis were facilitated by the autophosphorylation of the ABCDE cluster of DNA-PKcs (Goodarzi et al., 2006), consistent with our findings that phosphorylation in the ABCDE cluster is necessary for DNA end processing in living cells (Reddy et al., 2004).

Artemis was initially identified in a subset of patients exhibiting severe combined immunodeficiency (SCID) in addition to radiosensitivity (Moshous et al., 2000), characterized by defective coding joint and reduced, but precise signal joint formation during V(D)J recombination (Li et al., 2002). Subsequent studies using Artemis deficient murine models showed that Artemis was an essential factor in processing hairpin

structures of coding joints during V(D)J recombination (Li et al., 2005). However, the role of Artemis in processing DNA ends damaged by IR is substantially smaller (Darroudi et al., 2007), suggesting that DNA end processing of such ends could be provided by alternative factors yet unidentified.

Connecting noncomplementary ends: Polymerases mu and lambda

In the second part of DNA processing, non-complementary 3' DNA ends may be extended with one or two nucleotides to provide sequence microhomology – and stabilize the alignment of opposing ends – by the activities of polymerases (pol) of the X family of Polymerases, pol lambda and pol mu, in a template -dependent and -independent manner (Ma et al., 2004; Nick McElhinny et al., 2005; Nick McElhinny and Ramsden, 2004). Both pol mu (Aoufouchi et al., 2000) and pol lambda (Garcia-Diaz et al., 2002) have been shown to be widely expressed in the mammalian system, and they have been implicated in repair mechanisms other than NHEJ (Nick McElhinny and Ramsden, 2003; Tano et al., 2007; Trivedi et al., 2005). Pol mu and pol lambda compete during V(D)J recombination, however, they also have unique DNA end substrates (Nick McElhinny et al., 2005; Nick McElhinny and Ramsden, 2004). For instance, while pol mu promotes accurate rearrangement of immunoglobulin kappa light chain (Bertocci et al., 2003), pol lambda is important for the heavy chain gene rearrangement (Bertocci et al., 2006). Consequently, deficiencies in either pol mu or pol lambda do not present common phenotypes associated with NHEJ deficiencies (Bertocci et al., 2002). Instead, mice deficient in pol mu are not radiosensitive, but exhibit reduced splenic B cell population due to defective kappa-chain (Bertocci et al., 2003). On the other hand, pol lambda

deficient mice were most notable in displaying a reduction of 5 bp for either V_HDJ_{H1} or V_HDJ_{H4} for the heavy chain gene rearrangement, which is more related to the function of terminal deoxynucleotidyltransferase (TdT), another Pol X member, in inserting N-nucleotides (Bertocci et al., 2006).

It is not clear whether trimming or fill in happens first. Lieber's lab is promoting the idea that processing is flexible, that either trimming or fill in can happen first (Ma et al., 2005a).

Sealing the joints: XRCC4, DNA Ligase IV and XLF

The DNA backbone is resealed in a chemical reaction that requires the transfer of AMP by DNA ligase IV to the 5'-phosphate group of a nicked DNA strand. *In vivo*, Ligase IV is complexed with XRCC4, its obligate partner (Bryans et al., 1999; Grawunder et al., 1998) in a 1:2 stoichiometry (Dahm, 2008). In cells, the interaction of XRCC4 and Ligase IV (XL) is tight (Critchlow et al., 1997), but without the presence of Ku and activated DNA-PKcs, XL exhibits only modest ligase IV activity (Grawunder et al., 1997; Modesti et al., 1999).

Like DNA-PKcs, XL is recruited to DNA ends by Ku (Nick McElhinny et al., 2000), where it readily complexes with an activated DNA-PK (Costantini et al., 2007). Constantini et al also remarked that disassembly of the Ku/DNA ligase IV/Xrcc4 complex occurs as a result of the kinase activity of DNA-PKcs, further implicating the regulatory role of DNA-PKcs in complex dissociation.

The newest identified NHEJ core factor, XRCC4-like factor (XLF) or Cernunose, is structurally similar to XRCC4 (Andres et al., 2007; Li et al., 2008)

although its sequence is surprisingly dissimilar to XRCC4 (Ahnesorg et al., 2006). XLF interacts avidly with XRCC4. Like XRCC4, XLF exists as a homodimer and is capable of binding to DNA by itself (Hentges et al., 2006; Li et al., 2008). However, the presence of XL at the DSB promotes efficient accumulation of XLF at DNA damage sites (Wu et al., 2007). *In vitro* assays show that XLF contributes to the DNA end-to-end stability and promoting DNA end ligation of incompatible ends, those with little or no microhomology and blunt ends through its interaction with XRCC4 (Gu et al., 2007; Tsai et al., 2007).

Figure 1. Current model of NHEJ pathway.

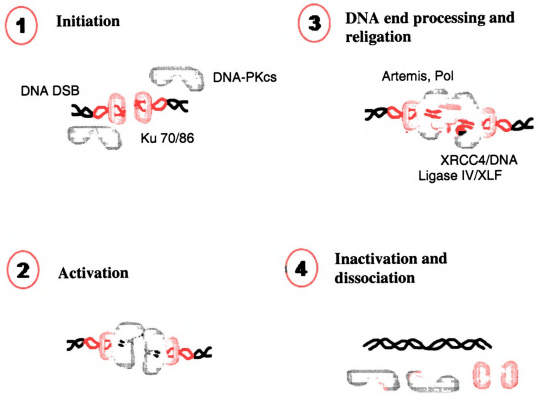
(1) In mammalian cells, DNA ends are immediately bound and protected by the Ku heterodimer, which is a ring structure, of Ku70 and Ku86 proteins. Once bound to DNA, Ku recruits the large DNA-PKcs to the DNA ends, which in effect, blocks HR factors from accessing the ends, and initiate NHEJ.

(2) Activation is characterized by the synapsis of two DNA-PKcs from each DNA end, leading to a series of phosphorylation and autophosphorylation events by DNA-PKcs.

(3) DNA ends are processed in a regulated manner by the autophosphorylation of DNA-PKcs. Nucleases, including Artemis, remove damaged nucleotides from DNA ends and open hairpin loops at the ends if there are any. Polymerase mu can add 1 non-templated nucleotide at the end while pol lambda is known to fill in gaps at DNA ends during this time to stabilize the joint for religation process. The religation step is mediated by the XRCC4/DNA Ligase IV/XLF complex.

(4) Then, in the final step, autophosphorylation of DNA-PKcs causes inactivation of its kinase abilities and the disassembly of the complex from the repaired DNA heteroduplex.

Figure 1. Current model of NHEJ



Modified from Meek, Gupta et al. 2004.

The current model of Homologous Recombination

Homologous recombination (HR) is a fundamental process conserved in all organisms that maintains genome stability and contributes to genomic diversity in evolution through its pivotal roles in the exchange of chromatids during meiosis (Haber, 1999). HR is implicated in the repair of a variety of damaged DNA, including single-stranded DNA (ssDNA) gaps, interstrand crosslinks, and DSBs. In mammalian cells, HR plays a major role for the resolution of IR-induced DSBs during the late S and G2 phases of the cell cycle (Sonoda et al., 2006), and particularly for DSBs that arise during DNA replication due to a stalled or collapsed replication fork to ensure error-free replication (Daboussi et al., 2008; Paques and Haber, 1999).

The HR pathway is much more complex than NHEJ. However, relatively little detailed information has been published on the nature of genetic changes occurring in mammalian HR-deficient cells, as compared to studies of NHEJ-deficient mouse knockout cells, because deficiencies in core HR factors are generally embryonically lethal as a result of genomic instability and apoptosis activation by the p53 pathway (Benson et al., 1998). Hence, our most intimate understanding of the homologous recombination pathway comes from studies of yeasts, *Saccharomyces cerevisia,* for which the genes RAD50, RAD51, RAD52, RAD54, RAD55, RAD57, RAD59, RDH54/TID1, MRE11, and XRS2 have been functionally identified, and their counterparts in vertebrates are being elucidated (Sung et al., 2000; Thompson and Schild, 2001).

A model of the HR pathway is shown in figure 2. A critical step in the initiation of HR involves DSB resection and ssDNA formation, which is catalyzed by the Mre11-

Rad50-Nbs1 (MRN) complex and CtIP (CtBP-interacting protein) (Sartori et al., 2007). MRN and CtIP have been shown to function together; both play a critical role in sensing DNA damage, recruit ATR, and catalyze the 5' → 3' single stranded resection that is required for repair by HR (Sartori et al., 2007; van den Bosch et al., 2003). Consequently, cells depleted of CtIP are like those deficient in MRN, which display hypersensitivity towards DSB-inducing agents, chromosome abnormalities, and an S-phase checkpoint defect (Shiloh and Kastan 2001), indicating the crucial role of HR in response to DSBs.

Single stranded DNA tails are initially coated transiently by Replication Protein A (RPA), a trimer of p70, p32, and p14 subunits (Krejci and Sung, 2002). The presence of RPA has been shown to be essential for the accumulation of RAD51 proteins at the ssDNA region (Sleeth et al., 2007). In vertebrates, the *RAD51* gene and the five RAD51 paralogs, *RAD51B, RAD51C, RAD51D, XRCC2, and XRCC3* constitute core HR factors. While they exhibit certain degrees of sequence homology to the yeast *RAD51* and bacterial *Rec*A genes, they are functionally non-redundant, and the loss of any one of them leads to extensive chromosomal instability (Baumann and West, 1998; Game, 1993; Park et al., 2008; Smiraldo et al., 2005). RAD51 binds to ssDNA and is important for the formation of a nucleoprotein filament, which has shown to be essential in homologous pairing and strand exchange reactions (Baumann and West, 1997, 1999; Shinohara et al., 1993).

In mammalian cells, HR is further regulated by proteins encoded by the tumor suppressor genes, *BRCA1* and *BRCA2* (Marquis et al., 1995). BRCA1 and BRCA2 have been shown to interact and colocalize with Rad51 (Bhattacharyya et al., 2000; Scully et

al., 1997) and MRN complex (Wu et al., 2001; Zhong et al., 1999) in response to DNA damage. Consistent with impaired DNA repair by the HR pathway, cells lacking BRCA2 exhibit a heightened sensitivity to genotoxins that are traditionally repaired by HR, such as ultraviolet light, methyl methanesulfonate (Chen et al., 1998; Sgagias et al., 2004), and DNA cross-linking agent, MMC (Moynahan et al., 2001) and cisplatin, but are only mildly more sensitive to IR, which is predominantly repaired by NHEJ (Patel et al., 1998). Studies using a chromosomally integrated I-*Sce*I – based HR substrate further confirmed that cells lacking BRCA1 or BRCA2 were unable to repair DSBs by the HR pathway, but that they were still able to utilize the NHEJ pathway (Moynahan et al., 1999; Stark et al., 2004; Tutt et al., 2001), strongly implicating the exclusive roles of BRCA1/2 in HR.

Women with germline mutations in *BRCA1* or *BRCA2* display significantly increased predisposition toward ovarian and breast cancers, and to a lesser degree, stomach cancer and leukemias/lymphomas (King et al., 2003; Risch et al., 2001). Male carriers of the *BRCA* mutations are also found to be at increased risk for cancer, particularly that of breast, prostate, and pancreas (Liede et al., 2004). The inheritance of one mutant allele invariably leads to the loss of heterozygosity, and tumors that develop frequently contain two mutant alleles (Begg, 2002; Satagopan et al., 2002).

Even though both BRCA1 (208 kDa) and Brca2 (384 kDa) mediate the HR pathway, they are not believed to be directly associated with each other *in vivo* (Wang et al., 2000) but rather with different complexes and are functionally distinct from each other (Liu and West, 2002). In brief, BRCA1 has been shown to be part of a genome surveillance complex termed BASC, which contains a number of proteins that are

important for efficient DNA repair (the MRE11/RAD50/NBS1) repair complex, and the DNA-damage-signaling protein kinase ATM, BLM helicase and replication factor-C (Wang et al., 2000). On the other hand, BRCA2 has been shown to play an intimate role in the assembly of the nucleoprotein filament by its tight association with RAD51 proteins (Davies and Pellegrini, 2007; Esashi et al., 2007; Galkin et al., 2005). In fact, a recent study demonstrated the critical role of BRCA2 in protecting RAD51 from caspase-3 proteolytic degradation, and that BRCA2 deficient cells can be completely complemented in response to IR treatment when a caspase-3 resistant RAD51 is overexpressed (Brown and Holt, 2008).

Figure 2. Current model of HR

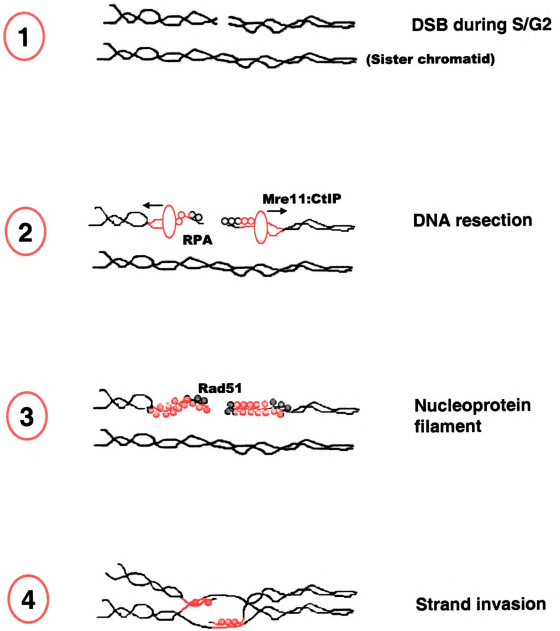
(1) The presence of DSBs does not necessarily initiate HR, especially if it occurs outside of the S to G2 phases of the cell cycle. HR is more likely to occur after DNA replication, when a sister chromatid is available to serve as a template.

(2) HR is initiated when DNA DSBs are resected into 3' ssDNA tails by the Mre11:CtIP complex. Mre11 is a DNA damage signaling protein that is known to accumulate at DSBs, and is present in all phases of the cell cycle. CtIP, on the other hand, is a recently discovered factor that is expressed in the late S and G2 phases of the cell cycle, and possesses the required 5' to 3' endonuclease activities on DNA ends.

(3) The ssDNA region is initially coated by RPA, or Replication protein A, which has been shown to interact with Mre and CtIP. At one point, RPA is replaced by Rad51 proteins, which is known to form a nucleoprotein filament of the ssDNA region. This structure catalyzes the strand invasion reaction of the ssDNA tails toward a homologous region on the sister chromatid.

(4) Then, using the homologous region as template, the missing segment is repaired by DNA synthesis.

Figure 2. Current model of HR.



V(D)J Recombination

V(D)J recombination is a cellular process unique to lymphocyte development, whereby B and T cells acquire their antigen specific receptors, immunoglobulins (B cells) and T cell receptors (T cells). The process can be roughly divided into two phases: 1) creation of DSBs and 2) religation of joints by NHEJ. During the initial step, the lymphoid-specific proteins Rag1 and Rag2 (recombination activation genes 1 and 2) (Oettinger et al., 1990; Schatz et al., 1989) recognize recombination signal sequences (RSSs), 12mers and 23mers, that flank all V, D, and J gene segments, and introduce a nick at the 5' border of RSS of each V, D, and J segment. The Rag complex then uses the 3' hydroxyl on each coding segment as a nucleophile in a transesterfication attack (McBlane et al., 1995; van Gent et al., 1996) on the antiparallel DNA strand that result in a DSB with the ends sealed like a hairpin structure (McBlane et al., 1995). While the signal ends, where the RSS are located, are usually religated directly without modification, the ends of coding joints consist of a hairpin structure, necessitating the endonuclease activity of Artemis (Moshous et al., 2001; Moshous et al., 2000). Hence, a subset of SCID patients is caused by Artemis deficiency rather than Rag deficiency.

Apoptosis

Apoptosis is a morphological description of a form of cell death that involves nuclear condensation and DNA fragmentation, executed through the activation of caspases. IR-induced apoptosis is a p53-mediated process (Lips and Kaina, 2001). The phosphorylation of Ser15 by ATM results in the stabilization and accumulation of p53, a transcription factor that activates a large panel of genes, and depending on the cellular

context, may modulate intracellular redox status, or induce the apoptosis pathway (Yu et al., 1999). Activated p53 stimulates the transcription of pro-apoptotic genes, notably *PUMA* and *NOXA* (Roos and Kaina, 2006), which further signal the cell to prepare for apoptosis. However, we still do not know why a cell chooses to commit suicide. Even when a homogeneous cell population has received the same dose of IR, the apoptotic response is only present in a select set of cells, as commonly demonstrated in IR survival assay.

CHAPTER 2

Analysis of 7 Putative Phosphorylation Sites of DNA-PKcs for Kinase Inactivation and Dissociation

INTRODUCTION

DNA double strand breaks (DSBs) are one of the most toxic lesions sustained by the cell. In all cells, DSBs are primarily repaired by one of two major mechanisms: non-homologous end-joining (NHEJ) or homologous recombination (HR). The NHEJ pathway, facilitated by 7 proteins – Ku70/86 heterodimer, DNA-dependent protein kinase catalytic subunit (DNA-PKcs), Artemis, XRCC4, XLF, and DNA Ligase IV – is the most important DSB repair pathway in mammalian cells. The presence of free DNA ends readily initiate NHEJ in mammalian cells. The nuclear abundant Ku70/86 heterodimers

are known to rapidly recognize and bind to DSBs and recruit the catalytic subunit, DNA-PKcs, to the DSB site, thus forming the essential component of the NHEJ pathway, DNA-PK. Structural studies reveal that one DNA-PK is situated at each DNA terminal while associating with each other through the N-terminal HEAT repeats in such a manner that allows the two DNA ends to be in close proximity to each other (DeFazio, Stansel et al. 2002; Spagnolo, Rivera-Calzada et al. 2006). Activation of DNA-PKcs occurs in this conformation (Rivera-Calzada et al., 2005), which results in structural conformational changes at its FAT and FATC domains, the sites that are in direct contact with the Ku proteins (Jin et al., 1997). These structural analyses are consistent with earlier studies that showed autophosphorylation of DNA-PKcs induced kinase inactivation and disassembly from Ku-bound DNA (Chan et al., 1996; Costantini et al., 2007; Merkle et al., 2002).

Major efforts have been put forth by at least 6 different labs (Meek, Lees-Miller, Hache, Chen, Mann, and Lieber) to identify autophosphorylation sites within DNA-PKcs that lead to the observed kinase dissociation. This has led to the identification of some 20 phosphorylation sites, some of which show functional relevance and others that do not. Mass spectrometry (MS) reveals at least another 20 phosphorylation sites that have not been functionally described. Furthermore, when I joined the lab, 13 autophosphorylation sites on DNA-PKcs have been studied and analyzed for their roles in kinase dissociation. In fact, they showed that DNA-PKcs with 13 autophosphorylation sites ablated could still undergo further autophosphorylation (Douglas et al., 2007), indicating that the relevant sites for kinase dissociation have not be identified.

In collaboration with Lees-Miller, our lab has used other approaches to define additional phosphorylation sites of DNA-PKcs when MS fails to do so. For instance, in the identification of the functional relevance of T3950, we first looked at the protein sequences of several species for sequence conservation of a kinase activation loop. We demonstrated that the substitution of T3950 with an aspartic acid caused inactivation of DNA-PKcs whereas substituting it with an alanine did not alter its kinase activity (Douglas et al., 2007). We subsequently showed that T3950 was an *in vivo* autophosphorylation site situated at the activation loop of the kinase domain that regulates kinase activity but not dissociation of the protein from Ku-bound DNA, indicating that T3950 is not responsible for kinase dissociation.

In this study, we will again identify relevant phosphorylation sites within DNA-PKcs by carefully examining its protein sequence for highly conserved target sites of DNA-PKcs: serine or threonine residues that are followed by a glutamine or a hydrophobic amino acid (denoted as S/T-Q) (Chan et al., 1999; Lees-Miller et al., 1992). Hence, by sequence conservation, we identified 3 putative phosporylation sites: threonine 946 (named J), serine 1004 (K), and threonine 1865 (L). In addition, I also analyzed how phosphorylation in regions known for DNA-binding would affect the protein's interaction with DNA. Previous studies done in our lab showed that the first 100 amino acids of the N terminus of DNA-PKcs was important for DNA binding (Douglass, Cui et al. 2007), so by examining that region, we found 2 putative phosphorylation sites: serine 56 and serine 72. In addition, I will also explore the effects of phosphorylation in the leucine zipper region (residues 1503 – 1602), previously shown to be important for DNA binding in our lab (Gupta, Meek 2005).

In all, I explored 7 putative phosphorylation sites for their roles in kinase dissociation and the NHEJ pathway: JK (T946 and S1004), L (T1865), N (S56 and S72), and LZ (S1470 and S1546) (fig. 3). In order to study how phosphorylation affects function, I substitute the putative phosphorylation site with an aspartic acid, which carries a negative charge, to mimic phosphorylation. These mutant constructs of DNA-PKcs are denoted as "site>D". Conversely, to block phosphorylation at the putative site, I substitute it with an alanine, which is neutral and cannot become phosphorylated. These mutants are denoted as "site>A". Then, the reconstructed mutant DNA-PKcs plasmids were stably transfected into the DNA-PKcs deficient cell line, V3 CHO cells. In order assess their role in kinase dissociation, we first determined whether the mutant's constructs were able to bind to DNA. If the phosphorylation caused dissociation, we expect that the phospho-mimicking mutant constructs would lose their ability to be pulled down by DNA-cellulose beads. However, if the mutations do not disrupt the protein's ability to bind to DNA, then we wanted to know if the mutation affected the NHEJ pathway. To assess the NHEJ pathway, we use three well developed assays commonly used in the field: IR survival assay, V(D)J recombination assay, and kinase activity assay.

In an IR assay, cells are irradiated at 0, 200, 400, and 600 rads, followed by incubation in complete media at 37 °C with 5% CO_2 for 7 days to allow cells that survived the irradiation to form colonies that could be stained and counted. Hence, the IR assay assesses whether cells could be repair DSBs caused by IR. Since the NHEJ pathway is the most important repair mechanism in mammalian cells, V3 cells not transfected with DNA-PKcs are radiosensitive, whereas those transfected with wild-type DNA-PKcs are radioresistant. If a phosphorylation site is important for the NHEJ

pathway, we would expect that ablating that site from phosphorylation would impair NHEJ and cause the cells to become radiosensitive. Conversely, if the phosphorylation of a putative site impairs the NHEJ pathway, we would expect that mimicking phosphorylation at that site would make the cells radiosensitive.

NHEJ is an essential step in the V(D)J recombination process, whereby DSBs created by the enzymatic reactions of RAG1/2 (Recombination Activation Genes) are repaired only by the NHEJ pathway. By evaluating V(D)J recombination, we could directly determine the role of the various mutant constructs of DNA-PKcs in mediating NHEJ. V3 cells that have not been transfected with DNA-PKcs are extremely inefficient in supporting V(D)J recombination, indicating the essential role of DNA-PKcs in the NHEJ pathway.

Finally, the kinase activities of DNA-PKcs are essential to NHEJ. If a mutation disrupts the protein's folding, it may lose its kinase domain. Importantly, if a putative site is responsible for kinase dissociation, the mutant construct should not possess kinase activities. Hence, in measuring the kinase activities of DNA-PKcs, we could infer whether the protein was folding appropriately as well as kinase dissociation.

Results were determined by 3 independent experiments, and the average and standard error of mean (SEM) were plotted on a graph using Windows Excel, where applicable. Statistical significance was established by a student's t-test where applicable ($p<0.05$).

Figure 3

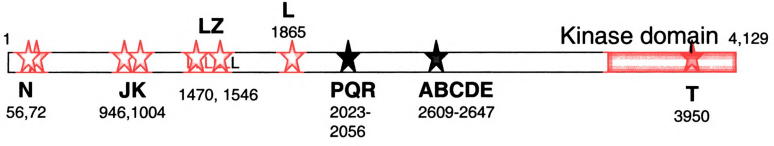


Figure 3 Schematic of DNA-PKcs. The human DNA-PKcs consists of 4,129 amino acids. It has a distinct kinase motif in the C-terminus, which places it among the protein kinases of the PI-3K related kinase superfamily (PIKK). Phosphorylation at clusters ABCDE and PQR, and threonine 3950 have been described previously (blue stars, referenced in text). 7 additional sites were analyzed in this study for their roles in kinase dissociation. 3 putative phosphorylation sites were identified by sequence conservation: threonine 946, named J, serine 1004 (K), and threonine 1865 (L). Furthermore, two putative phosphorylation sites were identified in the 100 amino acids of the N terminus, named N, which consists of S56 and S72. The leucine zipper region, which spans from residues 1503 – 1602, has two phosphorylation sites, as identified by MS: S1470 and S1546.

MATERIALS AND METHODS

Construction and transfection of expression plasmids. Construction of wild-type human DNA-PKcs expression vector was performed in our lab and described previously (Gupta and Meek, 2005). To make the JK and L mutants, the wild-type expression plasmid was initially digested with Kas I, and a 4.5 kb segment of the DNA-PKcs cDNA was isolated and subcloned into pUC19. The aspartic acid and alanine substitutions were performed by site-directed mutagenesis using the following oligonucleotides:

J>D top: 5'GTTGGGCAAAGCTGATCAGATGCCAGAA3'

J>D bottom: 5'TTCTGGCATCTGATCAGCTTTGCCCAAC3'

K>D top: 5'CAACAAGAAATTCGAAGATCAGGATACTGTT3'

K>D bottom: 5'AACAGTATCCTGATCTTCGAATTTCTTGTTG3'

L>D top: 5'TCTACCTTTGATGATCAAATCACCAGG3'

L>D bottom: 5'CTTGGTGATTTGATCATCAAAGGTAGA3'

J>A top: 5'GTTGGGCAAAGCTGCTCAGATGCCAGAA3'

J>A bottom: 5'TTCTGGCATCTGAGCAGCTTTGCCCAAC3'

K>A top: 5'CAACAAGAAATTCGAAGCTCAGGATACTGTT3'

K>A bottom: 5'AACAGTATCCTGAGCTTCGAATTTCTTGTTG3'

L>A top: 5'TCTACCTTTGATGCTCAAATCACCAGG3'

L>A bottom: 5'CTTGGTGATTTGAGCATCAAAGGTAGA3'

The mutations were confirmed by sequencing analysis, and the 4.5 kb segments containing the mutations were religated back into the expression vector.

The N mutations were constructed from a 2kb fragment of the N terminus of the human DNA-PKcs cDNA and subcloned into PCR2.1 plasmid. Site-directed mutagenesis was performed using the following oligonucleotides:

N>A top: (KAM642) 5' CAATACTGTTGAGTGCCTTCCGGACAAATACAAGCAAA CCGAAATCTCTGGAAAAAACTAGTGCTGTCTGTAATGCCAG3'

N>A bottom: (KAM645) 5' CTGGCATTACAGACAGCACTAGTTTTTTCCAGAGA TTTCGGTTTGCTTGTATTTGTCCGGAAGGCACTCAACAGTATTG3'

N>D top: (KAM643) 5' CTGGCATTACAGACAGATCTAGTTTTTTCCAGAGAT TTCGGTTTGCTTGTATTTGTCCGGAAGGATCTCAACAGTATTG3'

N>D bottom: (KAM644) 5' CAATACTGTTGAGATCCTTCCGGACAAATACAA GCAAACCGAAATCTCTGGAAAAAACTAGATCTGTCTGTAATGCCAG3'

The LZ mutants were constructed using the following oligonucleotides:

S1470→A Forward: 5'GGGCTTCTGCATAATATTTTACCGGCTCAGTCCACAG3'

S1470→A Reverse: 5'CTGTGGACTGAGCCGGTAAAATATTATGCAGAAGCCC

S1470→D Forward: 5'GGGCTTCTGCATAATATTTTACCGGATCAGTCCACAG3'

S1470→D Reverse: 5'CTGTGGACTGATCCGGTAAAATATTATGCAGAAGCCC3'

S1546→A Forward: 5'CGGCGTCCTTGGGCAGCGCACAGGGTAGCGTCATCC3'

S1546→A Reverse: 5'GGATGACGCTACCCTGTGCGCTGCCCAAGGACGCCG3'

S1546→D Forward: 5'CGGCGTCCTTGGGCAGCGACCAGGGTAGCGTCATCC3'

S1546→D Reverse: 5'GGATGACGCTACCCTGGTCGCTGCCCAAGGACGCCG3'

Cell lines and culture conditions. We used a variant of the V3 Chinese hamster ovarian (CHO) cell line, Vd7, which were cells derived from V3 cell line, with the integrated

substrate plasmid for HR assays (Allen et al., 2002). Cells were maintained in α-MEM with 10% fetal calf serum. Stable transfectants of Vd7 cells were maintained in 2.5 μg/ml blasticidin. Cells were kept in a 37°C incubator with 5% CO_2.

Western Blot analysis and DNA-cellulose pull-down. Whole cell extracts (WCE) were prepared as follows: Cells were grown to 90% confluence in a T75 tissue culture flask, harvested with Trypsin-Versene mix (Lonza), and washed with PBS. Thereafter, cell pellets were resuspended in 50 μl of extraction buffer (20 mM HEPES [pH 7.8], 450 mM NaCl, 50 mM NaF, 25% [vol/vol] glycerol, 0.2 mM EDTA) with Complete protease inhibitor (EDTA free; Roche Molecular Biochemicals). The resuspended pellets were then subjected to three freeze-thaw cycles (liquid nitrogen; 37°C) and centrifuged at 14,000 RPM for 10 min at 4°C. Supernatants were stored at -80°C. Protein concentration was determined using the Bradford method (BioRad), and samples were loaded at 20 μg into each lane of a 4.5% SDS-polyacrylamide gel. DNA-PKcs was detected using a mouse monoclonal antibody raised against DNA-PKcs (42-27; a generous gift of Tim Carter, St. John's University, New York, NY) at a 1:1000 dilution in PBS-0.1% Tween20 containing 1% milk. A goat anti-mouse immunoglobulin G conjugated to horseradish peroxidase at 1:5000 dilution was used as the secondary antibody (Abcam). The secondary antibodies were detected using the ECL chemiluminescent substrates (Sigma) according to manufacturer's recommendations.

Samples that were pulled down with DNA-cellulose beads were prepared as follows: 1 mg protein from WCE of V3 transfected cells were incubated with 50 μl (50:50 slush) DNA-cellulose beads (Amersham) in buffer A (25 mM HEPES [pH7.9], 50

mM KCl, 10 mM $MgCl_2$, 10% [v/v] glycerol, 1 mM EDTA, 1 mM EGTA, 1 mM DTT) containing protease inhibitors, (EDTA free, Roche Molecular Biochemicals) at 4°C for 1 hr in a rocking motion. The mixes were washed three times with Buffer A. Then 50 µl of 4X loading buffer containing β-2-mercaptoethanol were added to the beads, and the mix was heat denatured at 100°C for 1 minute. 10 µl of the sample mix were analyzed by Western blot.

Assessment of radiation sensitivity. Cells (1000/ml) were exposed to 200, 400, and 600 rads of ionizing radiation using a ^{60}Co source in plain α-MEM and immediately seeded in complete medium containing 10% fetal calf serum in triplicates. After 7 days, cell colonies were stained with 1% (w/v) crystal violet in ethanol, and colony numbers were assessed. Survival was plotted as percentage of the number of colonies in each radiation group over the number of colonies in untreated samples.

V(D)J recombination assay. Extrachromosomal recombination assays were performed as described previously (Kienker et al., 2000). To asses V(D)J recombination in V3 cells, RAG1 and RAG2 expression constructs (2 µg each), expression plasmids encoding DNA-PKcs, or the pCMV6 vector control (4 µg) and a recombination substrate (pJH201 or pJH290; 1 µg) were transfected using FuGENE6™ transfection reagent (Roche). Plasmid substrates were recovered from the cells 48 hours later by alkaline lysis. Alkaline lysates were digested with *Dpn*I to remove plasmid replicates but leaving the original plasmid intact. Then, the samples were used to transform chemically competent *Escherichia coli*. Transformed bacteria were spread onto two LB agar plates, one

containing 100 μg/ml ampicillin and the other containing 100 μg/ml ampicillin plus 22 μg/ml chloramphenicol. The percent recombinant ratio is calculated by taking the total number of colonies in the ampicillin-chloramphenicol resistant plates and dividing that with the total number of colonies in the ampicillin plates alone. Sequencing analyses were performed by the Research Technologies Support Facility at Michigan State University.

DNA-PKcs kinase activity. The SignaTECT DNA-PK assay system™ (Promega Corp.) was used to assay DNA-PK activity with the following modifications: Whole cell extracts were incubated with 20 μl of preswollen double-stranded DNA-cellulose beads (Amersham) for 60 minutes at 4°C. The beads were then washed three times with 1 ml Buffer A. Protein kinase reactions were conducted with 10 μl-aliquots of the resuspended cellulose beads and performed either in the presence or absence of [γ-^{32}P]ATP. Reactions were terminated after 60 minutes of incubation at room temperature, and spotted on a SAM™ membrane. After membranes were washed, ^{32}P activity was evaluated using a scintillation counter to determine ^{32}P-incoorporation. All assays were performed in duplicate with at least 3 different cell extract preparations.

Protein-protein interaction assay. Sf9 insect cells were infected with the baculovirus carrying the expression vector for human Artemis, Ku 70/86, and XRCC4 (Baculogold-linearlized viral DNA; Pharmingen), and the proteins were harvested after 3 days as noted before (Cui et al., 2005; Ding et al., 2003). An in-frame V5-His tag was added to the C-terminus. For the pull-down experiments, 1 mg of Sf9 whole cell extracts (in

buffer X) were briefly incubated in 50 µl Ni-NTA beads (50:50 slush, prewashed in buffer A plus 50mM imidazole) at room temperature. 0.5 mg V3 cell extracts were added to 500 µl buffer A+50 mM imidazole containing protease inhibitors (PI), and spun down for 10 minutes to remove excess precipitation. Then, the V3 cell extracts in buffer A were allowed to incubate with the Sf9 extracts and Ni-NTA beads at 4°C on a rocker for 1 hour. Subsequently, the beads were washed three times with buffer A containing 75 mM imidazole. The beads were resuspended in 40 µl 4X SDS-loading buffer, heat denatured, and 10 µl was electrophoresed on a 4.5% SDS polyacrylamide gel as described.

RESULTS

The JK site We examined the protein sequences of DNA-PKcs of 11 species that have been sequenced through this region, and noted that threonine 946 (J) and serine 1004 (K) were remarkably conserved in many of them (fig 4A). Mutations of DNA-PKcs were constructed as described, and their expressions in V3 cells were confirmed by Western blot (fig 4B). The cell extracts were then partially purified with DNA-cellulose beads to "pull down" proteins that bind to DNA and the samples were subsequently analyzed by Western blot for DNA-PKcs. If phosphorylation induces kinase dissociation, one would predict that a phospho-mimicking mutant would assemble onto DNA less efficiently than wild type does. As shown in figure 4B, these data do not support a role for JK phosphorylation in kinase dissociation.

Nonetheless, we wanted to determine whether the mutations impaired their NHEJ functions. Transfected V3 cells were irradiated at 0, 200, 400, or 600 rads and their % survival after 7 days was computed and plotted on the Y-axis. Cells expressing the J>A, K>A, and K>D mutations were radioresistant, indicating that these constructs fully complemented the DNA-PKcs deficiency in these cells (data not shown). In contrast, cells expressing the J>D, JK>A, and JK>D mutants were not completely complemented, which led to varying degrees of sensitivity to IR (fig 4C). Although J>A or K>A were similarly radioresistant as cells expressing WT DNA-PKcs, cells expressing JK>A were mildly more radiosensitive; similarly, we observed that the JK>D were significantly more radiosensitive than the J>D mutants. Hence, we considered that J and K might function as a phosphorylation cluster and that phosphorylation within the cluster was more functionally relevant than either one site alone.

An important function of NHEJ is its role in rejoining DSBs created by the enzymatic reactions of Rag1/2 proteins (recombination activation genes 1 and 2), as a part of V(D)J recombination process in developing lymphocytes. In an *in vivo* assay developed by Marty Gellert's lab, a substrate plasmid containing either the coding or signal joint sequences flanked by RSSs (recombination signal sequences) (Hesse et al., 1987; Lieber et al., 1987) was transiently transfected into V3 cells along with DNA-PKcs constructs and Rag1/2 expression plasmids. The percent recombination is a ratio of the number of recombinants over the experimental efficiency. As shown in figure 4D, the JK>D mutants showed severe impairment of V(D)J recombination, consistent with the results of the IR assays. These findings indicate that the JK>D mutants are severely deficient in NHEJ function.

Next, we wanted to investigate whether their kinase activities were impaired by the mutation. If JK phosphorylation induces kinase inactivation, one would predict that the phospho-mimicking mutant would exhibit reduced kinase activity. However, this was not observed, as both JK>A and JK>D retained full enzymatic activity (fig 4E). Moreover, the fact that JK>D possesses kinase activity suggests that substitution of the site to aspartic acid did not disrupt its overall function.

I conclude that the putative J and K phosphorylation sites function in concert as a cluster. While we did not find any evidence of kinase dissociation mediated by the JK>D mutants, the fact that NHEJ is substantially impaired in cells expressing the JK>D mutants suggest that phosphorylation at J and K are functionally important, and that they likely negatively control NHEJ. The JK phosphorylation site will be further explored in Chapter 3.

Figure 4 JK region. (**A**) The DNA-PKcs protein sequences of 11 species aligned to the putative J and K phosphorylation sites (shown in red). From available sequences, the putative J site is observed to be conserved to the level of the frog, whereas the putative K site is conserved to the level of the fugu blow fish. (**B**) Western blot of V3 transfected cell extracts, showing expression level of DNA-PKcs, either wild-type (WT), or the various mutant J and K constructs (samples in "Extracts"). Each lane consists of 20 μg total protein. Proteins from DNA cellulose pull-down, showing that all constructs bind to DNA like WT (samples in "pull-down"). (**C**) IR survival assay. Stably transfected V3 cells were irradiated at 0, 200, 400, and 600 rads, and incubated in a cell culture dish for 7 days, after which time, cells that survived the irradiation form colonies that can be stained and counted. The result of 3 experiments is shown here, with error bars showing the standard error of mean (SEM). (**D**) Result of 3 V(D)J recombination assay. In the absence of DNA-PKcs, V3 cells are significantly ineffective in producing recombinants. However, when they have been transfected either with WT or the JK>A mutants, both signal and coding joints are repaired with great efficiency. In contrast, cells transfected with the JK>D mutants were significantly impaired, with at least a 6-fold reduction in recombinants, indicating that phosphorylation at J and K inhibited NHEJ activities. Error bars represent the SEM of 3 experiments. (**E**) Kinase activities of both JK mutants were similar to WT, indicating that their kinase abilities were not impaired.

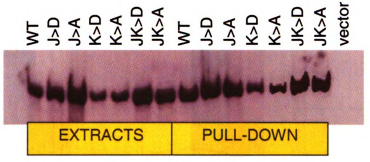
Figure 4A. Protein sequences surrounding putative sites J and K

```
                    J
human     MVMFMLGKATQMPEG-GQGAPPMYQLYKRTFPVLLRLACDVDQVTRQLYEPLVMQLIHWF  995
cow       VVMFMLGKATQMPDG-GQGPPPLYQLYKRTFPVLLRLACDVDQVTRQLYEPLVMQLIHWF  998
dog       MVMFTLGKATQMPEC-GQGFPPMYQLYKRTFPALLRLACDVDQVTRQLYEPLVMQLIHWF  1005
horse     MVMFMLGKATQMPED-GQGSPPMYQLYKRTFPVLLRLACDVDQVTRQLYEPLVMQLIHWF  968
mouse     MVMFMLGRATQMPE--GQGLPPMYQLYKHTFPVLLQLACDVDQVTRQLYEPLVMQLIHWL  992
rat       MVMFMLGRATQMPE--GQGLPPMYQLYKHMFPVLLKLACDVDQVTRQLYEPLVMQLIHWF  1002
chicken   IVAYMLGKASQMPER-QQGPPPMHQLYKRIFPVLLRLACDVDQVTRQLYEPLVMQLIHWF  991
frog      IVAFMLGKATQMPDDKKTGSSPMYKIYKRTFPVLLRLACDVDKVTEQLYKPLVMSLIHWF  1017
tetra     LVIYMVGKSAQISEG-ENSLPPMYKLHKRLFPVLMRLACDVDQVTRQLFEPLVMQLIHWF  778
fugu      LVIYTVGKNAQIVEG-ENTLPPMYKLHKRLFPVLMRLACDVDQVTRQLFEPLVMQLIHWF  997
dicty     VLLMMIGNSANQSQANQISYS---RLYRKIFPSLICLSTDVEQVTKQLFQPLVFQMIHWF  1080

                  K
human     TNNKKFESQDTVALLEAIL----------------------------------------  1014
cow       TNNKKFESQDTVALLEAIL----------------------------------------  1017
dog       TNNKKFESQDTVALLETIL----------------------------------------  1024
horse     TNNKKFESQDTVALLETIL----------------------------------------  987
mouse     TNNKKFESQDTVALLEAIL----------------------------------------  1011
rat       TNNKKFESQDTVALLEAIL----------------------------------------  1021
chicken   TNNKKFESQDTVTFLEAIL----------------------------------------  1010
frog      TNNKKFESQDTVALLEAIL----------------------------------------  1036
tetra     TNNKKFESQDTVAVLEAIMVTVWLSIRIVYPNPPFNPLTFNPSTLSFFFFLVKSVIKSQR  838
fugu      TNNKKFESQDTVAVLEAIM----------------------------------------  1016
dicty     TRNKKQESDDTMNLLNAIV----------------------------------------  1099
```

Figure 4 (cont.)

B. Western blot



C. IR survival assay

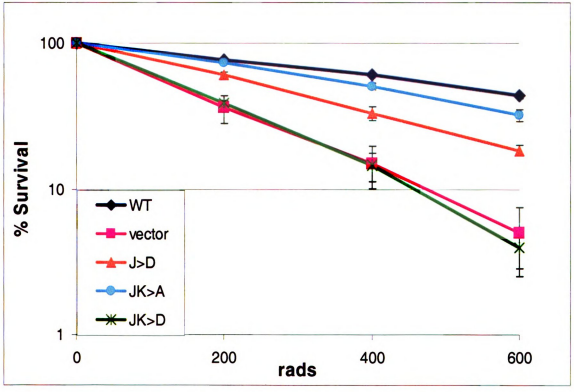
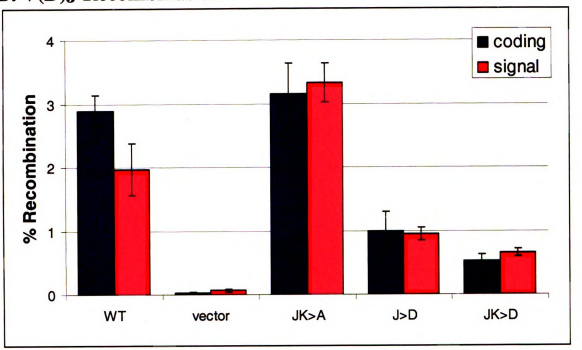
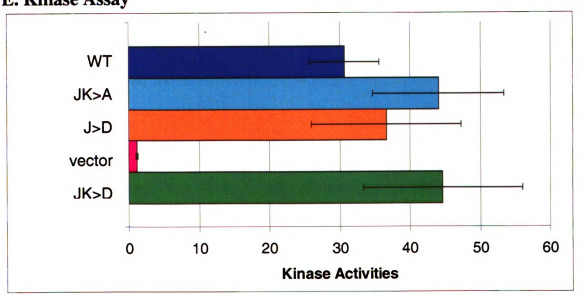


Figure 4 (cont.)

D. V(D)J Recombination



E. Kinase Assay



The L site Threonine 1865 is a strongly conserved autophosphorylation site (fig 5A), and confirmed by mass spectrometry experiments by our collaborator, S.P. Lees-Miller (personal comment). Protein expression of the L mutants was established by Western blot analysis (fig 5B). DNA-cellulose pull-down experiments demonstrated that mimicking phosphorylation of the L site by mutation was not sufficient to cause dissociation, suggesting that this site was not responsible for kinase dissociation. However, we proceeded to determine the radiosensitivity of cells complemented with the L mutants. As shown in figure 5C, cells expressing the L>D mutants were more sensitive to IR than the L>A mutants, suggesting that phosphorylation at L may negatively regulate NHEJ.

Next, we wanted to determine whether the moderate radiosensitivity of the L>D mutants would be reflected in reduced levels of V(D)J recombination. However, this was not the case. As shown in figure 5D, cells expressing the L>D mutant displayed normal V(D)J recombination activity for both coding and signal joints, suggesting that phosphorylation at L does not disrupt its function in V(D)J recombination. Our lab had previously shown that increased radiosensitivity does not necessarily translate into poor progress in V(D)J recombination because much less DNA-PKcs kinase activity is required for V(D)J recombination than is required for restoring full radioresistance (Kienker et al., 2000). Therefore, we also investigated the kinase activities of the L mutants and discovered that both L>D and L>A exhibited normal levels of kinase activities (fig 5E).

Our data suggest that phosphorylation of L may provide a weak negative regulation of NHEJ; however, the basis for this effect is not clear. The fact that the L>D

mutants were able to bind to DNA and exhibited normal levels of kinase activity convinced us that phosphorylation at L is not important for kinase dissociation.

Figure 5 L mutants. (**A**) Protein sequences of 11 species at the L region, showing that the phosphorylation site at 1865 is conserved (bold red letters) in 8 of them. (**B**) Western blot analysis of the L mutant construct, in the whole cell extracts (E) and in samples "pulled down" by DNA cellulose beads (P), demonstrating that both L mutants bind to DNA. Each lane contains 20 μg total proteins. (**C**) Irradiation survival assay of V3 cells transfectants. Cells expressing the L>D mutation were significantly more radiosensitive than those expressing L>A, suggesting that phosphorylation at L may serve to negatively regulate NHEJ. Error bars represent SEM of 3 experiments. (**D**) V(D)J recombination assay showed that both L>A and L>D mutants were able to support NHEJ like WT. Error bars represent SEM of 3 independent experiments. (**E**) Kinase assay. Both L>A and L>D mutants exhibited within normal levels of kinase activities. Error bars represent the SEM of 3 experiments.

Figure 5

A. Protein sequences

```
                                        L
human    TIVVDAIDVLKSRFTK--LNESTFDTQITKKMGYYKILDVMYSRL 1884
cow      RTVVEAIDVLKSRFTK--LNESAFDTQITKKMGYYKMLEVMYSRL 1887
dog      KIVVETIDVLKSRFTK--LNESTFDTQITKKMGFYKMLDVMYSRL 1897
horse    KIVVEAINVLKSRFIK--LNESAFDTQITKKMGYYKMLDVMYSRL 1857
mouse    RIVVDAIDVLKSRFTK--LNEFTFDTQITKKMCYYKMLAVMYSRL 1881
rat      RIVVEAIDVLKSRFTK--LND-TFDTQITKKMCYYKMLAVMYSRL 1889
chicken  KIIVQAMDTLNSRFTK--SNECVFDTQITKKMGYYKMLEVMYIRL 1882
frog     NIISLAMDTLKSRFTK--VPEAAFDSQITKKWGYYKMLEVQYSRL 1906
tetra    SNISDISAVLLGRFTK--TNSKEYEIQVIKKTGCYKLMELMYSRL 1756
fugu     SNICDISAVLLGRFTK--TNVAEFEVQILKKIGCYKLMELMYSRL 1873
dicty    QHLSSLMSIIQPLQPKYLSDSQERKSSIIEKICCFHLIEALYQSL 2052
```

B. Western blot

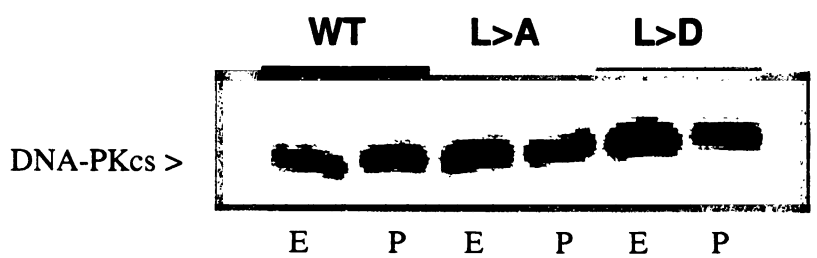
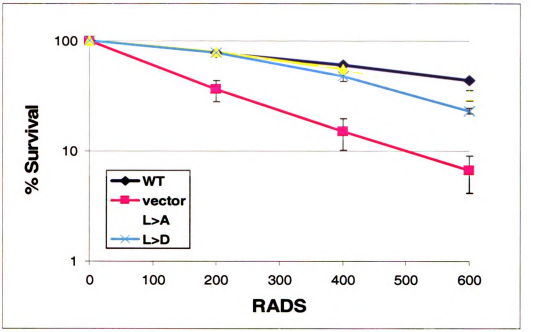


Figure 5 (cont.)

C. IR survival assay



D. V(D)J Recombination assay

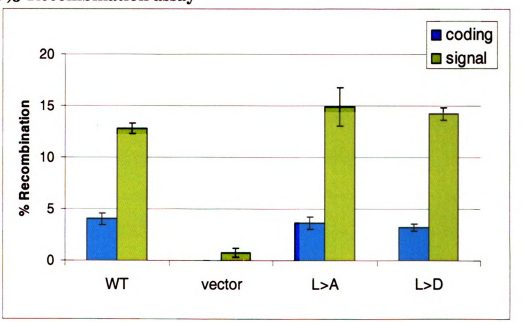
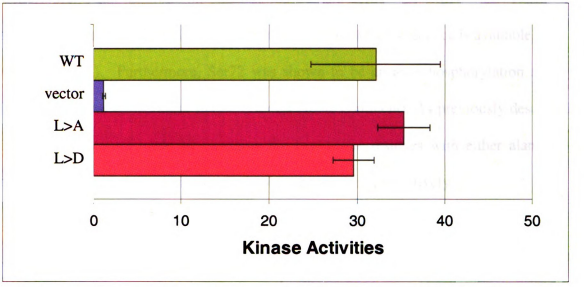


Figure 5 (cont.)

E. Kinase Assay



The N site We previously showed that deleting the first 100 amino acids from the N-terminus of the DNA-PKcs (denoted as ΔN) effectively impaired the protein's ability to bind to DNA cellulose beads (Douglass, Cui et al. 2007). By examining this region, we identified two phosphorylation sites: serine 56 and serine 72, which were conserved among the 10 and 9 of the 10 species, respectively, for which sequence is available in this region (fig 6A). Furthermore, Ser72 was shown to be an autophosphorylation site by MS, done by our collaborator Lees-Miller (personal comment). As previously described, the N mutants were made by changing the two serine residues with either alanine or aspartic acid residues to ablate or mimic phosphorylation, respectively.

Following verification by Western blot analysis of their expression in V3 cells (fig 6B), cellular extracts of the N mutants were assayed for DNA-binding. As shown, both forms of the N mutants were pulled down with DNA cellulose beads, demonstrating that N>A and N>D were able to bind to DNA like WT. As argued before, these data were not consistent with kinase dissociation.

Nonetheless, there is evidence that phosphorylation at N serves an important function in the NHEJ pathway. Cells expressing the N>D mutant exhibited severe radiosensitivity (fig 6C) and significantly reduced V(D)J recombination (fig 6D), suggesting that NHEJ is negatively regulated by the N>D mutants. Interestingly, signal joints are less affected than coding joints, suggesting a possible role for N phosphorylation in end processing. Therefore, we proceeded to analyze the sequences of coding joints from the V(D)J recombination assays. We isolated 56 substrate plasmids from the N>A mutant clones and 46 substrate plasmids from the N>D mutants; and found that on average, 5.9 nucleotides were lost in cells expressing the N>A mutants, as

compared to 6.8 nucleotides lost in cells expressing the N>D mutants. By conventional criteria, this difference is considered to be not statistically significant. Furthermore, these results are within the range of nucleotide loss for WT DNA-PKcs, indicating that N phosphorylation was not relevant to end processing. Work with the N mutants is ongoing in our lab to identify the functional relevance of N phosphorylation.

Figure 6 N mutants. (**A**) Protein sequences of 10 species encompassing the N region, showing the conserved Ser56 and Ser72 residues (in bold red letters). (**B**) Western blot analysis of whole cell extracts (E) and pull-downs (P), showing that the N mutants are stably expressed in V3 cells and that they interact with DNA. Each lane consists of 20 μg total protein. (**C**) IR assay of V3 transfectants, demonstrating that cells expressing the N>D mutation exhibited moderate radiosensitivity, whereas those expressing the N>A mutation were radioresistant like WT. Error bars represent the SEM of 4 experiments. (**D**) V(D)J recombination assays. Cells expressing the N>A mutants behaved like WT. However, cells expressing the N>D mutant exhibit significantly reduced coding joint recombinants but not signal joint recombinants. Error bars represent the SEM of 4 experiments.

Figure 6

A. Protein sequences

```
                56                  72
human     PAVLALQTSLVFSRDFGLLVFVRKS--LNSIEFRECREEILKFLC  90
cow       PALLALHTSLVFSKDFGLLVFVRKS--LSIDEFRDCREEALKFLC  90
dog       PAVLALQTSLVFSKDFGLLVFVRKS--LSIDEFRDCREEVLKFLY 100
horse     PAVLALQTSLVFSKDFGLLVFVRKS--LSIDEFRDCREEILKFLY  63
mouse     SAVQALQISLVFSRDFGLLVFIRKS--LSIEDFRDCREEALKFLC  90
rat       SAVQALQISLVFSKEFGLLVFIRKS--LSIDEFRDCREEALKFLC  90
chicken   GGALALHVSLVFAPERGLLAFVCRS--LGVEEFRECREEALKFLC  83
frog      SSALDLNTSLIFSKEFGLLAFVRKS--LSSDEFKDCREEALKFLY 110
fugu      ENKLAFQTSLLFSKELGLLSFLRKS--LGREELRETRVEVLCFLE  87
dicty     EEEIGLVASLLFTGDHSLLKYLEKSSTISNKENVKIKVSILNLIA 104
```

B. Western blot

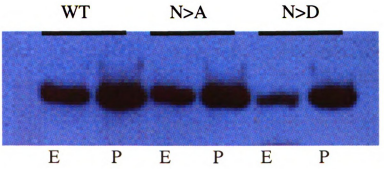
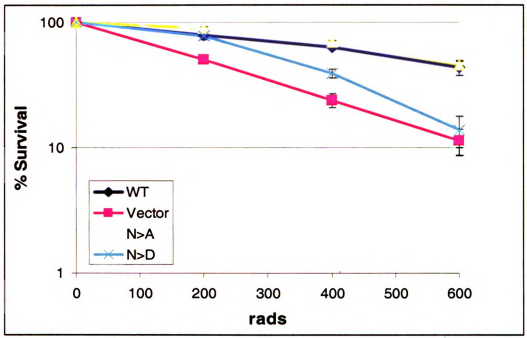
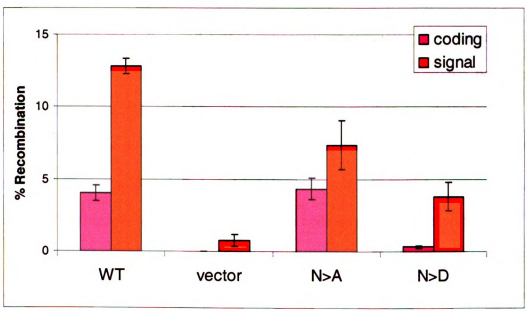


Figure 6 (cont.)

C. IR survival assay



D. V(D)J recombination assay



The LZ site Previous studies involving the leucine zipper region (LZ) of DNA-PKcs established that this region was important for DNA binding and we showed that changing two leucine residues with a proline (LRR) reduced the binding efficiency of the protein kinase by 50% (Gupta and Meek 2005). We examined the protein sequences of 11 species at the LZ region (residues 1503 – 1602), and identified two conserved phosphorylation sites, S1470 and S1546 (fig. 7A). As with S72 (N) and T1865 (L), both S1470 and S1546 were shown to be DNA-PKcs autophosphorylation sites by MS experiments done by our collaborator (personal comment).

V3 cells were stably transfected with the LZ>A and LZ>D mutants as described previously, and their expression was confirmed by Western blot analysis. To determine whether the phospho-mimicking mutants would exhibit reduced binding, we performed DNA-cellulose pull-down assay (fig 7B). However, unlike the LRR mutants, the LZ phospho-mutants were purified with DNA cellulose beads like WT, demonstrating that phosphorylation of the LZ region was not important for DNA binding.

Next, we wanted to determine the role of phosphorylation at LZ in NHEJ. V3 cells expressing the LZ>A and LZ>D mutants were irradiated as described previously and the results of at least 3 experiments were plotted (fig 7C). As shown, both constructs complemented the V3 cells like WT, suggesting that phosphorylation at the leucine zipper region probably did not have a role in regulating NHEJ. Consistent with the results of IR assays, both LZ>A and LZ>D constructs demonstrated complete ability to support V(D)J recombination like WT for both coding and signal joints (fig 7D).

Due to the intimate role of the LZ region in DNA binding, we wanted to ask whether this region participates in DNA end processing. Hence, we examined the coding

joints of recombinants sequences from cells expressing the LZ>A and LZ>D mutants. The result, with 6.34 and 6.06 nucleotides losses on average from LZ>A and LZ>D mutants, respectively, indicated that the LZ region was not important for DNA end processing.

In conclusion, LZ phosphorylation was not essential to NHEJ despite the fact that both serine residues were readily autophosphorylated during DNA-PKcs activation, as confirmed by MS. Both LZ mutants behaved like WT, indicating that phosphorylation is dispensable.

Figure 7 LZ mutants. (**A**) Protein sequences of DNA-PKcs of 11 species aligned. Yellow highlighted region indicate the leucine zipper (LZ) region, spanning from residues 1503 – 1602. Leucine residues that constitute the leucine zipper are bolded, and a "•" denotes their locations. The LZ site, S1470 and S1546, demonstrated by MS to be autophosphorylation sites, are conserved in 7 of the 11 species examined (shown in bold red letters). (**B**) Western blot analysis of extracts (E) and pull-down samples (P), showing that LZ mutant DNA-PKcs was able to bind to DNA like WT. Each lane consists of 20 μg total protein. (**C**) Cell irradiation assay, error bars represent the standard error of mean (SEM) of 3 experiments. V3 cells complemented with either LZ>A or LZ>D mutants were radioresistant like WT cells, indicating that the mutations did not change the function of DNA-PKcs in NHEJ. (**D**) V(D)J recombination experiments. Both LZ>A and LZ>D mutants supported NHEJ like WT. Error bars represent the SEM of 4 experiments.

Figure 7A

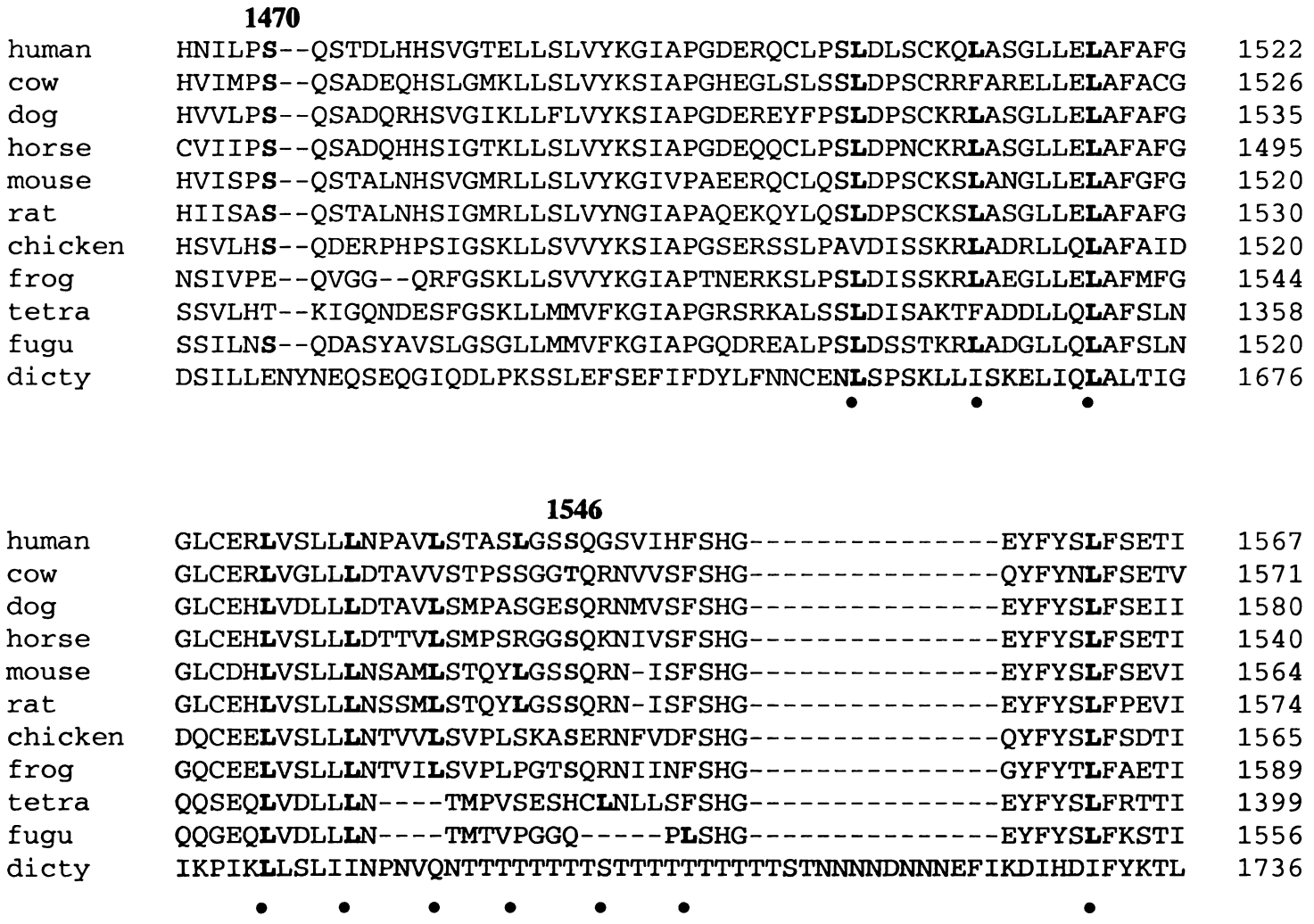
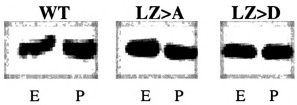

1470
human HNILPS--QSTDLHHSVGTELLSLVYKGIAPGDERQCLPSLDLSCKQLASGLLELAFAFG 1522
cow HVIMPS--QSADEQHSLGMKLLSLVYKSIAPGHEGLSLSSLDPSCRRFARELLELAFACG 1526
dog HVVLPS--QSADQRHSVGIKLLFLVYKSIAPGDEREYFPSLDPSCKRLASGLLELAFAFG 1535
horse CVIIPS--QSADQHHSIGTKLLSLVYKSIAPGDEQQCLPSLDPNCKRLASGLLELAFAFG 1495
mouse HVISPS--QSTALNHSVGMRLLSLVYKGIVPAEERQCLQSLDPSCKSLANGLLELAFGFG 1520
rat HIISAS--QSTALNHSIGMRLLSLVYNGIAPAQEKQYLQSLDPSCKSLASGLLELAFAFG 1530
chicken HSVLHS--QDERPHPSIGSKLLSVVYKSIAPGSERSSLPAVDISSKRLADRLLQLAFAID 1520
frog NSIVPE--QVGG--QRFGSKLLSVVYKGIAPTNERKSLPSLDISSKRLAEGLLELAFMFG 1544
tetra SSVLHT--KIGQNDESFGSKLLMMVFKGIAPGRSRKALSSLDISAKTFADDLLQLAFSLN 1358
fugu SSILNS--QDASYAVSLGSGLLMMVFKGIAPGQDREALPSLDSSTKRLADGLLQLAFSLN 1520
dicty DSILLENYNEQSEQGIQDLPKSSLEFSEFIFDYLFNNCENLSPSKLLISKELIQLALTIG 1676
1546
human GLCERLVSLLLNPAVLSTASLGSSQGSVIHFSHG----------EYFYSLFSETI 1567
cow GLCERLVGLLLDTAVVSTPSSGGTQRNVVSFSHG----------QYFYNLFSETV 1571
dog GLCEHLVDLLLDTAVLSMPASGESQRNMVSFSHG----------EYFYSLFSEII 1580
horse GLCEHLVSLLLDTTVLSMPSRGGSQKNIVSFSHG----------EYFYSLFSETI 1540
mouse GLCDHLVSLLLNSAMLSTQYLGSSQRN-ISFSHG----------EYFYSLFSEVI 1564
rat GLCEHLVSLLLNSSMLSTQYLGSSQRN-ISFSHG----------EYFYSLFPEVI 1574
chicken DQCEELVSLLLNTVVLSVPLSKASERNFVDFSHG----------QYFYSLFSDTI 1565
frog GQCEELVSLLLNTVILSVPLPGTSQRNIINFSHG----------GYFYTLFAETI 1589
tetra QQSEQLVDLLLN----TMPVSESHCLNLLSFSHG----------EYFYSLFRTTI 1399
fugu QQGEQLVDLLLN----TMTVPGGQ-----PLSHG----------EYFYSLFKSTI 1556
dicty IKPIKLLSLIINPNVQNTTTTTTTTSTTTTTTTTTTSTNNNNDNNNEFIKDIHDIFYKTL 1736

Figure 7 (cont.)

B. Western blot



C. IR survival assay

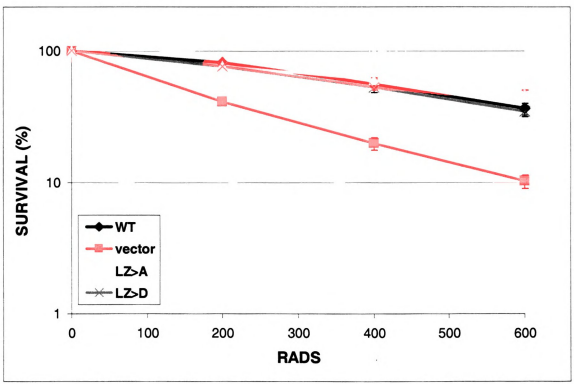
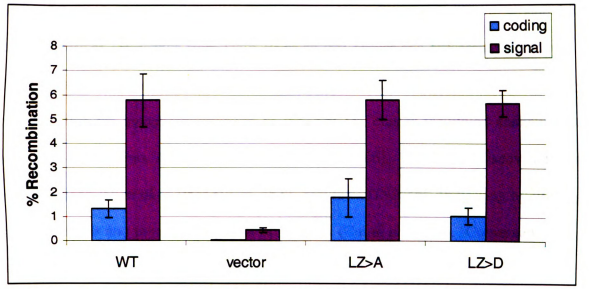


Figure 7 (cont.)

D. V(D)J recombination assay



DISCUSSION

Multiple laboratories, including ours, use phospho-mutants to demonstrate important regulatory roles by autophosphorylation of DNA-PKcs. For instance, we showed that autophosphorylation of the ABCDE cluster (2609 – 2647) was essential to allow access of DNA ends from DNA-PKcs so that proper end-processing could occur (Ding et al., 2003; Reddy et al., 2004). Consistent with this finding, another laboratory revealed that the autophosphorylation of the ABCDE cluster facilitated endonuclease activities of Artemis *in vitro* (Goodarzi et al., 2006). We also discovered that autophosphorylation of another region, PQR, regulates DNA-end processing by limiting nucleotide loss and promoting ligation of the ends (Cui et al., 2005). Conversely, when we blocked phosphorylation at PQR, we noted excessive end processing. Moreover, when PQR phosphorylation is blocked, the cells' utilization of the other pathway, HR, was increased. Although ABCDE and PQR represent the largest phosphorylation clusters of DNA-PKcs, they are not relevant to kinase dissociation of the complex.

There are many potential phosphorylation sites within the immense size of the DNA-PKcs that are presumed to serve different regulatory roles of NHEJ. Recently, we identified a novel autophosphorylation site, threonine 3950, with a unique function in regulating kinase activities. Using the phospho-mimicking mutant (T>D), we showed that this mutant protein exhibited severely reduced kinase abilities but without complex dissociation (Douglas et al., 2007). We demonstrated that this site was not responsible for kinase dissociation, however, by showing that the T>A mutants could become further autophosphorylated *in vitro*, and that it could still result in kinase dissociation. If the T

site was important for kinase dissociation, we expected that by blocking phosphorylation at T, we would prevent kinase dissociation from occurring.

In this study, we wanted to continue the quest to identify autophosphorylation sites responsible for inducing kinase inactivation and disassembly of the DNA-PKcs from Ku-bound DNA, which we believe to be a crucial step in NHEJ. Theoretically, the complex should disassemble from DNA at the end of NHEJ, so that DNA could be reassembled into chromatin structures and the components could be reused. By sequence comparisons of DNA-PKcs of several species, we focused our investigation on seven potential sites, T946 and T1004 (JK), T1865 (L), S56 and S72 (N), and S1470 and S1546 (LZ). MS studies done by our collaborator later confirmed that these sites – with the exception of T946, S1004, and S56 – were indeed autophosphorylation sites.

Although T946 and S1004 (JK) were not shown by MS to be autophosphorylation sites, we used phospho-specific antibodies raised in rabbits against a phospho-peptide of the J region to show that J was phosphorylated *in vitro* and *in vivo* in response to DSBs (data shown in chapter 3 of this dissertation). Since both the JK>A and JK>D mutants have more potent effects than the single site mutants, we believe that the J and K sites function as a cluster. In particular, cells expressing the JK>D mutants were as radiosensitive as vector cells, indicating severe impairment of NHEJ activities by the mutation. However, the phosphomimic construct possessed normal levels of kinase activity and were pulled down with DNA-cellulose beads like WT, indicating that phosphorylation at JK did not cause kinase dissociation. We will further investigate the properties of the JK cluster in chapter 3.

Next, we investigated the L site. DNA-cellulose pull-down assays demonstrated that both L>A and L>D mutants could bind to DNA, thereby ruling them out as candidates for kinase dissociation. However, substitution of the L site with aspartic acid and alanine conferred moderate and mild deficits in radioresistance, respectively. This phenotype exhibited by the L>D mutants was not due to lack of kinase activity, however, which was comparable to that of WT. In fact, both L>A and L>D constructs supported high levels of end joining similar to WT, suggesting that the residual function of the L>D mutants was sufficient for V(D)J recombination. These data suggest that L phosphorylation serve an important function in NHEJ, however, it is beyond the scope of this chapter to discuss the function of L phosphorylation. In short, we found that phosphorylation at L was not functionally related to kinase dissociation.

In addition to the JK and L sites, I investigated the roles of N and LZ phosphorylation in causing kinase dissociation. As described, the N>D mutants, which consisted of 2 phosphomimic substitutions, exhibited impaired NHEJ function, whereas the N>A mutants behaved like WT. However, we were able to purify the N>D mutants with DNA cellulose beads, indicating that they interacted with DNA. Therefore, phosphorylation at N was unlikely to cause complex disassembly from DNA. The N site will be further studied in our lab for its role in NHEJ but is beyond the scope of this dissertation. Conversely, phosphorylation at LZ did not present any observable phenotype, and cells expressing both LZ>A and LZ>D mutants behaved like those expressing WT DNA-PKcs. The LZ region, facilitated by abundant leucine residues, is important for DNA binding. However, phosphorylation within this region was not important for DNA binding.

In conclusion, we examined 7 novel phosphorylation sites for their role in kinase dissociation. While some of them were functionally relevant to NHEJ, none of them mediate kinase dissociation. These results add to an emerging consensus – that autophosphorylation of DNA-PKcs occurs on many different sites and has many functionally distinct outcomes. We have shown here that phosphorylation of JK, N, or L could potentially serve an evolutionarily important function by downregulating NHEJ.

CHAPTER 3

DNA-PKcs Modulates DNA Double Strand Break Repair Pathway Choice By Phosphorylation At T946 and S1004

INTRODUCTION

A DNA double strand break (DSB) represents a serious breach to the integrity of a cell's genome and is probably the most dangerous lesion that it can encounter. In response, cells have evolved distinct repair mechanisms to resolve DSBs as quickly as possible. One such mechanism, and also the primary DSB repair pathway in mammalian cells, is the nonhomologous end-joining (NHEJ) pathway. The goal of NHEJ is to rejoin ends as quickly as possible at the expense of losing sequence fidelity. The reason that NHEJ is error-prone lies in the fact that DNA ends broken by high energy irradiation or oxidative radicals consist of various configurations of damaged nucleotides, which

necessitate removal. In addition, during "end-processing", other NHEJ factors, such as polymerases mu and/or lambda may add a few nucleotides at the joints before they are rejoined. Consequently, joints repaired by NHEJ in these cases are rarely error-free but contain short deletions and additions (Jackson, 2002). Alternately, DSBs that occur during the S and G2 phases of the cell cycle can be repaired by another major mechanism, homologous recombination (HR). Repair by HR is precise and faithful to its original sequence because this mechanism uses the sister chromatid as template during the repair of the damaged region.

Numerous authors on the subject suggest that the two repair pathways exist in some type of competition for DSBs, and that the balance between the two pathways depend on a variety of factors, including the type and species of the cell (Shrivastav et al., 2008). For instance, in CHO cells, NHEJ has been shown to outcompete HR by at least 3-fold in one study (Roth and Wilson, 1985) and by as much as 50-fold in another study (Golding et al., 2004). In the human lymphoblastoid cell line, TSCE, NHEJ may dominate over HR by as much as 270 times in the event of DSBs (Honma et al., 2003). Yet, this balance between HR and NHEJ may shift in favor of HR in cells that are deficient in core NHEJ factors, such as DNA-PKcs and XRCC4 (Allen et al., 2003; Allen et al., 2002; Delacote et al., 2002). There is supportive evidence that DNA-PKcs (DNA-dependent protein kinase catalytic subunit), the 465 kDa protein kinase in the NHEJ pathway, is the determining factor of pathway choice. For instance, while the level of HR activities are enhanced in cells that are deficient in DNA-PKcs, this level remains low in wild type (WT) cells with DNA-PKcs chemically inactivated, suggesting that the mere presence of DNA-PKcs suppresses HR activities (Allen et al., 2003; Delacote et al.,

2002). Furthermore, previous work in our lab demonstrated that HR can be enhanced by blocking DNA-PKcs from autophosphorylation at the PQR cluster (Cui et al., 2005). Studies using fluorescent-tagged proteins show that while Ku and DNA-PKcs are found at DSB sites within minutes post-irradiation, HR factors such as RAD51 and Replication Protein A (RPA) show up much later (Kim et al., 2005). Therefore, in order for HR to take place, DNA-PK (the heterotrimer complex consisting of DNA-PKcs, Ku70, and Ku86) would have to allow HR factors to gain access to DNA ends, since the complex always arrives at the site first.

Cell cycle plays an important regulatory role for the initiation of HR. In order for HR to commence, DNA ends have to be resected into 3' single stranded DNA (ssDNA) tails by MRE11 and CtIP (Limbo et al., 2007), the latter of which is expressed only during the late S and G2 phases of the cell cycle (Sartori et al., 2007) and possibly the reason for the cell cycle dependence of HR. The long ssDNA region, initially coated and stabilized by RPA (Zou et al., 2003), is then replaced by Rad51, which forms a nucleoprotein filament on ssDNA and catalyzes the strand exchange step of HR (Sigurdsson et al., 2001). In addition, it is known that long stretches of ssDNA inhibit DNA-PK activity (Hammarsten et al., 2000; Pawelczak and Turchi, 2008), probably to limit NHEJ activity at that point. Hence, it seems logical that DNA repair pathway choice occurs before DNA resection occurs, when DNA ends are bound and protected by DNA-PK.

I had previously created two phosphorylation mutations at T946 and S1004 (named J and K, respectively) of the DNA-PKcs by substituting the phosphorylation sites either to alanine to ablate phosphorylation (JK>A), or aspartic acid to mimic

phosphorylation (JK>D). Here, I will demonstrate that JK is an *in vitro* phosphorylation site and that it becomes slowly phosphorylated *in vivo* in response to DSBs. The phospho-mimic mutant, JK>D readily accumulates at DNA ends in response to IR despite the fact that its ability to support NHEJ function is significantly impaired. In chapter 2, I demonstrated that cells expressing the JK>D mutation exhibited severe radiosensitivity and diminished V(D)J recombination. Here, I will show that even though the JK>D construct possesses full kinase activities, it is inefficiently phosphorylated at the PQR cluster. More importantly, cells expressing the phospho-mimic exhibit significantly enhanced HR activities, suggesting a positive regulation of HR activities by the JK>D mutant. In light of these findings, I propose a model whereby phosphorylation of the JK site functions to inhibit NHEJ and promote HR.

MATERIALS AND METHODS

Procedures and techniques as described in chapter 2 of this dissertation, with the following amends:

Cell culture. HEK293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% non-essential amino acids (Life Technologies), 2 mM L-glutamine (Life Technologies), 1 mM sodium pyruvate, 20 mM HEPES, and 1X Pen/Strep at 37°C and 5% CO_2 at 37°C and 5% CO_2.

Mobilization assay. Cells (2 X 10^6) were treated with Zeocin (2 mg/ml; Invitrogen) in plain α-MEM, at 37°C for 1 hr. Cells were pelleted and washed with PBS. Cell fractionation was carried out as described previously (Drouet et al., 2005) with the following modifications. The first supernatant (labeled S1) is collected from the supernatant after the cell pellet has been resuspended in 100 µl of extraction buffer (50 mM HEPES [pH 7.5], 150 mM NaCl, 1 mM EDTA) containing 0.1% Triton X-100 supplemented with protease inhibitor cocktail (EDTA-free, Roche) for 15 minutes on ice followed by centrifugation at 14,000 X g for 3 min at 4°C. The pellet that remained was further extracted with fresh buffer without Triton but supplemented with 200 µg/ml RNase A (Qiagen) at room temp for 30 minutes. This is followed by another spin at 14,000 X g for 3 min at 4°C, and the supernatant (S2) was discarded. The pellet is further extracted with fresh buffer containing protease inhibitors, DNase I (300 µg/ml)

and 5 mM magnesium chloride, and incubated at 37°C for 45 minutes. Following centrifugation at 14,000 X g for 3 min at 4°C, the pellet (P3) was dissolved in 25 µl 2X SDS loading buffer and analyzed with SDS-PAGE gel.

***In vitro* phosphorylation assays.** Whole cell extracts from HEK293 cells, containing 200 µg total protein, were incubated at room temperature for 15 minutes in buffer (50 mM Tris, pH 8.0, 10 mM MgCl2, 0.1 mM EDTA, 0.2 mM EGTA, 1 mM DTT) in the presence of ATP (0.2 mM) and DNA (40 µg/ml DNA from Calf thymus) to allow autophosphorylation of DNA-PKcs. The same conditions without DNA and ATP were performed as negative controls. The reactions were stopped by addition of SDS-loading buffer. 50 µg total protein was loaded into each lane, and the phosphorylation of DNA-PKcs was determined by Western blot.

***In vivo* phosphorylation.** HEK293 cells were grown to 80% – 90% confluence in a T75 flask prior to experimental treatment. On the day of treatment, the media is replaced with fresh complete media containing 2 mg/ml Zeocin (Invitrogen), 10 µg/ml Aphidicolin (Sigma), 1 µM Okadaic Acid (Sigma), or an comparable volume of DMSO where indicated. The cells were incubated at 37°C and 5% CO_2 for the times indicated, then whole cell extracts were prepared as described previously. Whole cell extract samples were analyzed by Western blot, each lane consisting of 80 µg total proteins.

Antibodies. Samples were loaded at the indicated concentrations. Antibody against an unphosphorylated region (monoclonal, 42-27) were used at 1:1000 dilution. Phosphospecific antibody against residue 2056 (phos-R, Abcam) were used at 1:1000 dilution. Phosphospecific antisera were raised in rabbits (Open BioSystems, Inc) using the phospho-peptide: LGKA[pT]QMPEGGQC, a region covering the J site, and purified by peptide affinity (Open BioSystems). For the detection of phospho-J, following protein transfer, the PVDF membrane was pre-blocked in 1% milk-PBST for 45 minutes at room temperature. The membrane was incubated in the presence of Phos-J antisera (1:200 dilution) and 1 μg/ml cold peptide, LGKATQMPEGGQC, at 4°C overnight. The following day, the membrane was washed and processed as described previously.

HR assay. Vd7 CHO cells which have the neo substrate gene incorporated into the genome (Allen et al., 2002) were seeded into p60 culture dishes at a density of 60% confluence and allowed to "sit down" fully (about 2 hours). The cells were then transiently transfected with a DNA-PKcs expression vector (wild-type or J/K mutants; 2-8 μg), pCDNA5-I-*Sce*I (2 μg), and pCMV*puro* (2 μg) using FuGENE6™ (Roche) at a ratio of 3 μl FuGENE6™: 1 μg DNA. After 48 hours, cells were harvested and seeded at low densities into either puromycin (1 μg/ml) or G418 (600 μg/ml) selection media (which is added 24 hours post seeding). Cell colonies are assessed by crystal violet staining after 7 days for the puromycin selection plates. G418 selection plates receive media change containing G418 after 7 days and are stained with crystal violet after 14 days.

RESULTS

As discussed in chapter 2, residues Thr946 and Ser1004 (here forth referred to as J and K) were initially identified by sequence conservation although phosphorylation at these sites has not been detected by mass spectrometric analyses. The two residues were changed to alanine to block phosphorylation (JK>A) and to aspartic acid to mimic phosphorylation (JK>D) by site-directed mutagenesis techniques. The constructs were subsequently stably transfected into DNA-PKcs deficient V3 CHO cells.

V3 cells expressing JK>D mutants are NHEJ deficient. In chapter 2, I showed that cells expressing the JK>D mutants were as radiosensitive as cells completely lacking DNA-PKcs while cells expressing the JK>A mutants were mildly radiosensitive as compared to cells expressing WT DNA-PKcs. Western blot analyses showed that the JK>D mutants were expressed at a comparable level to WT and JK>A mutants and that they interacted with Ku-bound DNA. I also showed that the radiosensitivity in JK>D expressing cells was indeed due to impaired NHEJ function, as cells expressing the JK>D mutants were severely deficient in supporting V(D)J recombination. However, JK>D still exhibited normal levels of kinase activities, indicating that the aspartic acid substitutions did not disrupt protein folding.

In order to further evaluate the JK>D construct, I first wanted to find out whether the mutations in JK>D disrupted its protein-protein interactions with other NHEJ factors. Therefore, I performed a pull-down assay, whereby the JK>D mutant constructs were allowed to interact with his-tagged NHEJ factors, such Ku, Artemis, and XRCC4, and the samples were then washed. Samples that interacted with the his-tagged proteins were

thus "pulled down" and the presence of DNA-PKcs was determined by Western blot (fig 8). The results indicate that the mutations within the JK>D construct did not disrupt gross protein-protein interactions, as the JK>D constructs were pulled down by Artemis, Ku, and XRCC4, but not by the negative control (empty vector containing a his-tag), like WT and the JK>A constructs.

JK>D mutants were recruited to DSBs. Factors in the NHEJ pathway have been shown to colocalize within the nucleus into distinct foci in response to DNA damaging agents and IR. Drouet et al. described a detergent-based cellular fractionation protocol that separates whole cell extracts into a soluble fraction (S1) and a chromatin-dense fraction (P3), and showed that γH2AX, the phosphorylated form of the histone variant, and NHEJ factors are only present in the P3 fraction when cells are treated with DNA damaging agents, such as Zeocin (Drouet et al., 2005).

In chapter 2, I showed that the JK>D mutant was able to bind to DNA *in vitro* despite its impaired NHEJ function. However, the question remains whether JK>D are recruited to DSBs *in vivo*. Therefore, V3 transfectants were treated with Zeocin (Zeo) for 2 hours to induce DSBs. Whole cell extracts are fractionated as describes by Drouet et al. A representation of 3 experiments is shown in figure 9. Like wild-type, both JK mutants were mobilized to the chromatin dense fraction, P3, demonstrating that JK>D are recruited to DSBs in response to DSBs.

Fig. 8 Protein-protein interaction was assessed using Ni-NTA bead pull-down assay. V3 cell extracts were incubated with Sf9 cell extracts containing his-tagged Ku, Artemis, XRCC4, or empty vector. Following incubation, samples were washed 3 times in buffer A containing 75mM imidazole, and analyzed by Western blot for the presence of DNA-PKcs. Whole cell extracts: crude extracts prior to pull-down, 20 μg/lane.

Fig. 8 Protein-protein Interaction assay

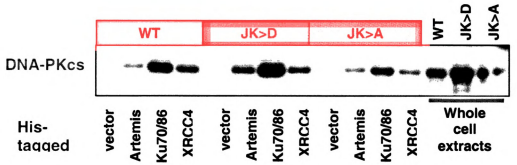
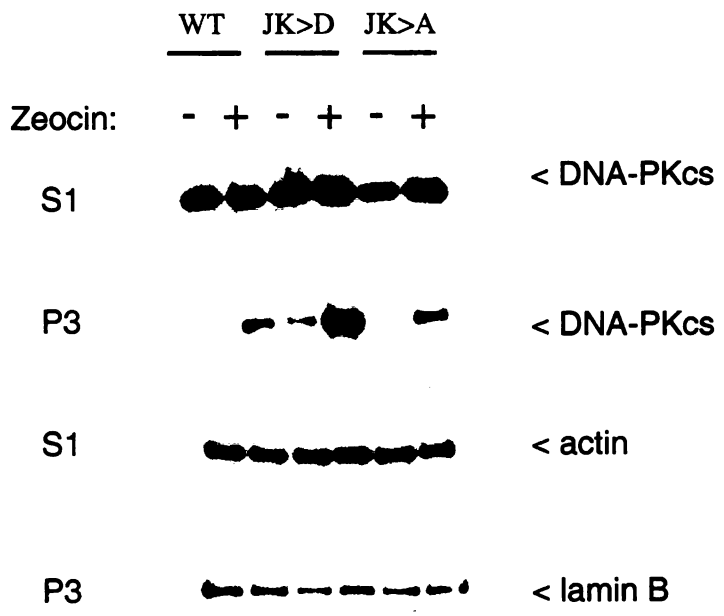


Figure 9. Nuclear mobilization of DNA-PKcs in response to DNA damage. Transfected V3 cells were treated with 2 mg/ml Zeocin for 1 hour at 37°C to induce DSBs. Whole cell extracts (WCE) were separated into an S1 fraction, consisting of Triton X - soluble proteins, and a P3 fraction, consisting of proteins embedded in chromatin that resisted solubilization by 0.1% Triton X-100 buffer. Samples were analyzed by Western blot analysis, and probed against DNA-PKcs (42-27). Samples were also probed against actin and lamin B to show loading control of both fractions. WT, JK>A, and JK>D were mobilized to the P3 fraction in response to Zeocin treatment, indicating that they were recruited to DSBs in vivo. The JK>D construct were expressed at a higher level than WT and JK>A.

Figure 9



JK is phosphorylated *in vitro*. We initially tried to identify phosphorylation at JK (T946 and S1004) by mass spectrometry (MS). However, JK phosphorylation was not detected. However, it should be noted that T3950 was also not identified by MS despite numerous attempts were made in 4 different laboratories. *In vivo* phosphorylation of T3950 was confirmed by generating a phospho-specific antibody (Douglas et al., 2007). Thus, phospho-specific anti-sera were generated using a phospho-peptide of 13 amino acids spanning the J site (T946) (fig 10A), raised in rabbits (Open BioSystems, Inc.). The anti-sera were further purified by column affinity to the peptide. As shown, the purified anti-sera were specific toward the phosphorylated but not the unphosphorylated peptides by at least 100 fold, even detecting the phospho-peptide at 0.08 μg (fig. 10B).

To determine whether J could be phosphorylated in vitro, HEK293 cells were utilized because DNA-PKcs is much more abundant in human cells than hamster cells. Whole cell extracts were treated with ATP and DNA where indicated, and the samples were allowed to become phosphorylated at room temperature for 10 minutes (fig 10C). The reaction was terminated by the addition of 4X SDS-loading buffer. A Western blot analysis, probed with the phospho-specific antibodies against the PQR cluster (phos-R, Abcam), JK site (phos-J), and a non-phosphorylated region of DNA-PKcs (DNA-PKcs, 42-27), showed that phosphorylation at J occurred only in samples that contained DNA and ATP. However, the signal from phosphorylation at J was considerably weaker than that for PQR; and detection of J phosphorylation required more protein extract than necessary for PQR. We speculate this difference in phosphorylation signal could be due to any of the following: 1) the phospho-J antisera had a lower affinity than the phospho-R

antisera; 2) phosphorylation at J might be an infrequent event; and 3) the antisera were degrading during storage.

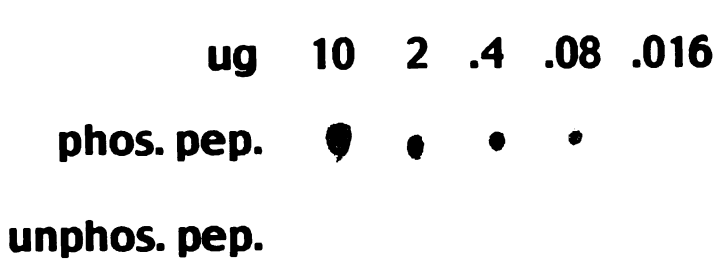
Figure 10. *In vitro* phosphorylation of J. (**A**) Protein sequences of the phosphorylated and unphosphorylated peptides covering the J site (Thr946). The phosphorylated peptide was used to generate phospho-specific anti-sera in rabbits (Open Biosystems, Inc.). (**B**) Anti-sera specificity was analyzed by dot-blot. Phosphorylated and unphosphorylated peptide dotted in decreasing concentration, and probed with anti-sera raised against the phosphorylated peptide. As shown, the anti-sera were able to detect the phosphorylated peptide even at a level of 0.08 ug, without cross-reacting with the unphosphorylated peptide. (**C**) HEK293 WCE, 200 μg total protein, was incubated in buffer (as described) with or without ATP and DNA for 15 minutes at room temperature. Samples were analyzed by Western blot, and probed against 3 different antibodies: DNA-PKcs (42-27, unphosphorylated region); Phos-J (phosphorylated JK); and Phos-R (phosphorylated PQR). Phospho-J antisera detected phosphorylation of JK in samples with ATP and DNA.

Figure 10

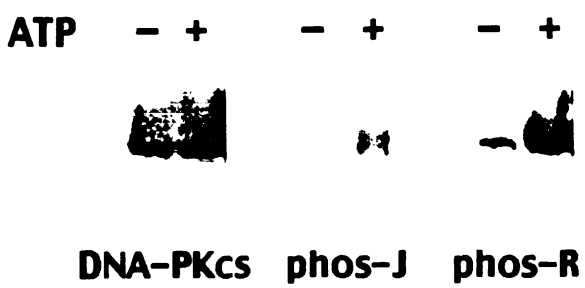
A

phos. peptide: LGKA[pT]QMPEGGQC
unphos. peptide: LGKA T QMPEGGQC

B



C



JK is phosphorylated *in vivo*. To determine whether JK is phosphorylated in response to DNA damage, HEK293 cells were treated with various DNA damaging agents. To enhance sensitivity, cells were also incubated with Okadaic acid (OA), a specific inhibitor of protein phosphatases 1 and 2A, which was necessary to facilitate the detection of phosphorylation at ABCDE (Douglas et al., 2002).

Zeocin is a member of the bleomycin/phleomycin family of antibiotics, known to bind and cleave DNA. At a concentration of 100 μg/ml, it is known to induce DSBs comparable to 250 Gy of radiation in *Chlamydomonas reinhardtii* (Chankova et al., 2007). Previous experiments using Zeocin revealed that HEK293 cells treated at 2 mg/ml for 2 hours induced DSBs, and subsequently autophosphorylation of DNA-PKcs at ABCDE and PQR (Meek et al., 2007). The experiment was repeated in this study; however, we found that phosphorylation at JK was detectable only at later time points, after 4 and 8 hours of treatment (fig 11). In addition, it should be noted that approximately 4 times more cellular proteins were loaded into each lane in order for the signals by phospho-J anti-sera to be detectable by Western blot, consistent with the results of the *in vitro* phosphorylation assay mentioned.

Another variation of DSBs is produced when a replication fork collapses, which can happen when it encounters a nick, or when the replication polymerase is chemically inhibited by Aphidicolin (APH) whereby only one free double-stranded end (DSE) is present. Without another DNA end, these types of DSBs are not repaired by NHEJ but are exclusively repaired by HR (Rothstein et al., 2000). However, there has not been any study to date that investigates how DNA-PKcs autophosphorylation is regulated in response to stalled replication forks which subsequently produce DSEs. Here, I show

that PQR is phosphorylated in response to APH treatment (fig 11), suggesting that DNA-PKcs is activated by DSE. In addition, phosphorylation at JK also occurs, but at a later time point than that at PQR, suggesting that phosphorylation at JK is indiscriminate to the type of DSBs.

Phosphorylation at JK suppresses autophosphorylation at PQR. Since JK is phosphorylated later than PQR, we next investigated whether phosphorylation at the two clusters was cooperative. V3 cells expressing JK mutants were treated with Zeocin for 2 hours as described previously and the samples were analyzed for phosphorylation at PQR by Western blot. Phosphorylation at the PQR cluster was consistently and significantly weaker in samples that contained the JK>D DNA-PKcs constructs (fig 12), suggesting a possible regulatory role for the JK site on the phosporylation at PQR.

Figure 11 ***In vivo*** **phosphorylation of JK and PQR in response to Zeocin and Aphidicolin.** HEK293 cells grown in T75 to 90% confluence were treated with 2 mg/ml Zeocin (Zeo) to induce DSBs, or 10 μg/ml Aphidicolin (APH) to induce DSEs, for 2, 4, and 8 hours in complete media containing 1 μM okadaic acid (OA), to stabilize phosphorylation and enhance detection. Negative control: cells were treated with DMSO. After treatment periods, cells were trypsinized and the whole cell extracts were prepared as described. Each lane consisted of 80 μg protein. Top panel: phosphospecific antibodies against PQR (phos-R) show that PQR is phosphorylated in response to Zeo and APH as early as 2 hours post-treatment. Middle panel: phosphorylation at JK (phos-J) occurred after 4 hours of treatment, in response to both Zeocin and Aphidicolin. Bottom panel: DNA-PKcs antibodies (42-27), as loading control.

Figure 11

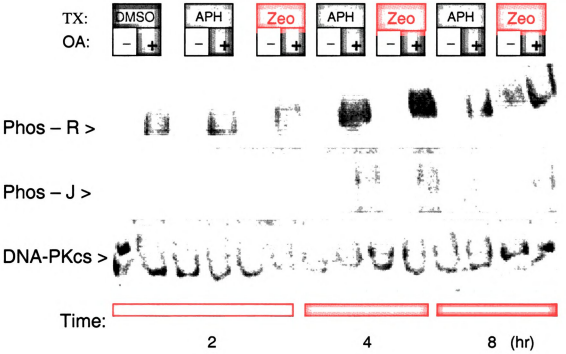
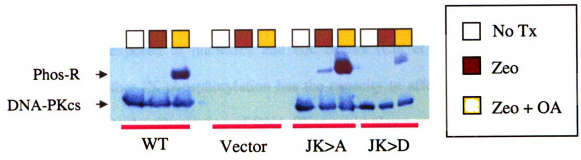

TX:
OA:
DMSO
APH
Zeo
APH
Zeo
APH
Zeo
Phos – R >
Phos – J >
DNA-PKcs >
Time:
2
4
8 (hr)

Figure 12. Phosphorylation at PQR is inefficient in the JK>D mutants. Transfected V3 cells were left untreated, treated with Zeocin (Zeo) alone, or with Zeocin and okadaic acid (OA) for 4 hours. Samples were analyzed for phosphorylation at PQR by Western blot. Top panel, the WT and the JK>A constructs exhibit robust phosphorylation at PQR in response to Zeocin and okadaic acid. However, phosphorylation at PQR is inefficient in the JK>D constructs. Bottom panel, the same blot was used to probe against DNA-PKcs (unphosphorylated region) as loading control.

Figure 12



JK>D constructs enhanced HR activities. Previous studies using DNA-PKcs mutants that were blocked from phosphorylation at ABCDE (ABCDE>A) demonstrated that DNA-PKcs can act as a "dominant negative" by inhibiting both repair pathways (Convery et al., 2005). In contrast, ablating phosphorylation at PQR stimulated HR activities, possibly by forcing the protein kinase to take an "open" conformation whereby DNA ends are exposed to nucleases and HR factors (Cui et al., 2005). We next investigated whether JK phosphorylation has the capacity to affect HR. These studies suggested that autophosphorylation of DNA-PKcs could play a role in affecting pathway choice.

The HR assay that I use relies on a chromosomally integrated substrate plasmid that contains two non-functional neo resistance genes (Allen et al., 2003) (in the V3 cells, subsequently named as Vd7 cell line.) The first nonfunctional neo gene is disrupted by the insertion of an 18-bp I-*Sce*I recognition site, and the other neo gene does not have a promoter and is not expressed (fig 13A). A functional neo gene can be generated only if an I-*Sce*I-induced break in the first neo gene is repaired by HR using the second neo gene as template. Conversely, if the DSB is repaired by NHEJ, the result is still a non-functional neo gene.

V3 cells harboring the substrate were transfected with the I-*Sce*I-expression plasmid and 0, 2, 4, or 8 μg of DNA-PKcs plasmids bearing either no mutations (WT), JK ablated (JK>A), or JK phosphorylation mimic (JK>D). Their expression levels after 48 hours of transfection are shown in a Western blot (fig 13B). The results of HR activities are graphed in fig 13C.

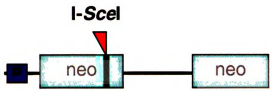
Consistent with our previous findings, the presence of wild-type DNA-PKcs in V3 cells can effectively reduce HR activities by at least 50%, as shown by the higher frequencies of HR usage in cells deficient in DNA-PKcs. Interestingly, we also observed a dose dependency of HR suppression, whereby the presence of increasing DNA-PKcs availability further suppress HR activities. This has important implications (beyond the scope of this dissertation) because of the extremely high levels of DNA-PKcs expressed in human cells (Kienker et al., 2000) and is an ongoing project in our lab.

Shown in figure 13 is a representative of 3 HR assays that consistently shows the positive effects of the JK>D mutant on HR activities. (Additional HR data are shown in Supplemental section of this dissertation.) More importantly, the presence of more JK>D constructs have the opposite effects on HR than WT and JK>A DNA-PKcs. These data demonstrate that the JK>D construct actively enhance the HR pathway.

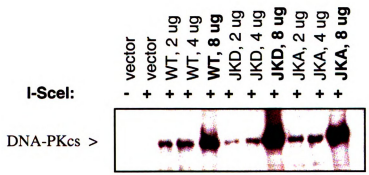
Figure 13 HR assay. (**A**) A single copy of the substrate plasmid containing two *neo*-resistance genes was integrated into the genome of V3 cells. The first *neo* gene is non-functional due to an 18-bp disruption of the I-*Sce*I recognition site whereas the second *neo* gene is non-functional due to the absence of a promoter (P). (**B**) V3 cells were transiently transfected with vector alone, JK>A, JK>D, or WT DNA-PKcs at 2, 4, and 8 μg, along with the I-*Sce*I expression plasmid and a *puromycin*-resistance expression plasmid. After 2 days, expression level was determined by Western blot analysis. Loading control was established by total protein concentration of whole cell extracts. Each lane consisted of 10 μg total protein. (**C**) Graph of HR activities from one representative experiment of 3. % HR was determined by calculation of G418 resistant colonies, which indicated repair by HR, over the number of puromycin resistant colonies, which indicated transfection efficiency of the experiment. RESULT: Spontaneous HR was observed in samples that did not express I-*Sce*I but possessed G418 resistant colonies. V3 cells that were transfected with empty vector do not express DNA-PKcs, and they exhibited higher HR repair than those transfected with WT DNA-PKcs as we expected. Interestingly, increasing the expression of WT DNA-PKcs in V3 cells caused further suppression of HR activities. The JK>A clones demonstrated HR suppression like WT. However, the JK>D mutants displayed greater use of HR with increasing JK>D protein expression, indicating that HR activities were enhanced by the JK>D construct.

Figure 13. HR

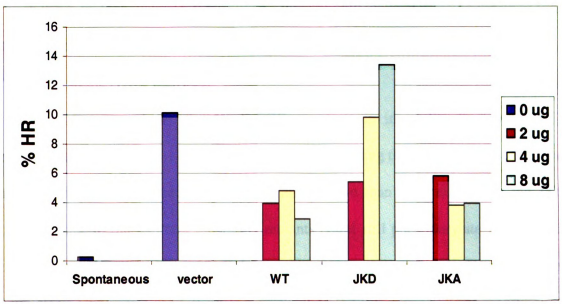
A



B. Western blot



C. HR assay



JK>D/PQR mutant construct. We previously showed that HR is enhanced when PQR phosphorylation is blocked (Cui et al., 2005). Since mimicking phosphorylation also enhanced HR activities, we wanted to determine whether the JK site is mechanistically distinct from the PQR cluster. Therefore, I created a DNA-PKcs construct that combined both JK>D and PQR>A mutations (here forth called JK>D/PQR). If JK operates through PQR, then the combined mutant construct should not enhance HR more so than the JK>D or PQR mutants. Conversely, if phosphorylation at JK activates another means of promoting HR, then the combined mutant construct should enhance HR additively.

As with the other mutants, expression of JK>D/PQR was confirmed by Western blot analysis (data not shown). First, we wanted to determine how this construct affected the NHEJ pathway. This was accomplished by the IR survival assay and the V(D)J recombination assay. The results of 3 IR experiments are plotted in figure 14A. As expected, cells complemented with the JK>D mutants exhibited a degree of radiosensitivity that was similar to cells deficient in DNA-PKcs, whereas cells expressing the PQR>A mutants were only mildly so. Interestingly, cells expressing the JK>D/PQR construct behaved like V3 DNA-PKcs deficient cells, indicating that the JK>D phenotype dominates in the NHEJ pathway.

Next, we analyzed the JK>D/PQR construct in its ability to support V(D)J recombination (fig 14B). Not surprisingly, cells expressing the JK>D/PQR mutants were also deficient in V(D)J recombination, even more so than cells expressing the JK>D construct due to the fact that the JK>D mutants could still phosphorylate PQR – which exhibited near normal levels of V(D)J recombination – whereas the JK>D/PQR construct couldn't phosphorylate at PQR, further implicating the importance of phosphorylation at

PQR in the NHEJ pathway. Hence, by mimicking phosphorylation at JK and blocking phosphorylation at PQR, we effectively suppressed NHEJ activities.

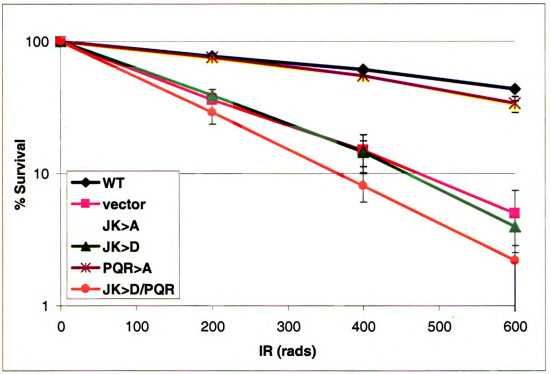
Interestingly, the JK>D/PQR construct has the opposite effect on HR activities. As demonstrated in figure 14C, the JK>D/PQR construct greatly enhances HR. In fact, we noted that the HR enhancement by JK>D/PQR is an additive effect of JK>D and PQR, indicating that JK and PQR are mechanistically independent of each other in the HR pathway. Equally important, we observed that JK>D and JK>D/PQR positively promotes HR in a dose dependent manner, and that having more of either mutant construct expressed in the cell resulted in greater HR utilization.

Previously, we showed that a distinct phenotype of PQR>A is in creating longer deletions at the joints (Cui et al., 2005). Therefore, we wanted to determine whether the JK site also participates in DNA end processing. I analyzed the sequences of coding joints from V(D)J recombination of the JK mutants as well as the JK>D/PQR mutants. As shown in Table 1, cells expressing the JK mutants exhibited similar nucleotide losses at the joints as cells expressing wild-type DNA-PKcs (WT). In contrast, cells expressing the JK>D/PQR repaired coding joints with an average of 13.5 nucleotide losses, which is comparable to the 14.1 nucleotide loss exhibited by the PQR>A mutants, confirming that JK phosphorylation does not affect DNA end processing. In conclusion, phosphorylation of the JK cluster is functionally different from blocking PQR phosphorylation, but both are able to increase HR activities.

Figure 14 Combined JK>D/PQR mutants show that phosphorylation of JK and PQR have distinct functional effects. (**A**) IR survival assay. Cells expressing the PQR>A and JK>A constructs were mildly radiosensitive, as compared to WT. However, cells expressing the JK>D and JK>D/PQR constructs exhibited severe radiosensitivity. Error bars represent the SEM of 3 experiments. (**B**) V(D)J recombination assay. As shown previously, PQR>A and JK>D exhibit moderate and severe deficits, respectively, in V(D)J recombination. However, cells transfected with the JK>D/PQR mutation were significantly more impaired in their ability to support V(D)J recombination than either PQR>A or JK>D constructs, indicating that the JK>D/PQR construct is severely inhibited from the NHEJ pathway. Error bars represent the SEM of 3 experiments. (**C**) HR assay. V3 cells were transfected either with 2 or 6 μg of DNA-PKcs constructs (WT, JK>D, PQR>A, JK>A, and JK>D/PQR) or no DNA-PKcs (-). JK>D/PQR exhibited greater utilization of HR activities than either PQR>A (PQR) or JK>D alone.

Figure 14

A. IR survival assay



B. V(D)J recombination assay

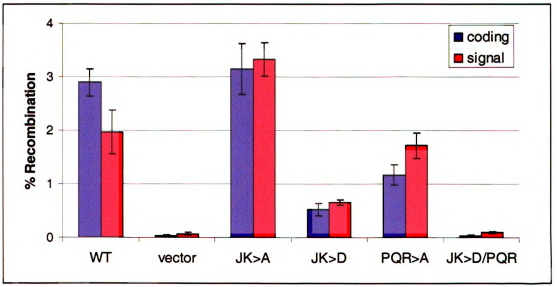


Figure 14 (cont.)

C. HR assay

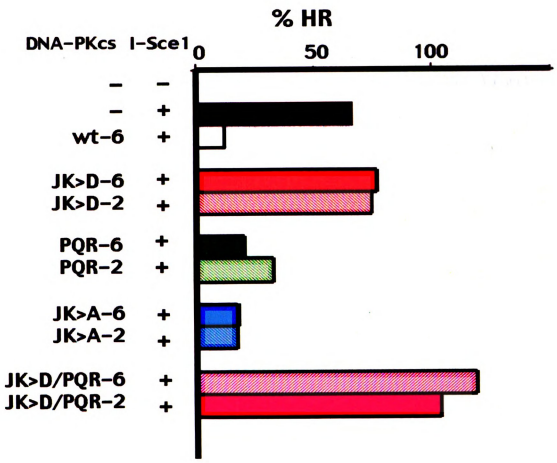


Table 1. Coding joint analysis. Following V(D)J recombination assay, coding joint substrates were analyzed for nucleotide losses. As shown, the JK>A and JK>D mutants exhibited unremarkable increases in nucleotide losses at the joints that were statistically insignificant as defined by student t-test. But the JK>D/PQR mutant demonstrated huge losses that were comparable to the PQR>A mutants, indicating that the JK phosphorylation site was not important for DNA end processing. (*) Data for WT and PQR>A were retrieved from previous publication (Cui, Yu et al. 2005).

Table 1

DNA-PKcs	No. sequences	average nt loss
WT*	48	5.9
JK>A	42	6.0
JK>D	66	7.4
PQR>A*	76	14.1
JK>D/PQR	28	13.5

DISCUSSION

In chapter 2, I showed that V3 cells expressing the JK>D mutation did not complement DNA-PKcs deficiency in response to IR, and that this defect was related to the cells' inability to perform NHEJ functions. However, I did not identify the culprit for the observed defect. The JK>D construct interacted with NHEJ factors such as Artemis, Ku70/86, and XRCC4 normally in a Ni-NTA bead pull-down assay (data shown in chapter 2) showing that the substitution did not disrupt its overall structure. More importantly, the JK>D mutant exhibited kinase activities at a level comparable to wild-type, suggesting that this protein kinase would still be able to phosphorylate itself at other sites (data shown in chapter 2). Hence, it appeared that JK>D presented a genuine phenotype that mimics the phosphorylation at JK.

However, *in vivo* studies using Zeocin and Aphidicolin to induce DSBs and DSEs, respectively, demonstrate that JK is phosphorylated only after prolonged DNA damage. Furthermore, results of Western blot analysis show that phosphorylation of JK was much weaker than that at the PQR, suggesting that phosphorylation at JK is heavily regulated, possibly by phosphatase activities. Conversely, the weak signal at JK could be due to the poor binding affinity of the anti-sera to its target However, the anti-sera were definitely very specific to the phosphorylated form of J, as it was able to detect even 0.08 μg of the peptide on membrane.

Phosphorylation at JK probably does not occur in a normal response to DSBs, when NHEJ should function at its highest peak. Cells expressing the phosphomimic construct, JK>D, show significantly reduced NHEJ activities in response to DNA damage, suggesting that phosphorylation at JK suppresses the NHEJ pathway, which

could be detrimental for the cell. Although we have not determined the mechanistic basis for the inability of JK>D to promote NHEJ, we have eliminated defects in enzymatic activity, DNA binding, appropriate targeting to DSBs, and appropriate interaction with XRCC4, Ku, and Artemis as potential explanations. Work is ongoing in the laboratory to understand how JK>D perturbs NHEJ. Surprisingly, we found that cells expressing the phosphomimic form were able to promote significantly higher HR activities than cells without any DNA-PKcs, suggesting a possible role for JK phosphorylation in regulating pathway choice.

Detection of phosphorylation at JK presented the most difficult obstacle in this study. For instance, due to significantly lower levels of DNA-PKcs present in V3 CHO cells, detection of JK phosphorylation in these cells was impossible. The only cell types that we could use were human cell lines, such as HEK293 and MO59K cells, which had more DNA-PKcs proteins. Because phosphorylation at J required a longer incubation with DNA damaging agents, cells retrieved from such conditions often appeared sick, making detection even more difficult.

Despite difficulties in technique, we confirmed by Western blot analysis that JK was phosphorylated *in vitro* and *in vivo* in response to DNA damage. Phosphorylation at JK probably serves an evolutionarily conserved function since this cluster is conserved at least from the level of the frog. In this study, we demonstrated that phosphorylation at PQR in response to DNA damage was reduced in the JK>D construct, suggesting that phosphorylation of PQR could be regulated by that at JK. This finding was consistent with our previous study, which demonstrated that blocking phosphorylation at the PQR cluster led to increased coding joint loss and enhanced HR activities. Furthermore, data

from cells expressing the combined mutation, JK>D/PQR, showed that DNA processing was a distinct feature of PQR that was not shared with JK phosphorylation.

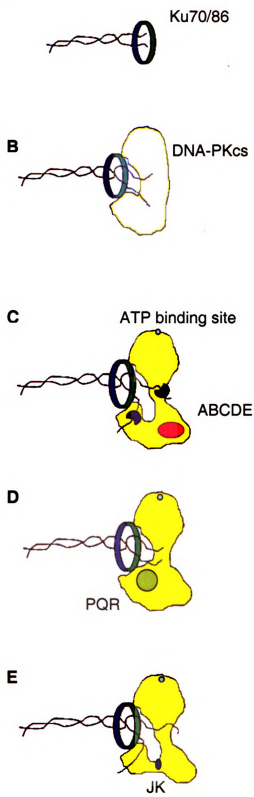
In light of our findings, we propose the following phosphorylation model (fig 15): in a normal cellular response to DSBs, phosphorylation at ABCDE occurs soon after the activation of DNA-PKcs, resulting in proper processing of DNA ends, whereby hairpin structures are opened, and nucleotides with damaged bases are removed. This step is further modified by the phosphorylation of PQR, which suppresses excessive trimming at the ends, thereby decreasing nucleotide loss at the joints. Phosphorylation of PQR has been suggested to promote ligation (Cui et al., 2005) although nobody has shown this. Phosphorylation at JK may not occur during a normal NHEJ response. However, when there are more DSBs than the cell can handle by NHEJ alone, or if DNA ends are so severely damaged that end processing is not successful (for example, cross-linking agents), it may decide to switch to a different mode of repair, particularly to HR. While it is not known yet where this signal to abandon the NHEJ pathway comes from, it may be manifested as phosphorylation at JK, which, judging from coding joint experiments, probably occurs prior to the phosphorylation at PQR and after the activation at ABCDE. The JK>D mutants exhibited an unremarkable increased nucleotide loss at the coding joints, possibly due to its tendency to suppress phosphorylation at PQR, thereby weakly mimicking the effects of PQR>A.

However, we do not know how phosphorylation at JK controls pathway choice. We speculate that DNA-PKcs could direct DSB repair toward HR by two modes of mechanisms: first, since DNA-PKcs and Ku70/86 arrive at DNA ends earliest, DNA-PKcs could promote HR by allowing DNA end resection to progress. Secondly, DNA-

PKcs, which has been shown to phosphorylate RPA *in vivo* (Wang et al., 2001), may recruit RPA and possibly other HR factors to DSB sites. Studies in yeast show that long ssDNA ends inhibit NHEJ activity (Daley and Wilson, 2005), and DNA end resection has been suggested to be the switch between NHEJ and HR (Frank-Vaillant and Marcand, 2002). Recent biochemical studies showed that DNA resection activities are increased in NHEJ deficient yeast cells (DNL4-/-) (Zierhut and Diffley, 2008), consistent with another study that showed extensive ssDNA formation in NHEJ deficient mammalian cell lines (Karlsson and Stenerlow, 2007). The second possibility is that phosphorylation at JK could actively recruit HR factors that participate in initiation, such as CtIP or RPA. However, at this time, we do not know how phosphorylation at JK promotes HR.

Figure 15. Model of phosphorylation events during DNA DSBs. Hypothetical structures of DNA-PKcs in response to site-specific phosphorylation at ABCDE (red circle), PQR (green circle), and JK (purple circle). ATP binding site (light blue circle) is on top of the "head" structure of DNA-PKcs, and autophosphorylation of ABCDE and PQR occurs by *trans* (Meek et al., 2007). At this time, it is not known what phosphorylates JK. Note that only one side of DNA ends is shown.

Figure 15



A. Initiation:
DNA ends are immediately recognized and bound by the Ku70/86 heterodimer.

B. Recruitment and Activation
DNA-PKcs is recruited to DNA termini by Ku. DNA-PKcs kinase activation leads to Ku translocation inward DNA termini, and synapsis of DNA-PKcs of opposing ends.

C. ABCDE:
Phosphorylation of ABCDE promotes DNA end processing, including endonuclease activities of Artemis.

D. PQR:
Phosphorylation of PQR inhibits further end processing.

E. JK:
Phosphorylation of JK suppresses phosphorylation at PQR, inhibits NHEJ, and promotes HR.

CHAPTER 4

Phosphorylation at JK: Conclusions and Future Directions

The evolution of DNA-PKcs occurs rather late. Ku proteins, XRCC4, and DNA Ligase IV – but not DNA-PKcs – are present in yeast and prokaryotic NHEJ pathway. Yet, where DNA-PKcs is present, it has adopted a central role in the NHEJ pathway. DNA-PKcs has high affinity toward Ku-bound DNA, and is known to "cap off" DNA ends from nucleases as well as HR factors. But if "capping" of DNA ends were absolute, the HR pathway would be completely inhibited in human cells, which have the most DNA-PKcs. Yet, we know that some DSBs are repaired by HR, especially during stalled replication forks.

Many laboratories have indirectly shown that DNA-PKcs directs DNA DSB repair toward the NHEJ pathway, by demonstrating that HR could either be enhanced or suppressed by the absence or presence of DNA-PKcs, respectively. Our lab first showed that blocking phosphorylation at PQR promoted repair by HR in V3 cells expressing the PQR>A mutant construct (Cui et al., 2005). However, we speculate that the PQR>A mutant construct enhanced HR by leaving DNA ends exposed, rather than it being an active regulatory site for pathway choice. From our understanding of the JK>D mutant constructs, we believe that the JK site is the regulatory site that promotes HR. When JK is phosphorylated, DNA-PKcs can no longer support NHEJ and HR is enhanced.

Of utmost interest – in addition to the relationship between JK phosphorylation and HR activities, as mentioned in chapter 3 – would be to determine how the JK site is regulated. We still do not know what roles, if any, phosphorylation of other sites of DNA-PKcs play in regulating the JK site from becoming phosphorylated. A way to answer this question would be to make combined mutations of several relevant sites and analyze for the presence of JK phosphorylation by Western blot.

In addition, an important regulator of JK phosphorylation is probably the protein kinase responsible for its phosphorylation. As mentioned, we did not identify JK as an autophosphorylation site by MS; therefore, it is highly likely that JK is phosphorylated by another serine-threonine kinase, such as ATM or ATR. We can answer this question by treating cells with specific ATM inhibitors and determine whether JK was phosphorylated by Western blot. If JK is phosphorylated by ATM, then cells that were treated with the ATM inhibitor should remain unphosphorylated. Conversely, if JK is

phosphorylated by another protein kinase, then it should still be phosphorylated even in treated cells.

It makes sense that JK phosphorylation should be prevented during a normal response to DSBs because NHEJ is the most effective DSB repair pathway in eukaryotes. In fact, sustained JK phosphorylation is further regulated by nuclear phosphatases. As mentioned, the use of Okadaic acid, a potent protein phosphatase, is instrumental to the detection of JK phosphorylation. A question that we could ask is whether the phosphatase activities at JK is diminished during late S and G2 phases of the cell cycle, thereby facilitating HR repair. In order to answer this question, we could treat S-G2 synchronized cells with DNA damaging agents without Okadaic acid, and analyze for phosphorylation at JK with Western blot. If phosphatase activities are reduced, then JK should remain phosphorylated even in the absence of Okadaic acid.

In conclusion, phosphorylation at JK is an evolutionarily conserved mechanism to direct some DSBs toward HR, probably when the damages cannot be efficiently repaired by NHEJ. Phosphorylation at JK may recruit HR factors to DSBs or stabilize DNA ends for resection, thereby promoting HR.

Supplemental Data

Figure 16. Additional HR data

(A) Assay conditions were as follows: 4 μg DNA-PKcs plasmid (vector, WT, JK>D, and JK>A) along with I-*Sce*I expression plasmid and puromycin-resistance plasmid were transiently transfected into V3 cells. %HR determined by the number of G418-resistant colonies divided by puromycin-resistant colonies (transfection efficiency). Spontaneous HR: V3 cells were not transfected with I-*Sce*I expression plasmid.

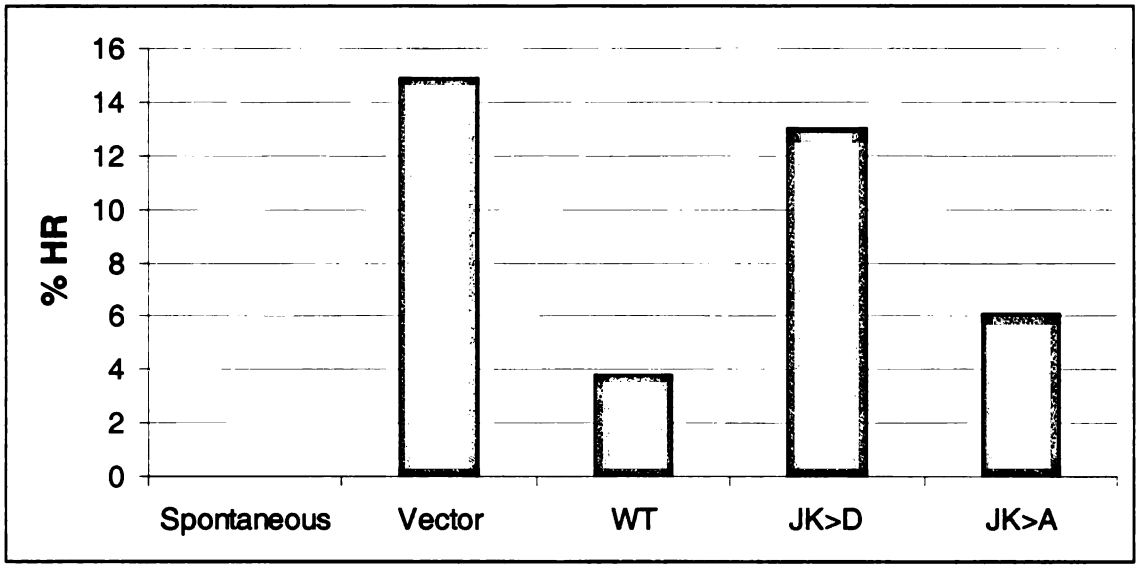
(B) Same conditions as described above (A).

(C) As above, but V3 cells were transfected either with 4 or 8 μg DNA-PKcs plasmid (vector, WT, JK>D, and JK>A).

(D) As above, but V3 cells were transfected either with 6 or 8 μg DNA-PKcs plasmid (vector, WT, JK>D, JK>A, and JK>D/PQR>A).

Figure 16. Additional HR data

A.



B.

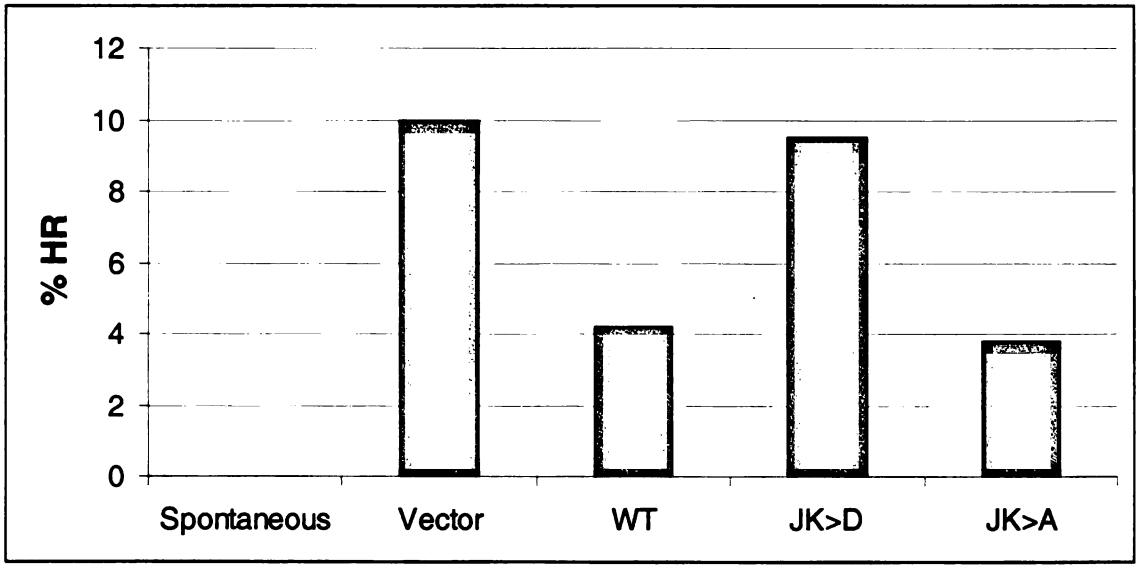
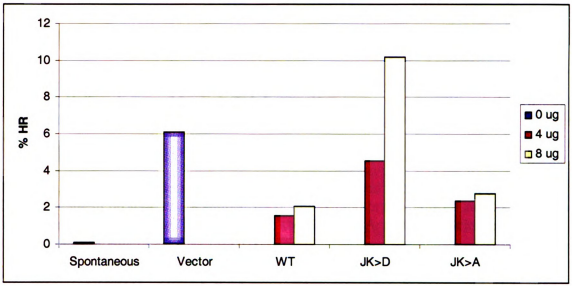
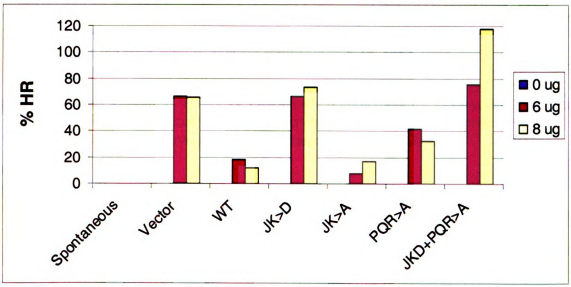


Figure 16 cont.

C.



D.



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