Sequential phosphorylation of Nedd1 by Cdk1 and Plk1 is required for targeting of the γTuRC to the centrosome

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Summary

Nedd1 is a new member of the γ-tubulin ring complex (γTuRC) and targets the γTuRC to the centrosomes for microtubule nucleation and spindle assembly in mitosis. Although its role is known, its functional regulation mechanism remains unclear. Here we report that the function of Nedd1 is regulated by Cdk1 and Plk1. During mitosis, Nedd1 is firstly phosphorylated at T550 by Cdk1, which creates a binding site for the polo-box domain of Plk1. Then, Nedd1 is further phosphorylated by Plk1 at four sites: T382, S397, S637 and S426. The sequential phosphorylation of Nedd1 by Cdk1 and Plk1 promotes its interaction with γ-tubulin for targeting the γTuRC to the centrosome and is important for spindle formation. Knockdown of Plk1 by RNAi decreases Nedd1 phosphorylation and attenuates Nedd1 accumulation at the spindle pole and subsequent γ-tubulin recruitment at the spindle pole for microtubule nucleation. Taken together, we propose that the sequential phosphorylation of Nedd1 by Cdk1 and Plk1 plays a pivotal role in targeting γTuRC to the centrosome by promoting the interaction of Nedd1 with the γTuRC component γ-tubulin, during mitosis.

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Key words: Cdk1, Nedd1, Plk1, Centrosome, Phosphorylation, γTuRC

Introduction

The centrosome is the primary microtubule-organizing centre (MTOC) in most somatic cells. It consists of two barrel-shaped microtubule assemblies, the centrioles, which are surrounded by electron-dense pericentriolar material (PCM). PCM is the main site for nucleation of both cytoplasmic and spindle microtubules (Bornens, 2002; Doxsey, 2001). Within the pericentriolar material, γ-tubulin ring complexes (γTuRCs) promote the nucleation of microtubules, probably by acting as structural templates for the minus end microtubules (Job et al., 2003). During mitosis, the microtubule network undergoes a profound rearrangement in which the spindle is the masterpiece. The intricate process requires centrosome maturation at G2-M transition, wherein the PCM expands through a recruitment of additional centrosomal proteins to nucleate enough microtubules for spindle organization (Blagden and Glover, 2003; Palazzo et al., 2000). Protein phosphorylation is one of the key post-translational mechanisms regulating PCM expansion and centrosome function (Fry et al., 2000). Indeed, the mitotic accumulation of the γTuRC at the centrosome is controlled by certain protein kinases such as Plk1 and Aurora A, which are enriched at the centrosome and involved in centrosomal regulation (Hannak et al., 2001; Lane and Nigg, 1996; Sumara et al., 2004; van Vugt et al., 2004).

Nedd1 (neural precursor cell expressed, developmentally downregulated gene 1) is an evolutionarily conserved protein (Manning and Kumar, 2007) that has previously been characterized as a potential mediator of γTuRC attachment to the mammalian centrosome (Haren et al., 2006; Luders et al., 2006). It was initially identified as a growth suppressor gene in mouse neuroblastoma cells (Kumar et al., 1994), but recent studies have shown that it acts as a centrosomal protein that plays a critical role, together with γ-tubulin, in microtubule nucleation and spindle assembly. Nedd1 consists of a highly conserved N-terminal WD40 repeat region responsible for its centrosomal localization, and a less conserved C-terminus, which is crucial for the γ-tubulin interaction. In interphase cells, small amounts of Nedd1 are localized at the centrosome. At the onset of mitosis, a larger amount of Nedd1 is recruited not only on the spindle poles, but also along the spindle microtubules. Depletion of Nedd1 reduces recruitment of the γTuRC to the centrosome and leads to the failure of microtubule nucleation around the centrosome and the spindle assembly (Haren et al., 2006; Luders et al., 2006). Although *Xenopus* Nedd1 plays an important role in microtubule organization, it is dispensable for targeting γ-tubulin to centrosomes (Liu and Wiese, 2008). Similarly, knockdown of the *Drosophila* ortholog of Nedd1, Dgp71 WD, had no effect on the recruitment of γ-tubulin to the centrosome (Verollet et al., 2006), indicating that important functional differences exist between human and other animal models.

Despite the discovery of the important function of human Nedd1 in γTuRC recruitment to centrosome, little is known about the upstream regulator(s) of Nedd1 for both localization and function on the centrosome, except for the clue that phosphorylation of Nedd1 during mitosis might be involved in the regulation process (Luders et al., 2006). It is speculated that a crucial phosphorylation site S411

on Nedd1 (referred to as S418 by Luder et al., which is further discussed in the Materials and Methods) is phosphorylated by Cdk1, but so far this presumption has not been confirmed. In addition to S411, there might be other phosphorylation sites involved in the regulation of Nedd1 activity (Luders et al., 2006).

In this report, we show that Nedd1 is sequentially phosphorylated by Cdk1 and Plk1. In contrast to the speculated phosphorylation at S411, we found that Cdk1 phosphorylates Nedd1 at T550 and this phosphorylation enhances the subsequent phosphorylation of Nedd1 at T382, S397, S426 and S637 by Plk1, during mitosis. We also show that Plk1 is required for Nedd1 recruitment onto the centrosome and that the sequential phosphorylation of Nedd1 by Cdk1 and Plk1 enhances the recruitment of the γTuRC to the centrosome by promoting the interaction of Nedd1 with γ-tubulin during mitosis. Our data demonstrate that Nedd1, Cdk1 and Plk1 act together to target the γTuRC to the centrosome and regulate centrosome maturation and mitotic spindle formation.

Results

Nedd1 and Plk1 colocalize at the centrosome and mitotic spindle and their interaction is polo box domain dependent

To search for the upstream regulators for Nedd1, we first carried out a co-immunoprecipitation (Co-IP) experiment in mitosis-arrested cell lysate of HeLa cells with specific Nedd1 antibody (Fig. 1A), followed by western blot analysis to identify Nedd1-binding proteins using a number of protein kinase antibodies. We observed that Plk1 was specifically co-immunoprecipitated with Nedd1 (Fig. 1A), and this Co-IP is reciprocal (Fig. 1B). These Co-IP experiments indicate that Nedd1 may interact with Plk1 at mitosis. Next, we examined the localization of endogenous Nedd1 in HeLa cells transiently expressing GFP-Plk1 with Nedd1 antibody (supplementary material Fig. S1) and found that both Nedd1 and Plk1 colocalized at the centrosomes and spindles of mitotic cells (Fig. 1C). Taken together, these data suggest that Nedd1 and Plk1 might interact and colocalize at centrosome and spindle during mitosis.

Plk1, the best characterized member of the Polo-like kinase family, is composed of an N-terminal catalytic domain and a unique C-terminal polo box domain (PBD). The binding of PBD with Plk1 substrate has been suggested to be phosphorylation dependent and is essential for both Plk1 subcellular distribution and function (Elia et al., 2003b; Lowery et al., 2004). To test the binding specificity of Nedd1 with Plk1, we generated two truncated fragments of Plk1 fused with GFP, the GFP-Plk1-kinase domain (1-400) and GFP-Plk1-PBD (326-end), and transiently coexpressed each fragment with Myc-Nedd1 in HeLa cells. In Co-IP experiments using anti-GFP antibody, we found that, like full-length GFP-Plk1, the GFP-Plk1-PBD domain, but not the GFP-Plk1-kinase domain, specifically immunoprecipitated with Nedd1 from the mitotic HeLa cell lysates (Fig. 1D). This result indicates that Nedd1 binds to Plk1 specifically through the PBD domain during mitosis.

We also generated two truncated fragments of Nedd1 fused with Myc, the N-terminal region (1-350) and C-terminal region (341-end), and transiently expressed them in HeLa cells. By western blot analysis using anti-Myc antibody, we observed two bands only in the C-terminal-expressing mitotic cells and speculated that the upshifted band was very probably phosphorylated (Fig. 1E,F, Fig. 2). Nedd1 phosphorylation in mitosis was also reported previously (Haren et al., 2006; Luders et al., 2006). Through a GST pull-down assay using Plk1-PBD fused with GST or GST only as control, we found that the Plk1-PBD fragment could specifically pull-down two protein

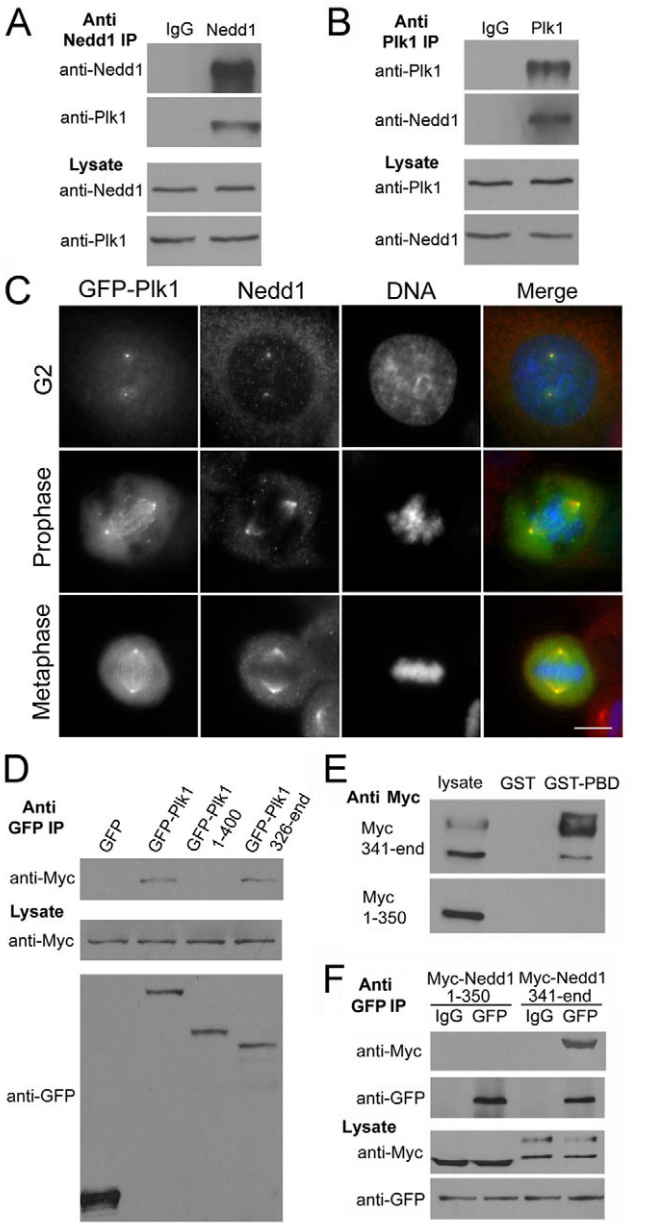


Fig. 1. Nedd1 and Plk1 colocalize at the centrosome and mitotic spindle and their interaction is PBD dependent. (A) Immunoprecipitations (IPs) with rabbit IgG as a control and anti-Nedd1 antibodies were performed in lysates of mitosis-arrested HeLa cells, followed by western blotting with antibodies against the indicated proteins. (B) IPs with mouse IgG as a control and anti-Plk1 antibodies were performed in lysates of mitosis-arrested HeLa cells, followed by western blotting with antibodies against the indicated proteins. (C) Immunofluorescence microscopy of HeLa cells to show colocalization of Nedd1 and Plk1 from G2 to metaphase. Merged images show GFP-Plk1 in green, Nedd1 in red and DNA (DAPI staining) in blue. Scale bar: 10 μm. (D) IPs with rabbit anti-GFP antibodies were performed in lysates of mitosis-arrested HeLa cells transiently coexpressing Myc-Nedd1 and GFP, GFP-Plk1, GFP-Plk1 (1-400) or GFP-Plk1 (326-end), followed by western blotting with antibodies against the indicated proteins. (E) HeLa cells were transfected with plasmids encoding Myc-Nedd1 (1-350), Myc-Nedd1 (341-end) and arrested in mitosis by nocodazole. The cell lysates were incubated with glutathione-agarose beads coated with GST, or GST-PBD-WT. After washing, the proteins bound to the beads and the cell lysates were blotted with mouse anti-Myc. (F) IPs with rabbit IgG as a control and anti-GFP antibodies were performed in lysates of mitosis-arrested HeLa cells transiently coexpressing GFP-Plk1 and Myc-Nedd1 (1-350) or Myc-Nedd1 (341-end). The cell lysates and anti-GFP IP proteins were blotted with anti-Myc and anti-GFP.

bands in Nedd1 C-terminus-expressing mitotic cell lysate and the upshifted band was much thicker (more than five-fold) than the other one. This result may suggest that Plk1-PBD preferentially interacts with the phosphorylated Nedd1 C-terminus, but not with the unphosphorylated N-terminus (Fig. 1E). To test this hypothesis, we transiently coexpressed GFP-Plk1 with the Myc-Nedd1 C-terminus (341-end) and with the Myc-Nedd1 N-terminus (1-350), and performed a Co-IP assay using anti-GFP antibody in the mitotic cell lysates. The western blot analysis demonstrated that only the upshifted C-terminus could bind to GFP-Plk1 during mitosis (Fig. 1F).

Binding of Nedd1 to Plk1 at mitosis is Cdk1 kinase activity dependent

To determine whether the interaction between Nedd1 and Plk1 is mitosis-specific and Nedd1 phosphorylation dependent, we checked the phosphorylation status of endogenous Nedd1 and performed a GST pull-down assay in the lysate of asynchronous (dominantly interphase) or mitosis-arrested HeLa cells using GST-Plk1-PBD or GST-Plk1-PBD H538A/K540A (GST-PBD-2A, in which His538 and Lys540, two residues crucial for PBD phosphopeptide binding, were mutated to alanine) (Elia et al., 2003b) as a negative control. In both asynchronous and mitosis cell lysates, the endogenous Nedd1 was clearly probed by anti-Nedd1 antibodies that had been raised in rabbit. In the asynchronous cell lysate, one positive band of the expected size was clearly seen, whereas in the mitosis cell lysate, the band was obviously upshifted. When λ-phosphatase (λPPase) was added into the mitotic lysates, the upshifted band disappeared and the normal band was enriched, indicating the conversion of the phosphorylated form of Nedd1 to the non-phosphorylated form (Fig. 2A). This result confirmed that, as reported previously (Haren et al., 2006; Luders et al., 2006), the endogenous Nedd1 was phosphorylated during mitosis. When GST-Plk1-PBD and GST-PBD-2A were used to pull-down Nedd1 in both asynchronous and mitosis cell lysates, Nedd1 was clearly detected in mitotic cell lysates, but not in asynchronous cell lysates (Fig. 2B). This result indicates that the binding of Nedd1 with Plk1 was mitosis-specific.

To test if the mitosis-specific interaction between Nedd1 and Plk1 is Cdk activity dependent, we performed a GST pull-down assay between the GST-Plk1 PBD domain and endogenous Nedd1 in mitotic HeLa cell lysates in the presence of the Cdk kinase inhibitor roscovitine. It was found that in the presence of the inhibitor, about half of the endogenous Nedd1 was downshifted, indicating that this fraction of Nedd1 was dephosphorylated, and the binding of the endogenous Nedd1 with the PBD domain was greatly reduced compared with the level of binding in the absence of the inhibitor (Fig. 2C). This result suggests that phosphorylation of Nedd1 by Cdk is essential for its interaction with Plk1. This notion was also supported by a protein-protein interaction experiment in vitro. His-Nedd1 (341-end) was pre-incubated with or without Cdk1 in the presence of ATP. The reaction mixtures were then incubated with beads coated with GST, GST-PBD or GST-PBD H538A/K540A. After washing, the proteins bound to the beads were subjected to SDS-PAGE. A pre-incubation of His-Nedd1 (341-end) with Cdk1 in the presence of ATP enhanced its interaction with GST-PBD. This Cdk1-enhanced interaction between the C-terminus of Nedd1 and Plk1-PBD required the intact phosphopeptide binding pocket of the PBD, as the GST-PBD H538A/K540A mutant showed a great decrease of the binding ability with Nedd1 (341-end) (Fig. 2D,E). These results suggest that phosphorylation of Nedd1 by Cdk1 facilitates the interaction of Nedd1 with Plk1-PBD domain.

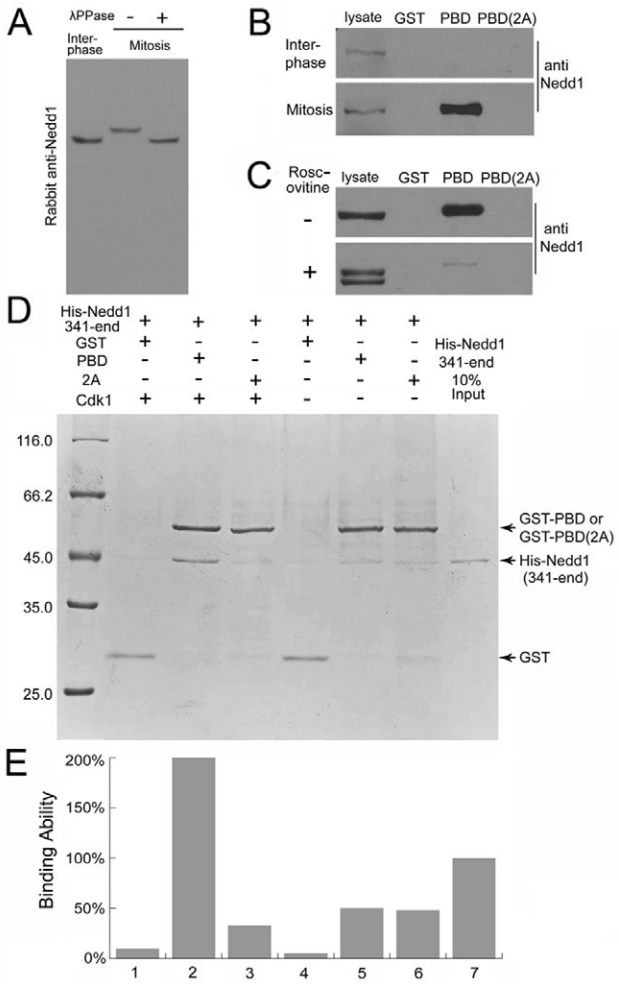


Fig. 2. Binding of Nedd1 to Plk1 at mitosis is Cdk1 kinase activity dependent. (A) Western blot analysis of interphase and mitotic HeLa cell lysates with an anti Nedd1 antibody raised in rabbit against the C-terminus of Nedd1 (amino acids 341-end). Mitotic HeLa cell lysates were treated with λ-phosphatase (400 U for 30 minutes at 30°C) or left untreated as controls. (B) Lysates of HeLa cells that were asynchronously cultured or arrested at mitosis by double thymidine and nocodazole, were incubated with glutathione-agarose beads coated with GST, GST-PBD WT or GST-PBD H538A/K540A. After washing, the proteins bound to the beads and cell lysates were blotted with rabbit anti-Nedd1. (C) Mitosis-arrested HeLa cells were treated with 10 μM roscovitine for 1 hour at 37°C or left untreated, then the cell lysates were incubated with glutathione-agarose beads coated with GST, GST-PBD-WT or GST-PBD-H538A/K540A. After washing, the proteins bound to the beads and cell lysates were blotted with rabbit anti-Nedd1. (D) His-Nedd1 (341-end) was first incubated with or without Cdk1 in the presence of ATP. The reaction mixtures were then incubated with glutathione-agarose beads coated with GST, GST-PBD or GST-PBD H538A/K540A. After washing, the proteins bound to the beads were subjected to SDS-PAGE. Coomassie Blue staining of the gel showed the loading of GST, GST-PBD WT and GST-PBD H538A/K540A in the reactions. (E) Quantification of the in vitro binding assay from D by densitometry. 10% of the input for His-Nedd1 (341-end) WT was taken as 100%.

Phosphorylation of Nedd1 on T550 by Cdk1 recruits Plk1 to Nedd1

To further determine whether Nedd1 was phosphorylated by Cdk1, and if so, at which site, GST-tagged Nedd1 fragments were purified and subjected to an in vitro protein phosphorylation assay. Autoradiography results indicated that the C-terminus of Nedd1 (341-end) can be phosphorylated by Cdk1 in vitro (Fig. 3A), which

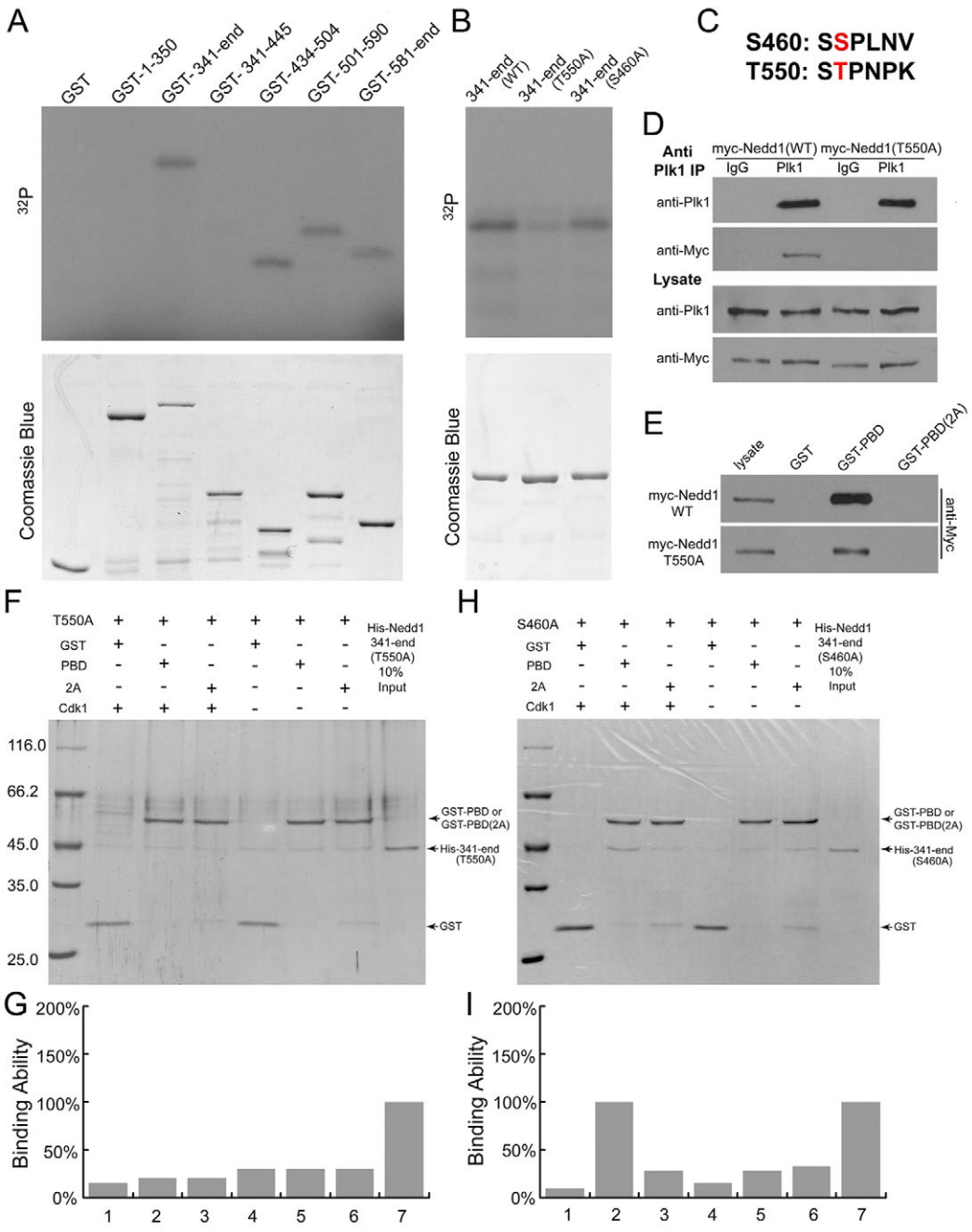


Fig. 3. Phosphorylation of Nedd1 at T550 by Cdk1 recruits Plk1. (A) Various truncated fragments of GST-tagged Nedd1 were subjected to a Cdk1 kinase assay in vitro followed by autoradiography (top). Coomassie Blue staining showed the loading of GST-tagged proteins in the reactions (bottom). (B) His-tagged Nedd1 (341-end) WT, S460A and T550A were subjected to an in vitro Cdk1 kinase assay followed by autoradiography (top). Coomassie Blue staining showed the loading of His-tagged proteins in the reactions (bottom). (C) Two potential Cdk1 phosphorylation sites: S460 and T550. (D) IPs with mouse IgG as a control and anti-Plk1 antibodies were performed in lysates of mitosis-arrested HeLa cells transiently expressing Myc-Nedd1 WT or Myc-Nedd1 T550A followed by western blotting with antibodies against the indicated proteins. (E) HeLa cells were transfected with plasmids encoding Myc-Nedd1 WT or Myc-Nedd1 T550A and arrested in mitosis by nocodazole. The cell lysates were incubated with glutathione-agarose beads binding GST, GST-PBD WT or GST-PBD H538A/K540A. After washing, the proteins bound to the beads and the cell lysates were blotted with mouse anti-Myc. (F,H) His-Nedd1 (341-end) T550A (F) and His-Nedd1 (341-end) S460A (H) were first incubated with or without Cdk1 in the presence of ATP. The reaction mixtures were then incubated with beads coated with GST, GST-PBD or GST-PBD H538A/K540A. After washing, the proteins bound to the beads were subjected to SDS-PAGE. Coomassie Blue staining showed the loading of GST, GST-PBD-WT and GST-PBD-H538A/K540A in the reactions. (G,I) Quantification of the in vitro binding assay from F and H, respectively, by densitometry. 10% of the input for His-Nedd1 (341-end) T550A (G) or His-Nedd1 (341-end) S460A (I) was taken as 100%.

is consistent with our aforementioned result that Nedd1 (341-end) was upshifted in mitotic cell lysates (Fig. 1E,F). Yaffe and colleagues have reported that Plk1-PBD binds preferentially to S[S(*P*)/T(*P*)]P motifs (Elia et al., 2003a). An examination of the amino acid sequence of the C-terminus of human Nedd1 revealed two such S[S/T]P motifs, in which S460 and T550 could be Cdk1 phosphorylation sites (Fig. 3C). To test if these two sites were phosphorylated by Cdk1, His-tagged Nedd1 (341-end) WT, S460A and T550A were subjected to an in vitro protein phosphorylation assay followed by autoradiography. The results unambiguously indicated that T550 is a Cdk1 phosphorylation site and the corresponding Coomassie Blue-stained gel showed a subtle low shift of T550A because of the lack of phosphorylation on this mutational site (Fig. 3B). Furthermore, mass spectrometry (MS) analysis of in vitro phosphorylated Nedd1 (341-end) by Cdk1 also revealed that a peptide consisting of the amino acids 524-554 was phosphorylated at T550 (supplementary material Fig. S2A).

To examine the function of T550 in vivo, HeLa cells were transfected with plasmids encoding Myc-Nedd1 WT or Myc-Nedd1 T550A and arrested in mitosis with nocodazole. The mitotic cell lysates were then subjected to a Co-IP assay using an anti-Plk1 antibody. The results showed that, compared with the interaction of Nedd1 WT and Plk1, the interaction between Nedd1 T550A and endogenous Plk1 was attenuated (Fig. 3D). The mitotic cell lysates were also subjected to a GST pull-down assay by incubating with Sepharose beads coated with GST, GST-PBD WT or GST-PBD 2A for 3 hours at 4°C. The beads were isolated and the proteins bound to the beads were separated on a gel and blotted with mouse anti-

Myc for Myc-Nedd1. Similar to the result in Fig. 2B showing that endogenous Nedd1 could be pulled-down by the Plk1 PBD, the exogenous Myc-Nedd1 WT also had a strong binding ability with the phosphopeptide-binding domain of Plk1. By contrast, the interaction between Myc-Nedd1 T550A and the Plk1 PBD domain was greatly decreased (Fig. 3E).

To further confirm the influence of phosphorylation of Nedd1 at T550 on Plk1-Nedd1 interaction, an in vitro protein binding assay was performed as described above. His-Nedd1 (341-end) T550A (Fig. 3F) or S460A (Fig. 3H) was pre-incubated with Cdk1 in the presence of ATP, followed by introduction of the GST proteins, and the protein binding state was determined by SDS electrophoresis. The result showed that, the binding ability of His-Nedd1 (341-end) T550A with GST-Plk1-PBD was very weak, regardless of the presence of Cdk1 (Fig. 3F,G). By contrast, the binding of His-Nedd1 (341-end) S460A with GST-Plk1-PBD could be promoted by Cdk1 in the presence of ATP (Fig. 3H,I).

Together these results demonstrate that the phosphorylation of Nedd1 at T550 by Cdk1 creates a binding site for the PBD of Plk1. Although there may be other Cdk1 phosphorylation sites on Nedd1 (Fig. 3A,B), our results indicate that T550 phosphorylation plays a major role in regulating Plk1-Nedd1 interaction.

Phosphorylation of Nedd1 by Cdk1 facilitates the further phosphorylation of Nedd1 by Plk1 in vitro

Next, we tested whether Nedd1 is phosphorylated by Plk1. GST (as a control), GST-Nedd1 (1-350) and GST-Nedd1 (341-end) were subjected to a protein phosphorylation assay with Plk1 followed by autoradiography. Results indicated that the C-terminus of Nedd1 (341-end), but not the WD40 repeat-containing N-terminus (1-350), was phosphorylated by Plk1 in vitro (Fig. 4A).

Combining with the in vitro protein phosphorylation assay with Plk1 kinase, we performed a MS analysis and revealed that Nedd1 T382, S397 and S637 were three Plk1 phosphorylation sites (supplementary material Fig. S2B-D). The sequence analysis also revealed that S426, which conformed to the consensus sequence for Plk1 phosphorylation: [D/E]x[S/T] (x, any amino acid) (Nakajima et al., 2003), was a potential Plk1 phosphorylation site. By comparing the sequences between human and mouse Nedd1, we found that, with the exception of T382, the other three putative Plk1 phosphorylation sites were conserved (Fig. 4B). To test the results of MS and sequence analyses, we constructed seven plasmids expressing the His-tagged C-terminus of Nedd1 in which the presumed phosphorylation sites for Plk1 were mutated to Ala (T382A, S397A and S637A referred to as 3A, and T382A, S397A, S426A and S637A referred to as 4A). These mutant proteins were purified and subjected to an in vitro phosphorylation assay with Plk1 followed by autoradiography (Fig. 4C). The result revealed that when the four points were mutated individually, any one of the four mutants T382A, S397A, S637A and S426A could significantly reduce the phosphorylation state of Nedd1 by Plk1 in vitro. However, S426A had less effect on the whole phosphorylation state than the other three point mutations. It was also found that the 4A mutant was hardly phosphorylated, and the 3A mutant, which lacked the S426A mutation, could be phosphorylated weekly. We believe that all the four amino acids, T382, S397, S637 and S426, are the phosphorylation sites for Plk1 in vitro (Fig. 4C). In addition, our results showed that S411, whose mutation to alanine was reported to affect the association of Nedd1 and γ-tubulin with the mitotic spindle (Luders et al., 2006), was not a phosphorylation site for Plk1, as mutation of this site had almost no effect on the protein phosphorylation state by Plk1 (Fig. 4C).

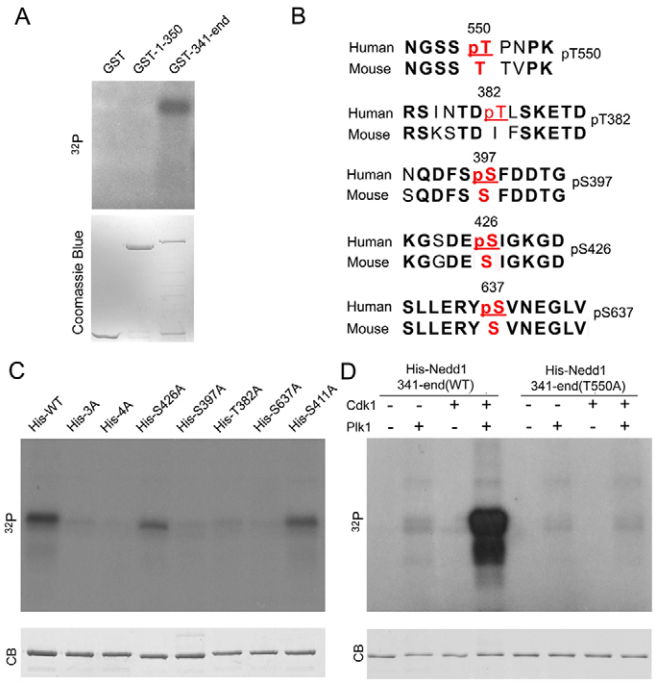


Fig. 4. Phosphorylation of Nedd1 by Cdk1 facilitates its further phosphorylation by Plk1 in vitro. (A) GST, GST-Nedd1 (1-350) and GST-Nedd1 (341-end) were subjected to a protein phosphorylation assay with Plk1 kinase in vitro, followed by autoradiography (top). Coomassie Blue staining showed the loading of GST-tagged proteins in the reactions (bottom). (B) Comparison between the conserved phosphorylation motifs of human and mouse Nedd1. (C) A few mutants of His-tagged Nedd1 (341-end) were subjected to Plk1 kinase assay in vitro followed by autoradiography (top). Coomassie blue staining showed the loading of His-tagged proteins in the reactions (bottom). (D) Sequential assay of Nedd1 (341-end) phosphorylation by Cdk1 and Plk1. His-tagged Nedd1 (341-end) WT and Nedd1 (341-end) T550A were first incubated with or without Cdk1 for 30 minutes at 30°C, in the presence of nonradioactive ATP. After inhibition of Cdk1 by roscovitine, Nedd1 proteins were further incubated with or without Plk1 in the presence of [γ-^{32}P]ATP. After 30 minutes, the reactions were stopped and analyzed by SDS-PAGE followed by autoradiography (top). Coomassie Blue (CB) staining showed the loading of His-tagged proteins in the reactions (bottom).

Furthermore, through a two-step sequential protein phosphorylation assay by Cdk1 and Plk1, we tested whether phosphorylation of Nedd1 by Cdk1 affected its phosphorylation by Plk1. The increased amount of incorporated ^{32}P in His-Nedd1 (341-end) revealed that Cdk1 largely promoted the phosphorylation of Nedd1 by Plk1. In the absence of Cdk1, the phosphorylation of Nedd1 by Plk1 is very limited. If His-Nedd1 (341-end) T550A was used, the mutant was hardly phosphorylated by Plk1, even when it was pre-incubated with Cdk1 (Fig. 4D). His-Nedd1 (341-end) S411A, S426A and 4A were also subjected to the same two-step sequential protein phosphorylation assay. The result further confirmed that S426, T382, S397 and S637 were Plk1 phosphorylation sites in vitro. At the same time, S411 was not involved in the regulation of the Nedd1-Plk1 interaction, as shown by nearly the same level of phosphorylation in S411A and the wild type (supplementary material Fig. S3).

Together, these results demonstrate that phosphorylation of Nedd1 at T550 by Cdk1 is required for its further phosphorylation by Plk1, at least in vitro.

Phosphorylation of Nedd1 by Cdk1 and Plk1 is required for targeting γ-tubulin to spindle poles by promoting the interaction of Nedd1 with γ-tubulin

Phosphorylation of Nedd1 by Plk1 and colocalization of Nedd1 with Plk1 suggests a functional interaction between Nedd1 and Plk1 during mitosis. To verify this, we first tested whether Nedd1 localization might be regulated by Plk1 or vice versa. In an RNAi assay, the empty RNAi vector pSuper and GFP-H2B were co-transfected as a control (Fig. 5A-C). To better visualize the effect of Plk1 or Nedd1 depletion, monastrol was added to the control RNAi cells to induce monopolar spindles. Nedd1 or Plk1 was depleted by co-transfected HeLa cells with RNAi vector pSuper-Nedd1 or pSuper-Plk1 and GFP-H2B, which was a marker to indicate the RNAi-vector-transfected cells. The results showed that Nedd1 or Plk1 depletion led to bipolar spindle assembly failure and monopolar spindle formation, and reduced the amount of γ-tubulin on spindle poles as previously reported (Haren et al., 2006; Lane and Nigg, 1996; Luders et al., 2006). Importantly, we found that when Plk1 was depleted, Nedd1 staining was largely reduced on the spindle pole and distributed to the cytoplasm when compared to control bipolar cells or monastrol-induced monopolar cells. However, when Nedd1 was silenced, no significant effect was observed on Plk1 localization to the spindle pole (Fig. 5A). In addition, Plk1 depletion in the RNAi assay decreased Nedd1 phosphorylation during mitosis, thus strengthening our previous conclusion that Plk1 is responsible for Nedd1 phosphorylation (Fig. 5C). To observe the detailed effect of Nedd1 and Plk1 depletion on the cell, with a time-lapse imaging technique, we followed the mitotic process in GFP-α-tubulin HeLa cells co-transfected with pSuper vector (control), pSuper-Nedd1 or pSuper-Plk1 and RFP-H2B as a marker to identify the pSuper plasmid-transfected cells. We observed that both depletions resulted in poorly separated spindle poles and interrupted centrosome function as shown by attenuated microtubule nucleation around the centrosomes (Fig. 5D). These observations are consistent with previous reports that depletion of Nedd1 (Luders et al., 2006) or Plk1 (Lane and Nigg, 1996) induced monopolar spindles and thus affected mitotic processes.

As it has been reported that Nedd1 binds directly to γ-tubulin through its highly conserved C-terminal region during targeting of the γTuRC to spindle poles (Haren et al., 2006; Luders et al., 2006), we asked whether phosphorylation of Nedd1 by Cdk1 and Plk1 is essential for its interaction with γ-tubulin in the γTuRC targeting process. To answer this question, we performed a Co-IP assay to examine the interaction status of Nedd1 and γ-tubulin using anti-GFP antibody in the mitotic lysate of the cells transiently expressing GFP-Nedd1 WT, GFP-Nedd1 4A or GFP-Nedd1 4E. Our results showed that the association of Nedd1-4A with γ-tubulin was decreased, compared with that of Nedd1 WT or Nedd1 4E with γ-tubulin (Fig. 6A). This result suggests that the phosphorylation of Nedd1 by Plk1 is required for the interaction of Nedd1 with γ-tubulin in vivo and the 4E mutant is indeed a functional phospho-mimicking mutant. To test whether the priming phosphorylation by Cdk1 was important for γ-tubulin recruitment, the mitotic lyaste of the cells transiently expressing GFP-Nedd1 WT, GFP-Nedd1 T550A or GFP-Nedd1 T550E was subjected to the same Co-IP assay (Fig. 6B). The result showed that the association of Nedd1-T550A with γ-tubulin was decreased, compared with that of Nedd1 WT or Nedd1

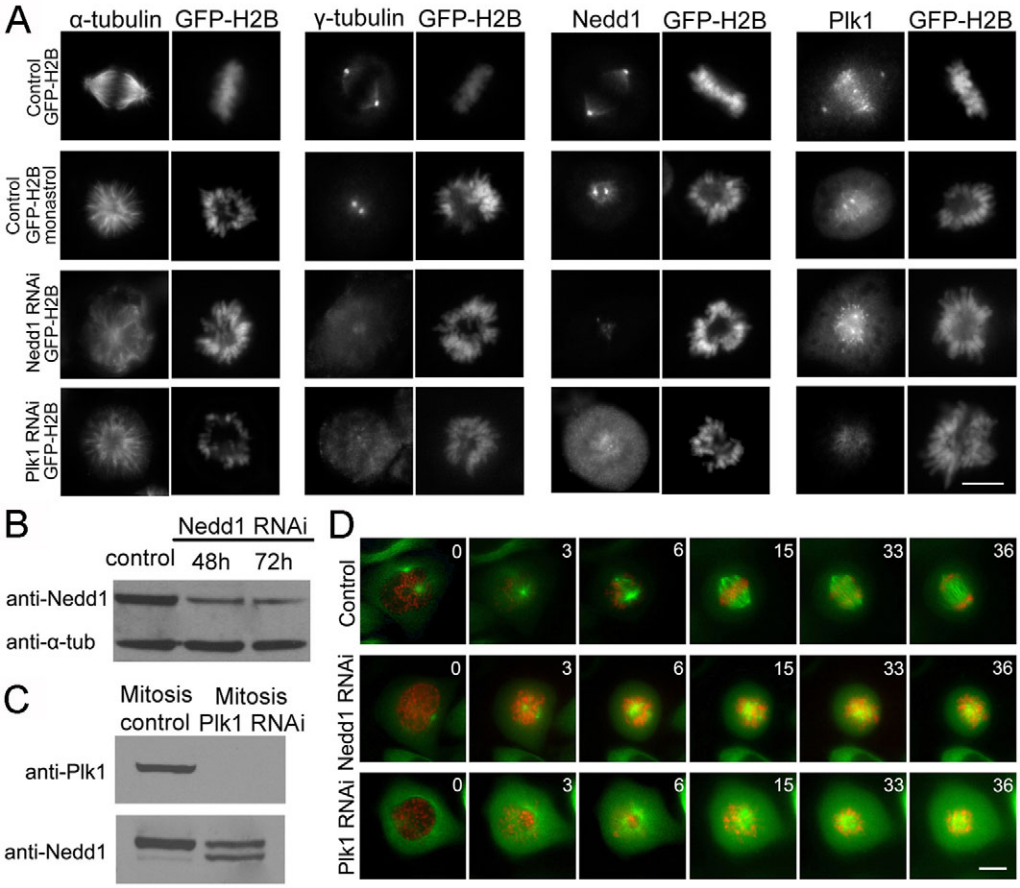


Fig. 5. Plk1 is required for Nedd1 recruitment to spindle poles. (A) HeLa cells were co-transfected with an empty vector pSuper (control), pSuper-Nedd1 or pSuper-Plk1 and GFP-H2B at a ratio of 20:1 and labeled with antibodies against α-tubulin, γ-tubulin, Nedd1 and Plk1. GFP-H2B was used as a marker to identify the pSuper-plasmid-transfected cells. 100 nM monastrol was added to the control cells for 2 hours before fixation. Scale bar: 10 μm. (B) Depletion efficiency of Nedd1 from HeLa cells was shown by western blotting. α-tubulin was used as a loading control. (C) Depletion efficiency of Plk1 from HeLa cells was shown by western blotting. Nedd1 was used as a loading control. (D) GFP–α-tubulin-expressing HeLa cells were co-transfected with pSuper vector (control), pSuper-Nedd1 or pSuper-Plk1 and RFP-H2B at the ratio of 20:1 and then subjected to time course fluorescence microscopy. RFP-H2B was used as a marker to identify the pSuper-plasmid-transfected cells. Images were acquired every 3 minutes from the beginning of the nuclear envelope brake down (0 minutes). Scale bar: 5 μm.

T550E. This result strongly supports the notion that two-step phophorylation of Nedd1 in mitosis is important for the binding of Nedd1 with γ-tubulin.

To better understand the roles of Nedd1 phosphorylation by Cdk1 and Plk1 in vivo, we carried out a Nedd1 RNAi rescue assay by expressing RNAi-insensitive exogenous GFP-Nedd1 WT, GFP-Nedd1 4A, GFP-Nedd1 4E, GFP-Nedd1 T550A or GFP-Nedd1 T550E in cells depleted of endogenous Nedd1 (Fig. 6C). In this assay, the endogenous Nedd1 was specifically depleted, and simultaneously, the exogenous GFP-Nedd1 were expressed at the level comparable to the endogenous Nedd1 (supplementary material Fig. S4A). The result showed that phosphorylation site mutation did not affect Nedd1 localization to spindle poles. This result could indicate that the N-terminus of Nedd1 is responsible for its centrosome localization and this localization is not affected by the mutation at the C-terminus. More importantly, we observed that γ-tubulin staining was attenuated in 4A- or T550A-expressing cells compared with that of WT-, 4E- or T550E-expressing cells (Fig. 6C). Quantification of the γ-tubulin fluorescence intensity indicated that the 4A and T550A mutants reduced the amount of γ-tubulin on the centrosomes by about 66% and 36%, respectively (Fig. 6D).

To further confirm the roles of Nedd1 phosphorylation in vivo, we carried out a Nedd1 RNAi rescue assay combined with a mitotic microtubule regrowth experiment. The result showed that the microtubule nucleation and spindle assembly were severely impaired in Nedd1 RNAi cells compared with control cells. The

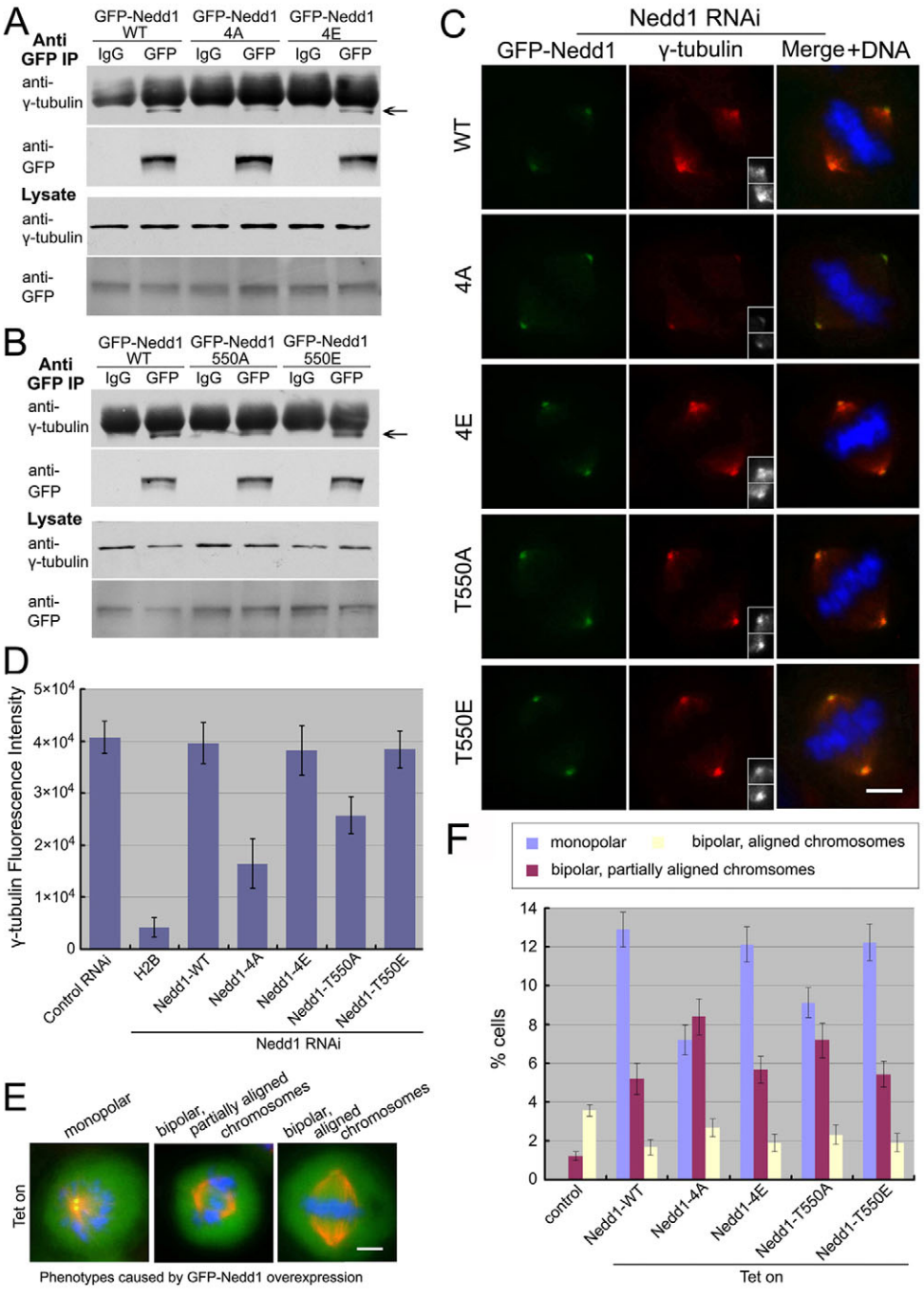


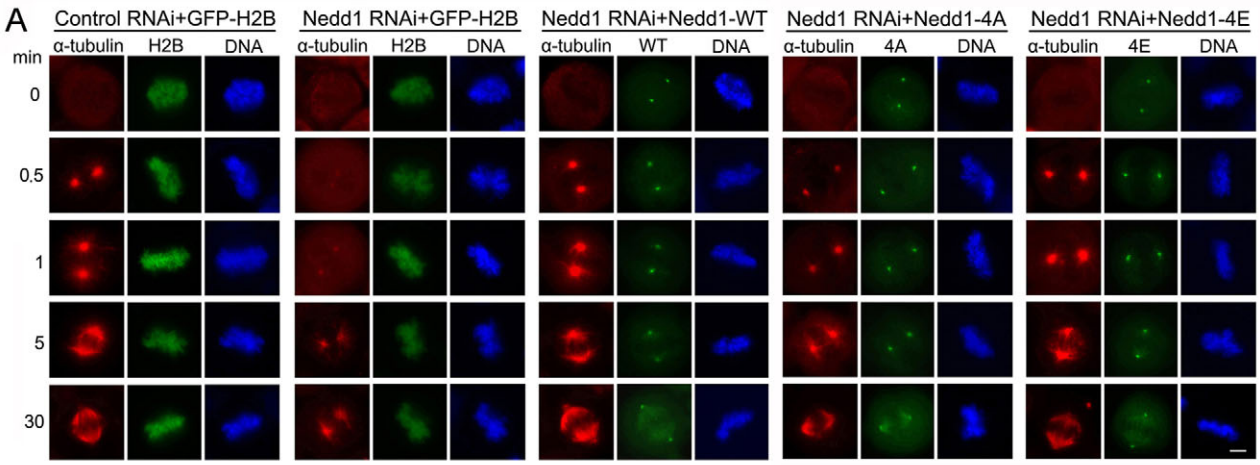
Fig. 6. Phosphorylation of Nedd1 by Cdk1 and Plk1 is required for the interaction between Nedd1 and γ-tubulin during mitosis. (A,B) HeLa cells were transfected with plasmids encoding GFP-Nedd1 WT (A,B) GFP-Nedd1 4A (A), GFP-Nedd1 4E (A), GFP-Nedd1 T550A (B) or GFP-Nedd1 T550E (B) and arrested in mitosis with nocodazole. The cell lysates and anti-GFP IP were blotted with anti-γ-tubulin and anti-GFP. The arrow indicates the band of γ-tubulin. (C) HeLa cells were co-transfected with pSuper-Nedd1 and plasmids encoding RNAi-insensitive GFP-Nedd1 WT, GFP-Nedd1 4A, GFP-Nedd1 4E, GFP-Nedd1 T550A or GFP-Nedd1 T550E at the ratio of 20:1, and were stained with anti γ-tubulin (red) and DAPI to show DNA (blue). Insets show views of γ-tubulin staining of two poles of the bipolar spindle. Scale bar: 5 μm. (D) Quantification of γ-tubulin fluorescence intensity of the spindle poles described in C. Co-transfection of an empty pSuper vector with GFP-H2B was as control RNAi. Values are means from ≥40 spindle poles from different cells. Error bars indicate the standard deviation. (E) Tet-on HeLa cells stably transfected with RNAi-insensitive pcDNA4/TO-GFP-Nedd1 WT, pcDNA4/TO-GFP-Nedd1 4A, pcDNA4/TO-GFP-Nedd1 4E, pcDNA4/TO-GFP-Nedd1 T550A or pcDNA4/TO-GFP-Nedd1 T550E were cultured in the presence of tetracycline for 24 hours and stained for α-tubulin (red). DNA was shown by DAPI staining (blue). Cells were categorized as having either a monopolar spindle, a bipolar spindle but with partially aligned chromosomes, or a bipolar spindle with aligned chromosomes. Scale bar: 5 μm. (F) Histogram showing percentage of each cell type described in E, determined from three independent experiments, in which 650-880 cells were counted. Error bars indicate the standard deviation. HeLa cells were used as a control.

defect of the microtubule nucleation and spindle assembly by Nedd1 RNAi could be rescued by expressing the wide-type or 4E Nedd1, whereas the 4A mutant could only partially restore the defect (Fig. 7A). In this assay, the microtubule nucleation status of prometaphase or metaphase cells were analyzed for each sample. Statistical results in Fig. 7B showed the percentages of the cells that have the similar microtubule nucleation status to that in Fig. 7A. These results indicated that the images in Fig. 7A accurately reflected the process of mitotic microtubule regrowth in different conditions.

Previous studies by Merdes and colleagues have shown that overexpression of Nedd1 affected γ-tubulin accumulation at spindle poles and therefore caused a dominant-negative phenotype during mitosis (Haren et al., 2006). To investigate the significance of Nedd1 phosphorylation in vivo, we generated adjustable Tet-on cell lines stably transfected with GFP-Nedd1 WT, GFP-Nedd1 4A, GFP-Nedd1 4E, GFP-Nedd1 T550A or GFP-Nedd1 T550E. We could ensure the same protein expression level of different cell lines by adjusting the tetracycline levels (supplementary material Fig. S4B). Overexpression of Nedd1 affected mitotic progression and generated many monopolar spindles and prometaphase-like spindles (bipolar, partially aligned choromosomes) (Fig. 6E). The overexpression assay showed that Nedd1 4A and T550A had less effect on mitotic progression than WT, 4E, T550E and control cells in this system, showing fewer monopolar spindles and more prometaphase-like spindles (Fig. 6F). Together, these results demonstrate that phosphorylation of Nedd1 by Cdk1 and Plk1 is required for targeting γ-tubulin to spindle poles by promoting the interaction of Nedd1 with γ-tubulin.

As aforementioned results showed that the GFP-Nedd1 4E construct was a functional phospho-mimicking mutant, we wanted to examine whether expression of the mutant could rescue γ-tubulin recruitment to the centrosome in the cells depleted of both Plk1 and Nedd1. We performed a double RNAi assay against endogenous Nedd1 and Plk1 and adjusted the amount of tetracycline to ensure that the exogenous RNAi-insensitive GFP-Nedd1 were expressed at a comparable level to that of the endogenous Nedd1 (Fig. 8A). The result showed that neither GFP-Nedd1 WT nor the phospho-mimicking mutant 4E could rescue the failure of γ-tubulin recruitment to the spindle poles (Fig. 8B). This result suggested that, although Plk1 is required for Nedd1 and γ-tubulin localization to the centrosome (Fig. 5A), the sequential localization of Nedd1 and γ-tubulin to the centrosome was not directly regulated by Plk1 phosphorylation of Nedd1. Therefore, when both Plk1 and Nedd1 were depleted, GFP-Nedd1 4E itself was unable to localize to the centrosome, and consequently, γ-tubulin could not be recruited to the centrosome (Fig. 8B).

Considering the fact that the phosphorylation of Nedd1 by Plk1 could promote the interaction of Nedd1 and γ-tubulin (Fig. 6A) and that Nedd1 and the γTuRC function in microtubule nucleation, we performed a microtubule regrowth assay by expressing GFP-Nedd1 4E in Plk1 and Nedd1 double-depleted Tet-on HeLa cells (Fig. 8C) to see if the phospho-mimicking mutant Nedd1 4E could nucleate microtubules. The percentages of the cells that have a similar microtubule nucleation status to that in Fig. 8C are shown in Fig. 8D. The results indicated that, compared with Nedd1 WT and control cells, the 4E mutant was sufficient to nucleate



B Quantification of the cells subjected to mitotic microtubule re-growth assay

	Control RNAi	Nedd1 RNAi	Nedd1 RNAi+WT	Nedd1 RNAi+4A	Nedd1 RNAi+4E
0 min	100%	100%	100%	100%	100%
0.5min	97.2%(±0.85%)	80.9%(±3.4%)	93.5%(±1.99%)	88.6%(±2.6%)	93%(±1.4%)
1 min	95.8%(±1.1%)	76.6%(±2.9%)	90.8%(±1.94%)	83.1%(±2.35%)	90.6%(±2.3%)
5 min	85.4%(±2.8%)	71.7%(±3.4%)	80.6%(±3.3%)	76.1%(±3.79%)	79.6%(±2.81%)
30 min	77.5%(±4.72%)	59.4%(±4.26%)	74.1%(±4.48%)	64.5%(±4.11%)	72.6%(±3.35%)

Fig. 7. Phosphorylation of Nedd1 by Plk1 is required for microtubule nucleation and spindle assembly. (A) HeLa cells were co-transfected with pSuper vector (control) or pSuper-Nedd1 and GFP-H2B, RNAi-insensitive GFP-Nedd1 WT, GFP-Nedd1 4A or GFP-Nedd1 4E at the ratio of 20:1. The cells were synchronized and arrested in mitosis. After cold treatment for 30 minutes, the cells were transferred into pre-warmed medium at 37°C for 30 seconds, 1 minutes, 5 minutes and 30 minutes before staining for α-tubulin (red). DNA was shown by DAPI staining (blue). Scale bar: 5 μm. (B) The microtubule nucleation status of at least 50 cells at prometaphase or metaphase were analyzed for each sample in A, and the percentages of the cells with a similar microtubule nucleation status to that in A were determined from three independent experiments. Error bars indicate the standard deviation.

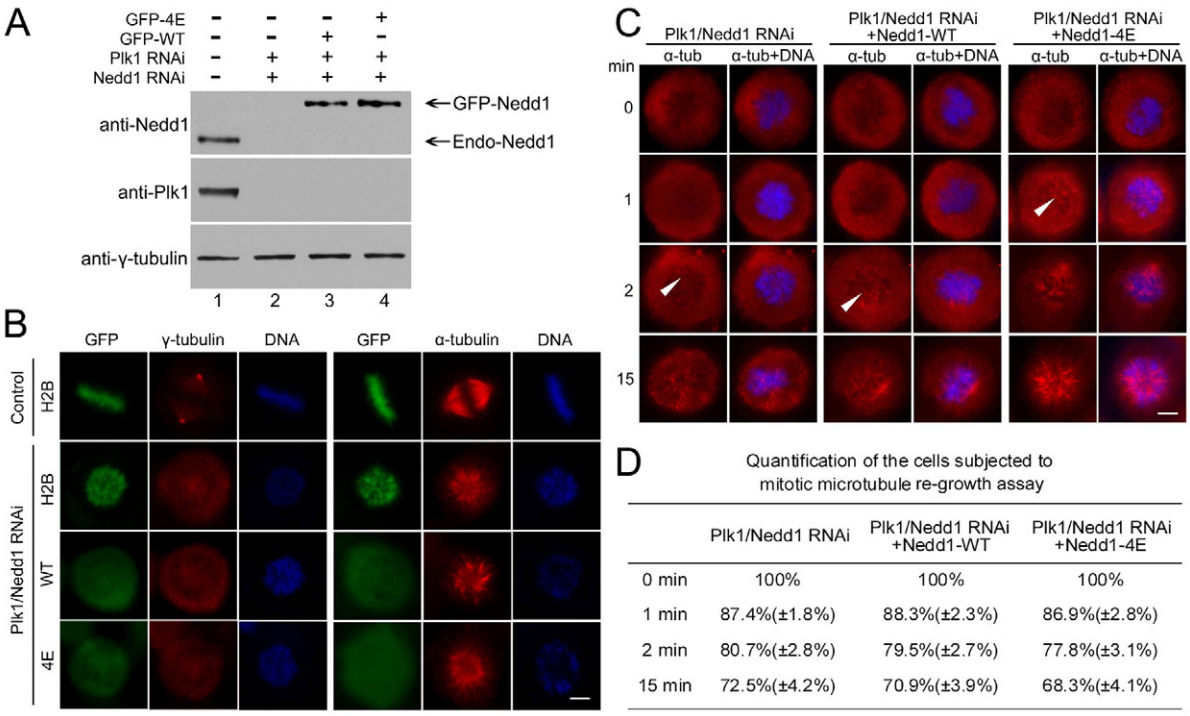
microtubules, suggesting that the four-sites phosphorylation by Plk1 is crucial for Nedd1 in regulating microtubule nucleation and spindle assembly.

Discussion

The γTuRC acts as a microtubule nucleation core at the centrosome. The recruitment of the γTuRC to the centrosome is significantly increased before mitosis in somatic cells and thus supports the formation of new spindle microtubules (Raynaud-Messina and Merdes, 2007). Thus far, a number of centrosomal proteins including pericentrin (Zimmerman et al., 2004), AKAP450/CG-NAP (Takahashi et al., 2002), ninein (Stillwell et al., 2004) and ninein-like protein Nlp (Casenghi et al., 2003), cofactor D (Cunningham and Kahn, 2008) and CDK5RAP2 (Fong et al., 2008) have been identified as regulators of the localization of the γTuRC to the centrosome. Mitotic accumulation of the γTuRC is regulated by a few protein kinases, such as Aurora A, Plk1 (Casenghi et al., 2003) and Cdk1. For instance, phosphorylation of centrosomal protein Nlp (ninein-like protein) by Plk1 results in the dissociation of Nlp from the centrosome and the subsequent recruitment of γ-tubulin to centrosome during mitosis (Casenghi et al., 2003).

Nedd1 is a newly identified key member of γTuRC components and contributes to γTuRC attachment to the centrosome. Nedd1 depletion induces a dramatic decrease of γTuRC attachment to the centrosome (Haren et al., 2006; Luders et al., 2006), leading to an increased number of abnormal spindles during mitosis, with predominant phenotypes of monopolar spindles (Haren et al., 2006; Luders et al., 2006). Although the downstream function of Nedd1 is relatively clear, the upstream regulator(s) of the localization and function of Nedd1 remains unknown, except for the indication that Nedd1 is phosphorylated during mitosis. In this work, we identified that Cdk1 and Plk1 are two primary upstream regulators for Nedd1.

The Cdk1-cyclin B complex is a master mitotic regulator. It is reported that a fraction of Cdk1 accumulates on centrosomes during the G2-M transition (Bailly et al., 1989; Bailly et al., 1992), where Cdk1 is activated (Lindqvist et al., 2005; Jackman et al., 2003), and contributes to centrosome separation and subsequent mitotic events by phosphorylating its substrates (Blangy et al., 1997; Lindqvist et al., 2007). In this work, we found that Cdk1 phosphorylates the centrosomal protein Nedd1, and T550 of Nedd1 has been identified as the Cdk1 phosphorylation site. Inhibition of Cdk1 by roscovitine in mitosis-arrested cells caused the dephosphorylation of Nedd1. The fact that the sequence context of T550 matches the consensus phosphorylation motif for Cdk1 ([S/T]Px[R/K]) (Songyang et al., 1994) indicates the specificity of Nedd1 phosphorylation by this kinase.



D

Quantification of the cells subjected to mitotic microtubule re-growth assay

	Plk1/Nedd1 RNAi	Plk1/Nedd1 RNAi +Nedd1-WT	Plk1/Nedd1 RNAi +Nedd1-4E
0 min	100%	100%	100%
1 min	87.4%(±1.8%)	88.3%(±2.3%)	86.9%(±2.8%)
2 min	80.7%(±2.8%)	79.5%(±2.7%)	77.8%(±3.1%)
15 min	72.5%(±4.2%)	70.9%(±3.9%)	68.3%(±4.1%)

Fig. 8. Phosphorylation of Nedd1 by Plk1 promotes microtubule nucleation. (A) Endogenous Nedd1 and Plk1 double-RNAi and exogenous GFP-Nedd1 expression efficiency assay using western blot analysis. The Tet-on HeLa cells stably transfected with RNAi-insensitive pcDNA4/TO-GFP-Nedd1 WT or pcDNA4/TO-GFP-Nedd1 4E were transiently transfected with pSuper-Plk1 and pSuper-Nedd1, and then treated with tetracycline for 10 hours to induce GFP-Nedd1 expression (lane 3 and 4). Normal HeLa cells were transfected without or with pSuper-Plk1 and pSuper-Nedd1 as a control (lane 1 and 2). The cell lysates were blotted with anti-Nedd1, anti-Plk1 and anti-γ-tubulin. The positions of the endogenous (Endo) Nedd1 and GFP-Nedd1 are indicated by arrows. (B) HeLa cells transiently co-transfected with pSuper vector (control) or both pSuper-Nedd1 and pSuper-Plk1 and GFP-H2B at a ratio of 20:1, and Tet-on HeLa cells described in A were stained with anti-γ-tubulin (red) or anti-α-tubulin (red) and DAPI to show DNA (blue). Scale bar: 5 μm. (C) Tet-on HeLa cells described in A (middle and right panels) and normal HeLa cells (left panel) that were transfected with pSuper-Plk1 and pSuper-Nedd1 were cold-treated for 30 minutes to depolymerise microtubules and then transferred to pre-warmed medium at 37°C for 1 minutes, 2 minutes and 15 minutes to allow microtubule nucleation before fixation and staining for α-tubulin (red). DNA was stained with DAPI (blue). The arrowheads indicated the regrown microtubules. Scale bar: 5 μm. (D) The microtubule nucleation status of at least 55 mitotic cells was analyzed for each sample in C, and the percentages of the cells with a similar microtubule nucleation status to that in C were determined from three independent experiments. The numbers in parentheses are standard deviations.

Plk1 is the best characterized member of the Polo-like kinases (Plks) and participates in several important events throughout the M phase progression, including centrosome maturation, bipolar spindle formation, chromosome segregation, mitotic exit and cytokinesis (Barr et al., 2004; Liu and Maller, 2005). Plk1 performs its function by phosphorylating its substrates and regulating their functions. Malfunction of Plk1 affects a broad range of mitotic events, such as the recruitment of several proteins to the spindle poles and the spindle pole formation (Barr et al., 2004; Liu and Maller, 2005). In this report, we have provided evidence that Nedd1 interacts with Plk1 and is phosphorylated by Plk1 in targeting the γTuRC to centrosomes. Plk1 and Nedd1 colocalize at the centrosome in interphase and at spindle poles and spindle microtubules during mitosis. Biochemical experiments indicated that phosphorylated Nedd1 C-terminus specifically binds to Plk1-PBD during mitosis. Through mass spectrometry and protein sequence analyses, we identified four major Plk1 phosphorylation sites in Nedd1 (T382, S397, S426 and S637), which, with the except of T382, are conserved in human and mouse. Through an in vitro protein phosphorylation assay, we verified that all of the four sites could be phosphorylated by Plk1.

Our results indicate that sequential phosphorylation of Nedd1 by Cdk1 and Plk1 is important for the targeting of the γTuRC to the centrosome through Nedd1. Sequential phosphorylation of Cdk1 and Plk1 on their substrates including vimentin (Yamaguchi et al., 2005), the origin recognition complex 1 (Hbo1) (Wu and Liu, 2008), BubR1 (Elowe et al., 2007), Bub1 (Qi et al., 2006) and Cep55 (Fabbro et al., 2005) have been reported previously. By sequence analysis, we found that the sequence context of T550 (SST550PNPK) corresponds to the optimal Plk1 PBD binding motif. Indeed, in this work, we have provided experimental evidence that phosphorylation of Nedd1 at T550 by Cdk1 enhances the binding between Nedd1 and Plk1 and promotes the subsequent phosphorylation of Nedd1 by Plk1. It has been reported that, in mitosis, the substitution of alanine at the possible Cdk1 phosphorylation site, S411, of Nedd1 decreases Nedd1 enrichment on the spindle and concomitantly induces a lower distribution of γ-tubulin on the spindle (Haren et al., 2006; Luders et al., 2006). However, our present work indicates that S411 is not involved in the regulation of the Plk1-Nedd1 interaction.

Up to now, how Nedd1 is recruited to the centrosome remains unknown. Our present work demonstrates that Plk1 regulates the recruitment of Nedd1 to the centrosome. Our results also showed that both the GFP-Nedd1 4A, a Plk1 non-phospho-mimicking mutant and GFP-Nedd1 T550A, a Cdk1 non-phospho-mimicking mutant localize to the centrosomes normally; meanwhile, the Plk1 phospho-mimicking mutant GFP-Nedd1 4E could not rescue the failure of γ-tubulin recruitment to the centrosomes in Plk1 and Nedd1 double-depleted cells. These results might suggest that the sequential localization of Nedd1 and γ-tubulin to the centrosome is not due to the phosphorylation of Nedd1 by Plk1 or to the direct interaction between Plk1 and Nedd1. Therefore, we speculate that Plk1 regulates Nedd1 recruitment to the centrosome by regulating other centrosomal protein(s). We expect that future studies will reveal the exact sequence of events that lead to the prominent recruitment of Nedd1 and the γTuRC to centrosome and the mitotic spindle.

We also demonstrate that the sequential phosphorylation of Nedd1 by Cdk1 and Plk1 functions through promoting the interaction of Nedd1 with γ-tubulin in mitosis in vivo. When T550 and the other four sites were mutated to T550E and 4E, these mutants could functionally mimic phosphorylated Nedd1. Our experiments proved that T550 is the Cdk1 phosphorylation site, and T382, S397, S426 and S637 are the Plk1 phosphorylation sites in vivo. However, as to why phosphorylation of Nedd1 at these sites by Plk1 is required for its interaction with γ-tubulin remains unclear.

In summary, we found that two key kinases in cell cycle, Cdk1 and Plk1, are two major upstream regulators of Nedd1 for its localization and function during mitosis. Phosphorylation of Nedd1 at T550 by Cdk1 creates a binding site for the PBD of Plk1 and promotes the subsequent phosphorylation of Nedd1 by Plk1. The sequential phosphorylation of Nedd1 by Cdk1 and Plk1 promotes the interaction of Nedd1 and γ-tubulin and the targeting of the γTuRC to centrosome, which in turn, facilitates the mitotic spindle assembly.

Materials and Methods

Plasmids and antibodies

Human Nedd1 and Plk1 were cloned from a cDNA library by RT-PCR and confirmed by DNA sequencing (Nedd1, NM_152905 which is an isoform of GCP-WD, NM_001135175 according to Luder et al. (Luder et al., 2006) and lacks seven amino acids at the N-terminus compared with GCP-WD; Plk1, NM_005030). In this study, cDNAs encoding full-length Nedd1 WT/T550A/T550E/4A (T382A, S397A, S426A, S637A)/4E (T382E, S397E, S426E, S637E), Nedd1 (1-350 or N-terminus), Nedd1 (341-end or C-terminus), Nedd1 (341-445), Nedd1 (434-504), Nedd1 (501-590), Nedd1 (585-end), mutants of Nedd1 (341-end), full-length Plk1, Plk1 (1-400), Plk1 (326-end or PBD) and Plk1-PBD-H538A/K540A were subcloned into pEGFP-C2, pCMV-Myc, pET-28a or pGEX-4T-1 vectors. All the His-tagged or GST-tagged Nedd1 proteins were expressed in *Escherichia coli.*

For RNAi experiments, a pSuper plasmid expressing shRNA was designed to target a region in the Nedd1 or Plk1 open reading frame: nucleotides 237-256 of Nedd1 and nucleotides 1581-1601 of Plk1. To obtain RNA interference (RNAi)-insensitive Nedd1 constructs, four silent point mutations within the RNAi targeting region were introduced through site-directed mutagenesis.

Anti-Nedd1 rabbit and mouse polyclonal antibodies were raised against the C-terminus (341-end) of human Nedd1 and affinity-purified. Mouse anti-α-tubulin, anti-γ-tubulin and anti-Myc antibodies were purchased from Sigma-Aldrich. Mouse anti-Plk1 antibody was from Upstate. Rabbit anti-GFP antibody was from Proteintech Group.

Cell culture, transfection and generation of stable cell lines

Cells were grown at 37°C in a 5% CO_2 atmosphere in DMEM, supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were synchronized in G1-S phase by a double-thymidine block. Mitosis-arrested cells were obtained by treatment with 100 ng/ml nocodazole (Sigma-Aldrich) about 5 hours after release from the thymidine block.

Plasmid transfection was performed at 50% cell efficiency using a standard calcium phosphate transfection protocol.

Tet-on HeLa cell lines were generated according to the supplier's instructions (plasmids and drugs from Invitrogen). HeLa cells were transfected with pcDNA6/TR, and cultured in the presence of 10 μg/ml blasticidin for 2 weeks. Positive clones were selected for further transfection of Nedd1 RNAi-insensitive pcDNA4/TO-GFP-Nedd1 WT, pcDNA4/TO-GFP-Nedd1 4A, pcDNA4/TO-GFP-Nedd1 4E, pcDNA4/TO-GFP-Nedd1 T550A or pcDNA4/TO-GFP-Nedd1 T550E followed by selection with 200 μg/ml Zeocin for 14 days. Again, positive clones were selected, tested and cultured for use. To compare the effects of the overexpression of Nedd1 WT, Nedd1 4A, Nedd1 4E, Nedd1 T550A and Nedd1 T550E impartially, we adjusted the protein expression to the same level during the same period by changing the amount of tetracycline added to each stable cell line.

Immunoprecipitation and GST fusion protein pull-down assay

Cells were washed once with cold PBS and lysed in cell lysis buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 2 mM EGTA, 0.5 mM EDTA, 0.5% NP-40, 5 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, 10 μg/ml aprotinin and 5 μg/ml pepstatin A) for 20 minutes on ice and centrifuged at 15,000 ***g*** for 15 minutes to obtain clear cell lysates.

For immunoprecipitation assays, the clarified cell lysates were incubated with the indicated antibodies for 60 minutes on ice. Then 15 μl of protein A or G Sepharose (75% slurry) were added and the mixtures were rotated for 2 hours at 4°C. The beads were washed three times with lysis buffer and harvested by brief centrifugation and finally suspended in gel sample buffer.

For GST pull-down assays, 5 μg of soluble GST or GST fused proteins bound to 15 μl of glutathione-Sepharose beads (75% slurry) were incubated with lysates from

HeLa cells for 3 hours at 4°C. The beads were washed four times with lysis buffer and harvested by brief centrifugation and finally suspended in gel sample buffer. The assays were analyzed by immunoblotting (IB) with indicated antibodies.

Immunofluorescence microscopy

HeLa cells were grown on glass coverslips and fixed with –20°C methanol for 5 minutes. Fixed cells were incubated with the appropriate primary antibodies for 1 hour at room temperature or overnight at 4°C, washed with PBS, and then incubated with the fluorescently labeled secondary antibodies for 1 hour at room temperature. The coverslips were mounted with mowiol (Sigma-Aldrich) containing 1 μg/ml DAPI (Sigma-Aldrich) for DNA staining. Cells were observed using a ×63 oil objective on an Axiovert 200M microscope (Zeiss) and the Axiovert software was used for acquisition and analysis.

For quantification of γ-tubulin intensity, images of the mitotic spindles were captured at the same exposure, and the γ-tubulin staining was measured in a 2 μm circular area around the spindle poles using ImageJ software.

For the microtubule regrowth assay, microtubules were completely depolymerized with cell dishes on the ice for 30 minutes and then the cells were transferred into pre-warmed medium at 37°C. Microtubule regrowth was stopped at different time points by methanol fixation.

Live imaging

GFP–α-tubulin-expressing HeLa cells grown in a glass-bottomed dish were co-transfected with an empty pSuper vector (control), pSuper-Nedd1 or pSuper-Plk1 and RFP-H2B at a ratio of 20:1. From 8 to 15 hours after the release of the second thymidine block, the images were acquired every 3 minutes on an Axiovert 200M microscope and the Axiovert software was used to collect and process data. Experiments were performed in a temperature-controlled chamber at 37°C with 5% CO_2 in air.

In vitro protein phosphorylation assays

2 μg of GST-tagged truncated Nedd1 proteins were incubated with either Plk1 (Cell Signaling Technology) or cyclin B/Cdc2 (New England Biolabs) in 50 mM Tris pH 7.5, 10 mM $MgCl_2$, 2 mM EGTA, 5 mM DTT, 100 mM ATP, 0.25 mCi/ml [γ-^{32}P]ATP and 1 μCi [γ-^{32}P]ATP (10 mCi/ml, 6,000 Ci/mmol; Amersham) for 30 minutes at 30°C. The reactions were stopped by adding gel sample buffer, and analyzed by SDS-PAGE and autoradiography.

For in vitro protein binding assays, 2 μg of purified substrate proteins, His-Nedd1 (341-end)-WT/T550A/S460A, were pretreated with cyclin B/Cdc2 in kinase buffer in the presence of ATP for 30 minutes at 30°C, or left untreated, as a control. The reaction mixtures were then incubated with beads binding GST, GST-PBD or GST-PBD H538A/K540A for 2 hours at 4°C. Pelleted beads were washed three times with PBS. Captured proteins were analyzed by SDS-PAGE.

For two-step sequential protein phosphorylation assays, His-tagged Nedd1 (341-end) WT, Nedd1 (341-end) T550A/S411A/S426A/4A were first incubated with or without cyclin B/Cdc2 for 30 minutes at 30°C, in the presence of nonradioactive ATP. After inhibition of Cdk1 with roscovitine, the Nedd1 proteins were further incubated with or without Plk1 in the presence of [γ-^{32}P]ATP for 30 minutes at 30°C. After stopping the kinase reactions by addition of gel sample buffer, the samples were resolved by SDS-PAGE and subjected to autoradiography.

For mass spectrometry (MS) analysis of the phosphorylated proteins, 2 μg of His-Nedd1 (341-end) WT were incubated with either cyclin B/Cdc2 (New England Biolabs) or Plk1 (Cell Signaling Technology) in 50 mM Tris pH 7.5, 10 mM $MgCl_2$, 2 mM EGTA, 5 mM DTT, 100 mM ATP and 0.25 mCi/ml [γ-^{32}P]ATP for 30 minutes at 30°C. The reactions were subjected to SDS-PAGE and the gels were processed for MS analysis.

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