

Polo-like Kinase 1 Regulates Nlp, a Centrosome Protein Involved in Microtubule Nucleation

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Summary

In animal cells, most microtubules are nucleated at centrosomes. At the onset of mitosis, centrosomes undergo a structural reorganization, termed maturation, which leads to increased microtubule nucleation activity. Centrosome maturation is regulated by several kinases, including Polo-like kinase 1 (Plk1). Here, we identify a centrosomal Plk1 substrate, termed Nlp (ninein-like protein), whose properties suggest an important role in microtubule organization. Nlp interacts with two components of the γ -tubulin ring complex and stimulates microtubule nucleation. Plk1 phosphorylates Nlp and disrupts both its centrosome association and its γ -tubulin interaction. Overexpression of an Nlp mutant lacking Plk1 phosphorylation sites severely disturbs mitotic spindle formation. We propose that Nlp plays an important role in microtubule organization during interphase, and that the activation of Plk1 at the onset of mitosis triggers the displacement of Nlp from the centrosome, allowing the establishment of a mitotic scaffold with enhanced microtubule nucleation activity.

Introduction

The centrosome is the major microtubule-organizing center (MTOC) of animal cells. In vertebrates, it is composed of two barrel-shaped centrioles that are embedded in a protein matrix known as pericentriolar material (PCM; for reviews, see Bornens, 2002; Doxsey, 2001). During the cell cycle, centrosomes are duplicated and segregated in synchrony with the genome, and aberrations in the centrosome cycle have been implicated in cancer progression (Brinkley, 2001; Doxsey, 2001; Hinchcliffe and Sluder, 2001; Nigg, 2002). In preparation for mitosis, centrosomes undergo a striking structural reorganization termed maturation. This event is characterized by the recruitment of additional γ -tubulin ring complexes (γ -TuRCs) and a concomitant increase in microtubule (MT) nucleation activity (Khodjakov and

Rieder, 1999; Palazzo et al., 2000). Although centrosome maturation is important for mitotic spindle formation, the underlying mechanisms remain largely unknown. Two protein kinases, Polo-like kinase 1 (Plk1; Lane and Nigg, 1996; Sunkel and Glover, 1988) and Aurora-A (Berdnik and Knoblich, 2002; Hannak et al., 2001), as well as protein phosphatase 4 (Helps et al., 1998; Sumiyoshi et al., 2002), have been implicated in the regulation of centrosome maturation, but the substrates of these enzymes await identification. Also acting at the G2/M transition, the protein kinase Nek2 and a member of the phosphatase 1 family contribute to regulate centrosome separation, in part through phosphorylation of the centriole-associated protein C-Nap1 (Fry et al., 1998b; Helps et al., 2000; Mayor et al., 2000).

The discovery of γ -tubulin and γ -tubulin-containing multiprotein complexes has greatly advanced our understanding of MT nucleation (for a review, see Moritz and Agard, 2001; Oakley, 2000). In *Saccharomyces cerevisiae*, two molecules of γ -tubulin associate with one molecule each of Spc97 and Spc98 (Schiebel, 2000). Orthologs of Spc97 and Spc98, termed GCP2 and GCP3 (γ -tubulin complex proteins 2 and 3), respectively, have also been identified in metazoan organisms, and animal γ -TuRCs contain at least three additional proteins, named GCP4, GCP5, and GCP6 (Fava et al., 1999; Murphy et al., 2001).

In animal cells and yeast, most MTs nucleate from MTOCs, centrosomes and spindle pole bodies (SPBs), respectively. Yet, the bulk of γ -tubulin occurs in the cytoplasm, where it is devoid of significant MT nucleation activity (Moudjou et al., 1996). Thus, γ -tubulin complexes may be activated only after their recruitment to the MTOC, raising the question of how this recruitment is regulated. In *S. cerevisiae*, the two SPB components Spc72p and Spc110p act as receptors for γ -tubulin complexes on the cytoplasmic and nuclear side, respectively (Knop and Schiebel, 1998; Schiebel, 2000; Francis and Davis, 2000), but the mechanism(s) underlying γ -TuRC recruitment to the centrosome in animal cells remains unknown. In *Drosophila*, the Asp (Abnormal spindle protein) gene product displays properties suggesting that it could act as a γ -tubulin complex binding protein (GTBP; do Carmo and Glover, 1999). On the other hand, Asp has also been implicated in the tethering of MTs to the centrosome and in central spindle formation (Riparbelli et al., 2002; Wakefield et al., 2001). In mammalian cells, A-kinase anchoring proteins, notably kendrin/pericentrin/CG-NAP, may provide a structural scaffold for MT nucleating complexes (Dictenberg et al., 1998; Doxsey et al., 1994; Takahashi et al., 2002).

A thorough analysis of MT nucleation and dynamics requires consideration of additional centrosome-associated activities, notably MT capping and MT anchoring. Recent evidence in fact suggests that the centrosome harbors two distinct multiprotein complexes, one involved in MT nucleation, the other in MT anchoring (Bornens, 2002; Mogensen, 1999; Mogensen et al., 2000). Whereas MT nucleation clearly depends on the γ -TuRCs, the mechanisms underlying MT anchoring are

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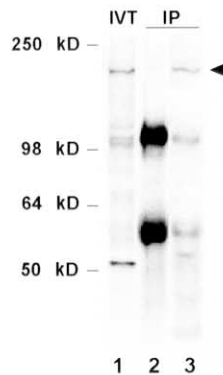
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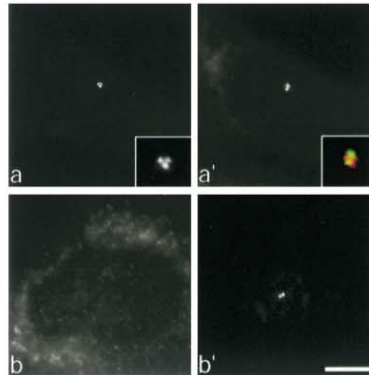
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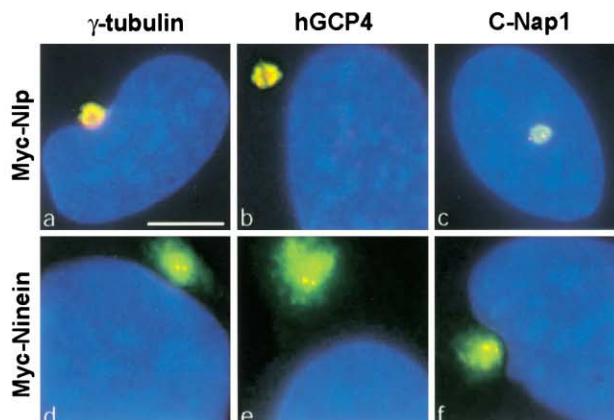
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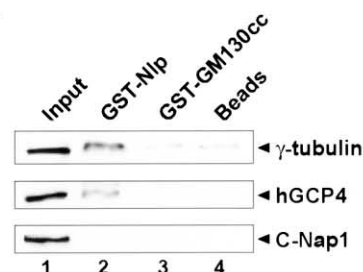
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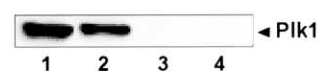


Figure 1. Nlp, a Centrosomal Protein Able to Recruit γ -TuRCs

(A) Schematic comparison between Nlp and ninein. Light gray bars indicate predicted EF-hand Ca^{2+} domains (residues 11–39 and 237–265 in Nlp; only the former is conserved in ninein), dark bars indicate coiled-coil domains, and the white bar in ninein refers to a potential GTP binding site (not conserved in Nlp). The human ninein depicted here corresponds to the most abundant isoform (ninein-Lm; Hong et al., 2000). (B) Western blot with anti-Nlp antibodies on in vitro translated Nlp (lane 1) and on immunoprecipitates with anti-Nlp (lane 2) or preimmune (lane 3) antibodies. (C) Immunofluorescent staining of U2OS cells with anti- γ -tubulin (a' and b') and anti-Nlp antibodies without (a) or with (b) competing antigen. Insets show an enlarged view of the centrosome stained with anti-Nlp (a and a'; red) and anti- γ -tubulin antibodies (a'; green). (D) U2OS cells expressing myc-Nlp (a–c) and myc-ninein (d–f; green) were counterstained with antibodies against γ -tubulin (a and d), hGCP4 (b and e), and C-Nap1 (c and f; red). The scale bars represent 10 μm . (E) U2OS cell extracts (lane 1) were incubated with GST-Nlp (lane 2), GST-GM130cc (lane 3), or glutathione-sepharose beads alone (lane 4). Pull-downs were probed by Western blotting with anti- γ -tubulin, hGCP4, or C-Nap1 antibodies. (F) To assay for an interaction between GST-Nlp and Plk1, extracts from G2-synchronized U2OS cells were treated as described in (E). The input lanes in (E) and (F) show 1/20 of the material loaded for pull-downs.

only beginning to emerge. Interest in MT anchoring has been sparked by the identification of a 249 kDa protein, termed ninein (Mogensen et al., 2000). Ninein localizes

to the appendages of mature centrioles, which are implicated in MT anchoring. Furthermore, in highly polarized cochlear epithelial cells ninein localizes to the apical

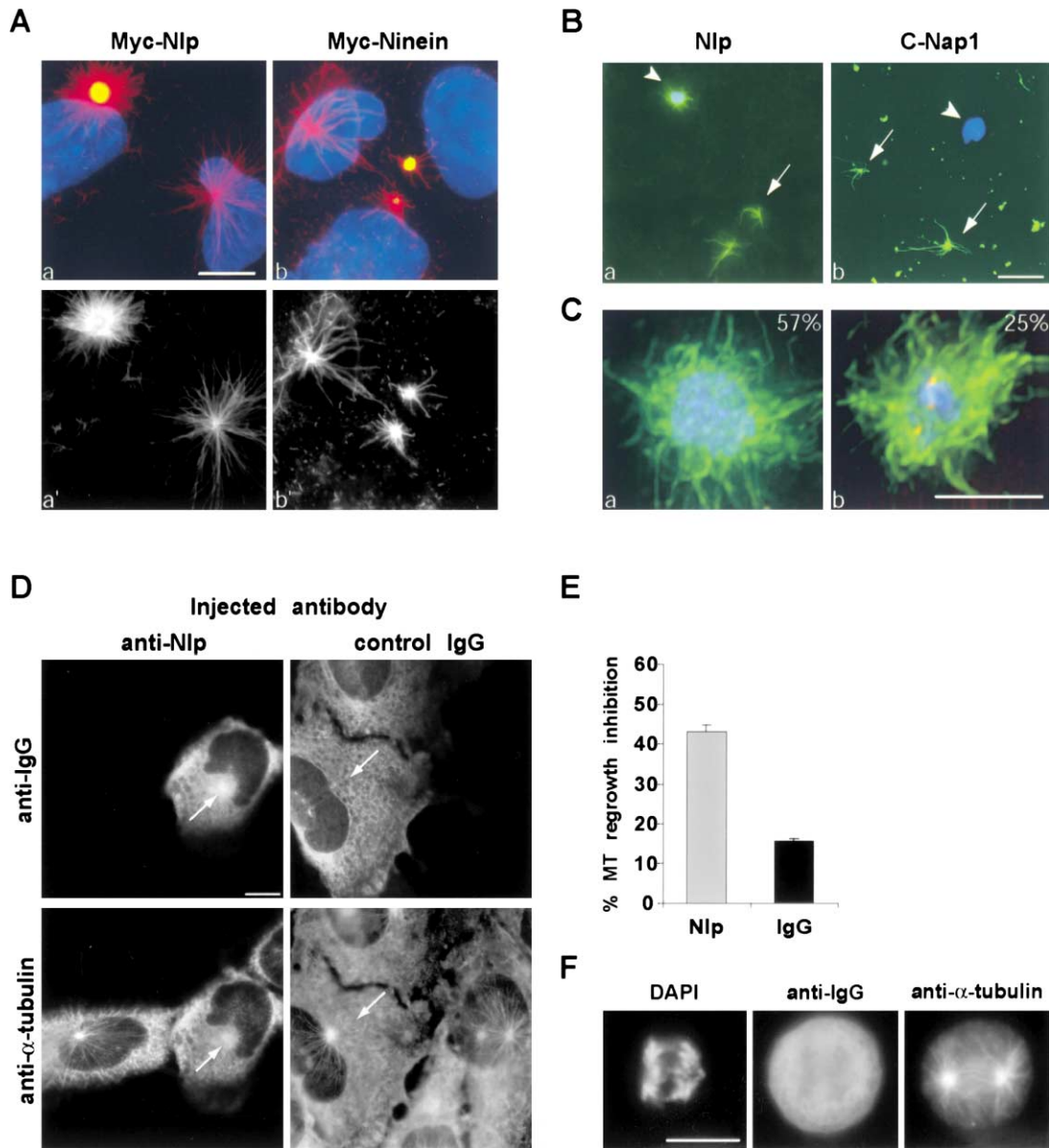


Figure 2. Nlp Stimulates MT Nucleation

(A) U2OS cells were transfected with myc-Nlp (a and a') and myc-ninein (b and b'; green) and subjected to MT regrowth assays. MTs were stained with anti- α -tubulin antibodies (a' and b'; red).

(B) Purified EGFP-Nlp (a) and EGFP-C-Nap1 (b) assemblies were incubated in *Xenopus* egg extracts and analyzed by IF microscopy. Assemblies (arrowheads) were detected by GFP fluorescence (blue) and MTs with anti- α -tubulin antibodies (green). Arrows point to MT asters triggered by copurifying centrosomes.

(C) Centrosomes in EGFP-Nlp assemblies with MT asters were detected by anti-C-Nap1 antibodies (red), analyzing a series of Z-sections with a DeltaVision microscope. Shown is one aggregate with centrosomes (b) and one without (a).

(D) U2OS were microinjected with anti-Nlp antibodies or rabbit IgG and subjected to an MT regrowth assay 6 hr later. Microinjected antibodies were detected with anti-IgG antibodies and MTs with anti- α -tubulin antibodies.

(E) Injected cells were counted as showing either a normal MT aster or inhibited MT regrowth. Histogram shows results from three independent experiments (total of 400 cells). The error bars indicate standard deviations.

(F) Nlp antibody microinjection did not inhibit MT nucleation in mitotic cells (left, DNA; middle, anti-IgG; right, anti- α -tubulin). The scale bars represent 10 μ m in (A), (D), and (F), and 5 μ m in (C).

membrane, where the vast majority of MT minus ends are anchored to noncentrosomal sites.

Here we report the characterization of a candidate GTBP. This 156 kDa human protein was named Nlp (ninein-like-protein) because it shares significant structural similarity to ninein. We show that Nlp recruits

γ -tubulin as well as hGCP4, suggesting an association with the whole γ -TuRC, both in vitro and in vivo. Functional studies indicate that Nlp plays an important, cell cycle-dependent role in MT nucleation. Our data further indicate that Nlp is a substrate of Plk1 and that Nlp phosphorylation at the G2/M transition displaces it from

the centrosome. Overexpression of Nlp caused aberrant spindle formation, suggesting that the replacement of Nlp by a mitotic MT nucleation scaffold is important for proper centrosome maturation and spindle assembly.

Results

Identification of a Ninein-Related Centrosomal Protein

To isolate proteins interacting with Polo-like kinases, a yeast two-hybrid screen was performed using the catalytically inactive *Xenopus laevis* Polo-like kinase 1 (Plx1^{N172A}) as bait. One partial cDNA isolated in this screen displayed sequence similarity to murine ninein (Bouckson-Castaing et al., 1996) and 52% sequence identity to a cDNA (KIAA0980) coding for a previously uncharacterized human protein. Because of the important role of Plk1 in centrosome maturation and the implication of ninein in the organization of MTs (Mogensen et al., 2000), this human protein was chosen for detailed analysis. Complete sequencing of KIAA0980 predicted a 156 kDa protein whose N-terminal half showed 37% sequence identity to the corresponding domain of ninein. Hence, this gene product is hereafter referred to as Nlp (*ninein-like protein*). The C-terminal domains of Nlp and ninein showed no significant sequence similarities other than predicted coiled-coil regions, but their N termini contained putative EF-hand Ca²⁺ binding domains (Figure 1A). The apparent mouse homolog of Nlp displays 52% sequence identity to human Nlp (not shown).

Overexpression of myc epitope-tagged Nlp in U2OS cells resulted in striking intracellular assemblies that formed primarily around the centrosome and resisted both nocodazole and taxol treatment (Supplemental Figure S1A at <http://www.developmentalcell.com/cgi/content/full/5/1/113/DC1>, and data not shown), suggesting that Nlp is a centrosomal protein. To study endogenous Nlp, antibodies were raised against both the N- and C-terminal domains. By immunoprecipitation-Western blotting, a 156 kDa protein could be detected in U2OS cells that comigrated with in vitro translated recombinant Nlp (Figure 1B). A protein of the same size could also be seen in Western blots performed on purified centrosomes, but not in total cell lysates, suggesting that Nlp is a low-abundance protein (data not shown). By immunofluorescence (IF) microscopy, anti-Nlp antibodies produced strong labeling of centrosomes in U2OS or HeLa cells (Figure 1C, panel a; Supplemental Figure S1B). This staining was independent of the fixation method (not shown) and readily competed by antigen (Figure 1C, panel b). Preimmune serum produced no centrosomal staining (not shown). The staining patterns of Nlp and γ -tubulin overlapped substantially (Figure 1C, panels a and a'), but a detailed comparison revealed that Nlp was often concentrated in distinct dots that appeared to surround primarily one of the two centrioles (Figure 1C, panel a, inset; Supplemental Figure S1B). This staining is reminiscent of ninein, where immunoelectron microscopy has demonstrated an association with multiple centrosomal substructures, including the appendages that are typical of the older, mature centriole (Mogensen et al., 2000). Unfortunately, our antibodies did not allow us to reliably localize Nlp by electron microscopy. So it is not clear whether Nlp and ninein localize to similar centrosomal substructures.

Nlp Recruits the γ -TuRC and Stimulates MT Nucleation

The functional consequences of Nlp overexpression were examined by staining Nlp assemblies with antibodies against known centrosomal components. Regardless of their size, Nlp assemblies recruited two distinct subunits of the γ -TuRC, γ -tubulin and hGCP4 (Fava et al., 1999), but not the unrelated centrosomal protein C-Nap1 (Fry et al., 1998a; Figure 1D, panels a–c). Similar assemblies formed by myc-ninein did not significantly alter the staining patterns of γ -tubulin, hGCP4, or C-Nap1 (Figure 1D, panels d–f). The ability of Nlp to bind γ -TuRCs, directly or indirectly, could also be demonstrated by pull-down experiments. Following incubation with total cell extracts, recombinant GST-Nlp specifically brought down both γ -tubulin and hGCP4, but not the centrosomal protein C-Nap1 (Figure 2E). Interestingly, Plk1 was also recovered specifically by GST-Nlp, confirming and extending the yeast two-hybrid data (Figure 2F). A GST-fusion to the coiled-coil domain of the Golgi protein GM130 (Barr et al., 1998) and glutathione beads alone served as negative controls (Figures 2E and 2F).

To further examine the role of Nlp in the organization of the MT network, U2OS cells expressing assemblies of either myc-Nlp or myc-ninein were subjected to MT regrowth assays. MTs were depolymerized through cold treatment and allowed to regrow for 45 s. In cells overexpressing myc-Nlp, formation of MT asters was very prominent (Figure 2A, panels a and a'). However, compared to the MT asters formed from centrosomes of nontransfected cells, asters in cells harboring Nlp assemblies were poorly focused (Figure 2A, panels a and a'). This suggested that Nlp assemblies acted as platforms for recruitment of γ -TuRCs, thereby stimulating MT nucleation. In comparison, MT nucleation in cells overexpressing myc-ninein appeared to be modest, possibly due to steric hindrance (Figure 2A, panels b and b'). Similar results have previously been obtained when analyzing MT regrowth in cells overexpressing C-Nap1 (Mayor et al., 2002).

We also purified assemblies of recombinant EGFP-Nlp from U2OS cells (Blomberg-Wirschell and Doxsey, 1998) and tested their ability to trigger MT aster formation in M phase extracts prepared from eggs of *Xenopus laevis*. Because EGFP-ninein assemblies proved unstable during purification and could not be tested in this assay, purified EGFP-C-Nap1 assemblies were used as controls. Upon incubation in *Xenopus* egg extracts, most of the Nlp assemblies (82%), but only few of the C-Nap1 assemblies (25%), were able to trigger aster formation (Figure 2B). About 25% of all Nlp assemblies and 25% of all C-Nap1 assemblies contained the centrosomal markers C-Nap1 and γ -tubulin, respectively, indicating that they had formed around U2OS centrosomes; all other assemblies lacked centrosomes, indicating that they had either formed at noncentrosomal sites in U2OS cells or been produced through fragmentation of assemblies during purification (Figure 2C and data not shown). It follows that aster formation by C-Nap1 assemblies depended on the presence of centrosomes, whereas the majority of the Nlp assemblies triggered MT aster formation in the absence of centrosomes. These latter assemblies all stained positive for γ -tubulin (not shown), indicating that MT nucleation was

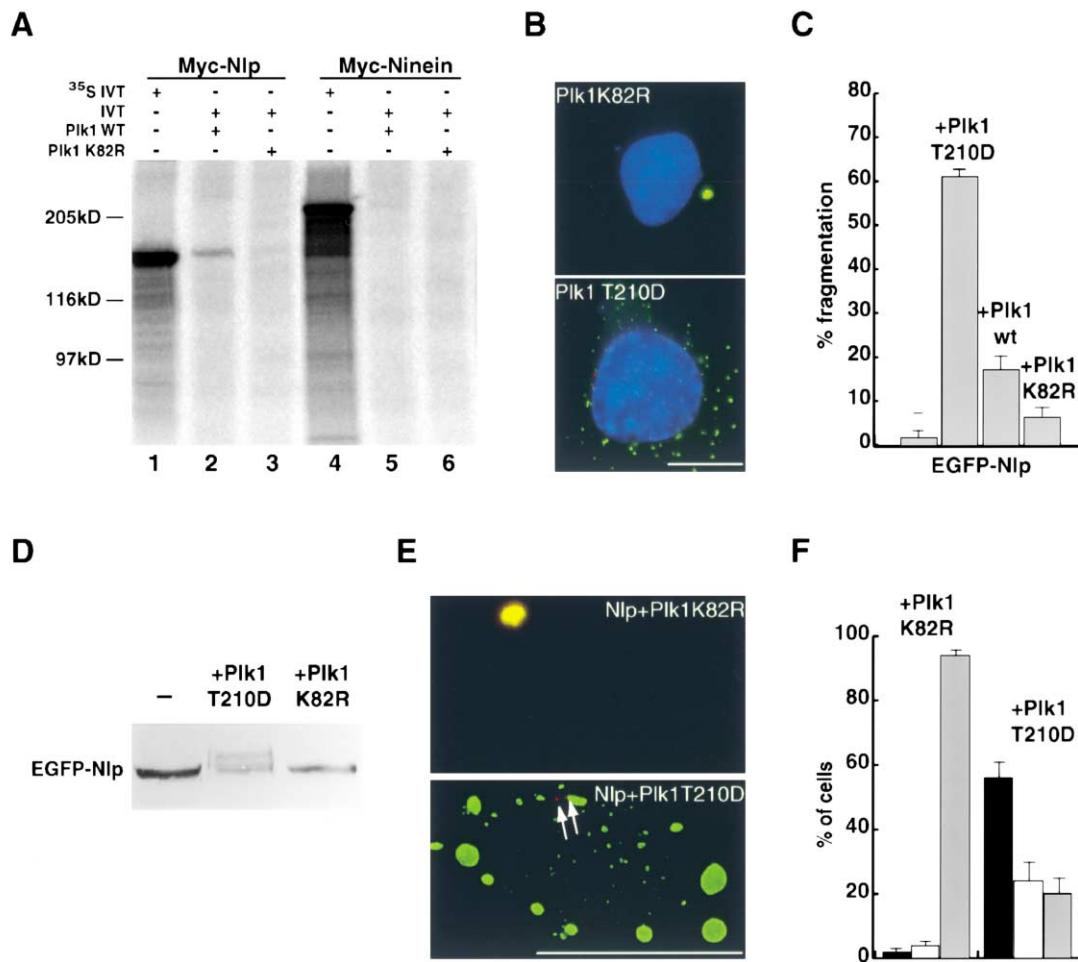


Figure 3. Plk1 Regulates Centrosome Association and γ -Tubulin Interaction of Nlp

(A) Myc-Nlp and myc-ninein were in vitro translated with (lanes 1 and 4) or without (lanes 2, 3, 5, and 6) [³⁵S]methionine, immunoprecipitated with anti-myc antibodies, subjected to in vitro phosphorylation in the presence of wild-type Plk1 (lanes 2 and 5) or catalytically inactive Plk1^{K82R} (lanes 3 and 6), and analyzed by phosphorimager.

(B) U2OS cells were cotransfected with EGFP-Nlp (green) and Plk1^{K82R} (upper panel) or Plk1^{T210D} (lower panel) and counterstained with GT335 antibodies to detect centrosomes (red).

(C) Cells transfected with EGFP-Nlp alone or cotransfected with either Plk1^{T210D}, Plk1 wt, or Plk1^{K82R} were analyzed for the appearance of EGFP-Nlp assemblies. The histogram shows percentages of transfected cells exhibiting fragmented Nlp assemblies (defined as multiple Nlp fragments distinct from the centrosome) determined from three independent experiments, counting 400–600 cells.

(D) 293 cells were transfected with EGFP-Nlp alone or together with either Plk1^{T210D} or Plk1^{K82R}, and cell lysates were analyzed by Western blotting with anti-GFP antibodies.

(E) U2OS cells were cotransfected with EGFP-Nlp (green) and either Plk1^{K82R} or Plk1^{T210D}, and the localization of γ -tubulin was analyzed by IF microscopy (red).

(F) Cells were categorized as showing either γ -tubulin only at centrosomes (black), on both centrosomes and Nlp assemblies (light gray), or only on Nlp assemblies (dark gray). Variability most likely reflects differences in the stoichiometry of Nlp phosphorylation. Histogram shows results from three independent experiments, counting 400–600 cells.

The error bars indicate standard deviations and the scale bars represent 10 μ m.

promoted through γ -TuRCs. Furthermore, nocodazole treatment of Nlp assemblies did not detectably reduce their ability to organize MT asters, arguing against mere elongation of copurifying MT fragments (not shown). These data thus indicate that Nlp assemblies could stimulate MT aster formation in both mammalian cells and *Xenopus* egg extracts.

Our attempts to deplete Nlp by RNA interference have not been successful. We thus asked whether the inhibition of endogenous Nlp by antibody microinjection would interfere with MT nucleation. Affinity-purified anti-Nlp antibodies were microinjected into asynchronously

growing U2OS cells, and MT regrowth assays were performed 6 hr later. Nearly 50% of the cells injected with anti-Nlp antibodies were devoid of detectable MT asters, whereas only 14% of cells injected with control rabbit immunoglobulins lacked obvious asters (Figures 2D and 2E). These results are quantitatively similar to those obtained after microinjection of antibodies against the γ -TuRC component GCP3/HsSpc98 (Tassin et al., 1998), and thus strengthen the conclusion that Nlp is involved in MT nucleation in vivo. Although it is difficult to rigorously exclude steric hindrance, we emphasize that antibodies against another centrosomal protein,

C-Nap1, did not significantly inhibit MT nucleation (Mayor et al., 2000). Furthermore, injection of anti-Nlp antibodies did not interfere with either entry into mitosis or MT nucleation during mitosis (Figure 2F), in line with our finding that Nlp is absent from mitotic spindle poles (see below).

Plk1 Regulates Centrosome Association and γ -TuRC Interaction of Nlp

The observed interaction between Nlp and Plk1 suggested that Nlp could be a substrate of Plk1. Indeed, wild-type Plk1, but not catalytically inactive Plk1 (K82R), readily phosphorylated *in vitro* translated myc-tagged Nlp (Figure 3A, lanes 2 and 3). Ninein was barely phosphorylated (Figure 3A, lanes 5 and 6), although it was immunoprecipitated in similar amounts (data not shown). To examine whether Nlp could also be a substrate of Plk1 *in vivo*, we analyzed the consequences of cotransfecting EGFP-tagged Nlp with myc-tagged Plk1^{T210D} or myc-tagged Plk1^{K82R} in 293 cells. The Plk1^{T210D} mutant used in these experiments displays a five times higher activity than wild-type Plk1 and mimics the activated, mitotic form of Plk1 (Smits et al., 2000). Twenty-four hours after cotransfection with Plk1^{T210D}, EGFP-Nlp showed markedly reduced gel electrophoretic mobility, suggestive of phosphorylation; no upshifted bands were seen when EGFP-Nlp was expressed alone or together with Plk1^{K82R} (Figure 3D). Similarly, EGFP-ninein showed no change in mobility in response to coexpression with Plk1^{T210D} (data not shown).

Plk1-dependent phosphorylation caused EGFP-Nlp assemblies to fragment and redistribute throughout the cytoplasm. As shown by counterstaining with anti-centriole antibodies (GT335), EGFP-Nlp was displaced from centrosomes in 65% of cells expressing Plk1^{T210D}, but only in 5% of cells expressing Plk1^{K82R} (Figures 3B and 3C). Coexpression of wild-type Plk1 produced only a moderate effect (Figure 3C), indicating that fully activated Plk1 was required to displace Nlp from the centrosome. No significant effects of Plk1^{T210D} were observed on assemblies formed by EGFP-ninein (not shown). Most interestingly, Plk1-dependent phosphorylation profoundly affected the interaction between Nlp and γ -tubulin (Figures 3E and 3F). In 55% of cells expressing activated Plk1^{T210D}, γ -tubulin staining was prominent at the centrosome but undetectable on cytoplasmic Nlp assemblies, and in a further 25% of cells, γ -tubulin was present on both centrosomes and fragments. Upon expression of Plk1^{K82R}, γ -tubulin was present throughout Nlp assemblies in virtually all cells, with no detectable enrichment at the centrioles (Figures 3E and 3F). These results indicate that Plk1 activity modulates not only the propensity of Nlp to self-associate but also its association with the centrosome and its ability to interact with the γ -TuRC.

Nlp Is Removed from the Centrosome during Centrosome Maturation

We next asked whether Plk1 regulates the centrosome association of endogenous Nlp during the cell cycle. Remarkably, the expression of Plk1^{T210D} in U2OS cells completely abolished Nlp staining at the centrosome,

whereas Plk1^{K82R} had no effect and wild-type Plk1 produced a partial reduction (Figure 4A and data not shown). This suggests that mitotic activation of Plk1 is required for efficient displacement of Nlp from the centrosome. Furthermore, the centrosome association of endogenous Nlp was cell cycle dependent, as predicted if Plk1 were to regulate Nlp under physiological conditions (Figure 4B). Whereas Nlp could readily be seen at the centrosome throughout interphase, centrosomal staining for Nlp became virtually undetectable as soon as Plk1 was activated at the onset of mitosis (Golsteyn et al., 1995): as γ -tubulin levels increased during prophase, indicative of centrosome maturation (Khodjakov and Rieder, 1999), centrosome staining by anti-Nlp antibodies was strongly reduced. During metaphase and anaphase, spindle poles were also strongly positive for γ -tubulin but almost completely negative for Nlp, before Nlp staining reappeared during telophase (Figure 4B). These results indicate that Nlp is transiently displaced from centrosomes at the onset of mitosis. Epitope masking is unlikely, as two antibodies raised against nonoverlapping parts of Nlp produced identical results (data not shown).

Plk1 Regulates the Interaction between the N-Terminal Half of Nlp and the γ -TuRC

To understand the relationship between Nlp phosphorylation by Plk1 and γ -TuRC binding in more detail, GST-fusions of the N-terminal and C-terminal halves of Nlp were tested as *in vitro* substrates of Plk1. The N-terminal half was an excellent substrate, but virtually no phosphate was incorporated into the C-terminal half (Figure 5A). The corresponding Nlp domains were then expressed as EGFP-fusions, and examined for their ability to recruit γ -TuRCs *in vivo*. The N-terminal part of Nlp formed many small assemblies throughout the cytoplasm, and all these assemblies recruited γ -tubulin (Figure 5B, panel a). Assays of MT regrowth in these cells revealed no distinct MT asters; instead, extensive MT nucleation throughout the cytoplasm was seen, presumably reflecting MT nucleation from the Nlp-associated, dispersed γ -TuRCs (Figure 5C, upper panels). In contrast, the C-terminal half of Nlp formed few large assemblies in the cytoplasm without affecting centrosomal γ -tubulin staining (Figure 5B, panel b) and the corresponding cells formed typical MT arrays from their centrosomes (Figure 5C, lower panels). These results indicate that the N-terminal domain of Nlp is responsible for recruiting γ -TuRCs and that Plk1 directly regulates this interaction.

Expression of Phosphorylation Site Mutant Nlp Causes Aberrant Mitotic Spindles

To determine whether the displacement of Nlp from the centrosome at the onset of mitosis is critical for spindle assembly, we proceeded to map Plk1 phosphorylation sites and explore the physiological consequences of expressing nonphosphorylatable Nlp mutants. Sequence analysis suggested the presence of eight potential *in vitro* Plk1 phosphorylation sites (Kelm et al., 2002) within the N-terminal half of Nlp (Figure 6A), and three peptides, containing four of these sites (S87 or S88, T161, and S686), were found to be phosphorylated by

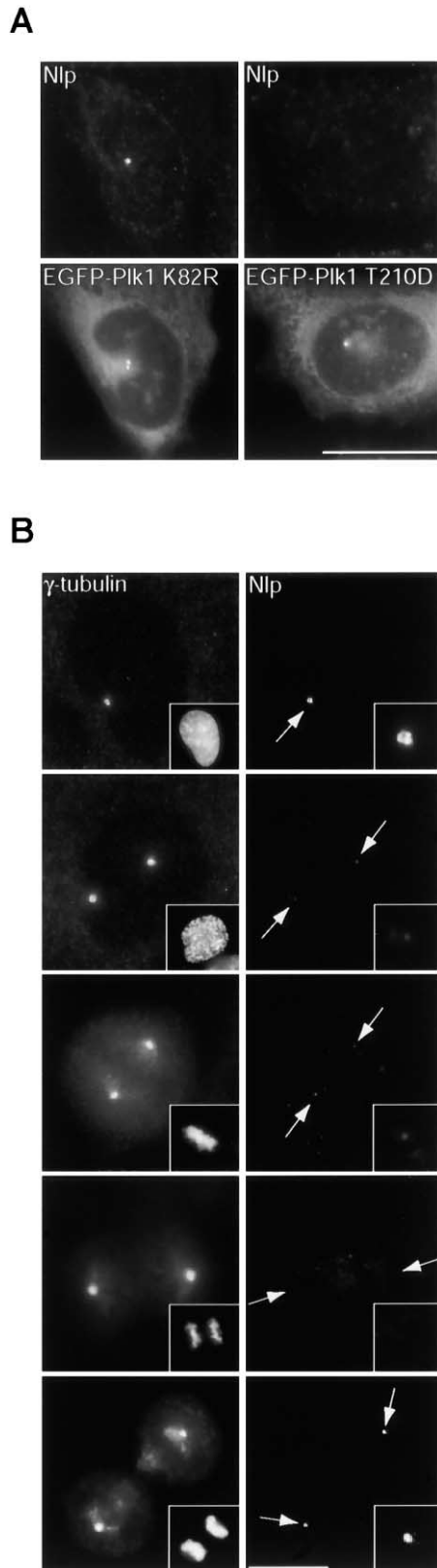


Figure 4. Nlp Is Removed from the Centrosomes during Centrosome Maturation

(A) U2OS cells overexpressing EGFP-Plk1^{K82R} or Plk1^{T210D} were analyzed by IF microscopy with anti-Nlp antibodies.

mass spectrometry (Supplemental Figure S2). To explore the consequences of Nlp phosphorylation, three Nlp mutants were constructed (Figure 6C). A mutant with all eight putative Plk1 phosphorylation sites altered to alanines (GST-NlpΔ8) was barely phosphorylated, indicating that most Plk1 phosphorylation sites had been made inaccessible (Figure 6B). This mutant was still able to recruit γ -tubulin and stimulate MT nucleation, arguing against severe misfolding (data not shown). All three mutants were then coexpressed with either Plk1^{T210D} or Plk1^{K82R}. Whereas hyperactive Plk1 dispersed most of the centrosome-associated assemblies of wild-type Nlp, assemblies formed by NlpΔ8 were almost completely resistant to fragmentation and the NlpΔ4A and Δ4B mutants showed partial resistance (Figures 6D and 6E). These results indicate that the N-terminal half of Nlp is phosphorylated directly by Plk1. They also show that phosphorylation disrupts the association of Nlp with the centrosome and that complete disassembly of Nlp requires phosphorylation at multiple sites.

Finally, we examined the consequences of unscheduled expression of wild-type Nlp and NlpΔ8 for spindle formation. Overexpression of wild-type Nlp caused the formation of aberrant spindles in about 40% of mitotic cells (Figures 6F and 6G), including monopolar, tripolar, or tetrapolar spindles and detached MT asters (Figure 6F). In all cases, the extra spindle poles and MT asters were associated with overexpressed Nlp, suggesting that Nlp was directly responsible for their formation. Overexpression of NlpΔ8 caused qualitatively similar mitotic defects, but in this case, the phenotype concerned up to 85% of all mitotic cells (Figures 6F and 6G). Although caution is required when interpreting overexpression studies, these results indicate that the persistence of excess Nlp at the onset of mitosis perturbs mitotic spindle formation. Furthermore, the resulting phenotypes are exacerbated if Plk1 cannot phosphorylate Nlp.

Discussion

MT nucleation from the animal centrosome clearly depends on γ -TuRCs, but the mechanisms regulating the recruitment of these complexes to the centrosome remain poorly understood. Here we describe a 156 kDa centrosomal protein, termed Nlp, whose properties suggest that it functions as a docking protein for γ -TuRCs during interphase of the cell cycle. Nlp displays significant structural similarity to ninein, a protein implicated in the capping and anchoring of MT minus ends at both centrosomal and noncentrosomal sites (Mogensen et al., 2000). Thus, the mammalian ninein family comprises at least two members, both of which appear to play important roles in the organization of MT arrays. We also show that the centrosome association of Nlp is

(B) U2OS cells in (from top to bottom) interphase, prophase, metaphase, and telophase were stained with antibodies against γ -tubulin (left panels) and Nlp (right panels, arrows indicate the position of the centrosome). Insets show DNA stained with DAPI and enlargements of Nlp staining, respectively. The scale bars represent 10 μ m.

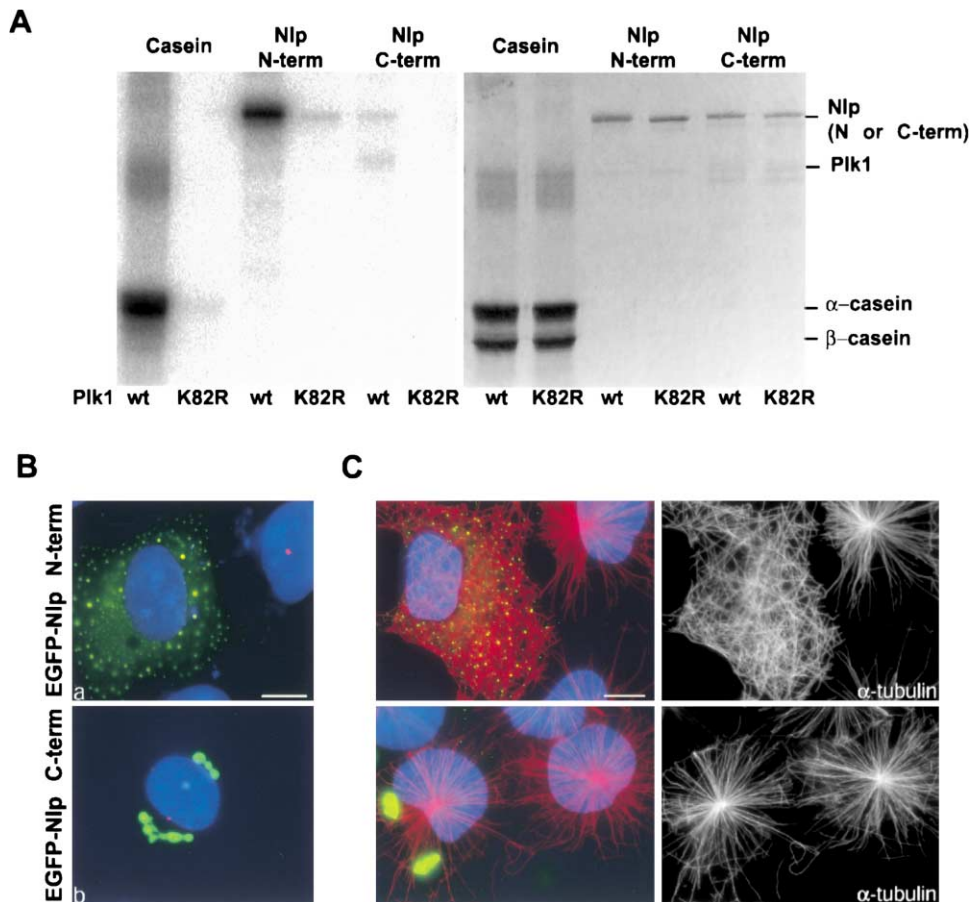


Figure 5. Plk1 Regulates Interaction between the N Terminus of Nlp and γ -TuRCs

(A) GST-Nlp (N- or C-terminal halves) and casein were incubated with recombinant wt Plk1 or Plk1^{K82R}, and phosphorylation was determined by phosphorimager (left panel); protein abundance was determined by Coomassie staining (right panel).

(B) U2OS cells expressing EGFP-Nlp (N- or C-terminal halves, respectively; green) were counterstained with anti- γ -tubulin antibodies (red) and DAPI (blue).

(C) Transfected cells were subjected to MT regrowth assays and stained with anti- α -tubulin antibodies (red).

The scale bars represent 10 μ m.

regulated during the cell cycle. Nlp is a substrate of Plk1 and dissociates from centrosomes in response to phosphorylation, suggesting that Plk1 triggers an exchange of GTBPs at the centrosome. Such an exchange of critical PCM components is likely to constitute a key aspect of centrosome maturation.

Nlp Is a Candidate GTBP

With Spc110p and Spc72p, two GTBPs have been identified and characterized in *S. cerevisiae* (Francis and Davis, 2000; Schiebel, 2000), but the identification of GTBPs in other organisms has proven difficult. Spc110 displays some sequence similarity with kendrin/pericentrin-B, but this similarity is largely restricted to a putative calmodulin binding domain (Flory et al., 2000; Li et al., 2001). Additional mammalian proteins, including members of the pericentrin/kendrin/CG-NAP family, Cep135 and CPAP (centrosomal P4.1-associated protein), have been proposed to bind to γ -tubulin, but their precise contributions to MT organization remain to be clarified (Dietenberg et al., 1998; Hung et al., 2000; Li et al., 2001; Ohta et al., 2002; Takahashi et al., 2002). Our present

study identifies Nlp (the product of cDNA KIAA0980) as a candidate GTBP in human cells. Nlp recruited both γ -tubulin and hGCP4, both in vitro and in vivo, suggesting that Nlp binds the entire γ -TuRC. Indeed, Nlp assemblies promoted MT nucleation both in mammalian cells and in *Xenopus* egg extracts. Conversely, microinjection of antibodies against Nlp severely suppressed MT nucleation.

Nlp Is a Second Member of the Ninein Protein Family

Over its N-terminal half, Nlp shares 37% identity with ninein. However, ninein is substantially larger than Nlp, and the C termini of the two proteins show no structural homology, except for the presence of predicted coiled-coil domains. Ninein has been proposed to function in MT anchoring rather than MT nucleation (Mogensen et al., 2000). It is difficult to rigorously distinguish various MT minus end-associated activities and it would be premature to exclude that Nlp may also contribute to MT anchoring. Our immunolocalization data suggest a preferential association of Nlp with one of the two centrioles,

which might be consistent with an anchoring function. On the other hand, our data strongly indicate that Nlp plays an important role in the recruitment of γ -TuRCs to the centrosome. Thus, while it is clear that both Nlp and ninein play important roles in the organization of MT networks in mammalian cells, the two proteins may have functionally diverged during evolution.

Nlp Is a Centrosomal Substrate of Plk1

Both yeast two-hybrid and direct biochemical data identify Nlp as a physiological substrate of Plk1. Furthermore, our results suggest that phosphorylation by Plk1 regulates the interaction of Nlp with both centrosomes and γ -TuRCs. In contrast, we have no evidence that Plk1 phosphorylates ninein, suggesting that Nlp and ninein are regulated differently. Our analysis of Nlp mutants with in vitro Plk1 phosphorylation sites altered to alanine strongly suggests that Nlp is a direct substrate of Plk1 not only in vitro but also in vivo. These results also strengthen the view that the motif [E/DxS/T] constitutes a consensus for Plk1 phosphorylation sites (Kelm et al., 2002). Although still tentative, the availability of such a consensus sequence may facilitate the future analysis of Plk1 substrates.

Nlp Is Regulated during Centrosome Maturation

The abrupt increase in the MT nucleation activity of centrosomes at the onset of mitosis is expected to require substantial changes in PCM composition (Dictenberg et al., 1998; Khodjakov and Rieder, 1999). These are apparently controlled by several protein kinases, including Plk1, Aurora-A, and Nek2 (Berdnik and Knoblich, 2002; Fry et al., 1998b; Hannak et al., 2001; Lane and Nigg, 1996), but only few substrates of these kinases have so far been identified. Here we have characterized a centrosomal protein, Nlp, whose properties suggest that it functions in MT nucleation at the centrosome. Remarkably, however, Nlp is displaced from the centrosome at the onset of mitosis, when centrosomal MT nucleation activity increases dramatically. It follows that Nlp functions in centrosomal MT nucleation specifically during interphase (G1, S, and G2) of the cell cycle, but not during M phase. This implies that structurally distinct GTBPs function as interphasic and mitotic scaffolds for γ -TuRC recruitment. Our data further suggest that the activation of Plk1 at the G2/M transition results in the displacement of Nlp from the maturing centrosome and that this event is important for mitotic spindle formation. As illustrated schematically in Figure 7, we envision that the removal of Nlp from the centrosome constitutes a prerequisite for the recruitment of an as yet unidentified mitotic GTBP, which then confers enhanced microtubule nucleation capacity to the centrosome. According to this model, the activation of Plk1 at the onset of mitosis triggers the replacement of the interphasic MT nucleation scaffold by the mitotic scaffold.

Our results raise several provocative questions for future studies. If it is correct that the removal of Nlp from maturing centrosomes is required for the recruitment of a distinct GTBP, then the identification of this hypothetical mitotic GTBP constitutes a high-priority task. Furthermore, it will be important to identify the phosphatase(s) and possibly other kinases that contribute to the

regulation of Nlp and to determine the fate of Nlp during mitosis. In particular, it will be interesting to determine whether proteolysis contributes to the regulation of this protein (Figure 7). The thorough characterization of Nlp and other centrosomal substrates will be indispensable for a complete understanding of the switch between interphasic and mitotic MT nucleation.

Experimental Procedures

Preparation of Plasmids

KIAA0980/Nlp cDNA, obtained from the Kazusa DNA Research Institute, was sequenced and subcloned into pEGFP-C1 and pBlue-scriptKSII-myc vectors. The myc-tagged Nlp was then introduced into the pCDNA4/TO vector to generate tetracycline-inducible stable cell lines. Corresponding expression constructs were also generated for ninein, using a cDNA kindly provided by M. Bornens. The N-terminal (residues 1–702) and C-terminal (residues 694–1382) halves of Nlp were inserted into the pEGFP-C1 vector. GST- and His₆-tagged Nlp fragments were prepared using pGEX-6P-3 and pQE-30 bacterial expression vectors, respectively. Phosphorylation site mutants of Nlp were prepared by substituting serine/threonine residues by alanines in positions 20, 87, 88, and 161 (pEGFP-Nlp Δ 4A), in positions 349, 498, 670, and 686 (pEGFP-Nlp Δ 4B), or in all eight positions (pEGFP-Nlp Δ 8). N-terminal Nlp Δ 8A (residues 1–702) was further subcloned into pGEX-6P-3. Wild-type and mutant (K82R, T210D) Plk1 plasmids have been described (Meraldi et al., 2002; Smits et al., 2000).

Antibody Production

Rabbit antibodies were raised against GST-N-terminal (1–702) and His₆-C-terminal (980–1382) Nlp expressed in *E. coli*. Anti-N-terminal antibodies were affinity purified on recombinant antigen (after removal of GST by Prescission Protease; Amersham Pharmacia Biotech), bound to an AffiGel 15 (Bio-Rad) column; anti-C-terminal antibodies were purified by elution from nitrocellulose strips bearing EGFP-Nlp expressed in 293 cells. Both antibodies produced qualitatively similar results; most data shown were obtained with anti-N-terminal antibodies.

Cell Culture, Transfections, and Generation of Stable Cell Lines

Human U2OS osteosarcoma, 293 epithelial kidney, and KE37 T-lymphoblastoid cells were grown at 37°C in a 5% CO₂ atmosphere in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% heat-inactivated fetal calf serum and penicillin-streptomycin (100 i.u./ml and 100 μ g/ml, respectively, all GIBCO-BRL). The Tet-On U2OS myc-Nlp and myc-ninein stable cell lines were grown additionally under selection of 200 μ g/ml zeocin and 50 μ g/ml hygromycin. These lines were obtained by transfection of Tet-On U2OS cells (Invitrogen) with 20 μ g of plasmid DNA (Krek and Nigg, 1991), followed by zeocin selection for 14 days and dilution for single-cell colony formation. Transient transfection of U2OS and 293 cells was performed as described (Meraldi et al., 2002; Seelos, 1997).

Cell Extracts and Pull-Down Experiments

For pull-down experiments (adapted from Murphy et al., 2001), cells were washed once with cold HBS (50 mM HEPES [pH 7.4], 150 mM NaCl) and collected into cold HBS plus 1 mM PMSF. Cells were lysed in 50 mM HEPES (pH 7.4), 150 mM NaCl, 2 mM EDTA, 1 mM DTT, 0.5% Triton X-100, 0.25 mM GTP, 1 mM MgCl₂, 1 mM PMSF, and aprotinin, leupeptin, and pepstatin at 1 μ g/ml each and incubated for 10 min on ice. Lysates were clarified (10,000 rpm, 4°C, 20 min) and incubated with glutathione beads carrying recombinant GST-fusion proteins. The glutathione beads were then washed once in lysis buffer, once in 50 mM HEPES (pH 7.4), 250 mM NaCl, once in HBS, and finally resuspended in gel sample buffer.

Purification of Recombinant Proteins and Aster Formation

Assays in *Xenopus* Egg Extracts

The GST-N- and C-terminal halves of Nlp and GST-N-terminal Nlp Δ 8 were expressed in *E. coli* BL21 RIL and purified by glutathione affinity

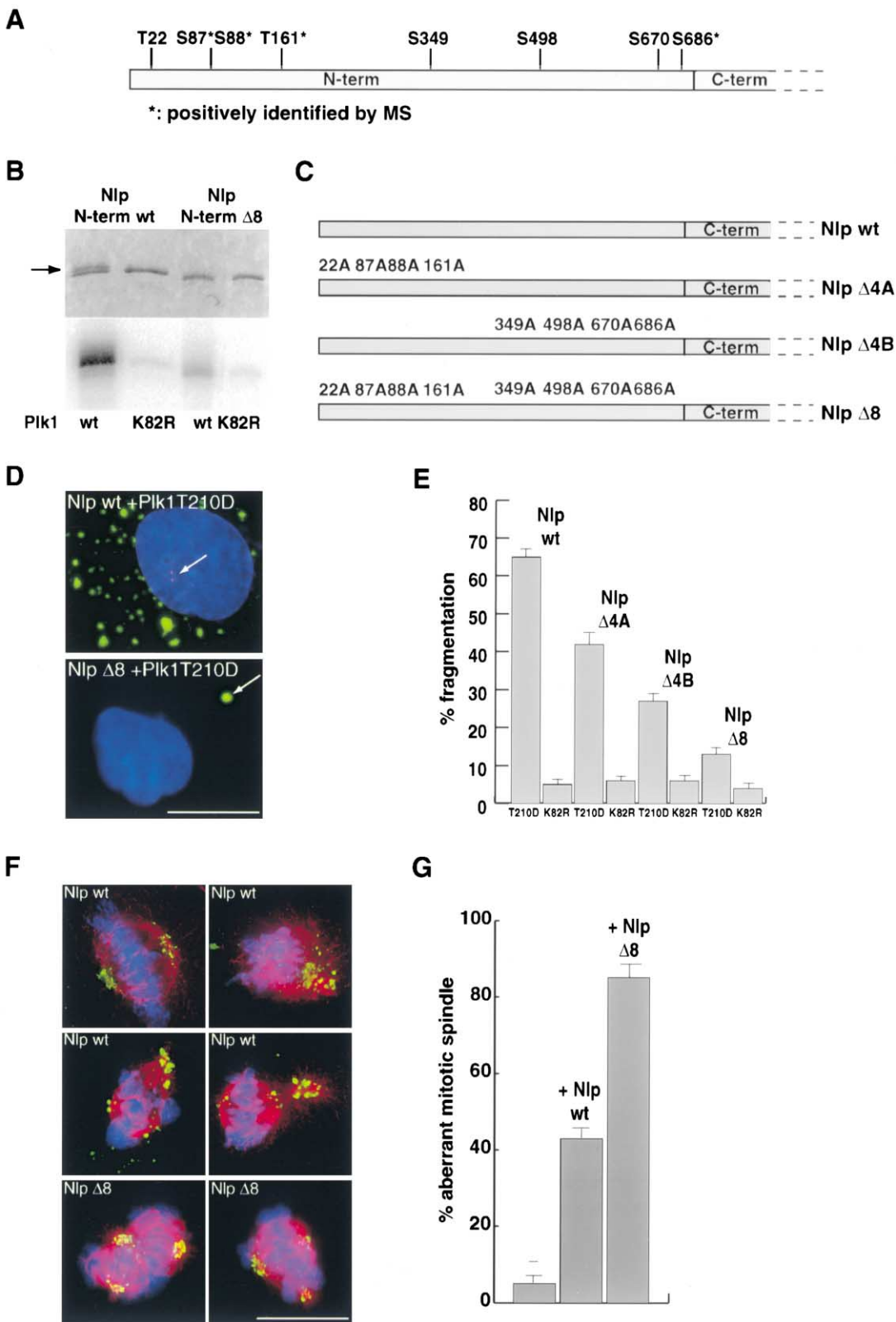


Figure 6. Overexpression of Phosphorylation Site Mutant Nlp Causes Aberrant Mitotic Spindle Formation
(A) Schematic representation of potential Plk1 phosphorylation sites in the N-terminal half of Nlp (but not ninein), conforming to an E/DxS/T consensus. Phosphopeptides derived from trypsin or LysC proteolytic cleavage that could be identified by mass spectrometry are marked by asterisks.
(B) GST-Nlp (N-terminal half wt or Δ8 mutant) was incubated with wt Plk1 or Plk1^{K82R} and phosphorylation was determined by phosphorimager

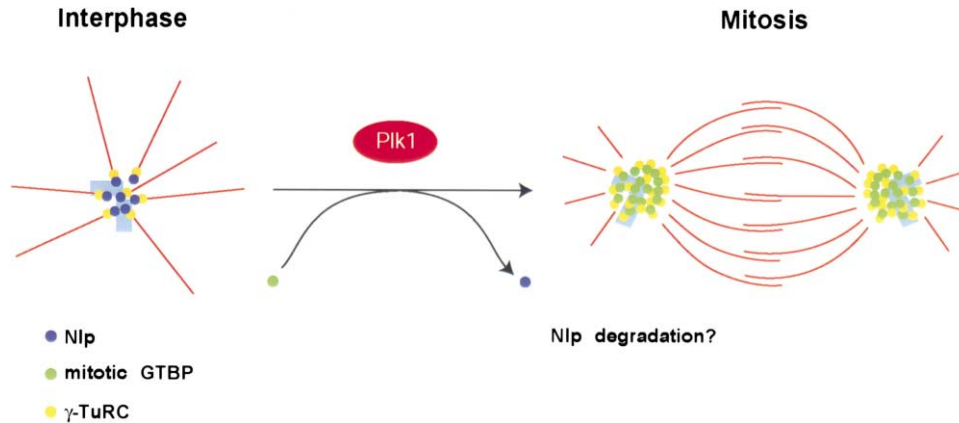


Figure 7. Model for the Regulation of Nlp during Centrosome Maturation

This model proposes that Nlp is specifically involved in tethering γ -TuRCs to the centrosome during interphase. To allow spindle assembly, Nlp needs to be replaced by an as yet unidentified mitotic GTBP at the onset of mitosis. Our data indicate that this centrosome maturation step is dependent on Plk1, but additional layers of regulation—such as protein degradation or phosphorylation by other kinases—are not excluded.

chromatography. Recombinant EGFP-Nlp and EGFP-C-Nap1 assemblies were purified from transfected U2OS cells, using a rapid centrosome purification method (Blomberg-Wirschell and Doxsey, 1998). Cytostatic factor (CSF)-arrested *Xenopus* egg extracts (M phase extracts) were prepared as described (Murray, 1991). Purified recombinant EGFP-Nlp and EGFP-C-Nap1 assemblies, as well as copurifying centrosomes, were added to 20 μ l of extract, and the formation of MT asters was monitored by IF microscopy with anti- α -tubulin antibodies (Sawin and Mitchison, 1991).

Antibody Microinjection

Antibody microinjection experiments were performed as described (Lane and Nigg, 1996). Both anti-N-terminal Nlp antibodies and non-immune rabbit IgG (Sigma-Aldrich) were extensively washed with PBS and concentrated with Ultrafree-0.5 centrifugal filter (Amicon bioseparation; Millipore) before injection at 2 mg/ml.

Immunofluorescence Microscopy

IF microscopy was performed as described previously (Meraldi et al., 2002). Antibody reagents were anti-Nlp antibodies (affinity purified, 1 μ g/ml), GT335 monoclonal antibodies (1:10,000 dilution from ascites; gift of B. Eddé; Bobinnec et al., 1998), rabbit anti-C-Nap1 antibodies (affinity purified, 1 μ g/ml; Fry et al., 1998a), anti- α -tubulin monoclonal antibodies (1:10,000 dilution from ascites, clone B-5-1-2; Sigma), rabbit anti- γ -tubulin antibodies (purified IgG, 5 μ g/ml; Fry et al., 1998b), and anti- γ -tubulin monoclonal antibodies (1:1000 dilution from ascites, clone GTU-88; Sigma). For high-resolution images, a Deltavision microscope on a Nikon TE200 base (Applied Precision), equipped with an APOPLAN 60 \times 1.4 oil immersion objective, was used for collecting optical sections at distances of 0.2 μ m in the Z-axis. Pictures at individual focal planes were deconvoluted and projected onto one plane using Softworx (Applied Precision).

Plk1 Kinase Assays

Myc-Nlp and myc-ninein were expressed and immunoprecipitated with anti-myc monoclonal antibody (9E10) as described (Fry et al., 1998a). Immunoprecipitates or recombinant proteins were subjected to in vitro kinase assays with wild-type or catalytically inactive His₆-Plk1 purified from baculovirus-infected insect cells (Kelm et al., 2002).

Miscellaneous Techniques

Human centrosomes were purified from the T-lymphoblastic cell line KE37 according to Moudjou and Bornens (1994). MT regrowth assays were performed as described (Fry et al., 1998b). For visualization of MT asters, cells were preextracted for 40 s in 80 mM K-PIPES (pH 6.8), 1 mM EGTA, 1 mM MgCl₂, and 0.1% Triton X-100, fixed with cold methanol, and processed for IF microscopy. Protocols for immunoprecipitation and Western blotting, yeast two-hybrid screening, and mass spectrometry are provided in Supplemental Experimental Procedures at <http://www.developmentalcell.com/cgi/content/full/5/1/113/DC1>.

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(lower panel); protein abundance was determined by Coomassie staining (upper panel). The arrow indicates the slower migrating, phosphorylated form of wt Nlp.

(C) Schematic representation of serine/threonine-to-alanine mutants of Nlp.

(D and E) U2OS cells were cotransfected with Plk1^{T210D} and the indicated EGFP-Nlp mutants (green) and analyzed 24 hr later by IF microscopy with GT335 antibodies (red). Cells were categorized as showing either one Nlp assembly at the centrosome or multiple Nlp fragments distant from the centrosome. Histogram shows results from three independent experiments, counting 400–600 cells.

(F) U2OS cells were transfected for 48 hr with EGFP-Nlp wt or EGFP-Nlp Δ 8 (green) and analyzed by IF microscopy with anti- α -tubulin antibodies to reveal the mitotic spindle (red) and DAPI to show DNA (blue).

(G) Transfected mitotic cells were counted as having either normal or aberrant mitotic spindles. Histogram shows result from three independent experiments, counting 300 cells.

The error bars indicate standard deviations and the scale bars represent 10 μ m.

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