

Deoxycytidine kinase regulates the G2/M checkpoint through interaction with cyclin-dependent kinase 1 in response to DNA damage

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ABSTRACT

Deoxycytidine kinase (dCK) is a rate limiting enzyme critical for phosphorylation of endogenous deoxynucleosides for DNA synthesis and exogenous nucleoside analogues for anticancer and antiviral drug actions. dCK is activated in response to DNA damage; however, how it functions in the DNA damage response is largely unknown. Here, we report that dCK is required for the G2/M checkpoint in response to DNA damage induced by ionizing radiation (IR). We demonstrate that the ataxia-telangiectasia-mutated (ATM) kinase phosphorylates dCK on Serine 74 to activate it in response to DNA damage. We further demonstrate that Serine 74 phosphorylation is required for initiation of the G2/M checkpoint. Using mass spectrometry, we identified a protein complex associated with dCK in response to DNA damage. We demonstrate that dCK interacts with cyclin-dependent kinase 1 (Cdk1) after IR and that the interaction inhibits Cdk1 activity both *in vitro* and *in vivo*. Together, our results highlight the novel function of dCK and provide molecular insights into the G2/M checkpoint regulation in response to DNA damage.

INTRODUCTION

As the human genome is constantly challenged by environmental DNA damaging agents, cells have developed

comprehensive control systems to deal with these insults in order to maintain genome stability and promote survival. Among the protective mechanisms, cell cycle checkpoints exist to halt cell cycle progression at the G1/S border (the G1 checkpoint), throughout the S-phase (the intra-S checkpoint) or at the G2/M border (the G2/M checkpoint) (1). The overall function of cell cycle checkpoints is to provide time for the cell to process DNA repair before division and to determine whether programmed cell death is necessary should the damage be beyond repair. Cell cycle checkpoints are regulated by signaling cascades involving evolutionally conserved proteins that include damage sensors, signal transducers, mediators and effectors (2). For example, the Mre11-Rad50-Nbs1 (MRN) complex acts as a sensor to recruit the ataxia-telangiectasia-mutated (ATM) kinase in the event of DNA double strand breaks (3). It is well documented that the ATM kinase is a central element critical for signal amplification to facilitate cell cycle checkpoints (4). Indeed, ATM belongs to a conserved protein kinase family related to phosphatidylinositol 3 kinase-like kinases. ATM phosphorylation of downstream targets is essential to initiate cell cycle checkpoints (5). Critical understanding of the cell cycle checkpoint regulatory network relies on elucidating the crosstalk between DNA damage response pathways and cell cycle regulators. Since cell cycle progression is driven by cyclin-dependent kinases (Cdks) (6), Cdks are prime targets of DNA damage response proteins for initiation of cell cycle arrest. Among them, Cdk1, encoded by the *CDC2* gene, functions as a critical Cdk for the G2/M transition and mitosis (7). Mediated by Wee1, Cdk1 is inhibited by

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phosphorylation at a conserved tyrosine 15 residue (8,9). This inhibition can be counteracted by Cdc25 phosphatase family members (10). ATM-mediated Chk2 phosphorylation/activation antagonizes the function of Cdc25 phosphatases, thereby indirectly inhibiting Cdk1 activity and leading to activation of the G2/M checkpoint (11). Besides these functions, Cdk1 is also involved in a feedback loop with PLK1, 53BP1 and Chk2 which serves to inactivate the G2/M DNA damage checkpoint (12).

Deoxycytidine kinase (dCK) is a critical enzyme responsible for phosphorylation of 2'-deoxycytidine, 2'-deoxyadenosine and 2'-deoxyguanosine to their corresponding monophosphorylated forms (13). This reaction is the first and rate limiting step in deoxyribonucleoside salvage, which provides deoxynucleoside triphosphates for DNA replication and repair. dCK is also critical for activation of a number of anticancer and antiviral nucleoside analogues, such as fludarabine, cladribine, gemcitabine, clofarabine, zalcitabine and lamivudine (14). Due to its broad role in DNA synthesis and drug action, dCK has been suspected of playing a role in the cellular response to DNA damage, though a detailed mechanism has yet to be described. dCK activity is enhanced in response to treatment with IR, UV and genotoxic drugs such as aphidicolin, etoposide and certain nucleoside analogues (15,16). Post-translational modification, especially phosphorylation, of dCK is critical for its enzymatic activity. Among several potential phosphorylation sites, Serine 74 has emerged as the most critical site for activated dCK (17). The kinases responsible for *in vivo* dCK Serine 74 phosphorylation are largely unknown; although Casein kinase 1 δ (CK1 δ) has been reported to phosphorylate dCK on Serine 74 *in vitro* (18).

Given that dCK is activated in response to DNA damage and plays a critical role in DNA synthesis, we hypothesized that dCK is essential for optimal DNA damage responses. Here we report that ATM phosphorylation of dCK on Serine 74 is essential to activate the G2/M checkpoint in response to DNA damage. We have identified a complex that associates with dCK in response to DNA damage and we demonstrate that dCK interacts with and inactivates Cdk1 to initiate the G2/M checkpoint.

MATERIALS AND METHODS

Cell culture

Human cervical carcinoma cell line HeLa and human embryonic kidney cell line HEK293T (The American Type Culture Collection, Manassas, VA, USA) were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum and 50 μ g/ml of penicillin/streptomycin (Thermo Fisher, Waltham, MA, USA) at 37°C under 5% CO₂ and a 95% relative humidity atmosphere. The isogenic cell lines stably expressing ATM and control shRNA were maintained in DMEM supplemented with 1 μ g/ml puromycin as described previously. EBV-transformed lymphoblast GM0536 (ATM^{+/+}) and GM1526 (ATM^{-/-}) cells (The NIGMS Human Mutant Cell

Repository, Camden, NJ, USA) were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium (Thermo Fisher, Waltham, MA, USA) supplemented with 10% fetal bovine serum and 50 μ g/ml of penicillin/streptomycin.

Construction of dCK knockdown stable cell lines

Stable cell lines with knockdown of dCK were generated according to a vector-based knockdown strategy. siRNA target sites (siRI: 436-454 and siRII: 148-166) were designed according to siRNA Target Finder (http://www.ambion.com/techlib/misc/siRNA_finder.html).

ShRNA was subcloned into the pHTsiRNA vector at *Bgl*III and *Xho*I sites. HeLa cell lines were transfected with the pHT-dCK-siRNA vector and empty vector using Effectene Transfection Reagent (Qiagen, Valencia, CA, USA) and cells were cultured under puromycin (4 μ g/ml) selection. Puromycin-resistant colonies were individually picked up and detected by RT-PCR and western blotting. Stable cell clones were maintained in DMEM supplemented with 1 μ g/ml Puromycin.

Plasmids

Full-length coding sequences of dCK were amplified by RT-PCR and subcloned into the vector pcDNA3. The dCK-S74A, dCK-S74E mutants and siRNA-resistant dCK-WT, S74A and S74E plasmids were generated using the QuikChange II XL site-directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's protocol. The primers used are shown in the following table.

Antibodies

Anti-ATM, anti-CDK1-Y15p and anti-beta actin antibodies were purchased from Abcam Inc (Cambridge, MA, USA). Anti-dCK was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-Cdk1 was purchased from Millipore (Billerica, MA, USA). The rabbit dCK-Ser74p antibody, generated as described previously (17).

Western blotting

Whole-cell lysates were extracted, quantified and subjected to electrophoresis with Criterion 4-12% precast XT Bis-Tris gels (Bio-Rad, Hercules, CA, USA) in

Primer's name	Sequences
dCK-WT-F	5'-tcactagatggccaccgcccaagagaagc-3'
dCK-WT-R	5'-acgctcgatcacaagaatctcaaaaactctt-3'
dCK-S74A-F	5'-cttacaatggcacagaaaaatggtgg-3'
dCK-S74A-R	5'-ccaccattttctgtgccattgtaag-3'
dCK-S74E-F	5'-cttacaatggaacagaaaaatggtgg-3'
dCK-S74E-R	5'-ccaccattttctgttccattgtaag-3'
dCK-siRI-Res-F	5'-gaagattgggaagtgtcccagagccagtcgccagatg-3'
dCK-siRI-Res-R	5'-catctggcgactggctctgggacgacttccaatcttc-3'
dCK-siRII-Res-F	5'-gtatgaatctgaatgtatgaacgaaactgaatggacaat-3'
dCK-siRII-Res-R	5'-attgtccattcagtttcgttcatactcagattcacaac-3'

XT MOPS Running Buffer (Bio-Rad), and subsequently transferred to nitrocellulose membranes (Pall Life Sciences, Ann Arbor, MI, USA). Membranes were then blocked with 5% non-fat dry milk solution (Lab Scientific, Inc., Livingston, NJ, USA) at room temperature for 1 h, incubated with primary antibodies overnight, secondary antibodies at room temperature for 1 h, and visualized with the Pierce chemiluminescence detection system (Thermo Scientific Pierce, Rockford, IL, USA).

Immunoprecipitation followed by mass spectrometry

Whole-cell lysates were incubated with Anti-FLAG-M2 Agarose beads (Sigma, St Louis, MO, USA) overnight at 4°C. The samples were then washed three times with 500 µl TBS buffer, eluted in 40 µl Laemmli buffer and placed in a boiling water bath for 5 min. The eluted proteins were analyzed on an SDS-PAGE gel followed by silver staining. The bands which showed different patterns among dCK-WT and S74A treated with/without IR were processed for identification using mass spectrometry (Synapt HDMS; Waters Corporation, Milford, MA, USA).

In vitro kinase assay

For the Cdk1 *in vitro* kinase assay, recombinant dCK (Novus Biologicals, Littleton, CO, USA) and Cdk1/CyclinA2 (Promega, Madison, WI, USA) were incubated with 1 µg of Histone H1 (Promega) in the presence of 50 µM ATP in kinase buffer for 30 min at room temperature. Cdk1 inhibitor CAS220749-41-7 (Santa Cruz Biotechnology) was used as a positive control. The kinase activity of Cdk1 was measured by quantifying the amount of ADP produced using the ADP-GLO™ kinase assay (Promega). Briefly, 5 µl of ADP-GLO™ reagent were incubated with the reaction products at room temperature for 40 min. Luminescence was recorded after incubation with 10 µl of the kinase detection reagent for 30 min at room temperature.

The ATM *in vitro* kinase assay was performed according to the method described previously (19), using the dCK-Ser74p antibody.

dCK activity assay

dCK activity in GM0536 and GM1526 cells was determined by measuring and comparing the activation of Ara-C to Ara-CTP using an assay described by Parker *et al.* (20). Briefly, acid-soluble extracts were obtained after cells were incubated with 100 µM Ara-C for 30 min. Intracellular nucleotides were eluted from a Nucleosil SB silica SAX column (Interchim, Montlucon, France) with a 30 min linear gradient from 7 to 500 mM KH₂PO₄ (pH 4.0), followed by 20 min of 500 mM KH₂PO₄ (pH 4.0) at a flow rate of 2 ml/min. Intracellular nucleotides including Ara-CTP were detected by UV absorbance at 260 nm.

Irradiation

Cells were irradiated at various doses using an XRAD 320 X-ray irradiator (Precision X-Ray, Inc., CT, USA) at a dose rate of 75 cGy/min at room temperature.

Clonogenic survival assay

Cells were seeded into 6-well plates in triplicate and then incubated for 24 h to allow settling. Cells were treated with Ara-C (0–1 µM) for 24 h or irradiated (0–6 Gy). After 7–10 days, the culture dishes were stained with 0.5% crystal violet and the number of colonies (more than 50 cells) was counted.

G2/M checkpoint assay

A total of 70% ethanol-fixed cells were permeabilized with 0.25% Triton X-100 in PBS on ice for 5 min. Cell pellets were blocked in PBS containing 1% bovine serum albumin (BSA) for 30 min followed by incubation with the primary antibody (anti-phospho-Histone H3-Ser10) (1: 200) (Millipore) at room temperature for 3 h and with the secondary antibody (goat anti-rabbit IgG-FITC) (1:200) (Santa cruz Biotechnology) in the dark for 30 min. Cells were stained with 25 µg/ml propidium iodide (Life Technology, Grand Island, NY, USA) and the percentage of mitotic cells was quantified by flow cytometry (BD FACS LSRII, CA, USA), as previously described (21).

RESULTS

dCK is involved in the optimal DNA damage response

To study the function of dCK in the DNA damage response, we generated isogenic HeLa cell lines in which control shRNA or dCK shRNA was stably expressed. After confirming successful knock-down (Figure 1A), we conducted a cytotoxicity assay using cytarabine (Ara-C), a nucleoside analogue that requires dCK for drug action (14). We found that cells with depleted dCK showed significant resistance to Ara-C at lower doses (Figure 1B). However, at higher doses of Ara-C treatment, the resistance became less significant. To assess this dose response discrepancy, we hypothesized that the resistant phenotype of dCK knock-down to Ara-C could have been partially reversed by hypersensitivity of the cells to DNA damage induced by higher doses of Ara-C. Indeed, colony formation assays indicated that dCK knock-down cells displayed hypersensitivity in response to DNA damage induced by ionizing radiation (IR) (Figure 1C), indicating that dCK is required for the DNA damage response.

We further characterized the DNA damage response of dCK knock-down cells by measuring the mitotic index. In response to IR, we found that dCK knock-down cells, unlike control cells, did not show a dramatic reduction in the percentage of mitotic cells, indicating a defective G2/M checkpoint (Figure 1D). A time course experiment showed that dCK knock-down cells displayed significant defects in the IR-induced G2/M checkpoint within the first 8 h following IR (Supplementary Figure S1).

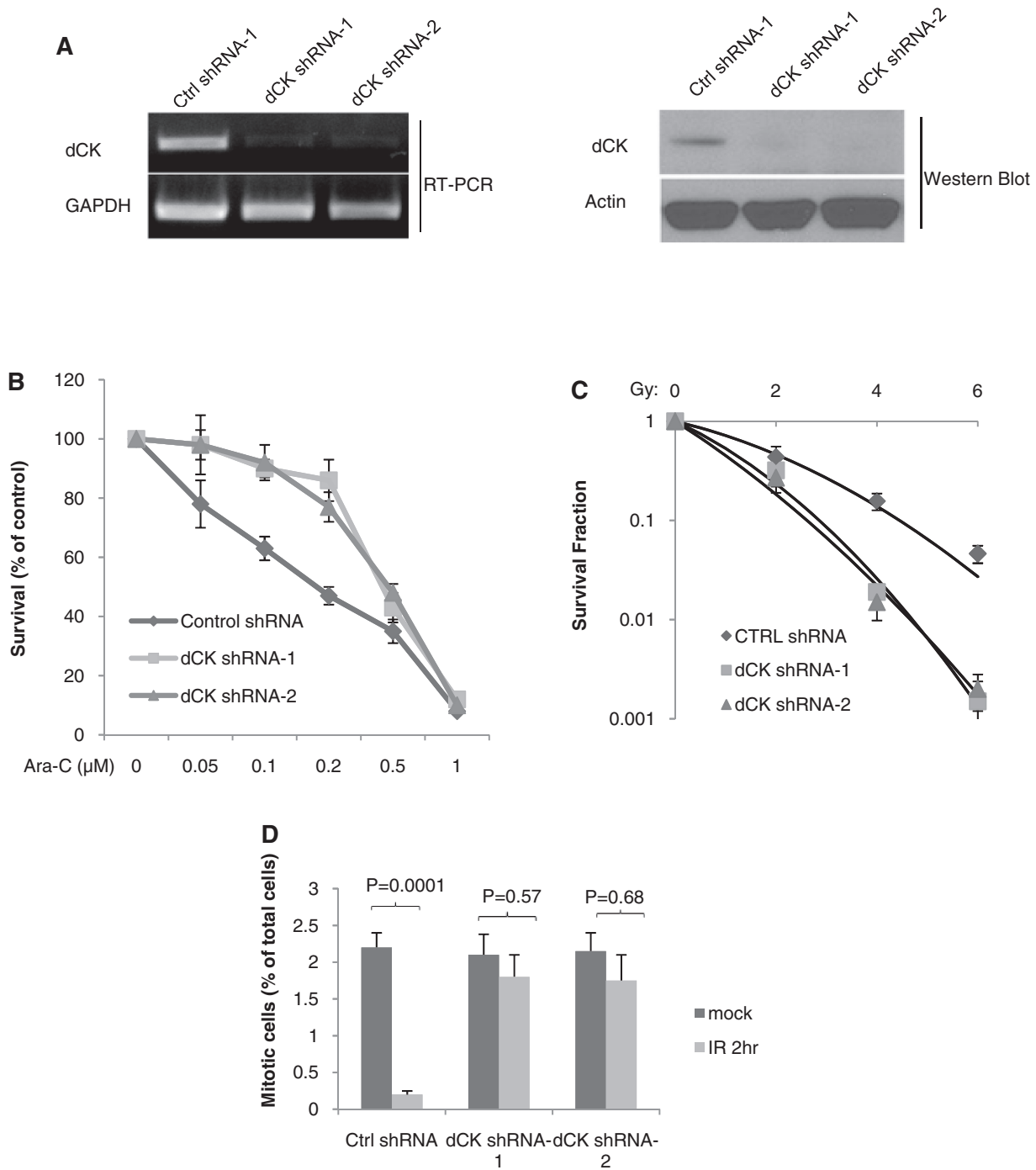


Figure 1. dCK is required for the G2/M checkpoint in response to ionizing radiation. (A) Establishment of isogenic cell lines with stable dCK knock-down. HeLa cells were stably transfected with control or dCK shRNA. Individual clones were obtained under puromycin selection. Knock-down effects were confirmed by RT-PCR (left panel) and western blot (right panel). (B) The isogenic cell lines with control or dCK knock-down were treated with Ara-C at indicated doses for 24h, and clonogenic survival was assessed. (C) Radiosensitivity was assessed by the colony formation assay in the isogenic cell lines after radiation treatment (0–6 Gy). (D) Isogenic HeLa cells were treated with mock or IR treatment (6 Gy). Two hours after treatment, cells were harvested and fixed followed by staining for Histone H3 Ser10 phosphorylation for flow cytometry. The average and standard deviations of at least triplicate samples are shown. Statistic analysis was done by Student's *t*-test.

ATM is required for dCK activation in response to IR

dCK is activated in response to DNA damage induced by IR, UV and chemotherapeutic drugs (15,16). As dCK activation in response to DNA damage might play a

critical role in the DNA damage response, we intended to investigate regulators of dCK activation. We measured dCK activity in a pair of lymphoblastoid cells lines in which ATM is either proficient (GM0536) or deficient (GM1526). We observed that GM0536 cells showed

dCK activation in response to IR, but GM1526 cells failed to do so (Figure 2A). Interestingly, stably transfected HeLa cells with shRNA against ATM (22) were significantly resistant to Ara-C treatment (Figure 2B).

ATM is required for dCK Serine 74 phosphorylation both *in vitro* and *in vivo*

To further study ATM-mediated dCK activation, we hypothesized that ATM might phosphorylate dCK. Among four potential *in vivo* phosphorylation sites (Threonine 3, Serine 11, Serine 15 and Serine 74) (17), Serine 74 fit into the SQ motif of the ATM consensus sequence (23). Using a polyclonal antibody for dCK Serine 74 phosphorylation, we first checked dCK Serine 74 phosphorylation in GM0536 and GM1526 cells. As shown in Figure 2C, we observed a weak Serine 74 phosphorylation signal in the absence of DNA damage in GM0536 cells. In cells treated with IR, a significant increase in Serine 74 phosphorylation was observed. In contrast, GM1526 cells displayed diminished dCK Serine 74 phosphorylation in the absence or presence of IR-induced DNA damage. We also utilized the isogenic HeLa cell lines with stably transfected control or ATM shRNA to measure dCK Serine 74 phosphorylation. Similarly, we observed that in ATM shRNA transfected cells, IR-induced dCK Serine 74 phosphorylation is defective (Figure 2D, left panel). These results indicate that ATM is required for dCK Serine 74 phosphorylation. It is noted that the phospho-Serine 74 antibody did not recognize any signal in dCK shRNA knock down cells (Figure 2D, right panel). To study whether dCK is directly phosphorylated by ATM, we conducted an *in vitro* kinase assay using a GST-ATM C-terminal fragment as kinase and dCK peptides containing Serine 74 as substrate followed by western blot using the anti-phospho-Serine 74 dCK antibody. As shown in Figure 2E, we found that dCK Serine 74 is directly phosphorylated by ATM *in vitro*. Together, these results strongly indicate ATM phosphorylates dCK on Serine 74. Additionally, during our initial study of ATM-mediated dCK phosphorylation, dCK was reported to be among the proteins phosphorylated by ATM in a large scale proteomic study (24).

ATM-mediated dCK Serine 74 phosphorylation is required for the G2/M checkpoint

To study the functional significance of ATM-mediated dCK phosphorylation, we transfected either vector only, wild-type, serine 74 to alanine substitution (which abrogates phosphorylation) or the serine 74 to glutamic acid substitution (which mimics phosphorylation) form of dCK into dCK knock-down cells (Figure 3A). We then conducted the G2/M checkpoint assay to assess whether the serine to alanine mutation (S74A) would have an effect on activation of the checkpoint. We found that wild-type dCK rescued the G2/M checkpoint defect in dCK knock-down cells (Figure 3B), further demonstrating the essential role of dCK in the checkpoint. Interestingly, unlike wild-type, the S74A mutant dCK was not capable of restoring the checkpoint. Notably, unlike other

previously reported serine to alanine mutants of ATM target proteins (21,25–27), S74A dCK does not possess a dominant negative activity since over-expression of S74A did not affect G2/M checkpoint activation in the control shRNA cells. Note the level of dCK overexpression compared to the endogenous dCK is shown in Figure 3C. To further study the role of ATM-mediated dCK Serine 74 phosphorylation, we introduced the various dCK constructs into the isogenic HeLa cell lines stably transfected with control or ATM shRNA. We found that neither wild-type nor S74A dCK was able to restore the G2/M checkpoint defect in ATM knock-down cells. However, phospho-mimicking dCK (S74E) complemented the G2/M checkpoint defect (Figure 3D). These results strongly support the conclusion that ATM-mediated dCK Serine 74 phosphorylation is required for the activation of the IR-induced G2/M checkpoint.

We also conducted the colony formation assay in dCK shRNA knock-down cells expressing either vector only, dCK WT, S74A or S74E. We found that similar to dCK-WT, either S74A or S74E can rescue the radiosensitivity phenotype of dCK knock-down cells (Supplementary Figure S2), indicating that ATM-mediated dCK phosphorylation is not required for the maintenance of radioresistance and that dCK-mediated G2/M checkpoint regulation is independent of IR-induced radiosensitivity.

Identification of a dCK complex in response to DNA damage

To further study the functional significance of dCK Serine 74 phosphorylation in the DNA damage response, we aimed to identify a potential dCK complex that is associated with DNA damage-induced phosphorylation. To achieve this goal, we transfected flag-tagged wild-type or the S74A mutant dCK into HeLa cells and immunoprecipitated the exogenous dCK using an anti-flag antibody. By mass spectrometry we identified a list of proteins that specifically bind to wild-type but not S74A dCK in response to IR (Supplementary Figure S3). Noticeably, among the identified proteins, there are two associated with cell cycle progression: Cdk1 and Prohibitin 2.

ATM-mediated Serine 74 phosphorylation is required for dCK and Cdk1 interaction

Given that ATM-mediated dCK phosphorylation is essential for activation of the G2/M checkpoint and that Cdk1 is an essential protein driving the G2/M transition, we focused on the potential interaction of dCK and Cdk1 (Figure 4A). First, we validated the interaction of dCK and Cdk1 *in vivo*. We transfected flag-tagged wild-type or S74A dCK into HeLa cells and immunoprecipitated exogenous dCK. We observed that Cdk1 associated with dCK even in unperturbed cells (Figure 4B), including a slight binding to the S74A form. In cells treated with IR, the interaction was significantly increased as more Cdk1 was brought down by wild-type dCK. More interestingly, the S74A mutant dCK failed to coimmunoprecipitate with Cdk1 (Figure 4B). We also

conducted a reciprocal experiment by immunoprecipitation of Serine 74 phosphorylated dCK and looked for Cdk1 interaction. Similarly, we found that Cdk1 only interacted with the Serine 74 phosphorylated form of dCK (Figure 4C). Furthermore, we found that in ATM-deficient cells, the dCK-Cdk1 interaction in the absence or presence of IR was significantly impaired (Figure 4D). Together, these results indicate that the dCK-Cdk1 interaction is enhanced in response to DNA damage and that ATM-mediated dCK Serine 74 phosphorylation is required for the interaction.

dCK interaction with Cdk1 inhibits Cdk1 activity *in vitro* and *in vivo*.

To study the functional role of the interaction of dCK with Cdk1, we conducted an *in vitro* kinase assay using the recombinant Cdk1/Cyclin A2 as kinase and Histone H1 as substrate. We found a dose-dependent inhibition of Cdk1 activity by recombinant dCK (Figure 5A). The inhibitory effect of dCK at the dose of 10 μg is similar to that of a known Cdk1 inhibitor CAS220749-41-7 (28). Because the inhibitory effect of dCK

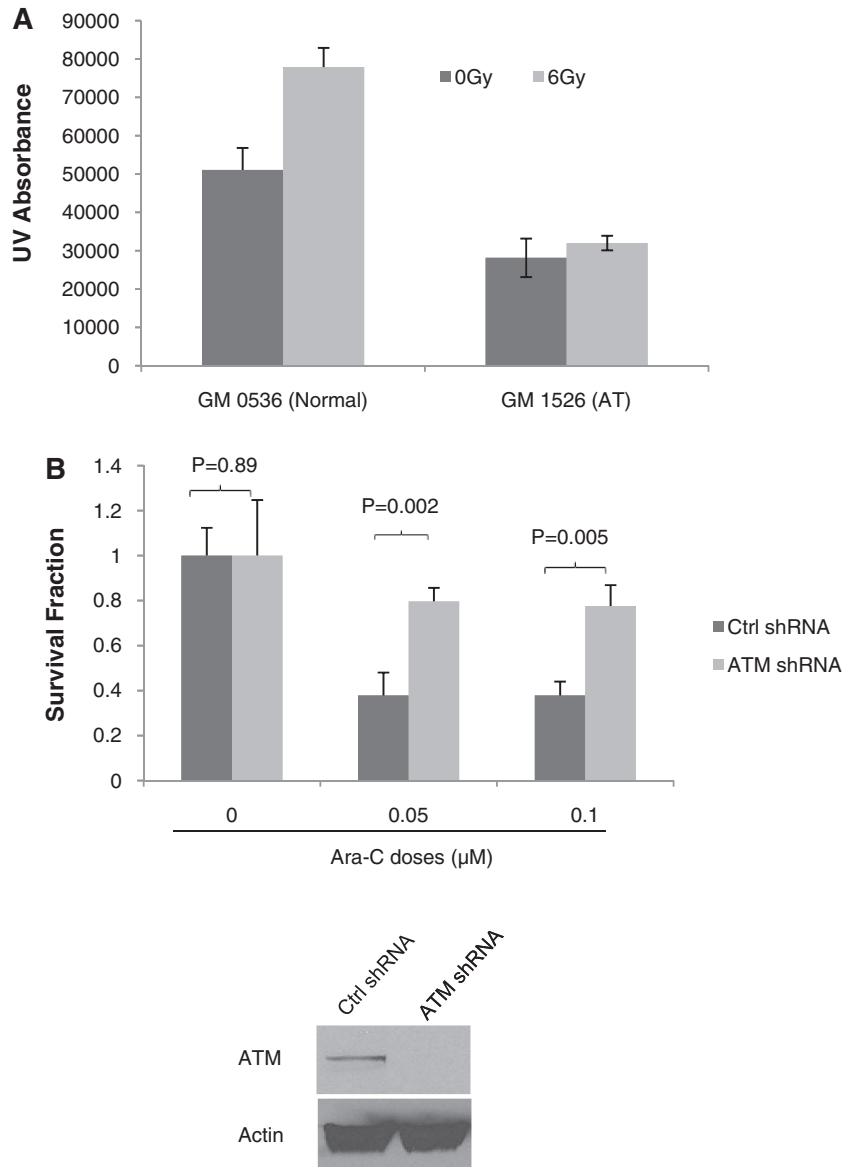


Figure 2. ATM phosphorylates dCK on Serine 74 in response to IR. (A) Lymphoblast cell lines GM0536 (ATM^{+/+}) and GM1526 (ATM^{-/-}) were treated with mock or IR treatment (6Gy) followed by the dCK kinase assay as described in the methods. (B) Isogenic HeLa cells treated with indicated doses of Ara-C for 24h were assessed for colony formation ability. Survival fractions are shown. (C) EBV-transformed lymphoblast cell lines, GM0536 and GM1526, were treated with mock or IR (6 Gy). 2h after radiation, whole cell lysates were harvested and subjected to immunoblotting using indicated antibodies. (D) HeLa cells stably transfected with control or ATM shRNA were treated with mock or IR (6 Gy). Two hours after radiation, whole-cell lysates were harvested and subjected for immunoblot using indicated antibodies. (E) The *in vitro* kinase assay was performed using ATM fragments (either GST-tagged N-terminal amino acids 248–522 or C-terminal fragments of ATM amino acids 2709–2964) in the presence of peptides containing the non-phosphorylated form of dCK (FEELTMSQKNGGNVL). The gel was immunoblotted with anti-dCK Ser74p antibody. The phosphor-dCK peptide (FEELTMS_pQKNGGNVL) was loaded as a positive control.

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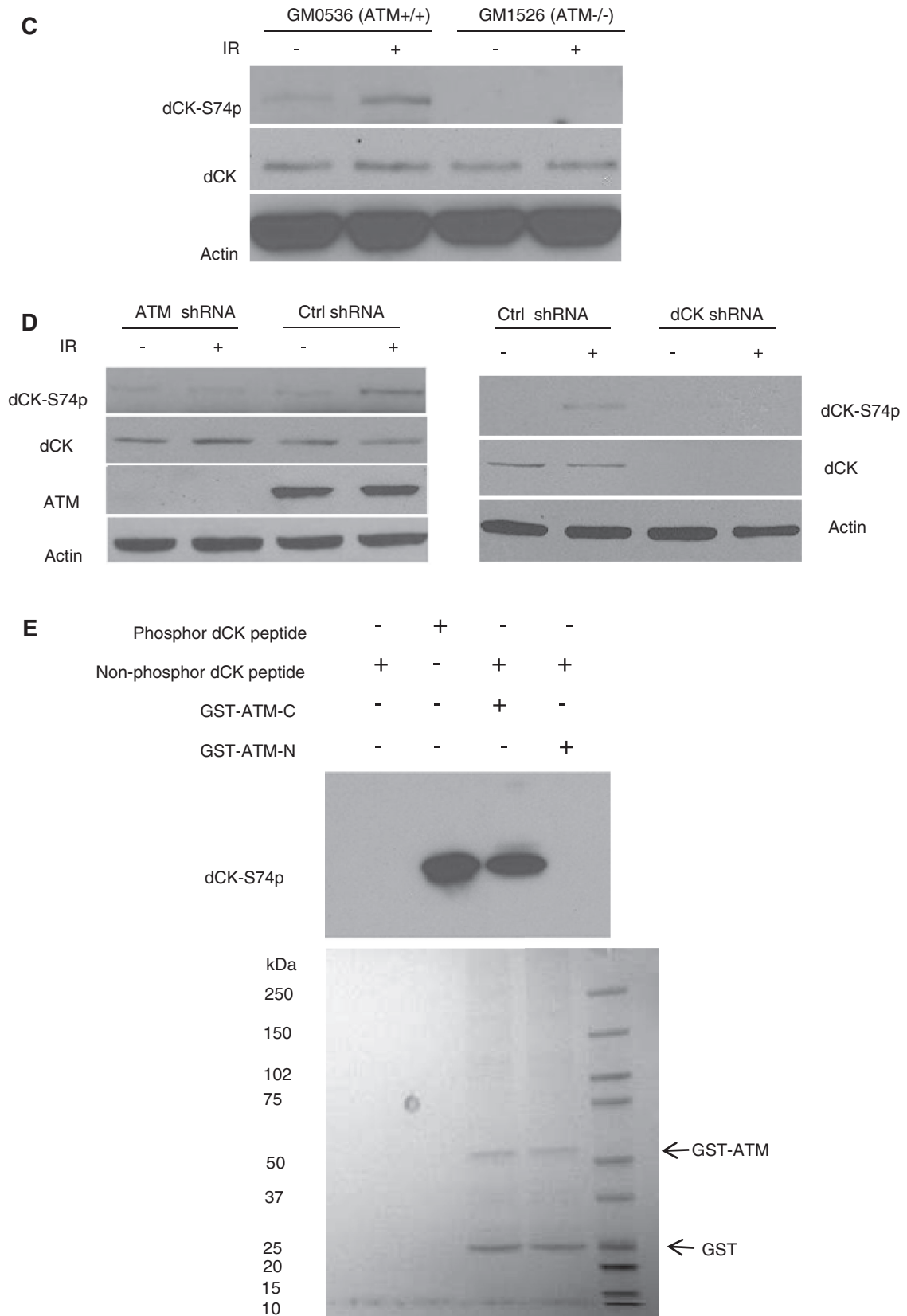


Figure 2. Continued.

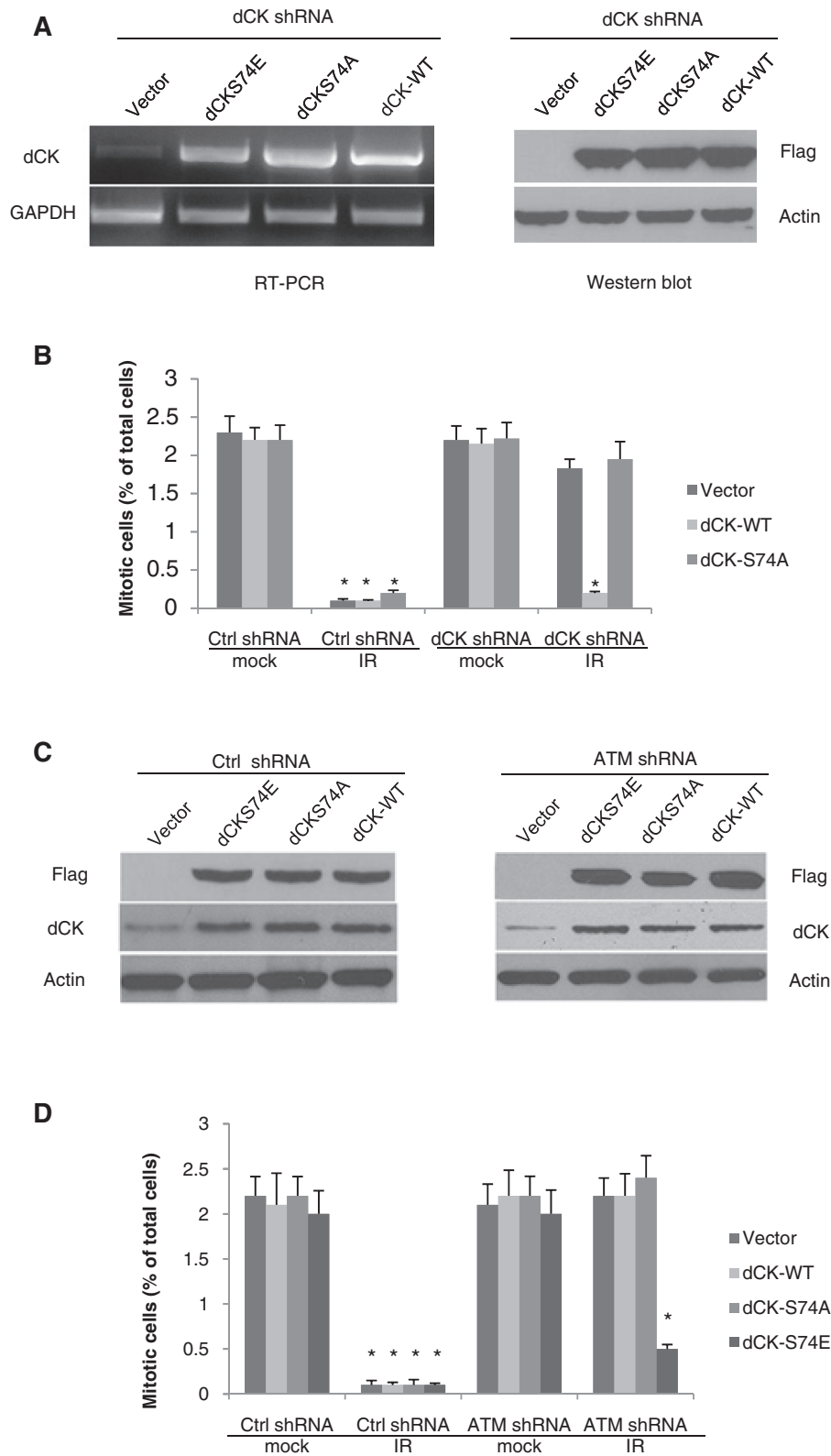


Figure 3. ATM-mediated dCK Serine 74 phosphorylation is required for the IR-induced G2/M checkpoint. (A) dCK knock-down HeLa cells were re-introduced with vector control, dCK wild-type, dCK Serine 74 to alanine (S74A) mutation or Serine 74 to Glutamic Acid (S74E) mutation. The complementation effects using RT-PCR and western blot are shown. (B) The dCK complemented cell lines were treated with mock or IR (6Gy). Two hours after treatment, cells were harvested and stained for Histone H3 Ser10 phosphorylation followed by flow cytometry. (C) Expression of flag-tagged dCK as compared to endogenous dCK in control shRNA and ATM shRNA cells. (D) HeLa cells with control or ATM shRNA were transiently transfected with vector, wild-type (WT), S74A or S74E of dCK and treated with IR (6Gy). Two hours after treatment, cells were harvested and stained for Histone H3 Ser10 phosphorylation followed by flow cytometry analysis. The average and standard deviations of at least triplicate samples are shown. Statistic analysis was done by Student's *t*-test; * $P \leq 0.05$.

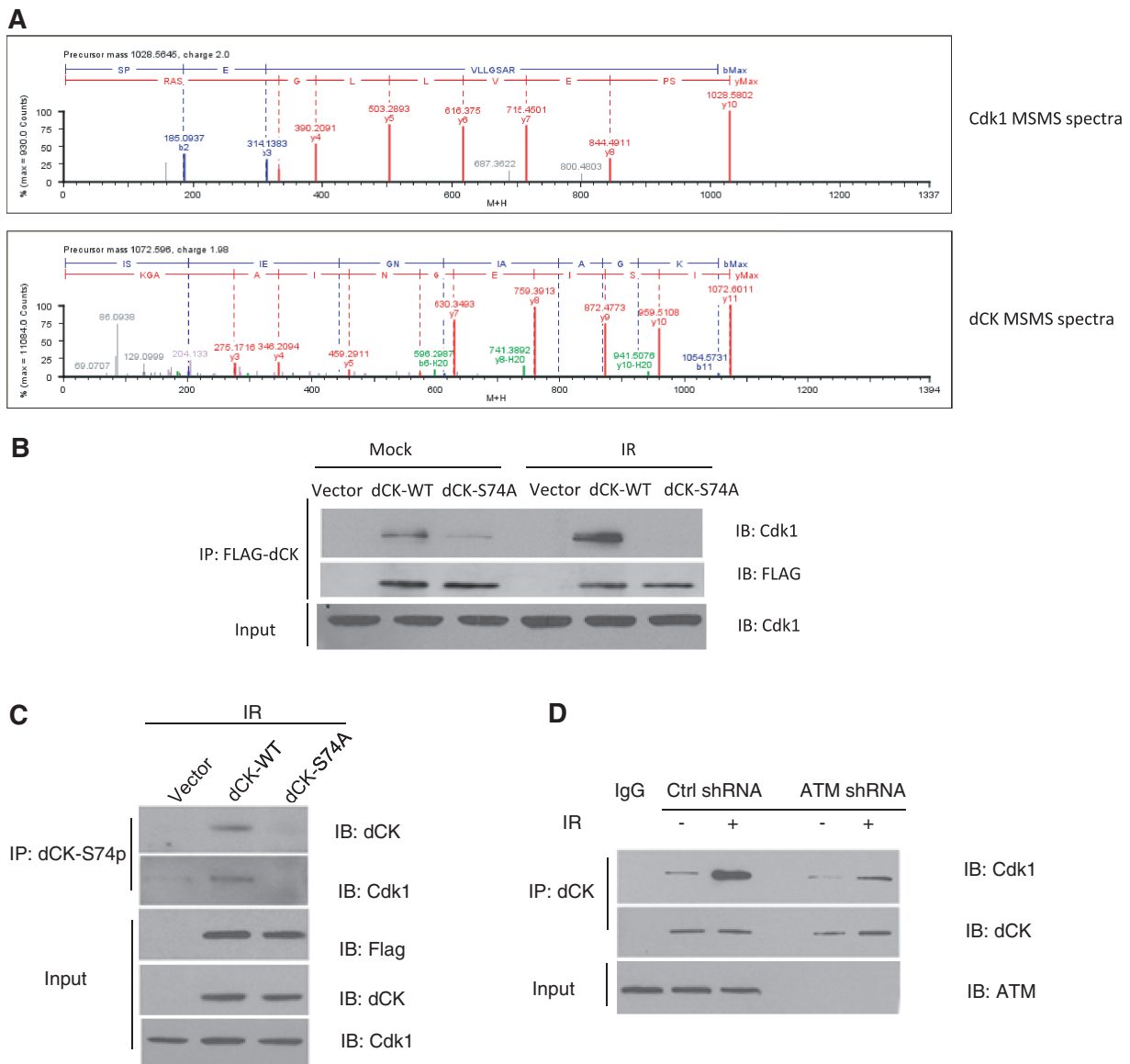


Figure 4. dCK interacts with Cdk1 in response to IR. (A) Mass spectrometry identified Cdk1 as one of the dCK association proteins. (B) Vector control, flag-tagged wild-type or S74A dCK were transiently transfected into HeLa cells. After 36 h of transfection, the cells were treated with mock or IR (6 Gy). Two hours after IR, cells were harvested and subjected to immunoprecipitation using an anti-Flag antibody. The immunoprecipitates were then blotted with indicated antibodies. (C) dCK knock-down HeLa cells were transfected with vector control, flag-tagged dCK-WT or dCK-S74A and irradiated (6 Gy). Two hours after IR, cells were harvested and immunoprecipitated with the anti-dCK Ser74p antibody. The immunoprecipitates were then blotted with indicated antibodies. (D) Isogenic HeLa cells stably expressing control or ATM shRNA were treated with mock or IR (6 Gy). Two hours after IR, cells were harvested and endogenous dCK was immunoprecipitated followed by immunoblotting with the indicated antibodies.

might not be solely dependent on interaction, we do not rule out non-specific inhibition of dCK on Cdk1 in this assay.

To study the *in vivo* effect of the dCK–Cdk1 interaction, we investigated Cdk1 Tyrosine 15 phosphorylation, a marker representing inhibition of Cdk1 activity. We found that IR-induced Tyrosine 15 phosphorylation is significantly impaired in dCK knock-down cells (Figure 5B). Furthermore, reintroduction of wild-type or S74E dCK significantly enhanced Tyrosine 15 phosphorylation in response to IR in dCK knock-down cells (Figure 5C). On the contrary, the S74A mutant dCK showed

significantly lowered Tyrosine 15 phosphorylation as compared to that in vector cells. Taken together, our results demonstrate that dCK interaction with Cdk1 directly inhibits Cdk1 activity, resulting in activation of the G2/M checkpoint. It is noted that unlike Cdk1 Y15p, Chk2 Threonine 68 phosphorylation was not affected by dCK S74A mutation.

DISCUSSION

Our study presented here highlights a critical, mechanistic link between a DNA damage response signaling cascade

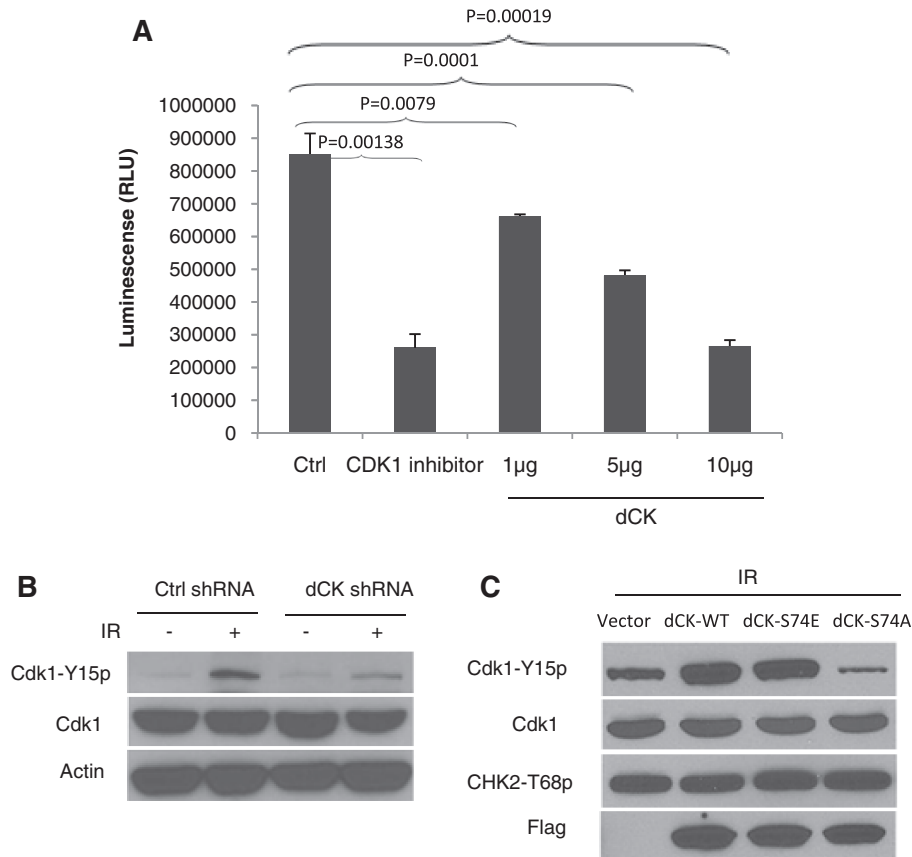


Figure 5. The dCK–Cdk1 interaction inhibits Cdk1 activity both *in vitro* and *in vivo*. **(A)** Cdk1 kinase activity was measured by an *in vitro* kinase assay using the recombinant Cdk1/CyclinA2 complex incubated with 1 µg of Histone H1 as substrate in the presence of 50 µM ATP. Recombinant dCKs at indicated doses were added into the kinase reaction, and the luminescence signal indicative of Cdk1 activity was recorded. The average and standard deviations of at least triplicate samples are shown. **(B)** Isogenic HeLa cell lines with control or dCK shRNA were treated with mock or IR (6 Gy). Two hours after IR, total cell lysates were obtained and subjected to western blot analysis using indicated antibodies. **(C)** HeLa cells transiently transfected with vector, flag-tagged wild-type, S74A or S74E were irradiated (6 Gy). Total cell lysates were obtained and subjected to western blot with indicated antibodies.

(i.e. the ATM–dCK pathway) and the cell cycle regulator Cdk1. While DNA damage-induced cell cycle checkpoints have been extensively studied, many unsolved puzzles in terms of critical links between DNA damage response pathways and essential cell cycle activators still remain. Cdk1, in complex with its cyclin partners, governs the mitotic entry (7). Previous studies have demonstrated that Cdk1 activation involves Tyrosine 15 dephosphorylation, which is partly governed by Cdc25A. Chk1 and Chk2 phosphorylate Cdc25A and Cdc25C, leading to Cdc25A ubiquitination and proteasomal degradation and Cdc25C nuclear exclusion/cytoplasmic sequestration via binding to 14-3-3 proteins (29). Our data presented in this manuscript demonstrate that dCK functions as a critical regulator of the G2/M checkpoint by interaction and inhibition of Cdk1 activity upon DNA damage. Though it is still not known to which dCK domains Cdk1 binds to, it is likely that Serine 74 phosphorylation leads to a conformational change that opens the accessibility of dCK to Cdk1 or that enhances the affinity of existing binding in unperturbed cells. The increased interaction of dCK and Cdk1 further inhibits Cdc25 phosphatase dependent dephosphorylation of Tyrosine 15.

We demonstrate that dCK is a downstream kinase regulated by ATM in the DNA damage response. dCK is among as many as 700 proteins phosphorylated by ATM in the DNA damage response (24). The functional significance of ATM-mediated dCK phosphorylation is demonstrated in this study.

The identification of the dCK complex reveals a more comprehensive picture of the functional role of dCK in the DNA damage response. In the dCK complex, there are a number of interesting interacting proteins worthy of further investigation. For example, the involvement of Elongation Factor 1 (EEF-1) delta, which is essential for the enzymatic delivery of aminoacyl tRNAs to the ribosome (30), might indicate a cytosolic function of dCK in response to DNA damage. Since EEF-1 is also a substrate of Cdk1 (31), the complex containing dCK, EEF-1 and Cdk1 might balance cell cycle regulation in the presence of DNA damage. In addition, prohibitin 2, also shown in the dCK complex after DNA damage, is involved in various cellular functions such as cell cycle, apoptosis, mitochondrial enzyme assembly, nuclear receptor signaling and senescence (32,33–35). Interaction with prohibitin 2 indicates broader functions of dCK in

cellular processes and stress responses. The complex might also shed light on cellular phenotypes such as prolonged G2/M accumulation in cells lacking dCK (unpublished data). Since prolonged G2 accumulation results from a lack of the intra-S-phase checkpoint (36), this potential link might indicate that dCK also participates in the intra-S-phase checkpoint in response to DNA damage.

The significance of these findings extends beyond molecular insights into cell cycle checkpoint regulation. Since dCK activity is required for many chemotherapeutic drugs for toxicity, the mechanism of dCK activation can be potentially explored to optimize chemo-radiation combination therapy. This is supported by findings that many nucleoside analogues display synergistic effects when combined with radiation therapy (14). Many earlier studies have suggested that nucleoside analogues can inhibit DNA repair to serve as radiosensitizers (37). While this is still a valid conclusion, our study indicates a different mechanism for the synergistic effect as IR can activate dCK through ATM and promote chemosensitization. These observations might have a significant impact in optimization of the treatment schedule for combination treatment in the clinical setting.

In summary, our data demonstrated that dCK is activated by ATM-mediated Serine 74 phosphorylation in response to DNA damage and is required for the G2/M checkpoint. Further, we demonstrated that dCK interacts with and inhibits Cdk1 to activate the G2/M checkpoint.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Figures 1–3.

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