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Cdc2-mediated Phosphorylation of CLIP-170 Is Essential for Its Inhibition of Centrosome Reduplication*

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CLIP-170, the founding member of microtubule "plus ends tracking" proteins, is involved in many critical microtubule-related functions, including recruitment of dynactin to the microtubule plus ends and formation of kinetochore-microtubule attachments during metaphase. Although it has been reported that CLIP-170 is a phosphoprotein, neither have individual phosphorylation sites been identified nor have the associated kinases been extensively studied. Herein, we identify Cdc2 as a kinase that phosphorylates CLIP-170. We show that Cdc2 interacts with CLIP-170 mediating its phosphorylation on Thr^{287} *in vivo*. Significantly, expression of CLIP-170 with a threonine 287 to alanine substitution (T287A) results in its mislocalization, accumulation of Plk1 and cyclin B, and block of the G2/M transition. Finally, we found that depletion of CLIP-170 leads to centrosome reduplication and that Cdc2 phosphorylation of CLIP-170 is required for the process. These results demonstrate that Cdc2-mediated phosphorylation of CLIP-170 is essential for the normal function of this protein during cell cycle progression.

Microtubule dynamics consist of alternating phases of growth and shortening, a pattern of behavior known as dynamic instability (1). This process is tightly regulated by a group of proteins that bind specifically to the plus ends of the growing microtubules (2). Cytoplasmic linker protein (CLIP)[3]-170, the founding member of the microtubule plus end family (3), is composed of three separate regions: N terminus, central coiled-coil region, and C terminus. In addition to two conserved cytoskeleton-associated protein glycine-rich (CAP-Gly) domains, the N terminus has three serine-rich regions. The N-terminal domain plays an essential role in microtubule targeting (4), the long central coiled-coil domain is responsible for dimerization of the protein, and the C-terminal region, which contains two zinc-finger domains interferes with microtubule binding by interacting with the N terminus (5). Experiments in a variety of organisms have demonstrated that CLIP-170 plays an important role in microtubule dynamics (6, 7). In addition to its positive role in regulating microtubule growth in both yeast and humans (8, 9), CLIP-170 is involved in recruitment of dynactin to the microtubule plus ends and in linking microtubules to the cortex through Cdc42 and IQGAP (10, 11). The role of CLIP-170 during mitosis was recently examined by loss-of-function approaches. It was shown that CLIP-170 localizes to unattached kinetochores in prometaphase and that such localization is essential for the formation of kinetochore-microtubule attachments (12, 13).

It was previously reported that CLIP-170 is a phosphoprotein and that overall phosphorylation of CLIP-170 affects its microtubule binding ability (14). More recently, metabolic labeling experiments indicated that CLIP-170 is phosphorylated at multiple sites (15). However, individual phosphorylation sites have not been identified. Moreover, the FKBP12-rapamycin-associated protein (FRAP) is the only kinase identified to date for CLIP-170 (15). Therefore, to fully understand the regulation of CLIP-170, it is important to identify individual phosphorylation sites and the responsible kinases. In this communication, we describe a novel kinase/substrate partnership between Cdc2 and CLIP-170. We provide evidence that Cdc2 phosphorylates CLIP-170 at Thr^{287}, and the Cdc2-mediated phosphorylation of CLIP-170 is essential for its localization at microtubule plus ends in the G2 phase and the G2/M transition.

EXPERIMENTAL PROCEDURES

Cell Culture, Synchronization, and DNA Transfections—HEK293T and U2OS cells were cultured in Dulbecco modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, 100 units ml^{-1} penicillin, and 100 units ml^{-1} streptomycin at 37 °C in 8% CO_2. For synchronization, U2OS cells were treated with 2.5 mM thymidine for 16 h, released for 8 h, then blocked with 2.5 mM thymidine for another 16 h to arrest at the G1/S boundary. After release into fresh medium for different times, cells were stained with various antibodies. DNA transfection was performed using MegaTran 1.0 (ORIGENE) with the protocol as described by the manufacturer.

Generation of U2OS Cells Stably Expressing CLIP-170 Constructs—U2OS cells transfected with rat GFP-CLIP-170 (WT, T287A, or T287D) by MegaTran 1.0 were selected with 400 μg ml^{-1} of G418 for 6 weeks. Positive transfectants were confirmed by anti-GFP immunoblotting.

Recombinant Protein Purification—Various domains of CLIP-170 were subcloned into pGEX-KG vector and expressed in *Escherichia coli*. Expression was induced by 0.5 mM isopropyl

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[3] The abbreviations used are: CLIP, cytoplasmic linker protein; RNAi, RNA interference; Plk1, Polo-like kinase 1; GST, glutathione *S*-transferase; GFP, green fluorescent protein; WT, wild type; FACS, fluorescence-activated cell sorter; DAPI, 4′,6-diamidino-2-phenylindole; PIPES, 1,4-piperazinediethanesulfonic acid.

β-D-1-thiogalactopyranoside at 37 °C for 3 h after the cell density had reached 0.6 at 600 nm. Recombinant GST fusion proteins were affinity-purified by incubation with glutathione-agarose beads (Sigma, catalogue number G4510), followed by extensive washes with STE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 150 mM NaCl) and elution with glutathione elution buffer (40 mM glutathione, 50 mM Tris-HCl, 10 mM dithiothreitol, 200 mM NaCl, pH 8.0).

Kinase Assays—Purified recombinant CLIP-170 protein fragments prepared from bacteria were incubated with commercial Cdc2/cyclin B in TBMD buffer (50 mM Tris, pH 7.5, 10 mM $MgCl_2$, 5 mM dithiothreitol, 2 mM EGTA, 0.5 mM sodium vanadate, 20 mM *p*-nitrophenyl phosphate) supplemented with 125 μM ATP and 10 μCi of [γ-^{32}P]ATP (3000 Ci mmol^{-1}, PerkinElmer Life Sciences). The reaction mixtures were incubated at 30 °C for 30 min and resolved by SDS-PAGE. Gels were stained with Coomassie Brilliant Blue, dried, and subjected to autoradiography.

Metabolic Labeling and Phosphopeptide Mapping—HEK293T cells were transfected with GFP-CLIP-170 (WT or T287A) and treated with 100 ng ml^{-1} of nocodazole for 10 h to arrest cells in mitosis. Cells were labeled for 3 h with [^{32}P]orthophosphate (ICN) at 1 mCi ml^{-1} in phosphate-free DMEM in the absence or presence of roscovitine, followed by incubation with 1.0 μM okadaic acid for 30 min. Total cell lysates were prepared and incubated with an anti-GFP antibody overnight at 4 °C, followed by incubation with protein A PLUS-agarose beads for an additional 3 h. The beads were washed at least five times with TBSN buffer containing 500 mM NaCl, and at least 10 times with TBSN buffer containing 150 mM NaCl. Samples were resolved by SDS-PAGE and stained with Coomassie Brilliant Blue, followed by autoradiography. For phosphopeptide mapping, phosphoproteins were immunoprecipitated with anti-GFP antibodies, resolved by SDS-PAGE, and transferred to nitrocellulose filters. Labeled GFP-CLIP-170 was detected by autoradiography, excised, exhaustively digested with trypsin, and separated by 40% alkaline acrylamide gel (16).

Immunoprecipitation and Western Blot—Total cell lysates were incubated with Cdc2 antibody (Santa Cruz Biotechnology, catalogue number sc-53) overnight at 4 °C, followed by 2 h of incubation with protein A PLUS-agarose beads (Santa Cruz Biotechnology). Immunocomplexes were resolved by SDS-PAGE and transferred to Immobilon-P membranes (Millipore). Immunoblot analyses were performed using antibodies for GFP (Invitrogen, catalogue number A11122), CLIP-170 (Santa Cruz Biotechnology, catalogue number sc-25613), Cdc2, Plk1 (Santa Cruz Biotechnology, catalogue number sc-17783), cyclin B (Santa Cruz Biotechnology, catalogue number sc-245), and Mek1 (Santa Cruz Biotechnology, catalogue number sc-6250), followed by anti-mouse or anti-rabbit horseradish peroxidase-linked secondary antibodies (Amersham Biosciences) and detection with ECL reagent (Amersham Biosciences).

Immunofluorescence Staining Analysis—Cells grown on coverslips were incubated with 0.1% Triton X-100 in PEM buffer (20 mM PIPES, pH 6.8, 0.2% Triton X-100, 1 mM $MgCl_2$, 10 mM EGTA) for 1 min, fixed in 3.7% formaldehyde in PEM for 5 min, and subsequently permeabilized with 0.3% Triton X-100 in PEM buffer for 30 min. After blocking in phosphate-buffered saline with 3% bovine serum albumin for 1 h, cells were incubated with various antibodies (phospho-H3, α-tubulin, and γ-tubulin) at room temperature overnight, followed by incubation with a secondary antibody for 2 h at room temperature. Finally, DNA was stained with 4′,6′-diamidino-2-phenylindole (DAPI).

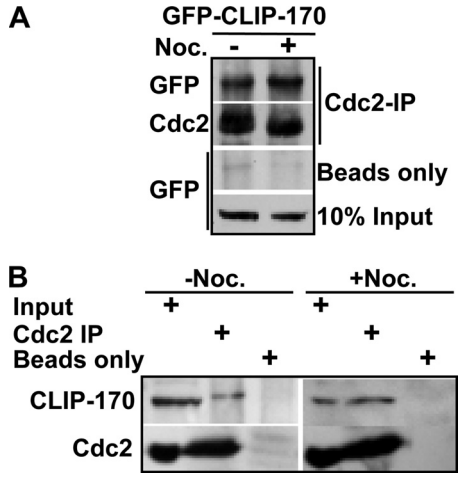


FIGURE 1. **Physical association between Cdc2 and CLIP-170 in mammalian cells.** *A*, HEK293T cells were transfected with GFP-CLIP-170, treated with or without 100 ng ml^{-1} nocodazole (*Noc.*) for 12 h, and harvested. Cell lysates were subjected to anti-Cdc2 immunoprecipitation (*IP*) and analyzed by anti-GFP Western blotting. *B*, endogenous CLIP-170 binds to endogenous Cdc2. HEK293T cells were treated with or without nocodazole and harvested. Lysates were subjected to anti-Cdc2 IP, followed by anti-CLIP-170 Western blotting.

RESULTS AND DISCUSSION

Physical Interaction between Cdc2 and CLIP-170—Although CLIP-170 was reported to be a phosphoprotein almost 20 years ago (14), kinases responsible for the multiple phosphorylations are largely unknown (15). Recently, it was shown that knockdown of CLIP-170 with RNAi results in an almost complete block of cells in mitosis (13). Considering the essential role of Cdc2 in mitosis, we explored the possibility of a potential direct connection between CLIP-170 and Cdc2.

To test whether CLIP-170 is a binding partner of Cdc2, a co-immunoprecipitation experiment was performed. Briefly, HEK293T cells were transfected with GFP-CLIP-170, treated with or without nocodazole, harvested, and immunoprecipitated with a Cdc2 antibody, followed by anti-GFP Western blot. Nocodazole is a drug to block cells at mitosis through microtubule depolymerization (17). As shown in Fig. 1*A*, overexpressed GFP-CLIP-170 protein was specifically pulled down by the Cdc2 antibody, indicating an association between CLIP-170 and Cdc2. The increased level of GFP-CLIP-170 that co-immunoprecipitates with Cdc2 in nocodazole-treated cells suggests a substrate/kinase partnership in mitosis. Furthermore, we also examined the interaction between endogenous CLIP-170 and Cdc2. As shown in Fig. 1*B*, CLIP-170 co-immunoprecipitates with a Cdc2 antibody, confirming an interaction between these two proteins.

Cdc2 Phosphorylates CLIP-170 at Thr^{287}—Considering the *in vivo* association between CLIP-170 and Cdc2, we asked

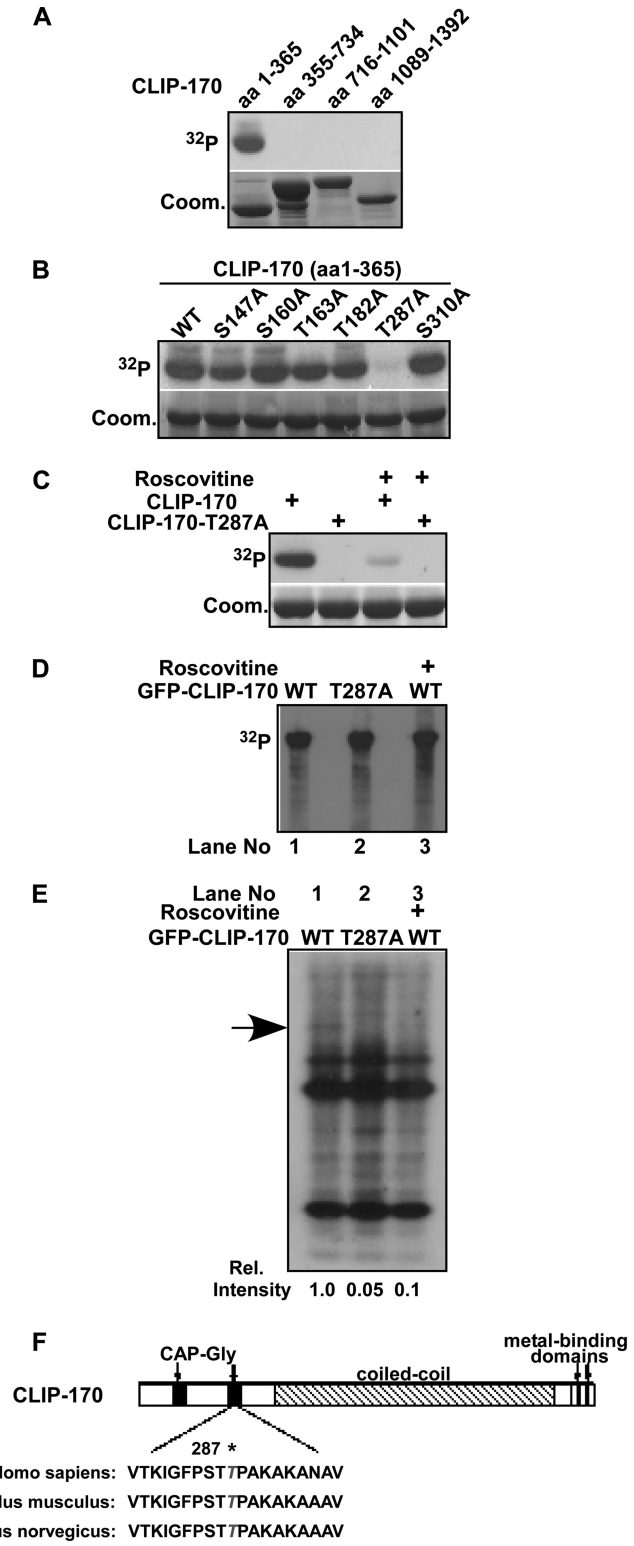


FIGURE 2. **Cdc2 phosphorylates CLIP-170 at Thr^{287} both *in vitro* and *in vivo*.** *A*, Cdc2 phosphorylates the N-terminal domain of CLIP-170 *in vitro*. Purified Cdc2/cyclin B was incubated with purified GST-CLIP-170 fragments (amino acids 1–365, 355–734, 716–1101, 1089–1392) for 30 min at 30 °C in the presence of [γ-^{32}P]ATP (*^{32}P*). The reaction mixtures were resolved by SDS-PAGE, followed by autoradiography. *Coom.*, Coomassie Blue. *B*, Thr^{287} of CLIP-170 is a Cdc2 phosphorylation site *in vitro*. Cdc2 was incubated with various GST-CLIP-170 (amino acids 1–365) serine to alanine mutants. *C*, commercial Cdc2/cyclin B was incubated with purified GST-CLIP-170-(1–365) (WT or T287A) in the presence or absence of roscovitine, resolved by SDS-PAGE, and subjected to autoradiography. *D*, HEK293T cells were transfected with GFP-CLIP-170-WT (*lanes 1* and *3*) or GFP-CLIP-170-T287A (*lane 2*) and treated with roscovitine (*lane 3*). After overnight incubation, cells were treated with nocodazole for 10 h and metabolically labeled with [^{32}P]orthophosphate. Phosphoproteins were immunoprecipitated with anti-GFP antibodies, resolved by SDS-PAGE, and subjected to autoradiography. *E*, Cdc2 phosphorylates CLIP-170 at Thr^{287} *in vivo*. Samples were prepared as in *D*. Phosphoproteins were immunoprecipitated with anti-GFP antibodies, resolved by SDS-PAGE, and transferred to nitrocellulose filters. Labeled GFP-CLIP was detected by autoradiography, excised, exhaustively digested with trypsin, and separated on a 40% alkaline acrylamide gel. The *arrow* indicates a peptide containing phosphorylated Thr^{287}. Numbers on the *bottom* are quantification results to indicate relative intensity (*Rel. Intensity*) of the peptide containing Thr^{287}. *F*, *diagram* indicates the position of Thr^{287} in CLIP-170.

whether Cdc2 directly phosphorylates CLIP-170. Commercial Cdc2/cyclin B was incubated with four purified GST-CLIP-170 fragments (amino acids 1–365, 355–734, 716–1101, and 1089–1392) in the presence of [γ-^{32}P]ATP. The reaction mixtures were resolved by SDS-PAGE, followed by autoradiography. As shown in Fig. 2*A*, only the fragment containing amino acids 1–365 was phosphorylated by Cdc2, indicating that this N-terminal region of CLIP-170 is a Cdc2 substrate *in vitro*. To map the potential phosphorylation site(s) for Cdc2, we generated a series of serine to alanine mutants in CLIP-170 spanning amino acids 1–365. Compared with the phosphorylation levels of wild-type CLIP-170 and other mutants, the phosphorylation level of the T287A mutant was completely abolished, indicating that Thr^{287} is the major phosphorylation site for Cdc2 *in vitro* (Fig. 2*B*). The consensus phosphorylation motif for Cdc2 is (S/T)P*X*(R/K), in which the Pro at +1 position is absolutely required, and a basic residue at the +3 position is preferred but not essential (18). The sequence context of Thr^{287} (^{287}TPAK) matches this consensus phosphorylation motif (Fig. 2*F*). To confirm specificity of the kinase assay, we repeated the experiment described above in the absence or presence of roscovitine, a Cdc2 inhibitor (19). Addition of roscovitine strongly inhibited the phosphorylation of CLIP-170-WT by Cdc2, supporting the specificity of the kinase assay (Fig. 2*C*).

To examine whether Cdc2-dependent phosphorylation of CLIP-170 at Thr^{287} also occurs *in vivo*, metabolic labeling experiments were performed. In brief, HEK293T cells were transfected with GFP-CLIP-170-WT (wild type) or T287A. At 16-h post-transfection, roscovitine was added to inhibit Cdc2 activity. After an additional overnight incubation, cells were treated with nocodazole for 10 h, and metabolically labeled with [^{32}P]orthophosphate for 3.5 h. To inhibit possible dephosphorylation of CLIP-170, cells were further treated with okadaic acid for 0.5 h before harvesting. GFP-CLIP-170 protein was immunoprecipitated with anti-GFP antibody, resolved by SDS-PAGE, and subjected to autoradiography. As indicated in Fig. 2*D*, the total phosphorylation level of GFP-CLIP-170 was not significantly reduced in cells expressing the T287A mutant, suggesting that Thr^{287} is only one of multiple sites phosphorylated *in vivo*. Moreover, treatment of cells with roscovitine did not dramatically reduce the phosphorylation level of CLIP-170-WT, indicating that Cdc2 is only one of multiple enzymes responsible for phosphorylation of CLIP-170 *in vivo* (Fig. 2*D*). These results are consistent with previous analyses that suggest that CLIP-170 is phosphorylated at multiple sites by multiple kinases

(15). To further analyze phosphorylation of CLIP-170-T287 *in vivo*, samples prepared as in Fig. 2*D* were excised from the filter, digested with trypsin, and resolved on a 40% alkaline gel. We observed disappearance of one specific band (indicated by the *arrow*, Fig. 2*E*) in the lanes loaded with the T287A mutant and with roscovitine-treated CLIP-170-WT, supporting the notion that Cdc2 does phosphorylate CLIP-170-Thr^{287} *in vivo*. Quantification of the relative ^{32}P intensity of the peptide containing Thr^{287} further confirmed the conclusion. Finally, in a separate study using mass spectrometry to analyze CLIP-170 phosphorylation, Thr^{287} is also identified as one of multiple sites phosphorylated *in vivo*. A detailed description of additional phosphorylation sites and additional kinases using this approach will be reported later.[4] In sum, the data show that Cdc2 phosphorylates CLIP-170 at Thr^{287} *in vivo*.

Cdc2-mediated Phosphorylation of CLIP-170 at Thr^{287} Regulates Its Binding Pattern with Tubulin—To explore the role of Cdc2-mediated phosphorylation of CLIP-170, U2OS cell lines were generated that stably express rat GFP-CLIP-170 (WT, T287A, or T287D). As shown in Fig. 3*A*, the expression levels of these GFP-CLIP-170 constructs are similar in the stably transfected cells.

Because CLIP-170 is a plus end microtubule-binding protein and the Cdc2 phosphorylation site is located at the microtubule binding domain (Fig. 2*F*), we asked whether the Cdc2-mediated phosphorylation of CLIP-170 regulates its microtubule binding ability. To address this question, U2OS cells stably expressing GFP-CLIP-170 (WT, T287A, or T287D) were analyzed by immunofluorescence microscopy using α-tubulin antibody. As indicated in Fig. 3, *B* and *C*, both GFP-CLIP-170-WT and the GFP-CLIP-T287D mutants localized to plus ends of microtubules. In striking contrast, 75% of cells expressing GFP-CLIP-170-T287A showed a diffuse pattern of GFP signal, rather than localization at microtubule plus ends (see the enlarged images of the *far-right column* in Fig. 3*B*). Because Cdc2 is critical for the later stages of the cell cycle, U2OS cells stably expressing GFP-CLIP-170-T287A were synchronized by the double thymidine block protocol in the G1/S boundary, released for different times, and stained with α-tubulin antibody (Fig. 3*D*). In late G1/early S phase (0 h of release) when Cdc2 activity is low, most GFP-CLIP-170-T287A was observed at plus ends of microtubules. In late S phase (6 h of release), G2 phase (8 h of release), and mitosis (10 h of release), most GFP-CLIP-T287A had lost its ability to localize at the plus ends of microtubules; instead, it localized throughout the cytoplasm. In the next G1 phase (12 h of release), a significant portion of GFP-CLIP-T287A relocalized back to plus ends of microtubules. All together, we conclude that Cdc2-associated phosphorylation of CLIP-170 is essential for its correct localization at the plus ends of microtubules at the later stages of the cell cycle. Neither the α-tubulin nor the γ-tubulin staining pattern was affected in cells expressing different forms of GFP-CLIP-170 (WT, T287A, or T287D) (Fig. 3, *B* and *E*).

Cdc2-mediated Phosphorylation of CLIP-170 Is Essential for the G2/M Transition—To further analyze the functional significance of Cdc2-associated phosphorylation of CLIP-170, we compared cell cycle progression of U2OS cells, stably expressing different forms of GFP-CLIP-170 (WT, T287A, or T287D) in the absence of endogenous protein. Vector-based RNAi was used to deplete CLIP-170. Specifically, U2OS cells were co-transfected with pBS/U6-CLIP-170 and pBabe-puro, a vector expressing a puromycin resistance gene. After puromycin selection, cells were harvested and analyzed by anti-CLIP-170 Western blots. As indicated in Fig. 4*A*, this approach can efficiently deplete CLIP-170. Next, U2OS cells stably expressing rat GFP-CLIP-170 (WT, T287A, or T287D) were depleted of endogenous CLIP-170 using vector-based RNAi as in Fig. 4*A*, treated with or without nocodazole, and harvested for Western blots using various antibodies as indicated (Fig. 4, *B–D*). Because of the high specificity of RNAi, rat GFP-CLIP-170 was not targeted by RNAi designed to deplete the human sequence. Compared with cells expressing CLIP-170-WT and -T287D, cells expressing CLIP-170-T287A showed significantly higher levels of Plk1 and cyclin B (compare *lanes 1*, *2*, and *3* and Fig. 4*B*). Because the protein levels of both Plk1 and cyclin B are significantly elevated in the G2/M phase, these results suggest that overexpression of CLIP-170-T287A arrests cells in the G2 or M phase. Upon depletion of endogenous CLIP-170, the levels of Plk1 from the three cell lines became similar. However, the difference between the levels of cyclin B from the three cell lines remained significant (compare *lanes 4*, *5*, and *6*, Fig. 4*B*). Addition of nocodazole to the three cell lines in the absence of endogenous protein resulted in identical levels of cyclin B. Interestingly, in this case the Plk1 level in cells expressing CLIP-T287A was clearly lower than that of cells expressing CLIP-170-WT and -T287D (compare *lanes 7*, *8*, and *9* in Fig. 4, *B–D*). We interpret these results to indicate that CLIP-170-T287A expression causes a G2 block under this condition, as the level of Plk1 continues to increase in mitosis, whereas the level of cyclin B remains the same after G2 (20).

Next, control or CLIP-170-depleted U2OS cells were synchronized with the double thymidine block to arrest in the G1/S boundary, released for different times, and stained with phospho-H3 antibodies. We found that both control and CLIP-170-depleted cells started to enter mitosis at 10 h after release, reached a peak at 12 h after release, and exited mitosis at 16 h after release. Although CLIP-170 depletion did not affect mitotic entry and exit, CLIP-170-depleted cells had significantly higher levels of phospho-H3-positive staining at 10, 12, and 14 h postrelease, indicating that CLIP-170 is involved in mitotic progression (data not shown). This is consistent with the published CLIP-170 loss-of-function phenotypic analysis (12). To assess the contribution of Cdc2 phosphorylation of CLIP-170 during mitosis, similar experiments were performed using U2OS cells stably expressing GFP-CLIP-170 (WT, T287A, or T287D) in the absence of endogenous CLIP-170 (Fig. 4*E*). Compared with cells expressing CLIP-170-WT and -T287D, a significant lower percentage of cells expressing CLIP-170-T287A could go through mitosis, indicating that

[4] H. Li, X. Yang, Y. Wang, and X. Liu, unpublished data.

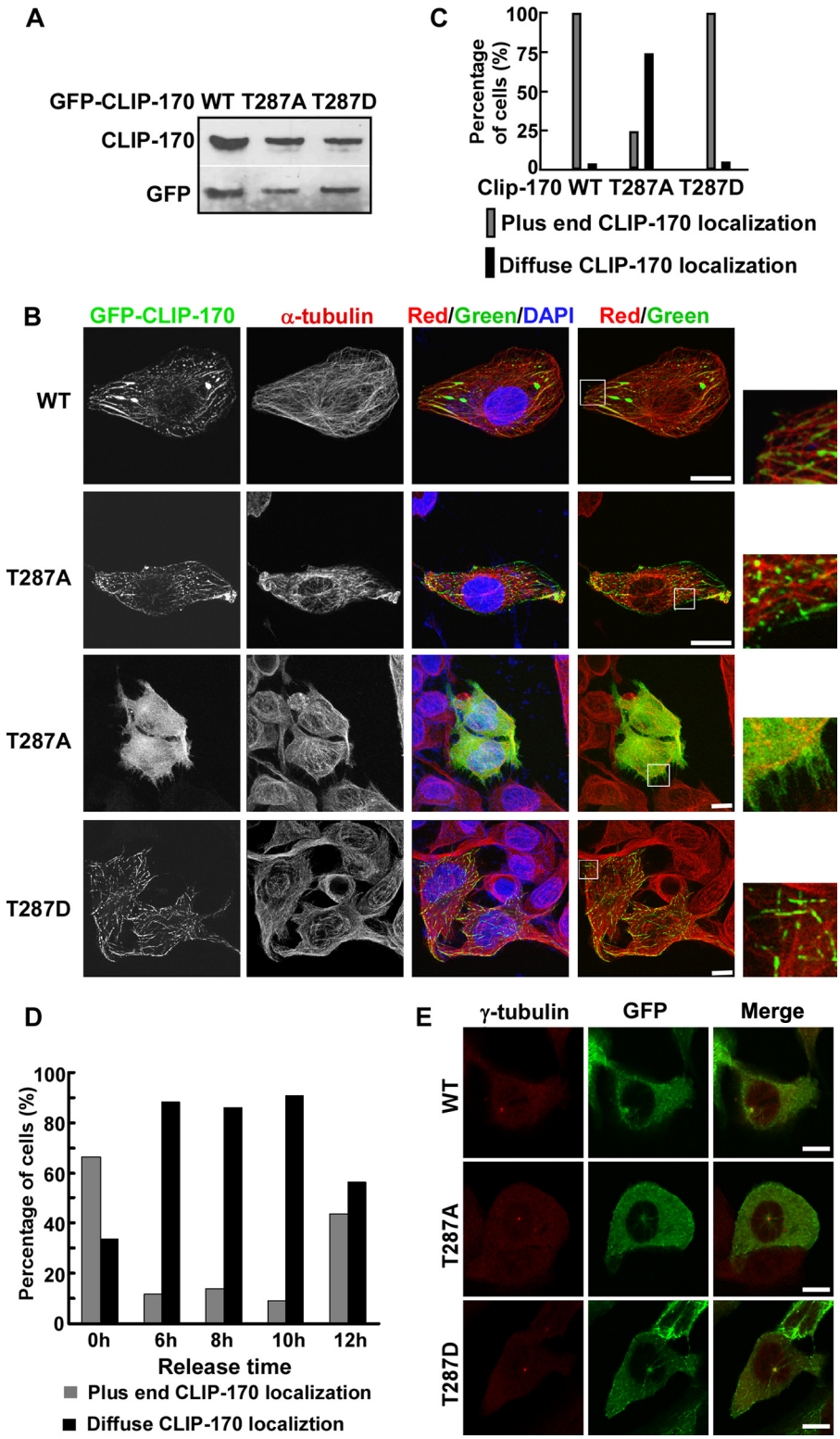


FIGURE 3. **Cdc2-mediated phosphorylation of CLIP-170 regulates its binding pattern with tubulin.** *A*, U2OS cells stably expressing rat GFP-CLIP-170 (WT, T287A, or T286D) were harvested for Western blotting using antibodies indicated. *B*, U2OS cells stably expressing GFP-CLIP-170 (WT, T287A, or T287D) were stained with α-tubulin antibodies. DNA was stained with DAPI to mark the nucleus. The *far-right column* is the enlargement of areas marked by *white squares*. *Scale bars*, 10 μm. *C*, quantification of *B* indicates relative percentages of cells with CLIP-170 localization at microtubule plus ends *versus* a diffuse pattern. *D*, U2OS cells stably expressing GFP-CLIP-170-T287A were synchronized with a double thymidine block (16 h treatment with thymidine, 8 h release, and a second thymidine block for 16 h), released for different times as indicated, and stained with α-tubulin antibodies. Quantification indicates relative percentages of cells with CLIP-170 localization at microtubule plus ends *versus* a diffuse pattern. *E*, U2OS cells stably expressing GFP-CLIP-170 (WT, T287A, or T287D) were stained with γ-tubulin antibodies. *Scale bars*, 10 μm.

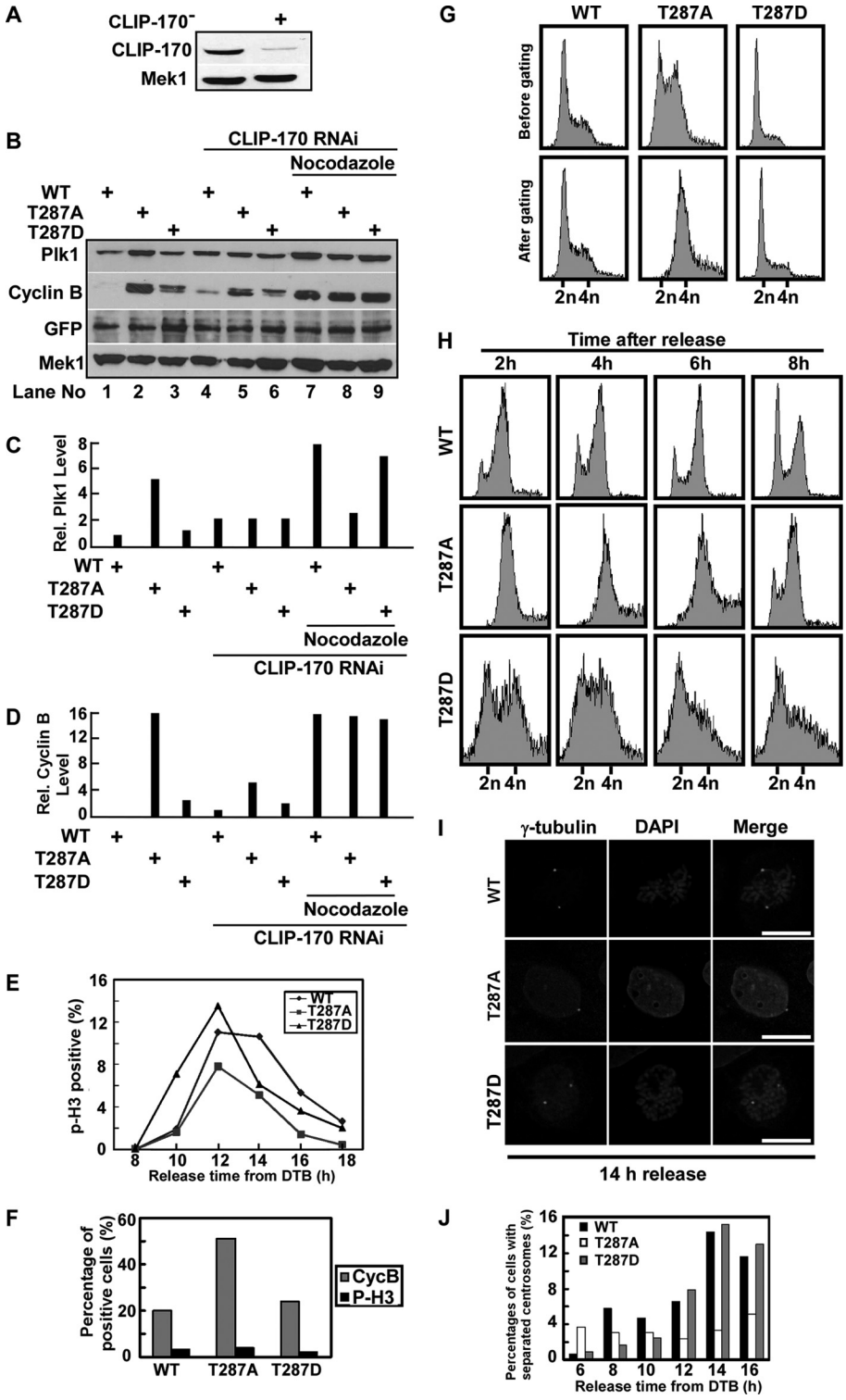
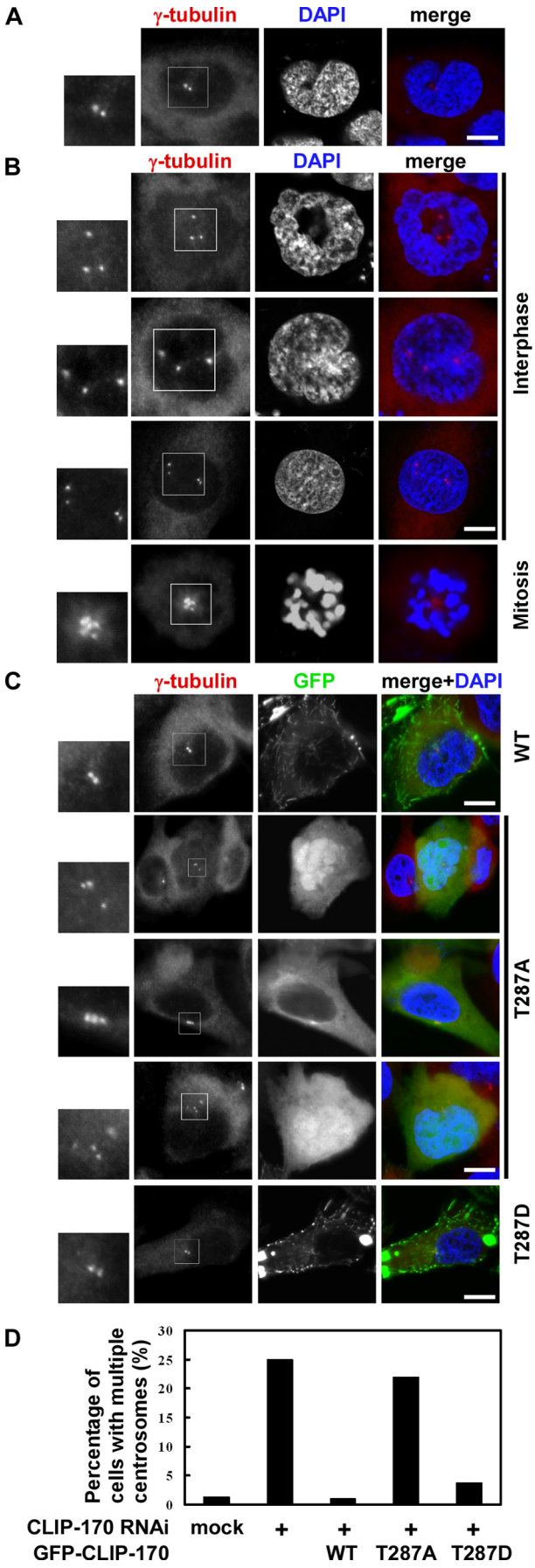


FIGURE 4. **Expression of a Cdc2 unphosphorylatable CLIP-170 mutant (T287A) leads to a G2 block.** *A*, U2OS cells were transfected with pBS/U6-CLIP-170 and pBabe-puro at a ratio of 7:1, incubated overnight, and selected with puromycin for 2 days. After floating cells were removed, the remaining attached cells were harvested and analyzed by anti-CLIP-170 Western blotting. *B,* U2OS cells stably expressing rat GFP-CLIP-170 (WT, T287A, or T287D) were depleted of endogenous CLIP-170 as in *A*, treated with nocodazole, and harvested for Western blotting using various antibodies indicated on the *left*. *C* and *D*, experiments were performed as in *B* and relative levels of Plk1 (*C*) and cyclin B (*D*) were quantified. *E*, U2OS cells stably expressing rat GFP-CLIP-170 (WT, T287A, or T287D) were depleted of endogenous CLIP-170, synchronized with the double thymidine block, released for different times as indicated, and stained with phospho-H3 antibodies. *F*, U2OS cells stably expressing GFP-CLIP-170 (WT, T287A, or T287D) were grown on coverslips and stained with either cyclin B (*CycB*) or phospho-histone H3 (*P-H3*). *G*, random growing U2OS cells stably expressing GFP-CLIP-170 (WT, T287A, or T287D) were harvested for FACS analysis. *H*, U2OS cells stably expressing rat GFP-CLIP-170 (WT, T287A, or T287D) were synchronized with thymidine block for 24 h, released for 6 h, and incubated for additional 6 h in the presence of roscovitine to arrest cells at the G2/M boundary. Upon washing away roscovitine, cells were released into fresh medium for the times indicated and harvested for FACS analysis. *I* and *J*, U2OS cells stably expressing rat GFP-CLIP-170 (WT, T287A, or T287D) were synchronized with the double thymidine block, released for different times as indicated, and stained with γ-tubulin antibodies. *I*, representative images of cells after 14 h of release. *Scale bars*, 10 μm. *J*, quantification results of centrosome separation during cell cycle progression.



Cdc2 phosphorylation of CLIP-170 is essential for its function during cell cycle progression.

To further confirm the phenotypes we described above, U2OS cells stably expressing GFP-CLIP-170 (WT, T287A, or T287D) were grown on coverslips and stained with cyclin B or phosphohistone H3 antibodies. In agreement with the Western results in Fig. 4*B*, cells expressing CLIP-170-T287A show significantly higher levels of cyclin B (50%) than those of cells expressing CLIP-170-WT (19%) or -T287D (23%). In striking contrast, the levels of phosphohistone H3, a mitotic marker, of these cells are the same (Fig. 4*F*), arguing that cells expressing CLIP-170-T287A tend to arrest in G2, but not M phase.

Next, a series of FACS analysis was performed with U2OS cells stably expressing GFP-CLIP-170 (WT, T287A, or T287D). In randomly growing cultures, cells expressing CLIP-170-T287A show a dramatic increase in population with 4N DNA content even before the gating. After gating the GFP-positive cells, we detected only the population with 4N DNA content in cells expressing CLIP-T287A. This enrichment was not observed in cells expressing GFP-CLIP-170-WT and -T287D (Fig. 4*G*). We also tried to follow the cell cycle progression after synchronization of these cells. Toward that end, cells were blocked at the G2/M boundary with roscovitine and released for different times. Both cells expressing GFP-CLIP-170-WT and -T287D were able to go through mitosis, as indicated by a gradual decrease of 4N DNA population and an increase of 2N DNA population at the same time. As expected, no obvious cell cycle progression was detected in cells expressing GFP-CLIP-170-T287A even after 8 h of release from the G2 block (Fig. 4*H*).

During the G2/M transition, centrosome separation is one essential step to ensure subsequent bipolar spindle formation in metaphase. We thus monitored centrosome separation in synchronized U2OS cells by staining with anti-γ-tubulin, a centrosome marker (Fig. 4*I*). As expected, U2OS cells stably expressing both rat GFP-CLIP-170-WT and GFP-CLIP-170-T287D showed gradual increases of centrosome separation as cells went through the G2/M transition. In striking contrast, centrosome separation was strongly inhibited in cells stably expressing CLIP-170-T287A (Fig. 4*J*).

As CLIP-170 is a centrosomal protein (Fig. 3*E*), we continued to analyze its loss-of-function phenotypes using γ-tubulin staining. Strikingly, depletion of CLIP-170 in U2OS cells leads to formation of supernumerary γ-tubulin dots (compare Fig. 5, *A versus B*). Similar experiments were performed in U2OS cells stably expressing GFP-CLIP-170 (WT, T287A, or T287D) to assess the contribution of Cdk-mediated phosphorylation of CLIP-170 in the process. While both cells expressing GFP-CLIP-170-WT and -T287D still have normal centrosomal numbers in the absence of endogenous protein, cells expressing

FIGURE 5. **Cdc2-mediated phosphorylation of CLIP-170 is required for its inhibition of centrosome reduplication.** *A*, U2OS cells were stained with γ-tubulin antibodies. *B*, U2OS cells were depleted of CLIP-170 and immunostained. *C* and *D*, U2OS cells stably expressing rat GFP-CLIP-170 (WT, T287A, or T287D) were depleted of endogenous CLIP-170, immunostained (*C*), and quantified (*D*). *A–C*, the images in the *far-left column* are high-resolution presentations of areas indicated by *white squares* in γ-tubulin staining patterns. *Scale bars*, 10 μm.

GFLP-CLIP-170-T287A failed to reverse the phenotype (Fig. 5, *C* and *D*).

It has been reported that CLIP-170 is phosphorylated at multiple sites, most likely by multiple kinases (15). To date, specific phosphorylation sites have not been determined, and information about the upstream kinases is very limited. In this communication, we provide evidence that Cdc2/cyclin B is responsible for phosphorylation of Thr^{287} of CLIP-170, and that Cdc2-mediated phosphorylation of CLIP-170 is required for its plus end microtubule localization. More significantly, we show that the Cdc2-mediated phosphorylation of CLIP-170 is essential in the G2/M transition. Specifically, cells expressing the Cdc2 unphosphorylatable mutant of CLIP-170 accumulate Plk1 and cyclin B and fail to separate the centrosomes in late G2. As the critical role of CLIP-170 in mitosis was established using both dominant negative and RNAi approaches, we have shown that CLIP-170 function during the G2/M transition is also regulated by Cdc2/cyclin B, the master kinase that controls cell division. The mechanism by which the Cdc2-mediated phosphorylation of CLIP-170-Thr^{287} regulates the G2/M transition is currently unknown. Different possibilities are currently under investigation.

Centrosomes duplicate once and only once per cell cycle, although how this process is controlled is still elusive. To our knowledge, nucleophosmin is the only protein that has been identified as yet to prevent centrosome duplication (21, 22). It was shown that nucleophosmin dissociates from the centrosomes upon Cdk2/cyclin E-mediated phosphorylation in late G1, thus allowing centrosome duplication in the S phase (21). Based on its subcellular locations during different phases of the cell cycle and its inhibitory role of centrosome duplication, it was further proposed that nucleophosmin acts as a licensing factor to ensure that the centrosome duplicates once and only once per cell cycle (21, 22). However, nucleophosmin does not bind to centrosomes during S and G2 phases, only re-associating with centrosomes in mitosis (21), suggesting that additional factors rather than nucleophosmin must act to inhibit centrosome reduplication during the G2 phase. CLIP-170 clearly localizes at the centrosomes throughout the entire cell cycle, including S and G2 phases (Fig. 3*E*), and its depletion leads to centrosome reduplication, indicating that CLP-170 might be a factor that prevents centrosome reduplication after the S phase. Consistent with the cell cycle regulation of the levels of cyclin B, the essential role of CLIP-170 to inhibit centrosome reduplication during G2 is Cdc2/cyclin B phosphorylation-dependent. Our data provide a novel mechanism of the centrosome cycle.

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