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Identification and characterization of a novel phosphoregulatory site on cyclin-dependent kinase 5

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Abstract

Cyclin-dependent kinase 5 (CDK5) is a serine/threonine kinase essential for embryonic development whose overactivation has been implicated in several pathologies including neurodegeneration, cancer cell metastasis and type II diabetes. Therefore, it is important to investigate molecular mechanism(s) that mediate regulation of CDK5 activity. Here we identify and characterize a novel phosphoregulatory site on CDK5. Our mass spectrometry analysis identified seven putative phosphorylation sites on CDK5. Using phosphomimetic and non-phosphorylatable mutants, we determined that phosphorylation of S47, one of the identified sites, renders the kinase catalytically inactive. The inactivation of the kinase due to the phosphomimetic change at S47 results from inhibition of its interaction with its cognate activator, p35. We connect the effect of this regulatory event to a cellular phenotype by showing that the S47D CDK5 mutant inhibits cell migration and promotes cell proliferation. Together, these results have uncovered a potential physiological mechanism to regulate CDK5 activity. The evolutionary placement of a phosphorylatable residue (S/T) at this position not only in CDK5 but also in the majority of other CDK family members suggests that this phosphosite may represent a shared regulatory mechanism across the CDK family.

Keywords

Phosphorylation; Cyclin-dependent kinase (CDK); Cell migration; Cell proliferation

INTRODUCTION

Cyclin dependent kinases (CDKs) are a family of proline-directed serine/threonine kinases with roles in regulation of crucial cellular processes such as cell cycle and transcription (1). One of the unifying features of this family is its dependence on association with cyclins/cyclin-like regulatory proteins for activation (1,2). In addition, some CDKs also require

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phosphorylation of a key threonine residue in the activation loop to stabilize the CDK-Cyclin binding leading to complete activation of the kinase (1-3).

CDK5 is one of the unique members of this family with a primary role in cell migration rather than cell cycle (4,5). It was initially identified for its essential role in neuronal development including neuronal migration and differentiation; and normal synaptic function post-natally (4,5) but has since been implicated in non-neuronal functions as well (6,7). Its primary cognate neuronal activator is p35 which has no sequence similarity with classical cyclin proteins, however, structural analysis has revealed that it adopts a cyclin-like fold that mediates interaction with CDK5 (8-9). Unlike other CDKs, the activation loop phosphorylation is dispensable for activation of CDK5 and it solely depends on binding to its activator to adopt a catalytically active conformation (10). While CDK5 is a relatively stable protein, p35 has a short half-life owing to its proteasomal degradation which is triggered upon its phosphorylation by CDK5, a feedback mechanism likely to control the duration and level of CDK5 activation (11).

Given its role in important cellular processes, it is not surprising that deregulation of CDK5 activity has been implicated in many pathophysiological conditions including neurodegenerative diseases, cancer and type II diabetes (12-14). One of the main mechanisms that results in overactivation of CDK5 is calpain mediated cleavage of p35 to generate its 10 kDa shorter counterpart, p25 (15). This shorter version maintains the CDK5 binding region and has a ~5-10 times longer half-life than p35 resulting in prolonged activation and mislocalization of CDK5 leading to sustained phosphorylation of its substrates (5). Therefore, it is important to investigate mechanisms that regulate CDK5 activity in a manner independent of the degradative clearance of its activator. Here we identify a novel phosphosite and provide the biochemical basis of how phosphorylation of this residue may negatively regulate CDK5 by abolishing its interaction with p35. Together, our study uncovers a phosphoregulatory mechanism that renders CDK5 inactive leading to inhibition of cell migration and promotion of cell proliferation.

MATERIALS AND METHODS:

Reagents, Antibodies and Plasmid Constructs:

All reagents and chemicals were of analytical grade. The purified recombinant CDK5:p35 complex (Catalog#14-477) was purchased from Millipore-Sigma. Recombinant shrimp alkaline phosphatase (rSAP) was from New England Biolabs (NEB). Protease and phosphatase inihibitors were from Fisher Scientific and Sigma, respectively. ProLong Gold Antifade Mountant with DAPI was from ThermoFisher Scientific. Antibodies against CDK5 (Cat#sc-6247) and p35 (Cat#sc-820) were from Santa Cruz Biotechnology. Antibodies against Tubulin (Cat#2128), Glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Cat#5174) and phospho-Histone H3 Serine 10 (Cat#53348) were from Cell Signaling. IRDye 680RD and IRDye 800CW secondary antibodies for western blotting were from Li-COR Biosciences and goat anti-rabbit Alexafluor-647 conjugated antibody for immunofluorescence was from Invitrogen. CDK5 (WT and D144N) and p35-HA constructs were gifts from David S. Park (University of Ottawa, Ottawa) and Edward Giniger (NIH/NINDS), respectively. Point mutants were generated using the QuikChange Lightning Site-

Directed Mutagenesis Kit (Agilent Technologies). The mutations and the integrity of the rest of the insert were confirmed by sequencing.

Cell Culture and Transfection:

Cos7 cells (American Type Culture Collection) were cultured in Dulbecco's Modified Eagle Medium (DMEM; HyClone) supplemented with 10% Fetal Bovine Serum (FBS; HyClone) and Penicillin, Streptomycin, and Glutamine (Corning). Cells were maintained in a humidified atmosphere containing 5% CO_2 at 37°C. Transfections were performed using TransIT®-LT1 (MirusBio) according to the manufacturer's protocol.

Western blotting:

Western blotting was performed following the standard protocol as previously described (16). All the images were collected using the Odyssey Fc imaging system (Li-COR Biosciences).

In vitro Kinase Assay and Mass Spectrometry:

In vitro kinase assays were performed on ~1 μg of purified CDK5/p35 complex in 30 μL of kinase buffer (50 mM HEPES pH 7.4, 1 mM DTT, 10 mM $MgCl_2$, 5 mM ATP and Phosphatase Inhibitors) at 30°C for 2h. The samples were electrophoretically separated followed by staining with Instant Blue. The CDK5 band was excised and sent to MS Bioworks (Ann Arbor, MI) to be analyzed using their Post Translational Modification (PTM)-Profiling service.

Co-immunoprecipitation Assay:

Cos7 cells co-transfected with CDK5 (WT, D144N, S47A or S47D) and p35 constructs were lysed in the immunoprecipitation buffer (25 mM HEPES pH 7.4, 0.5% Triton X-100, 2 mM DTT, 150 mM NaCl, 10 mM $MgCl_2$, Protease and Phosphatase Inhibitors) on ice. The cleared cell lysates were incubated with 1 μg of anti-rabbit p35 antibody overnight at 4°C followed by incubation with 20 μL of Protein A Sepharose beads for 1h at 4°C. The beads were then washed, eluted and analyzed by western blotting.

Shrimp Alkaline Phosphatase (SAP) Assay:

Lysates of Cos7 cells co-expressing p35 and CDK5 (WT or D144N) were immunoprecipitated as described above except after incubation the Protein A sepharose beads were washed thrice with the immunoprecipitation buffer without the phosphatase inhibitors, resuspended in 300 μL of 1X Cut-Smart Buffer (NEB) and distributed evenly into three microcentrifuge tubes. The beads were incubated at 37°C for 15 minutes either without, with SAP (0.5U/μ,l) or with SAP and EDTA (50 mM). After incubation, the beads were eluted and analyzed by western blotting.

Scratch Wound Healing Assay.

Cells expressing p35 and CDK5 (WT, S47A or S47D) were grown to 90% confluency. Cells were starved in reduced serum medium (DMEM containing 0.2% FBS) overnight before the day of wounding and were maintained in reduced serum medium throughout the remainder

of the experiment. The wound was made by scratching the center of the plate with a sterile tip. Images were captured using the Leica DM IRB Inverted Modulation Contrast Microscope (Leica Microsystems). To quantify cell migration, images were analyzed using ImageJ (NIH) to calculate the % wound closure at 40 h post-scratching.

Immunofluorescence Microscopy:

Cells expressing p35 and CDK5 (WT, S47A or S47D) were fixed with 4% paraformaldehyde and permeabilized (0.2% TX100 in PBS) for 20 min at room temperature. Cells were then blocked (1% BSA and 0.2% TX100 in PBS) followed by subsequent incubations with the primary (anti-phospho Histone H3; 1:500 dilution in the blocking buffer) and secondary antibody. Cells were mounted on glass slides using the ProLong Gold Antifade Mountant with DAPI. Images were taken using the Olympus IX81 inverted confocal microscope with the 40X oil immersion objective. The images were analyzed using ImageJ.

Statistical Analysis:

All graphical data were prepared and statistical analyses were performed using GraphPad Prism. Statistical significance between the differences of means was determined by performing a One-way Analysis of Variance followed by the Bonferroni's *post-hoc* test.

RESULTS:

CDK5 is phosphorylated in an activation-dependent manner:

Reversible phosphorylation is a key regulatory mechanism to control kinase activity. In this study, we wished to identify specific sites and study phosphoregulation of CDK5. Since phosphorylation is known to retard electrophoretic mobility of modified proteins, we first determined if we could detect phosphomodified CDK5 in cell lysates using a mobility shift based western blot. Cos7 cells were transfected with CDK5 - either WT or D144N (catalytically inactive) mutant with or without its cognate activator, p35. Cos7 cells do not have detectable levels of endogenous p35, therefore, they serve as an excellent system to control CDK5 activation by simply including or omitting p35 from the transfections (17). Cells were lysed 48h post-transfection and lysates were analyzed by western blotting. As shown in Fig. 1A, when expressed without p35 (lanes 1 and 3), both WT and D144N CDK5 were detected as a single band. D144N CDK5 also showed a single band even in the presence of p35 (lane 4). In contrast, WT CDK5 showed slower migrating bands in addition to the main band when co-expressed with p35 (lane 2) suggesting that CDK5 is post-translationally modified in a p35 *i.e.* activation dependent manner. p35 is also a substrate for CDK5 (17) and it showed a broader/more smeared band (suggestive of its phosphorylation) when coexpressed with WT but not D144N CDK5 further confirming that WT CDK5 was activated in the presence of p35.

To ascertain that the slower migrating bands represented phosphorylated CDK5, we performed a phosphatase assay. Lysates of cells co-expressing p35 and either WT or D144N CDK5 were immunoprecipitated using an anti-p35 antibody. The immunoprecipitates were then dephosphorylated using SAP along with appropriate controls. As shown in Fig. 1B

(*Top*), D144N CDK5 samples showed a single band in all three conditions (lanes 1-3) suggesting the absence of any post-translational modification. The WT CDK5 on the other hand, showed the slower migrating bands in the control lane without SAP (lane 4). Upon treatment with SAP, the slower migrating bands disappeared and instead showed a single band (lane 5) comparable in mobility to that of D144N CDK5 suggesting that the mobility shift seen in lane 4 was due to phosphorylation of CDK5. As a negative control, when SAP was added along with EDTA (SAP inhibitor), the slower migrating phosphorylated bands of CDK5 were restored (lane 6). As expected, we also observed the same pattern for p35 *i.e.* modified p35 bands in lanes 4 and 6 but not in lanes 1-3 and 5. Together, these results confirmed that the bands showing retarded electrophoretic mobility represent phosphorylated CDK5/p35 and that the phosphorylation of CDK5 is dependent on its catalytic activity.

Identification of the phosphosites on CDK5:

Next we wished to identify the phosphosites on CDK5. To do so, we performed an *in vitro* kinase assay on the commercially available recombinant His_6-CDK5 and GST-p35 complex (Fig. S1A). A total of seven residues on CDK5 were identified as putative phosphosites using mass spectrometry (Fig. S1B). Next, we mapped these sites on the CDK5-p25 crystal structure (PDB ID-3O0G; Fig. 2A) (18,19). Out of all the sites, S47 seemed the most interesting as it is part of the cyclin binding element and is within favorable distance to make a H-bond with the backbone of I241 in p25 (Fig. 2A, Inset). If phosphorylated, the phosphate group at S47 may perturb the H-bond and possibly may also pose a steric clash with the p25-binding interface. When we aligned the partial sequence encompassing S47 in CDK5 from different species, it appeared to be evolutionarily conserved with a serine at this position in human, bovine, rat, mouse and drosophila CDK5 and a substitution with threonine in yeast (Fig. 2B).

Phosphomimetic mutation at S47 abolishes CDK5-p35 binding:

In order to directly test the effect of phosphorylation on S47, we generated phosphomimetic (S47D) and non-phosphorylatable (S47A) mutations on CDK5. The CDK5 constructs were co-transfected with p35 in Cos7 cells and the cell lysates were analyzed by western blotting. As expected, the modified bands were absent in the D144N mutant (Fig. 2C; lane 1). Both WT and S47A CDK5, on the other hand, showed the slower migrating bands suggesting that the S47A mutant retains its kinase activity (Fig. 2C; lanes 2,3). Interestingly, the S47D mutant lacked the phospho-modified bands (lane 4) suggesting that this mutation may render CDK5 inactive. We next performed a co-immunoprecipitation assay to test if the inactivity of the S47D mutant is due to the loss of binding to p35. Lysates of cells co-expressing p35 and CDK5 constructs (D144N, WT, S47A and S47D) were immunoprecipitated using the p35 antibody and analyzed by western blotting. The D144N mutant bound to p35 as expected (Fig. 2D; lane 2) because the D144N mutation inactivates the kinase due to its inability to coordinate Mg^{2+} ion but does not affect the CDK5-p35 interaction (2, 20). The WT and S47A CDK5 also co-immunoprecipitated with p35 (lanes 3,4). Notably, in both WT and S47A CDK5 expressing lysates, p35 preferentially bound to the phosphorylated form of CDK5 represented by the slower migrating band suggesting that one or more of the phosphosites on active CDK5 enhance its interaction with p35. In contrast, the S47D mutant

failed to bind to p35 (Fig. 2D; lane 5). Based on these results, it appeared that while the H-bond made by S47 may be dispensable (p35:S47A CDK5 binding was comparable to p35:WT CDK5 binding), phosphorylation at S47 may sterically hinder CDK5-p35 interaction resulting in loss of its kinase activity.

Cells expressing the CDK5 S47D mutant exhibit impaired migration and enhanced proliferation:

Finally, we wished to determine the functional significance of this phosphoevent. Multiple studies have implicated active CDK5 in promotion of cell migration (9, 16, 21-24) and in one study the CDK5-p35 complex has been shown to be required to suppress cell proliferation (25). Since the S47D mutation impedes the formation of a functional CDK5-p35 complex thus inhibiting its catalytic activity, we reasoned that the cells expressing this mutant should display retarded migration and higher proliferation. We found that such was indeed the case where in a scratch wound assay (Fig. 3), the WT and S47A CDK5 expressing cells were able to migrate and close the wound effectively. On the other hand, the cells expressing S47D CDK5 were significantly impaired in their ability to migrate and close the wound (Fig. 3). Conversely, S47D harboring cells showed a significantly higher mitotic index compared to the WT or S47A expressing cells as determined by the phospho-Histone 3 immunostaining (Fig. 4). Together, these data indicate that the inhibition of CDK5 activity by S47 phosphorylation negatively influences cell migration and promotes cell division.

DISCUSSION

Identification of a novel phosphoregulatory mechanism to inactivate CDK5:

The results presented in this study reveal a novel site-specific, phosphorylation based regulation of CDK5 activity. There are two other residues – Y15 and S159 - that have been previously implicated in phosphoregulation of CDK5 (26). Our study adds to the phosphoregulatory mechanisms to control CDK5 activity. The data presented here elucidate the mechanism whereby a phosphomimetic change at S47, a residue in the cyclin binding element, disrupts the binding of CDK5 to its activator resulting in failure to adopt an active conformation (Fig. 2). This potential phosphorylation based regulatory mechanism results in a loss of CDK5 activity comparable to that of the catalytically inactive D144N mutant. In fact, since the S47D mutant simulates regulation imparted by phosphorylation, it provides a more physiologically relevant mutation to control CDK5 activity. In addition, it can serve as an experimental tool to use with the D144N mutant in studies aiming to dissect the kinase activity independent roles of the CDK5-p35 complex. Notably, there is another conserved serine at position 46 (Fig. 2B) that also interfaces with the p25 binding surface (Fig. S2A). Although we did not identify S46 as a putative phosphosite in our screen, it has been previously reported in a high throughput proteomics study (34). The phosphomimetic mutation at S46 showed a similar effect on binding to p35 (Fig. S2B) as we observed in case of S47 (Fig. 2D) suggesting that phosphorylation of either residue is sufficient in abolishing binding to p35. An alignment of the cyclin binding element from all human CDKs revealed a phosphorylatable residue (Serine in 6 members and Threonine in 14 members) at the position equivalent of S47 in 20 out of the 21 members of the family whereas position S46 is

only conserved in 4 members (Fig. S3). As it is commonly observed in kinase families to adopt shared regulatory mechanisms and considering that the cyclin binding element plays a crucial role in binding of CDKs to their respective activators, it is possible that phosphorylation of S47 may represent a broad regulation strategy shared among the CDK family members.

Is S47 an autophosphorylation site?

Our mobility shift based western blotting assay from cellular lysates showed that the slower migrating phosphorylated bands of CDK5 appear only if the kinase is catalytically active (Fig. 1). Thus, it can be inferred that CDK5 may undergo autophosphorylation. However, an alternative explanation could be that CDK5 is phosphorylated by another kinase(s) that gets activated downstream of CDK5. The recombinant CDK5-p35 complex purified from eukaryotic insect cells (purity 84.4% as per the datasheet provided by the vendor) allowed us to capture all the phosphosites including any that might be a result of self-phosphorylation and those phosphorylated by other kinases copurified with CDK5-p35 complex. That the latter is at least partly the case is supported by the fact that we identified Y15 in our screen. Since CDK5 is a serine/threonine kinase, the most likely explanation is that Y15 was phosphorylated by a co-purified tyrosine kinase. It also seems unlikely that S47 or the other sites we identified are autophosphorylation sites because they do not satisfy the minimum consensus site requirement for CDK5 *i.e.* a serine or threonine residue followed by a proline (S/T-P motif) (35). Nevertheless, we cannot completely rule out the possibility of autophosphorylation as kinases can phosphorylate non-consensus sites.

CDK5-S47 phosphorylation status influences the differential cellular proliferation versus migration response:

Previous work from our laboratory has shown CDK5 to be a critical determinant of the cellular proliferation-migration dichotomy whereby CDK5 activation downstream of stimulated epidermal growth factor receptor triggers cell migration and decreases cell proliferation (16). Our present work further adds to the repertoire of mechanisms that a cell may employ to differentially regulate a pro-mitotic *vs* pro-migratory response by controlling the phosphorylation status of S47 on CDK5. The formation of the CDK5-p35 complex has been shown to be required to suppress neuronal cell cycle regardless of CDK5 activity (25). Based on our current work, phosphorylation of S47 also achieves the same phenotype *i.e.* relieves the cell cycle suppression by disrupting the CDK5-p35 interaction (Fig. 4). Taken together with the inhibition of cell migration in cells expressing the S47D CDK5 mutant (Fig. 3), reversible phosphorylation of S47 on CDK5 presents a quick, physiologically relevant mechanism to help cells conform to the proliferation-migration dichotomy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- • Identification of a novel phosphosite on Cyclin-dependent kinase 5 (CDK5)
- • Elucidation of the mechanism by which the phosphosite negatively regulates CDK5 activity
- • Potential role for this phosphoevent in regulating cellular proliferation-migration dichotomy

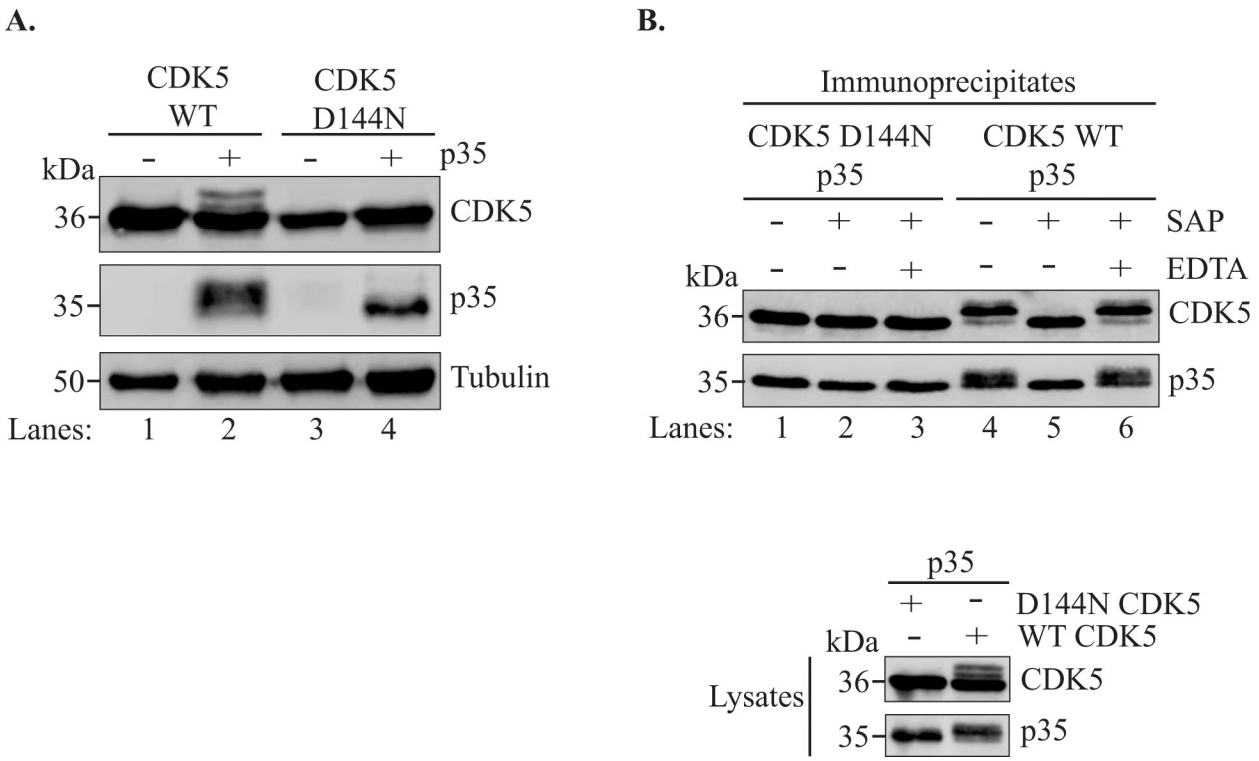

Figure 1. CDK5 is phosphomodified in an activity dependent manner.

A. Lysates of Cos7 cells expressing either WT or the catalytically inactive (D144N) CDK5 with or without its activator p35 were analyzed by western blotting. Tubulin served as a loading control. **B.** Lysates of Cos7 cells co-expressing CDK5 (WT or D144N) and p35 were immunoprecipitated using an anti-p35 antibody. The immunoprecipitates were either mock-treated (lanes 1,4) or treated with SAP alone (lanes 2,5) or SAP+EDTA (lanes 3,6). The immunoprecipitates (*Top*) and lysates (*Bottom*) were analyzed by western blotting.

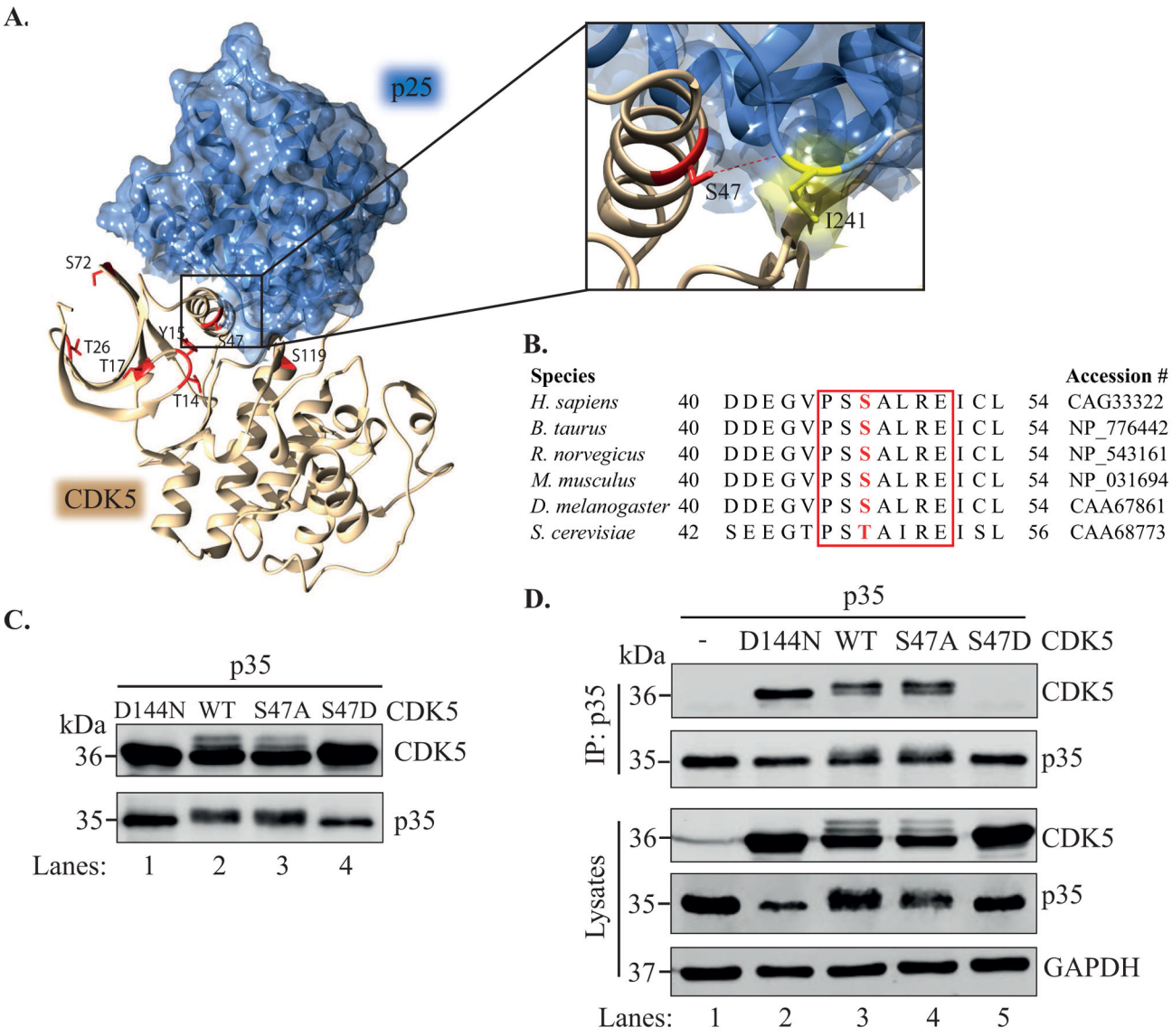

Figure 2. Serine 47, a conserved residue, contributes to binding of CDK5 to p35.

A. The phosphosites were mapped onto the crystal structure of CDK5-p25 (PDB ID-3O0G) using the UCSF Chimera program. Inset - S47 on CDK5 is shown making a H-bond with the backbone of the I241 residue on p25. **B.** The sequence encompassing the cyclin binding element in CDK5 (boxed in red) was aligned among various species. The residue numbers for the aligned region and the NCBI accession numbers are listed. S47 is shown in red. **C.** Lysates of cells co-expressing p35 and CDK5 (D144N, WT, S47A or S47D) were analyzed by western blotting. **D.** Lysates of cells expressing p35 alone or with CDK5 (D144N, WT, S47A or S47D) were immunoprecipitated using an anti-p35 antibody. The immunoprecipitates and lysates were analyzed by western blotting. GAPDH in the lysates served as a loading control.

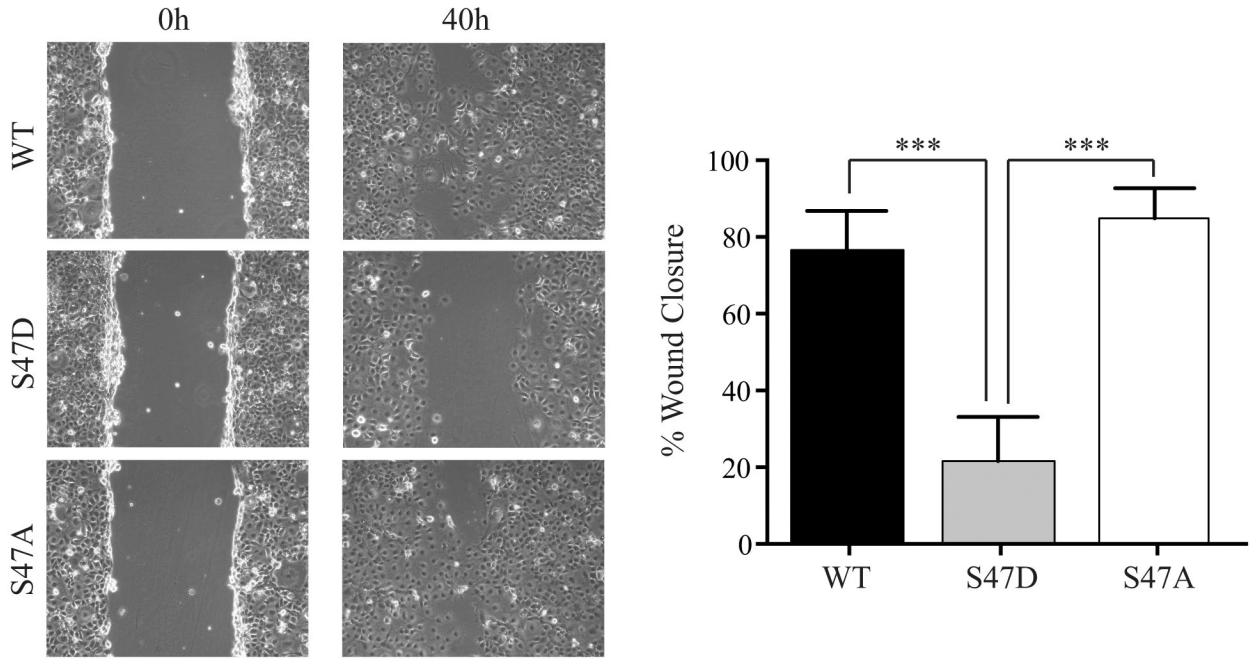

Figure 3. CDK5-S47D impairs cell migration.

Left. Monolayers of cells co-expressing p35 and CDK5 (WT, S47D or S47A) were wound scratched and imaged at 0 and 40 h. Representative images are shown from three independent experiments. *Right*. The area of the wound was measured at time 0 and 40h using the ImageJ software and % wound closure was calculated for each set of cells. Data are presented as mean ± S.D. ***p <0.001.

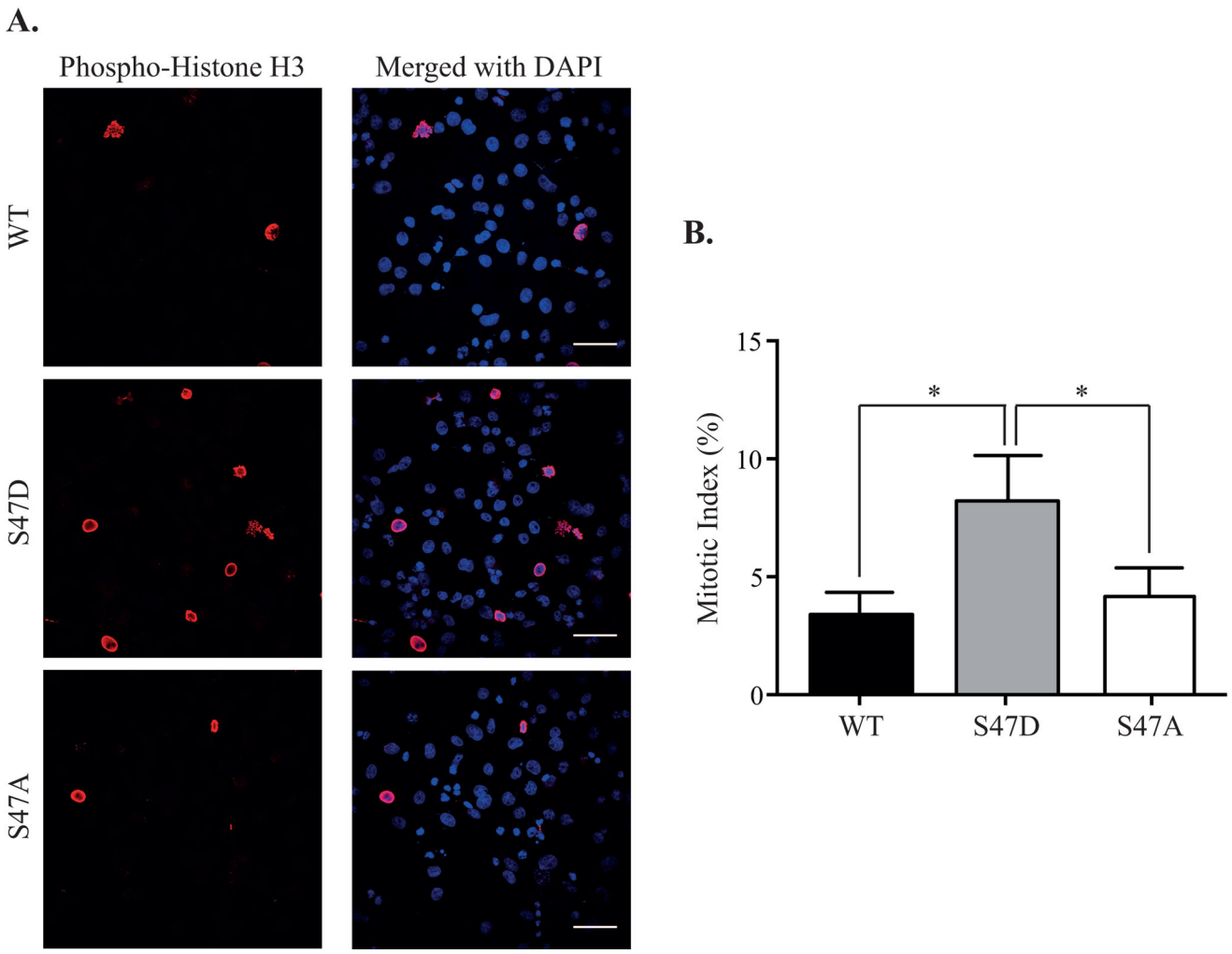

Figure 4. CDK5-S47D promotes cell proliferation.

A. Cells co-expressing p35 and CDK5 (WT, S47D or S47A) were immunostained with the anti-pS10-Histone H3 antibody (Red). The panels on the right show merged images of DAPI (blue) and pS10-Histone H3 (red) channels. Three independent sets of coverslips were examined for each transfection with ~400-500 cells scored per experiment. All the images were captured under identical settings. Scale bars=50 μm. **B.** The mitotic index was determined by calculating the percentage of phospho-Histone H3 positive nuclei for each set of images. Data are presented as mean ± S.D.; *$p < 0.05$.