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# The Catalytic Subunit of DNA-Dependent Protein Kinase Coordinates with Polo-Like Kinase 1 to Facilitate Mitotic Entry[1]

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## Abstract

DNA-dependent protein kinase catalytic subunit (DNA-PKcs) is the key regulator of the non-homologous end joining pathway of DNA double-strand break repair. We have previously reported that DNA-PKcs is required for maintaining chromosomal stability and mitosis progression. Our further investigations reveal that deficiency in DNA-PKcs activity caused a delay in mitotic entry due to dysregulation of cyclin-dependent kinase 1 (Cdk1), the key driving force for cell cycle progression through $G_2$/M transition. Timely activation of Cdk1 requires polo-like kinase 1 (Plk1), which affects modulators of Cdk1. We found that DNA-PKcs physically interacts with Plk1 and could facilitate Plk1 activation both *in vitro* and *in vivo*. Further, DNA-PKcs–deficient cells are highly sensitive to Plk1 inhibitor BI2536, suggesting that the coordination between DNA-PKcs and Plk1 is not only crucial to ensure normal cell cycle progression through $G_2$/M phases but also required for cellular resistance to mitotic stress. On the basis of the current study, it is predictable that combined inhibition of DNA-PKcs and Plk1 can be employed in cancer therapy strategy for synthetic lethality.

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## Introduction

DNA-dependent protein kinase catalytic subunit (DNA-PKcs), a key component of the non-homologous end joining pathway, plays a pivotal role in DNA double-strand break repair [1]. In addition, DNA-PKcs is critical for normal cell cycle progression through mitosis as cells lacking a functional DNA-PKcs display a delay in mitotic transition and increases in abnormal spindle formation and chromosomal instability [2,3]. Although the precise mechanism leading to mitotic DNA-PKcs activation remains to be clarified, recent evidence indicates that DNA-PKcs activation stimulates the Chk2-Brca1 signaling pathway in mitosis to facilitate microtubule dynamics regulation [3].

Cell cycle progression from the $G_2$ phase to mitosis is tightly regulated through a mitosis-promoting factor complex consisting of Cdk1 and cyclin B1 [4]. Accumulation of cyclin B1 before mitotic entry promotes the formation of the Cdk1–cyclin B1 complex [5,6]. However, Cdk1 is kept in the inactive status by Wee1 and Myt1 kinase through inhibitory phosphorylation of Cdk1 [7,8]. The dual-specific phosphatase Cdc25C is required to remove the inhibitory phosphorylation of Cdk1 and to facilitate Cdk1 activation [9]. The activated Cdk1–cyclin B1 complex then accumulates in the nucleus and triggers chromosome condensation and nuclear envelop breakdown [10].

Polo-like kinase 1 (Plk1) is also a key mitotic regulator and is crucial for multiple processes throughout mitosis transition until

Abbreviations: DNA-PKcs, DNA-dependent protein kinase catalytic subunit; Plk1, polo-like kinase 1; PBD, polo box domain

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cytokinesis [11]. Plk1 contains an N-terminal canonical kinase domain and a C-terminal polo box domain (PBD), which recognizes phospho-serine or phospho-threonine sequences and is crucial for Plk1 association with its substrates [12,13]. For example, several Plk1 docking partners are phosphorylated by Cdk1 during mitosis to facilitate Plk1 recruitment and subsequent Plk1-dependent phosphorylations [14]. Conversely, Plk1 could regulate Cdk1 activity through multiple mechanisms. First, Plk1-dependent phosphorylation activates Cdc25C and its nuclear translocation [15]. Plk1 also phosphorylates Wee1 and promotes its degradation through β-TrCP–dependent ubiquitination [16,17]. Additionally, Plk1 could phosphorylate cyclin B1 to stimulate Cdk1–cyclin B1 at the centrosome and promote its nuclear import [18,19].

The current study further reveals that DNA-PKcs is involved in the timely activation of Cdk1 and mitotic entry through Plk1. DNA-PKcs directly interacts with Plk1 and could stimulate its kinase activity *in vitro* and *in vivo*. Our findings demonstrate that the crosstalk between DNA-PKcs and Plk1 plays a vital role in cellular progression and survival through mitosis.

## Materials and Methods

### *Cell Culture, Synchrony, and Small Inhibitory RNA Transfection*

All cell cultures, including human cervical cancer HeLa cells, human colon cancer HCT116 and derivative DNA-PKcs$^{-/-}$ cells [20], and Ligase 4$^{-/-}$ [21], were maintained in α-minimum essential medium supplemented with 10% FBS. Mitosis synchronization was performed as described previously [2]. Small inhibitory RNA (siRNA) oligonucleotides designed against DNA-PKcs [22] were transfected with RNAiMax (Invitrogen, Carlsbad, CA).

### *Immunoblot Analysis and Antibodies*

Whole-cell lysate preparation and Western blot analysis were performed as described [23,24]. Phospho-specific anti–DNA-PKcs antibodies were described previously [23–25]. Antibodies used in this study include anti-Cdk1, anti–phospho-Cdk1 (pY15), anti-Cdc25C, anti-Wee1, anti-Myt1, anti–cyclin B1, anti–poly(ADP-ribose) polymerase 1 (PARP-1) (Cell Signaling Technology, Danvers, MA), anti–phospho-histone H3 (pH3), anti–cyclin A (Upstate), anti-Plk1 (Bethyl Laboratories, Montgomery, TX), and anti–β-actin (Sigma-Aldrich, St. Louis, MO) and were commercially available as indicated.

### *Flow Cytometry Analysis*

Flow cytometry analysis was performed as described [26]. In brief, harvested cells were fixed in 70% ethanol, washed with phosphate-buffered saline, and incubated in propidium iodide (PI) solution (0.1 mg/ml RNase A, 0.1% Triton X-100, and 20 mg/ml PI in phosphate-buffered saline) for 30 minutes at 37°C. DNA content was measured by an FS500 flow cytometer, and cell cycle compartments were analyzed using the CXP cytometry analysis program (Beckman Coulter, Brea, CA). For mitotic indicator pH3 analysis, cells were incubated with anti-pH3 for 1 hour followed by fluorescein isothiocyanate–conjugated secondary antibody for 1 hour. Mitotic cell population was analyzed with the CXP cytometry analysis program. Mitotic index analysis was performed as previously described [2].

### *Immunoprecipitation and Glutathione S-Transferase Pull-Down Assays*

For immunoprecipitation (IP), cells were lysed in lysis buffer [50 mM Tris HCl (pH 7.5), 150 mM NaCl, 1% Tween 20, 0.5% NP-40, and protease inhibitor cocktail]. Cell lysates were incubated with 2 μg of control IgG or indicated specific antibodies at 4°C overnight, incubated with protein A/G agarose beads (Roche, Branford, CT) for 1 hour, followed by three washes in IP buffer and two washes in kinase reaction buffer (IP-kinase assay only, see below). For glutathione *S*-transferase (GST) pull-down assay, GST–DNA-PKcs fusion proteins were incubated with glutathione-sepharose beads (GE Healthcare Life Sciences, Piscataway, NJ) and then recombinant Plk1 proteins followed by immunoblot analysis.

### *Protein Purification and In Vitro Kinase Assays*

DNA-PKcs and Ku proteins were purified as described before [27]. Recombinant His-tagged wild-type and kinase dead (KD) Plk1 proteins were prepared from a bacterial expression system and purified through Ni-NTA agarose (Qiagen, Valencia, CA) and Superdex200 and MonoQ 5/50 column chromatography (GE Healthcare Life Sciences). *In vitro* DNA-PKcs kinase assay was performed as described elsewhere [27]. For *in vitro* Plk1 kinase assay, purified recombinant Plk1 or immunoprecipitated endogenous Plk1 proteins were incubated in Plk1 kinase reaction buffer [20 mM HEPES (pH 7.4), 100 mM KCl, 10 mM $MgCl_2$, 1 mM EDTA, 0.5 mM DTT, 5% glycerol, 10 μM ATP, and 0.17 μM γ-$^{32}$P ATP] with recombinant GST-Cdc25C or casein (Sigma) as substrates. The reaction was incubated at 30°C for 30 minutes and stopped by adding sodium dodecyl sulfate (SDS) sample buffer. In the combined DNA-PKcs and Plk1 kinase reaction, Plk1 kinase buffer was used. After kinase reaction, samples were subject to SDS–polyacrylamide gel electrophoresis analysis. Incorporation of $^{32}$P was analyzed by Typhoon 9410 Imager (GE Healthcare Life Sciences).

### *Clonogenic Survival Assay*

Clonogenic survival assay was performed as described elsewhere [23]. In brief, cells were plated onto 60-mm culture dishes followed by incubation with Plk1 inhibitor BI2536 for 7 to 10 days. Cells were fixed and stained with crystal violet. Colonies containing more than 50 cells were scored under a microscope.

## Results

### *DNA-PKcs Is Required for Cell Cycle Progression into Mitosis*

We have previously reported that DNA-PKcs is a critical regulator of mitosis progression and chromosomal stability maintenance [2,3]. Here, we further investigate whether DNA-PKcs is required for cell cycle progression into mitosis. HeLa cells transfected with either siRNA against DNA-PKcs or control siRNA were subjected to microtubule destabilizer nocodazole. Nocodazole treatment caused a dramatic increase in mitotic index in control HeLa cells but not in DNA-PKcs knockdown HeLa cells. In comparison, greater than 75.5 ± 10.0% of control cells were arrested in mitosis at 16 hours, whereas only 37.3 ± 1.8% of DNA-PKcs knockdown cells entered into mitosis (Figure 1*A*). A similar result was generated when control and DNA-PKcs knockdown cells were subjected to flow cytometry analysis using pH3 as a mitotic indicator (Figure 1*B*). At 16 hours after nocodazole treatment, a $G_2$ population in DNA-PKcs knockdown cells remained, whereas most control cells entered into mitosis and were positively stained with pH3. We also observed that, in the absence of nocodazole, there was a slight increase in mitotic index in DNA-PKcs knockdown HeLa cells. This increase in mitotic index is likely due to a defect in chromosome segregation,

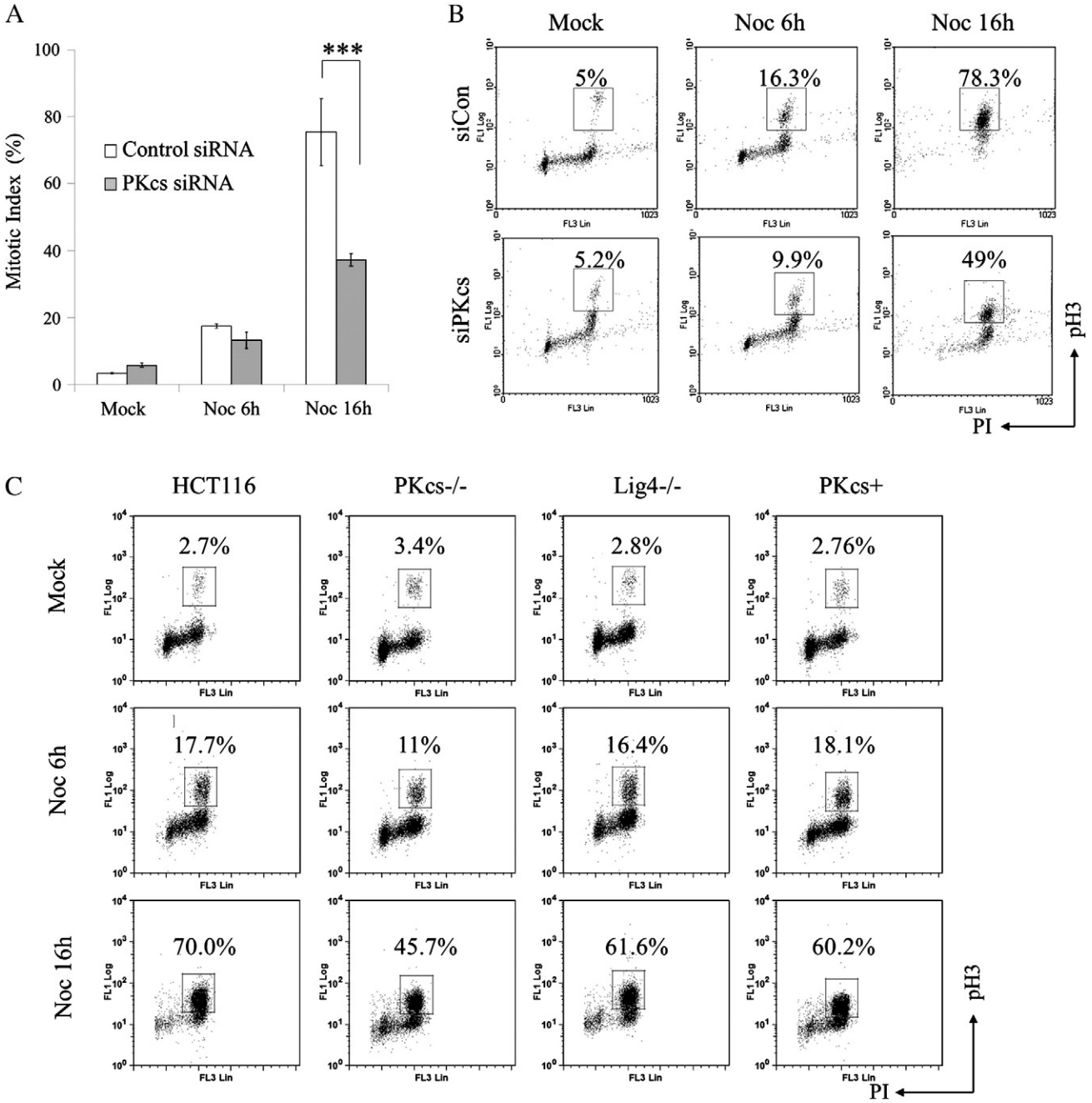


**Figure 1.** DNA-PKcs is required for cell cycle progression into mitosis. (A) HeLa cells were transfected with control siRNA or DNA-PKcs siRNA for 48 hours and were incubated with nocodazole (50 ng/ml) for the indicated time. After harvest, cells were processed for chromosome spread and mitotic index analysis. The result was generated from three independent experiments. ***$P < .001$. (B) The harvested HeLa cells were analyzed by flow cytometry against anti-pH3 antibody and PI staining. (C) Human colon cancer HCT116 cells and derivative DNA-PKcs knockout cells (PKcs$^{-/-}$), DNA Ligase IV knockout cells (Lig4$^{-/-}$), as well as PKcs$^{-/-}$ cells complemented with wild-type DNA-PKcs (PKcs+) were treated with nocodazole. Mitotic index were analyzed as described.

which causes the delay in mitosis progression in DNA-PKcs knockdown cells [2].

To further validate the role of DNA-PKcs in mitotic entry, human colon cancer HCT116 and the derivative DNA-PKcs knockout (DNA-PKcs$^{-/-}$) cells were analyzed for nocodazole-induced mitotic indices. Consistent with DNA-PKcs knockdown HeLa cells, DNA-PKcs$^{-/-}$ cells displayed decreased mitotic indices than the parental HCT116 cells in response to nocodazole treatment (Figure 1*C*). This reduction was rescued by restoration of DNA-PKcs expression in DNA-PKcs$^{-/-}$ cells (PKcs+) and was not observed in DNA Ligase IV knockout (Lig4$^{-/-}$) HCT116 cells. These results underscore a distinct role of DNA-PKcs to facilitate mitotic entry independently of its function in non-homologous end joining or DNA double-strand break repair.

### *DNA-PKcs Deficiency Delays Cdk1 Activation and $G_2$/M Transition*

Cell cycle progression from the $G_2$ phase entering into mitosis is primarily governed the cyclin-dependent kinase 1 (Cdk1). Cdk1

activation requires its complex with cyclin B1 and removal of Cdk1 inhibitory phosphorylation at Tyr15 (pY15). We further examined whether DNA-PKcs depletion alters Cdk1 activation during $G_2$/M transition. Nocodazole synchrony caused a transient increase in Cdk1 pY15 in control HeLa cells before mitotic entry followed by removal of Cdk1 pY15 at later time points (Figure 2*A*), when the majority of cells have already entered into mitosis (Figure 1). The levels of Cdk1 pY15, however, were higher in DNA-PKcs knockdown HeLa cells before and after nocodazole treatment even at later time points. Dysregulation of Cdk1 pY15 was also found in DNA-PKcs$^{-/-}$ cells. Similar to DNA-PKcs knockdown in HeLa cells, the overall levels of Cdk1 pY15 were significantly elevated in DNA-PKcs$^{-/-}$ cells compared to the parental wild-type HCT116 cells (Figure 2*B*). Contrary to the change in Cdk1 pY15 expression, Cdk1 total protein levels remained constant in control and DNA-PKcs–deficient cells at various time points.

The timely expression of Cdk1 pY15 is regulated through the balance between Wee1-type kinases [7,8] and Cdc25C phosphatase [9]. Consistent with the elevation of Cdk1 pY15 in DNA-PKcs–defective cells, we observed that activation of Cdc25C phosphatase, as indicated by its hyperphosphorylation [28], was significantly attenuated in DNA-PKcs–depleted HeLa cells (Figure 2*A*). Additionally, we observed that DNA-PKcs is involved in the timely inactivation of Wee1 and Myt1 kinases. Nocodazole treatment caused a transient increase in Wee1 and Myt1 protein levels in HCT116 cells at 6 hours followed by their depletion at later time points when cells entered into mitosis, whereas increased expression levels of Wee1 and Myt1 were found in DNA-PKcs$^{-/-}$ cells (Figure 2*B*), suggesting that mitosis-specific degradation of Wee1 and Myt1 was significantly delayed in the absence of DNA-PKcs (Figure 2*B*). To further determine whether DNA-PKcs kinase activity is involved in mitosis entrance, thymidine-synchronized HeLa cells were released into the S phase and were subjected to DNA-PKcs inhibitor Nu7441 incubation. We observed that there is a delay in Cdk1 phosphorylation as well as histone H3 Ser10 phosphorylation (Figure 2*C*), indicating that DNA-PKcs kinase activity is involved in mitosis transition.

It has been documented that DNA-PKcs is required for the optimal expression of ataxia telangiectasia mutated (ATM) [29,30]. It is possible that the reduction of ATM expression in DNA-PKcs–

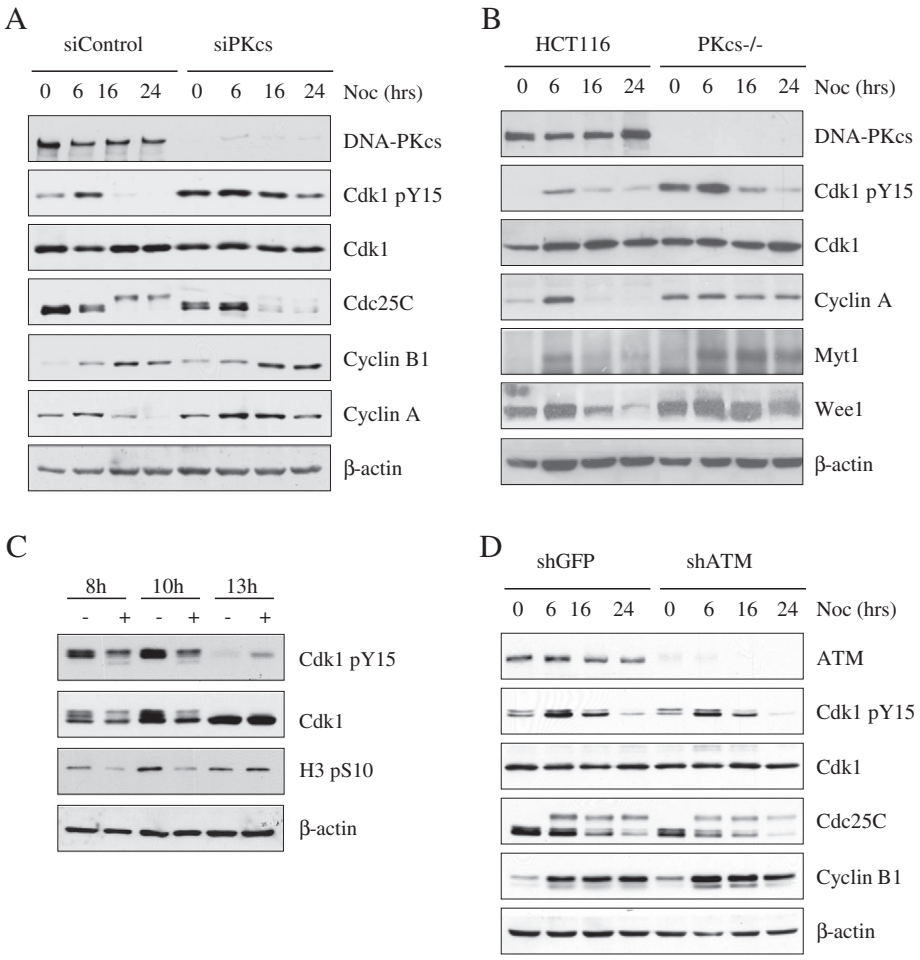


**Figure 2.** DNA-PKcs deficiency causes misregulation of Cdk1 activation. (A) HeLa cells transfected with either control or DNA-PKcs siRNAs were incubated with nocodazole (50 ng/ml) for the indicated durations. Whole-cell lysates were subjected to Western blot against the indicated antibodies. (B) HCT116 and derivative PKcs$^{-/-}$ cells treated with nocodazole were harvested at different time points and were analyzed by Western blot. (C) HeLa cells were synchronized and released from a double thymidine block. At 6 hours after release, cells were incubated with DMSO or Nu7441 (10 $\mu$M) and were harvested at the indicated time points for Western blot analysis. (D) HeLa cells stably expressing shGFP or shATM were incubated with nocodazole for the indicated durations and analyzed by Western blot.

defective cells might contribute to the impairment in mitotic entrance. To determine this possibility, HeLa cells expressing shRNA against ATM (shATM) or green fluorescent protein (shGFP) were subjected to nocodazole treatment and were analyzed for mitotic markers. As shown in Figure 2*D*, both shATM and shGFP cells displayed similar kinetics of Cdk1 and Cdc25C phosphorylations as well as cyclin B1 accumulation. These results suggest that expression levels of ATM are not critical for mitotic entrance.

### *DNA-PKcs Interacts with Plk1*

Impaired Cdc25C phosphorylation and Wee1 degradation have been reported in cells with defective Plk1 activity [15–17]. Thus, we speculated that DNA-PKcs might have a functional link with Plk1. This notion is further supported by our previous report that mitosis-specific indication of DNA-PKcs phosphorylation overlaps with Plk1 at centrosomes, kinetochores, and mid-body during mitosis [2]. To test this hypothesis, asynchronous and nocodazole-treated HeLa cell lysates were subjected to IP with anti–DNA-PKcs or anti-Plk1 antibodies. Our analysis revealed that Plk1 and DNA-PKcs could be reciprocally co-immunoprecipitated and that their complex formation increased in mitosis-synchronized cells (Figure 3*A*). The interaction between DNA-PKcs and Plk1 was also detected from

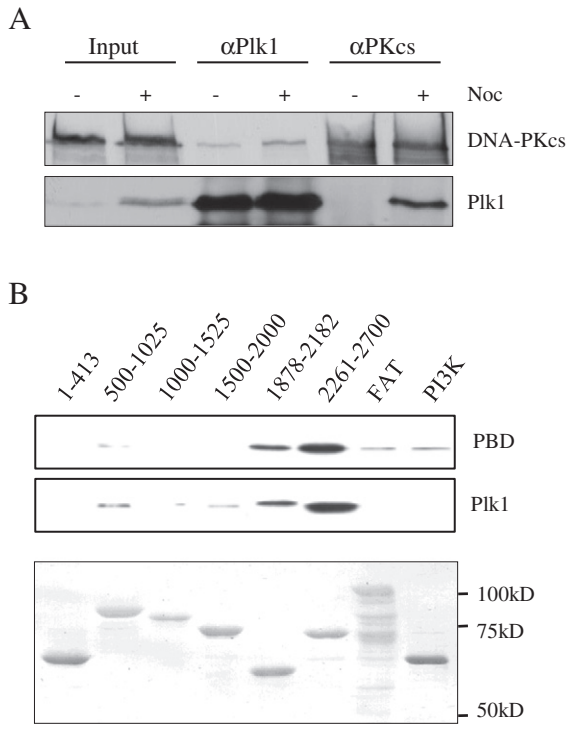


**Figure 3.** DNA-PKcs directly interacts with Plk1. (A) Sham or nocodazole (50 ng/ml, 16 hours) treated HeLa cell lysates were immunoprecipitated using anti-Plk1 or anti–DNA-PKcs antibodies. Immunoprecipitated proteins were analyzed by immunoblot analysis to determine the association between DNA-PKcs and Plk1. (B) Domain mapping of direct protein-protein interaction between DNA-PKcs and Plk1. GST fusion proteins carrying different regions of DNA-PKcs (bottom panel, stained by Ponceau S) were incubated with His-tagged full-length Plk1 or Plk1 PBD domain followed by glutathione agarose pull-down. The bound Plk1 or PBD domain was detected by anti-His antibody. Numbers on the top indicate the coverage of DNA-PKcs residues in each GST fusion protein.

overexpression of flag-tagged Plk1 in 293 cells that DNA-PKcs preferentially interacts with Plk1 than the Ku70/80 complex in the absence of DNA damage (Supplementary Figure 1). To further investigate whether there is direct protein-protein interaction between DNA-PKcs and Plk1, full-length recombinant Plk1 was expressed and purified from *Escherichia coli* (Supplementary Figure 2) and was subjected to GST pull-down assay using a series of GST fusions carrying different fragments of DNA-PKcs. GST pull-down assay revealed that full-length Plk1 preferentially binds to both 1878-2182 and 2261-2700 regions of DNA-PKcs covering Ser2056 and the Thr2609 phosphorylation cluster, respectively (Figure 3*B*). GST pull-down was further investigated using the C-terminal PBD of Plk1. Plk1 PBD also interacted with DNA-PKcs at the same regions with stronger affinity to the 2261-2700 fragment (Figure 3*B*).

### *DNA-PKcs Promotes Plk1 Activation*

The direct protein-protein interaction between DNA-PKcs and Plk1 kinases prompted us to speculate that DNA-PKcs could modulate Plk1 activity. To test this hypothesis, recombinant Plk1 was subjected to *in vitro* kinase reaction together with the endogenously purified DNA-PK protein complex from HeLa cells. The DNA-PK kinase, consisting of DNA-PKcs and Ku70/80 heterodimer, was kinase active and further stimulated in the presence of DNA (Figure 4*A*, lanes 4-5 with Xrcc4 as substrate). Recombinant wild-type Plk1 was also kinase active alone and could autophosphorylate itself under the *in vitro* kinase reaction (lane 1). Such Plk1 phosphorylation was significantly enhanced in the presence of active DNA-PK kinase (lane 6). Conversely, we observed that the level of DNA-PKcs phosphorylation increased in the presence of Plk1 (lane 5 *vs* lane 6), which is consistent with recent report that Plk1 phosphorylates DNA-PKcs [31]. It is possible that DNA-PKcs and Plk1 association stimulates Plk1 kinase and autophosphorylation *in vitro*, or DNA-PKcs could play a direct role and phosphorylate Plk1 *in vitro*. To delineate the mechanism, recombinant KD mutant Plk1 was examined in *in vitro* kinase assay. Plk1 KD mutant was unable to phosphorylate by itself alone (lane 2) but was phosphorylated by the DNA-PK kinase (lane 7). Further analysis revealed that DNA-PKcs could directly phosphorylate the C-terminal PBD domain of Plk1 (lane 8). To determine whether DNA-PKcs could stimulate Plk1 kinase activity *in vitro*, Plk1 activity was further examined with recombinant Cdc25C as substrate (Figure 4*B*). Our analyses revealed that Plk1 could phosphorylate Cdc25C *in vitro* (lane 3) and the levels of Cdc25C phosphorylation slightly enhanced in the presence of Ku or DNA-PKcs alone (lanes 5 and 6, respectively). However, in the presence of the active DNA-PK kinase (lane 8), Plk1-mediated Cdc25C phosphorylation decreased, which was contrary to a significant increase in Plk1 and DNA-PKcs phosphorylation. These results suggest that DNA-PKcs and Plk1 association mutually stimulates each other *in vitro*. It is possible that DNA-PKcs and Plk1 are preferable substrates of each other.

To validate the *in vitro* result generated with recombinant Plk1, endogenously expressed Plk1 was immunoprecipitated from HeLa cells for evaluation of its kinase activity. Our analysis revealed that immunoprecipitated Plk1 could phosphorylate Cdc25C and that Plk1-mediated Cdc25C phosphorylation was attenuated in the presence of Plk1 inhibitor BI2536 but not DNA-PKcs inhibitor NU7441 (Figure 4*C*). Similarly, NU7441 incubation did not alter the kinase activity of recombinant Plk1 *in vitro* (Supplementary Figure 3). However, when HeLa cells were pretreated with NU7441

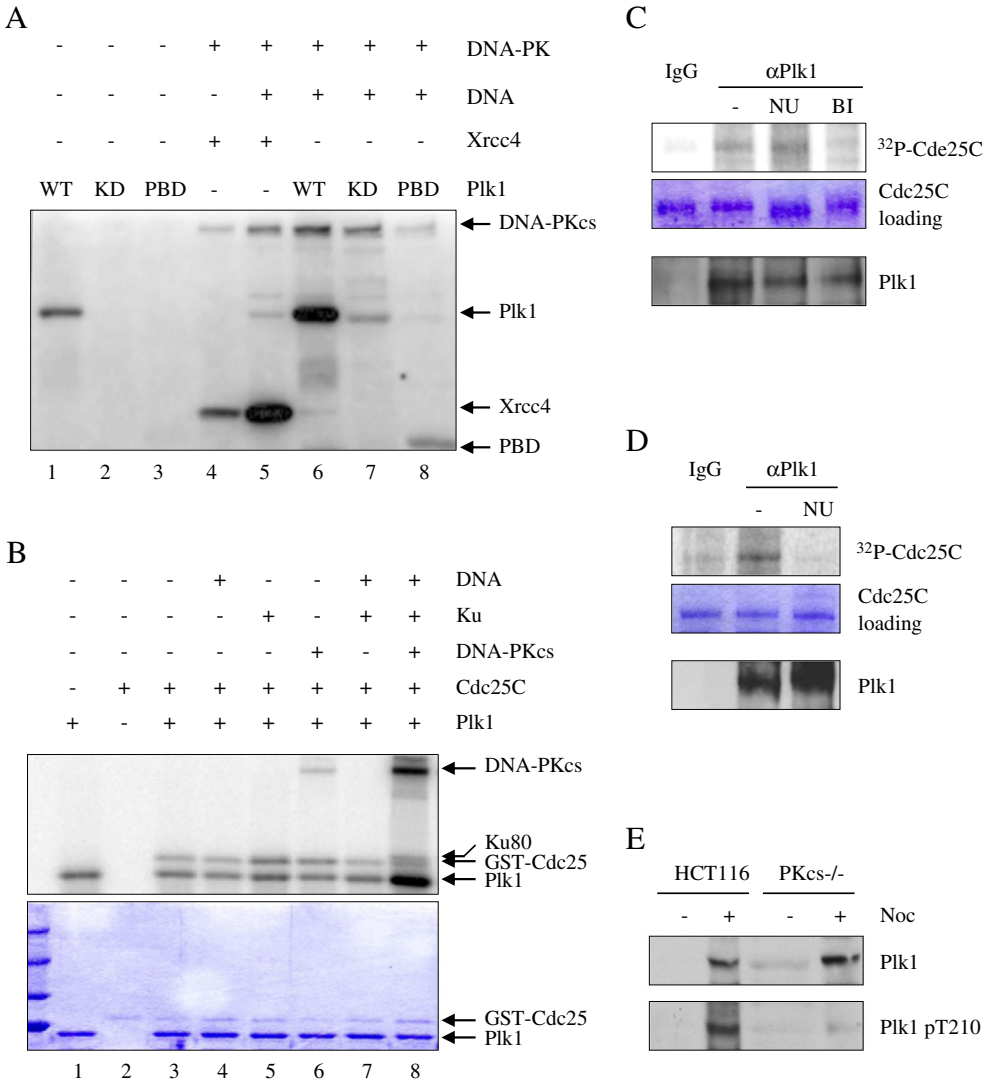


**Figure 4.** DNA-PKcs stimulates Plk1 kinase activity *in vitro* and *in vivo*. (A) Recombinant His-tagged wild-type Plk1, KD mutant, and PBD domain were subjected to *in vitro* kinase reactions in the presence or absence of HeLa-purified DNA-PK complex. C-terminal fragment of XRCC4 was included as a positive control of DNA-PK kinase activity. (B) Similar *in vitro* kinase reactions were performed with recombinant GST-Cdc25C as Plk1 substrate. The bottom panel shows the loadings of recombinant Plk1 and GST-Cdc25C. The amounts of DNA-PKcs and Ku proteins were not sufficient to be visualized by Coomassie blue staining. (C) Endogenous Plk1 proteins were immunoprecipitated with control IgG or α-Plk1 antibodies from HeLa cell extracts and were subjected to kinase reaction in the presence of γ-$^{32}$P ATP, purified GST-Cdc25C (substrate), and inhibitors against DNA-PKcs or Plk1 (NU7441 and BI2536, respectively, 10 μM). Immunoblot analysis of Plk1 showed similar amounts of Plk1 in the kinase reactions. (D) HeLa cells were pretreated with NU7441 (10 μM) for 2 hours. Endogenous Plk1 proteins were immunoprecipitated from whole-cell extracts followed by *in vitro* kinase assay with GST-Cdc25C as substrate. (E) HCT116 and DNA-PKcs$^{-/-}$ cells were subjected to nocodazole treatment and were analyzed for Plk1 total protein and Thr210 phosphorylation.

before harvest, the Plk1 activity measured was significantly decreased compared to that of sham retreated HeLa cells (Figure 4*D*). Furthermore, we observed that mitotic induction of Plk1 phosphorylation at Thr210 was significantly decreased in DNA-PKcs$^{-/-}$ cells compared to that in the parental HCT116 cells (Figure 4*E*). Taken together, these results indicate that the kinase activity of DNA-PKcs is required for optimal Plk1 activation *in vivo*.

### *Combined Inhibition of DNA-PKcs and Plk1 Results in Synthetic Lethality*

The coordination between DNA-PKcs and Plk1 is likely critical for cell cycle progression through $G_2$/M transition, various steps in mitosis, as well as cellular resistance against mitotic stress. We also expect that DNA-PKcs–deficient cells will be susceptible on Plk1 inhibition. To test this hypothesis, HCT116 and DNA-PKcs$^{-/-}$ cells were treated with Plk1 kinase inhibitor BI2536 and were analyzed for mitotic index. Overnight incubation with BI2536 at 10 nM concentration induced significant mitotic arrest in DNA-PKcs$^{-/-}$ cells but only mild mitotic arrest in HCT116 cells as measured by pH3 in flow cytometry analysis and immunofluorescence staining (Figure 5*A*). In addition, we observed that BI2536 treatment induced a sub-$G_1$ population (representing cells undergo apoptosis) in DNA-PKcs$^{-/-}$ cells but not in HCT116 cells (Figure 5*B*). BI2536-induced apoptosis was also evidenced by cleavage of PARP-1 in immunoblot analysis. The result showed that BI2536 treatment resulted in severe PARP-1 cleavage and apoptosis in DNA-PKcs$^{-/-}$ cells but not in HCT116 cells (Figure 5*C*).

Susceptibility of DNA-PKcs$^{-/-}$ cells toward BI2536 was further determined using the clonogenic survival assay. Our analysis revealed

that DNA-PKcs$^{-/-}$ cells were highly sensitive to BI2536 compared to the parental HCT116 cells (Figure 6*A*). This result also suggested that combined inhibition of both DNA-PKcs and Plk1 kinases could achieve synthetic lethality with potential implication in cancer treatment. To test this possibility, DNA-PKcs–proficient HCT116 cells were subjected to increasing concentrations of BI2536 alone or were treated in conjunction with specific DNA-PKcs kinase inhibitor NU7441. As expected, NU7441 treatment further augmented the cell killing effect of BI2536 in clonogenic survival assay (Figure 6*B*) as well as in 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assay (Supplementary Figure 4). These results indeed support the therapeutic potential to target DNA-PKcs and Plk1 kinases simultaneously for achieving combined modality in cancer treatment.

## Discussion

In the current study, we reveal that DNA-PKcs plays a crucial role in cell cycle progression through the $G_2$/M transition. Depletion or inactivation of DNA-PKcs results in a delay in mitotic entry due to dysregulation of Cdk1 activation and its upstream modulators including Cdc25C phosphatase and Wee1-type kinases. Both Cdc25C and Wee1 kinases are subjects being regulated by Plk1 kinase, which is one of the key mitotic regulators and is involved in multiple processes throughout the entire course of mitosis [32]. Our results further demonstrate that there is a direct association between DNA-PKcs and Plk1 in mitosis (Figure 3) and that DNA-PKcs could stimulate Plk1 activation both *in vitro* and *in vivo* (Figure 4). Thus, in the absence of a functional DNA-PKcs, inadequate Plk1 activation fails to support the timely activation of Cdk1 kinase and results in delay of mitotic entry. Consistent with this notion, our results also demonstrate that DNA-PKcs–defective cells are sensitive to Plk1 kinase inhibitor BI2536. Taken together, these results demonstrate that the interaction between DNA-PKcs and Plk1 facilitates the optimal activation of Plk1 and is required for timely mitotic entry as well as cellular resistance to mitotic stress.

We have previously reported that DNA-PKcs is activated and required for proper chromosomal segregation and that mitotic

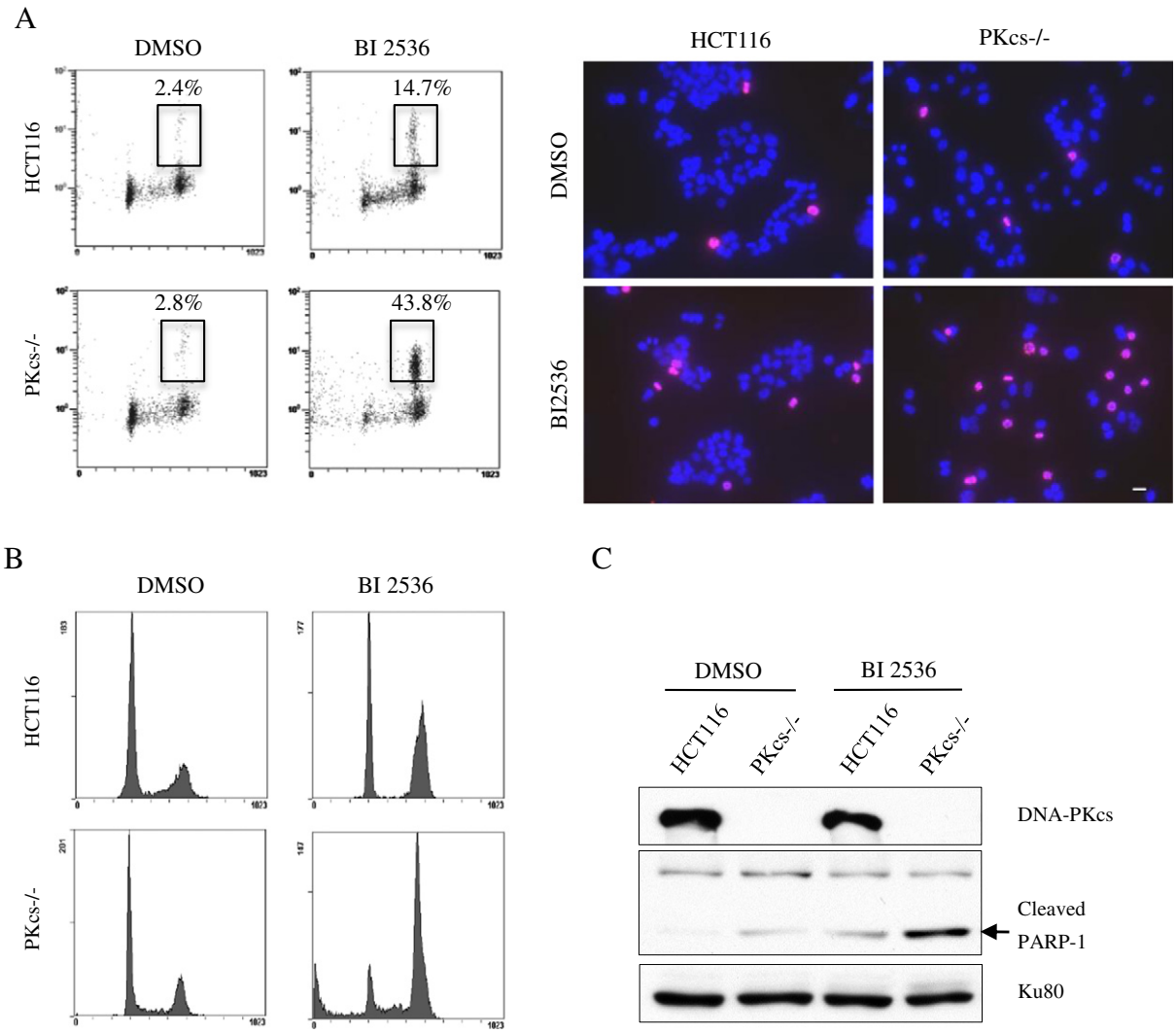


**Figure 5.** DNA-PKcs–deficient cells are susceptible to Plk1 inhibitor BI2536. (A) HCT116 and DNA-PKcs$^{-/-}$ cells were incubated with DMSO or 10 nM BI2536 for 16 hours and were analyzed against mitotic indicator pH3 in flow cytometry (left panel) and immunofluorescence staining (right panel). (B) HCT116 and DNA-PKcs$^{-/-}$ cells treated with BI2536 (10 nM, 16 hours) were analyzed by flow cytometry against PI staining. (C) Apoptosis was determined by PARP-1 cleavage product (indicated by an arrow) in the immunoblot analysis.

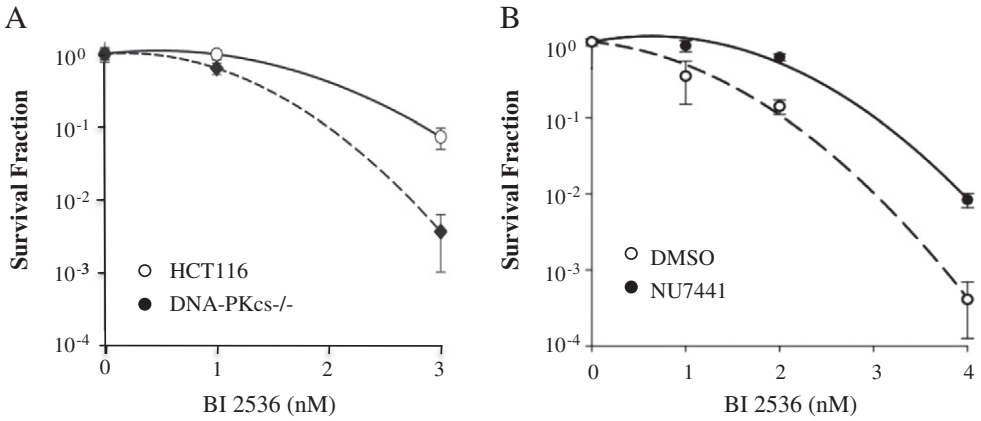


**Figure 6.** Combined inhibition of DNA-PKcs and Plk1 resulted in synthetic lethality. (A) Clonogenic survival of HCT116 and DNA-PKcs$^{-/-}$ cells were determined in the presence of indicated concentrations of BI2536 for 7 to 10 days. (B) HCT116 cells were subjected to increasing dosages of BI2536 alone or in conjunction with NU7441 (10 $\mu$M) in clonogenic survival analysis.

DNA-PKcs autophosphorylation is associated with spindle apparatus and completely overlaps with mitotic Plk1 at centrosomes, kinetochores, and mid-body [2]. These results also imply that there are both functional and physical links between these two kinases. Consistent with our report, the association between DNA-PKcs and Plk1 has been verified recently by independent groups [31,33]. Here, we further demonstrate that there is a direct protein-protein interaction between PBD domain of Plk1 and fragments of DNA-PKcs that cover two key phosphorylation regions, the Thr2609 cluster and Ser2056 [23–25]. Crystallographic studies of Plk1 reveal that the C-terminal PBD directly interacts with the N-terminal kinase domain to block its own kinase activation [12,34]. Such auto-inhibition can be reversed through Plk1 phosphorylation at Thr210 or through direct interaction between Plk1 PBD and Plk1 target proteins [35]. Although recent evidence suggested that Aurora-A kinase can phosphorylate Plk1 at Thr210 and promote its activation [36], it was initially reported that Plk1 can autophosphorylate itself at the same residue [37]. Consistent with this notion, our *in vitro* kinase assay showed that recombinant wild-type Plk1 could phosphorylate itself and that Plk1 phosphorylation was further stimulated in the presence of DNA-PKcs. We speculate that the association of Plk1 PBD with DNA-PKcs Thr2609 cluster or Ser2056 regions may relieve the inhibitory effect of Plk1 PBD on its kinase domain. Alternatively, it is possible that DNA-PKcs phosphorylates Plk1 and alleviates the inhibitory effect of Plk1 PBD, which is supported by our observation that DNA-PKcs could phosphorylate Plk1 KD mutant protein and the C-terminal PBD fragment (Figure 4*A*). These two possible yet distinctive scenarios are not mutually exclusive and could both contribute to DNA-PKcs–dependent Plk1 activation.

The crosstalk between DNA-PKcs and Plk1 is unlikely to be unidirectional as we observed that DNA-PKcs phosphorylation *in vitro* was stimulated in the presence of an active Plk1 kinase (Figure 4*A*), indicating that Plk1 could either phosphorylate DNA-PKcs directly or stimulate DNA-PKcs autophosphorylation. A recent study by Douglas et al. reported that Plk1 phosphorylates DNA-PKcs at Ser3205 during mitosis and that mitotic DNA-PKcs activation and phosphorylation occurs independent of Ku70/80 heterodimer [31]. This leads to a possible scenario that Plk1-mediated DNA-PKcs phosphorylation at Ser3205 further enhances DNA-PKcs kinase activation and results in Ku-independent DNA-PKcs activation. However, treatment with Plk1 inhibitor BI2536 blocked only Ser3205 phosphorylation, but it did not affect other DNA-PKcs downstream phosphorylation events in mitosis. Douglas et al. further reported that DNA-PKcs phosphorylation at Ser3205 is removed by protein phosphatase 6 (PP6) as siRNA depletion of PP6 improves Ser3205 phosphorylation in mitosis and after ionizing radiation (IR) [31]. Independent studies have also reported that PP6 is a modulator of DNA-PKcs and is required for IR-induced DNA-PKcs activation and radioresistance [38,39]. Thus, further investigation will be needed to delineate the impact of Plk1-dependent DNA-PKcs phosphorylation and whether Plk1 directly modulates DNA-PKcs activity in mitosis. Nonetheless, these data support our findings that association between DNA-PKcs and Plk1 reciprocally affects both kinases.

The coordination between DNA-PKcs and Plk1 is critical for preserving chromosomal stability and completion of cell division. We have previously reported that, in DNA-PKcs–defective cells, there is a delay in mitotic progression and an increase in mitotic index due to impairment in chromosome alignment and segregation [2,3]. Our analyses further revealed that DNA-PKcs is involved in modulation of microtubule dynamics and attachment to kinetochores through direct regulation of the Chk2-Brca1 signaling pathway, which is known to regulate mitotic spindle formation and maintenance of chromosomal stability [40]. Similarly, Plk1 is also capable of modulating microtubule nucleation and attachment to kinetochores through multiple downstream effector molecules [41–43]. It is unlikely that Plk1 could affect DNA-PKcs downstream Chk2-Brca1 signaling since mitosis-induced Chk2 phosphorylation was not affected by Plk1 inhibitor [31]. In contrast, DNA-PKcs might influence Plk1 function in microtubule dynamics as DNA-PKcs could facilitate Plk1 activation (Figure 4). Further, DNA-PKcs–deficient cells are sensitivity toward the Plk1 inhibitor (Figure 6*A*). Their coordination is likely required for optimal regulation of microtubule to ensure proper chromosome segregation during mitosis and successful cell division.

Chromosome instability is one of the key hallmarks of cancer cells and the driving force for carcinogenesis [44]. It is conceivable that an elevation in either Plk1 or DNA-PKcs activity would promote chromosome stability and/or mitigate the intrinsic chromosome instability character in most cancer cells to support continuous cell

division. Indeed, dysregulation of Plk1 has been linked to development of various cancer types and correlates with poor patient prognosis [45]. Evidence further indicates that Plk1 could contribute to chemoresistance [46]. Thus, molecular targeting of Plk1 has emerged as an attractive therapeutic strategy for a wide range of cancers [12,47]. For example, BI2536 is a highly selective and potent Plk1 inhibitor identified by a diverse organic compound screening and had passed phase II clinical trials [48]. Similarly, a growing body of evidence has implicated a role of DNA-PKcs in carcinogenesis and development of radioresistance in tumor, and thus, various anti-DNA-PKcs strategies have evolved [49]. Here, we further investigate whether synthetic lethality can be achieved with combination of both Plk1 and DNA-PKcs inhibitors in their sublethal dosage. Consistent with this hypothesis, our result demonstrated that combined inhibition of both DNA-PKcs and Plk1 increased mitotic arrest and cellular apoptosis (Figure 5) and reduced clonogenic survival ability (Figure 6). This combined modality strategy could be beneficial in particularly targeting tumors with elevated Plk1 or DNA-PKcs activities.

In conclusion, our works reveal that there is a direct association between DNA-PKcs and Plk1 and that their coordination is required for timely activation of Cdk1 kinase and normal cell cycle progression through $G_2$/M transition. Our results also shed new light on the therapeutic potential of targeting both DNA-PKcs and Plk1 for synthetic lethality in cancer treatment.

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## Appendix A. Supplementary Materials

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.neo.2015.02.004.

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