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RESEARCH ARTICLE

Dynamic Phosphorylation and Dephosphorylation of Cyclase-Associated Protein 1 by Antagonistic Signaling through Cyclin-Dependent Kinase 5 and cAMP Are Critical for the Protein Functions in Actin Filament Disassembly and Cell Adhesion

Haitao Zhang,[a] Auburn Ramsey,[b] Yitong Xiao,[c] Uddhab Karki,[a,d] Jennifer Y. Xie,[c] Jianfeng Xu,[a,d,e] Thomas Kelly,[f,g] Shoichiro Ono,[h] Guo-Lei Zhou[a,b]

[a]Molecular Biosciences Graduate Program, Arkansas State University, Jonesboro, Arkansas, USA
[b]Department of Biological Sciences, Arkansas State University, Jonesboro, Arkansas, USA
[c]Department of Basic Sciences, New York Institute of Technology College of Osteopathic Medicine at Arkansas, Jonesboro, Arkansas, USA
[d]Arkansas Biosciences Institute, Arkansas State University, Jonesboro, Arkansas, USA
[e]College of Agriculture and Technology, Arkansas State University, Jonesboro, Arkansas, USA
[f]Department of Pathology, University of Arkansas for Medical Sciences, Little Rock, Arkansas, USA
[g]Winthrop P. Rockefeller Cancer Institute, University of Arkansas for Medical Sciences, Little Rock, Arkansas, USA
[h]Department of Pathology and Department of Cell Biology, Winship Cancer Institute, Emory University, Atlanta, Georgia, USA

ABSTRACT Cyclase-associated protein 1 (CAP1) is a conserved actin-regulating protein that enhances actin filament dynamics and also regulates adhesion in mammalian cells. We previously found that phosphorylation at the Ser307/Ser309 tandem site controls its association with cofilin and actin and is important for CAP1 to regulate the actin cytoskeleton. Here, we report that transient Ser307/Ser309 phosphorylation is required for CAP1 function in both actin filament disassembly and cell adhesion. Both the phosphomimetic and the nonphosphorylatable CAP1 mutant, which resist transition between phosphorylated and dephosphorylated forms, had defects in rescuing the reduced rate of actin filament disassembly in the CAP1 knockdown HeLa cells. The phosphorylation mutants also had defects in alleviating the elevated focal adhesion kinase (FAK) activity and the enhanced focal adhesions in the knockdown cells. In dissecting further phosphoregulatory cell signals for CAP1, we found that cyclin-dependent kinase 5 (CDK5) phosphorylates both Ser307 and Ser309 residues, whereas cAMP signaling induces dephosphorylation at the tandem site, through its effectors protein kinase A (PKA) and exchange proteins directly activated by cAMP (Epac). No evidence supports an involvement of activated protein phosphatase in executing the dephosphorylation downstream from cAMP, whereas preventing CAP1 from accessing its kinase CDK5 appears to underlie CAP1 dephosphorylation induced by cAMP. Therefore, this study provides direct cellular evidence that transient phosphorylation is required for CAP1 functions in both actin filament turnover and adhesion, and the novel mechanistic insights substantially extend our knowledge of the cell signals that function in concert to regulate CAP1 by facilitating its transient phosphorylation.

KEYWORDS CAP1, CDK5, actin cytoskeleton, cell adhesion, cyclic AMP, protein phosphorylation

The actin cytoskeleton is essential for a variety of fundamental cell functions, such as migration, morphogenesis, cytokinesis, and phagocytosis. On the other hand, an aberrant actin cytoskeleton underlies pathological conditions like cancer and neuro-

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Address correspondence to Guo-Lei Zhou, gzhou@astate.edu.

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logical disorders. Assembly and disassembly of actin filaments must be precisely regulated spatially and temporally for actin cytoskeletal rearrangements, which are required for the actin-dependent cell functions and biological processes. A number of actin-binding proteins play important roles in actin cytoskeletal rearrangements (1, 2). Among them, the cyclase-associated protein (CAP) is a critical factor across eukaryotes for regulating actin filament dynamics through binding to both actin monomer (G-actin) and actin filament (F-actin). CAP was originally identified in the yeast *Saccharomyces cerevisiae* (where it is also known as SRV2), where it forms a complex with adenylyl cyclase to mediate regulation of the enzyme by Ras (3, 4). Whereas evidence is lacking for a role of CAP in mediating Ras signaling in higher eukaryotes, the actin-regulating functions of CAP appear to be conserved in all eukaryotes (5, 6). CAP promotes actin filament turnover through multiple mechanisms, performing much more versatile roles than the initially identified role in binding and sequestering actin monomers, which is believed to help maintain a pool of actin monomers readily available for dynamic actin cytoskeletal rearrangement (6). First, CAP binds to the side of actin filaments to promote cofilin-mediated actin filament depolymerization (7–10). Second, CAP catalyzes nucleotide exchange of actin monomers from ADP–G-actin to ATP–G-actin, which is required before the depolymerized G-actin can be polymerized efficiently into filaments again (7, 8, 11–14). Third, CAP promotes actin monomer dissociation from filament ends, in cooperation with twinfilin (15, 16).

Studies so far have found roles for CAP homologues, including mammalian CAP1, in regulating the actin cytoskeleton, cell morphology, adhesion, and migration (17). Not surprisingly, dysregulated CAP1 is also implicated in a growing list of human cancers, largely in the invasiveness of cancer cells (18–21). Depletion of CAP1 in mammalian cells universally leads to enhanced actin stress fibers, and in some cell types, it leads to increased cell size (22–24), which is comparable to a disrupted actin cytoskeleton and a swollen cell morphology observed in budding yeast with the deletion of the *CAP* gene (25). The phenotype of enhanced stress fibers is believed to derive from the loss of CAP1 function in promoting the actin filament turnover, as well as in sequestering actin monomers, since CAP1 is a key facilitator of the actin dynamics driven by cofilin/actin depolymerization factor (ADF) (8, 26). Repeated rounds of actin filament turnover drive cell movement, and accordingly, loss of the CAP1 function is expected to reduce cell motility. While it appears to be the case in certain mammalian cell types tested (18, 22), we found that knockdown of CAP1 in HeLa and metastatic breast cancer cells led to activated cell adhesion signaling, which was more than sufficient to overcome the negative effect on cell migration from the reduced actin filament turnover. As a net outcome, knockdown of CAP1 actually led to substantially increased motility in these cells (21, 23). The function of CAP1 in cell adhesion appears to be cell context dependent, leading to distinct and even opposing roles in cell migration and invasiveness (21, 23). Consistently, we demonstrated that CAP1 interacts with focal adhesion kinase (FAK) and talin (23), which likely facilitates the CAP1 function in cell adhesion. Moreover, CAP1 was recently found to also bind the small G protein Rap1 (27), which regulates cell proliferation, as well as adhesion (28), providing further support for CAP1 function in cell adhesion. Cell adhesion is critical for cell movement as well, since it generates tensile force essential for pulling the cell body forward. Therefore, CAP1 plays profound and more complex roles in cell migration and cancer cell invasiveness than initially thought, by functioning in both actin cytoskeletal rearrangement and cell adhesion.

Given the fundamental cellular functions of CAP1 and its translational potential, it is of critical importance to obtain a better understanding of the regulation of CAP1 functions. We previously identified the first regulatory mechanism for CAP, through phosphorylation at the Ser307/Ser309 (S307/S309) tandem regulatory site on mouse CAP1, where glycogen synthase kinase 3 (GSK3) phosphorylates the S309 residue (24). It was previously reported that in order to facilitate all steps in the cycle of actin filament turnover, CAP1 needs to undergo alternate associations with cofilin and actin (8). Our findings suggest that transient phosphorylation at both serine residues controls

the alternate associations of CAP1 with partner proteins cofilin and actin, which are critical for CAP1 function in the actin cytoskeleton. The cycling of CAP1 between phosphorylated and dephosphorylated forms would thus allow alternate binding with cofilin and actin. Moreover, the phosphoregulation of CAP1 also suggests that CAP1 likely mediates relevant cell signals to control the actin cytoskeleton and actin-dependent cell functions.

A number of critical gaps remain in further establishing the roles of phosphoregulation in the cellular functions of CAP1, to build a more complete and mechanistic picture of how cell signals phosphoregulate CAP1 functions. First, while biochemical evidence and actin cytoskeletal phenotypes suggest that transient S307/S309 phosphorylation is important for CAP1 function in actin dynamics (24), a role in directly regulating actin filament turnover in the cell has yet to be demonstrated. Second, whether the transient phosphorylation also regulates CAP1 function in cell adhesion remains to be tested. Third, whereas GSK3 is a kinase for S309, the kinase(s) that phosphorylates S307 and additional ones that may also phosphorylate S309 remain to be identified. On the other hand, cell signals that regulate dephosphorylation of CAP1 at S307/S309 remain elusive. Here, we report our findings that both the phosphomimetic and the nonphosphorylatable mutant of CAP1 had defects in rescuing the reduced rate of actin filament disassembly in the CAP1 knockdown HeLa cells. Moreover, the mutants also had defects in suppressing the elevated FAK activity and alleviating the focal adhesion phenotypes in the knockdown cells. These results support the idea that transient phosphorylation is critical for CAP1 functions in actin filament turnover and cell adhesion. Finally, we identify novel cell signals that phosphoregulate CAP1: cyclin-dependent kinase 5 (CDK5) phosphorylates both the S307 and S309 residues, whereas cAMP signaling induces dephosphorylation of CAP1 at the tandem site. These cell signals antagonistically regulate the phosphorylation status at S307/S309; along with the previously identified CAP1 phosphoregulatory cell signals, they likely function in concert to regulate CAP1 functions by facilitating the transient phosphorylation of CAP1 at the S307/S309 tandem regulatory site.

RESULTS

Phosphorylation mutants of CAP1 reexpressed in CAP1 knockdown HeLa cells had defects in rescuing the reduced rate of actin filament disassembly driven by latrunculin A. We previously reported that expression of the phosphomimetic (DD) or nonphosphorylatable (AA) mutants of CAP1 in NIH 3T3 cells or reexpression of them in the CAP1 knockdown HeLa cells led to an actin cytoskeletal phenotype of most characteristically enhanced actin stress fibers (24). Here, we designed and conducted experiments to directly test the capability of the AA and DD mutants reexpressed in the CAP1 knockdown HeLa cells in rescuing the reduced rate of actin filament disassembly driven by latrunculin A (LA). These mutants resist the physiological regulation through transient phosphorylation and dephosphorylation, and the experiment would thus provide insights on the importance of the transient phosphorylation for CAP1 function in facilitating actin filament disassembly. LA binds actin monomers and prevents them from polymerizing into filaments (29); as a result, treatment of cells with LA quickly disassembles the actin cytoskeleton when rapid actin filament turnover occurs. As we previously reported (23, 24) and also as shown by the results in Fig. 1A (top, 0 min), CAP1 knockdown HeLa cells develop enhanced stress fibers, and this phenotype was effectively rescued by the reexpressed wild-type (WT) CAP1 (R-WT) but was not rescued in the knockdown cells harboring an empty reexpression vector (R-Vec). In contrast, the reexpressed DD and AA mutants had clearly disrupted function in rescuing the enhanced stress fibers in the CAP1 knockdown HeLa cells compared to the results for WTCAP1 (Fig. 1A).

Consistent with the function of CAP1 in promoting actin filament depolymerization through collaboration with cofilin (8, 12), we found that CAP1 knockdown HeLa cells harboring an empty vector (Fig. 1A, leftmost column) had a remarkably reduced rate of actin filament disassembly induced by LA compared to that of the cells stably reex-

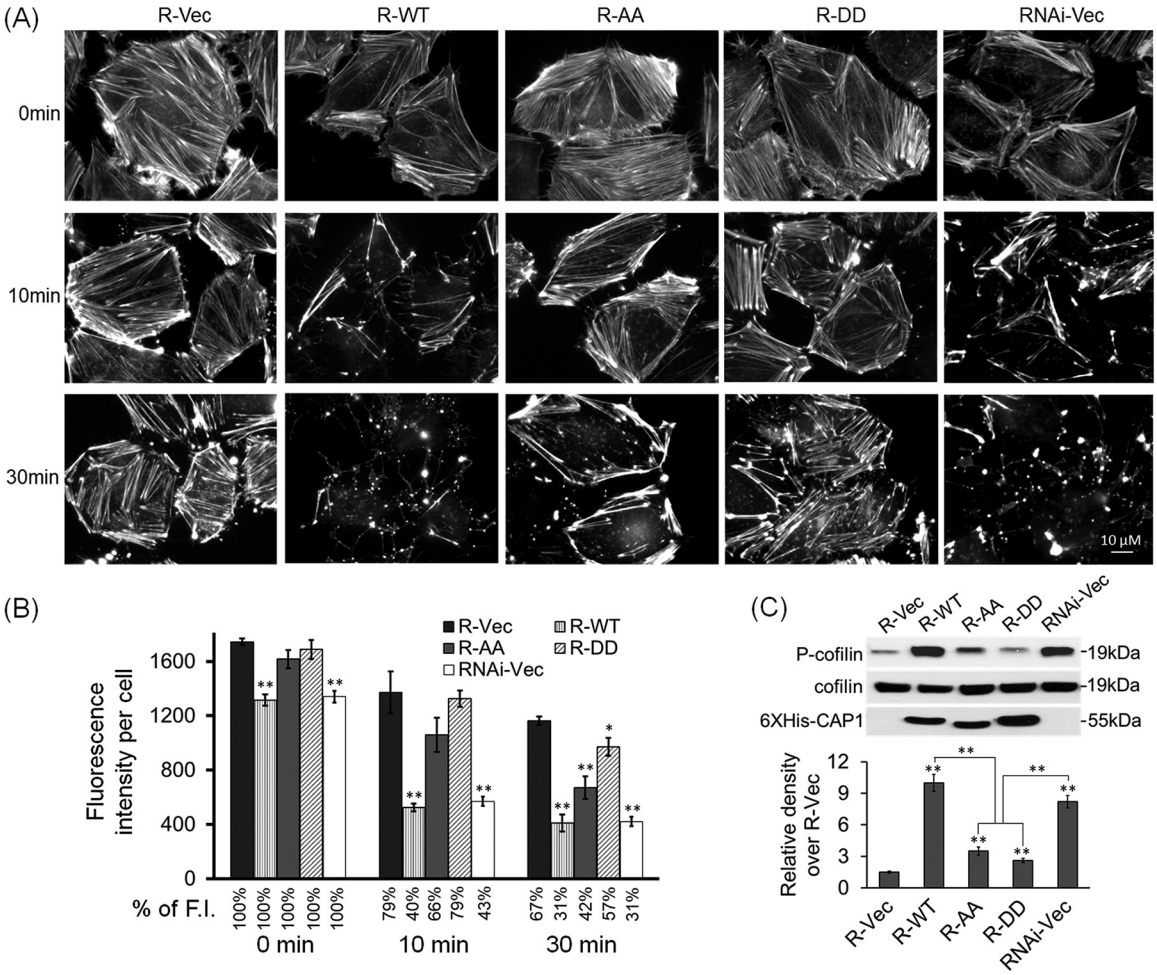

FIG 1 Phosphorylation mutants of CAP1 with point mutations at S307/S309 that resist transient phosphorylation had defects in rescuing the reduced actin filament disassembly in the CAP1 knockdown HeLa cells. (A) Both the phosphomimetic (DD) and nonphosphorylatable (AA) mutant had defects in rescuing the reduced actin filament disassembly in CAP1 knockdown HeLa cells driven by LA. CAP1 knockdown HeLa cells stably reexpressing WTCAP1 (R-WT) or the AA (R-AA) or DD mutant (R-DD) or harboring a control vector (R-Vec) were treated with 1 μM LA for 10 min and 30 min. The cells were stained with fluorescent phalloidin to visualize filamentous actin, and images were taken under fluorescence microscopy. HeLa cells harboring an empty shRNA vector (RNAi-Vec) were also included. (B) Fluorescence intensity per cell was measured from 25 cells using ImageJ and analyzed using Student's *t* test by comparing to that in the R-Vec cells. The percentages shown reflect the integrity of the actin cytoskeleton at the specific time points of LA treatment compared to that of the same cell type without LA treatment. Both phosphorylation mutants show reduced capability in rescuing the reduced rate of actin filament disassembly compared to the rate in WTCAP1, especially at the 10-min time point. The error bars in the graph represent standard deviations of data from three independent experiments. (C) The phosphorylation mutants also had compromised capability in restoring the level of inhibitory phosphorylation of cofilin at Ser3 in CAP1 knockdown HeLa cells, as detected in Western blotting. Reexpression of the 6×His-/Xpress-tagged WTCAP1 or the AA or DD phosphorylation mutant was confirmed in Western blotting using an antibody against the 6×His tag. Results from three experiments were quantified through densitometry and analyzed using Student's *t* test. The error bars in the graph represent standard deviations. *, $P < 0.05$; **, $P < 0.01$.

pressing WTCAP1 (Fig. 1A, 2nd column from left). We stained filamentous actin in cells with fluorescent phalloidin at the 10- and 30-min time points after beginning LA treatment to compare the degrees of functionality of the WTCAP1 and phosphorylation mutants in disassembling actin filaments. In cells reexpressing WTCAP1 (R-WT), at the 10-min time point of LA treatment, only very limited actin stress fibers and cortical stress fibers were left (Fig. 1A, middle row), and the cells also had an obscured cell periphery due to the loss of integrity of the actin cytoskeletal structure. The efficient disassembly of the actin cytoskeleton in these cells was even more evident at the 30-min time point of LA treatment, when stress fibers were essentially nonexistent (Fig. 1A, bottom row). In contrast, the actin filament disassembly in the R-Vec control cells was substantially less efficient. At both the 10- and 30-min time points of LA treatment,

the actin stress fibers and cell cortex remained largely intact, and the integrity of the actin cytoskeleton and cell morphology were also well preserved. In examining the effects in the cells reexpressing the AA (R-AA) or DD (R-DD) mutant, we found partially compromised functions for these mutants in promoting actin filament disassembly compared to these functions in cells reexpressing WTCAP1. The R-AA cells showed reduced disassembly of actin filaments compared to that in the R-WT cells (relatively more evident at the 30-min time point of LA treatment in Fig. 1), while the R-DD cells had further reduced actin filament disassembly, to a degree that was close to that in the R-Vec cells, suggesting that DD is even less functional in disassembling actin filaments. At the 10-min time point, the actin stress fibers in the R-AA cells remained largely intact and were comparable to those in the vector control and R-DD cells. At the 30-min time point, however, the R-AA cells had lost considerably more actin stress fibers than the R-DD and R-Vec cells, suggesting that dephosphorylated CAP1 is a relatively more functional form than phosphorylated CAP1. Finally, as expected, the HeLa cells harboring the control vector for small hairpin RNA (shRNA), and thus, without CAP1 knockdown (RNA interference vector [RNAi-Vec]) (Fig. 1A, right column), had actin filament disassembly comparable to that in the cells reexpressing WTCAP1. Together, these results support the notion that transient phosphorylation and dephosphorylation are critical for CAP1 function in disassembling actin filaments, which is a key step in the cycle of actin filament turnover. Moreover, the finding that the AA mutant is relatively more functional is consistent with our previous results supporting the idea that dephosphorylated CAP1 has enhanced cofilin binding and localizes to the cell periphery (24). However, the AA mutant still showed compromised function in disassembling actin filaments compared to the function of WTCAP1, as evident from the delay in the disassembly in cells reexpressing the AA mutant. Figure 1B presents the quantitative and statistically analyzed data, which show both mutants had significantly reduced capability in rescuing the reduced rate of actin filament disassembly compared to the capability of WTCAP1. These findings also suggest that CAP1 is the isoform important for regulating the actin cytoskeleton in HeLa cells, which express abundant levels of both CAP1 and CAP2, as we reported previously (23).

Knockdown of CAP1 in HeLa and metastatic breast cancer cells also led to reduced inhibitory phosphorylation of cofilin at serine 3 (21, 23). We next examined rescue of this by the stably reexpressed phosphorylation mutants in the CAP1 knockdown cells. As shown by the results in Fig. 1C, while WTCAP1 effectively increased the inhibitory phosphorylation of cofilin at Ser3, the AA and DD mutants had modest effects in this regard. The quantitative and statistically analyzed data in Fig. 1C show significant differences between them. These results suggest that phosphorylation also controls the CAP1 function in regulating cofilin.

Phosphorylation mutants of CAP1 had defects in rescuing the elevated-FAK-activity and focal adhesion phenotypes in the CAP1 knockdown HeLa cells. Cell adhesion to extracellular matrix, particularly the focal adhesions (FAs) that form in the protrusions, also plays an important role in cell migration. Strong traction forces reside at the cell front, as the cell pushes the protrusions ahead and pulls the cell body and the trailing edge forward. Recent progress has pointed to a signaling loop that links actin polymerization and the reorganization of cell adhesions. On one hand, actin polymerization at the leading edge orchestrates the architecture and dynamics of adhesions, and on the other hand, these adhesions mediate signals that regulate the polymerization and organization of actin (30, 31). In migrating cells, FAs are highly dynamic, with fast assembly and fast turnover, and the coordinated dynamics of the FAs and the actin cytoskeletal rearrangement are important for efficient cell movement. The adhesions that are not turned over grow in size and become stable, and thus, there is in general an inverse correlation between the sizes of focal adhesions and the migration rates of cells (32).

We previously reported molecular and functional interactions between CAP1 and FAK (23). Depletion of CAP1 led to activation of FAK and enhanced cell adhesion in HeLa and metastatic breast cancer cells (21, 23). Consistent with a role for FAK in

promoting lamellipodium formation (33), CAP1 knockdown HeLa cells also form large-sized lamellipodia, which are subcellular structures with highly dynamic actin cytoskeletal rearrangement. Expression of the AA or DD mutant did not fully rescue this lamellipodium phenotype (24) and also failed to effectively alleviate the enhanced stress fibers (Fig. 1A). It has been reported that FAK regulates the turnover of FAs in protrusions, and cells deficient in FAK develop large peripheral adhesions but have reduced migration (34). We tested the capabilities of the AA and DD mutants in suppressing the elevated FAK activity and the cell adhesion phenotypes. Results from Western blotting using a phosphorylation-specific anti-Y397 antibody against FAK show that both the AA and DD mutants had defects in suppressing the elevated FAK phosphorylation in the knockdown cells compared to its suppression by WTCAP1 (Fig. 2A). The quantitative and statistically analyzed data shown in Fig. 2B show significantly compromised capability of the AA and DD mutants in suppressing the elevated FAK activity. These results suggest that transient S307/S309 phosphorylation and dephosphorylation are also important for CAP1 to regulate FAK activity in HeLa cells.

We next examined and compared the numbers of FAs per cell and their sizes in CAP1 knockdown HeLa cells that reexpressed WTCAP1 or the AA or DD mutant. As shown in confocal images of FAs stained with an antivinculin antibody in cells cultured for 2.5 h on fibronectin, reexpression of WTCAP1 reduced the number of FAs (Fig. 2C, top middle) compared to the number in the R-Vec control cells (Fig. 2B, top left). Cells reexpressing the AA or DD mutant (Fig. 2C, bottom row) had remarkably increased numbers of FAs compared to the number in the cells reexpressing WTCAP1. We quantified the number of FAs in 25 cells using ImageJ and statistically analyzed the data using Student's *t* test with the cell area adjusted. Figure 2D shows the size comparison of FAs in the cells reexpressing WTCAP1 or the AA or DD phosphorylation mutant. Most of the FAs were found to cluster around the periphery in the control cells and the cells reexpressing the AA or DD mutant, which presented a challenge in the attempt to accurately discern the difference visually. To address this issue, we used the ImageJ software, employing two plugins to subtract the background and enhance the contrast. The sizes of FAs were measured using the Analyze Particle Tool, and the data were statistically analyzed using Student's *t* test. The size of FAs in the cells reexpressing WTCAP1 was significantly larger than that in the control R-Vec cells in both the 2.5-h ($P = 0.002$) and overnight cultures ($P = 0.007$). These results are consistent with the reduced motility in the WTCAP1-rescued cells (23). The sizes of FAs in the AA and DD mutant cells were closer to the size in the control R-Vec cells, with no statistical difference between them. The *P* value for the difference between the sizes of FAs in the R-AA cells and in the control R-Vec cells was 0.29; for the sizes of FAs in the R-DD cells compared to their sizes in the R-Vec cells, the *P* value was 0.51. We also cultured cells for an extended time duration (13 h) on fibronectin-coated surfaces and observed that the effects on the sizes of FAs were similar to the results for the cells cultured for 2.5 h (data not shown).

We next scored the numbers of FAs and compared them in cells reexpressing WTCAP1 and the phosphorylation mutants. As shown by the results in Fig. 2E, which were normalized by cell area because of the considerable difference in size among different types of cells, cells reexpressing the AA or DD mutant had a substantially increased number of FAs per cell compared to the number in cells reexpressing WTCAP1. The cells reexpressing either of the mutants had a number of FAs comparable to that in the control (R-Vec) cells. No significant difference between the number in the R-AA or R-DD cells and the number in the R-Vec cells was found (*P* values of 0.67 and 0.35, respectively). In contrast, cells rescued by WTCAP1 had a significantly reduced number of FAs ($P = 0.004$ compared to the number of FAs in R-Vec cells). Together, these results suggest that the phosphorylation mutants also had impaired functions in regulating FAK and the formation of FAs in HeLa cells. Again, cells cultured for 13 h on fibronectin-coated surfaces showed effects on the numbers of FAs similar to the results for the cells cultured for 2.5 h (data not shown).

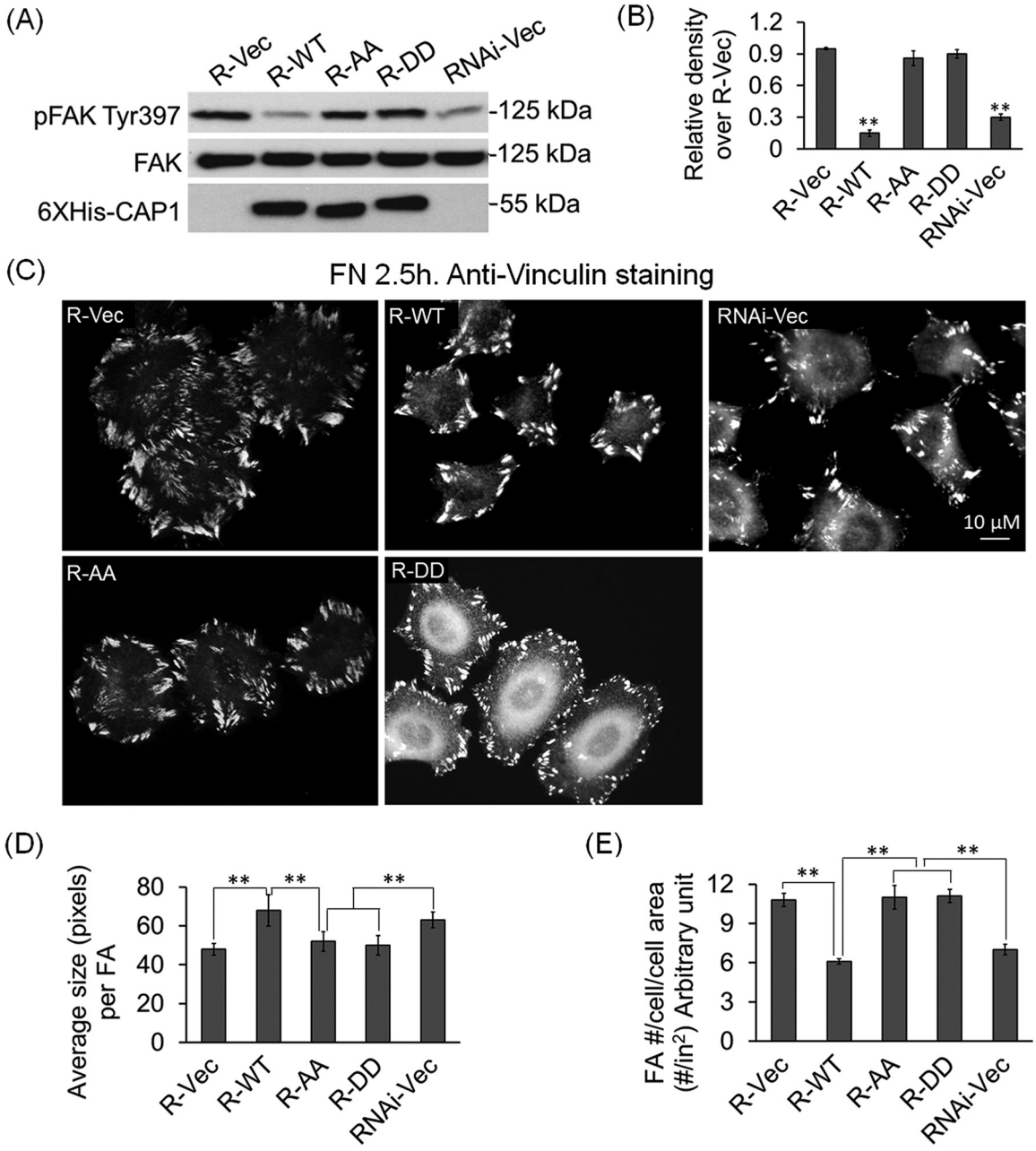

FIG 2 The phosphorylation mutants of CAP1 also had defects in suppressing the elevated FAK activity and focal adhesion phenotypes in the CAP1 knockdown HeLa cells. (A) The reexpressed AA and DD mutants had defects in suppressing the elevated FAK activity in the CAP1 knockdown HeLa cells compared to the results for WTCAP1. HeLa cells that harbor the empty shRNA vector were also included as a control. Phosphorylation-specific antibody against Tyr397 was used to assess FAK activity in Western blotting. (B) Phosphorylation signals from three independent Western blot experiments as shown in panel A were measured through densitometry and statistically analyzed using Student's *t* test. The phosphorylation mutants had significantly reduced capability in suppressing the elevated FAK activity. (C) The AA and DD mutants had defects in rescuing the focal adhesion phenotypes in the CAP1 knockdown HeLa cells compared to the results for WTCAP1. Cells were cultured on fibronectin (FN)-coated dishes with a glass bottom for 2.5 h, followed by fixation and staining with an antivinculin antibody. The focal adhesions (FAs) were visualized and images taken under a confocal microscope. (D) The sizes of FAs in 25 cells of each cell type in the experiment whose results are shown in panel C were measured using ImageJ and statistically analyzed using Student's *t* test. The error bars in the graph represent standard deviations of data from three independent experiments. (E) The numbers of FAs per cell were scored from 25 cells using ImageJ, normalized to the cell area, and then statistically analyzed using Student's *t* test. The error bars represent standard deviations of results from three independent experiments. **, $P < 0.01$.

Treatment of cells with inhibitors of CDK2 and CDK5 reduced S307/S309 phosphorylation on CAP1. We previously identified GSK3 as a kinase that phosphorylates the S309 residue (24). In an attempt to identify additional cell signals that phosphorylate the tandem site to regulate CAP1, we first conducted *in silico* analyses

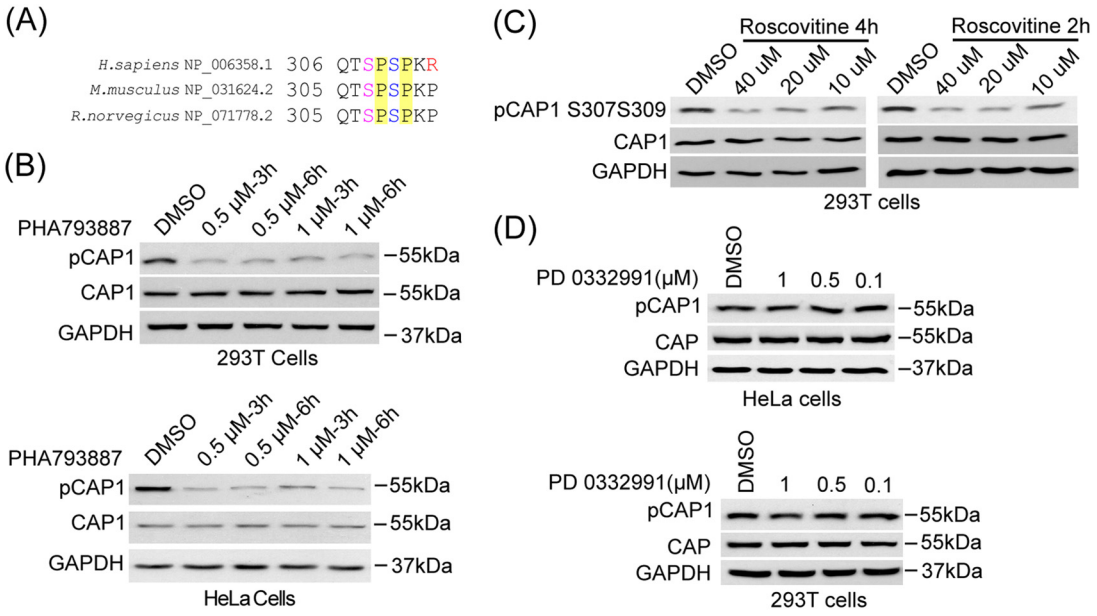

FIG 3 Inhibitors that target both CDK5 and CDK2 reduced S307/S309 phosphorylation on CAP1 in cells. (A) *In silico* analyses reveal that the S307/S309 tandem site is conserved in mammalian CAP1 homologues from humans (S308/S310), mouse, and rat (*Homo sapiens*, *Mus musculus*, and *Rattus norvegicus*). The two residues form part of the recognition motif for CDKs. (B) Treatment with a pan-inhibitor of CDKs, PHA-793887, reduced CAP1 phosphorylation at S307/S309 remarkably in both HEK293T and HeLa cells. The phosphorylation signals were detected using a phosphorylation-specific antibody that recognizes signals on both S307 and S309 residues. (C) Roscovitine, an inhibitor specific for CDK2 and CDK5, also considerably reduced CAP1 phosphorylation at S307/S309 in HEK293T cells. (D) PD 033299, an inhibitor specific for both CDK4 and CDK6, did not show detectable effect in reducing S307/S309 phosphorylation on CAP1 in HEK293T and HeLa cells, after treatment of cells for 16 h at the indicated concentrations. The Western blot results shown are representative of those from three independent experiments. DMSO, dimethyl sulfoxide.

by comparing the S307/S309 phosphoregulatory site and its surrounding sequences among mammalian CAP1 homologues, including those from humans, mouse, and rat. The analyses revealed a conserved SPSP motif (S307 and S309 in mouse and rat homologues and S308 and S310 in human CAP1). Both serine residues (highlighted in pink and blue text, respectively, in Fig. 3A) precede a proline (shaded in yellow), and for mouse CAP1, the (S/T)PXX sequence fits a nonclassical recognition motif of CDKs (35). For human CAP1, S310 also fits a classical recognition motif of CDKs, (S/T)PX(K/H/R) (35). These findings suggest that S307/S309 is a potential CDK site. To examine this possibility, we treated cells with PHA-793887, a pan-inhibitor of CDK that inhibits CDK2, CDK5, and CDK7 at low concentrations (50% inhibitory concentrations [IC_{50}s] of 8 nM, 5 nM, and 10 nM for CDK2, CDK5, and CDK7, respectively) and CDK1, CDK4, and CDK9 at higher concentrations (36). In both HEK293T (Fig. 3B, top panel) and HeLa (Fig. 3B, bottom panel) cells, treatment with 0.5 μM or 1 μM PHA-793887 for 3 or 6 h reduced the S307/S309 phosphorylation remarkably. Since CDK7 and CDK9 function as components of the transcription factor TFIIH, which is involved in cell cycle-related transcription initiation and elongation (37), these two CDK family members are likely to be functionally irrelevant and were thus dropped from further pursuit. Interestingly, the proline-directed serine/threonine kinase CDK5 has been reported to regulate cell functions overlapping with those of CAP1, including cytoskeletal organization and cell adhesion, contraction, and migration (38–43). Unlike the other CDK family members, CDK5 is an atypical member that does not function in cell cycle control but has functions related to the regulation of actin cytoskeleton dynamics, membrane trafficking and cell adhesion and migration (40, 41, 43, 44).

Most substrates of CDK5 are part of the cytoskeleton or have cytoskeleton-related functions, including p21-activated kinase (PAK) (45), FAK (44), talin (40), and a number of actin-associated proteins (46, 47). CDK5 is activated by the binding of its activating partners p35 and p39, which are localized at the cytoplasmic membrane mainly

through myristoylation (48, 49). We hypothesized that CDK5 may phosphorylate CAP1 to regulate the actin cytoskeleton and cell adhesion. To test this, roscovitine, a potent inhibitor of both CDK2 (IC_{50} of 0.7 μM) and CDK5 (IC_{50} of 0.16 μM) (50), was used to treat cells to test whether it reduces S307/S309 phosphorylation on CAP1 (an inhibitor specifically acting on CDK5 would be preferred, but roscovitine is the best option available). As shown by the results in Fig. 3C, treatment of HEK293T cells with roscovitine at concentrations ranging from 10 μM to 40 μM for 2 or 4 h reduced S307/S309 phosphorylation remarkably. For comparison, we also treated both HeLa and HEK293T cells with palbociclib isethionate (PD 0332991), a highly selective inhibitor of CDK4 and CDK6 (51), and no effect was detected in reducing the CAP1 phosphorylation (Fig. 3D). These results suggest that CDK2 and/or CDK5, but not CDK4 or CDK6, likely phosphorylates the S307/S309 regulatory site on CAP1 in the cell.

CDK5 phosphorylates CAP1 in the cell, and both S307 and S309 are CDK5 substrate sites. We next sought to determine whether CDK5 or CDK2 or both phosphorylate the S307/S309 sites on CAP1. *In vitro* kinase assays using active CDK2 and CDK5 were conducted to test whether they phosphorylate 6×His-tagged full-length CAP1 expressed and purified from *Escherichia coli*. Following the kinase reaction, the samples were analyzed in Western blotting to detect phosphorylation signals on both S307 and S309 residues. As shown by the results in Fig. 4A, CDK5 exhibited strong activity in phosphorylating CAP1, while the same amount of CDK2 also showed a modest kinase activity toward the tandem site *in vitro*.

We next sought to determine if CDK2 and CDK5 also phosphorylate CAP1 in the cell, by determining the effects on S307/S309 phosphorylation of manipulating CDK2 or CDK5 activity. CDK2 is activated by binding of several cyclins, such as cyclin D1 (52), whereas binding of p35 (or p39), a noncyclin protein, causes activation of CDK5. We first overexpressed CDK2 and cyclin D1 or a dominant-negative (DN) form of CDK2 that is kinase dead and also disrupts cellular CDK2 signaling in both HEK293T (Fig. 4B, top panel) and HeLa cells (Fig. 4B, bottom panel). Neither significant nor consistent alterations in S307/S309 phosphorylation were detected on CAP1 that would support phosphorylation of CAP1 by CDK2 in cells. In contrast, S307/S309 phosphorylation on CAP1 increased remarkably in HEK293T cells with CDK5 activated by the coexpression of p35 (endogenous CDK5 alone or exogenously expressed CDK5) (Fig. 4C, top panel). This effect was partially offset by the expression of a DN CDK5 that competitively binds p35 but does not possess the kinase activity toward its substrates. Similar results were obtained from HeLa cells as well (Fig. 4C, bottom panel). Finally, we silenced CDK5 through RNAi to further establish the role for the kinase in phosphorylating S307/S309 on CAP1. As shown by the results in Fig. 4D, efficient knockdown of CDK5 in HeLa cells derived from two independent shRNA constructs consistently led to significantly reduced S307/S309 phosphorylation. Taken together, our results support the idea that CDK5, but not CDK2, is a kinase that phosphorylates the S307/S309 tandem site on CAP1 in the cell.

The CDK5/p35 complex associates with CAP1 and phosphorylates both S307 and S309 residues. Since CDK5 phosphorylates S307/S309 on CAP1 both in kinase assays and in cells, we next tested whether the CDK5/p35 complex associates with CAP1. We conducted glutathione *S*-transferase (GST) pulldown assays from the lysates of HEK293T cells transiently expressing hemagglutinin (HA)-CDK5 along with the CDK5 activating partner p35, using GST-CAP1 purified from bacteria. As shown by the results in Fig. 5A, exogenously expressed CDK5 and p35 were both pulled down by GST-CAP1 but not by GST alone, supporting the idea that CAP1 indeed associates with CDK5/p35. We next performed coimmunoprecipitation assays using antibodies against p35 and the HA tag on CDK5, respectively, to test whether the reexpressed Xpress-tagged CAP1 in the CAP1 knockdown HeLa cells coprecipitates with p35 and HA-CDK5. Consistently, Xpress-CAP1 coprecipitated with both p35 (Fig. 5B) and HA-CDK5 (Fig. 5C). Furthermore, we tested whether endogenous CDK5 and CAP1 associate with each other and found that CDK5 and CAP1 indeed coprecipitated from HeLa cell lysates in coimmu-

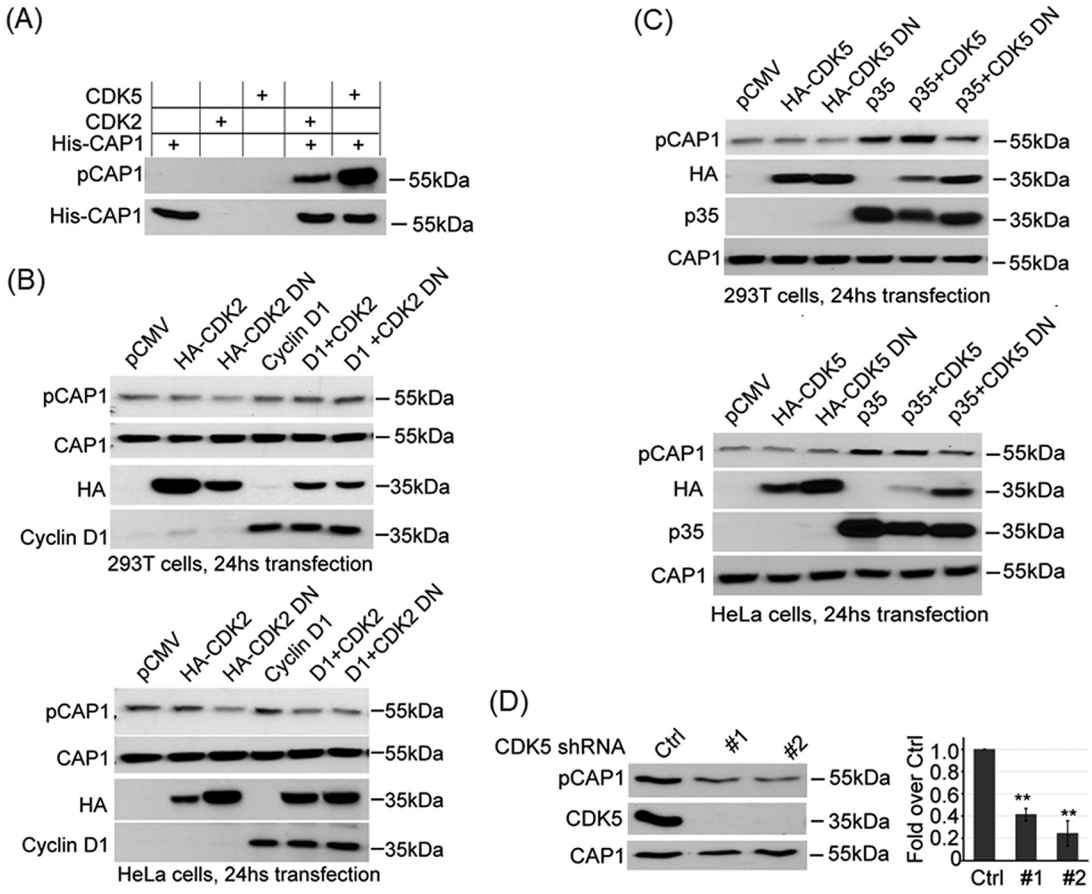

FIG 4 Results from *in vitro* kinase assays and manipulation of CDK5 activity in cells support the idea that CDK5 phosphorylates CAP1. (A) *In vitro* kinase assays show that CDK5 strongly phosphorylates S307/S309 on CAP1, while CDK2 exhibits a much more modest activity toward the tandem site. Recombinant and full-length proteins were incubated with the same amount of active CDK2 or CDK5 in the kinase buffer. The samples were then resolved on SDS-PAGE gels and blotted with a phosphorylation-specific antibody that detects phosphorylation signals on both S307 and S309. (B) Results from overexpression of CDK2 (HA-CDK2), its activating partner cyclin D1 (D1), or a dominant-negative (DN) HA-CDK2, alone or in combination, did not support a role for CDK2 in phosphorylating S307/S309 on CAP1 in the cell. HEK293T and HeLa cells were transiently transfected with either a control vector or plasmids that express the above-named proteins; cell lysates were prepared 24 h after transfection and analyzed in Western blotting for effects on S307/S309 phosphorylation. (C) Activation of CDK5 increased S307/S309 phosphorylation on CAP1 in cells. HEK293T and HeLa cells were transiently transfected with either a control vector or plasmids that express HA-CDK5, its activating partner p35, or a DN HA-CDK5, alone or in combination. Cell lysates were prepared 24 h after transfection and analyzed in Western blotting for effects on S307/S309 phosphorylation. (D) Stable silencing of CDK5 in HeLa cells with two independent lentivirus-based shRNA constructs consistently led to reduced CAP1 phosphorylation at S307/S309. CAP1 phosphorylation signals from three independent experiments were measured through densitometry and statistically analyzed using Student's *t* test. The graph shows significantly reduced phosphorylation in the knockdown cells. The error bars represent standard deviations. **, $P < 0.01$.

noprecipitation assays (Fig. 5D). Together, these results consistently support the idea that CAP1 associates with CDK5/p35 in the cell to facilitate the phosphorylation.

We next mapped the domain on CAP1 that is responsible for mediating the interaction of the protein with CDK5. For this, we first subcloned mouse CAP1 into the pcDNA4 vector and generated three additional constructs that expressed the Xpress-tagged N terminus (amino acids [aa] 1 to 226), middle domain (aa 217 to 319), and C terminus (aa 319 to 474) of CAP1 in mammalian cells. HEK293T cells were transfected with these constructs, and cell lysates were incubated with a bead-conjugated mouse anti-CDK5 antibody in coimmunoprecipitation assays, followed by Western blotting to detect coprecipitated CAP1 with the anti-Xpress antibody. As shown by the results in Fig. 5E, both the N terminus and full-length CAP1 coprecipitated with CDK5, suggesting that the N terminus of CAP1 interacts with CDK5. Notably, the expression of the middle domain was much lower than the expression of the other constructs and was detectable only with extended exposure in Western blotting (no coprecipitation was detected

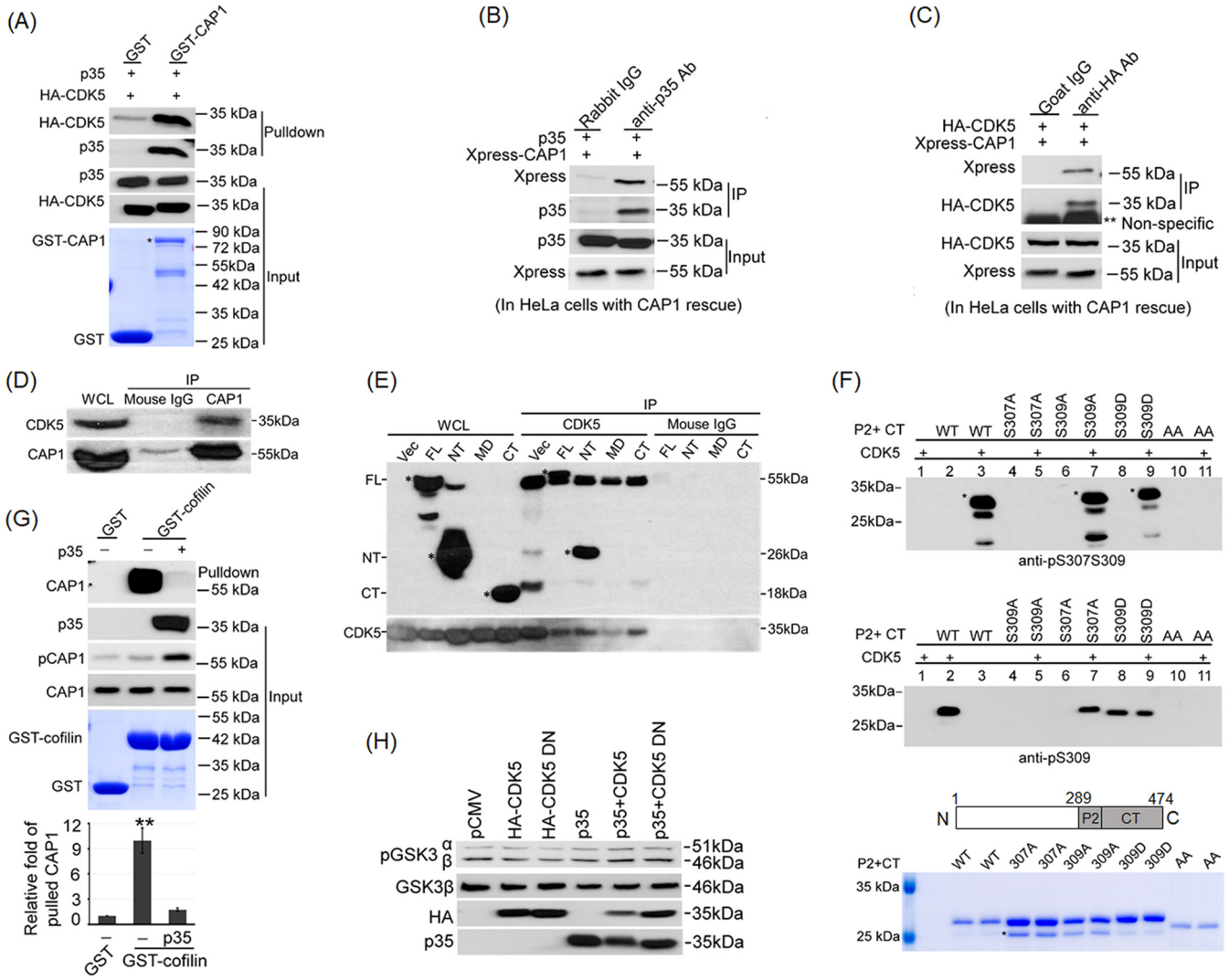

FIG 5 CAP1 associates with CDK5/p35 in the cell, and both the S307 and S309 residue on CAP1 are phosphorylated by CDK5. (A) Recombinant GST-CAP1, but not GST alone, coprecipitated with CDK5 and p35 in pulldown assays of lysates of HEK293T cells transiently expressing p35 and HA-CDK5. Bottom, input of GST and GST-CAP1, where an asterisk indicates the correct band of GST-CAP1 on the Coomassie blue-stained gel. (B) Immunoprecipitation with an anti-p35 antibody, but not the control IgG, coprecipitated Xpress-CAP1 from the lysate of CAP1 knockdown HeLa cells that stably reexpress WTCAP1. (C) Immunoprecipitation of transiently expressed HA-CDK5 with the anti-HA antibody coprecipitated stably expressed Xpress-CAP1 in the CAP1 knockdown HeLa cells. (D) Endogenous CAP1 and CDK5 coprecipitated in the immunoprecipitation assays. HeLa cell lysate was incubated with a bead-conjugated mouse anti-CAP1 antibody, and the coprecipitated CDK5 was detected with a rabbit anti-CDK5 antibody in Western blotting. WCL, whole-cell lysate. (E) The N-terminal domain of CAP1 is responsible for mediating the interaction between CAP1 and CDK5. HEK293T cells were transiently transfected with plasmids that express Xpress-tagged full-length CAP1 (FL), the N terminus (NT), the middle domain (MD), or the C terminus (CT). Cell lysates were incubated with a bead-conjugated anti-CDK5 antibody, and the coprecipitated Xpress-tagged CAP1 (FL or truncated domains) was detected in Western blotting with the anti-Xpress antibody. Asterisks indicate the correct signal bands for FL CAP1 and the truncated domains. Note that the expression level of the middle domain was much lower and only detected with extended exposure in Western blotting (not shown). (F) Results from *in vitro* kinase assays suggest that CDK5 phosphorylates CAP1 at both Ser307 and Ser309. Truncated CAP1 fragments (aa 289 to 474, which covers the CT domain and the P2 region of the middle domain, as WT or harboring indicated single or double point mutations) purified from bacteria were incubated with CDK5 in kinase buffer. The samples were analyzed in Western blotting using phosphorylation-specific antibodies that recognize either signals on both Ser307 and Ser309 residues (top) or the signal on Ser309 alone (middle). The Coomassie-stained gel at the bottom shows the 6×His-tagged truncated CAP1 proteins used in the kinase assays. Asterisks indicate protein fragments that are either nonspecific or a product derived from partial cleavage of the truncated fragments. The schematic representation highlights the CT domain and the P2 region in the middle domain covered in the truncated substrate (shaded) used in the kinase assays. (G) GST-cofilin pulldown assay shows that activation of CDK5 in HEK293T cells through overexpression of p35 inhibited the binding between CAP1 and cofilin. The Coomassie blue-stained gel in the middle shows the input of GST and GST-cofilin proteins used in the pulldown assays. The graph at the bottom shows the quantitated and statistically analyzed results from three independent experiments. The error bars represent standard deviations. **, $P < 0.01$. (H) Activation of CDK5 did not alter the activity of GSK3. Lysates from HEK293T cells that transiently express the indicated proteins were used in Western blotting. The activity of GSK3 was assessed using an antibody that detects inhibitory phosphorylation on both GSK3 isoforms.

for the middle domain [results not shown]). This is likely because overexpression of the proline-rich domain, which is heavily involved in cell signaling, was a burden for cells by interfering with cell signaling.

The next question that remained to be addressed was whether CDK5 phosphorylates either S307 or S309 or both. To determine this, we conducted *in vitro* kinase assays using truncated CAP1 that harbors the tandem site (aa 289 to 474, encompassing the C-terminal [CT] domain and the P2 region in the middle domain). The following versions of the 6×His-tagged truncated CAP1 were expressed in *E. coli* cells and purified: the wild type (WT), mutants that harbor a single point mutation (S307A [a change of S to A at position 307], S309A, or S309D; the attempt to express S307D in bacteria was unsuccessful), and the S307A/S309A double alanine mutant (AA). The S307A mutant harbors an intact S309 residue, and similarly, the S309A mutant harbors an intact S307. The S309D mutant harbors a phosphomimetic mutation at S309, while S307 remains intact. Finally, neither S307 nor S309 is phosphorylatable in the AA mutant. We first used a phosphospecific antibody that recognizes phosphorylation signals on both residues to test the samples from kinase assays in Western blotting. As expected, CDK5 readily phosphorylated the WTCAP1 fragment (Fig. 5F, top panel, lane 3). The antibody also detected phosphorylation signals on S307 when S309 had been mutated to an alanine (Fig. 5F, top panel, lane 7) or an aspartic acid (Fig. 5F, top panel, lane 9), suggesting that CDK5 phosphorylates the S307 residue. Also as expected, the antibody did not detect any phosphorylation signal on the AA mutant (Fig. 5F, top panel, lane 11). However, the antibody did not detect any phosphorylation signal on the S307A mutant either (Fig. 5F, top panel, lane 5), suggesting that either S309 is not a CDK5 site or the antibody failed to recognize phosphorylation signals on S309 with the nearby S307 mutated to an alanine. To clarify this, we used a second phosphorylation-specific antibody we previously developed that recognizes phosphorylation signals on the S309 residue alone (24). As shown by the results in the middle panel of Fig. 5F, interestingly, S309 phosphorylation signals were detected in the S307A mutant at levels similar to those in the WTCAP1 fragment (Fig. 5F, middle panel, lanes 2 and 7), supporting the idea that CDK5 phosphorylates S309 as well. Also, phosphorylation signals were detected from the S309D mutant, even in the absence of CDK5, suggesting that the antibody recognizes even mimicked phosphorylation signals on S309 (Fig. 5F, middle panel, lanes 8 and 9). The recombinant truncated CAP1 fragments used in the kinase assays were verified by SDS-PAGE and Coomassie staining, as shown in the bottom panel in Fig. 5F. Taken together, the kinase assay results support the idea that CDK5 phosphorylates CAP1 at both the S307 and S309 residues of the tandem regulatory site.

We previously reported evidence supporting the idea that simultaneous phosphorylation on both the S307 and S309 residues releases CAP1 from association with cofilin (24). Since CDK5 phosphorylates both residues, activation of CDK5 is expected to cause dissociation of CAP1 from cofilin. We tested this scenario by conducting GST-cofilin pulldown assays. As expected and as shown by the results in Fig. 5G, coexpression of p35 in HEK293T cells led to elevated S307/S309 phosphorylation and, moreover, essentially abolished the pulldown of CAP1 by GST-cofilin. However, this nearly complete abolishment is somewhat surprising, since it would require virtually all the CAP1 molecules to be in phosphorylated form, which is unlikely. We speculate that the binding of the CDK5/p35 complex may have competed with cofilin in binding CAP1, and as a result, the CDK5/p35 may have physically blocked cofilin binding with CAP1. The fact that the N terminus of CAP1 also binds CDK5, in addition to binding with cofilin, supports this scenario. Therefore, the reduced binding of cofilin and CAP1 caused by overexpression of p35 likely reflects a collective effect from increased phosphorylation of cellular CAP1 and the disruption of cofilin binding to CAP1 by the bound CDK5/p35. The quantitative and statistically analyzed results in the graph in Fig. 5G show significantly reduced CAP1 pulldown by GST-cofilin.

Since GSK3 also phosphorylates the S309 residue (24), we next tested whether activated CDK5 may have somehow activated GSK3 and, thus, indirectly led to elevated

S307/S309 phosphorylation on CAP1. As shown by the results in Fig. 5H, the activation of CDK5 did not cause any remarkable alterations in either the expression level or the inhibitory phosphorylation of GSK3, suggesting that CDK5 directly phosphorylates CAP1 at the regulatory site.

Activated cAMP signaling induces CAP1 dephosphorylation at S307/S309, and both PKA and exchange proteins directly activated by cAMP (Epac) are involved in mediating the dephosphorylation signals. We previously reported that CAP1 phosphorylation at S307/S309 undergoes a dynamic change that correlates with the activity of actin cytoskeletal rearrangement: suspension-cultured cells, which have a static actin cytoskeleton, had elevated S307/S309 phosphorylation, while reduced phosphorylation was detected in cells undergoing active spreading (24). Culture conditions, including serum starvation and stimulation with serum or platelet-derived growth factor (PDGF), remarkably alter CAP1 phosphorylation at S307/S309 as well (21, 53). Along with the subcellular localization and cofilin binding of the phosphorylation mutants, these results suggest that dephosphorylated CAP1 at S307/S309 is the relatively active form, although transient phosphorylation is required for optimal cellular functions of CAP1. We next aimed to identify cell signals that regulate dephosphorylation of CAP1. A literature search led to hints suggesting that cAMP may be a candidate molecule that acts in this role. First, protein kinase A (PKA), which is activated by cAMP signaling, is activated in spreading cells (54), where we previously detected a reduced level of phosphorylated CAP1. Second, cAMP signaling induces dephosphorylation of the actin-regulating protein WAVE1, also on residues phosphorylated by CDK5 (46, 55). Third, cAMP signaling is well documented to regulate the cellular processes in which CAP1 functions, including the actin cytoskeleton rearrangements, cell adhesion, and migration (55–59). To test the potential role of cAMP in inducing dephosphorylation of CAP1, we treated HEK293T cells with forskolin, an activator of adenylyl cyclase that converts ATP into the second messenger cAMP (60), and found that treatment with 5 μM forskolin indeed reduced S307/S309 phosphorylation (Fig. 6A). We further treated HEK293T cells with isoproterenol, a physiological agonist and external stimulus of cAMP signaling that activates the β2-adrenergic receptor (59), and found that treatment with 10 μM isoproterenol for 3 h remarkably reduced S307/S309 phosphorylation on CAP1 as well (Fig. 6B). These results suggest that activated cAMP signaling indeed induces dephosphorylation of CAP1 at S307/S309.

In most cells, there are two groups of effectors that bind cAMP and mediate intracellular cAMP signals, PKA and Epac (also called cAMP-regulated guanine nucleotide exchange factors) (61, 62). To dissect potential roles for each in mediating the cAMP signals to induce dephosphorylation of CAP1, we first pretreated HEK293T cells with a specific inhibitor of PKA, H89, and then stimulated them with forskolin. The preinhibition of PKA by treating cells with H89 for 2 h indeed blocked CAP1 dephosphorylation induced by forskolin to a considerable degree (Fig. 6C). We next employed two independent shRNA constructs to transiently knock down catalytic subunit alpha of PKA (PKAcα) in HEK293T cells and found that silencing of PKAcα resulted in increased basal levels of CAP1 phosphorylation and also partially blocked the CAP1 dephosphorylation induced by forskolin (Fig. 6D). Together, these results consistently support the idea that PKA mediates the cAMP signals that induce CAP1 dephosphorylation. In testing the potential role of Epac in mediating the cAMP dephosphorylation signal, we used a plasmid that expresses FLAG-tagged Epac1 (Epac1 is the more ubiquitously expressed isoform compared with the expression of Epac2) in both HEK293T and HeLa cells (Fig. 6E). In both cell lines, overexpression of Epac1 reduced phosphorylation of CAP1 at S307/S309. Taken together, our results suggest that both PKA and Epac1 function downstream to mediate cAMP signals to induce dephosphorylation of CAP1 at S307/S309.

The nonphosphorylatable AA mutant of CAP1 had increased capacity in binding cofilin (24). Since activation of cAMP signaling by forskolin induces dephosphorylation at S307/S309, the treatment was expected to lead to increased CAP1-cofilin association. We similarly tested this in the GST-cofilin pulldown assays, and indeed, forskolin

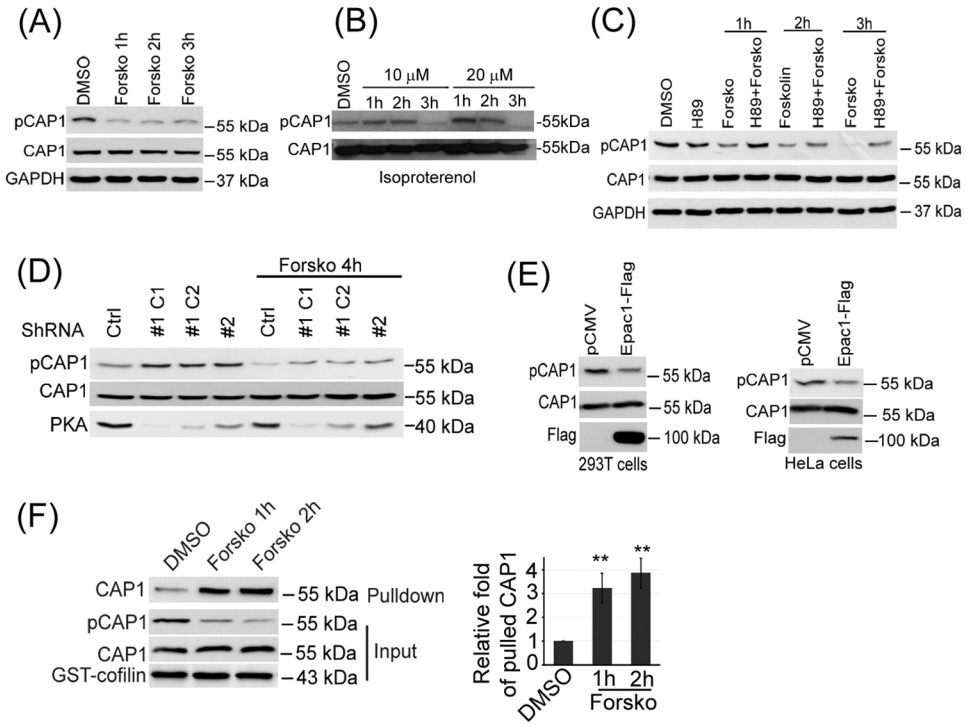

FIG 6 Activation of cAMP signaling in the cell induced dephosphorylation of CAP1 at S307/S309, and both PKA and Epac1 are involved in mediating the cAMP signals. (A) Treatment of HEK293T cells with 5 μM forskolin, an activator of adenylyl cyclase, induced dephosphorylation of CAP1 at S307/S309. (B) Treatment of HEK293T cells with isoproterenol, a physiological agonist and an external stimulus of cAMP signaling, caused dephosphorylation on CAP1 at S307/S309. (C) H89, a specific inhibitor of PKA, considerably blocked dephosphorylation of CAP1 induced by forskolin in HEK293T cells. (D) Stable knockdown of the catalytic subunit alpha of PKA in HeLa cells using two independent shRNA constructs increased CAP1 phosphorylation at S307/S309 and also partially blocked CAP1 dephosphorylation induced by forskolin. #1 and #2, two independent shRNA constructs; C1 and C2, two stable clones derived from construct #1. (E) Overexpression of Epac1 in both HEK293T cells and HeLa cells led to enhanced dephosphorylation of CAP1 at S307/S309. (F) GST-cofilin pulldown assays show that forskolin treatment, which induced CAP1 dephosphorylation, enhanced CAP1 binding with cofilin. The graph shows the quantitated results and statistical analysis using Student's *t* test. The error bars represent standard deviations. **, $P < 0.01$.

treatment significantly increased the binding of endogenous CAP1 with cofilin (Fig. 6F). These results indicate that the cofilin binding results derived from using phosphorylation mutants of CAP1 are consistent with those derived from using endogenous CAP1.

No evidence suggests involvement of activated protein phosphatase in the dephosphorylation of CAP1 induced by cAMP. The next question that needed to be addressed was how activated cAMP signaling, which cannot directly dephosphorylate a substrate itself as a protein phosphatase does, induces CAP1 dephosphorylation. The two most likely scenarios were tested: first, the activated cAMP signaling may have activated a protein phosphatase(s), which then directly dephosphorylated S307/S309 on CAP1, and second, the cAMP signaling may have somehow prevented phosphorylation of CAP1 by its kinases, including CDK5 and GSK3.

Protein phosphatases PP1 and PP2A are the major phosphatases that act on phosphorylated serine and threonine residues (63, 64). They are ubiquitously expressed in eukaryotic cells, and upon activation, execute prominent functions in a wide range of cellular processes, such as cell division, apoptosis, and cytoskeletal rearrangement, by dephosphorylating substrates that execute relevant functions. Studies have found that protein phosphatases PP1 and PP2A are responsible for dephosphorylating proteins downstream from the cAMP signaling (46, 55, 57, 63, 65). We tested the possibility that these phosphatases also dephosphorylate S307/S309 on CAP1 upon their activation by cAMP. Cells were first treated with a number of inhibitors of PP1 or PP2A and then by forskolin stimulation to determine if the pretreatment

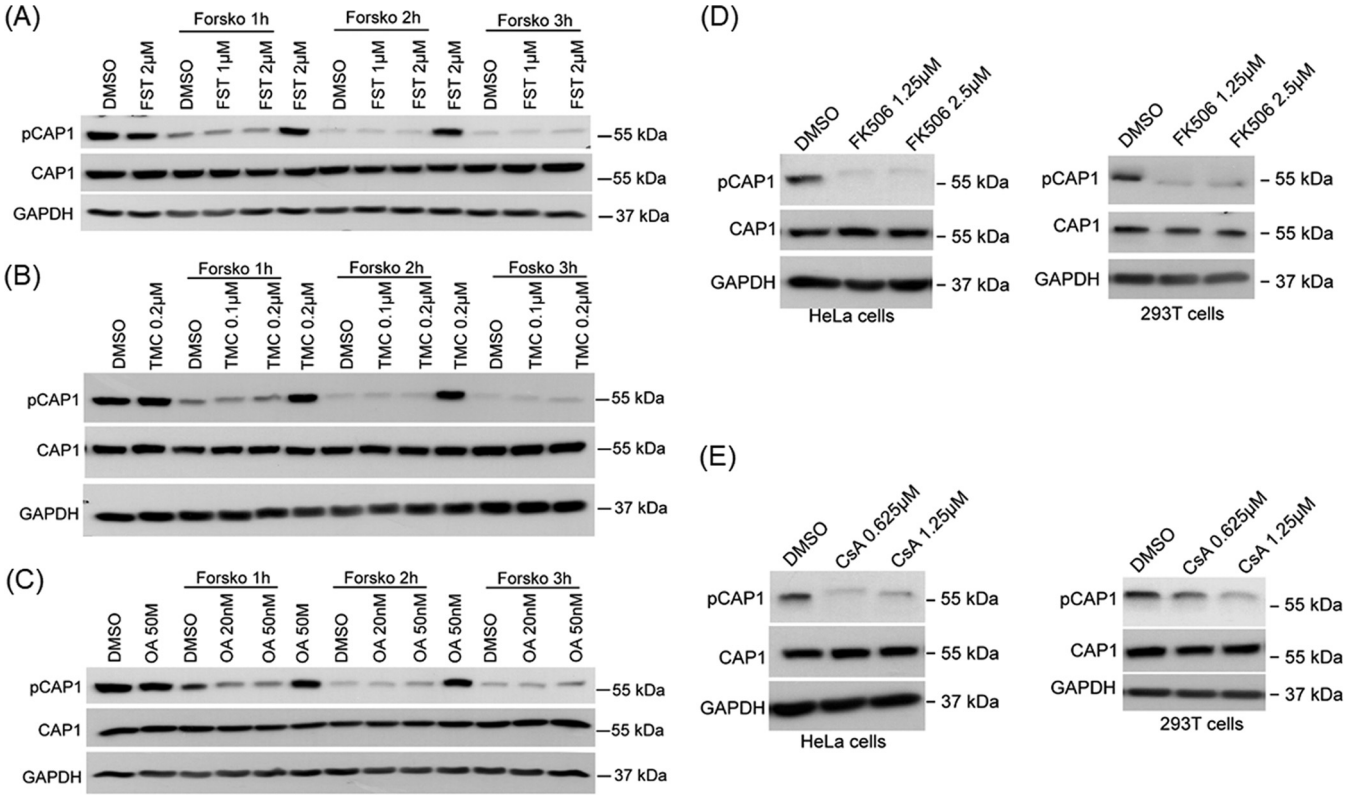

FIG 7 Inhibition of the activity of protein phosphatases in cells did not block the dephosphorylation of CAP1 induced by activated cAMP signaling. (A) Pretreatment of HEK293T cells with fostriecin (FST), an inhibitor of the protein phosphatases PP2A and PP4, did not block the dephosphorylation of CAP1 at S307/S309 induced by forskolin. (B) Pretreatment of HEK293T cells with protein phosphatase PP1 inhibitor tautomycetin (TMC) did not block the dephosphorylation of CAP1 at S307/S309 induced by forskolin either. (C) Inhibiting PP2A and PP1 using okadaic acid (OA) in HEK293T cells did not block the dephosphorylation of CAP1 induced by forskolin. The cells were pretreated with OA or the vehicle DMSO for 1 h, followed by forskolin treatment for the indicated time durations, and cell lysates were prepared for analysis in Western blotting. (D) Inhibition of protein phosphatase PP2B with the specific inhibitor FK506 did not inhibit but instead enhanced dephosphorylation of CAP1 at S307/S309 in both HeLa and HEK293T cells. (E) Inhibition of PP2B with another specific inhibitor, cyclosporine (CsA), similarly enhanced the dephosphorylation of CAP1 at S307/S309 in both HeLa and HEK293T cells.

blocks the effect of forskolin in inducing CAP1 dephosphorylation. We first tested fostriecin (IC_{50} 3.2 nM), a potent inhibitor specifically for PP2A (66). When HEK293T cells were pretreated with fostriecin (1 μM or 2 μM) for 1 h, followed by the forskolin treatment, no remarkable effect was detected in preventing dephosphorylation of CAP1 induced by forskolin at any of the three time points tested (Fig. 7A). Silencing of PP2A by RNAi was unsuccessful in achieving efficient depletion (not shown). We next tested tautomycetin, a selective inhibitor of PP1 (IC_{50} values of 1.6 nM and 62 nM for PP1 and PP2, respectively) (67). Similar to the case for fostriecin, inhibition of PP1 by tautomycetin (0.1 μM and 0.2 μM) did not block dephosphorylation of CAP1 induced by forskolin (Fig. 7B). Finally, we also tested okadaic acid (OA), a potent inhibitor of both PP1 (IC_{50}s of 15 to 20 nM) and PP2A (IC_{50} of 0.1 nM) (68). Treatment of either HEK293T or HeLa cells (results are shown for HEK293T cells only) with 20 nM and 50 nM OA did not have a remarkable effect in blocking the forskolin-induced dephosphorylation of CAP1 (Fig. 7C). Notably, treatment with OA caused pronounced cytotoxicity, with higher concentrations of OA leading to extensive cell death (not shown). Together, these results did not support a role for either PP1 or PP2A as a major phosphatase that functions downstream from cAMP to directly dephosphorylate CAP1.

We also tested the potential involvement of another serine/threonine phosphatase, calcineurin (also called PP2B), whose activity is dependent on calcium (Ca^{2+}) (69). Calcineurin has been reported to dephosphorylate cofilin at Ser3, although this is indirectly mediated by the cofilin phosphatase slingshot (70, 71). Given the functional link between CAP1 and cofilin (8, 12, 23), we wondered if calcineurin may also

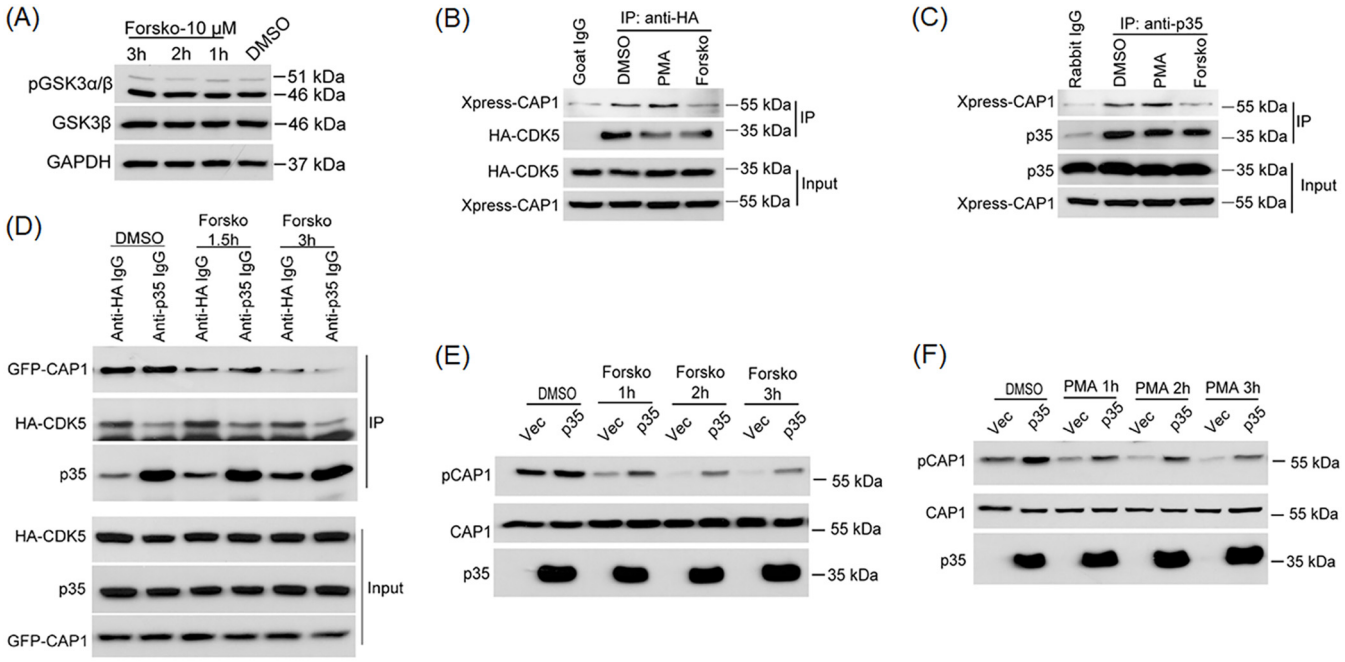

FIG 8 Prevention of access of CAP1 to CDK5/p35 is a mechanism underlying the dephosphorylation of CAP1 induced by cAMP signaling. (A) Treatment of HEK293T cells with forskolin did not affect the expression levels or activity of GSK3 isoforms, as assessed by inhibitory phosphorylation of GSK3 in Western blotting. (B and C) Coimmunoprecipitation of CAP1 with HA-CDK5 (B) and p35 (C) shows that treatment of HEK293T cells with forskolin prevented association of CAP1 with the CDK5/p35 complex. In contrast, the PKC activator PMA, which also induces dephosphorylation of CAP1 at S307/S309, as we reported previously, did not have this effect. (D) Activated cAMP signaling also inhibited the association between GFP-CAP1 and CDK5/p35, as detected by coimmunoprecipitation with the HA tag or p35. Longer treatment with forskolin led to further reduced association between CAP1 and CDK5/p35. HEK293T cells were transfected with GFP-CAP1, HA-CDK5, and p35. After 24 h, the cells were treated with forskolin for the indicated time durations before cell lysates were prepared and used in coimmunoprecipitation with either the anti-HA or anti-p35 antibody. The precipitates were analyzed in Western blotting to detect coprecipitated GFP-CAP1 using an anti-GFP antibody. (E and F) Activation of CDK5 through the coexpression of p35 attenuated or delayed dephosphorylation of CAP1 induced by both forskolin (E) and PMA (F).

dephosphorylate CAP1. To test this, two selective inhibitors, FK506 (72) and cyclosporine (73), were employed. As shown by the Western blot results in Fig. 7D and E, neither of these PP2B inhibitors increased CAP1 phosphorylation in the HeLa or HEK293T cells treated; instead, they actually reduced CAP1 phosphorylation. Therefore, our results did not support a role for PP2B either in dephosphorylating CAP1 at S307/S309.

Activated cAMP signaling prevents CAP1 from association with CDK5/p35. We next tested whether activated cAMP signaling may have compromised the capabilities of CDK5 or GSK3 in phosphorylating CAP1. As shown by the results in Fig. 8A, forskolin treatment altered neither the expression levels of GSK3 nor its inhibitory phosphorylation, at least throughout the first 3 h of the treatment tested. We next tested whether cAMP signaling may inhibit the phosphorylation of CAP1 by activated CDK5 in the cell. HEK293T cells were transfected with plasmids that express HA-CDK5, p35, and Xpress-CAP1; 24 h after transfection, cells were treated with forskolin for 4 h. For comparison, we also treated cells with phorbol 12-myristate 13-acetate (PMA), an activator of protein kinase C that we previously found to also induce dephosphorylation of CAP1 at S307/S309 (24). The cell lysates were incubated with antibodies against HA or p35, respectively, and the coprecipitated Xpress-CAP1 was detected with an antibody against the tag in Western blotting. As shown by the results in Fig. 8B and C, forskolin treatment caused remarkably reduced coprecipitation between Xpress-CAP1 and CDK5/p35 compared to that in the vehicle control. Similar results were obtained in the coimmunoprecipitation of p35 or HA-CDK5 with green fluorescent protein (GFP)-CAP1, where longer forskolin treatment led to further reduced association between CAP1 and CDK5/p35 (Fig. 8D). Interestingly, while PMA also induces CAP1 dephosphorylation at S307/S309 similarly as effectively as forskolin, PMA did not reduce the association between CAP1 and CDK5/p35, and the coimmunoprecipitation between them was

comparable to the case in the vehicle control (Fig. 8B and C). Taken together, these results consistently suggest that preventing complex formation between CAP1 and CDK5/p35 underlies the dephosphorylation of the cellular pool of CAP1 induced by cAMP, at least partially. In contrast, PMA likely induces CAP1 dephosphorylation through a different mechanism, potentially involving activated protein phosphatase(s).

We next looked into whether activation of CDK5 attenuates the effect of cAMP signaling in inducing dephosphorylation of CAP1. Cells were transfected with p35 or the control vector for 24 h, followed by forskolin treatment. At the time points indicated in Fig. 8E, cell lysates were prepared and analyzed in Western blotting. The results show that activation of CDK5 by the coexpression of p35 partially reduced or delayed the effect of forskolin in inducing CAP1 dephosphorylation (Fig. 8E). We also found that activation of CDK5 similarly attenuated PMA-induced CAP1 dephosphorylation (Fig. 8F). Together, these results suggest that CDK5 phosphorylation signals and the dephosphorylation signals activated by forskolin or PMA antagonistically control the S307/S309 phosphorylation state on CAP1. These CAP1 phosphoregulatory cell signals are believed to function in concert to achieve temporally and spatially controlled CAP1 phosphorylation and, consequently, the localized CAP1 activity that is important for facilitating local actin dynamics and actin-dependent cell functions.

DISCUSSION

We previously reported findings that support the idea that transient S307/S309 phosphorylation controls alternate associations of CAP1 with cofilin and actin, partners essential for CAP1 function in regulating the actin cytoskeleton. Here, we demonstrate further evidence supporting the idea that dynamic phosphorylation and dephosphorylation at S307/S309 is critical for CAP1 to promote actin filament disassembly and regulate focal adhesions in the cell. Phosphorylation mutants of CAP1 that resist dynamic phosphorylation and dephosphorylation at the S307/S309 regulatory site, both phosphomimetic and nonphosphorylatable, had defects in rescuing the reduced actin filament disassembly driven by latrunculin A (LA) and the focal adhesion phenotypes in CAP1 knockdown HeLa cells. Moreover, we identify novel cell signals that regulate the transient phosphorylation and dephosphorylation of CAP1: CDK5 phosphorylates both S307 and S309 residues, while cAMP signaling induces dephosphorylation at the tandem regulatory site by preventing association of CAP1 with the CDK5/p35 complex. Figure 9 shows a schematic model where CDK5 phosphorylates CAP1, whereas cAMP induces dephosphorylation of CAP1 at the tandem site. These phosphorylation and dephosphorylation signals are believed to function in concert to achieve spatially and temporally regulated phosphorylation states of CAP1 to control localized CAP1 activity and the actin dynamics that is important for actin-dependent cellular functions like adhesion, polarization, and migration.

The findings that the AA and DD phosphorylation mutants had defects in rescuing the reduced actin filament disassembly also suggest that reduced actin dynamics is an important mechanism underlying the phenotype of enhanced stress fibers in the CAP1 knockdown cells, although loss of CAP1 function in sequestering G-actin likely also contributes to the phenotype. Moreover, while cofilin is activated in the CAP1 knockdown HeLa cells (23), the reduced rate of actin filament disassembly in these cells is consistent with the findings that only the combination of CAP1 and cofilin, but neither cofilin nor CAP1 alone, is capable of rapidly severing actin filaments *in vitro* at the physiological pH range (9). It is noted, however, that the net effect of CAP1 depletion on the activity of the total cofilin pool in the cell is not clear, since the localization of cofilin to aggregates in these cells may contain its activity.

Knockdown of CAP1 leads to activated FAK and enhanced adhesion in HeLa cells (23). The phosphorylation mutants also had compromised capabilities in suppressing the elevated FAK activity and focal adhesion phenotypes in the knockdown cells. Cell adhesion and actin dynamics are intimately connected. Phosphorylation of FAK at Tyr397 is required for actin assembly catalyzed by the Arp2/3 complex (74), and the generation of actin filaments controlled by Arp2/3 promotes the formation of lamelli-

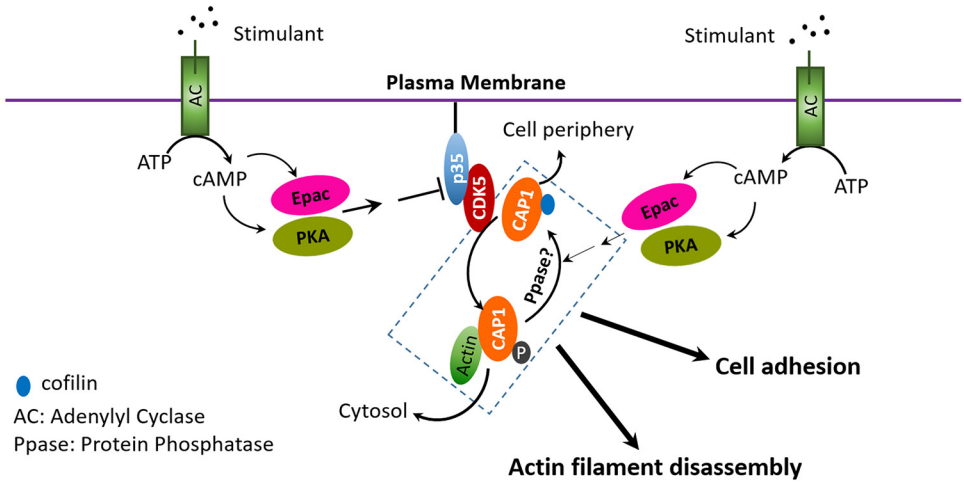

FIG 9 A schematic model of how CAP1-phosphoregulatory cell signals CDK5 and cAMP antagonistically regulate the phosphorylation state of CAP1 at S307/S309 to control the functions of the protein in actin filament disassembly and regulation of cell adhesion. CDK5 phosphorylates the S307/S309 regulatory site, while cAMP induces dephosphorylation of CAP1, with the cAMP signals being mediated by both of the cAMP effectors PKA and Epac. This transient phosphorylation, or the cycling between the phosphorylated and dephosphorylated forms, is essential to the regulation of CAP1 functions in promoting actin filament disassembly and regulating cell adhesion. Preventing this physiological regulation of CAP1 at the S307/S309 site, as in the case of the phosphorylation mutants that resist transition between phosphorylated and dephosphorylated forms, disrupts CAP1 functions in these cellular processes. Our data support the idea that activated cAMP signaling prevents the access of CAP1 to its kinase, CDK5, which at least partially underlies the dephosphorylation of the cellular pool of CAP1 induced by cAMP. No evidence was found supporting a role for activated serine/threonine protein phosphatase(s) in dephosphorylating CAP1 downstream from the cAMP signaling cascade; however, some degree of involvement of a protein phosphatase is not entirely ruled out.

podia (75, 76). Activation of the FAK-Arp2/3 signaling in the CAP1 knockdown HeLa cells that reexpress the mutants may explain, at least partially, why these mutants failed to suppress the increased cell size or the large-sized lamellipodia formed in these knockdown cells (24). Compared to cells rescued by WTCAP1, cells reexpressing the AA or DD mutant developed more FAs but in smaller sizes. These phenotypes suggest that these FAs likely derive from increased formation or delayed maturation or turnover, which are characteristic of cells with elevated motility.

The results from studies in HeLa and HEK293T cells consistently support the idea that CDK5 phosphorylates CAP1 at S307/S309, while cAMP induces dephosphorylation at the tandem regulatory site. Our *in vitro* kinase assays show that CDK5 phosphorylates both the S307 and S309 residues of the tandem site. Since simultaneous phosphorylation (and dephosphorylation) at both residues is required in regulating CAP1 function, CDK5 may have a more important role as part of the phosphorylation machinery for CAP1 than does GSK3, which phosphorylates CAP1 at S309 alone. It should not be surprising that CDK5 and GSK3, two kinases that have overlapping functions in regulating cell adhesion, polarization, and migration, have a shared role in phosphoregulating CAP1. Considering the critical importance of S307/S309 phosphorylation in regulating CAP1, a protein that controls fundamental cell functions, it is reasonable that multiple kinases may phosphorylate the same site, which would allow the cell to utilize a multitude of regulatory mechanisms to control its functions. A question that arises from this is how the cell distinguishes signals from distinct pathways that phosphorylate the same substrate site/residue on CAP1. A possible scenario would be that when one of these kinases (e.g., CDK5) is active toward a substrate residue (e.g., S309), it structurally blocks access for the other kinase (GSK3 here) to the same residue. Another possibility is that when one of these kinases is active, the activity of the other kinase is turned off; indeed, inhibition of CDK5 has been reported to lead to activation of GSK3 (77). Also, our finding that dephosphorylated CAP1 has increased binding with cofilin is consistent with our previous findings, including those from using the phosphoryla-

tion mutants, that suggest the phosphorylated form of CAP1 is inactive (24). We also reported previously that the nonphosphorylatable AA mutant localizes to the cell periphery, while the phosphomimetic DD mutant localizes to the cytosol; these results suggest that phosphorylation of CAP1 by CDK5 may serve to drive relocalization of CAP1 from the cell periphery to the cytosol, whereas cAMP may have an opposite function.

S307/S309 phosphorylation on CAP1 was reduced in cells undergoing dynamic spreading (24), which provided a hint that led to the identification of cAMP signaling as having a role in inducing dephosphorylation of CAP1. Interestingly, cAMP also induces dephosphorylation of another actin-regulating protein, WAVE1 (46, 55). Moreover, in the case of WAVE1, the cAMP signaling induces dephosphorylation specifically on the residues phosphorylated by CDK5. Through a combination of approaches using chemical activators and inhibitors, RNAi silencing, and exogenous expression, we show results that support the involvement of both cytoplasmic effectors of cAMP, PKA and Epac1, in mediating cAMP signals to induce dephosphorylation of CAP1. Neither PKA nor Epac, however, is capable of directly dephosphorylating CAP1; PKA is a kinase, while Epac is a guanine nucleotide exchange factor specifically activating Ras GTPase members Rap1/2 (61, 62). Therefore, we speculate that cAMP may have activated a certain serine/threonine protein phosphatase(s), which in turn executes the dephosphorylation of CAP1 at the S307/S309 regulatory site. However, results from using specific inhibitors of PP1, PP2A, and PP2B do not support the idea of any of these phosphatases playing a major role in dephosphorylating S307/S309 downstream from cAMP. Instead, we found that activation of cAMP signaling inhibited complex formation between CDK5/p35 and CAP1. Interestingly, while PMA also induces dephosphorylation of CAP1 at S307/S309 (24), it did not show an effect in preventing CAP1 association with CDK5/p35. These results suggest that blocking access of CAP1 to its kinase CDK5 is a mechanism underlying, at least partially, the dephosphorylation of CAP1 induced by forskolin. The highly dynamic turnover of CAP1 in cells, as we previously demonstrated (21), also supports this scenario, since preventing the newly synthesized CAP1 from being phosphorylated alone would lead to a pool of cellular CAP1 with considerably reduced phosphorylation. We cannot completely rule out the possibility that higher doses of okadaic acid (OA) show some effect in blocking CAP1 dephosphorylation induced by cAMP in cells that are more tolerant to the inhibitor. In short, we did not find any evidence supporting a major role for the activation of phosphatase by cAMP in dephosphorylating CAP1.

The identification of roles for CDK5 and cAMP signaling in antagonistically regulating CAP1 phosphorylation, which is of critical importance for CAP1 cellular functions in both actin filament turnover and cell adhesion, is also consistent with the reported functions of these cell signals. Both CDK5 and cAMP regulate the actin cytoskeleton and cell adhesion and migration. It is likely that CAP1 functions as one of the downstream targets of these cell signals, through which CDK5 and cAMP control relevant cell functions. Our results support the idea that both PKA and Epac mediate cAMP signaling to induce dephosphorylation on CAP1. It has been reported that Epac mediates cAMP signals to control cell adhesion through Rap1 (59, 78), whereas the PKA-mediated cAMP signal is more relevant to the regulation of the actin cytoskeleton and actin-based cell migration (56, 57). Accordingly, it is possible that the cAMP dephosphorylation signal mediated by cAMP controls CAP1 function in cell adhesion through Rap1, whereas dephosphorylation of CAP1 mediated by the cAMP/PKA axis controls CAP1 function in actin filament turnover and actin-based cell migration. An effort is under way to test these scenarios. Besides CDK5 and cAMP, we have previously reported that GSK3 phosphorylates CAP1, whereas the activation of protein kinase C and platelet-derived growth factor (PDGF) both induce dephosphorylation of CAP1. Therefore, a number of signaling pathways likely converge on CAP1 to regulate the relevant cell functions shared with CAP1.

In summary, the present study establishes critical roles for transient S307/S309 phosphorylation in CAP1 functions in regulating the actin cytoskeleton and adhesion in

the cell. The identification of CDK5 and cAMP signals that antagonistically regulate the phosphorylation state of CAP1 provides further mechanistic insights into how the signals that phosphorylate CAP1 at S307/S309 and those that induce its dephosphorylation function in concert to facilitate transient phosphorylation of CAP1. This regulation is critical for CAP1 functions in promoting actin cytoskeletal rearrangement and actin-dependent cell functions like cell adhesion and migration. Knowledge of how the cell signaling system controls the actin cytoskeleton is an important area that influences multiple disciplines in the life and biomedical sciences. Moreover, our recent studies in breast and pancreatic cancer cells show that S307/S309 phosphorylation also controls CAP1 functions in cancer cells, where its dysregulation leads to both morphological and proliferative transformations that are arguably the most prominent hallmarks of cancer (21, 53). Such novel mechanistic insights not only extend our knowledge of the regulation of CAP1 functions in the cell but also carry implications that may ultimately open up avenues for realizing the translational potential of the protein in targeted cancer therapeutics.

MATERIALS AND METHODS

DNA constructs. Lentivirus-based shRNA constructs for silencing human CDK5 and PKA catalytic subunit alpha (PKAcα) were purchased from Sigma-Aldrich (St. Louis, MO). The two sense oligonucleotides targeting PKAcα are 5′-CCGGCCCACTTGCTAAGGGCAAATGCTCGAGCATTTGCCCTTAGCAAGTGGGTTTTTG-3′ and 5′-CCGGTAGATCTCACCAAGCGCTTTGCTCGAGCAAAGCGCTTGGTGAGATCTATTTTTG-3′. The two sense oligonucleotides targeting CDK5 are 5′-CCGGCAGAACCTTCTGAAGTGTAACCTCGAGGTTACACTTCAGAAGGTTCTGTTTTTTG-3′ and 5′-CCGGCCTGAGATTGTAAAGTCATTCCTCGAGGAATGACTTTACAATCTCAGGTTTTTTG-3′. Plasmid pCMV-p35 was a gift from Li-Huei Tsai (Addgene plasmid number 1347). Plasmids pCMV-CDK2-HA, pCMV-CDK2-DN-HA, pCMV-CDK5-HA, and pCMV-CDK5-DN-HA were gifts from Sander van de Heuvel (Addgene plasmids number 1884, 1882, 1872, and 1871) (79). Plasmid pCMV-Cyclin D1 was a gift from Yue Xiong (Addgene plasmid number 19927) (80). Plasmid pCMV-Epac1-Flag has been described previously (81). The pcDNA4HisMaxA-based plasmids harboring wild-type mouse *CAP1*, the AA mutant, or the DD mutant and the enhanced green fluorescent protein (EGFP)-based plasmid harboring mouse *CAP1* were described previously (24). pGEX4T-2-cofilin for purifying GST-cofilin from *E. coli* was described previously (23). To express GST-tagged human CAP1 from *E. coli*, the PCR product amplified with forward primer 5′-CCGGAATTCGGATGGCTGACATGCAAAATC-3′ and reverse primer 5′-GGCCTCGAGCTTATCCAGCGATTTCTGTCACTGT-3′ was subcloned into the EcoRI and XhoI sites of pGEX4T-2. To express 6×His-tagged full-length mouse CAP1 (WT and mutants) in *E. coli*, forward primer 5′-CCGGAATTCATGGCTGACATGCAAAATCTTGTA-3′ and reverse primer 5′-GGCCTCGAGCTTATCCAGCGATTTCTGTCACTGT-3′ were used and the PCR product was subcloned into the EcoRI and XhoI sites of pET28a vector. To express the WT and mutant 6×His-tagged mouse CAP1 fragments (consisting of the P2 and CT domains, aa 289 to 474) in bacteria, forward primer 5′-CCGGAATTC CCTGCCCTGAAAGCTCAGAGCGGT-3′ and reverse primer 5′-GGCCTCGAGCTTATCCAGCGATTTCTGTCACTGT-3′ were used, and the PCR product was subcloned into the EcoRI and XhoI sites of pET28a vector. To express Xpress-tagged mouse CAP1 fragments (N-terminal [NT] domain, aa 1 to 226; middle domain, aa 217 to 319; and CT domain, aa 319 to 474) in mammalian cells, the following primer combinations were used, and the amplified fragments were subcloned into EcoRI and XhoI sites of pcDNA4 vector. For the NT domain, the primers were 5′-CCGGAATTCATGGCTGACATGCAAAATCTTGTA-3′ (forward [F]) and 5′-GGCCTCGAGATCCCACAGAGGGTCCAGATGG-3′ (reverse [R]). For the middle domain, the primers were 5′-CCGGAATTCTGAGTGGATTGCCATCTGGA-3′ (F) and 5′-CCGCTCGAGTAGCTGGTTCCTTCTTTGT-3′ (R). For the CT domain, the primers were 5′-CCGGAATTCTGGCTCTGCTGGAACTGGAA-3′ (F) and 5′-GGCCTCGAGCTTATCCAGCGATTTCTGTCACTGT-3′ (R). The constructs were sequenced to confirm the correct reading frames and the absence of mutations introduced in the PCRs.

Antibodies, chemical inhibitors, and activators. The monoclonal antibody against human CAP1, rabbit antibody against phospho-CAP1 S307/S309, and rabbit antibody against phospho-CAP1 S309 have been described previously (24, 82). Antibodies against phospho-cofilin S3, phospho-FAK Tyr397, and phospho-GSK3α/β Ser21/9 were purchased from Cell Signaling Technology (Danvers, MA). Rabbit antibodies against p35, GSK3β, cofilin, cyclin D1, PKA catalytic subunit alpha, and CDK5, mouse antibodies against GAPDH (glyceraldehyde 3-phosphate dehydrogenase), FAK1, and CDK5 (bead conjugated), and goat antibody against HA were from Santa Cruz Biotechnology, Inc. (Dallas, TX). Mouse antibody against FLAG and isoproterenol were from Sigma-Aldrich (St. Louis, MO). Mouse antibodies against 6×His and Xpress tags were from Invitrogen (Carlsbad, CA). Chemicals forskolin, PMA, H89, PHA-793887, roscovitine, and palbociclib isethionate (PD 0332991) were all from Selleckchem (Houston, TX). Okadaic acid, fostriecin, tautomycetin, FK506, and cyclosporine were from Tocris Bioscience (Bristol, UK).

Cell culture, treatment, and generation of shRNA knockdown clones. HeLa and HEK293T cells were maintained in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum and penicillin-streptomycin. The establishment of stable CAP1 knockdown HeLa clones has been described previously (83). The stable reexpression of Xpress-tagged mouse WTCAP1 or the phosphory-

lation mutants (S307A/S309A and S307D/S309D) in the CAP1 knockdown HeLa cells has also been described (23, 24).

To generate stable knockdown of PKA catalytic subunit alpha or CDK5 in HeLa cells, overnight-cultured cells were transfected with 1 μg of the shRNA construct. Twenty-four hours after transfection, the cells were subcultured onto 100-mm plates and selected with 0.4 μg/ml puromycin for 2 weeks. Isolated colonies were picked and amplified, and colonies with efficient knockdown of the target protein were identified through Western blotting.

For transient transfection, the indicated plasmid or a combination of plasmids were first mixed with FuGene 6 (Promega; Madison, WI), and the mixture was then added into a plate of cells cultured overnight. Twenty-four hours after transfection, the cells were either directly harvested or treated before harvesting.

For treatment with inhibitors, HeLa or HEK293T cells cultured for 24 h and at ~80% confluence were treated with the indicated inhibitors at the specified concentrations and time durations. The cells were washed with ice-cold phosphate-buffered saline (PBS) and lysed using radioimmunoprecipitation assay (RIPA) cell lysis buffer. The supernatant derived after centrifugation was collected as cell lysate and used for analysis in Western blotting or immunoprecipitation assays.

***In vitro* kinase assays.** Recombinant 6×His-tagged CAP1 (full length or truncated) purified from *E. coli* cells and conjugated to nickel beads (Qiagen, Hilden, Germany) was incubated with active CDK5 or CDK2 purchased from SignalChem (Richmond, Canada) in kinase assay buffer (5 mM MOPS [morpholinepropanesulfonic acid; pH 7.2], 2.5 mM β-glycerol-phosphate, 5 mM $MgCl_2$, 1 mM EGTA, and 250 μm ATP) for 30 min at 30°C. The reactions were stopped by adding SDS loading buffer. After boiling, 25-μl samples were loaded onto SDS-PAGE gels, transferred to nitrocellulose membranes, and blotted with either rabbit anti-phospho-S307/S309 antibody or rabbit anti-phospho-S309 CAP1 antibody.

GST pulldown and immunoprecipitation assays. To express GST-tagged cofilin and CAP1, *E. coli* strain BL21(DE3) cells harboring the pGEX-4T-2-cofilin or the pGEX-4T-2-CAP1 plasmid were cultured, and expression of the protein was induced with 0.2 mM IPTG (isopropyl-β-D-thiogalactopyranoside) at room temperature for 3 h (for cofilin) or at 17°C for 24 h (for CAP1). After induction, the cells were collected by centrifugation, suspended in PBS supplemented with 1 mM dithiothreitol (DTT) and 1% Triton X-100, and sonicated to rupture the cells. The lysates were then centrifuged, and the supernatant was incubated with glutathione-Sepharose 4B beads at room temperature for 1 h. The beads were then precipitated, washed twice, and resuspended in PBS.

For GST-cofilin pulldown assays, equal amounts of GST-cofilin beads were incubated with cell lysate containing ~200 μg total proteins. For GST-CAP1 pulldown assays, cell lysate containing ~200 μg of total proteins from HeLa cells transiently transfected with the indicated plasmids for 24 h was incubated with the same amount of GST-CAP1 beads. The remaining steps of the pulldown assays and Western blotting were conducted as previously described (23, 24). For immunoprecipitation, cell lysate containing ~200 μg of total proteins was incubated with the appropriate antibody at 4°C overnight with rotation, followed by further incubation with protein A/G beads for 1 h. The precipitated beads were washed three times and suspended in SDS loading buffer for analysis by Western blotting to detect the coprecipitated proteins.

Latrunculin A treatment and immunofluorescence. HeLa cells stably reexpressing WTCAP1 or the AA or DD phosphorylation mutant were plated onto fibronectin-coated MatTek glass-bottom dishes. After overnight culture, the cells were treated with 1 μM latrunculin A for 10 and 30 min, followed by fixation and staining with Alexa Fluor 488-conjugated phalloidin as previously described (23, 24). The fluorescence images were taken using a Nikon Eclipse TE2000 microscope, and the fluorescence intensity was measured using ImageJ. For staining the focal adhesions, cells were cultured as described above for either 2.5 h or overnight (13 h), followed by fixation and staining using an antivinculin antibody, and visualized with Alexa Fluor 594-conjugated goat anti-mouse IgG(H). Confocal images were acquired with the BD Pathway 855 imaging station.

Quantification of focal adhesions. The average size (pixels) and number of focal adhesions per cell were measured in 25 representative cells and calculated using the Analyze Particle Tool of ImageJ software. Before the quantification, the raw confocal images were processed by two plugins (CLAHE and Log3D) to subtract the background and enhance the contrast, following a detailed method described previously (84) with minor modifications.

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H.Z. conceived the study, designed and performed the majority of the experiments, analyzed the data, and contributed to figure organization and writing of the manuscript. A.R., U.K., Y.X., J.X., and J.Y.X. performed experiments and analyzed data. J.X. contributed to organizing figures. S.O. contributed to the design of experiments and edited the manuscript. T.K. made overall contributions to the project and edited the manuscript. G.-L.Z. conceived the study, directed the project, organized the figures, and wrote the manuscript.

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