Phosphorylation of Nlp by Plk1 negatively regulates its dynein-dynactin-dependent targeting to the centrosome

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Summary

When cells enter mitosis the microtubule (MT) network undergoes a profound rearrangement, in part due to alterations in the MT nucleating and anchoring properties of the centrosome. Ninein and the ninein-like protein (Nlp) are centrosomal proteins involved in MT organisation in interphase cells. We show that the overexpression of these two proteins induces the fragmentation of the Golgi, and causes lysosomes to disperse toward the cell periphery. The ability of Nlp and ninein to perturb the cytoplasmic distribution of these organelles depends on their ability to interact with the dynein-dynactin motor complex. Our data also indicate that dynactin is required for the targeting of Nlp and ninein to the centrosome. Furthermore, phosphorylation of Nlp by the polo-like kinase 1 (Plk1) negatively regulates its association with dynactin. These findings uncover a mechanism through which Plk1 helps to coordinate changes in MT organisation with cell cycle progression, by controlling the dynein-dynactin-dependent transport of centrosomal proteins.

Key words: Dynein, Dynactin, Plk1, Nlp, Centrosome

Introduction

Centrosomes are the primary MT-organising centres in most vertebrate cells (Nigg, 2004). In interphase, they organise a MT array that imparts shape and polarity to the cell. During mitosis they contribute to the assembly of the mitotic spindle (Bornens, 2002; Doxsey, 2001; Rieder et al., 2001). To accomplish such different tasks, the structure and the molecular composition of the centrosome is under cell cycle control. In preparation for mitosis, centrosomes undergo a maturation process resulting in increased MT nucleation activity (Blagden and Glover, 2003), which is characterised by the release of proteins required for interphase MT organisation and the recruitment of additional γ-tubulin ring complexes (γ-TuRC) (Khodjakov and Rieder, 1999). The mitotic kinases Plk1 and Aurora A have emerged as key regulators of centrosome maturation in a variety of different organisms (Blagden and Glover, 2003). In *Drosophila melanogaster*, Aurora A is required for association of centrosomin, γ-tubulin and a protein complex involved in MT growth and stabilisation during mitosis that is formed by D-TACC and Minispindles/XMAP215 with the mitotic centrosome (Giet et al., 2002; Lee et al., 2001; Terada et al., 2003). In *Caenorhabditis elegans*, Aurora A activity, together with cytoplasmic dynein, regulates accumulation of SPD-2, a protein required for centrosome duplication and maturation, within the pericentriolar material (PCM) (Kemp et al., 2004). *D. melanogaster* polo kinase is required for the recruitment of CP190 and γ-tubulin to the centrosome (Donaldson et al., 2001). It also directly phosphorylates the *abnormal spindle* gene product Asp, a protein involved in MT organisation, which becomes associated to the centrosome in mitosis (do Carmo et al., 2001). In mammalian cells, two related centrosomal proteins, ninein and Nlp, are involved in MT minus-end anchoring and nucleation events (Casenghi et al., 2003; Mogensen et al., 2000; Rapley et al., 2005). In contrast to Asp, Nlp is displaced from the centrosome upon phosphorylation by Plk1 (Casenghi et al., 2003; Rapley et al., 2005; Delgehyr et al., 2005). Thus, mitotic kinases regulate both the association and release of centrosomal proteins during centrosome maturation.

Assembly of centrosomal components occurs through both MT-independent (Khodjakov and Rieder, 1999) and MT-dependent pathways (Balczon et al., 1999; Khodjakov et al., 2002; Young et al., 2000). MT-dependent assembly of centrosomal components requires cytoplasmic dynein; a minus-end directed motor protein (Blagden and Glover, 2003). Dynein works in conjunction with a multi-subunit complex, dynactin, which enhances the processive movement of the motor protein along MTs and functions as an adaptor that allows dynein to bind cargo (Karki and Holzbaur, 1999; Schroer, 2004). Centrosomal targeting of multiple PCM components involved in MT organisation, including SPD-2, pericentrin, BBS4 and PCM-1 requires dynein activity in organism as diverse as *C. elegans*, *Xenopus laevis* and mammalian cells (Dammermann and Merdes, 2002; Kemp et al., 2004; Kim et al., 2004; Kubo et al., 1999; Young et al., 2000). γ-tubulin undergoes dynein-mediated transport to centrosomes through its association with pericentrin (Dictenberg et al., 1998; Young et al., 2000), whereas

inhibition of PCM-1 function leads to reduced amounts of centrin, pericentrin and ninein at the centrosome (Dammermann and Merdes, 2002). Pericentrin and PCM-1 may therefore function as adaptors that mediate the dynein-dynactin-dependent transport and assembly of multiple centrosomal components at centrosomes (Dictenberg et al., 1998; Young et al., 2000; Dammermann and Merdes, 2002).

The dynein-dynactin motor protein also plays a pivotal role in determining the position of membranous organelles such as the Golgi and lysosomes. Inhibition of cytoplasmic dynein function causes all these organelles to disperse toward the cell periphery (Burkhardt et al., 1997; Corthesy-Theulaz et al., 1992). One of the phenotypes observed upon expression of ninein and Nlp is the dispersal of organelles such as the Golgi and lysosomes, hinting at a link between ninein and Nlp, and the dynein-dynactin complex. Here we have investigated the function of the dynein-dynactin complex in the targeting of Nlp and ninein to the centrosome in interphase cells, and the role of mitotic kinases in regulating this process at the onset of mitosis.

Materials and Methods

Antibody reagents

Antibodies were used at the dilutions or concentrations stated, for both western blotting and immunofluorescence microscopy, unless indicated otherwise: affinity-purified rabbit antibodies to Nlp (1 μg/ml), mouse monoclonal anti-GFP (1:5), sheep antibodies to GM130 (FBA38; 1:1000), affinity-purified sheep antibodies to GFP (FBA35; 1 μg/ml), monoclonal antibodies to the His-tag (1:1000) were all purchased from Dianova, Hamburg, Germany; mouse monoclonal antibodies to α-tubulin (DM1a; 1:2000) and DIC (clone 70.1, D5167; 1:500) were purchased from Sigma-Aldrich, Taufkirchen, Germany; mouse monoclonal antibodies to $p150^{Glued}$ (clone 12, 612709; 1:500 for western blotting, 1:150 for immunofluorescence microscopy) and p50-dynamitin (clone 25, 611002; 1:400) were purchased from BD Transduction Laboratories, Heidelberg, Germany; mouse monoclonal antibodies to Arp1 (clone 45A) (Schafer et al., 1994), rabbit polyclonal antibodies to PCM1 (Dammermann and Merdes, 2002) and pericentrin (Takahashi et al., 2002) and rabbit polyclonal antibodies to the Myc epitope (1:1000) were purchased from Santa Cruz Biotechnology, Heidelberg, Germany (sc-789).

Molecular biology and purification of recombinant proteins

Mammalian expression constructs coding for EGFP- or Myc-tagged full length Nlp, N- and C-terminal halves of Nlp, and for full-length ninein have been previously described (Casenghi et al., 2003). The ΔEF-h I and ΔEF-h I-II deletion mutants of Nlp were generated, inserting the sequence encoding amino acids (aa) 38-1382 and 350-1382, respectively, into the pEGFP-C1 vector. The ninein deletion mutant was also produced, inserting the N-terminal domain (aa 1-693) into the pEGFP-C1 vector. GST-tagged Nlp fragments and the ninein N-terminal domain were prepared using pGEX-6P-3 and Gateway pDest15 expression vectors. EGFP-tagged NlpΔ8 and C-Nap1, Plk1 wild type, K82R and T210D mutants have been described previously (Mayor et al., 2002; Casenghi et al., 2003). The pDsRedp150^{Glued} CC1 and pEGFP-ninein constructs were kindly provided by T. Schroer (Quintyne and Schroer, 2002) and Y. R. Hong (Kaohsiung Medical University, Taiwan), respectively. The GST-tagged full length, N- and C-terminal halves of Nlp, N-terminal fragment of ninein, and GM130cc were expressed in *E. coli* BL21[pRIL] and purified by glutathione affinity chromatography.

Cell culture and transfections

All cell culture media and additives were obtained from Invitrogen. Human U2OS, HEK293T and HeLa S3 cells were grown at 37°C in a 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium, supplemented with 10% (vol/vol) heat-inactivated foetal calf serum, 100 i.u./ml penicillin, 100 μg/ml streptomycin. For HeLa S3 spinner cultures, cells were grown in RPMI1640 medium supplemented as described above. Tet-On U2OS Myc-Nlp, Myc-ninein and EGFP-C-Nap1 stable cell lines were grown as previously described (Casenghi et al., 2003; Mayor et al., 2002). Transient transfections of U2OS and HEK293T cells were performed using calcium phosphate (Casenghi et al., 2003).

Cell extracts, pull-down and immunoprecipitation experiments

For pull-down experiments cells were washed once in PBS and collected into cold PBS containing 1 mM phenylmethylsulphonyl fluoride (PMSF). Cells were lysed in 25 mM Tris-HCl, pH 8.0, 50 mM NaCl, 0.5% (vol/vol) Triton X-100, 1 mM PMSF, 1 mM NaF and aprotinin, leupeptin and pepstatin at 1 μg/ml each, and incubated for 20 minutes on ice. Lysates were clarified (10,621 ***g***, 4°C, 20 minutes) and incubated with glutathione beads carrying recombinant GST fusion proteins. For pull-down experiments performed with phosphorylated Nlp N terminus, okadaic acid was added to the lysate to a final concentration of 500 nM. The glutathione beads were then washed once in lysis buffer, once in 50 mM Tris, pH 8, 250 mM NaCl, 0.5% (vol/vol) Triton X-100 once again in lysis buffer and resuspended in gel sample buffer. For immunoprecipitation experiments, cell lysates were pre-absorbed on protein A beads (BioRad Laboratories, Munich, Germany) or protein-G beads (Amersham Biosciences, Freiburg, Germany) for 30 minutes at 4°C and then incubated at 4°C for 2 hours with beads bearing rabbit polyclonal anti-Nlp antibodies (Casenghi et al., 2003), sheep anti-GFP antibodies, or non-immune rabbit or sheep antibodies. Immunoprecipitates were then washed once in lysis buffer, once in 50 mM Tris pH 8.0, 250 mM NaCl, 0.5% (vol/vol) Triton X-100, once again in lysis buffer, and then resuspended in gel sample buffer.

Immunofluorescence microscopy

Samples for immunofluorescence microscopy were prepared as described previously (Casenghi et al., 2003). Images were collected using an Axioskop-2 microscope with a 63× Plan Apochromat oil immersion objective of NA 1.4, standard filter sets (Carl Zeiss MicroImaging, Inc.), a 1300 by 1030 pixel cooled CCD camera (model CCD-1300-Y; Princeton Instruments) and Metavue software (Visitron Systems). Images were cropped in Adobe Photoshop 7.0 then sized and placed for figures using Adobe Illustrator 10.0 (Adobe Systems).

Protein phosphorylation

For the phosphorylation of the recombinant GST-Nlp N terminus, glutathione beads carrying the recombinant protein were washed in 20 mM Hepes pH 7.7 and then incubated with Plk1 in a 20 μl total volume of kinase buffer (20 mM Hepes pH 7.7, 15 mM KCl, 10 mM $MgCl_2$, 1 mM EGTA, 5 mM NaF, 1 mM DTT, 2 mM ATP), for 30 minutes at 37°C. For mock-treated controls the GST-Nlp N terminus was incubated with Plk1 in 20 μl of 20 mM Hepes pH 7.7, 10 mM EDTA in the absence of ATP.

Results

Overexpression of Nlp and ninein cause fragmentation of the Golgi

While studying the function of Nlp, we discovered that transient overexpression of Nlp caused a striking dispersal of

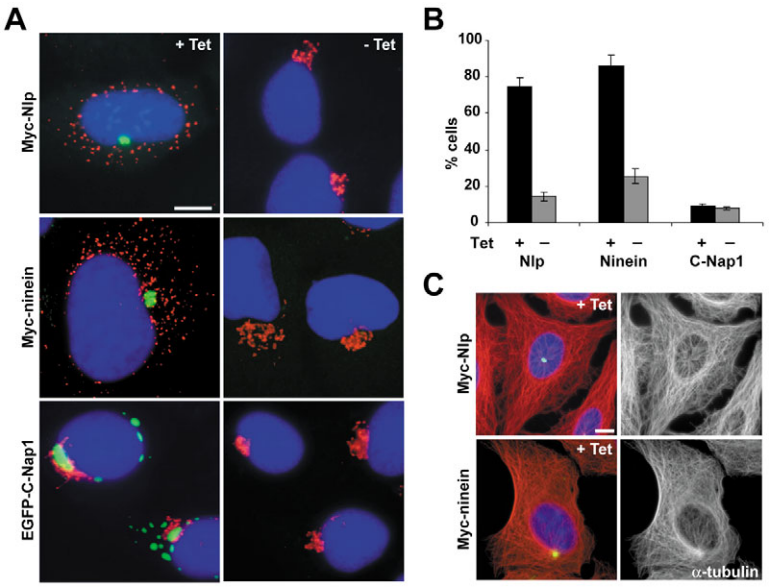

Fig. 1. Effect of Nlp and ninein overexpression on Golgi organisation. (A) Tet-On U2OS stable cell lines carrying Myc-Nlp, Myc-ninein or EGFP-C-Nap1 were either treated with tetracycline (+Tet) or left untreated (–Tet). Cells were then grown for 5 hours, fixed and analysed by immunofluorescence microscopy. Overexpressed proteins were detected with anti-Myc antibodies or using EGFP fluorescence (green). The Golgi was detected using antibodies against the Golgi matrix protein GM130 (red). DNA was stained with DAPI (blue). (B) Expression of Myc-Nlp, Myc-ninein or EGFP-C-Nap1 was either induced in Tet-On U2OS stable cell lines with tetracycline (black bars), or the cells were left untreated (grey bars). Histograms show the percentage of cells with a fragmented staining for the Golgi marker GM130, calculated from three independent experiments in which 400-600 cells were counted. Error bars indicate standard deviations. (C) Myc-tagged Nlp and ninein were expressed in U2OS cells. These were then stained with Myc antibodies to detect the overexpressed protein (green) and counterstained with antibodies against α-tubulin (red). Bars, 10 μm.

the Golgi apparatus. To further examine this phenotype, U2OS cell lines stably expressing tetracycline-inducible forms of three centrosomal proteins C-Nap1, Nlp and ninein were stained with antibodies to the Golgi matrix proteins GM130 and p115 (Fig. 1A; data not shown). Upon induction of Nlp and ninein expression a dramatic dispersal of the Golgi was observed in more than 70% of the cells (Fig. 1A,B). By contrast, induction of C-Nap1 expression had no discernable effect on the Golgi (Fig. 1A,B), indicating that there is a specific property of Nlp and ninein not shared by C-Nap1. The effect of Nlp and ninein induction on Golgi morphology is very similar to that induced by the MT-depolymerising drug nocodazole (Burkhardt et al., 1997). However, the structural integrity of the MT network was not obviously altered in cells expressing either Nlp or ninein (Fig. 1C), suggesting that Golgi dispersal was being induced by a different mechanism. One clue comes from the observation that lysosomes were also dispersed toward the cell periphery in cells expressing Nlp or ninein (data not shown). Since lysosomes, like the Golgi, depend on dynein for positioning, this suggests that the activity of the dynein-dynactin complex is perturbed by Nlp and ninein overexpression.

Nlp and ninein associate with the dynein-dynactin complex

To test whether the overexpression of Nlp or ninein could perturb the localisation of the dynein-dynactin complex, thereby interfering with its activity, cells expressing Nlp or ninein were stained with antibodies to the dynactin subunit p150^{Glued} and the dynein intermediate chain (DIC). Nlp and ninein assemblies recruited p150^{Glued} and DIC (Fig. 2A,B), while similar assemblies formed by C-Nap1 did not alter the staining patterns of the dynein and dynactin subunits (Fig. 2A,B). Moreover, the p150^{Glued} and p50-dynamitin subunits of dynactin were precipitated together with Nlp and ninein but not with C-Nap1 (Fig. 2C). Under the same conditions another centrosomal protein, pericentrin, was not precipitated with Nlp and ninein although it did come down with C-Nap1 (Fig. 2C), supporting the idea that Nlp and ninein specifically interact with the dynein-dynactin complex.

To identify the domains of Nlp and ninein responsible for this interaction, pull-down experiments were performed using GST-tagged recombinant proteins. Since the purification of the recombinant full-length and C-terminal ninein had proved difficult, only the N-terminal region of ninein was tested in these experiments. The p150^{Glued}, p50, and Arp1 subunits of dynactin bound to GST-tagged full-length Nlp and its N-terminal domain, as well as the N-terminal domain of ninein (Fig. 3A). The intermediate chain subunit of the motor protein dynein (DIC) was also present in the bound fractions, albeit with lower enrichment than dynactin subunits (Fig. 3A). By contrast, the C-terminal domain of Nlp, as well as a coiled-coil domain from the Golgi protein GM130 (Barr et al., 1998), did not bind any of the dynein-dynactin complex subunits tested (Fig. 3A). These results indicate that the N-terminal domains of Nlp and ninein are responsible for the interaction with the dynein-dynactin complex. Since pericentrin and PCM-1 have also been proposed to act as adaptors for the dynein-dynactin complex (Dammermann and Merdes, 2002; Young et al., 2000), we tested if they were present in the immunoprecipitated or bound fractions. Whereas no interaction of Nlp and ninein with pericentrin could be detected, PCM-1 was found to weakly associate with ninein but not with Nlp (X. Yan, personal communication; Fig. 2C). Nlp therefore either binds directly to the dynein-dynactin complex, or via a novel adaptor protein. Further support for this idea comes from observations that the dynactin subunits p150^{Glued}, p50-dynamitin and Arp1 specifically co-precipitated with endogenous Nlp (Fig. 3B). Dynein subunits could not be detected in the precipitated fractions, possibly because of the instability of the dynein-dynactin interaction (Schroer and Sheetz, 1991). Together these findings indicate that Nlp and dynactin interact, and that this interaction could be relevant for centrosome function.

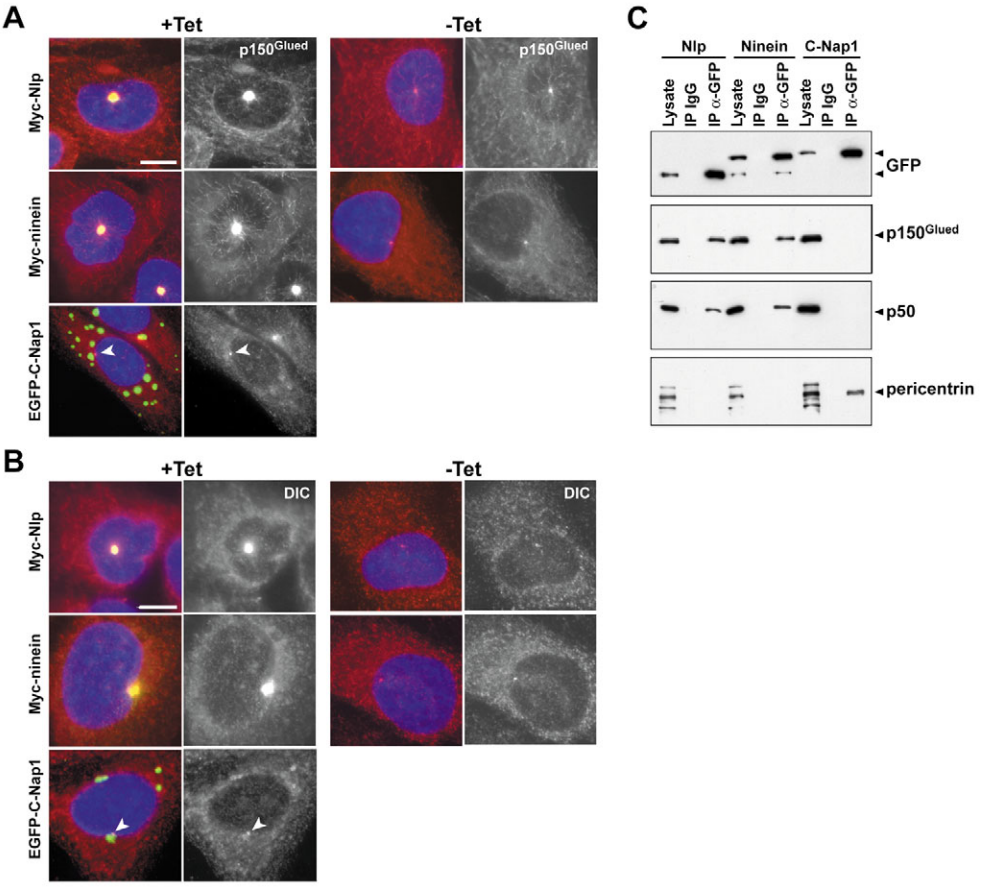

Fig. 2. Nlp and ninein associate with the dynein-dynactin complex. (A,B) U2OS cells expressing Myc-Nlp, Myc-ninein or EGFP-C-Nap1 were analysed by immunofluorescence microscopy. Overexpressed proteins were detected with anti-Myc antibodies or using EGFP fluorescence (green). Induced (+Tet) and control (–Tet) cells were counterstained with antibodies to (A) anti-p150^{Glued} or (B) the dynein intermediate chain (DIC) (red). DNA was stained with DAPI (blue). Arrowheads indicate centrosomes. Bars, 10 μm. (C) Immunoprecipitation experiments were performed from extracts of HEK293T cells transfected with EGFP-tagged Nlp, ninein or C-Nap1 using anti-GFP antibodies. Immunoprecipitates were analysed by blotting with antibodies against GFP, the dynactin subunits p150^{Glued} and p50-dynamitin, and pericentrin.

The dynactin-binding domains of Nlp and ninein induce Golgi fragmentation

Since the pull down experiments indicated that the N-terminal half of Nlp was responsible for the association with the dynactin complex (Fig. 3A), deletion mutants lacking portions of the N-terminal domain were tested for their ability to recruit dynactin and induce Golgi fragmentation. Removal of the first 38 amino acids of Nlp, containing the first EF-hand domain (EF-h I), did not affect either the centrosomal localisation of Nlp or its ability to disperse the Golgi and recruit p150^{Glued} (Fig. 4A,B). By contrast, deletion mutants lacking larger portions of the N-terminal domain (EGFP-NlpΔEF-h I-II and EGFP-Nlp C-terminal), formed large assemblies in the cytoplasm that did not have any effect on either p150^{Glued} localisation or Golgi morphology (Fig. 4A,B). Conversely, the N-terminal domains of Nlp and ninein containing the dynactin-binding site were sufficient to induce fragmentation of the Golgi (Fig. 4A), and the displacement of p150^{Glued} from the centrosome (Fig. 4B). However, they did not perturb p150^{Glued} localisation to MT plus-ends, indicating that not all aspects of dynactin function were disrupted. Under these conditions, localisation of the centrosomal marker C-Nap1 was unaltered (data not shown), excluding the possibility that the altered p150^{Glued} staining was due to disruption of the

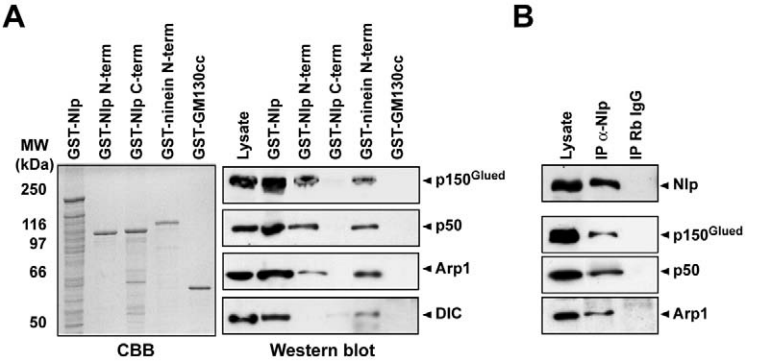

Fig. 3. The N-terminal domains of Nlp and ninein bind the dynein-dynactin complex. (A) Whole cell extracts were prepared as described in the Materials and Methods and incubated in the presence of GST-Nlp full length, GST-Nlp N terminus and C terminus, GST-ninein N terminus or GST-GM130cc. The bound fractions were analysed by blotting with antibodies against dynactin subunits. Dynein was detected using antibodies against the dynein intermediate chain (DIC). The amount of total protein extract loaded was 1/80 of the amount of the bound fractions. The left panel shows a representative Coomassie Blue-stained gel to illustrate GST-fusion proteins. (B) Endogenous Nlp was precipitated from HEK293T cell extracts using rabbit polyclonal anti-Nlp antibodies. Rabbit pre-immune antibodies were used as control. Immunoprecipitates were analysed by blotting using antibodies against the dynactin subunits p150^{Glued}, p50-dynamitin and Arp1.

centrosome. These results, summarised schematically in Fig. 4C, indicate that the N-terminal domains of Nlp and ninein interact with the dynein-dynactin complex, consistent with the biochemical data (Figs 2 and 3). Furthermore, they suggest that the fragmentation of the Golgi induced by Nlp and ninein is a consequence of their ability to interact with and disturb the function of the dynein-dynactin complex. Remarkably, apart from pericentrin (Purohit et al., 1999) other centrosomal proteins reported to interact with dynein and dynactin were unable to induce a similar phenotype in our hands (data not shown). One possibility is that this reflects different modes of interaction with the dynein-dynactin complex.

Targeting of Nlp and ninein to the centrosome depends on dynein-mediated transport

The observations presented above raise the question of what the interaction between Nlp/ninein and dynactin is used for. Nlp and ninein could require the dynein-dynactin complex for transport to the centrosome, be anchored to the centrosome via dynactin, or a combination of both mechanisms could occur. To investigate this we asked whether the localisation of these two proteins to the centrosome was MT dependent. Only a small fraction of endogenous and overexpressed Nlp or ninein could be displaced from the centrosome even after 4 hours of nocodazole treatment (data not shown). This suggests that once Nlp and ninein are associated with the centrosome, they do not require a MT-dependent transport mechanism to remain there. To follow what happens to newly synthesised Nlp and ninein, we made use of cell lines expressing Nlp and ninein under the control of inducible promoters. When Myc-Nlp and Myc-ninein expression was induced in cells pre-treated with nocodazole to depolymerise MTs, the overexpressed proteins did not form the typical assemblies around the centrosome (Fig. 5A, centre) but were dispersed throughout the cytoplasm (Fig. 5A, left). If nocodazole was added to cells already expressing Myc-Nlp or ninein the centrosomal assemblies of these proteins remained intact (Fig. 5A, right; the small dispersed assemblies reflect Nlp and ninein expressed in the 4 hours after addition of nocodazole). Similar to the overexpressed protein, endogenous Nlp remained at centrosomes even after prolonged periods of nocodazole treatment (Fig. 5B). MTs are therefore not required to maintain Nlp or ninein at centrosomes, but are required for the recruitment of the newly synthesised proteins to the centrosome. To determine whether the targeting of Nlp and ninein to the centrosome was dependent on dynein-dynactin-mediated transport, cells were transfected with the p150^{Glued} CC1 fragment, which inhibits dynein based motility (Quintyne and Schroer, 2002). Centrosomal assemblies of Nlp or ninein were absent from p150^{Glued} CC1-expressing cells (Fig. 5C). Thus, a functional MT network and the activity of the dynein motor protein are required for targeting newly synthesised Nlp and ninein to the centrosome.

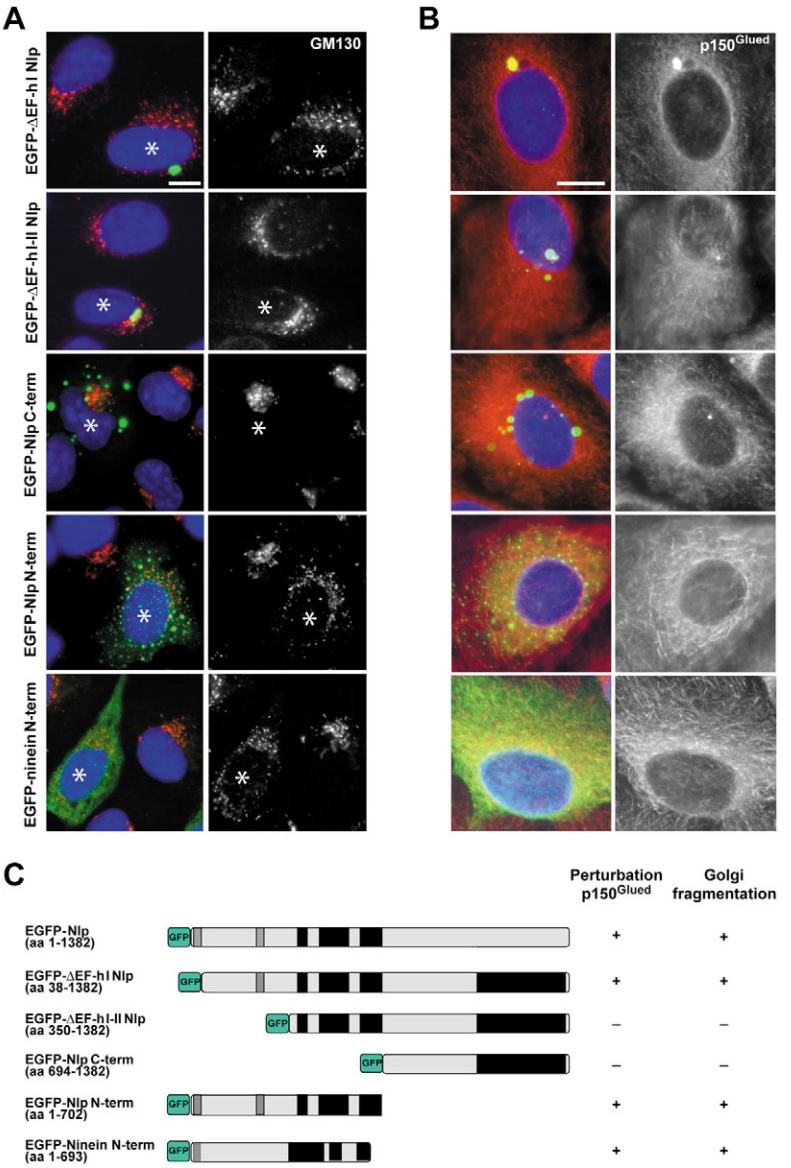

Fig. 4. The dynactin-binding domains of Nlp and ninein are required for the Golgi fragmentation phenotype. (A,B) U2OS cells were transfected with EGFP-tagged Nlp, NlpΔEF-h I (Δ1-38aa), EGFP-NlpΔEF-h I-II (Δ1-350aa), and the Nlp N- and C-terminal domains, or the EGFP-tagged N-terminal domain of ninein. Expressed proteins were detected using EGFP fluorescence (green). (A) The Golgi was stained with antibodies against GM130 (red), transfected cells are marked by an asterisk. (B) the dynactin complex was stained with antibodies against p150^{Glued} (red). DNA was stained with DAPI (blue). Bars, 10 μm. (C) A schematic representation of the Nlp and ninein deletion mutants, and a summary of their effects on p150^{Glued} and the Golgi.

Plk1 phosphorylation regulates the association of Nlp with the dynein-dynactin complex

We have previously shown that Nlp is a substrate of Plk1 and that phosphorylation by Plk1 triggers the displacement of Nlp from the centrosome at the onset of mitosis. This led to the

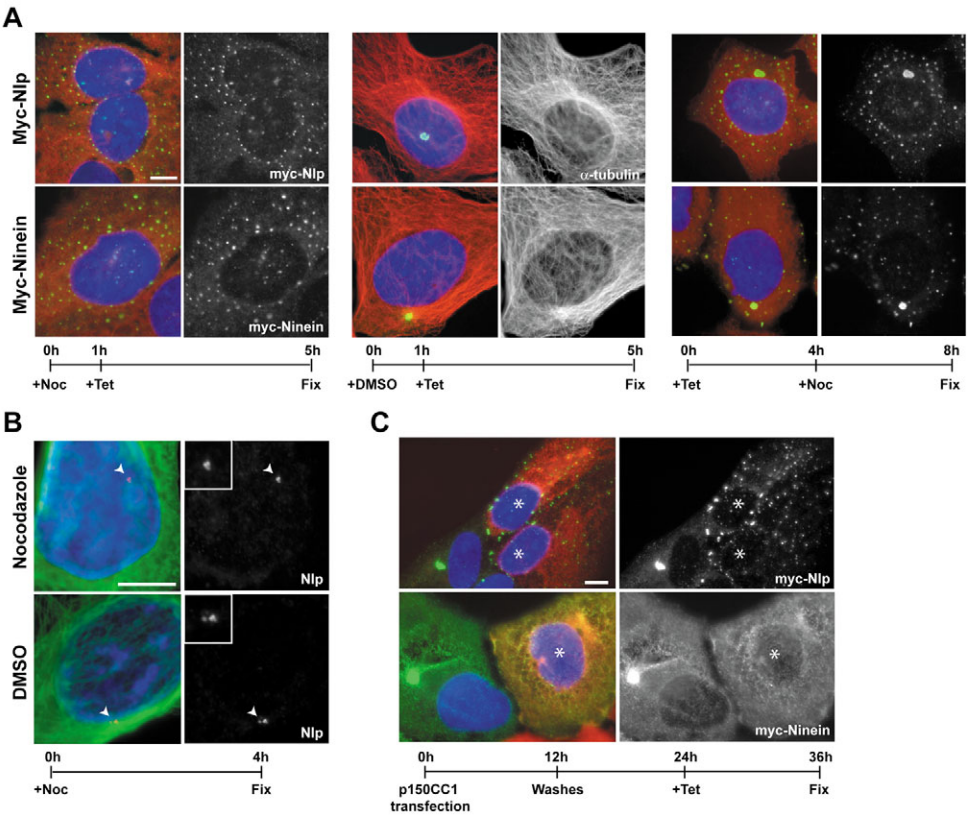

Fig. 5. Centrosomal targeting of Nlp and ninein depends on dynein-mediated transport. (A) Tet-On U2OS cell lines carrying Myc-tagged Nlp or ninein were treated with nocodazole (left panels) or DMSO (middle panels) for 1 hour before Nlp and ninein expression was induced for 5 hours by addition of tetracycline. Alternatively, Nlp and ninein expression was induced for 4 hours by addition of tetracycline, followed by a 4 hours treatment with nocodazole (right panels). Cells were fixed and then stained with antibodies to the Myc-epitope to detect Nlp and ninein (green), and α-tubulin (red). (B) U2OS cells treated with nocodazole or DMSO for 4 hours prior to fixation were stained with antibodies to Nlp and α-tubulin. (C) Tet-On U2OS stable cell lines carrying Myc-tagged Nlp or ninein were transfected with DsRed-p150 CC1. Addition of tetracycline to the culture medium 12 hours after transfection was used to induce expression of Nlp and ninein. Cells were then grown for 12 hours in the presence of tetracycline, fixed and analysed by immunofluorescence microscopy. Cells transfected with p150 CC1 were detected by DsRed fluorescence (asterisks). Cells were counterstained with anti-Myc antibodies and DNA was stained with DAPI (blue). Bars, 10 μm. A schematic representation of the experimental procedures is shown below each set of images.

hypothesis that Plk1 phosphorylation reduces the affinity of Nlp for the centrosome, and thus causes its release into the cytoplasm. However, if the dynein-dynactin motor complex is continuously transporting Nlp back to the centrosome, then an additional level of regulation may also exist. In this context it is significant that Plk1 phosphorylates Nlp on its N-terminal domain (Casenghi et al., 2003), which is the portion of the protein responsible for the association with the dynein-dynactin complex. To test if Plk1-mediated phosphorylation of Nlp regulates its association with the dynein-dynactin complex, EGFP-tagged wild-type Nlp or NlpΔ8, a mutant lacking all major Plk1 phosphorylation sites, were expressed together with activated $Plk1^{T210D}$ or inactive $Plk1^{K82R}$. Activated $Plk1^{T210D}$ caused a significant reduction in the ability of wild-type Nlp to recruit $p150^{Glued}$ (Fig. 6A), whereas

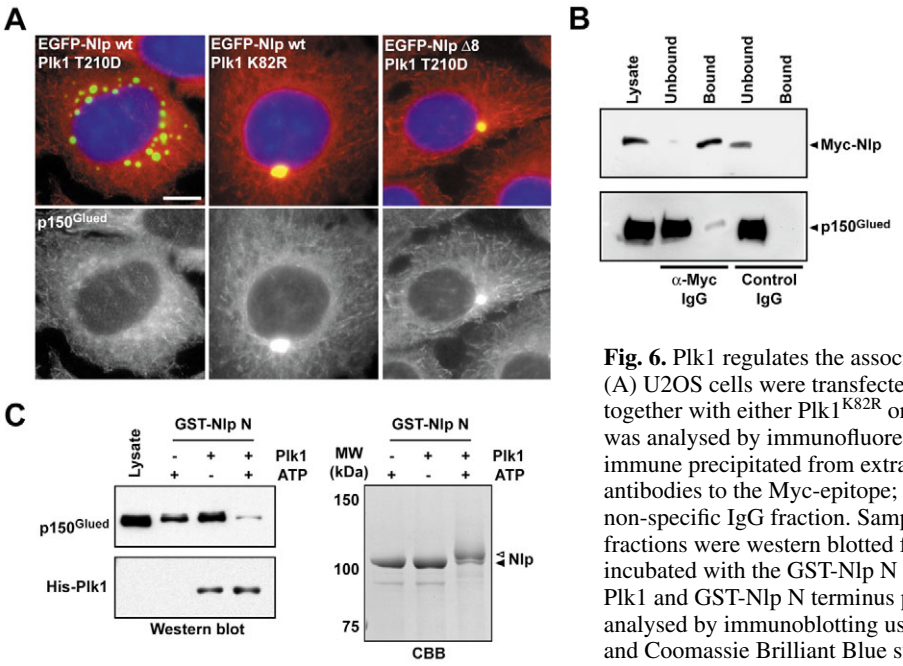

Fig. 6. Plk1 regulates the association of Nlp with the dynein-dynactin complex. (A) U2OS cells were transfected with EGFP-Nlp or EGFP-NlpΔ8 (green) together with either $Plk1^{K82R}$ or $Plk1^{T210D}$, and the localisation of $p150^{Glued}$ (red) was analysed by immunofluorescence microscopy. Bar, 10 μm. (B) Nlp was immune precipitated from extracts of 'Tet-On' Myc-Nlp-expressing cells using antibodies to the Myc-epitope; control precipitations were performed using a non-specific IgG fraction. Samples of the cell lysate, unbound, and bound fractions were western blotted for Nlp and $p150^{glued}$. (C) Total cell extracts were incubated with the GST-Nlp N terminus, GST-Nlp N terminus mock-treated with Plk1 and GST-Nlp N terminus phosphorylated by Plk1. Bound fractions were analysed by immunoblotting using antibodies to $p150^{Glued}$ and Plk1 (left panel), and Coomassie Brilliant Blue staining (right panel).

inactive Plk1^{K82R} had little effect on p150^{Glued} recruitment by Nlp (Fig. 6A). By contrast, the NlpΔ8 mutant lacking Plk1 phosphorylation sites was able to recruit dynactin even in the presence of activated Plk1^{T210D} (Fig. 6A). Immune precipitation of Nlp from cells expressing a Myc-tagged form of the protein showed that only a minor pool of the p150^{glued} dynactin subunit was precipitated together with Nlp (Fig. 6B). This is consistent with the fact that the major pool of dynactin at microtubule plus ends was unaltered in Nlp-expressing cells (Fig. 6A).

To confirm that Plk1-dependent phosphorylation of Nlp directly interferes with its ability to associate with dynactin we performed pull-down experiments using a recombinant form of the Nlp N-terminal domain phosphorylated by Plk1. The association of dynactin with the Plk1-phosphorylated Nlp N terminus was strongly reduced when compared with the control incubations using the Nlp N terminus treated with buffer or incubated with Plk1 in the absence of ATP and $MgCl_2$ (Fig. 6C). Taken together, these results suggest that the association of Nlp with the dynein-dynactin complex is regulated by Plk1-dependent phosphorylation. Furthermore, they indicate that interaction between Nlp and dynactin can be regulated by Plk1-dependent phosphorylation of Nlp alone, in the absence of dynactin phosphorylation.

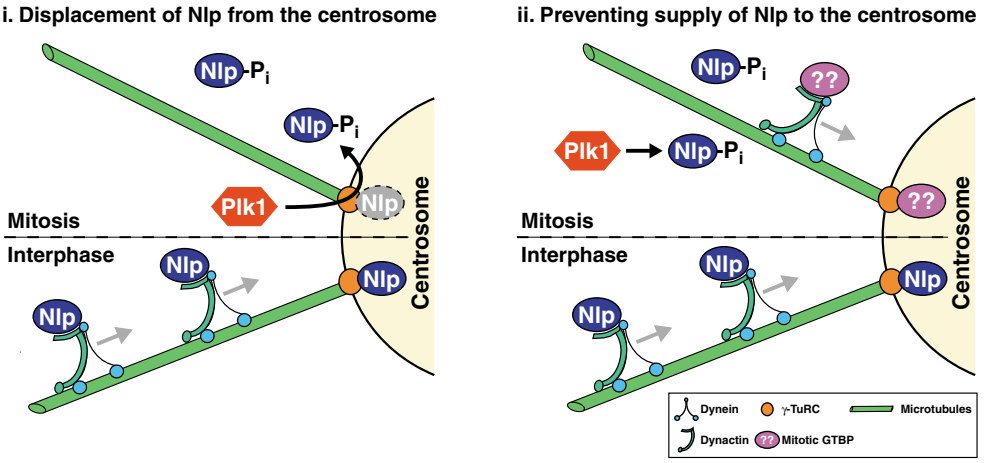

Fig. 7. Plk1 regulates Nlp transport and attachment to the centrosome. In interphase cells, Nlp is transported to the centrosomes by the dynein-dynactin motor complex. At the centrosome it then contributes to the attachment and nucleation of microtubules via the γ-tubulin ring complex (γ-TuRC). In mitosis, Plk1 phosphorylates Nlp and exerts a dual regulatory function. Phosphorylated Nlp is unable to interact with either (i) its binding partners at the centrosome or (ii) the dynein-dynactin motor complex. This dual action of Plk1 both promotes the release of Nlp from the centrosome and prevents its re-supply by the dynein-dynactin motor complex, and as a consequence Nlp redistributes into the cytoplasm during mitosis. Mitosis-specific γ-TuRC binding proteins (GTBPs) then replace Nlp at the centrosome as part of the centrosome maturation process required for the formation of the mitotic spindle.

Discussion

A model for the regulation of Nlp function by Plk1

Our data show that the association of Nlp with the dynein-dynactin complex is regulated by Plk1-dependent phosphorylation. This uncovers a second mechanism through which Plk1 controls the targeting of Nlp to the centrosome, and may explain our previous findings that overexpression of Nlp is capable of interfering with mitotic spindle assembly (Casenghi et al., 2003). During centrosome maturation, Plk1 directly phosphorylates Nlp resulting in its release into the cytoplasm. However, this would be insufficient to prevent Nlp being transported back to the centrosome and spindle poles during mitosis, since it is a cargo for the dynein-dynactin motor complex. We, therefore, propose that Plk1-dependent phosphorylation of Nlp regulates both these processes (Fig. 7). Moreover, phosphorylation-dependent regulation of cargo binding to the dynein-dynactin complex may represent a general mechanism controlling the exchange of centrosomal components, thereby coordinating changes in MT organisation with progression through the cell cycle.

Interestingly, although expression of Nlp and ninein cause dispersal of the Golgi apparatus and other organelles that require dynein and dynactin for their normal localisation, it does not disrupt the localisation of dynactin at microtubule plus ends. This is consistent with the idea that the mechanism of dynactin binding to microtubule plus ends is different to the mechanism of cargo binding. In addition, in Nlp-expressing cells, even though Nlp is overexpressed it does not complex all dynactin (Fig. 6B), which may explain why dynactin is still observed at microtubule plus ends. The concentrated, and thus prominent, centrosomal staining for dynactin in Nlp- and ninein-expressing cells probably reflects only a small pool of the protein. Together these findings suggest that there may in fact be two distinct pools of dynactin, one at microtubule plus ends, and a second, possibly cytosolic, pool required for transport of proteins to the centrosome and the organisation of organelles. Why Nlp and ninein, but not other dynactin binding proteins, can disrupt the Golgi apparatus is unclear, but this may reflect the fact that dynactin interacts with multiple cargo proteins at distinct sites. One attractive speculation is that the cargo site to which Nlp and ninein bind is also used by adaptors necessary for the motility of organelles such as the Golgi.

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