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Cyclin E/Cdk2 Phosphorylates Plant Homeodomain Finger Protein 8 (PHF8) and Regulates Its Function in Cell Cycle

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Running title: *Cyclin E/Cdk2 regulates PHF8 function*

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Keywords: cyclin E/Cdk2, PHF8, phosphorylation, cell cycle, rRNA transcription

Background: Cyclin E/Cdk2 is a key protein kinase in cell cycle.
Results: Phosphorylation of PHF8 by cyclin E/Cdk2 enhances its demethylase activity towards H3K9me2 and promotes rDNA transcription as well as S phase progression.
Conclusion: Cyclin E/Cdk2-dependent phosphorylation of PHF8 affects its function.
Significance: We unveiled the mechanism of how cyclin E/Cdk2 regulates the function of PHF8 in cell cycle regulation.

SUMMARY

Cyclin E/Cdk2 is a key regulator in G_1/S transition. Previously we identified a number of Cdk2 interacting proteins including PHF8 (Plant homeodomain finger protein 8). In this report, we confirmed that PHF8 is a novel cyclin E/Cdk2 substrate. By taking the approach of mass spectrometry, we identified that PHF8 Ser844 is phosphorylated by cyclin E/Cdk2. Immunoblotting analysis indicated that wt-PHF8 demethylates histone H3K9me2 more efficiently than the cyclinE/Cdk2 phosphorylation-deficient PHF8-S844A mutant. Furthermore, flow cytometry analysis showed that wt-PHF8 promotes S phase progression more robustly than PHF8-S844A. Real-time PCR results demonstrated that PHF8 increases transcription of cyclin E, E2F3 and E2F7 to significantly higher levels compared to PHF8-S844A. Further analysis by ChIP assay

indicated that PHF8 binds to cyclin E promoter stronger than PHF8-S844A, and reduces H3K9me2 level at the cyclin E promoter more efficiently than PHF8-S844A. In addition, we found that cyclinE/Cdk2-mediated phosphorylation of PHF8 Ser844 promotes PHF8-dependent rRNA transcription in luciferase reporter assays and real-time PCR. Taken together, these results indicate that cyclin E/Cdk2 phosphorylates PHF8 to stimulate its demethylase activity in order to promote rRNA transcription as well as cell cycle progression.

Cyclins and cyclin-dependent kinases (CDKs) play important roles during cell cycle progression (1). Different cyclin/Cdk complexes exert their function at different cell cycle phases. Among them, cyclin E/Cdk2 plays critical role at the G_1 /S transition (2, 3) via phosphorylating a number of downstream signaling proteins (4-7). Multiple cyclin E/Cdk2 substrates are cell division regulators. Cyclin E/Cdk2 assists cyclin D/Cdk4 in phosphorylating retinoblastoma protein (Rb), which leads to the release of E2F transcriptional factors from Rb-dependent inhibition in order to promote expression of genes (such as cyclin E, myc and DNA polymerase) that drive cells to enter the S phase (8, 9). Cyclin E/Cdk2 also phosphorylates Cdk inhibitor p27 to promote its degradation (10). Smad3 is a transcriptional factor that regulates TGF-β signal pathway. The phosphorylation of Smad3 by cyclin E/Cdk2 inhibits its transcriptional activity (11). Cyclin E/Cdk2 phosphorylates CBP/p300 to stimulate its histone acetyltransferase activity during cell cycle progression (12). NPAT is phosphorylated by the cyclin E/Cdk2 complex to increase histone transcription required for S-phase entry and progression (13). In addition to its function on promoting G_1/S transition, cyclin E/Cdk2 is also involved in the DNA damage checkpoint control by phosphorylating DNA helicase complex subunit MCM3 and MCM7 (14, 15).

As cyclins/Cdks are orderly activated during cell cycle progression, their phosphorylation substrates orchestrate the cellular changes that ultimately lead to cell division. However, many of these substrates remain unknown. In order to understand the molecular mechanisms of cyclin E/Cdk2 and its associated proteins in cell cycle regulation, we employed TAP (Tandem Affinity Purification) technique to identify 14 novel proteins interacting with Cdk2, including the histone demethylase PHF8 (16). PHF8 belongs to a class of PHD domain-containing zinc finger proteins, which play an important role in histone demethylation, gene transcription and chromatin remodeling. PHF8 can demethylate H3K9me2/1, H3K27me2 or H4K20me1, and relieve the transcriptional repression and promote the transcription of related genes (17, 18, 19, 20). It has been found that PHF8 can also bind to the promoter region of rDNA and regulate rDNA transcription (21, 22). As a histone demethylase, PHF8 regulates retinoic acid response in APL (acute promyelocytic leukemia) (23). The germline F279S mutation in PHF8 that disrupts the demethylase activity causes hereditary XLMR (X-linked mental retardation) (24, 25).

PHF8 is involved in the regulation of cell cycle. PHF8 can interact with E2F1, HCF-1 and SET1A, and promote cell cycle G_1/S transition (26), while loss of

PHF8 leads to prolonged G_2 phase and defective mitosis (27). However, the mechanism of PHF8 regulation is not clear and it remains to be elucidated whether phosphorylation influences PHF8 function. In this report, we demonstrate that cyclin E/Cdk2 phosphorylates Ser-844 of the PHF8 demethylase and regulates its cellular distribution. We found that mutation of the cyclinE/Cdk target site (Ser-844) negatively affects the ability of PHF8 to promote the S phase progression. Further analysis revealed that overexpression of PHF8 upregulates the cyclin E by affecting PHF8 and H3K9me2 levels at the cyclin E promoter. Additionally, we found that wt-PHF8 promotes rDNA transcription more effectively than the PHF8-S844A mutant. We conclude that phosphorylation of PHF8 by cyclin E/Cdk2 plays an important role in regulating gene transcription and and cell cycle progression.

EXPERIMENTAL PROCEDURES

Plasmids and Antibodies- pCMV-SPORT6-PHF8 was purchased from Openbiosystems. PHF8 and its mutant PHF8-S844A were cloned into pCMV FLAG. The truncated PHF8 (PHF8 (1-316), PHF8 (317-680), PHF8 (681-1024) and PHF8 mutants PHF8 (317-680) 381AGA, PHF8 (317-680) 641AKA, PHF8 (681-1024) S844A) and full-length Cdk2 were cloned into pET41b. The mammalian expression vectors for cyclin E and Cdk2 were generated as described previously (16).

The following primary antibodies were used: FLAG antibody (M2) was purchased from Sigma. PHF8 (P-15), myc (9E10), His (H-3), Cdk2 (D-12), cyclin E (E-4), β-actin (1-19), lamin B (M-20) antibodies were purchased from Santa Cruz Biotechnology. Phosphor-Ser antibody (2324S) was purchased from Cell Signaling. Histone H3 (ab1791) and H3K9me2 (ab1220) antibodies were purchased from Abcam.

Co-immunoprecipitation and GST Pulldown- For immunoprecipitation, 293T cells were transfected with indicated plasmids. The cell lysates were incubated with indicated antibodies at 4℃ for 2 h followed by the addition of protein A-agarose beads. After 4 h of incubation at 4℃, the beads were washed with lysis buffer. The immune complexes were separated by SDS-PAGE followed by immunoblotting analyses.

For the GST pull down assay, 1 μg of GST-PHF8, GST-PHF8 (1-316), GST-PHF8 (317-680), GST-PHF8 (681-1024) or GST as a negative control were incubated with cell lysates from 293T cells overexpressing myc-tagged cyclin E and Cdk2, or with His-Cdk2 purified from BL21. The glutathione beads were then added and incubated for 2 h. The bound proteins were eluted with sample loading buffer and analyzed by immunoblotting with anti-myc or Cdk2 antibodies.

Cell Culture and Synchronization- 293T and HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (PAA) and 100 μg/ml streptomycin and 100 U/ml penicillin at 37℃ with 5% CO_2. HeLa cells were synchronized at early S phase by treatment with double thymidine in the same way as for U2OS cells described in the previous report (16). Briefly, cells were synchronized at early S transition by treatment with 2 mM thymidine for 16 h in complete medium, released in fresh

medium for 8 h and then incubated with 2 mM thymidine for another 16 h. Cells were synchronized to early S phase at this point.

In Vitro Kinase Assay- 1 μg of GST-PHF8, GST-PHF8 (1-316), GST-PHF8 (317-680), and GST-PHF8 (681-1024) or GST-PHF8 mutants were incubated with GST-cyclin E/Cdk2 in kinase buffer (50 mM Tris-Cl, pH7.5, 10 mM $MgCl_2$, 1 mM DTT) with 1 mM cold ATP, 0.5 μCi of [γ-^{32}P]ATP at 30℃ for 30 min. The reaction was terminated with SDS-PAGE loading buffer. The samples were subjected to 8% SDS-PAGE gel and analyzed by autoradiography.

Mass Spectrometry- GST-PHF8 (681-1024) was incubated with GST-cyclin E and GST-Cdk2 for *in vitro* kinase assay as described above in the presence of cold ATP. Subsequently, GST-PHF8 (681-1024) was isolated using SDS-PAGE and then trypsinized. The tryptic peptides were analyzed by HPLC-ESI/MS/MS with a Thermo Finnigan LTQ adapted for nanospray ionization. All MS/MS spectra were processed using the SEQUEST (BioworksBrowser 3.3.1 SP1).

RNA Interference- RNA interference was carried out using double strand RNAs. The synthetic siRNA duplexes corresponding to the PHF8 and Cdk2 mRNA sequences and the control siRNA sequence were obtained from the manufacture (Ribobio). 293T cells or HeLa cells were plated in a six-well plate and transfected with 100 nM siRNA using the Lipofactamine 2000 transfection reagent (Invitrogen).

Transient Transfection and Flow Cytometry Analysis- Cells were plated in 60 mm dishes and transfected with indicated siRNAs or plasmids and pCMV GFP-H2B plasmid for the selection of transfected cells. Cells were harvested at indicated time, fixed in ethanol, and stained with propidium iodide, and then subjected to flow cytometry analysis.

Dual-luciferase reporter activity assay- 293T cells in a 24-well plate were transfected with plasmids for expressing PHF8, PHF8-S844A, or siRNA targeting PHF8 or Cdk2, and rDNA luciferase reporter pHrD-IRES-Luc (28) and pRL-TK for 48 h. The cell lysates were harvested for dual-luciferase assay according to the manufacturer's instructions. Three independent experiments were performed.

RNA extraction and real-time PCR- Total RNA was extracted from cells by using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Real-time PCR for cyclin E, E2F3 and E2F7 was performed using SYBR green premix reagent (Toyobo, Japan) with β-actin as the internal control. Real-time PCR analysis of rRNA was also performed using SYBR green premix reagent, with GADPH as the internal control. Relative amount of mRNA or rRNA was quantified using the comparative threshold cycle (*CT*) method. Primers are:
Cyclin E-For: 5' CTCCAGGAAGAGG AAGGCAA 3', Cyclin E-Rev: 5' TCGA TTTTGGCCATTTCTTCA 3', E2F3-For: 5'AAGAAGAAGTCTAAAAACAACGT CCAA 3', E2F3- Rev: 5' CTTGACACTG GGCCAGCAT 3', E2F7-For: 5' GGAAA GGCAACAGCAAACTCT 3',E2F7-Rev:, 5'TGGGAGAGCACCAAGAGTAGAAG A 3', β-actin-For: 5' GTGAAGGTGAC AGCAGTCGGTT 3', β-actin-Rev: 5'GA AGTGGGGTGGCTTTTAGGA 3', GAPDH For: 5' CGACCACTTTGTCA AGCTCA 3', GAPDH Rev: 5'AGGGG

AGATTC AGTGTGGTG 3', 47s rRNA precursor For: 5' TGTCAGGCGTTCTC GTCTC 3', 47s rRNA precursor Rev:5' GAGAGCAC GACGTCACCAC 3'.

Chromatin immunoprecipitation- HeLa Cells were treated with 1% formaldehyde for 10 minutes at room temperature. The cross-linking was stopped by the addition of 125 mM glycine. The cells were lysed and the lysates were sonicated at 30 w for 5 s and paused for 10 s up to 8 cycles to shear DNA to an average fragment size to 200-1000 bp. Immunoprecipitation was carried out with indicated antibody. Normal mouse IgG was used as the negative control. After immunoprecipitation, the eluates were incubated at 65℃ for to reverse the cross-linking for 4 h followed by treatment with proteinase K (0.2 mg/ml) 45℃ for 2 h. Then the DNA were precipitated and subjected to PCR with indicated primers. The tested promoters were quantified by SYBR Green-based real-time qPCR. All samples were subjected to PCR amplification with oligonucleotide primers specific for indicated promoter DNA.

Primers are:

Cyclin E promoter For: 5' CCCCGTCC CTGCGCCTCGCTG 3', Cyclin E promoter Rev: 5' CGGCGGCGGCGACG GCAGTGG 3', rDNA promoter For: 5' AGAGGGGCTGCGTTTTCGGCC3',

rDNA promoter Rev: 5' CGAGACAGAT CCGGCTGGCAG 3'.

RESULTS

PHF8 interacts with Cyclin E/Cdk2 in an RXL-dependent manner. We previously identified a number of novel Cdk2 associated proteins, including PHF8 (16). PHF8 is known as a H3K9me2 demethylase, which consists of a PHD domain and a JmjC domain. To confirm whether PHF8 is a *bona fide* Cdk2 interacting partner, we analyzed the association between PHF8 and Cdk2 by co-immunoprecipitation and GST pull-down. pCMV FLAG-PHF8 and pCMV myc-cyclin E or Cdk2 were co-transfected into 293T cells. The cell lysates were subjected to immunoprecipitation with FLAG antibody and immunoblotted with myc antibody. As shown in Fig. 1A, myc-cyclin E or myc-Cdk2 co-immunoprecipitated with FLAG-PHF8. Next, we examined whether endogenous PHF8 and Cdk2 are associated with each other. The cell lysates from 293T cells were immunoprecipitated with Cdk2 antibody and then immunoblotted with PHF8 antibody. As shown in Fig. 1B, PHF8 interacts with Cdk2. These results confirm that PHF8 can interact with Cdk2 *in vivo*. To examine whether there is direct interaction between PHF8 and Cdk2, GST-PHF8 and His-Cdk2 were purified for the GST pull-down assay. As shown in Fig. 1C, PHF8 directly binds Cdk2.

To determine which region of PHF8 is critical for its binding with cyclin E/Cdk2, we generated a set of truncated PHF8 mutants for GST pull-down assays. Our data showed that PHF8 (317-680) binds with Cdk2 much stronger than PHF8 (1-316) or PHF8 (681-1024) (Fig. 1D). The RXL motif has been previously shown to be the essential cyclin-binding motif in a wide range of cyclin/Cdk-interacting proteins (29). Therefore, we asked whether the interaction between PHF8 and cyclin E/Cdk2 is dependent on the RXL motif of PHF8. There are two RXL motifs in PHF8 (317-680) (aa381-383 and 641-643, respectively). To find out which RXL motif on PHF8 is important for its binding

with cyclin E/Cdk2, we generated the PHF8 (317-680) AXA mutants in which RXL motifs were converted into non-functional AXA motifs by site-directed mutagenesis. The data in Fig. 1E showed that PHF8 (316-680) 381AGA mutant but not PHF8 (316-680) 641AKA greatly weakened the interaction between PHF8 and Cdk2, indicating that PHF8 (381-383) RXL motif is critical for its interaction with Cdk2.

PHF8 is phosphorylated by cyclin E/Cdk2- To examine whether PHF8 can be phosphorylated by cyclin E/Cdk2, the GST-PHF8 was used in *in vitro* kinase assays. As shown in Fig. 2A, PHF8 is phosphorylated by cyclin E/Cdk2. To identify target cyclin E/Cdk2 phosphorylation region(s) within PHF8, we performed *in vitro* kinase assays with truncated GST-PHF8 proteins. As shown in Fig. 2B, GST-PHF8 (681-1024) is phosphorylated much stronger than GST-PHF8 (1-316) or GST-PHF8 (317-680). To determine the cyclin E/Cdk2 phosphorylation site(s) of PHF8, we employed mass spectrometry analysis of purified GST-PHF8 (681-1024) phosphorylated *in vitro* by the cyclin E/Cdk2 complex. The data in Fig. 2C indicate that PHF8 Ser844 is phosphorylated by cyclin E/Cdk2. To further verify the phosphorylation site, we generated GST-PHF8 (681-1024) S844A and performed *in vitro* kinase assay with cyclin E/Cdk2. As shown in Fig. 2D, cyclin E/Cdk2-dependent phosphorylation of the PHF8 (681-1024) S844A mutant is significantly reduced compared to the wild type PHF8 (681-1024). Then we transfected the 293T cells with cyclin E/Cdk2 or cyclin E/Cdk2-DN (Cdk2 dominant negative mutant), and analyzed the phosphorylation of endogenous PHF8 with phosphor-Ser antibody. As shown in Fig. 2E, phosphorylation of PHF8 at Ser was much stronger in Cdk2 overexpressed cells than that in control or Cdk2 DN overexpressed cells which indicated that endogenous PHF8 can be phosphorylated by cyclin E/Cdk2. Furthermore, we transfected 293T cells with FLAG-PHF8 and FLAG-PHF8-S844A expression plasmids with cyclin E/Cdk2 or cyclin E/Cdk DN expression plamids into 293T cells. Then the cell lysates were immunoprecipated with FLAG antibody by immunoblotting with phosphor-Ser antibody. The data indicated that phosphorylation on serine of FLAG-PHF8 but not FLAG PHF8-S844A can be enhanced by cyclin E/Cdk2 while cyclin E/Cdk DN did not affect the phosphorylation of FLAG-PHF8 (Fig. 2F), suggesting that cyclin E/Cdk2 can phosphorylate PHF8-Ser844 in cells. Taken together, these data suggest that PHF8 Ser844 is the specific site phosphorylated by cyclin E/Cdk2.

Effects of PHF8 knockdown on H3K9me2 demethylation and cell cycle progression- PHF8 has been reported to function as a H3K9me2 demethylase and affect cell cycle progression (21, 26). Consistent with previous reports, we found that level of H3K9me2 was elevated in 293T cells treated with 2 sets of PHF8 siRNAs (Fig. 3A). Also, We generated siRNA-resistant FLAG-PHF8 and FLAG-PHF8-S844A expression plasmids and transfected them with PHF8 siRNA into 293T cells. Then we analyzed the level of H3K9me2 in transfected cells by immunoblotting. As shown in Fig. 3B, only siRNA-resistant PHF8 but not PHF8 S844A mutant can reduce the level of H3K9me2. We also transfected HeLa cells with Cdk2 siRNA with or without PHF8 siRNA and

examined the level of PHF8 and H3K9me2 by immunoblotting. We found that knockdown of Cdk2 can elevate the level of H3K9me2 and further knockdown of PHF8 increased H3K9me2 in certain level (Fig. 3C), which suggested that Cdk2 may affect PHF8 demethylase activity. To examine the effect of PHF8 on cell cycle progression, we treated the HeLa cells with PHF8 siRNA. The immunoblotting data showed that PHF8 siRNA efficiently knocks down PHF8 expression in HeLa cells (Fig. 3D). The siRNA-transfected HeLa cells were synchronized at early S phase by double thymidine block, released for 3 h and 6 h respectively, and subjected to flow cytometry analysis. The data indicated that knockdown of PHF8 significantly inhibited S progression (Fig. 3E and 3F). These results suggest that knockdown of PHF8 affects H3K9me2 demethylation and cell cycle progression.

Phosphorylation of PHF8 promotes H3K9me2 demethylation and S Phase progression- Since we found that PHF8 Ser844 is phosphorylated by cyclin E/Cdk2, we wondered whether phosphorylation of PHF8 by cyclin E/Cdk2 will affect its function. To determine if the histone demethylation by PHF8 fluctuates with Cdk2 activity, we transfected 293T cells with FLAG-PHF8 and FLAG-PHF8-S844A expression plasmids with Cdk2 or Cdk DN expression plamids. Then we analyzed the level of H3K9me2 in transfected cells. The data showed that overexpression of PHF8, but not PHF8-S844A mutant, led to the reduction of H3K9me2. More importantly, overexpression of FLAG-PHF8 with cyclin E and Cdk2, but not with Cdk2 DN, demethylates H3K9me2 much more efficiently than overexpression of FLAG-PHF8 alone (Fig 4A). This data suggests that phosphorylation of PHF8 at Ser844 by cyclin E/Cdk2 enhances PHF8 demethylase activity and promotes H3K9me2 demethylation.

To determine the effect of PHF8 phosphorylation on the cell cycle, we transfected pCMV FLAG-PHF8 or pCMV FLAG-PHF8-S844A into HeLa cells. The cells were synchronized at the early S phase by double thymidine treatment, released for 3 h and 6 h, and subjected to flow cytometry analysis. As shown in Fig. 4B & 4C, at 6 h after release, 61% of PHF8 overexpressing cells entered S phase, (10% more compared to the PHF8-S844A overexpressed cells). These results indicated that phosphorylation of PHF8 at Ser844 positively regulates its ability to promote the S phase entry.

PHF8 regulates transcription of cyclin E, E2F3, and E2F7- To explore how phosphorylation of PHF8 by cyclin E/Cdk2 mechanistically regulates the cell cycle progression, we transfected HeLa cells with PHF8 or PHF S844A expressing plasmids and examined the mRNA levels of cyclin E, E2F3, E2F7, and GAPDH by real-time PCR. As shown in Fig. 5A, B, C and D, the amount of mRNAs (cyclin E, E2F3 and E2F7) in PHF8 overexpressed cells is significantly higher compared to PHF8-S844A overexpressed cells, although PHF8-S844A also modestly up-regulated cyclin E, E2F3 and E2F7 mRNAs. However, there is no change in the level of GAPDH. The levels of PHF8 and PHF8-S844A are comparable (Fig. 5E). In addition, we performed the similar experiments with knockdown of Cdk2 and PHF8. We found that knockdown of Cdk2 and PHF8 respectively can reduce the expression levels of cyclin E and E2F3 (Fig. 5F and 5G), but did not affect the level of E2F7 (Fig. 5H). Moreover, we

noticed that knockdown of Cdk2 and PHF8 together made the trend of cyclin E and E2F3 reduction more apparently. These results indicate that phosphorylation of PHF8 by cyclin E/Cdk2 enhances its ability to promote transcription of cyclin E and E2F3, which may contribute to the PHF8's ability to promote cell cycle progression. It is worth to point out that both overexpression of PHF8 causes the increase of E2F7 expression. It is known that induction of E2F7 during G0-G1/S causes cell cycle arrest whereas expression of E2F7 during G2/M failed to disturb cell cycle progression. In addition, it was reported that E2F7 is highly expressed during mid to late S-phase, and represses transcription of G1/S-regulated genes such as E2F1 (30). Together with our data, we postulate that E2F7 may play a positive role in promoting S phase progression while PHF8 could promote its function.

To understand how phosphorylation of PHF8 up-regulates transcription of cyclin E, we transfected the HeLa cells with PHF8 or PHF8-S844A expressing plasmids and examined the relative amount of PHF8 and H3K9me2 on cyclin E promoters by ChIP assay. The ChIP assays demonstrated that the amount of wt-PHF8 associated with cyclin E promoters is significantly higher compared to PHF8-S844A (Fig. 6A and 6B), while the amount of H3K9me2 on cyclin E promoters was reduced in PHF8 overexpressing cells compared to controls and PHF8-S844A overexpressing cells (Fig. 6C and 6D). The immunoblotting data indicated that expression levels of PHF8 and PHF8-S844A are similar (Fig. 6E). In addition, we performed the experiments with knockdown of Cdk2 by ChIP assay. The data indicated that knockdown of Cdk2 reduced the binding of PHF8 with cyclin E promoter, and correspondingly increased the level of H3K9me2 on cyclin E promoter (Fig. 6F and 6G). The efficiency of knockdown of Cdk2 was shown in Fig. 6H. These results demonstrated that phosphorylation of PHF8 by cyclin E/Cdk2 influences the levels of PHF8 and H3K9me2 at the promoter regions of cyclin E, which in turn promotes cyclin E transcription.

Phosphorylation of PHF8 at Ser844 enhances its ability to promote transcription of rDNA- It has been reported that PHF8 promotes transcription of rDNA and its binding with rDNA promoter can effect rDNA transcription (21). Consistently, we observed that knockdown of PHF8 cause the reduction of transcription of rDNA by rDNA luciferase reporter assay (Fig. 7A). In order to know whether phosphorylation of PHF8 regulates its ability to promote rDNA transcription, we transfected 293T cells with PHF8 or PHF8-S844A expressing plasmids together with rDNA luciferase reporter, and then performed luciferase assays. Our data showed that overexpression of wild type PHF8 activates rDNA promoters much more significantly compared to the phosphorylation-deficient PHF8-S844A mutant (Fig. 7B). Then we measured the levels of the pre-ribosomal RNA (pre-RNA) by quantitative PCR. The result showed that the relative amount of pre-rRNA in PHF8 overexpressing cells is significantly higher compared to PHF8-S844A overexpressing cells (Fig. 7C). Next we transfected HeLa cells with PHF8 or PHF8-S844A expressing plasmids and examined the relative amount of PHF8 and H3K9me2 on rDNA promoters by ChIP assay. The data demonstrated that the amount of wt-PHF8

associated with rDNA promoters is significantly higher compared to PHF8-S844A (Fig. 7D), while the amount of H3K9me2 on rDNA promoters was reduced in PHF8 overexpressing cells compared to controls and PHF8-S844A overexpressing cells (Fig. 7E). The immnoblotting data indicated that expression levels of PHF8 and PHF8-S844A are similar (Fig. 7F). In addition, we analyzed the rDNA promoter activity and pre-rRNA level in Cdk2 knockdown 293T cells. The data showed that knockdown of Cdk2 causes the reduction of rDNA promoter activity and pre-rRNA transcription (Fig. 7G, 7H and 7I)..These results indicate that phosphorylation of PHF8 at Ser844 by cyclin E/Cdk2 increases its binding with rDNA promoter and enhances its ability to promote rDNA transcription.

Taken together, we propose that phosphorylation of PHF8 by cyclin E/Cdk2 facilitates its loading onto chromatin and stimulates its demethylase activity, which enhances the expression of genes essential for the cell cycle progression, including cyclin E, E2Fs and rDNAs.

DISCUSSION

The cyclin E/Cdk2 serine/threonine kinase complex plays a critical role in coordinating both the onset of S phase and centrosome duplication during cell cycle progression. Cyclin E/Cdk2 activates multiple signaling networks by phosphorylating multiple downstream signal transducers. For example, cyclin E/Cdk2 phosphorylates NPAT to promote histone transcription (13, 31, 32, 33). CBP/p300 is phosphorylated by cyclin E/Cdk2 at the G_1/S transition to activate its histone acetyltransferase activity (34). We previously identified PHF8 as a novel Cdk2-interacting protein using tandem affinity purification (16). PHF8 is a H3K9me2 demethylase, and can regulate rDNA transcription and cell cycle progression. It has been reported that PHF8 is phosphorylated by Cdk1 and regulates G_2/M progression (26). However, the role of Cdk2 in regulating the function of PHF8 is unclear. Here we demonstrated that PHF8 Ser-844 is phosphorylated by cyclin E/Cdk2 and phosphorylation of PHF8 affects its ability to demethylate H3K9me2 and regulate S Phase progression. These results suggest that the function of PHF8 in cell cycle may be precisely controlled by cyclin/Cdks.

PHF8 is a H3K9me2 demethylase. Consistent with previous report (21), we showed that H3K9me2 level is reduced in PHF8 overexpressing cells. Interestingly, we found that cyclin E/Cdk2 enhances PHF8 ability to promote H3K9me2 demethylation, which suggests that phosphorylation of PHF8 by cyclin E/Cdk2 directs its demethylase activity towards H3K9me2. However, it remains to be determined whether phosphorylation of PHF8 influences the demethylation of other histones.

Multiple key regulators of the G_1/S transition include cyclin E as well as the E2F3 and E2F7 transcription factors. Their expression levels are precisely controlled during cell cycle. Our results demonstrate that overexpression of PHF8 causes the significant increase of their mRNA levels compared with overexpression of the phosphorylation-deficient PHF8-S844A PHF8 mutant. Meanwhile, ChIP analysis showed that wt-PHF8 is recruited to the cyclin E promoter more efficiently than the PHF8-S844A mutant. Wt-PHF8 reduces the H3K9me2 levels at the cyclin

E promoters more effectively than PHF8-S844A. These results indicate that phosphorylation of PHF8 by cyclin E/Cdk2 enhances its binding to the cyclin E promoter and directs its demethylase activity towards H3K9me2, which in turn promotes the transcription of cyclin E to enhance the positive feedback loop promoting cell cycle progression. Therefore, it will be worth to investigate whether PHF8 has similar effect on other genes involved in cell cycle regulation and whether phosphorylation of PHF8 also has direct impact on transcription of these candidate genes. In addition, comparing the transcriptome of PHF8 and PHF8-S884A overexpressing cells would generate valuable data to further our understanding of the signaling pathways regulated by cyclin E/Cdk2.

It has been reported that PHF6 as a PHD domain family member and tumor suppressor directly interacts with upstream binding factor (UBF) through its PHD domain and suppresses rRNA transcription (35). Our data show that overexpression of PHF8 promote rRNA synthesis. These results indicated that PHF family members function as both positive and negative regulators of rRNA transcription to control the overall level of rRNA. However, it needs to be elucidated whether PHF8 binds UBF to reverse the inhibitory effect of PHF6 on rRNA synthesis.

In summary, we identified PHF8 as a novel substrate of cyclin E/Cdk2. We confirmed the essential role of cyclin E/Cdk2-mediated Ser844 phosphorylation in regulating PHF8 function. Our studies revealed that cyclin E/Cdk2 regulates cell cycle progression through phosphorylation of PHF8, which consequently influences H3K9me2 level on promoters of specific G1/S-regulating genes as well as rDNA transcription. These findings unveiled the new roles of cyclin E/Cdk2 and PHF8 in cell cycle control.

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FOOTNOTES

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The abbreviations used are: PHD, plant homeodomain; PHF8, plant homedomain finger protein 8; Cdk, cyclin dependent kinase; APL, Acute Promyelocytic Leukemia; TAP, tandem affinity purification; GST, glutathione S-transferase; ChIP, Chromatin Immunoprecipitation; rRNA, ribosomal RNA.

FIGURE LEGENDS

FIGURE 1. PHF8 interacts with cyclin E/Cdk2. A, co-immunoprecipitation assay. 293T cells were transfected myc-tagged cyclin E and Cdk2 and FLAG-tagged PHF8. Cell lysates were harvested for immunoprecipitation (IP) with FLAG antibody followed by immunoblotting (IB) with myc antibody. B, 293T cell lysates were immunoprecipitated with Cdk2 antibody or normal mouse of IgG and then immunoblotted with PHF8 antibody. C, D and E, GST, GST-PHF8 or GST-PHF8 mutants as indicated immobilized on glutathione beads were incubated with His-Cdk2 in lysis buffer for 2 h. The associated protein was eluted with SDS loading buffer and immunoblotted with Cdk2 antibody. *TCE*, total cell extract; *NMS*, normal mouse serum; *CBB*, Coomassie Brilliant Blue; * represents GST or GST fusion proteins.

FIGURE 2. Cyclin E/Cdk2 phosphorylates PHF8 at Ser-844. A, GST or GST-PHF8 were incubated with GST-cyclin E and GST-Cdk2 for the *in vitro* kinase assay. B, GST-PHF8 (1-316), GST-PHF8 (317-680) and GST-PHF8 (681-1024) were incubated with GST-Cdk2 and GST-cyclin E for the *in vitro* kinase assay. C, Identification of phosphorylation site(s) of PHF8 (681-1024) by HPLC-ESI/MS/MS spectrometry. Purified GST-PHF8 (681-1024) was incubated with GST-Cdk2 for the *in vitro* kinase assay in the presence of cold ATP and then subjected to mass spectrometry analysis.[y3+HPO3] indicates phosphorylation with an increasement of 80 mass unit. D, Purified GST-PHF8 (681-1024) and GST-PHF8 (681-1024) S844A mutant were incubated with GST-Cdk2 in the presence of [γ-^{32}p]ATP for the *in vitro* kinase assay. E and F, 293T cells were transfected with indicated plasmids. The cell lysates were harvested for immunoprecipitation with PHF8 antibody (E) and FLAG antibody (F) followed by immunoblotting with phosphor-Ser antibody. *CBB*, Coomassie Brilliant Blue; * represents GST or GST fusion proteins.

FIGURE 3. Knockdown of PHF8 effects H3K9me2 demethylation and cell cycle progression. A, 293T cells were transfected with control, PHF8 siRNA #1 or PHF8 siRNA #2. The cell lysates were harvested for immunoblotting with indicated antibodies. B, 293T cells were transfected with FLAG-PHF8 and FLAG-PHF8 S844A or siRNA resistance PHF8 and PHF8 S844A, together with PHF8 siRNA. The cell lysates were harvested for immunoblotting with indicated antibodies. C, HeLa cells were transfected with control or Cdk2 siRNA or PHF8 siRNA. The cell lysates were subjected to immunoblot with indicated antibodies. D, E and F, HeLa cells were transfected with control or PHF8 siRNA. Then the cells were synchronized to early S phase and release for 3 h and 6 h, and then subjected to flow cytometry analysis (D). The percentages of S/G_2/M phase cells in different groups were calculated (E). The cell

lysates were subjected to immunoblot with indicated antibodies (F). *, $p<0.01$.

FIGURE 4. Phosphorylation of PHF8 S844 promotes H3K9me2 demethylation and S phase progression. A, 293T cells were transfected with indicated plasmids. The cell lysates were harvested and subjected to immunoblotting analysis. B and C, HeLa cells were transfected with control, pCMV FLAG-PHF8 or pCMV FLAG-PHF8-S844A and pCMV GFP-H2B as sorting marker. The cells were synchronized at early S phase by double thymidine treatment, then release for 3 h and 6 h and subjected to flow-cytometry analysis (B). The percentages of $S/G_2/M$ phase cells in different groups were calculated (C). *, $p<0.05$.

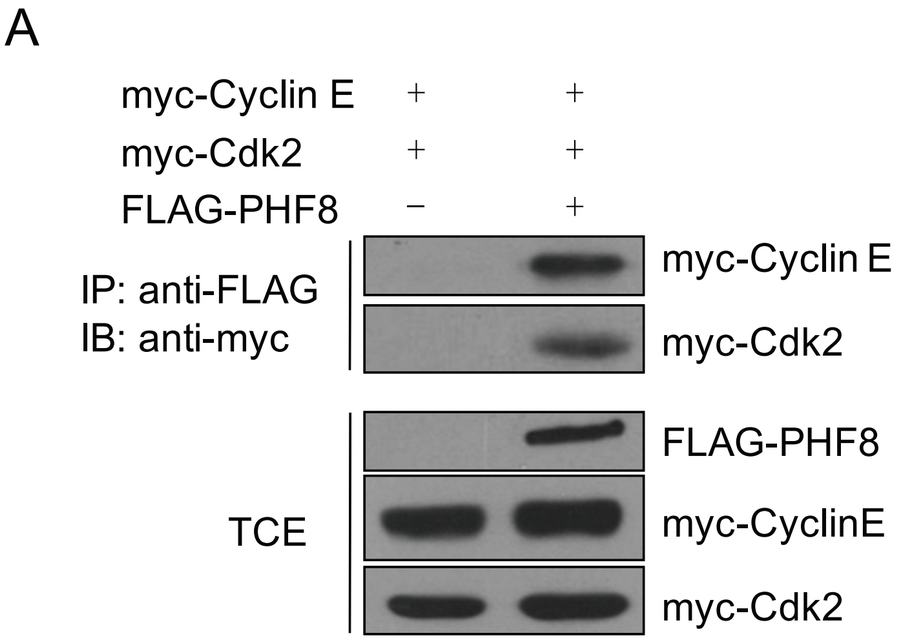
FIGURE 5. PHF8 regulates the transcription of cyclin E, E2F3 and E2F7**.** A, B, C, D and E, HeLa cells were transfected with pCMV PHF8, pCMV PHF8-S844A or empty vector as control. Total RNAs from transfected cells were extracted and subjected to real-time PCR for cyclin E (A), E2F3 (B) E2F7 (C) and GAPDH (D) with β-actin as internal control. HeLa cells were transfected with indicated plasmids. The cell lysates were subjected to immunoblot with indicated antibodies (E). F, G and H, HeLa cells were knocked down with siRNA control, Cdk2, PHF8 and Cdk2&PHF8. Total RNAs from transfected cells were extracted and subjected to real-time PCR for cyclin E (F), E2F3 (G) and E2F7 (H). *, $p < 0.05$; **, $p < 0.01$.

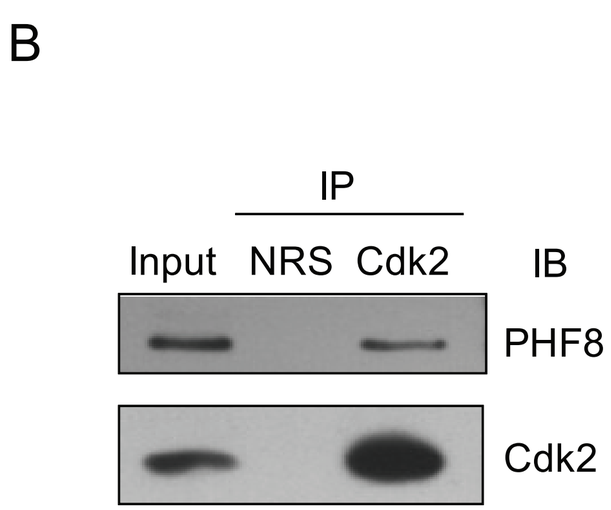
FIGURE 6. Phosphorylation of PHF8 influences its binding to cyclin E promoter. A, B, C, D and E, HeLa cells were transfected with pCMV FLAG-PHF8, pCMV FLAG-PHF8-S844A or empty vector as control. The cells were harvested and subjected to ChIP assays. Briefly, the cells were treated with formaldehyde and sonicated. The sheared DNAs were immunoprecipitated with FLAG or H3K9me2 antibodies, and then subjected to PCR with primers for cyclin E promoter (A, C) and quantitative PCR assay on cyclin E promoter (B, D). E, The cell lysates were subjected to immunoblotting analysis. F, G, and H, HeLa cells were transfected with Cdk2 siRNA. The Cdk2 knockdown efficiency was confirmed by immunoblotting analysis (F). The cells were harvested and subjected to ChIP assays with primers for cyclin E promoter as above (G, H).

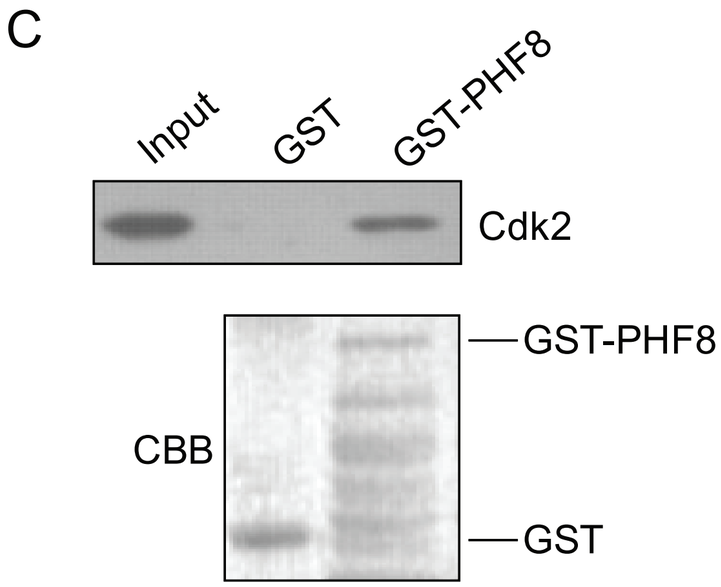
FIGURE 7. Phosphorylation of PHF8 promotes rRNA transcription. A, 293T cells were transfected with PHF8 siRNA or control siRNA. The total RNAs from transfected cells were extracted and subjected to real-time PCR for pre-rRNA. B and C, 293T cells were transfected with pCMV FLAG-PHF8, pCMV FLAG-PHF8-S844A or empty vector as control together with and pRL-TK as internal control. The cell lysates were harvested and subjected to dual-luciferase assay (B). The pre-rRNA in transfected cells were quantified by RT-PCR, with GADPH as internal control (C). D, E and F, HeLa cells were transfected with pCMV FLAG-PHF8, pCMV FLAG-PHF8-S844A or empty vector as control. The cells were harvested and subjected to ChIP assays with primers for rDNA promoter.(D, E) The immunoblotting analysis was performed to check the expression of PHF8 and PHF8-S844A (F). G, H and I, 293T cells were transfected with Cdk2 siRNA or control siRNA, and rDNA luciferase reporter. The cell lysates were subjected to luciferase assay (G). The total RNAs from transfected cells were extracted and used for real-time PCR for pre-rRNA (H). The

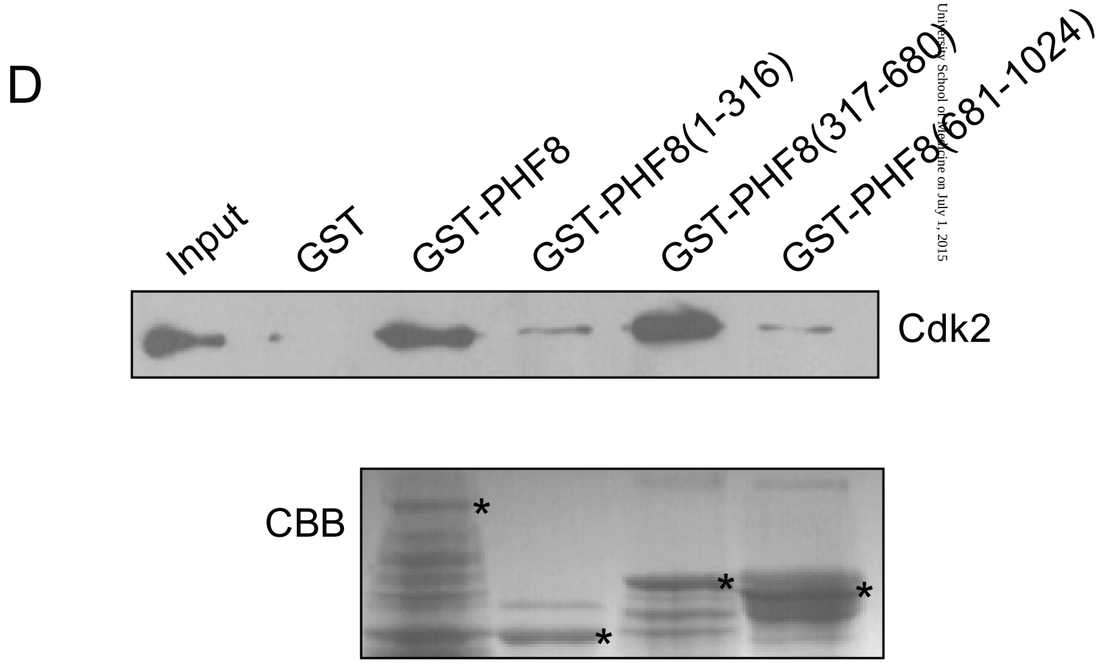
immunoblotting analysis was performed to check the expression of Cdk2 (I). *, $p<0.05$; **, $p<0.01$.

Figure 1









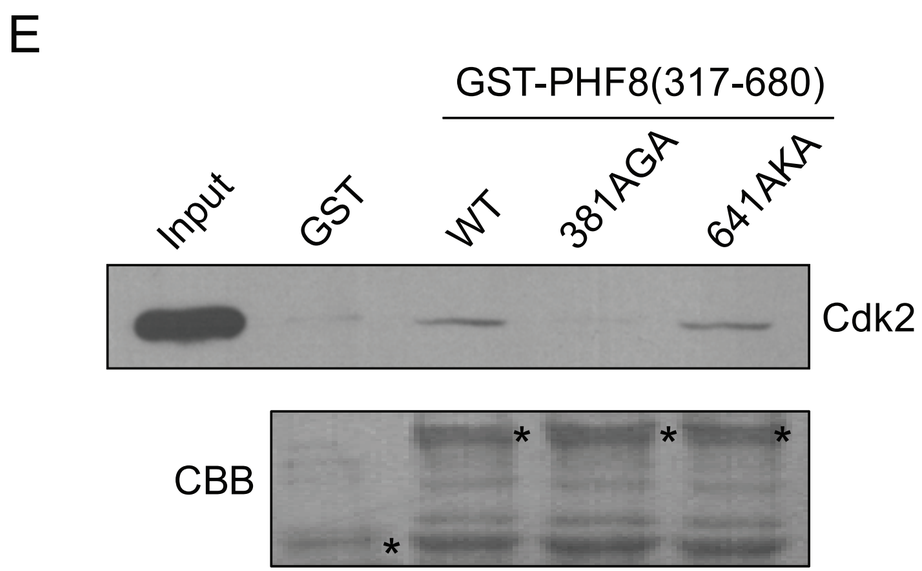
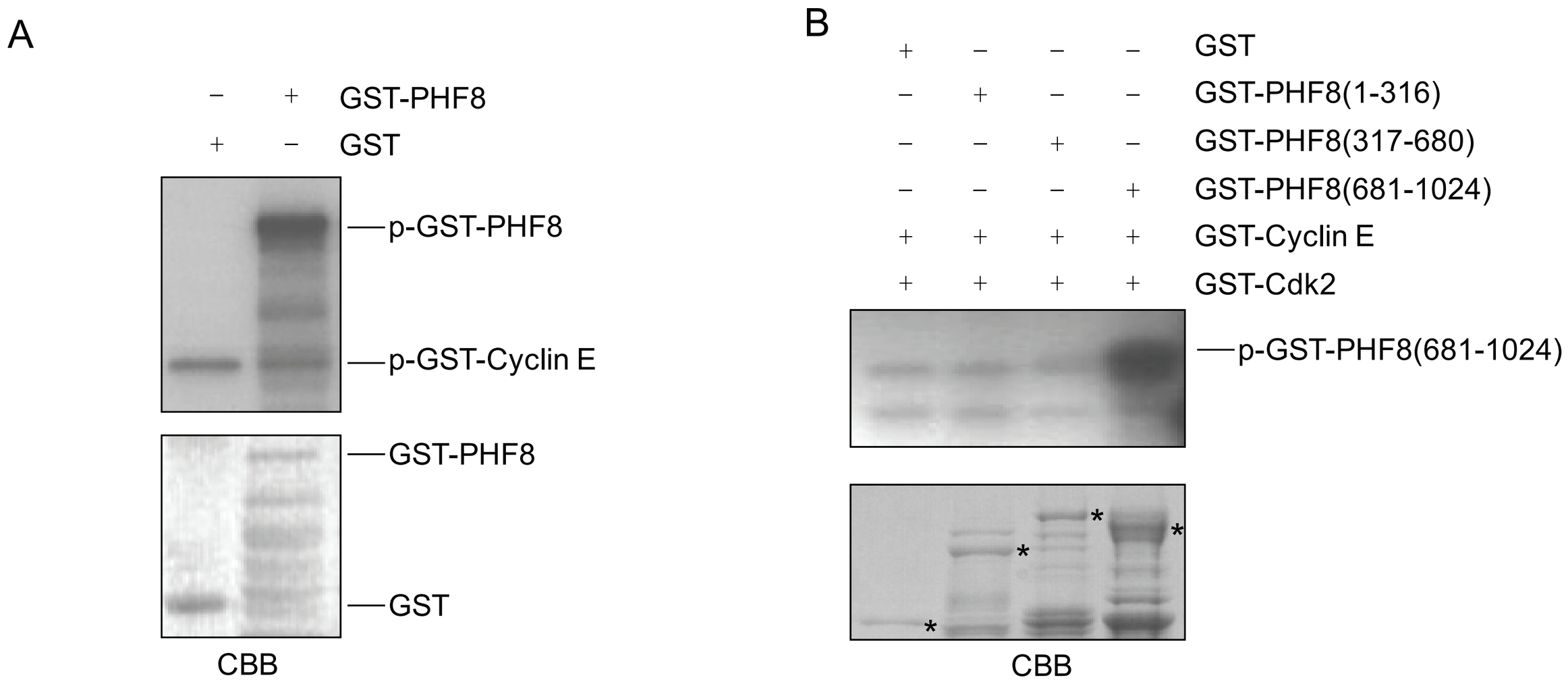
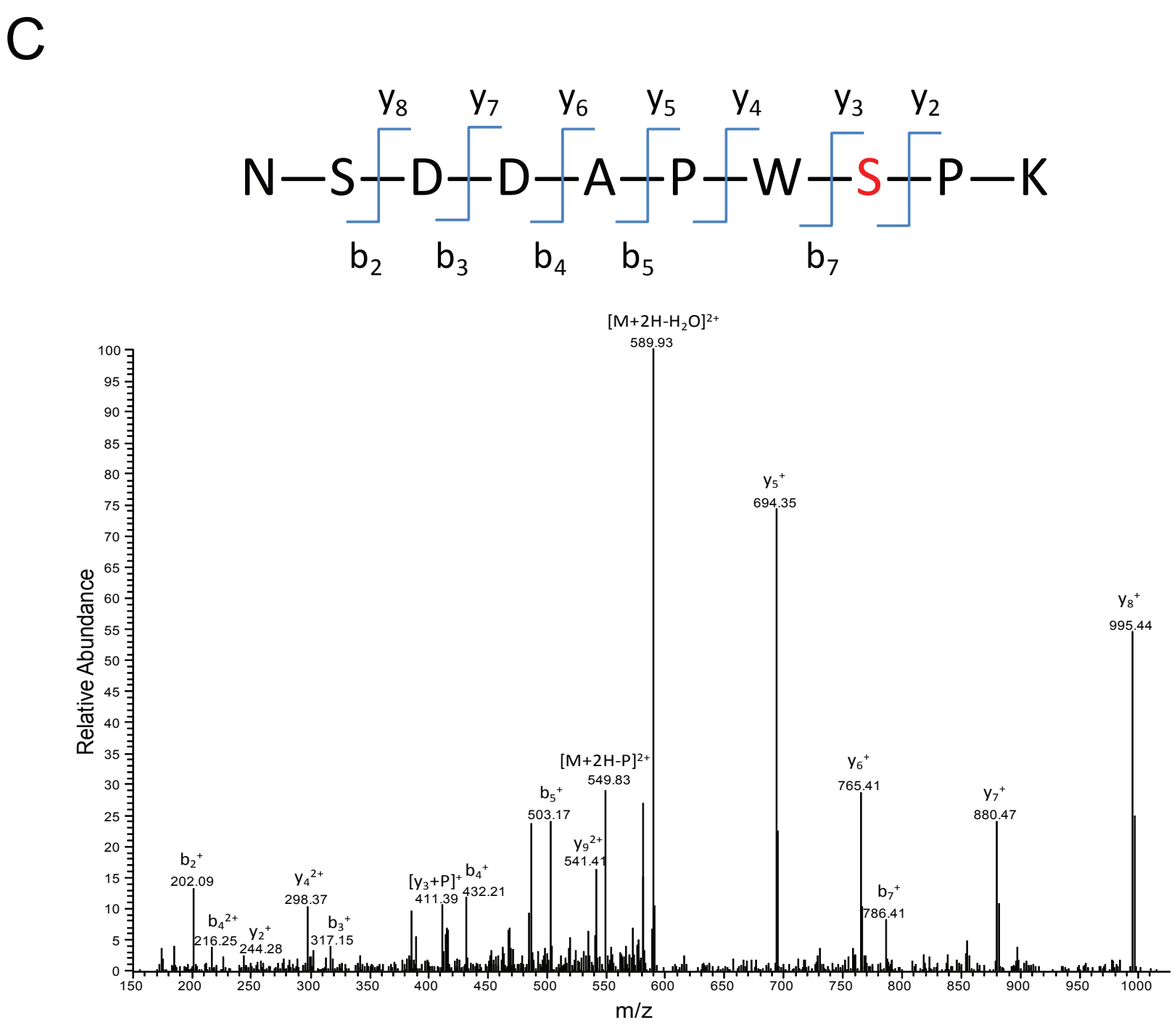
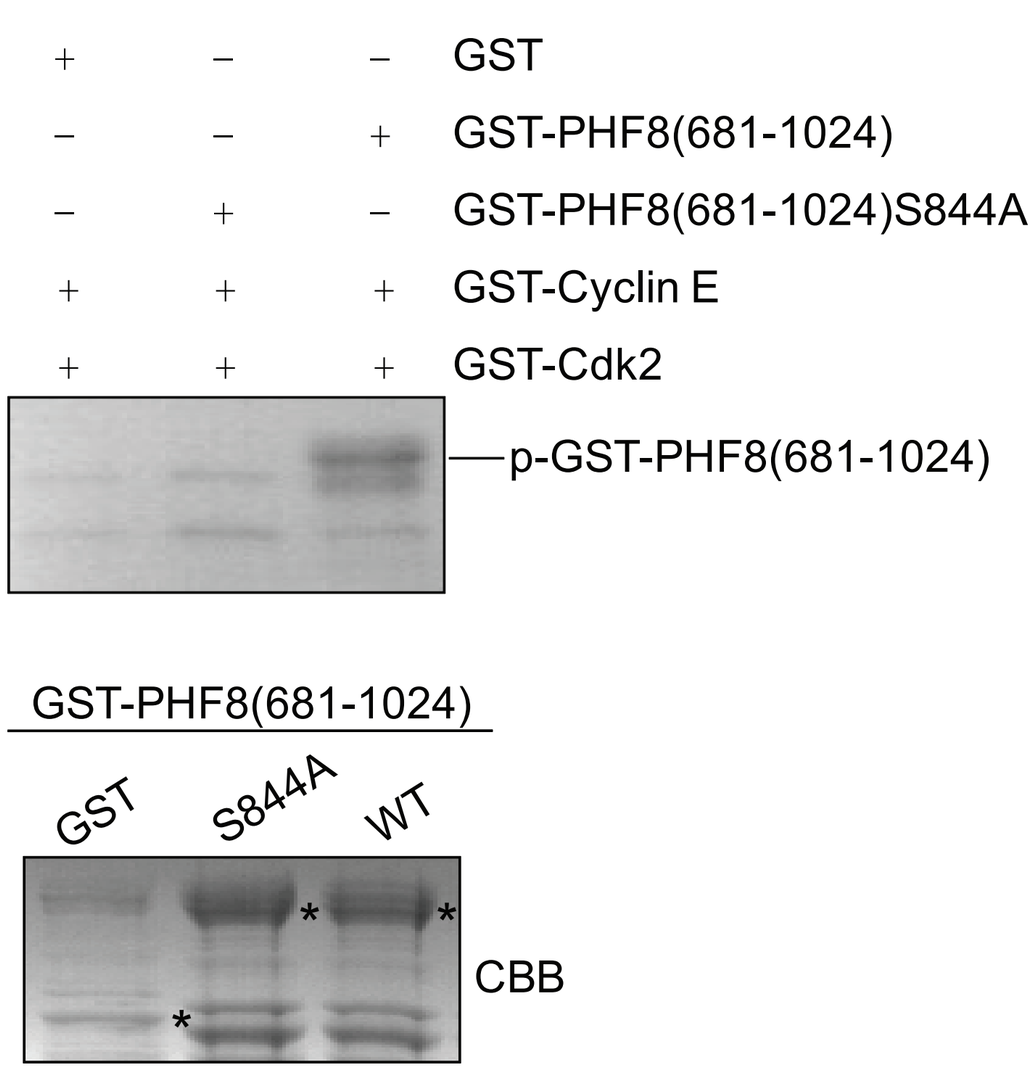
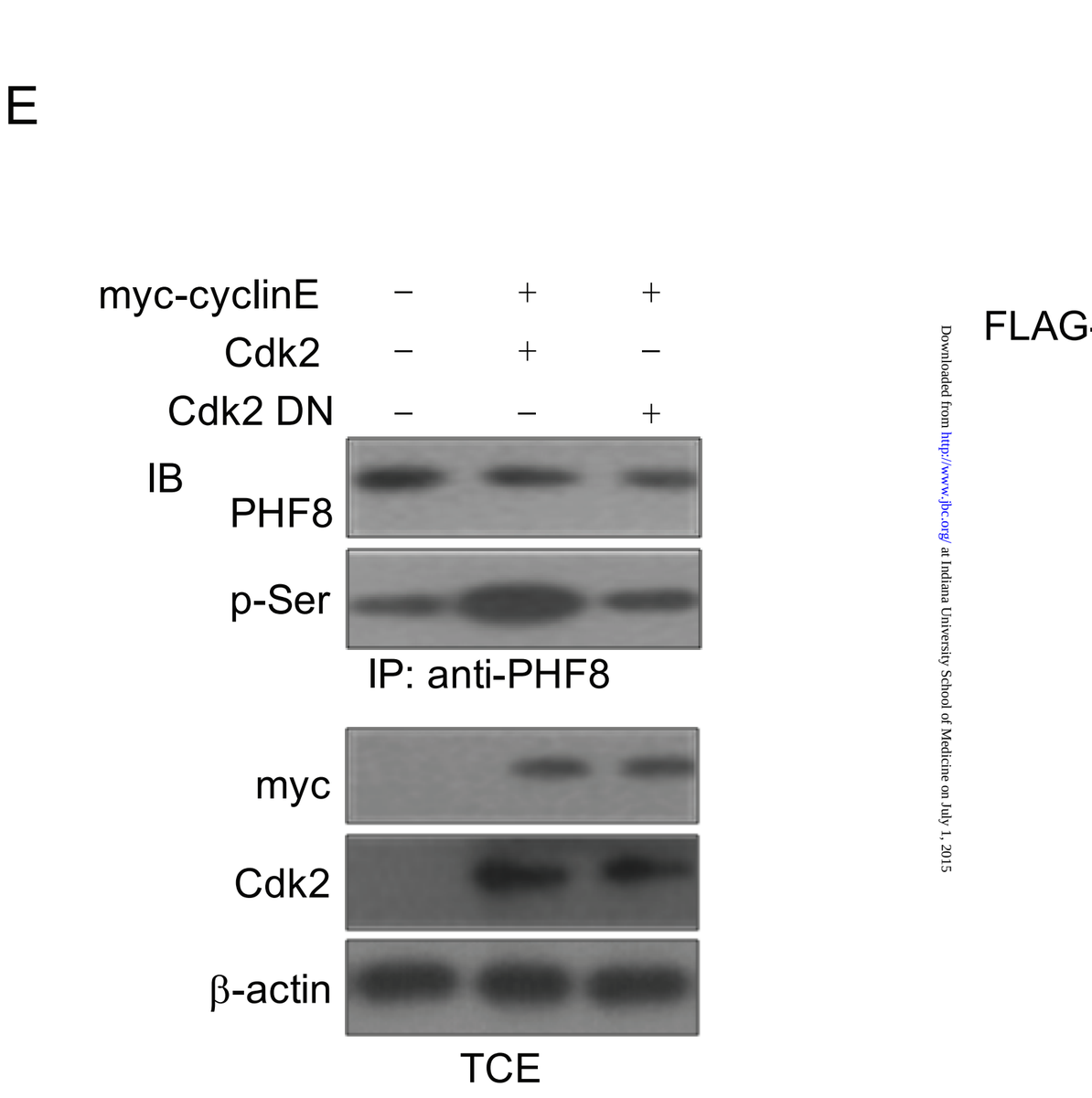


Figure 2









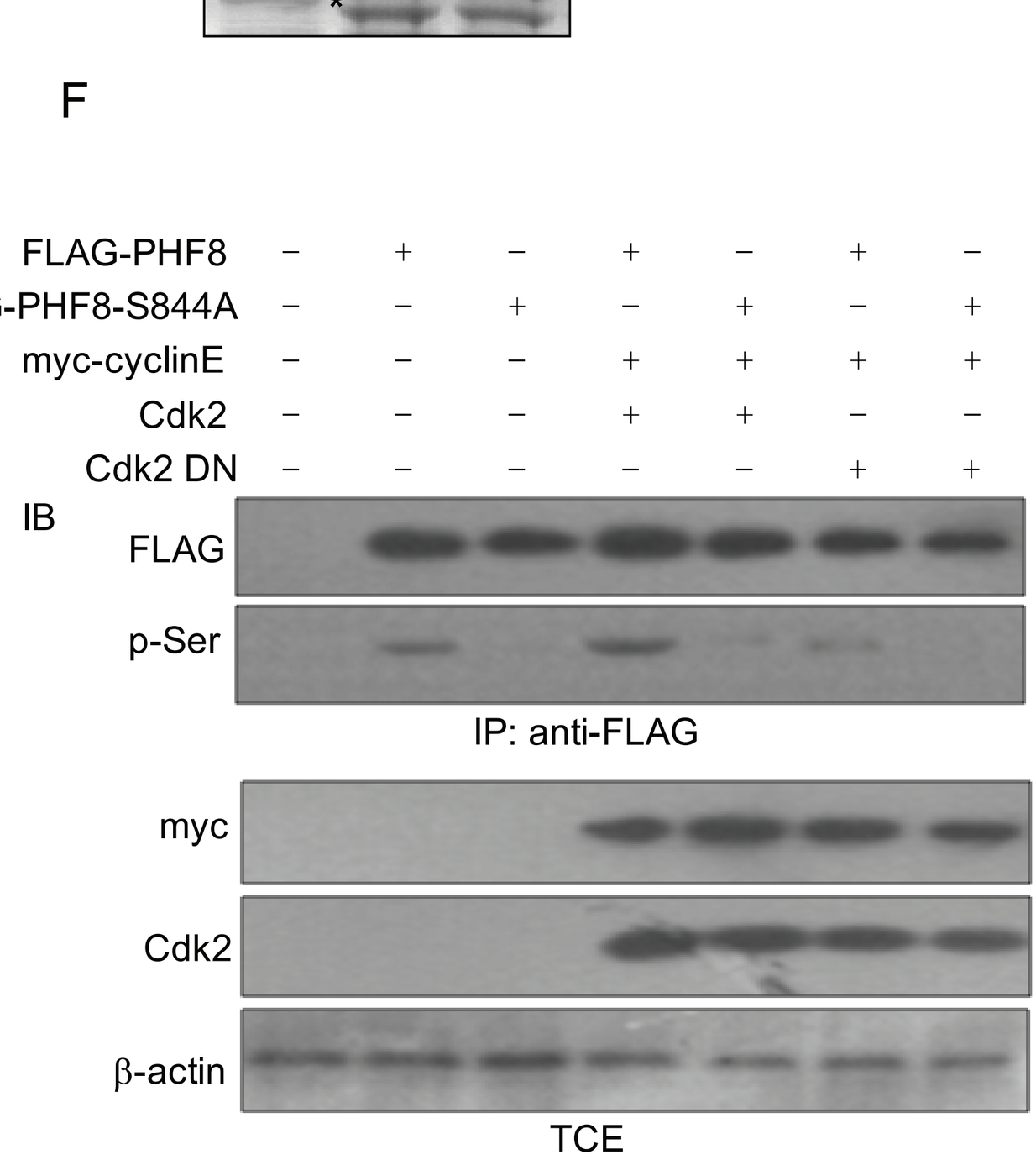
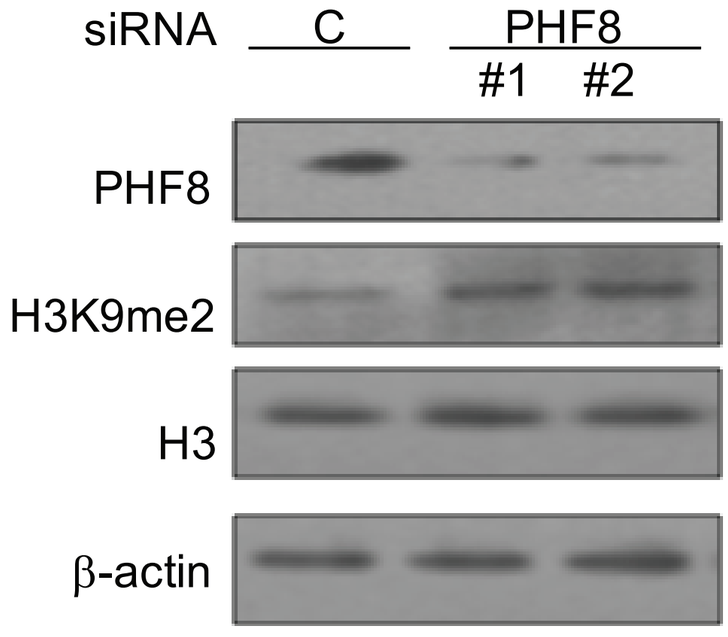


Figure 3

A



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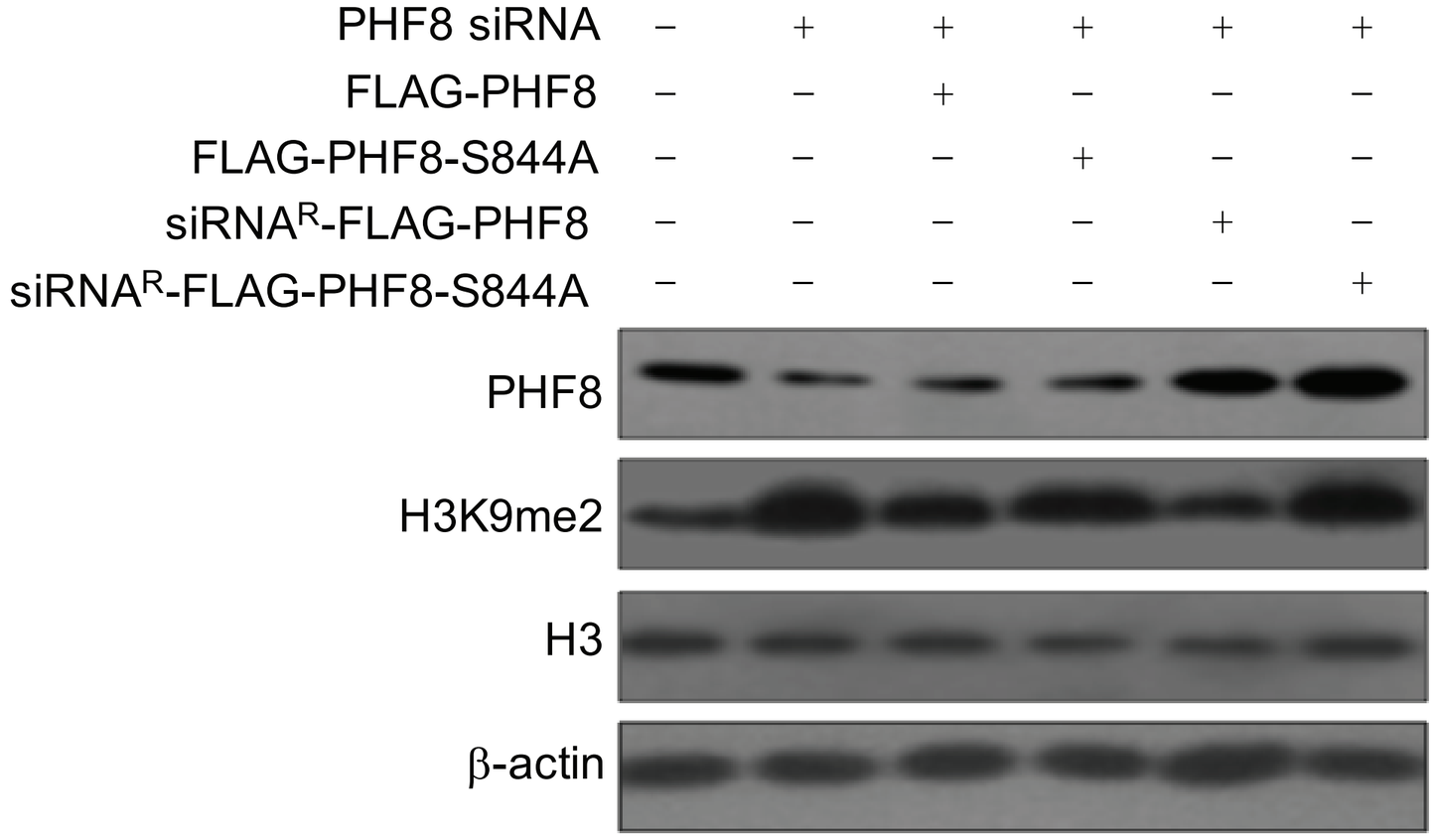

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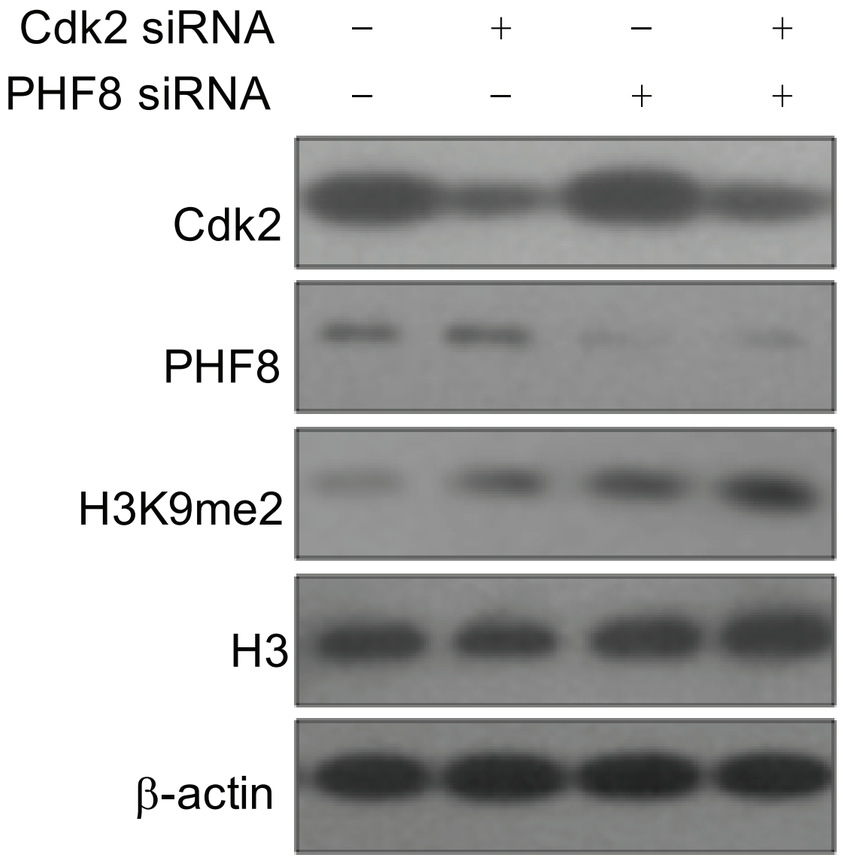

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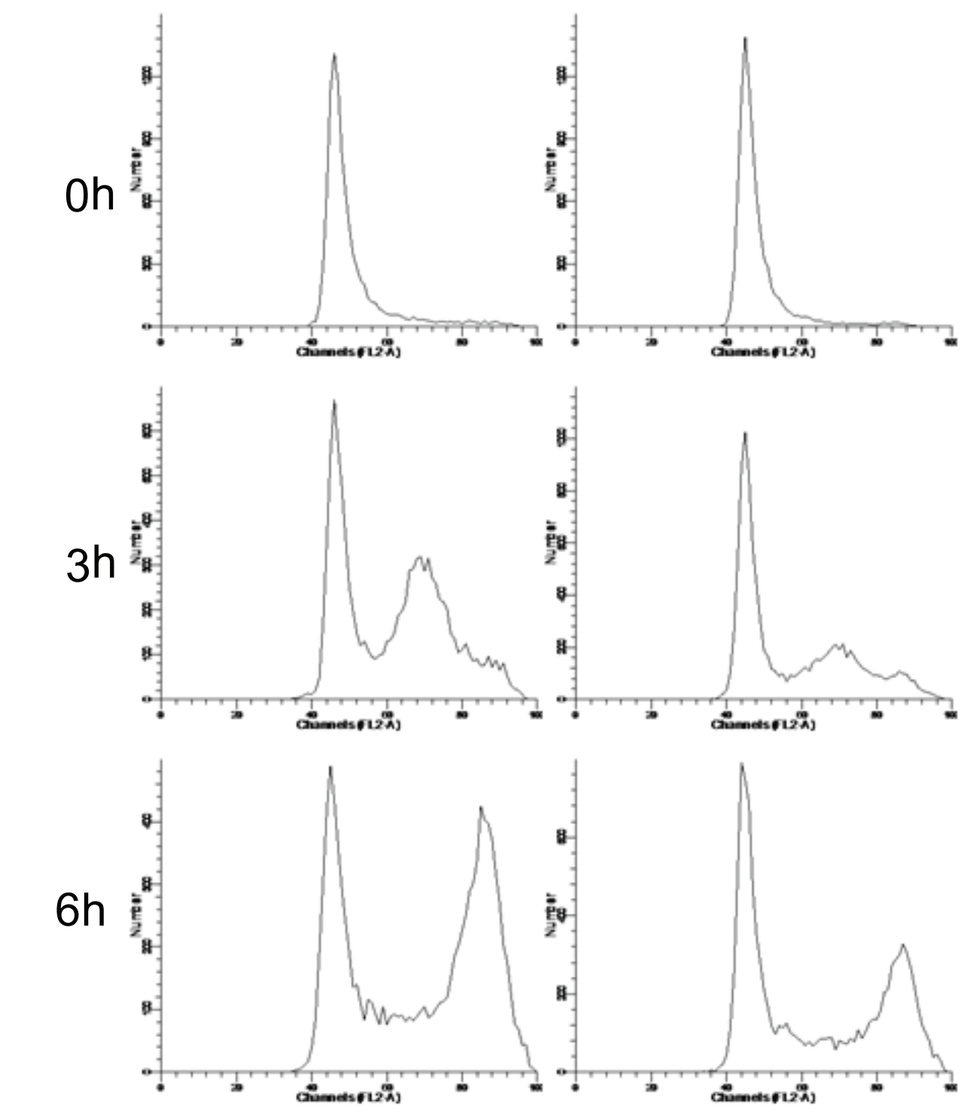

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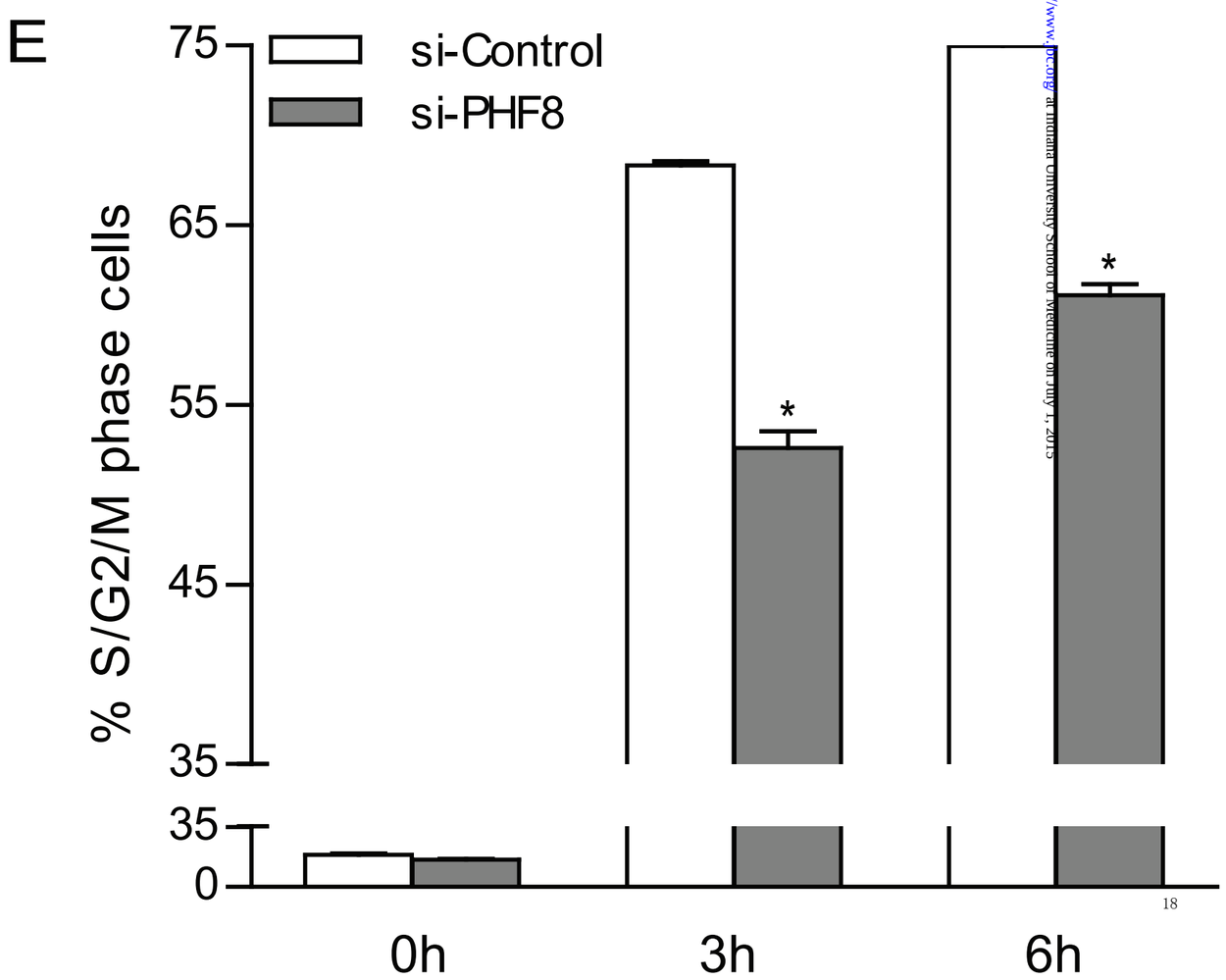

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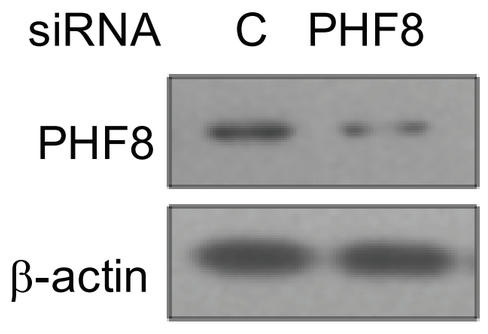

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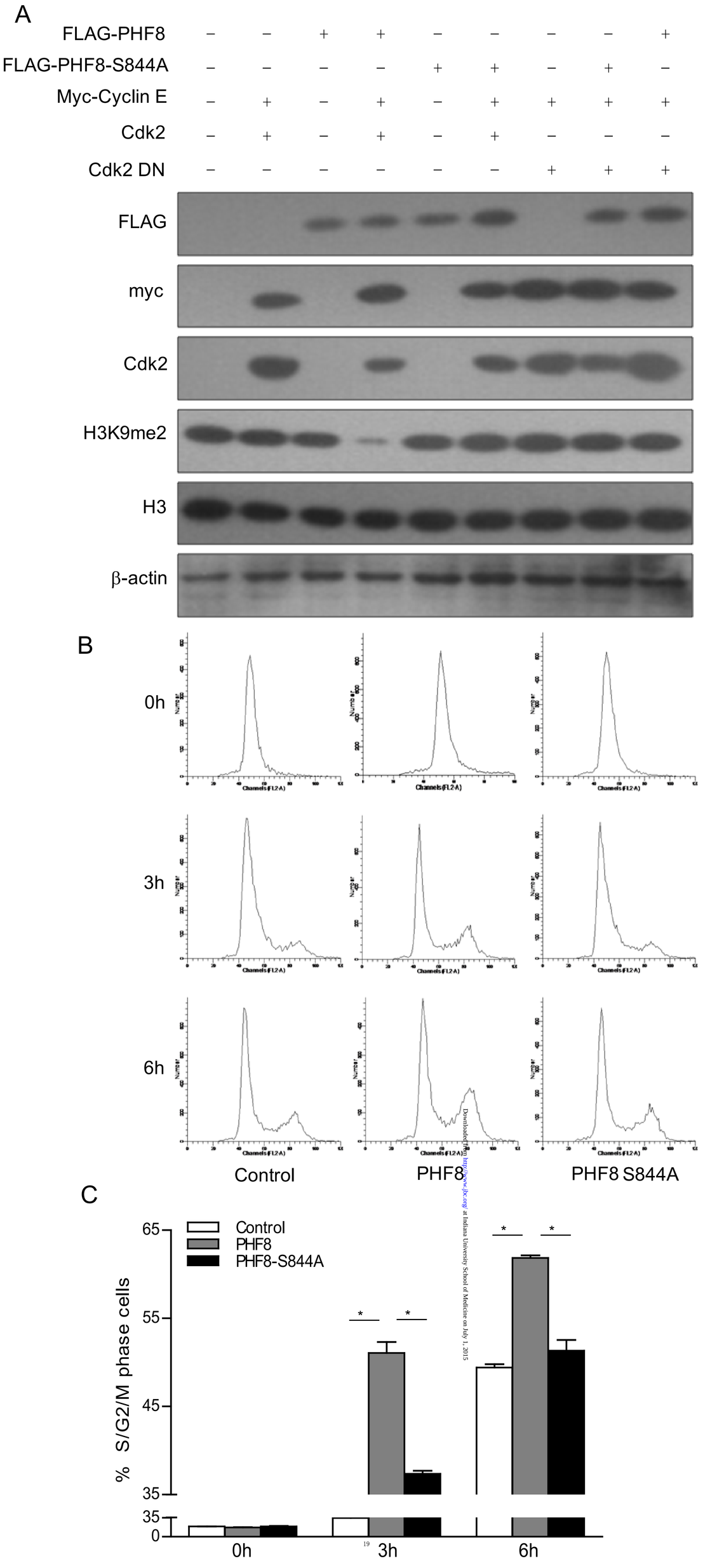
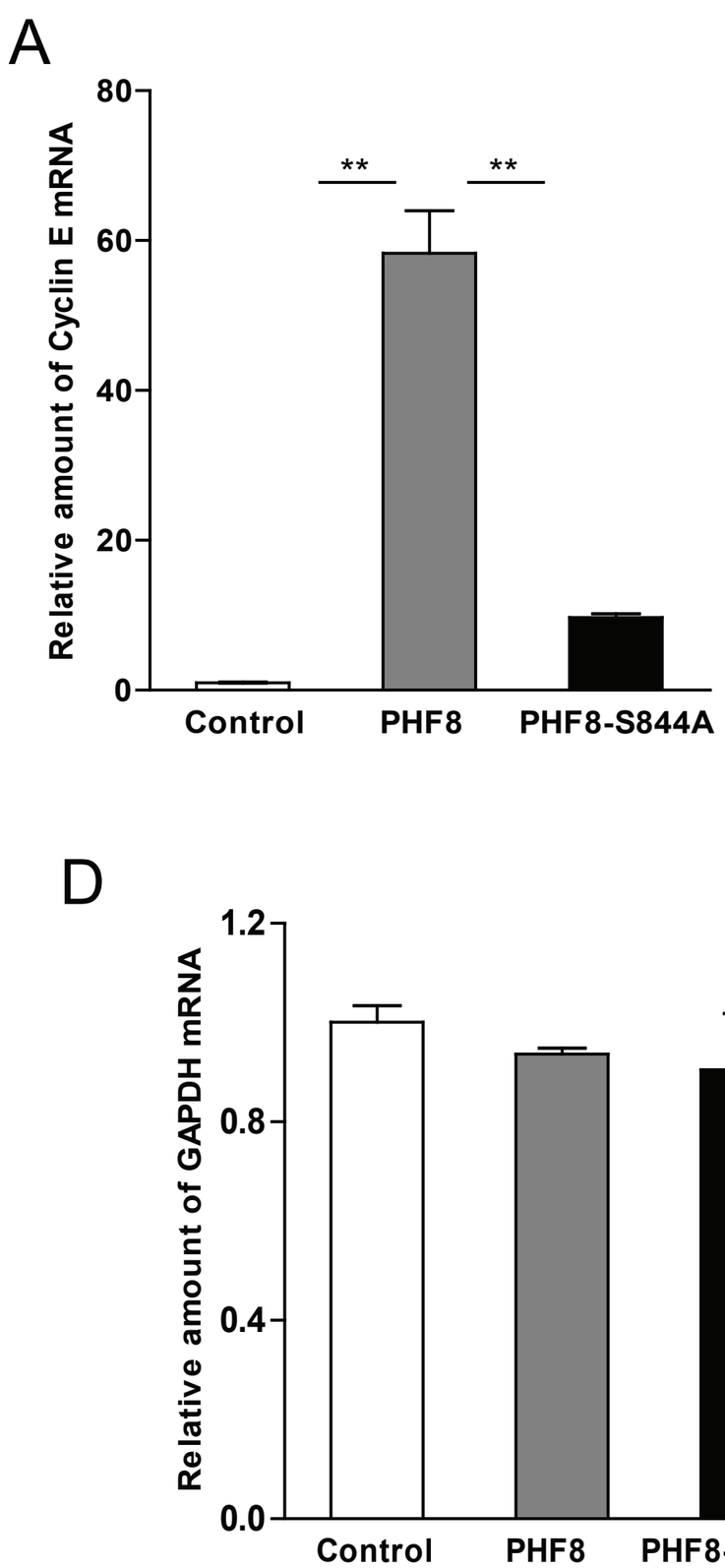
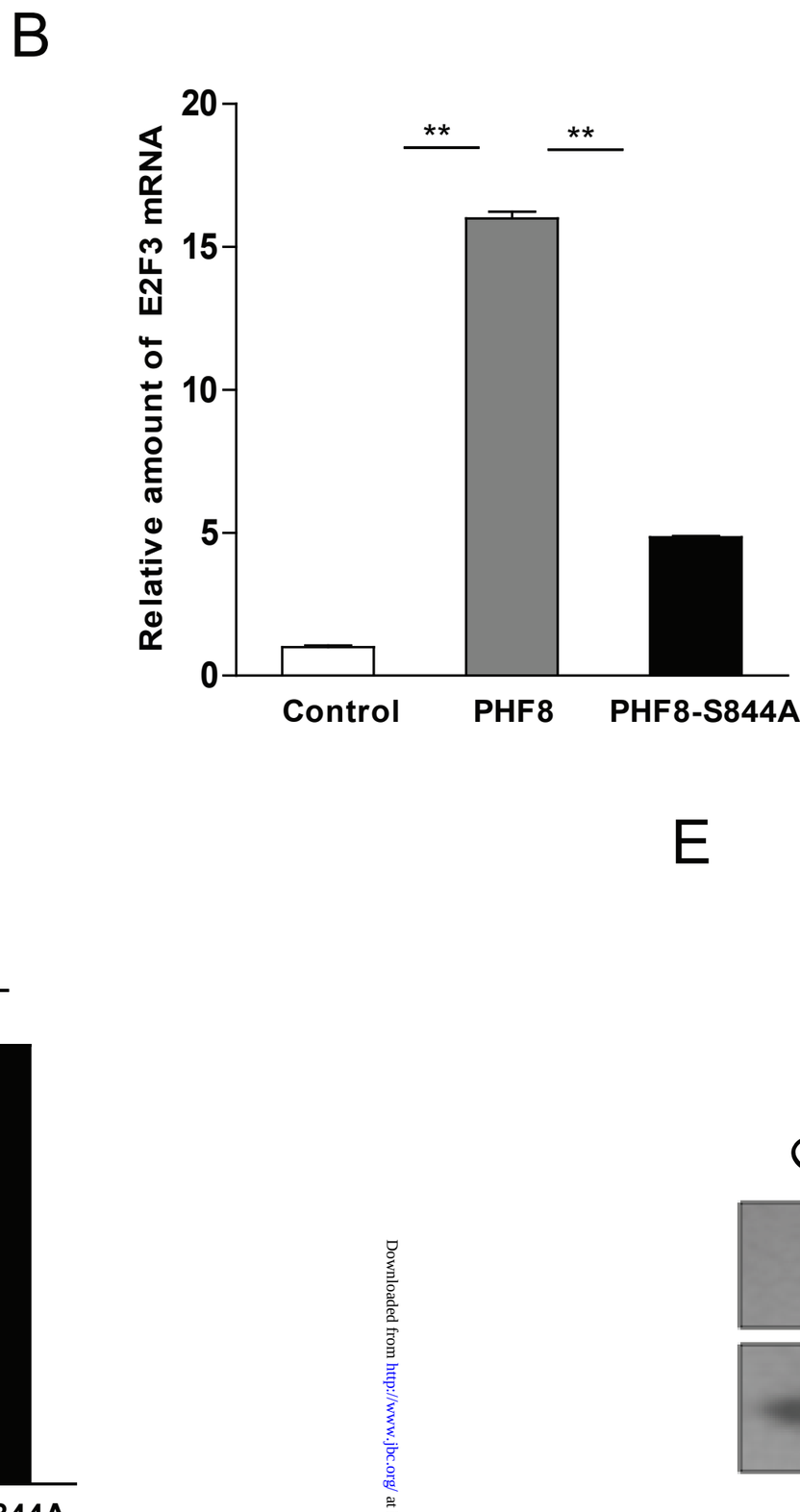
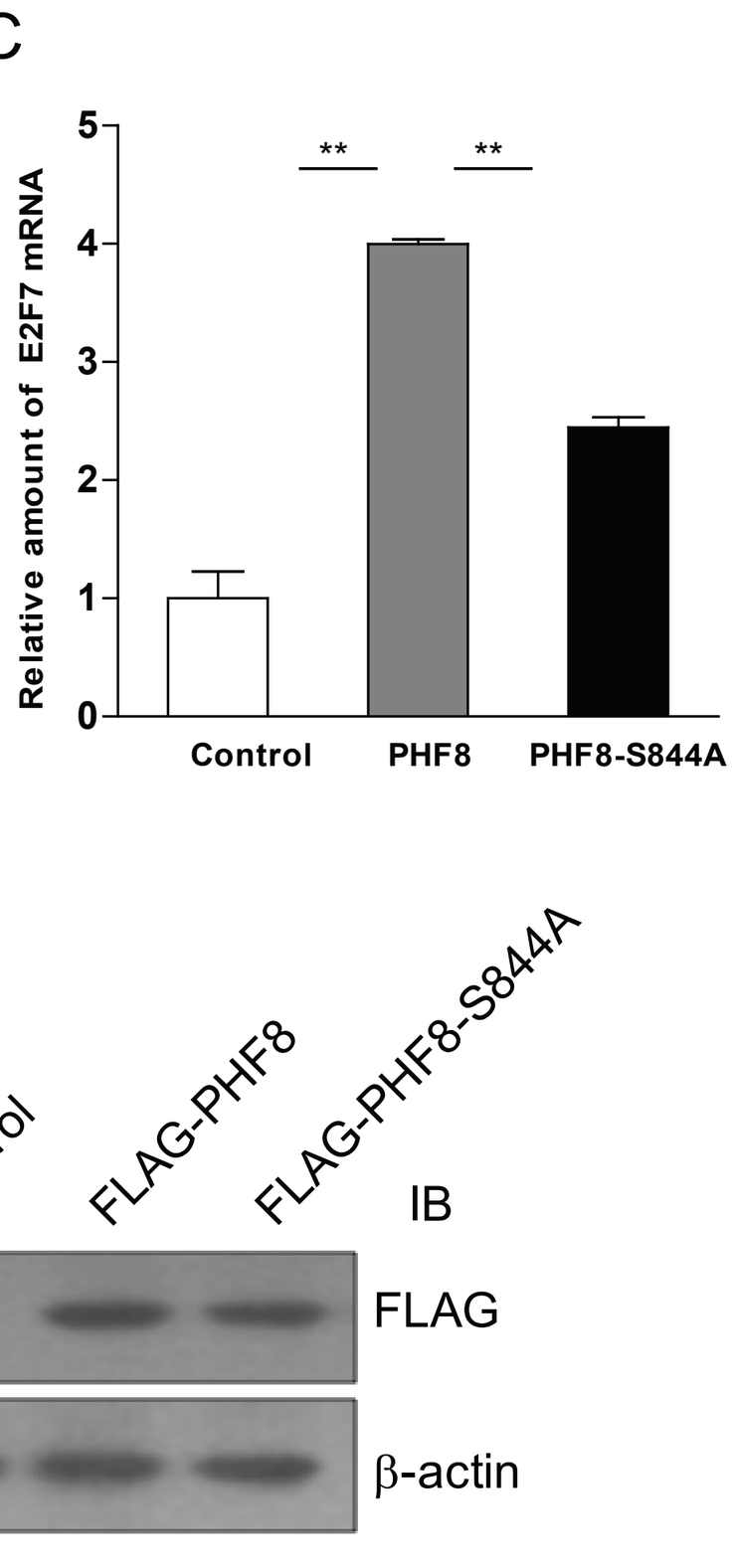
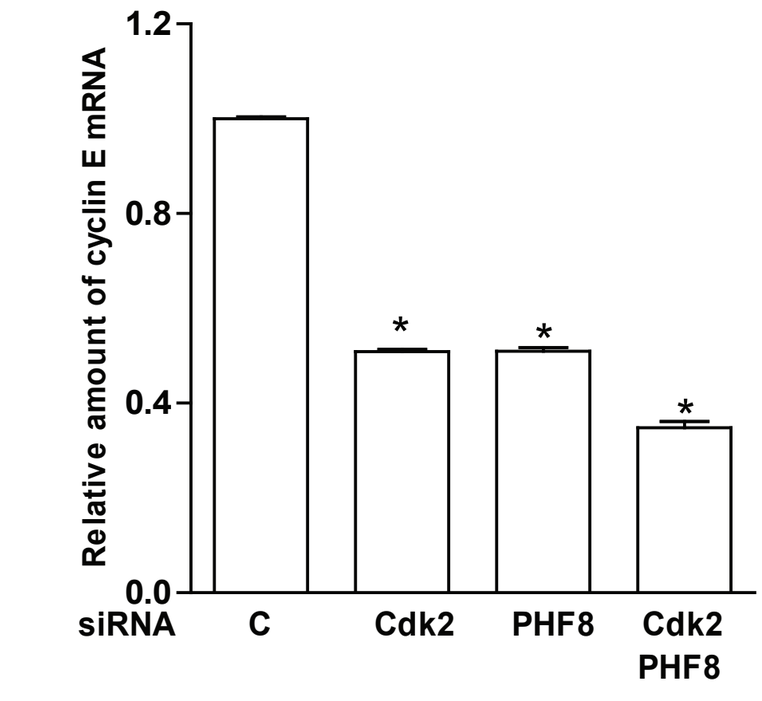


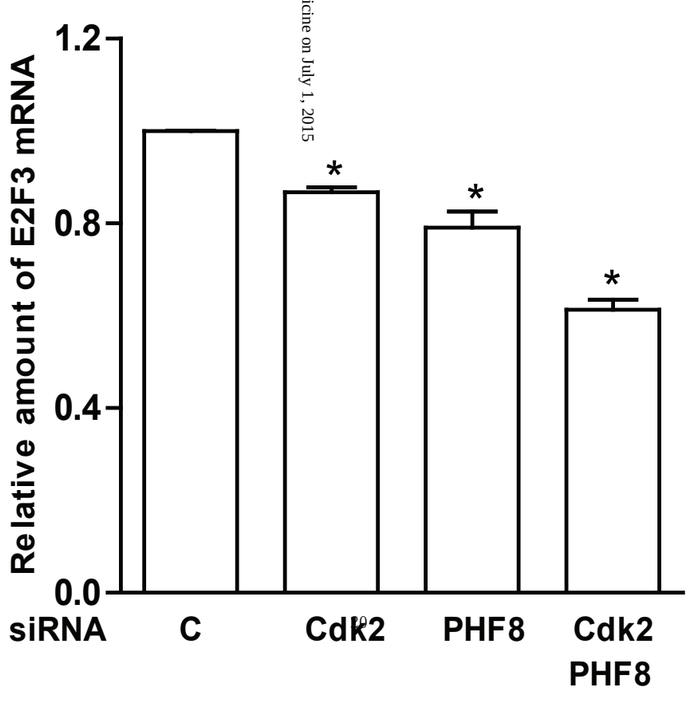
Figure 5











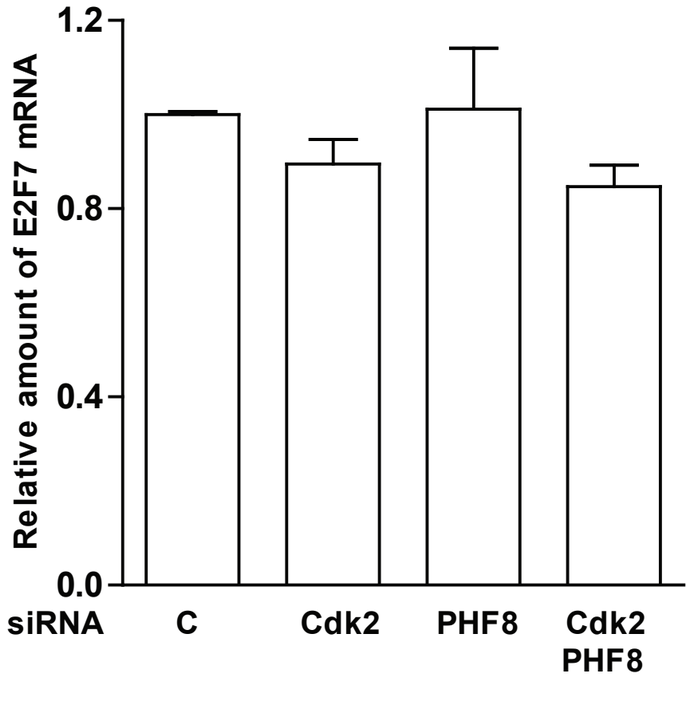
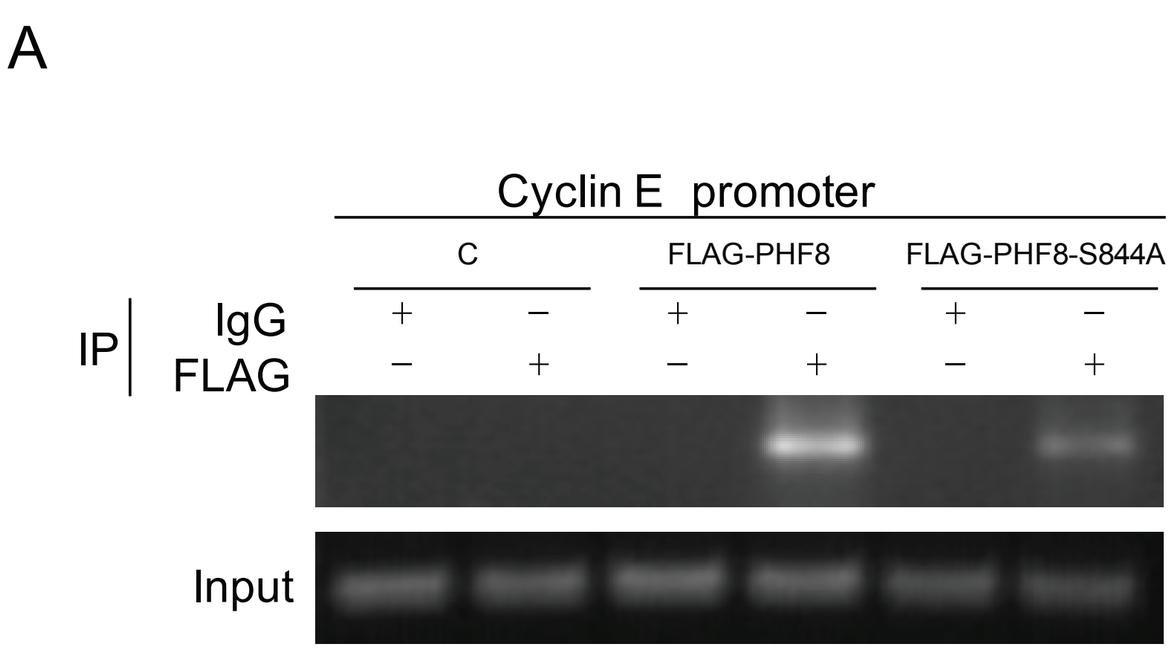
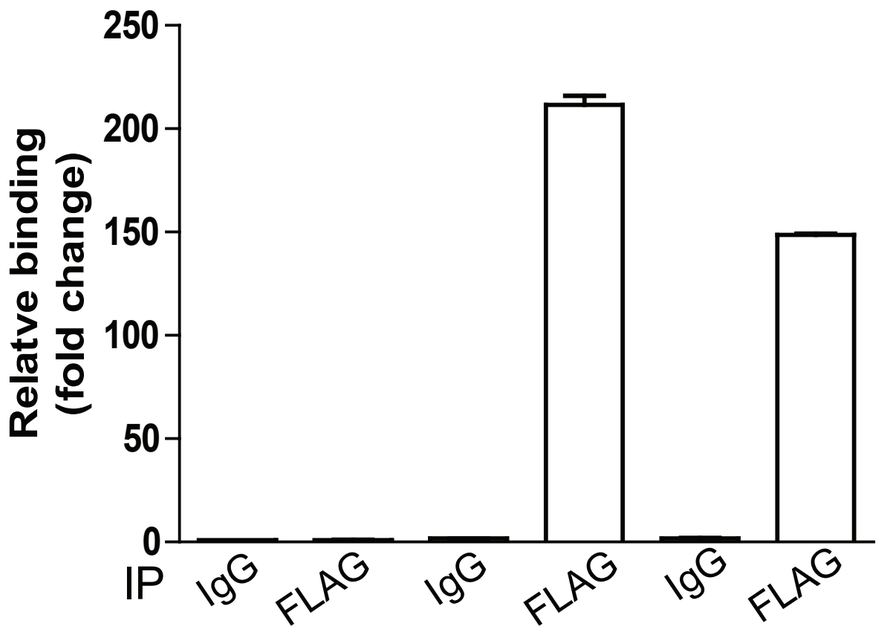
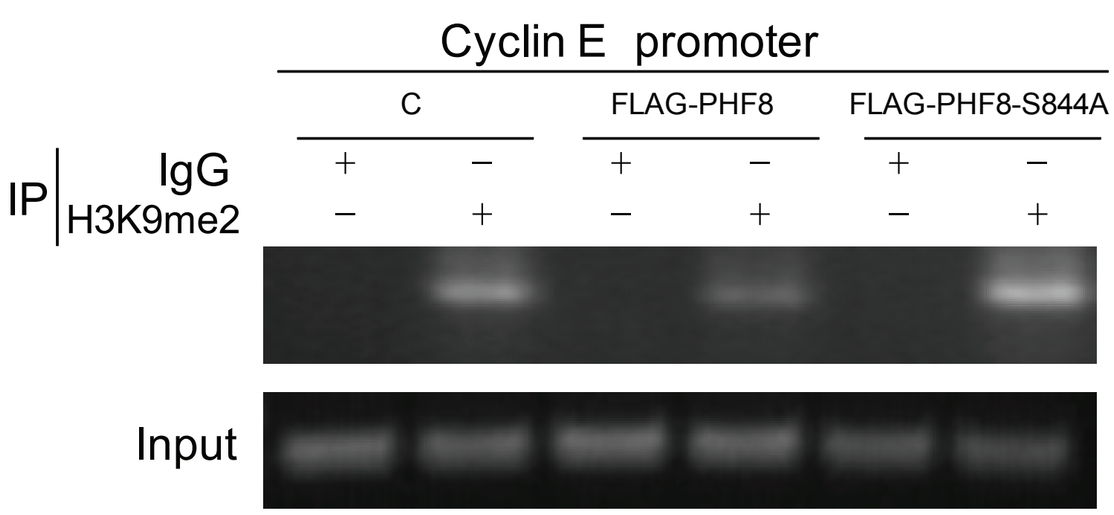
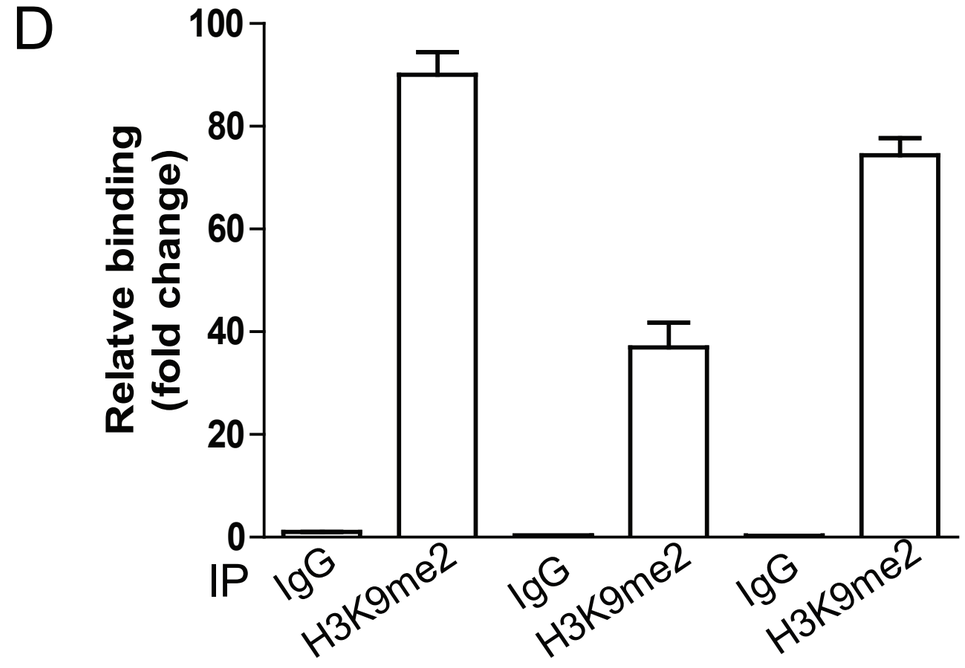


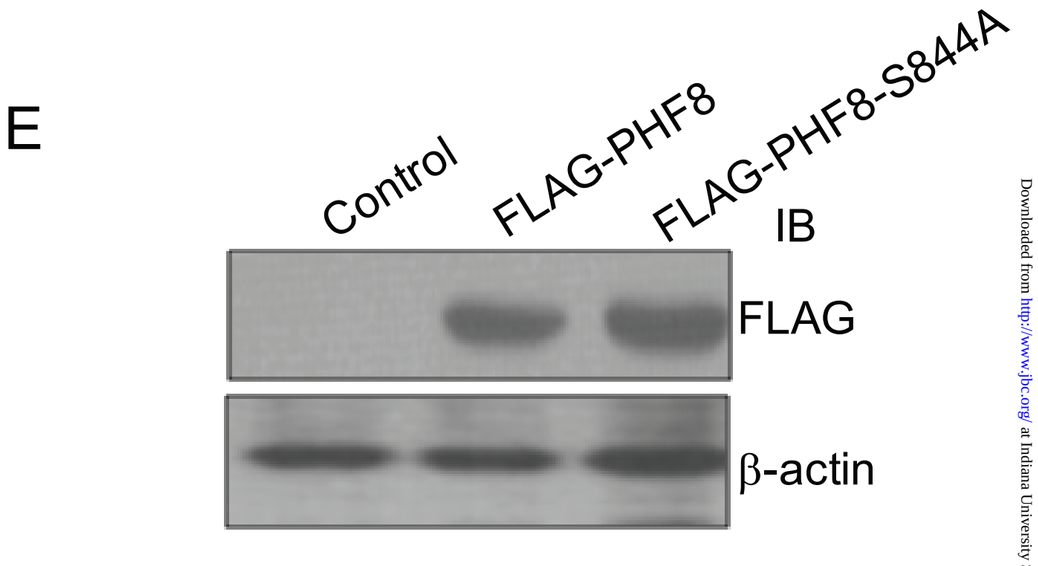
Figure 6

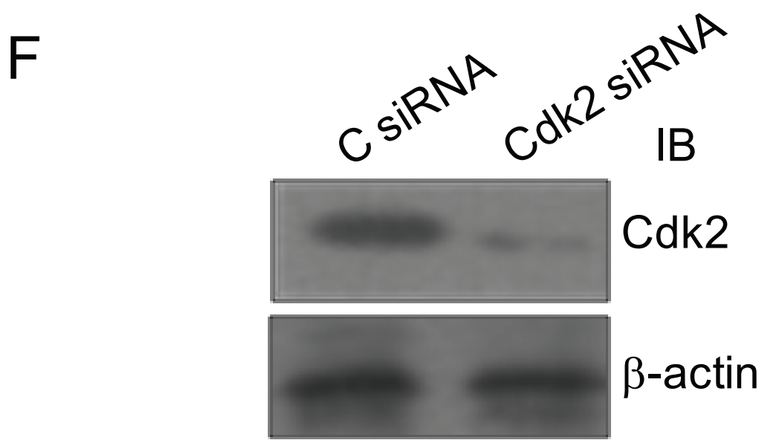


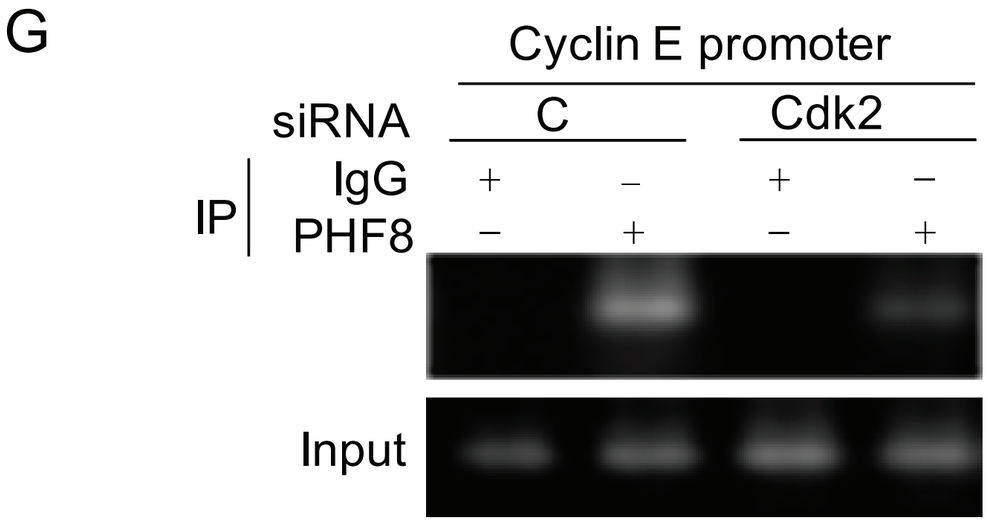












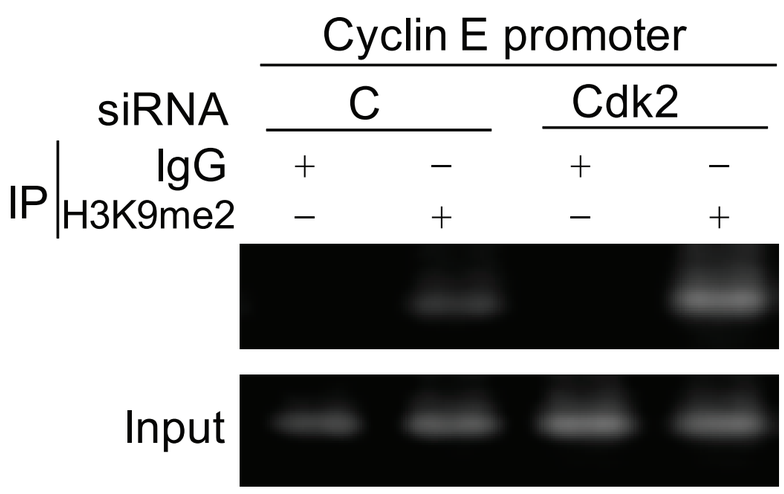
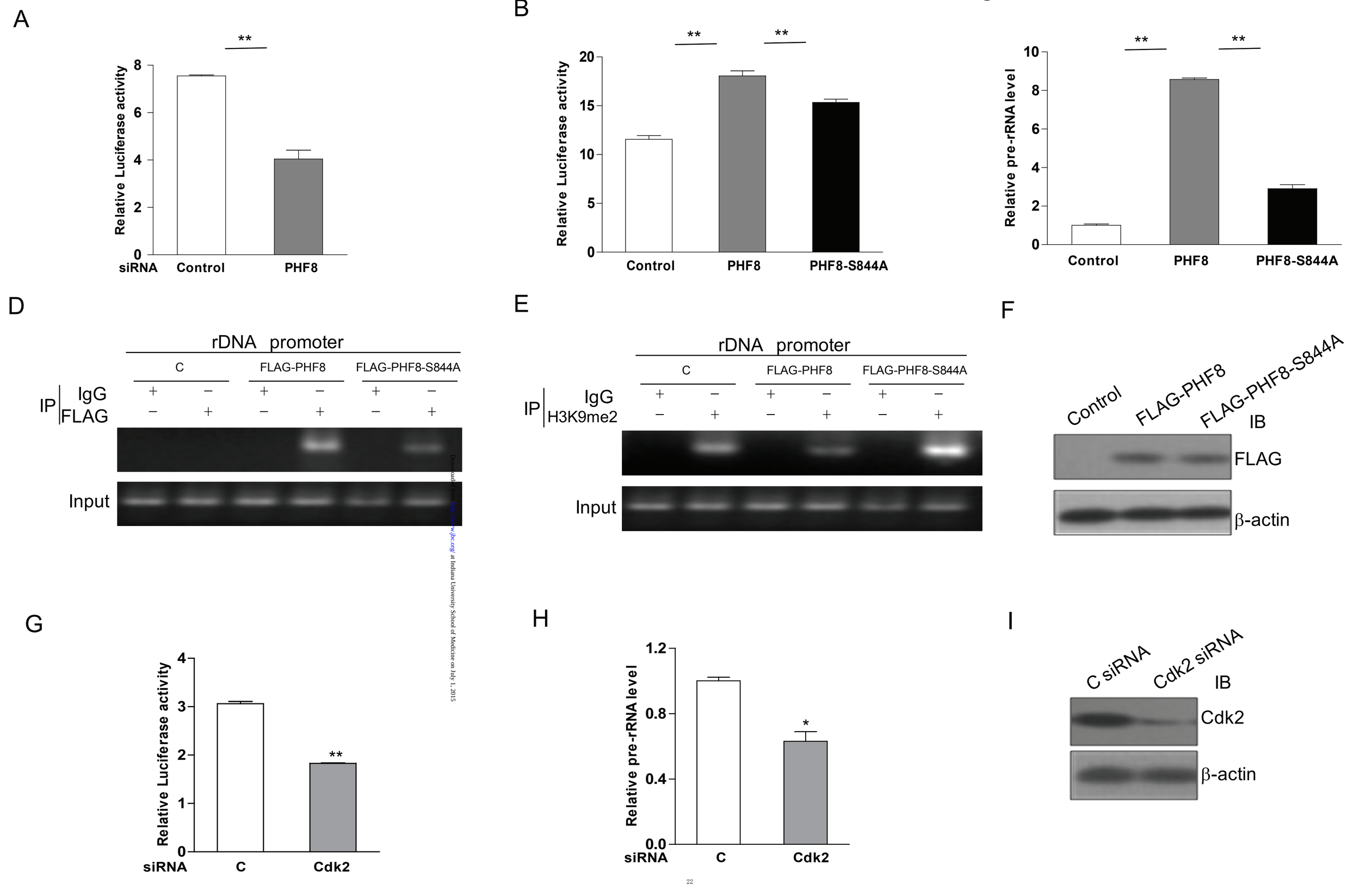
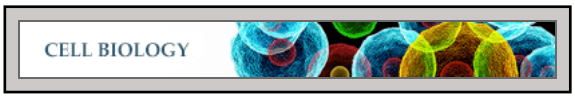


Figure 7



Cell Biology:

Cyclin E/Cdk2 Phosphorylates Plant Homeodomain Finger Protein 8 (PHF8) and Regulates Its Function in Cell Cycle



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