이학석사 학위 논문

CIP2A 에 의한 PLK4 분해 조절

The regulation of PLK4 stability by CIP2A

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The regulation of PL4K stability by CIP2A

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이 논문을 이학석사 학위 논문으로 제출함

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Abstracts

The overexpression of cancerous inhibitor of protein phosphatase 2A (CIP2A), a 90kDa oncoprotein, is observed in several human cancer and correlated with cancer progression. CIP2A interacts with Protein Phosphatase 2A (PP2A) which is known as a tumor suppressor and inhibits PP2A activity to c-MYC. Therefore, CIP2A promotes cancer cell proliferation. However, the other oncogenic function of CIP2A has not been studied well. In this study, PLK4 has been discovered as a new binding partner for CIP2A. I observed CIP2A inhibits PLK4 ubiquitylation and consequently increases protein half-life by stabilizing PLK4. Therefore, overexpression of CIP2A increases PLK4 levels. PLK4 is a master regulator of centriole duplication and high PLK4 levels are a cause of centriole amplification. But, contrary to expectation, CIP2A overexpression did not lead to centriole overduplication. Thus, further study is required to uncover the effect of overexpression of PLK4 by CIP2A.

Key words: CIP2A, PLK4, E3 ligase, Centriole

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INTRODUCTION

Cancerous inhibitor of protein phosphatase 2A (CIP2A), previously named KIAA1524 or p90 tumor-associated antigen, is a 90kDa oncoprotein that is encoded by the KIAA1524 gene [1]. Overexpression of CIP2A is correlated with cancer progression. High CIP2A levels are observed in several solid cancers, and some hematological tumors [2-4]. CIP2A interacts with Protein Phosphatase 2A (PP2A) which is known as a tumor suppressor by regulating several oncogenic pathways and inhibiting PP2A activity to c-MYC. Therefore, CIP2A stabilizes the oncogenic transcript factor c-MYC in human malignancies [5]. Also, CIP2A supports cancer cell growth by interrupting PP2A meditated dephosphorylation of mTORC1 substrates [6]. CIP2A overexpression increases an oncogenic transcript factor E2F1 which is attached to the CIP2A promoter and the inactivation of the P53 tumor suppressor stimulates the positive feedback loop between these two oncogenic proteins [7]. CIP2A prevents tumor cells from apoptosis and helps anchorage-independent cell growth, which are hallmarks of human malignant cells [8]. In addition, CIP2A is involved in cell cycle progression through the regulation of Polo-like kinase1 (PLK1) stability and in centrosome separation by interaction with NIMA-related kinase 2 (NEK2). CIP2A stabilizes PLK1 and enhances its kinase activity during the M phase by interrupting APC/C-Cdh1-dependent proteolysis activity. Depletion of CIP2A delays mitotic progression. High PLK1 levels have been reported in a wide range of human cancer and inhibition of PLK1 leads to cancer cell death. Therefore, CIP2A plays an important role in cell cycle progression in cancer cells [9, 10]. Overexpression of CIP2A is observed in many cancer patients, and patients with high CIP2A expression have lower survival rates than those with low expression levels. Thus, further studies on the oncogenic properties of CIP2A will help our understanding of the mechanism of tumors and approaches to cancer therapy.

TurboID is an efficient proximity labeling (PL) assay for mapping protein-protein interactions in live mammalian cells [11]. In this study, CIP2A was conjugated with TurboID to find new partners in human U2-OS cells and PLK4 is one of the most highly detected proteins in the TurboID assay.

Polo-like Kinase 4 (PLK4) is a member of the polo-like kinases family (PLKs), which are essential for cell cycle progression and DNA damage response. All polo-like kinase families have a kinase domain in N-terminus and a polo-dox domain (PBD) in C-terminus, which is responsible for binding substrates and subcellular localization [12]. Especially, PLK4 contains three PBDs (Crypto polo-box and polo-box 3) unlike other members. PLK4 PBDs help PLK4 localize in centriole in the G1/ S phase and crypto polo-box (CPB), which consists of PB1 and PB2, forms a homodimer that is required for regulation of PLK4 kinase activity. PLK4 is known that a master regulator of centriole duplication via interaction with STIL, SAS-6, and overexpression of PLK4 leads to centriole overduplication [13]. In the G1/S phase, PLK4 is recruited to the mother centriole by CEP192/152 interaction. PLK4 binds STIL via its PB3 and then leads to the phosphorylation of STIL on the STAN motif. Subsequently, SAS-6 is recruited to phosphorylated STIL and forms a nine-fold symmetric cartwheel structure for procentriole. The position of PLK4 was restricted to the centrosome until the M phase [14]. But phosphorylated PLK4 (the active PLK4) truncates its PBDs and relocalizes to the midbody in cytokinesis. PLK4 kinase activity is required to meditate cytokinesis [15].

In normal circumstances, PLK4 expression is low in abundance. PLK4 levels tightly controlled for the fidelity of centrosome duplication and high PLK4 levels lead to centrosome overduplication. Aberrant PLK4 expression was observed in various human cancer. overexpression of PLK4 was analyzed in human gastric cancer, lung adenocarcinomas, melanoma, renal carcinoma and correlated with poor prognosis [16-19].

In this study, I investigate the regulation of PLK4 stability by CIP2A. Data showed that CIP2A binds to PLK4. CIP2A prolongs PLK4 protein half-life by inhibiting PLK4 ubiquitylation. Overexpression of CIP2A leads to the increase of PLK4 levels and that action requires the kinase activity of PLK4.

Materials and methods

Cell culture and treatment

Human HEK293T and U2OS cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Hyclone) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco) and 1% penicillin-streptomycin solution (Gibco). Cultures were maintained at 37℃ in a humified atmosphere containing 5% CO_2.

The doxycycline-inducible U2OS cell line (U2OS: mEmerald CENTRIN-2 and U2OS: mEmerald CENTRIN-2 & MYC-CIP2A) was generated. Stable U2OS cell lines were established by selection with 25 μg/ml puromycin (InvivoGen) and 100 μg/ml blasticidin (InvivoGen). mEmerald CENTRIN-2 and MYC-CIP2A expression was induced by the addition of 100 ng/ml doxycycline.

To measure PLK4 stability, cells were incubated with 25 μg/ml cycloheximide (Sigma) for the indicated times. For ubiquitination assay, cells were incubated with 10 μM MG132 (MedChemExpress) for 6 hours.

Cell cycle synchronization and flow-cytometry analysis

For cell synchronization at the G2/M phase, HEK293T cells were treated with 300 nM nocodazole (Selleckchem) for 18 hours.

For flow cytometry analysis, cells were trypsinized, washed twice in PBS and fixed with ice-cold 70% ethanol. Fixed cells were washed twice with PBS and incubated with 10 μg/ml propidium iodide (PI; Invitrogen) and 100 μg/ml RNase (Thermo scientific) at 37℃ for 30 minutes. Then, cells were analyzed with a flow cytometer (BD Biosciences).

RNA extraction and cDNA synthesis

Total RNA was extracted from HEK293T and U2OS using Trizol reagent (Invitrogen) according to manufacturer's instructions. RNA was reversed transcribed to cDNA using reverse transcription master premix (Elpis Biotech) according to manufacturer's instructions.

Transfection and siRNA

Transfection of siRNA or plasmids into HEK293T and U2OS cells was performed using branched PEI (Sigma) or K4 transfection reagent (Biontex) according to the manufacturer's instructions.

CIP2A was depleted using described siRNA-duplex oligonucleotides targeting CIP2A.

Transfected cells were analyzed 24-48 hours after transfection with siRNA and 24 hours after transfection with DNA plasmids.

Ubiquitin assay

Cells were transfected with FLAG-tagged ubiquitin, HA-PLK4 and MYC-empty vector or MYC-CIP2A. Six hours before harvest, the cells were treated with MG132. Then, cells were lysed in Pierce IP lysis buffer (Thermo scientific), containing 1x Halt protease inhibitor cocktail (Thermo scientific) for 30 minutes on ice. After clearing by centrifugation at 13,000 g for 15 minutes, cell lysates were incubated with anti-HA antibodies (MBL international, m180-3) and then incubated for 3 hours at 4°C with protein A-agarose (GenDEPOT). The beads were washed at least three times with Pierce IP lysis buffer and resuspended in 2X SDS sample buffer, followed by loading onto an SDS–PAGE gel.

Immunofluorescence

U2OS cells were seeded in 8 well chamber slide (SPL) and fixed with 4% paraformaldehyde at 15 minutes. The cells were blocked with 0.5% BSA and 0.3% Triton X-100 in PBS. The fixed cells were incubated with primary antibodies against MYC-tag (MBL international, m192-3, 1:2,000) overnight at 4℃, washed with PBS three times and incubated with anti-mouse Alex Fluor 594 (Invitrogen). Slides were mounted in a medium containing 4,6-diamidino-2-phenylindole (DAPI) and images were obtained using a confocal laser-scanning microscope (LSM 880; Carl Zeiss, Inc.). All images were processed by using ZEN Light Edition (Carl Zeiss).

Immunoprecipitation

HEK293T cells were washed by cold PBS and lysed in ice-cold IP lysis buffer. The cell lysates were vortexed for 30 minutes at 4°C, and insoluble material was removed after centrifugation at 13,000 g for 15 minutes. For immunoprecipitation, whole-cell lysates (WCL) were incubated with HA antibody (MBL international, m180-3) and FLAG antibody (CST, 9A3) for overnight at 4 °C and then incubated for 2 hours at 4 °C with protein A-Agarose (GenDEPOT). The beads were washed at least three times with IP lysis buffer and resuspended in 2X SDS sample buffer before loading onto an SDS–PAGE gel.

Antibodies

The following primary antibodies were used in this study: rabbit antibodies against FLAG-tag (CST, D6W5B), β-TrCP (CST, D13F10, Immuno-blotting (IB) 1:1,000); beta-Actin (GeneTex, GTX109639), mouse antibodies against CIP2A (Santacruz, 2G10-3B5, IB 1:10,000), HA-tag (MBL, m180-3, IB 1:10,000), MYC-tag (MBL, m193-3, Immunofluorescent (IF) 1:2,000, IB 1:10,000), FLAG-tag (CST, 9A3, IB 1:5,000), GAPDH (Santacruz, sc-32233, IB 1:10,000).

Immuno-blotting

For the preparation of human cell lysates for immunoblotting, cells were harvested, washed in PBS and lysed in Pierce IP lysis buffer (Thermo scntific) containing 1x Halt protease inhibitor cocktail (Thermo scientific) for 30min on ice. Lysates were cleared by centrifugation for 15min at 13,000 g at 4 °C and protein concentration of cell lysates was measured using bicinchoninic acid (BCA) protein assay kit (Thermo scientific). SDS–polyacrylamide gel electrophoresis (SDS–PAGE) was performed using 8% or 10% polyacrylamide gels and transferred onto a nitrocellulose membrane. The membrane was washed with Tris-buffered saline (10 mM Tris, 150 mM NaCl) containing 0.1% Tween-20 (TBS-T) and blocked in TBS-T containing 5% Skim-milk. The membrane was incubated with primary antibodies. After rinsing with TBS-T solution, the membranes were incubated for 2-3 hours at room temperature in a blocking solution containing peroxidase-conjugated secondary antibodies. After washing, the signal was detected as Chemi Doc XRS+ (Bio-Rad).

RESULT

CIP2A regulates PLK4 levels

TurboID was performed using CIP2A as the bait to find novel binding partners. One of the candidates detected high was PLK4, which is known to be associated with centriole duplication. Co-immunoprecipitation was conducted to confirm that PLK4 bound to CIP2A (FIG. 1), and immunoblotting showed not only CIP2A bound to PLK4 but also increased PLK4 levels (FIG. 2A).

Then, to further verify CIP2A contributed to PLK4 stability regulation, I carried out siRNA transfection to deplete CIP2A and the level of HA-PLK4 was measured. As expected, siRNA mediated CIP2A knockdown decreased the PLK4 levels (FIG. 2B).

CIP2A is required for cell cycle progression by regulating mitotic kinase protein and overexpression of CIP2A affects cell proliferation [20]. PLK4 is also known as a cell cycle regulator [15, 21]. Previous studies demonstrated that PLK4 mRNA level is relatively low in G0 phase and increases beginning in the late G1 phase of the cell cycle. PLK4 mRNA expression is relatively high in the S, G2, and M phases [22]. Therefore, it was necessary to verify that increase in PLK4 levels was not due to cell cycle fluctuation by CIP2A overexpression. HEK293T cells were transfected with HA-PLK4, MYC-CIP2A, and incubated for an additional 24 hours, followed by a harvest for propidium iodide (PI) staining. Flow cytometry analysis showed that CIP2A transfection increased the G2/M Phase slightly (FIG. 3).

Next, HEK293T cells were transfected with HA-PLK4 to examine the levels of PLK4 in nocodazole treatment. HA-PLK4 levels did not show a significant change even when the G2/M phase was increased (FIG. 4). Although overexpression of CIP2A increased the G2/M phase slightly, it has nothing to do with changes in the PLK4 levels. Therefore, it seems that the increase in the levels of PLK4 was not due to the cell cycle changes caused by CIP2A transfection.

PLK4 kinase activity is required for the regulation of stability by CIP2A

Former studies reported that PLK4 kinase activity regulates its own stability by autophosphorylation. PLK4 auto-phosphorylates conserved phosphor-degron, which is commonly founded in $SCF^{\beta\text{-}TrCP}$ substrates and catalyzes its own degradation [23].

To investigate whether PLK4 kinase activity is necessary for regulating PLK4 stability by CIP2A, Lysine 41 within the kinase domain of human PLK4 was mutated alanine to generate a kinase-inactive form of PLK4 (PLK4 K41M).

HEK293T cells were transiently transfected with HA-PLK4 mutants and immuno-blotting was carried out. Comparison of PLK4 levels by immuno-blotting with the HA antibody clearly showed that mutation of the kinase domain had a great effect on the ability of CIP2A on regulating PLK4 stability. Unlike the PLK4 wild-type, the kinase-dead form of PLK4 (PLK4 K41M) was not increased even when CIP2A was overexpressed (FIG. 5). Therefore, PLK4 kinase activity is necessary for PLK4 stability regulation by CIP2A.

CIP2A reduces PLK4 ubiquitination and prolongs protein half-life

Next, to investigate how CIP2A increased PLK4 levels, I observed whether the half-life of protein PLK4 was affected by CIP2A. The transient transfection method was used for the overexpression of HA-PLK4, MYC-EV, and MYC-CIP2A in the presence of cycloheximide (CHX), a protein synthesis inhibitor that acts on the eukaryotic ribosome. Overexpression of CIP2A in HEK293T cells remarkably extended the protein half-life of PLK4 whereas MYC-EV had no effect on PLK4 (FIG. 6).

To test CIP2A extended PLK4 half-life by preventing proteolytic degradation, HEK293T cells were transfected with HA-PLK4, MYC-CIP2A, FLAG-Ubiquitin, and immuno-precipitation assay was conducted. Immuno-blotting data showed that MYC-CIP2A overexpression significantly reduced the ubiquitylation of PLK4 regardless of the presence of MG132, a 26S proteasome inhibitor. Whereas, in

striking contrast, MYC-EV did not affect PLK4 ubiquitylation (FIG. 7). Thus, CIP2A inhibits PLK4 ubiquitylation and prevents its degradation.

PLK4 binds to CIP2A via its Linker 1 region

The previous co-immunoprecipitation assay showed that CIP2A directly binds to PLK4 (FIG. 1). Next, PLK4 truncated mutants were made to determine the PLK4 region responsible for binding to CIP2A. PLK4 has a catalytically active kinase domain (KD) located in N-terminus and three polo-box domains (PBD) in C-terminus. PBD, which only PLKs have determined substrates, leads PLK4 to centrioles and regulates kinase activity. PLK4 form a homodimer via PB1, PB2 interaction and PB3 release PLK4 kinase activation loop (AL) from Linker 1 region (L1) to activate PLK4. The N-terminal portion of PLK4, which includes the kinase domain and L1 region, had a strong interaction with CIP2A than the PLK4 C-terminal portion (FIG. 8A). PLK4 has two linker-region and the first linker region (L1) located between KD and PBD. L1 region prevents the phosphorylation of the AL of KD upon completion of PLK4 synthesis [24]. Upon L1 was phosphorylated, PLK4 was fully activated. Immunoblotting data showed that the binding required an L1 region within the N-terminus domain of PLK4 (FIG. 8B).

PLK4 and E3 ligase: β-TrCP and FBXW2

PLK4 has three PEST motifs (rich in proline, aspartate, glutamate, serine, and threonine residue), which were thought to be associated with reduced protein stability [25]. The first PEST motif (aa272-318) was found to be more effective than the other two sequences located in C-terminus. Previous studies showed that PLK4 has 13 phosphorylable amino acids within a first PEST sequence (aa 282-305) [26].

The SCF(SKP1-Cullins-F box proteins) E3 ubiquitin ligase is the largest family of E3 ubiquitin ligase. SCF E3 ligase degraded more than 350 substrates and controlled several important biological processes

[27, 28]. It is well known that the stability of PLK4 was regulated by the ubiquitin-dependent proteasome pathway through $SCF^{\beta\text{-}TrCP}$ (SKP1-CUL1-FBXW1A complex). β-TrCP binding motif, known as Degron, was located in first PEST sequence. phosphorylation of Ser293 and Thr297 within degron motif was required for $SCF^{\beta\text{-}TrCP}$-meditated PLK4 degradation [29].

Recent research found FBXW2 degron sequence TSXXXS and PLK4 contain such a motif in the first PEST sequence [26, 30]. 300 threonine, 301 serine, and 305 serine mutated alanine to test PLK4 degradation were conducted by FBXW2 (PLK4 AA3).

To investigate whether CIP2A regulates the stability of PLK4 via disturbing E3 ligase activity, HEK293T cells were transfected with MYC-CIP2A, FLAG-FBXW2, HA-PLK4, and immuno-blotting was conducted. Immunoblotting showed that overexpression of FLAG FBXW2 reduces PLK4 levels even in the presence of CIP2A. (FIG. 9)

Because overexpression of CIP2A reduced PLK4 ubiquitylation, co-immunoprecipitation assay was conducted to find out that CIP2A affects PLK4/β-TrCP binding. But, endogenous β-TrCP and HA-PLK4 binding is unaffected by CIP2A overexpression. (FIG.10)

Also, the co-immunoprecipitation assay showed HA-PLK4 wild-type bound to Flag-FBXW2. But against expectation, mutations in the TSXXXS sequence within the first PEST motif was not affected PLK4/FBXW2 binding. (FIG. 11)

CIP2A overexpression does not increase centriole numbers

PLK4 plays a key role in centrosome duplication. The centrosome is composed of two centrioles surrounded by the pericentriolar material (PCM) and is required for the formation of cilia and microtubule-organizing centers (MTOC) [31, 32]. Previous studies reported that high expression of PLK4 leads to multiple formations of the centriole [33]. Centrosome amplification is closely linked to chromosome mis-segregation, aneuploidy and correlates with chromosome instability (CIN), which is frequently observed in many human cancer [34]. In this study, data showed that overexpression of

CIP2A increased PLK4 levels. To investigate how the elevated PLK4 levels caused by CIP2A overexpression affected the cell phenotype, MYC-CIP2A inducible U2-OS cells were made. Doxycycline (DOX) was applied for 72 hours to overexpress MYC-CIP2A and then, the number of centrioles was observed. But, unlike prediction, CIP2A overexpression did not lead to centrosome amplification (FIG. 12 A,B).

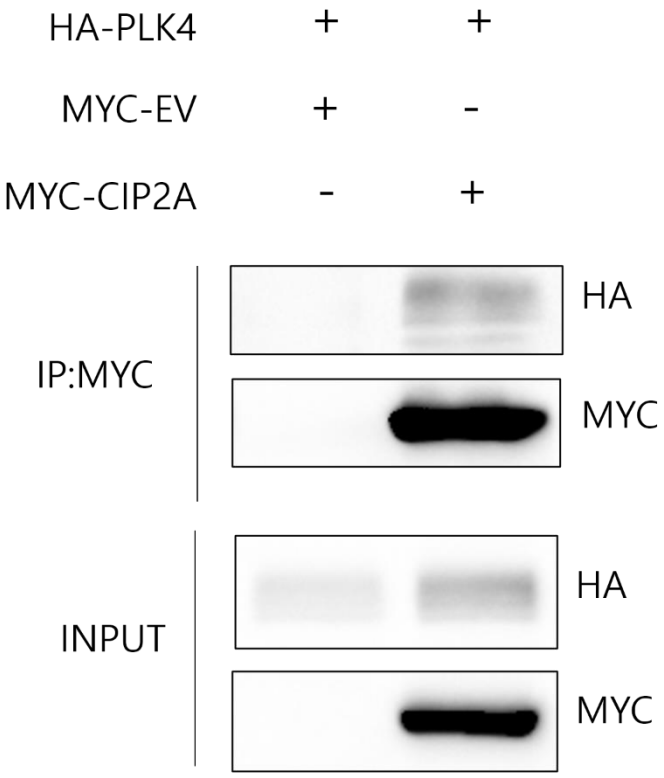


FIGURE 1. CIP2A binds to PLK4

HA-PLK4 was co-expressed with MYC-CIP2A or MYC-Empty Vector (EV) in HEK293T cells. Cell lysates were subjected to anti-MYC immunoprecipitations and analyzed by immunoblotting using the indicated antibodies.

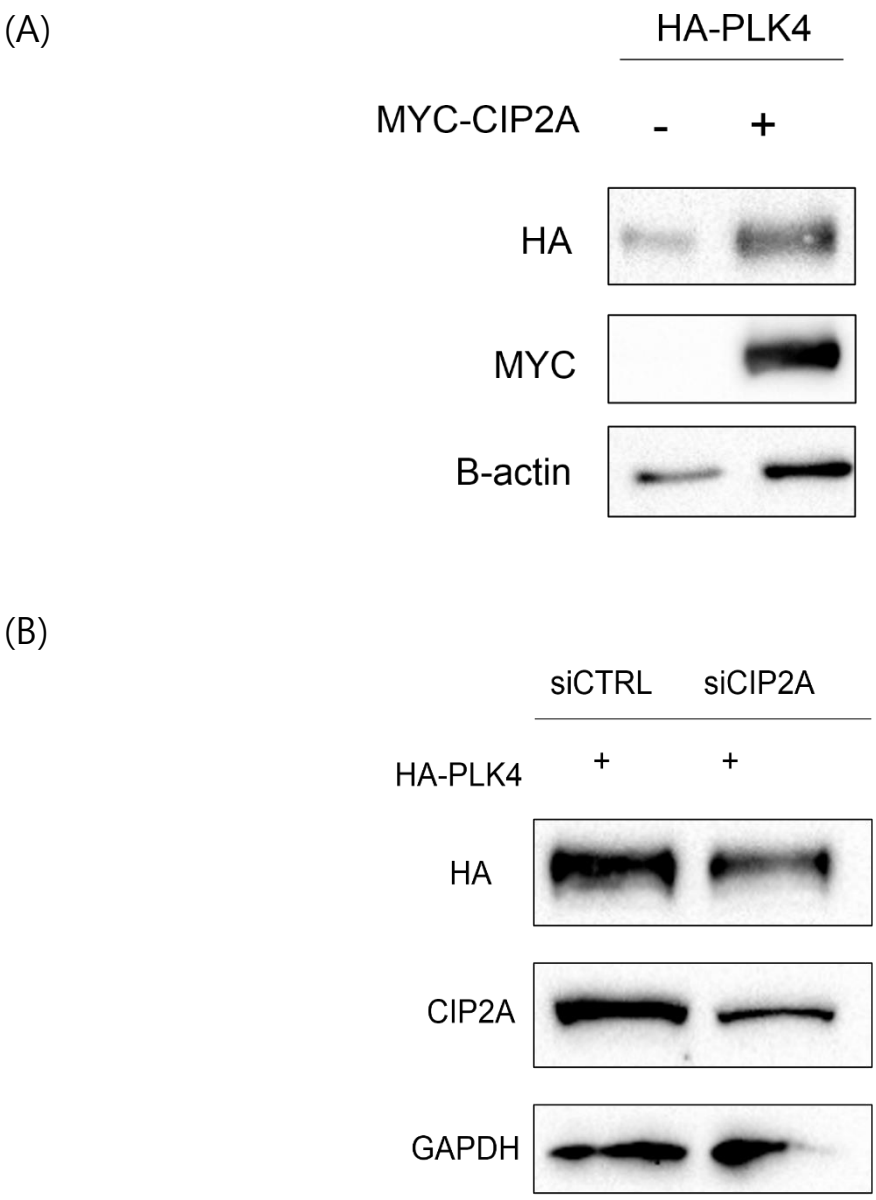


FIGURE 2. CIP2A regulates PLK4 protein levels

(A) HEK293T cells were transfected with HA-PLK4 and MYC-CIP2A for 24h. Cell lysates were analyzed by immunoblotting with indicated antibodies.

(B) HEK293T cells were transfected with siRNA targeting CIP2A. HA-PLK4 was transfected for 24 hours. Then, cells were collected and analyzed by immunoblotting using the indicated antibodies.

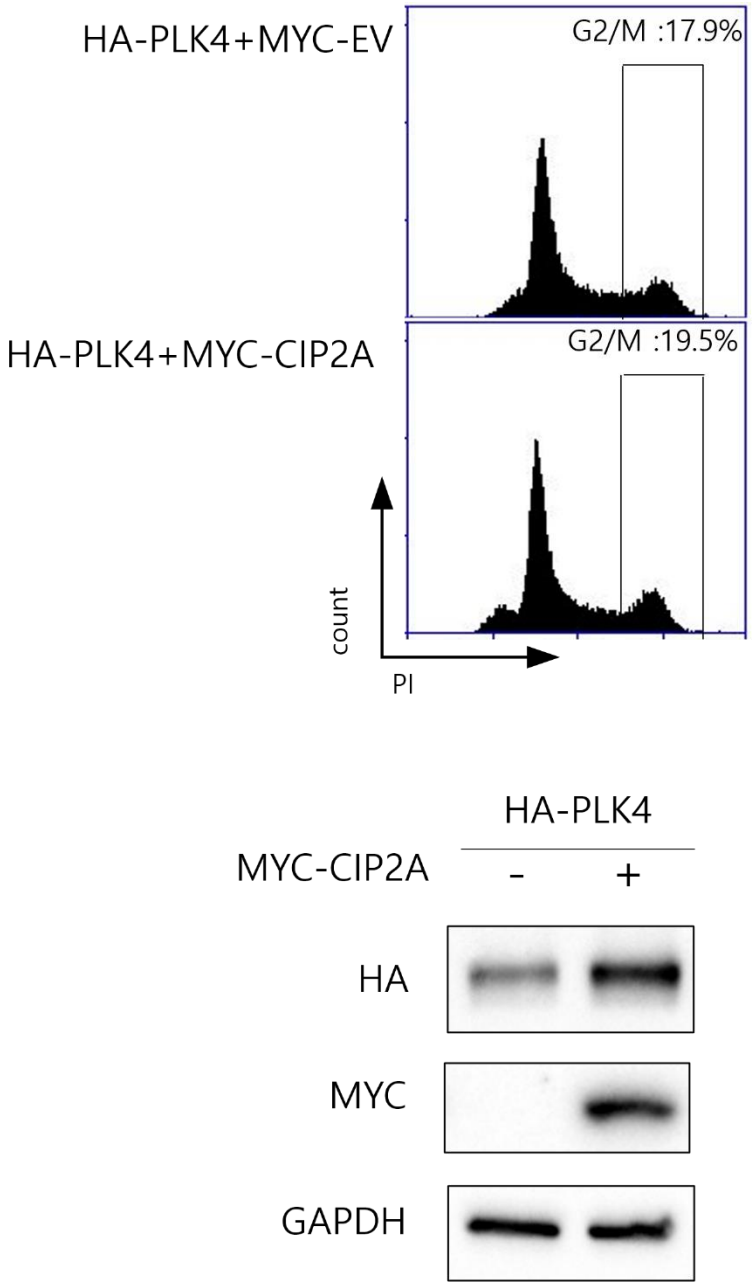


FIGURE 3. CIP2A overexpression slightly increases G2/M phase

HEK293T cells were transfected with HA-PLK4 and MYC- CIP2A. After transfected for 42 hours, cells were analyzed by immunoblotting with indicated antibodies or by fluorescence-activated cell sorting analysis.

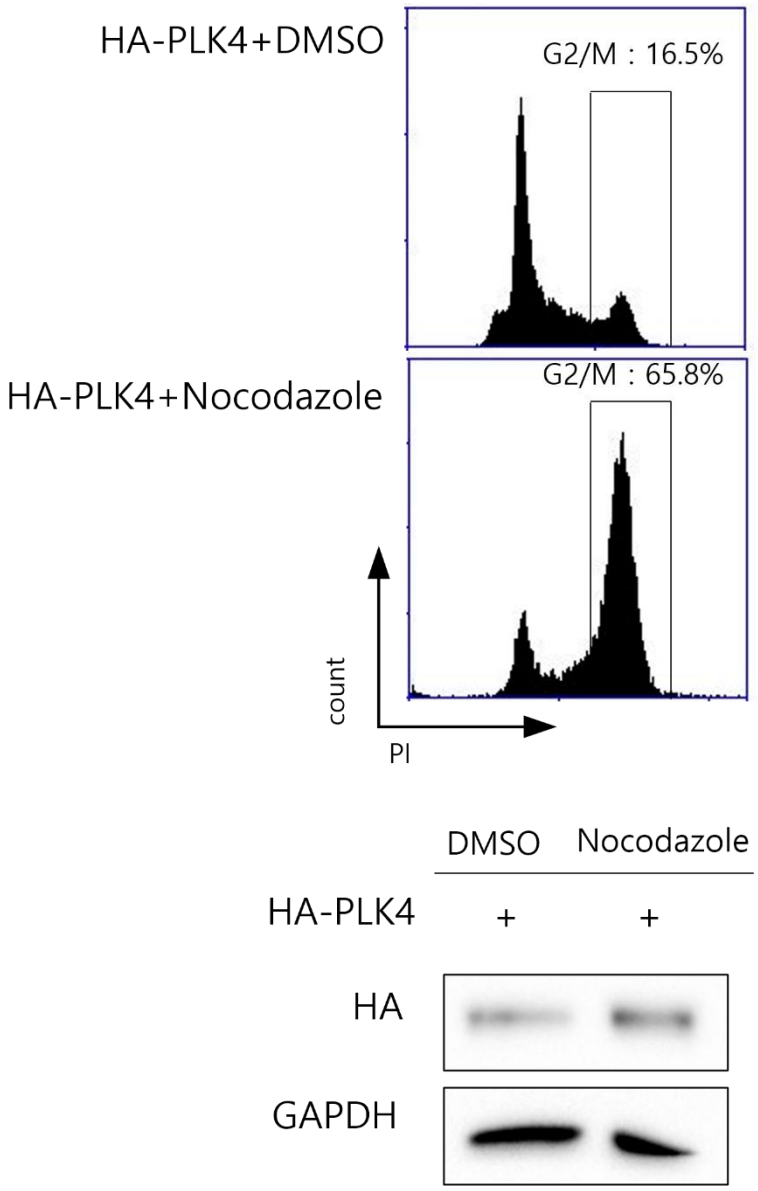


FIGURE 4. PLK4 protein levels in G2/M phase

HEK293T cells were transfected with HA-PLK4 for 24 hours and then incubated with 300nM nocodazole for 18 hours. Cells were analyzed by immunoblotting with indicated antibodies or fluorescence-activated cell sorting analysis.

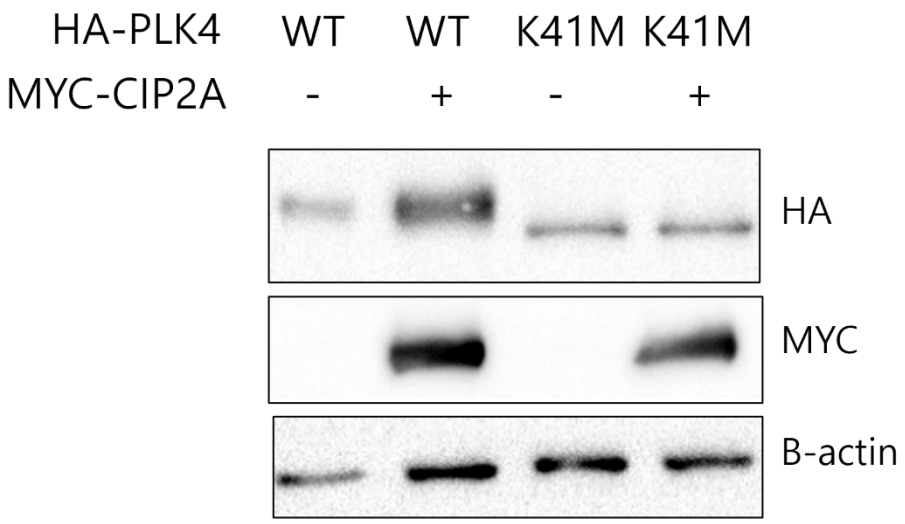


FIGURE 5. PLK4 kinase activity is required for PLK4 stability regulation by CIP2A

PLK4 Lysine 41 was mutated alanine to create PLK4 kinase-dead form. HA-PLK4-WILD TYPE (WT), HA-PLK4 K41M were co-expressed for 24 hours with MYC-Empty Vector or MYC-CIP2A. Whole cell lysates were analyzed by immunoblotting using the indicated antibodies.

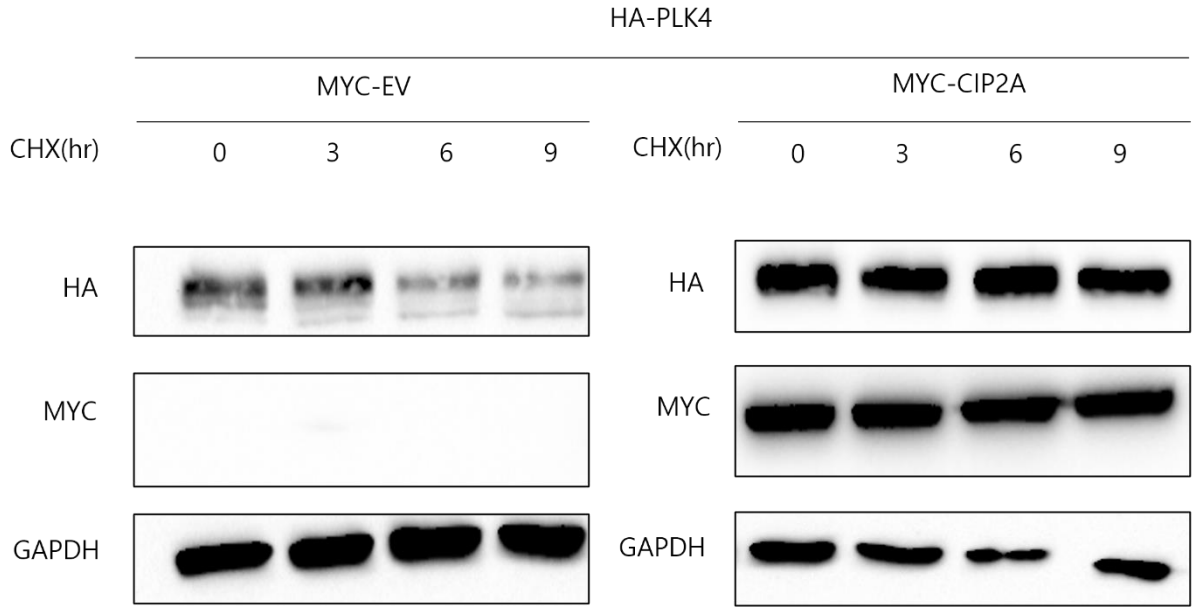


FIGURE 6. Overexpression of CIP2A prolongs PLK4 protein half-life

HEK293T cells were transfected with HA-PLK4, MYC-Empty Vector and MYC-CIP2A and treated with 25 μg/ml cycloheximide (CHX). At the indicated times after the addition of cycloheximide, cell extracts were collected and analyzed by immunoblotting with the indicated antibodies.

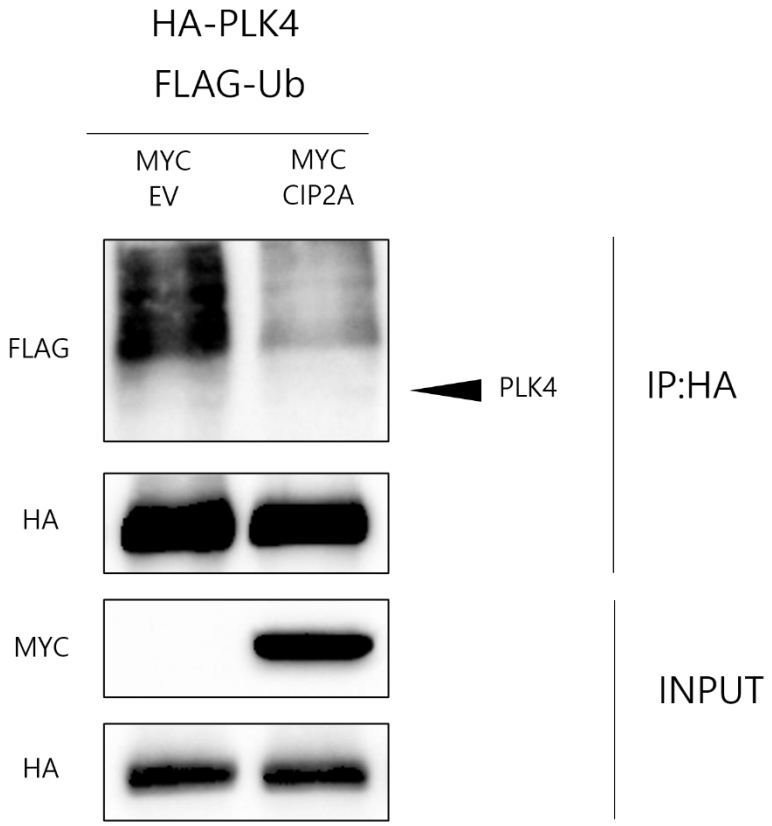
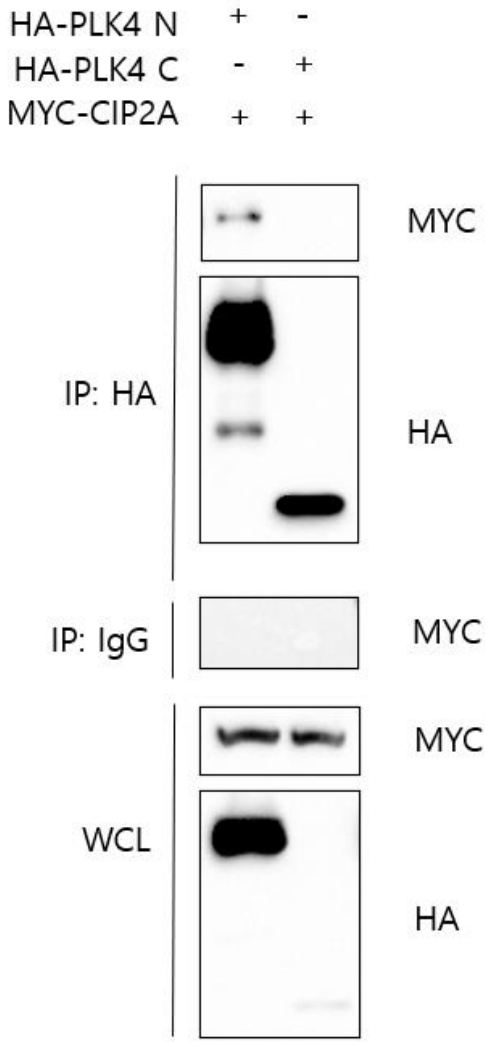


FIGURE 7. Overexpression of CIP2A reduces PLK4 ubiquitylation

HEK293T cells were transfected with HA-PLK4, FLAG-Ubiquitin (Ub), MYC-Empty Vector (EV), or MYC-CIP2A and treated with 10 μM MG132 for 6 hours. Cells were harvested and then immunoprecipitated (IP) with HA antibodies. IP was analyzed by immunoblotting using FLAG, HA, and MYC antibodies.

(A)



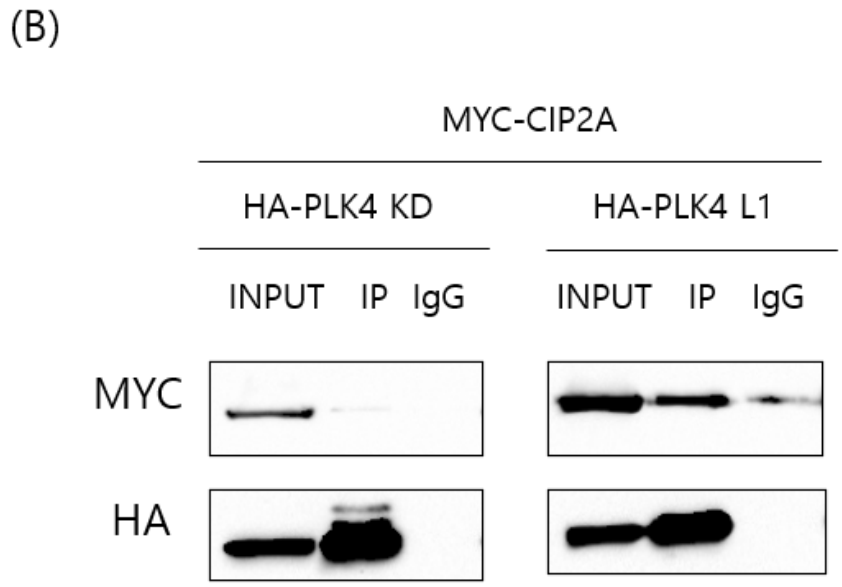


FIGURE 8. CIP2A binds Linker-1 region within PLK4 N-terminus

(A) HEK293T cells co-expression HA-PLK4 N-terminus or C-terminus and MYC-CIP2A were immunoprecipitated using HA-antibodies, and protein levels were detected using the indicated antibodies.

(B) HEK293T cells co-expression HA-PLK4 KD or L1 and MYC-CIP2A were immunoprecipitated using HA-antibodies, and protein levels were detected using the indicated antibodies.

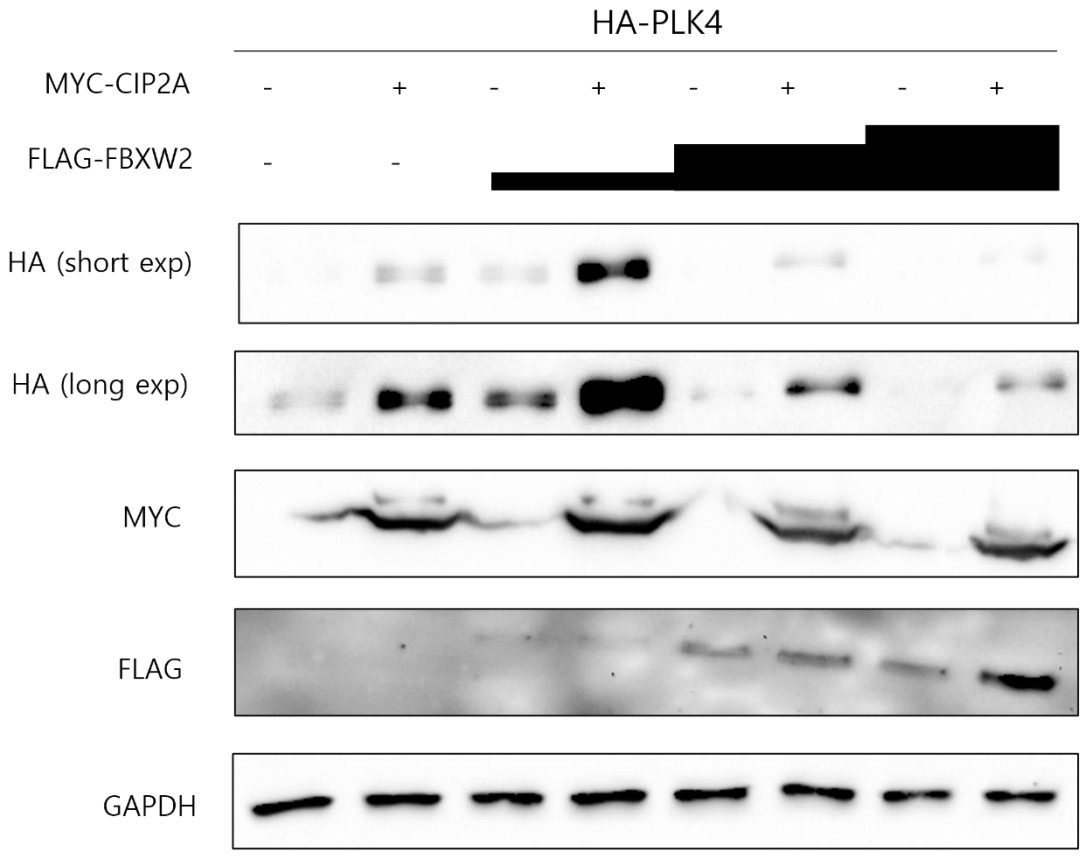


FIGURE 9. FBXW2 reduces PLK4 levels despite CIP2A overexpression

HEK293T cells were transfected with HA-PLK4, FLAG-FBXW2 and MYC-CIP2A. Cells were harvested and analyzed by immunoblotting using FLAG, HA, and MYC antibodies. Black square box represents FLAG-FBXW2 levels.

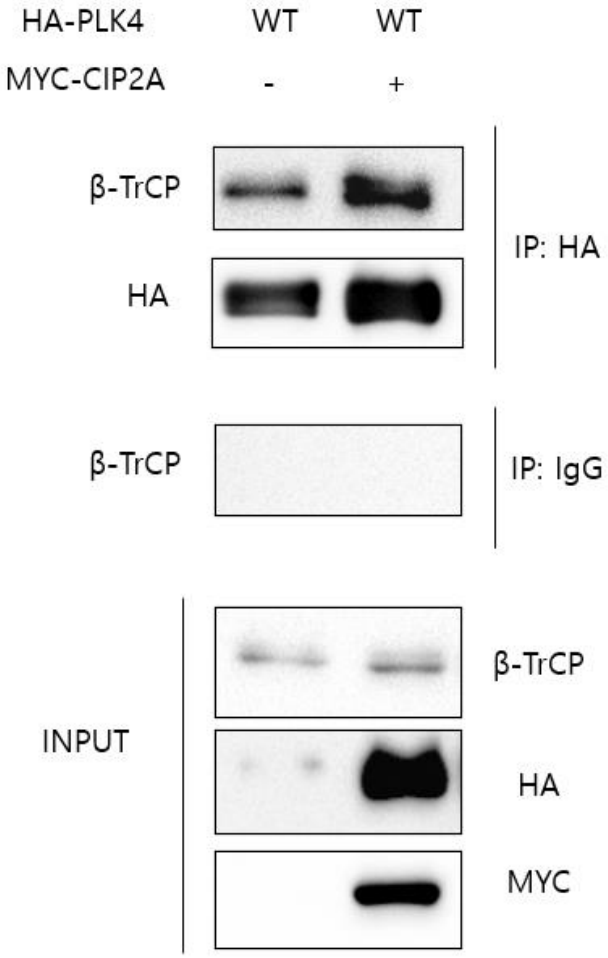


FIGURE 10. Endogenous β-TrCP and HA-PLK4 binding is unaffected by CIP2A overexpression

HEK293T cells were transfected with HA-PLK4, MYC-empty vector and MYC-CIP2A. Cells were collected and then immunoprecipitated (IP) with HA antibodies. IP was analyzed by immunoblotting using β-TrCP, HA, and MYC antibodies.

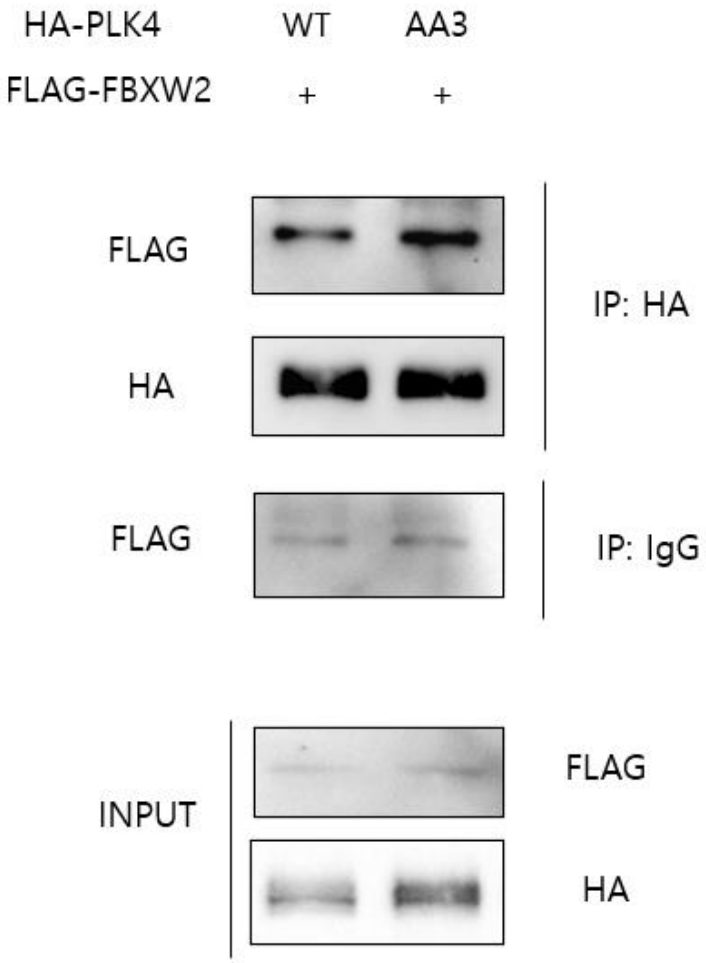
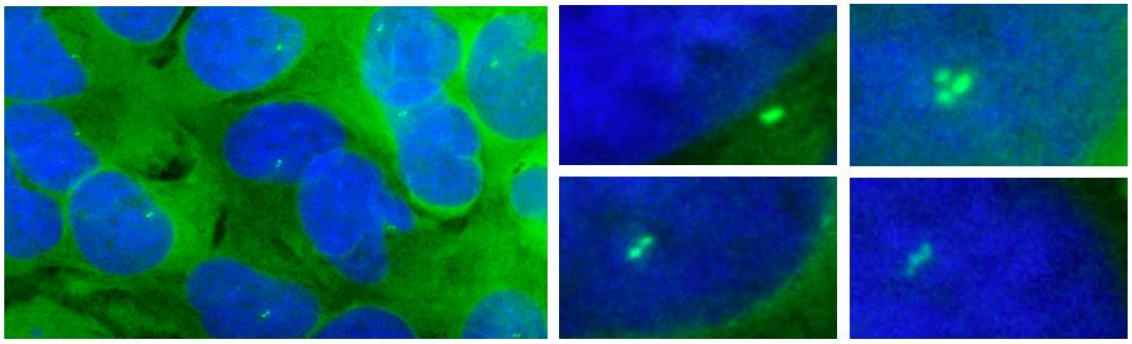


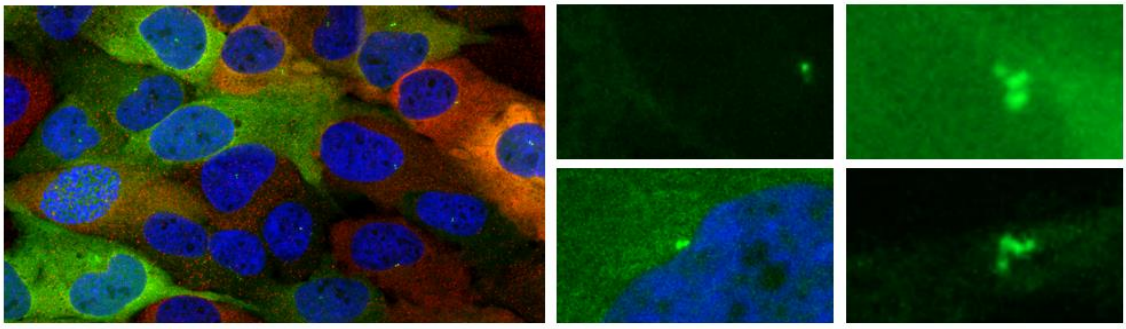
FIGURE 11. Mutation for TSXXS motif in PEST does not affect PLK4/FBXW2 binding

HEK293T cells were transfected with HA-PLK4 WT, HA-PLK4 AA3 and FLAG-FBXW2. Cells were collected and then immunoprecipitated (IP) with HA antibodies. IP was analyzed by immunoblotting using HA and FLAG antibodies.

(A)



U2OS:mEmerald-CETN2
mEmerald-CETN2



U2OS:mEmerald-CETN2&MYC-CIP2A
mEmerald-CETN2
MYC-CIP2A

(B)

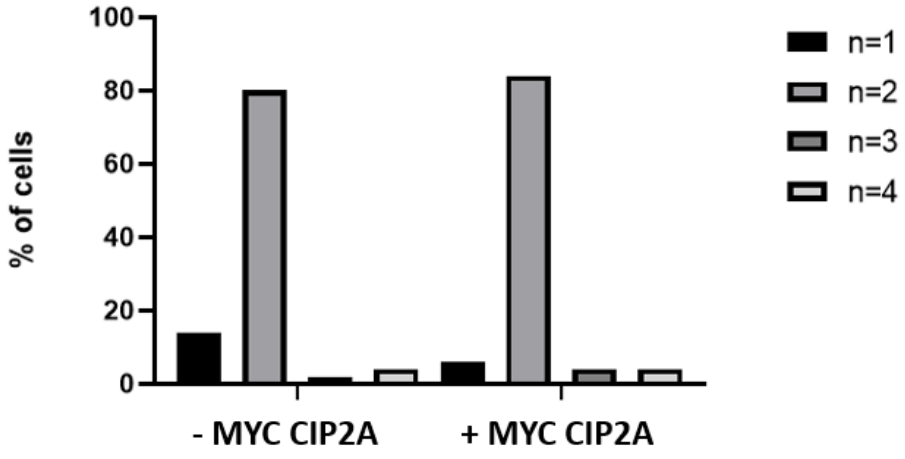


FIGURE 12. CIP2A overexpression does not increase centriole numbers

(A) Immunofluorescence images were acquired 72hr after mEmerald-CETN2 and MYC-CIP2A was induced with doxycycline. DNA was stained with DAPI.

(B) Comparisons of the percentage of the cells with different numbers of centrioles after MYC-CIP2A overexpression in A. More than 80 cells were quantified.

DISCUSSION

In this study, I revealed a novel function of CIP2A as a regulator of PLK4 stability. Overexpression of CIP2A led to the elevation of PLK4 levels and inversely depletion of CIP2A reduced PLK4 levels. This action of CIP2A required PLK4 kinase activity. Without PLK4 kinase activity, CIP2A cannot affect PLK4 stability. Results showed that CIP2A reduced PLK4 ubiquitylation and thereby, prolonged the half-life of the protein. Given that CIP2A was bound to the L1 region within the N-terminus of PLK4, I thought that CIP2A enhanced the stability of PLK4 by interfering with E3 ligase action on PLK4. β-TrCP which binds to the DSGHAT motif within the Linker-1 region and FBXW2 whose recently reported binding motif is located in the Linker-1 region were leading candidates but overexpression of CIP2A could not disturb neither PLK4/β-TrCP binding. In this study, I did not find E3 ligase that interfered with CIP2A to regulate PLK4 stability. It is necessary to further study how CIP2A regulates PLK4 stability and increases PLK4 levels.

One of the most widely studied PLK4 abilities is centriole duplication. PLK4 is recruited to CEP192/CEP152, which is located at the mother centriole. And then, STIL is phosphorylated on the STAN motif and binds to PLK4. SAS-6 is recruited via phosphorylated STIL and forms a nine-fold symmetric cartwheel structure for procentriole. But, contrary to what has been reported in previous studies, PLK4 overexpression did not lead to centriole overduplication. One study reported that the depletion of E3 ligase MIB1, which ubiquitinates PLK4, could not affect centriole duplication [35]. Perhaps the E3 ligase, which CIP2A disturbed to increase PLK4 levels, works through the unknown function of PLK4 or more than one E3 ligase is blocked by CIP2A. Also, consider the possibility that the unknown E3 ligase may be a target of CIP2A.

In addition, according to previous studies of CIP2A, it is observed that CIP2A was located centrosome during mitosis but not from the G1 to G2 phase. PLK4 is localized to the centrosome from G1/S to M phases and begins to degrade at G1 phase. CIP2A is also a protein involved in mitotic cell cycle progression that shows mitotic delay when depleted. And there is also a report that PLK4 kinase activity

reaches peaks at the centrosome in the M phase. Thus, PLK4 and CIP2A may interact at the centrosome during the M phase.

In summary, overexpression of CIP2A leads to an increase in PLK4 levels. But, unlike expectation, PLK4 overexpression could not cause centrosome amplification. Perhaps, the PLK4/CIP2A interaction is related to the unknown activity of PLK4 in the M phase and more follow-up studies are necessary.

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국문요약

Cancerous Inhibitor of Protein Phosphatase 2A (CIP2A)는 90kDa의 종양단백질로, KIAA1524 유전자에 의해 암호화되어 있다. CIP2A는 Protein Phosphatase 2A (PP2A)의 종양 억제 기능을 저해하여 c-MYC, E2F1을 비롯하여 많은 발암인자들을 탈 인산화 시킴으로써 종양의 증식, 분열을 촉진, 세포사멸을 회피하고 부착 비의존성 세포성장을 돕는 것으로 알려져 있다. 많은 연구를 통해 CIP2A가 인간의 여러 악성 종양에서 발현이 증가하였음이 보고되었으며, CIP2A의 과발현은 환자들의 예후에 부정적인 영향을 미치는 것으로 알려져 있다. CIP2A 단백질의 새로운 상호작용을 밝히기 위해 암 세포주를 이용한 Proximity labeling assay인 TurboID를 통하여 Polo-Like Kinase 4 (PLK4)가 발굴되었다.

PLK4는 N-말단에 kinase domain (KD)을 C-말단에 polo-box domain (PBD)을 가지고 있는 serine/threonine 인산화효소이다. PLK4의 발현량은 G0기에 가장 낮으며 S-G2-M기를 거치며 서서히 증가하며, 일반적인 상황에서 PLK4는 낮은 발현량을 유지하나 PLK4의 과발현이 여러 악성 종양에서 관찰되었으며 암 환자들의 poor prognosis와 밀접한 연관을 보인다고 보고되어있다.

본 연구에서 CIP2A가 PLK4 단백질의 발현량에 미치는 영향과 CIP2A의 과발현이 PLK4의 과발현을 일으킴을 확인하였다. 또한, 이러한 CIP2A와 PLK4의 상호작용은 PLK4의 kinase activity를 필요로 하였으며 PLK4의 kinase activity가 작용하지 못할 때에는 CIP2A는 PLK4의 발현량에 영향을 미치지 못했다. 두 단백질의 상호작용에 kinase activity가 필요하다는 점을 고려해 CIP2A가 PLK4의 발현량에 영향을 미친 것이 RNA수준이 아닌 단백질 수준에서 이루어 졌을 가능성을 염두 하여 단백질 합성 억제제인 cycloheximide를 이용, CIP2A의 과 발현 상황에서 PLK4 단백질의 반감기가 증가했음을 확인했다. CIP2A에 의한 PLK4 단백질 반감기의 증가 기작을 확인하기 위해 CIP2A를 과발현시킨 상황에서의 PLK4 ubiquitination을 확인하였으며 PLK4의 ubiquitination 정도가 감소하였음이 확인되었다.

CIP2A와 E3 ligase β-TrCP와 FBXW2가 결합하는 PLK4의 domain이 겹치기 때문에 CIP2A가 E3 ligase와 PLK4의 결합을 방해했는지 확인했으나 CIP2A는 PLK4와 β-TrCP 의 결합을 방해하지 않았다. 마지막으로, CIP2A에 의한 PLK4의 발현량 증가가 세포 표현형에 미친 영향을 확인하기 위해 PLK4의 가장 널리 알려진 기능이 centriole duplication이라는 점을 고려하여 centriole 개수 변화를 확인하였으나 centriole의 복제는 영향을 받지 않았다. PLK4와 CIP2A 모두 M기에 centrosome에 위치한다고 보고되었던 점을 고려할 때 두 단백질이 M기에 centrosome에서 상호작용할 가능성을 고려해 보아야 하며 이에 대한 후속 연구가 필요하다.