Effect of the inositol polyphosphate InsP₆ on DNA-PK-dependent phosphorylation

Running title: Effect of InsP₆ on DNA-PK

Author: Les Hanakahi

University of Illinois at Chicago
College of Pharmacy
Department of Medicinal Chemistry and Pharmacognosy
Rockford Health Science Campus
1601 Parkview Avenue
Rockford, Illinois 61107
email: hanakahi@uic.edu
Tel: (1+) 815-395-5924 (office)
Abstract

Inositol hexakisphosphate (InsP$_6$) is a member of the inositol polyphosphate group that participates in numerous intracellular signaling pathways. My colleagues and I previously reported that InsP$_6$ stimulated double-strand break repair by non-homologous end joining (NHEJ) in cell-free extracts and that InsP$_6$ binding by the Ku70/80 subunit of the DNA-dependent protein kinase (DNA-PK) was required for stimulation of NHEJ in vitro [1-3]. This report describes InsP$_6$-dependent phosphorylation of two NHEJ factors, XRCC4 and XLF, in partially purified human cell extracts. XRCC4 and XLF are known substrates for DNA-PK, which does not require InsP$_6$ for protein kinase activity. Consistent with a role for DNA-PK in these reactions, InsP$_6$-dependent phosphorylation of XRCC4 and XLF were DNA-dependent and not observed in the presence of DNA-PK inhibitors. Depletion of the Ku70/80 DNA-, InsP$_6$-binding subunit of DNA-PK resulted in loss of InsP$_6$-dependent phosphorylation and demonstrated a requirement of Ku70/80 in these reactions. Complementation of Ku70/80-depleted reactions with recombinant wild type Ku70/80 restored InsP$_6$-dependent phosphorylation of XRCC4 and XLF. In contrast, addition of a Ku70/80 mutant with reduced InsP$_6$ binding failed to restore InsP$_6$-dependent phosphorylation. While additional protein kinases may participate in InsP$_6$-dependent phosphorylation of XRCC4 and XLF, data presented here describe a clear requirement for DNA-PK in these phosphorylation events. Furthermore, these data suggest that binding of the inositol polyphosphate InsP$_6$ by Ku70/80 may modulate the substrate specificity of the phosphoinositide-3 kinase related protein kinase DNA-PK.
ABBREVIATIONS

NHEJ: non-homologous end joining

DNA-PK: DNA-dependent protein kinase

DNA-PKcs: DNA-PK catalytic subunit

PIKK: phosphoinositide-3 kinase related protein kinase

InsP: inositol polyphosphate

InsP$_6$: inositol hexakisphosphate

CK2: casein kinase 2
Introduction

The inositol polyphosphates (InsPs) are a group of ubiquitous, structurally similar small molecules that differ from one another in the placement of phosphate and pyrophosphate groups around a 6-carbon inositol ring. Study of the kinases and phosphatases responsible for InsP metabolism has demonstrated that the InsPs play important roles in many cellular functions [4-8]. Of particular importance to this paper is inositol hexakisphosphate (InsP₆), which is phosphorylated at all 6 positions. Studies in mice have shown that the pathway responsible for InsP₆ biosynthesis is required for viability. For example, failure to express the inositol polyphosphate multikinase Ipk2, which produces the InsP₆ precursor InsP₅, or the InsP₅ kinase that produces InsP₆ resulted in embryonic lethality [9, 10]. These reports indicate that production of the highly phosphorylated InsPs, InsP₆ in particular, are essential for growth and development, and exemplify the importance of the InsPs as a group.

InsP₆ has been mechanistically linked to biologically important pathways such as chromatin remodeling [11, 12], RNA editing [13] and mRNA export [14-17]. The well-studied role of InsP₆ in mRNA export has outlined a basic paradigm for the action of InsP₆, which alters the structure and/or surface charge topology of an InsP₆-binding protein that, in turn, modulates the activity of an enzyme. A variation of this paradigm is reflected in the regulation of casein kinase 2 (CK2) by InsP₆, in which formation of an InsP₆-CK2 complex prevents binding of the CK2 negative regulator nucleolar protein p140 [18-20]. Because a single protein kinase may act on several substrates, the discovery of CK2 regulation by InsP₆ sets an important precedence for a mechanism by which InsP₆ can exert a profound biological effect. My colleagues and I have shown that
InsP₆ interacts with the regulatory subunit of a protein kinase that is a key participant in DNA double-strand break (DSB) repair by non-homologous end joining (NHEJ), which implicates InsP₆ as a co-factor in this important process [1-3].

NHEJ plays an influential role in maintenance of genomic integrity. Studies of mammalian NHEJ employing mouse models have demonstrated that genomic stability depends upon the NHEJ mechanism and deficiencies in this pathway can lead to events that initiate or propagate tumorigenesis [21]. NHEJ rejoins DSBs during G0, G1 and early-S phases of the cell cycle and is therefore the dominant mechanism used for DSB repair in humans and other multicellular organisms. End joining is carried out by DNA ligase IV (ligase IV), which acts as part of the XLF/XRCC4/ligase IV complex [22-26]. In a direct display of the tumor-prevention function of the mammalian NHEJ apparatus Sharpless et al. [27] demonstrated that in tumor-prone mice, even a modest reduction in ligase IV production dramatically increased the accumulation of soft tissue sarcomas showing chromosomal rearrangements. Such data support the growing view that deficiencies in the NHEJ apparatus can provide a mechanism for the generation of oncogenic events that drive general mammalian tumorigenesis.

XRCC4 directly interacts with ligase IV and XLF and is required for stability and catalytic activity of the ligase [25]. All members of this complex are required for NHEJ in vivo and deficiency in XLF, XRCC4 or ligase IV results in a loss of NHEJ in cells [22-26]. Given the central role of the XLF/XRCC4/ligase IV complex in end joining, modification of XLF, XRCC4 or ligase IV has the potential to significantly impact NHEJ.

A second protein complex required for NHEJ is the heterotrimeric DNA-dependent protein kinase (DNA-PK). This serine/threonine kinase is comprised of the
phosphoinositide-3 kinase-related protein kinase (PIKK) DNA-PK catalytic subunit (DNA-PKcs) and the heterodimeric Ku70/80 regulatory subunit [28]. Knockout studies in mice have shown that all three components of DNA-PK are required for NHEJ in vivo and deficiency in DNA-PKcs, Ku70 or Ku80 results in genomic instability, immunodeficiency and radiosensitivity [28-30]. Because the catalytic activity of DNA-PK is required for NHEJ in vivo, inhibitors of DNA-PK radiosensitize cells and have potential as chemotherapeutic agents [28, 31]. While DNA-PK has been shown to act on the known NHEJ factors, many of which are phosphorylated in response to DNA damage, the biological roles of these phosphorylation events remain an open question [25].

DNA-PK has been shown to bind InsP$_6$, which acts as a stimulatory cofactor for NHEJ in vitro [2]. The Ku70/80 heterodimer was later identified as the InsP$_6$-binding component of DNA-PK [1, 32]. These observations were followed by the demonstration that Ku70/80 mobility was reduced in cells depleted of InsP$_6$ through treatment with calmodulin inhibitors, [33] and that InsP$_6$ binding by Ku70/80 is necessary for stimulation of NHEJ in vitro [3]. Taken together these findings are consistent with the hypothesis that InsP$_6$ binding by Ku70/80 acts to potentiate one or more steps in DNA-PK-dependent DNA repair by NHEJ.

In assays using partially purified human-cell extracts, InsP$_6$-dependent stimulation of NHEJ was coincident with InsP$_6$-dependent phosphorylation of the NHEJ factors XRCC4 and XLF. These InsP$_6$-dependent phosphorylation events required DNA-PK activity and, most significantly, InsP$_6$-binding by Ku70/80. Because Ku70/80 is the regulatory subunit of DNA-PK, these data imply that phosphorylation of NHEJ factors by DNA-PK may be modulated by InsP$_6$. 
Materials and Methods

Reagents

Tyrosine Phosphatase Inhibitor Cocktail (Upstate). Okadiac acid (Roche). Ku55933 and Nu7026 were a generous gift from Graham Smith, KuDos Pharmaceuticals, Astrazeneca. Expression and purification of recombinant Ku70/80 (wild type and Ku70DM/80TM) were performed as previously described [3]. The PhosphoProtein Purification Kit (Qiagen) was used to isolate phosphorylated proteins as per manufacturers instructions.

Rabbit polyclonal anti-XLF antibodies

Full-length human XLF in the plasmid pGEX-XLF (a generous gift from Steve Jackson, Cambridge, UK) was subject to direct DNA sequencing to confirm the sequence of human XLF then transformed into E. coli strain Rosetta 2 (Novagen). Cells were grown to log phase, cooled to 18°C, induced with 0.25 mM IPTG for 18 hours then collected by centrifugation and pellets were stored at -80°C. Cells were resuspended in Buffer A (50 mM tris pH 8.0, 5 mM EDTA, 0.8 M NaCl, 0.1% Triton X-100) with 1 mM DTT, 1 mM PMSF, 1 mM Benzamidine with 1x Complete Mini EDTA-free protease inhibitor cocktail (Roche) and lysed by sonication. Cell debris was removed by centrifugation and the resulting lysate was bound in batch to GST-sepharose (GE) for 1 hour on ice with gentle agitation. The resulting mixture was packed into a column, washed with 5 column volumes of Buffer A, eluted with 40 mM Glutathione in Buffer A and the bound fraction was dialyzed against Buffer B (20 mM Tris pH 8.0, 5% glycerol,
0.5 mM EDTA) with 50 mM NaCl and 1 mM DTT. Following dialysis the sample was fractionated over a 1 ml Hi-trap Q column (GE) in buffer B and eluted with a 50 mM to 1M NaCl linear gradient. Peak fractions were pooled, resolved on preparative SDS-PAGE and bands corresponding to full-length GST-XLF were excised and used as implants to immunize two New Zealand White Rabbits (JHU08 and JHU09) for antibody production. JHU08 antiserum was suitable for XLF immunoprecipitation (IP) and JHU09 antiserum was suitable for Western blot detection.

**Large-scale extract preparation**

Whole-cell extract was prepared essentially as previously described [34]. Briefly, HeLa cells were grown in suspension, 5 x 10⁹ cells were harvested by centrifugation, washed twice with cold phosphate-buffered saline (PBS) and stored as frozen (-80°C) cell pellets. To prepare extract, cell pellets were thawed and resuspended in 2.5x packed-cell volumes (PCV) of hypotonic lysis buffer (HPLB: 10 mM Tris-HCl pH 8.0, 1 mM EDTA, 1 mM DTT) and held on ice for 20 min. Cells were opened by dounce homogenization in the presence of protease inhibitors (1 mM PMSF, 2.2 ng/ml aprotinin, 1 ng/ml leupeptin, 1 ng/ml pepstatin A and 1 ng/ml chymostatin). High salt buffer (HSB: 83.5 mM Tris pH 7.5, 1.65 M KCl, 3.3 mM EDTA, 1 mM DTT) was added to adjust to final salt concentration to 0.33 M KCl and the sample was held on ice for 20 min. The sample was subject to ultracentrifugation (170,000 xg, 3 hours, 4°C, Beckman SW41 Ti rotor), the supernatant was collected, precipitated with 65% ammonium sulphate then dialyzed against L buffer (20 mM Tris pH 8.0, 10% glycerol, 0.5 mM EDTA and 1 mM DTT) with 0.3 M KOAc for 2 hours. The resulting extract was passed over...
phosphocellulose equilibrated in L buffer with 0.3 M KOAc, the column flow-through was collected and diluted to 0.1 M KOAc with L buffer and fractionated over phosphocellulose equilibrated in L buffer with 0.1 M KOAc. Step-elution (L buffer, 0.3 M KCl) was used to collect phosphocellulose-bound proteins, which were precipitated with 65% ammonium sulphate to concentrate the protein sample, which was dialyzed against L buffer with 0.1 M KOAc before being snap frozen on liquid nitrogen and stored at -80°C. Protein concentration: 15-20 mg/ml by Bradford assay.

**Small-scale extract preparation**

Small-scale whole-cell extract was prepared from MO59J and MO59K cells essentially as previously described for 120 min mini-NHEJ extracts [35]. Briefly, adherent cells were harvested at 70-80% confluency using a cell scraper, collected (centrifugation, 5 min, 800 xg, 4°C), washed twice in PBS and stored at -80°C. Frozen cell pellets were resuspended in 2 PCV of HPLB, incubated on ice for 20 min and lysed by vortexing for 30 sec. Nuclei were collected (microcentrifugation, 800 xg, 2 min, room temp) and the supernatant was retained. Nuclei were resuspended in 1 PCV of nuclear extract buffer (25 mM Tris pH 8.0, 0.33 M KCl, 1.5 mM EDTA) and incubated on ice for 20 min. The retained supernatant was added back to the nuclei and cell debris was removed (microcentrifugation, 10 min, 16,500 xg, 4°C). The resulting supernatant was collected and used without dialysis. Protein concentration: 5-10 mg/ml by Bradford assay.

**DNA end-joining assays**
Reactions were carried out essentially as previously described [2]. Briefly, 10 µl reactions that contained 50 mM HEPES pH 8.0, 0.1 M KOAc, 0.5 mM Mg(OAc)$_2$, 1 mM ATP, 1 mM DTT, 0.1 mg/ml BSA, 0.6 mg/ml partially purified extract and 10 ng $^{32}$P-labeled Hind III-linearized pBlueScript were incubated for 1 hour at 37ºC. DNA products were deproteinized by treatment with proteinase K and SDS, separated by agarose gel electrophoresis (0.6%, TAE) then visualized by autoradiography.

**XRCC4 phosphorylation**

Reactions were carried out using the same conditions as described for DNA end joining using unlabeled DNA as substrate. Reactions were terminated by the addition of 4x protein sample loading buffer and resolved on 9.4% 21.1:1 acrylamide:bis SDS-PAGE gels, which were run for 160 min at 150V when the 45 kDa marker reached the bottom of the gel. Samples were transferred to PVDF membrane (Immobilon P) and XRCC4 was detected using anti-XRCC4 rabbit polyclonal antiserum (Serotec) at 1:2000 dilution.

**XLF phosphorylation**

100 µl reactions were carried out as described for DNA end joining using unlabeled DNA, 0.1 mM ATP and $[^{32}$P]ATP. Reactions were terminated by the addition of 300 µl of IP binding buffer (14 mM Tris pH 8.0, 0.7 M NaCl, 0.35 mM EDTA, 1.33% NP-40 and 1 mM DTT). Samples were pre-cleared on Protein A Sepharose (25 µl 50% slurry, GE) for 1 hour at 4ºC with gentle agitation. Centrifugation was used to separate Protein A sepharose from the cleared extract, which was retained and combined with
0.5 µl of anti-XLF rabbit polyclonal antiserum (JHU08) at 4ºC for 2 hours with gentle agitation, after which the sample was transferred to a tube containing 12.5 µl washed Protein A Sepharose and binding was allowed to proceed for 1 hour at 4ºC with gentle agitation. Centrifugation was used to collect the Protein A Sepharose resin, which was then washed 3 times with 1 ml of IP wash buffer (50 mM Tris pH 8.0, 0.6 M NaCl, 5% glycerol, 1 mM EDTA and 1% NP-40). During the 2nd wash the sample was incubated at 4ºC for 15 min with gentle agitation to minimize background. Immune complexes were eluted with 2x SDS-PAGE buffer, resolved on 9% SDS-PAGE and transferred to PVDF membrane. ³²P was detected by phosphoimager after which XLF was detected using anti-XLF rabbit polyclonal antiserum (JHU09) at 1:1000 dilution.

Results

InsP₆-dependent XRCC4 phosphorylation

My colleagues and I previously reported mutational analysis of Ku70/80, which showed that Ku70/80 proteins with reduced affinity for InsP₆ (InsP₆-binding mutants) were impaired for participation in NHEJ in vitro [3]. Because Ku70/80 is an important functional part of DNA-PK, binding of InsP₆ might affect the ability of DNA-PK to phosphorylate protein targets. To test the hypothesis that InsP₆ may affect DNA-PK phosphorylation of full-length protein substrates, the effect of 1 µM InsP₆ on phosphorylation of the DNA-PK substrate XRCC4 [36, 37] was observed during the course of a DNA end-joining reaction. As shown in Figure 1A, Western blot analysis of
DNA end-joining reactions (top) revealed changes in XRCC4 mobility on SDS-PAGE that correlated with increased DNA end joining in the presence of InsP_6 (bottom).

Figure 1B shows that a reaction lacking InsP_6 contained one low-mobility form of XRCC4, while reactions carried out in the presence of InsP_6 contained two forms of XRCC4 with reduced mobility. Because phosphorylation can alter the mobility of a protein on SDS-PAGE the low-mobility forms of XRCC4 could be phosphorylated forms of XRCC4 [25]. Treatment of the InsP_6-containing reaction with lambda protein phosphatase resulted in loss of all low-mobility forms of XRCC4. Figure 1B identifies two species of phosphorylated XRCC4 in DNA end-joining reactions: one independent of InsP_6 (X4-P) and one that required InsP_6 (X4-P^{InsP_6}, InsP_6-dependent phosphorylation).

Finally, a specific requirement for a phosphorylated inositol was observed in an experiment that compared phosphorylation of XRCC4 in reactions containing InsP_6 with those containing inositol hexasulphate (IS_6), an inositol ring with sulphate rather than phosphate groups. InsP_6-dependent phosphorylation (X4-P^{InsP_6}) was only observed in reactions that contained InsP_6 and was not observed in reactions containing IS_6 (Fig. 1C).

It is plausible that the results shown in Figure 1 are the results of protein phosphatase inhibition rather than protein kinase stimulation. While InsP_6 has been shown to act as a competitive inhibitor of protein phosphatases [38], inhibition required more than 10 µM InsP_6; 10-fold higher than the 1 µM used in these experiments. Additionally, no change in InsP_6-dependent XRCC4 phosphorylation was observed in response to treatment with a general tyrosine protein phosphatase inhibitor cocktail.
(sodium orthovanadate, sodium molybdate, sodium tartrate and imidazole) or to the inhibitor of serine/threonine protein phosphatase 1 (PP1) and 2A (PP2A), okadiac acid (data not shown). Taken together, data presented in Figure 1 illustrates InsP$_6$-dependent XRCC4 phosphorylation that correlated with DNA end joining.

**InsP$_6$-dependent XRCC4 phosphorylation requires DNA-PK activity**

XRCC4 is a known substrate for the protein kinase DNA-PK [36, 37] and Ku70/80, the DNA-binding subunit of DNA-PK, is an InsP$_6$-binding protein [1, 32]. Together, these published observations suggested that DNA-PK activity might be required for InsP$_6$-dependent XRCC4 phosphorylation. This hypothesis was tested by examining of the sensitivity of XRCC4 InsP$_6$-dependent phosphorylation to the general PIKK inhibitor wortmannin [39-41]. Wortmannin inhibited both accumulation of low-mobility (e.g. phosphorylated) forms of XRCC4 and DNA-PK-dependent DNA end joining (Fig. 2A), and similar results were obtained with the general PIKK inhibitor LY29004 [39] (data not shown). These data implicate a member of the PIKK family in InsP$_6$-dependent phosphorylation of XRCC4, which is consistent with a role for DNA-PK in InsP$_6$-dependent XRCC4 phosphorylation.

Significantly, a control reaction that lacked double-stranded DNA (dsDNA) and InsP$_6$ showed no evidence of low-mobility XRCC4 (Fig. 2A, top). These data suggested a requirement for dsDNA in XRCC4 phosphorylation. To directly assess the role of DNA in XRCC4 phosphorylation, reactions were carried out with various amounts of linear dsDNA. As shown in Figure 2B (top), dsDNA was required for accumulation of both low-
mobility forms of XRCC4, which is consistent with participation of DNA-PK in these phosphorylation events.

DNA-PK specifically requires the exposed DNA termini presented by linear dsDNA. To further explore the DNA requirement shown in Figure 2B (top) phosphorylation of XRCC4 was carried out in the presence of linear double-stranded (ds), covalently closed circular (ccc) and single-stranded (ss) DNA to determine which form(s) of DNA would support InsP$_6$-dependent phosphorylation. Data presented in Figure 2B (bottom) shows that the phosphorylated form of XRCC4 was only observed in reactions that contained linear dsDNA. Taken together, data presented in Figure 2A and 2B show that InsP$_6$-dependent XRCC4 phosphorylation was sensitive to general PIKK inhibitors and required linear dsDNA as a co-factor, which implicates DNA-PK in InsP$_6$-dependent XRCC4 phosphorylation.

To determine if DNA-PKcs is required for InsP$_6$-dependent XRCC4 phosphorylation end-joining reactions were carried out using extracts prepared from DNA-PKcs wild type or DNA-PKcs-deficient cells. A comparison of XRCC4 phosphorylation in end-joining reactions that contained DNA-PKcs wild type HeLa and MO59K cell extracts or DNA-PKcs-deficient MO59J cell extracts is shown in Figure 2C. Both low-mobility form of XRCC4 were observed in the presence of InsP$_6$ in reactions containing DNA-PKcs wild type cell extracts, but not in reactions carried out with extracts prepared from DNA-PKcs-deficient cells (Fig. 2C).

While the data presented in Figure 2C strongly suggest that DNA-PKcs is required for InsP$_6$-dependent phosphorylation of XRCC4, it is important to note that MO59J cells are not only DNA-PKcs-deficient but have reduced expression of the
ataxia-telangiectasia mutated (ATM) protein kinase [42], which also functions in DSB repair. To determine whether the results in Figure 2C reflected a requirement for DNA-PKcs or for ATM, XRCC4 phosphorylation reactions were carried out in the presence of DNA-PKcs- or ATM-specific inhibitors. The DNA-PKcs-specific inhibitor Nu7026, which exhibits minimal off-target effects (IC50: DNA-PK, 230 nM; ATM >100 µM), has been described previously and used in the investigation of DNA-PK activity [43-45]. To corroborate the results presented in Figure 2C, XRCC4 phosphorylation reactions were treated with Nu7026, which markedly reduced XRCC4 phosphorylation (Fig. 2D, top). In contrast, treatment with the ATM-specific inhibitor Ku55933 [46] had no effect on phosphorylation of XRCC4 (Fig. 2D, bottom). Taken together the data in Figure 2 clearly demonstrate a requirement for DNA-PK activity in InsP6-dependent phosphorylation of XRCC4, and go on to implicate DNA-PKcs as an InsP6-responsive protein kinase.

DNA-PK-, InsP6-dependent phosphorylation of XLF

XRCC4 is known to interact functionally with ligase IV and XLF to form the XLF/XRCC4/ligase IV complex [22-26]. As XRCC4 is subject to InsP6-dependent phosphorylation other members of the XLF/XRCC4/ligase IV complex might similarly be phosphorylated in response to InsP6. To assess the effect of InsP6 on phosphorylation of ligase IV and XLF, phosphorylation reactions were carried out in the presence, or absence, of InsP6, after which phosphorylated proteins were isolated and Western blot analysis was used to detect ligase IV and XLF in the phosphoprotein fraction.

XRCC4 phosphorylation reactions were carried out in the presence, or absence, of dsDNA and 1 µM InsP6 then diluted to a protein concentration of 0.1 mg/ml in a buffer
containing high salt (1 M NaCl) and detergent (0.25% CHAPS) to abrogate protein-protein and protein-DNA interactions. Following dilution, samples were loaded onto a PhosphoProtein column (Qiagen) for collection of phosphorylated proteins. As shown in Figure 3A, ligase IV and XRCC4 were retained on the PhosphoProtein column under all conditions. These results indicate that XRCC4 and ligase IV were present in the input extract as phosphoproteins, modified in a dsDNA-, InsP₆-independent manner, or both. In the absence of dsDNA, XLF was not retained on the PhosphoProtein column. Significantly, XLF did not co-fractionate with ligase IV and XRCC4 under these conditions, which indicated that XLF had separated from the XLF/XRCC4/ligase IV complex and was fractionating independent of XRCC4 and ligase IV. A small amount of XLF was retained on the PhosphoProtein column when reactions contained dsDNA and retention increased when reactions also contained InsP₆. These data show DNA-dependent XLF phosphorylation that increased in the presence of InsP₆.

To confirm that XLF phosphorylation was stimulated by InsP₆, reactions were carried out in the presence of [γ³²P]ATP, anti-XLF antibodies were used to immunoprecipitate (IP) XLF and the immune complexes were examined for evidence of ³²P-labeled XLF. Figure 3B shows the results of the XLF IP (top) and phosphorimager analysis (bottom), which demonstrate radiolabeling of a 37 kD species that immunoprecipitated with anti-XLF antibodies and co-migrated with XLF and is therefore believed to be XLF. Data in Figure 3B show that the 37 kD species was phosphorylated in the absence of InsP₆, which may represent InsP₆-independent phosphorylation or InsP₆-dependent phosphorylation that was stimulated by residual endogenous InsP₆ in the reaction. Importantly, Figure 3B shows that phosphorylation increased with the
addition of 1 µM exogenous InsP₆. It is worth noting that these data cannot unequivocally identify the 37 kD species that is phosphorylated in response to InsP₆ as XLF and it is plausible that the radiolabeled species shown in Figure 3B is not XLF. Given the low probability that a completely unrelated polypeptide would be recognized by anti-XLF antibodies and co-migrate with XLF to yield the data presented in Figures 3A and 3B the 37 kD radiolabeled species will be referred to as XLF throughout this manuscript.

Figure 3C shows that phosphorylation of XLF (top) was specifically stimulated by InsP₆, but not by IS₆ and that IP of XLF was consistent for all reactions (bottom). In aggregate, data in Figure 3 show that phosphorylation of XLF is specifically stimulated by InsP₆ and provide evidence for InsP₆-dependent phosphorylation of XLF.

Data presented in Figures 1 and 2 provide evidence for InsP₆-, DNA-PK-dependent phosphorylation of XRCC4. Like XRCC4, XLF is a substrate for DNA-PK [47] and InsP₆-dependent phosphorylation of XLF might require also DNA-PK activity. InsP₆-dependent phosphorylation of XLF required dsDNA (Fig. 4A) and was sensitive to treatment with the general PIKK inhibitors wortmannin (Fig. 4B) and LY29004 (data not shown), which is consistent with a requirement for DNA-PK. Similarly, addition of the DNA-PKcs-specific inhibitor Nu7026 to XLF phosphorylation reactions markedly reduced XLF phosphorylation (Fig. 4C). In contrast, treatment with the ATM-specific inhibitor Ku55933 had no effect on phosphorylation of XLF (Fig. 4D). Taken together, the data presented in Figure 4 show that DNA-PK activity is required for InsP₆-dependent phosphorylation of XLF.
InsP$_6$-dependent phosphorylation of XRCC4 and XLF required InsP$_6$ binding by Ku70/80

Previously, my colleagues and I used mutational analysis to establish that InsP$_6$ binding by Ku70/80, which is the DNA-binding subunit of DNA-PK, is required for InsP$_6$ stimulation of NHEJ in vitro [3]. That study described the Ku70DM/80TM mutant, which carries five site-specific lysine-to-alanine mutations in the InsP$_6$-binding site of Ku70/80 [3]. These mutations reduce InsP$_6$-binding by Ku70DM/80TM to 1.22% of wild type, but did not affect the ability of the protein to bind DNA or to participate in DNA-PK phosphorylation of a peptide substrate [3]. Here, Ku70DM/80TM was used to assess the role of InsP$_6$ binding by Ku70/80 in InsP$_6$-dependent phosphorylation of XRCC4 and XLF. Briefly, endogenous Ku70/80 was removed from partially purified human cell extracts by immunodepletion to create Ku70/80-depleted extracts, which were complemented with purified recombinant Ku70/80 proteins, wild type or Ku70DM/80TM, and used in XRCC4 and XLF phosphorylation reactions.

As shown in Figure 5A, immunodepletion of Ku70/80 (lanes 1 and 2) resulted in significant reduction of XRCC4 modification, while mock depletion, with no anti-Ku70/80 antibodies, had no effect on XRCC4 phosphorylation (lanes 8-10). These data demonstrate a requirement for Ku70/80 in InsP$_6$-dependent phosphorylation of XRCC4, which is consistent with a requirement for DNA-PK. Addition of purified recombinant wild type Ku70/80 (lanes 3 and 4) restored InsP$_6$-dependent and -independent XRCC4 modification and demonstrated that the lack of phosphorylation observed in Ku70/80-depleted extracts was caused by immunodepletion of Ku70/80 and not removal of other factor(s). Addition of purified recombinant Ku70DM/80TM (lanes 5 and 6), which has
only 1.22% of wild type InsP$_6$-binding activity [3], restored InsP$_6$-independent (X4-P) XRCC4 phosphorylation, but failed to restore InsP$_6$-dependent modification (X4-P$^{InsP_6}$). Detection of Ku70 by Western blot confirmed immunodepletion of endogenous Ku70/80 and demonstrated that recombinant wild type and mutant Ku70/80 proteins were added in comparable amounts.

Ku70/80 was also found to be important in the InsP$_6$-dependent phosphorylation of XLF as immunodepletion of Ku70/80 resulted in reduced XLF phosphorylation (Fig. 5B, lanes 8 and 9). Complementation of Ku70/80-depleted extracts with wild type recombinant Ku70/80 restored InsP$_6$-dependent XLF phosphorylation to levels observed with the untreated extract (compare lanes 1 and 2 with 4 and 5). Complementation with Ku70DM/80TM did not significantly restore InsP$_6$-dependent modification of XLF. Western blot analysis showed that IP of XLF was comparable for all samples, confirmed immunodepletion of Ku70/80 and demonstrated that recombinant Ku70/80 proteins were added in comparable amounts.

Data presented in Figure 5 show that InsP$_6$ binding by Ku70/80, the regulatory subunit of DNA-PK, is required for InsP$_6$-dependent phosphorylation of XRCC4 and XLF. Taken together with Figures 2 and 4, which show that DNA-PK activity is required for InsP$_6$-dependent phosphorylation of XRCC4 and XLF, data in Figure 5 suggests the hypothesis that InsP$_6$ binding by DNA-PK may influence substrate selection by the PI3K-related protein kinase DNA-PKcs.

Discussion
Data presented here demonstrate InsP$_6$-dependent phosphorylation of the DNA repair factors XRCC4 and a species believed to be XLF. Experiments in which DNA-PKcs was absent or specifically inhibited revealed a requirement for DNA-PK in these InsP$_6$-dependent phosphorylation events. Finally, a mutant of Ku70/80 with reduced InsP$_6$ affinity was used to show that InsP$_6$ binding by Ku70/80, the regulatory subunit of DNA-PK, is necessary for InsP$_6$-dependent phosphorylation of XRCC4 and XLF.

These data suggest that binding of InsP$_6$ by Ku70/80 may affect the way in which DNA-PK phosphorylates XRCC4 and XLF. While it is not yet possible to attribute InsP$_6$-dependent phosphorylation to DNA-PK, the data presented in Figures 2 and 4, which show that specific inhibition of DNA-PKcs resulted in inhibition of InsP$_6$-dependent phosphorylation, implicate DNA-PK as the InsP$_6$-responsive protein kinase. The data shown in Figure 5, which directly link InsP$_6$-dependent phosphorylation with InsP$_6$ binding by Ku70/80, are consistent with the possibility that InsP$_6$ may affect protein phosphorylation by DNA-PK. Although it is possible that the 37 kD species that was phosphorylated in an InsP$_6$-dependent manner is not XLF, the radiolabeled polypeptide was phosphorylated in a DNA-PK$^-$, InsP$_6$-dependent manner and the data still support the hypothesis that InsP$_6$ may affect DNA-PK phosphorylation of full-length protein substrates.

How might InsP$_6$ modulate DNA-PK activity? Three basic models for InsP$_6$ regulation of enzymatic activity have been reported in biologically relevant systems. In the first model, direct binding of InsP$_6$ by an enzyme regulates catalytic activity. This is exemplified by the RNA editing enzyme ADAR2, which requires binding of InsP$_6$ for adenosine deaminase activity [13]. In this instance, InsP$_6$ is bound in the ADAR2 core
where a large number of hydrogen bonds between InsP₆ and ADAR2 are formed and presumably stabilize the protein fold [13]. This study showed that the structurally similar tRNA deaminase ADAT1 also required InsP₆ for catalytic activity [13]. Together, ADAR2 and ADAT1 show how binding of InsP₆ by an enzyme can directly regulate its catalytic activity.

In the second model, binding of InsP₆ by an enzyme does not directly affect catalytic activity, but affects binding of regulatory factors, that influence enzymatic activity. As previously noted, InsP₆ has been shown to regulate binding of CK2 by the CK2 negative regulator nucleolar protein p140 [18-20]. Binding of InsP₆ by CK2 prevents formation of inactive CK2-p140 complexes, thereby preserving CK2 activity [18-20].

In the final model, InsP₆ binds to a protein cofactor that regulates the activity of an enzyme. Nuclear export and translation of mRNA have been shown to require binding of InsP₆ by the factor Gle1, resulting in activation of the DEAD-box ATPase, Dbp5 [16, 17, 48]. The InsP₆-dependent regulation of Dbp5 by Gle1 in mRNA export and translation represent an example of cofactor-mediated indirect regulation of enzymatic activity by InsP₆.

My colleagues and I have previously shown that DNA-PKcs does not bind InsP₆ [1] and can therefore rule out the possibility that InsP₆ directly regulates DNA-PK activity. Specific binding of InsP₆ by the DNA-PK regulatory subunit Ku70/80 has been observed [1, 3, 32] and data presented here show that formation of a Ku70/80-InsP₆ complex is necessary for InsP₆-dependent phosphorylation of XRCC4 and XLF. These data fit a model for cofactor-mediated indirect regulation of enzymatic activity by InsP₆.
In the above examples, enzymatic activity varies in response to the amount of InsP$_6$ in a given reaction. DNA-PK is catalytically active in the presence and absence of InsP$_6$, and phosphorylation of a p53-peptide substrate by DNA-PK containing either the InsP$_6$-binding mutant Ku70DM/80TM or wild type Ku70/80 was comparable [3]. Yet InsP$_6$-dependent phosphorylation was observed only when InsP$_6$ was present. Under assay conditions used to visualize DNA-PK-, InsP$_6$-dependent phosphorylation of XRCC4 and XLF, comparable amounts of DNA-PKcs S5026 autophosphorylation were observed in the presence and absence of InsP$_6$ (data not shown). These results point toward a mechanism in which specific binding of InsP$_6$ by Ku70/80 regulates substrate selection by DNA-PK. Identification of the InsP$_6$-dependent protein kinase in this system is required to directly test this possibility, and work is proceeding toward this goal.

Ku70/80 has been shown to interact with the XLF/XRCC4/ligase IV complex and it is tempting to speculate that binding of InsP$_6$ by Ku70/80 might influence the nature of that interaction regulating accessibility of DNA-PK consensus phosphorylation sites on XRCC4 and XLF. While sites of DNA-PK phosphorylation have been identified for XRCC4 and XLF, there is no evidence to suggest that phosphorylation at these sites is critical for DSB repair in vivo [36, 47]. The possibility that biologically relevant DNA-PK sites may be phosphorylated in an InsP$_6$-dependent fashion is under investigation and I am in the process of identifying sites of InsP$_6$-dependent phosphorylation in XRCC4 and XLF with plans to assess the biological significance of these phosphorylation events.

Protein kinases are an important part of many cellular pathways, including signal transduction during the DNA damage response. Understanding the mechanisms that govern protein kinase function is central to understanding signaling pathways. While
interactions of kinases with ATP generally involves structural motifs that are common amongst protein kinases, the repertoire of interactions with the protein substrate is diverse and is rendered even more complex by participation of regulatory subunits. Findings presented here indicate that the small molecule InsP$_6$ can play a role in determining the substrate selectivity of an active protein kinase. These results expand the complexity of protein kinase/protein substrate interactions and identify InsP$_6$ as a participant in signal transduction.

Acknowledgements
Thanks to Drs. John Nitiss, William Beck, Karol S. Bruzik and Richard van Breemen for careful reading of this manuscript and insightful comments. Thanks also to Nicholas Okoro for purification the GST-XLF fusion protein used to produce anti-XLF antibodies and to Jae Choi for contributing data used in Figure 2B, bottom. This work supported by R01GM70639 and the University of Illinois at Chicago College of Pharmacy.

Literature Cited
3. Cheung, J.C., B. Salerno, and L.A. Hanakahi, Evidence for an inositol hexakisphosphate-dependent role for Ku in mammalian nonhomologous end


Figure Legends

Figure 1: InsP₆-dependent phosphorylation of XRCC4.

(A) Treatment of extracts with InsP₆ resulted in increased DNA end joining and in a novel form of XRCC4. DNA end-joining reactions were carried out in the presence or absence of 1 µM InsP₆ for the indicated amount of time. Half of the reaction was subject to SDS-PAGE, Western transferred and probed for XRCC4 (top). The remaining half of the reaction was deproteinized, after which DNA products were resolved by agarose gel electrophoresis and visualized by autoradiography (bottom).

(B) InsP₆-dependent phosphorylation of XRCC4. Extracts were incubated under DNA end-joining conditions in the presence (+) or absence (-) of 1 µM InsP₆, after which the InsP₆-containing reaction was split into equal fourths. One portion was left untreated. The remaining three portions were treated as indicated and incubated at 30°C for 30 min. Samples were resolved by SDS-PAGE, Western transferred and probed for XRCC4. C: control reaction that was not incubated at 37°C. Untreated: reactions that received no additional treatment. Buffer: inclusion of Lambda Phosphatase buffer. Mn⁺⁺: inclusion of 2 mM MnCl₂ required for lambda phosphatase activity. λ Pptase: inclusion of lambda phosphatase.

(C) Specificity of InsP₆-dependent phosphorylation of XRCC4. Extracts were incubated under XRCC4 phosphorylation conditions in the presence or absence (-) of 1 µM InsP₆ or 1 µM IS₆ as indicated. Samples were resolved by SDS-PAGE, Western transferred and probed for XRCC4. C: control reaction that was not incubated at 37°C. X4-P: phosphorylated XRCC4 that did not require InsP₆. X4-P<sup>InsP₆</sup>: species thought to be InsP₆-dependent phosphorylated form(s) of XRCC4.
Figure 2: InsP$_6$-dependent phosphorylation of XRCC4 requires DNA-PK activity.

(A) Wortmannin sensitivity of InsP$_6$-dependent XRCC4 phosphorylation. Extracts were incubated under DNA end-joining conditions in the presence of 1 µM InsP$_6$ with wortmannin (0.1, 0.3, 1 and 3 µM) and in the absence of InsP$_6$ with wortmannin (0.3 and 3 µM). Half of the reaction was subject to SDS-PAGE, Western transferred and probed for XRCC4 (top). The remaining half of the reaction was deproteinized, after which DNA products were resolved by agarose gel electrophoresis and visualized by autoradiography (bottom). No protein: control DNA end-joining reaction carried out in the absence of extract. No DNA: control reaction carried out in the absence of dsDNA. X4-P: phosphorylated XRCC4 that did not require InsP$_6$. X4-P$_{\text{InsP6}}$: species thought to be InsP$_6$-dependent phosphorylated form(s) of XRCC4.

(B) InsP$_6$-dependent phosphorylation of XRCC4 requires double-stranded DNA ends. (Top) Extracts were incubated as in (A) with varying amounts of linear, dsDNA and the presence or absence of 1 µM InsP$_6$. Samples were resolved by SDS-PAGE, Western transferred and probed for XRCC4. (Bottom) Extracts were incubated as in (A) with various forms of DNA and the presence or absence of 1 µM InsP$_6$. C: control reaction that was not incubated at 37ºC. dsDNA: double-stranded DNA. ccc dsDNA: covalently closer circular dsDNA. ssDNA: single stranded DNA. X4-P and X4-P$_{\text{InsP6}}$ as in (A).

(C) InsP$_6$-dependent phosphorylation of XRCC4 requires DNA-PKcs. Small-scale whole-cell extracts were prepared from DNA-PKcs wild type (HeLa and MO59K) and DNA-PKcs-deficient (MO59J) cells as described in experimental procedures and incubated as in (A) in the presence or absence of 1 µM InsP$_6$ as indicated. Samples
were resolved by SDS-PAGE, Western transferred and probed for XRCC4. X4-P and X4-P_{InsP6} as in (A).

(D) Sensitivity of InsP_6-dependent phosphorylation of XRCC4 to DNA-PKcs-specific inhibitor. Extracts were incubated as in (A) in the presence or absence of 1 µM InsP_6 with DNA-PKcs inhibitor Nu7026 (6.9 and 23 µM, top) or ATM inhibitor Ku55933 (0.13 and 0.65 µM, bottom), after which the reaction was subject to SDS-PAGE, Western transferred and probed for XRCC4. –DNA: control reactions carried out in the absence of DNA. X4-P and X4-P_{InsP6} as in (A).

**Figure 3:** InsP_6-stimulated phosphorylation of XLF.

(A) Treatment of extracts with InsP_6 and DNA resulted in increased retention of XLF on PhosphoProtein binding column. Extracts were incubated under XRCC4 phosphorylation conditions in the presence or absence of dsDNA and 1 µM InsP_6 as indicated. 1% of the reaction was reserved and designated Input (left). The remainder of the reaction was subject to PhosphoProtein binding column chromatography as per the manufacturer’s instructions and bound phosphoproteins were recovered (right). Input and bound samples were resolved by SDS-PAGE, Western transferred and probed for Ligase IV, XRCC4 and XLF.

(B) Treatment of extracts with InsP_6 resulted in increased phosphorylation of XLF. Extracts were incubated under XRCC4 phosphorylation conditions in the presence of [γ^{32}P]ATP and the presence or absence of 1 µM InsP_6 as indicated. 10% of the reaction was reserved and designated input. The remaining sample was split in half and subject to immunoprecipitation in the presence or absence of anti-XLF antibodies (αXLF
IP) as indicated. Immune complexes were resolved by SDS-PAGE, Western transferred and $^{32}$P was detected using a phosphorimag (bottom). Following $^{32}$P detection blots were probed for XLF (top). C: control reaction that was not incubated at 37°C. NE: reaction containing no extract.

(C) Specificity of InsP$_6$-dependent XLF phosphorylation. Extracts were incubated as described in (B) in the presence of InsP$_6$ or IS$_6$ (1 and 10 µM) as indicated then subject to immunoprecipitation (IP) using anti-XLF antibodies. Immune complexes were resolved by SDS-PAGE, Western transferred and $^{32}$P was detected using a phosphorimag (top). Following $^{32}$P detection blots were probed for XLF (bottom). –: reactions containing neither InsP$_6$ or IS$_6$.

**Figure 4:** Specific inhibition of DNA-PKcs, but not of ATM, resulted in loss of InsP$_6$-dependent XLF phosphorylation.

(A) InsP$_6$-dependent XLF phosphorylation requires dsDNA. Extracts were incubated under XRCC4 phosphorylation conditions in the presence of [γ$^{32}$P]ATP with InsP$_6$ (1 µM) and dsDNA (1 µg/ml) as indicated. 10% of each reaction was reserved as the input sample. The remainder of the reaction was subject to immunoprecipitation using anti-XLF antibodies ($\alpha$XLF IP). Immune complexes were resolved by SDS-PAGE, Western transferred and $^{32}$P was detected using a phosphorimag (bottom). Following $^{32}$P detection blots were probed for XLF (top).

(B) Wortmannin sensitivity of InsP$_6$-dependent XLF phosphorylation. Extracts were treated as in (A) with InsP$_6$ (1 µM) and wortmannin (0.04, 0.2 and 1 µM) as indicated, after which samples were subject to immunoprecipitation using anti-XLF
antibodies. Immune complexes were resolved by SDS-PAGE, Western transferred and
\(^{32}\)P was detected using a phosphorimager (top). Following \(^{32}\)P detection blots were
probed for XLF (bottom).

(C and D) InsP\(_6\)-dependent XLF phosphorylation is sensitive to DNA-PKcs-specific
inhibitor. Extracts were incubated as in (A) with InsP\(_6\) (1 \(\mu\)M) and the DNA-PKcs
inhibitor Nu7026 (C: 0.23, 1.15 and 5.75 \(\mu\)M) or the ATM inhibitor Ku55933 (D: 13, 65
and 325 \(\mu\)M). Samples were then subject to immunoprecipitation using anti-XLF
antibodies. Immune complexes were resolved by SDS-PAGE, Western transferred and
\(^{32}\)P was detected using a phosphorimager (top). Following \(^{32}\)P detection blots were
probed for XLF (bottom).

**Figure 5:** InsP\(_6\)-dependent phosphorylation or XRCC4 and XLF required InsP\(_6\) binding
by Ku70/80.

(A) Mutant of Ku70/80 with reduced affinity for InsP\(_6\) did not participate in InsP\(_6\)-
dependent phosphorylation of XRCC4. Ku70/80 monoclonal antibodies were used to
immunodeplete Ku70/80 from extracts to create Ku70/80-Depleted extract. For Mock-
Depleted extracts the immunodepletion protocol was carried out in the absence of anti-
Ku70/80 antibodies. Extracts were then incubated under XRCC4 phosphorylation
conditions in the presence or absence of 1 \(\mu\)M InsP\(_6\) and no exogenous Ku70/80
(none), wild type (WT) or InsP\(_6\)-binding mutant (Ku70DM/80TM) recombinant Ku70/80
(rKu70/80) as indicated. Following incubation the reactions were subject to SDS-PAGE,
Western transferred and probed for Ku70 (top) and XRCC4 (bottom). C: control reaction
that was not incubated at 37°C. X4-P: phosphorylated XRCC4 that did not require InsP₆.
X4-P_{InsP₆}: species thought to be InsP₆-dependent phosphorylated form(s) of XRCC4.

(B) Mutants of Ku70/80 with reduced affinity for InsP₆ were compromised for participation in InsP₆-stimulated XLF phosphorylation. Following Ku70/80 or mock immunodepletion, extracts were incubated under XRCC4 phosphorylation conditions in the presence of [γ³²P]ATP with or without InsP₆ (1 µM) and no exogenous Ku70/80 (none), wild type (WT) or InsP₆-binding mutant (Ku70DM/80TM) recombinant Ku70/80 (rKu70/80) as indicated. Following incubation 10% of the reaction was reserved as the input sample, which was resolved by SDS-PAGE, Western transferred and probed for Ku70 (bottom). The remainders of the reactions were subject to immunoprecipitation using anti-XLF antibodies (αXLF IP). Immune complexes were resolved by SDS-PAGE, Western transferred and ³²P was detected using a phosphorimager (top). Following ³²P detection blots were probed for XLF (middle).
FIGURES

### A

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>0</th>
<th>7.5</th>
<th>15</th>
<th>30</th>
<th>60</th>
<th>7.5</th>
<th>15</th>
<th>30</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>W. blot: XRCC4</strong></td>
<td><img src="image-url" alt="Image" /></td>
<td><img src="image-url" alt="Image" /></td>
<td><img src="image-url" alt="Image" /></td>
<td><img src="image-url" alt="Image" /></td>
<td><img src="image-url" alt="Image" /></td>
<td><img src="image-url" alt="Image" /></td>
<td><img src="image-url" alt="Image" /></td>
<td><img src="image-url" alt="Image" /></td>
<td><img src="image-url" alt="Image" /></td>
</tr>
<tr>
<td><strong>DNA end-joining</strong></td>
<td><img src="image-url" alt="Image" /></td>
<td><img src="image-url" alt="Image" /></td>
<td><img src="image-url" alt="Image" /></td>
<td><img src="image-url" alt="Image" /></td>
<td><img src="image-url" alt="Image" /></td>
<td><img src="image-url" alt="Image" /></td>
<td><img src="image-url" alt="Image" /></td>
<td><img src="image-url" alt="Image" /></td>
<td><img src="image-url" alt="Image" /></td>
</tr>
</tbody>
</table>

**Legend:**
- Low mobility XRCC4

### B

<table>
<thead>
<tr>
<th>Untreated</th>
<th>+</th>
<th>+</th>
<th>-</th>
<th>+</th>
<th>+</th>
<th>+</th>
<th>+</th>
<th>+</th>
<th>+</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>W. blot: XRCC4</strong></td>
<td><img src="image-url" alt="Image" /></td>
<td><img src="image-url" alt="Image" /></td>
<td><img src="image-url" alt="Image" /></td>
<td><img src="image-url" alt="Image" /></td>
<td><img src="image-url" alt="Image" /></td>
<td><img src="image-url" alt="Image" /></td>
<td><img src="image-url" alt="Image" /></td>
<td><img src="image-url" alt="Image" /></td>
<td><img src="image-url" alt="Image" /></td>
</tr>
<tr>
<td><strong>X4-P</strong></td>
<td><img src="image-url" alt="Image" /></td>
<td><img src="image-url" alt="Image" /></td>
<td><img src="image-url" alt="Image" /></td>
<td><img src="image-url" alt="Image" /></td>
<td><img src="image-url" alt="Image" /></td>
<td><img src="image-url" alt="Image" /></td>
<td><img src="image-url" alt="Image" /></td>
<td><img src="image-url" alt="Image" /></td>
<td><img src="image-url" alt="Image" /></td>
</tr>
</tbody>
</table>

**Legend:**
- λ PPTase
- Mn
- Buffer

### C

<table>
<thead>
<tr>
<th>C</th>
<th>IS6</th>
<th>InsP6</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image-url" alt="Image" /></td>
<td><img src="image-url" alt="Image" /></td>
<td><img src="image-url" alt="Image" /></td>
</tr>
</tbody>
</table>

**Legend:**
- X4-P
- X4-P

Hanakahi Figure 1
Hanakahi Figure 3
Hanakah Figure 4
### A

<table>
<thead>
<tr>
<th></th>
<th>Ku70/80-Depleted</th>
<th>Mock-Depleted</th>
</tr>
</thead>
<tbody>
<tr>
<td>rKu70/80</td>
<td>none</td>
<td>none</td>
</tr>
<tr>
<td>1 μM InsP₆</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>W. blot: Ku70</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>W. Blot: XRCC4</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>C</td>
<td>+</td>
<td>-</td>
<td>C</td>
<td></td>
</tr>
</tbody>
</table>

### B

<table>
<thead>
<tr>
<th>Extract</th>
<th>Untreated</th>
<th>Ku70/80-Depleted</th>
<th>Mock-Depleted</th>
</tr>
</thead>
<tbody>
<tr>
<td>rKu70/80</td>
<td>none</td>
<td>WT</td>
<td>none</td>
</tr>
<tr>
<td>1 μM InsP₆</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>αXLF IP</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>10% of IP input</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>X7P</td>
<td>-37</td>
<td>-32P</td>
<td>W. Blot: XLF</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>W. Blot: Ku70</td>
<td>W. Blot: Ku70</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Hanakahi Figure 5
Effect of the inositol polyphosphate InsP₆ on DNA-PK-dependent phosphorylation

Les Hanakahi

Mol Cancer Res  Published OnlineFirst August 19, 2011.