Functional characterization of the novel centrosomal protein Nlp (ninein-like protein)

Dissertation

der Fakultät für Biologie

der Ludwig-Maximilians-Universität

München

vorgelegt von

Martina Casenghi

München 2003

Dissertation eingereicht am 1.12.2003
Tag der mündlichen Prüfung: 16.03.2004

Erstgutachter: Prof. Dr. Erich A. Nigg
Zweitgutachter: Prof. Dr. Stefan Jentsch

Hiermit erkläre ich, dass ich die vorliegende Dissertation selbständig und ohne unerlaubte Hilfe angefertigt habe. Sämtliche Experimente sind von mir selbst durchgeführt, ausser wenn explizit auf dritte verwiesen wird. Ich habe weder anderweitig versucht, eine Dissertation oder Teile einer dissertation einzureichen bzw. einer Prüfungskommission vorzulegen, noch eine Doktorprüfung durchzuführen.

München, den 25.11.2003

TABLE OF CONTENTS

SUMMARY

The centrosome is the major microtubule organizing centre (MTOC) in animal cells. Most microtubules (MTs) emanate from the centrosome, where γ-tubulin ring complexes (γTuRCs) act as templates for MT nucleation. During interphase, the centrosome organizes a MT array that imparts shape and polarity to the cell and is essential for intracellular transport and positioning of organelles such as the Golgi apparatus. During mitosis, centrosomes ensure bipolarity and correct orientation of the spindle by forming the spindle poles. In order to switch from the interphasic to the mitotic state, the centrosome undergoes a structural reorganization, termed maturation, which is mainly characterized by an increase in MT nucleation activity.

A full appreciation of how centrosomes contribute to cellular functions requires the isolation and characterization of unknown centrosome-associated molecules. Here we describe the identification and characterization of a novel centrosomal component, the human protein Nlp (ninein-like protein) related to the previously characterized MT-anchoring protein ninein.

In the first part of the present thesis we describe the identification of Nlp as a novel centrosomal substrate of Polo-like kinase 1 (Plk1), an important regulator of mitosis whose activity is required for centrosome maturation. Nlp interacts with two distinct γTuRC components, γ-tubulin and hGCP4, and stimulates MT nucleation. Plk1 phosphorylates Nlp and disrupts its centrosomal association. Overexpression of an Nlp mutant lacking Plk1 phosphorylation sites induces defects in mitotic spindle formation. We propose that Nlp acts as a γTuRC binding protein (GTBP), contributing to the MT nucleation activity of the centrosome during interphase. At the onset of mitosis, the displacement of Nlp from the centrosome triggered by Plk1 phosphorylation could represent an important step in the maturation process which allows the centrosome to switch from the interphasic to the mitotic state.

Thus, we conclude that Nlp, as well as the related protein ninein, plays an important role in MT organization. However the function of these two proteins

possibly diverged during evolution: whilst Nlp gained a more prominent role in MT nucleation, ninein became principally involved in MT anchoring.

In the second part of this thesis we report the initial characterization of the molecular mechanisms underlying the ability of Nlp and ninein to induce the fragmentation of the Golgi apparatus when overexpressed in human cells. We show that the ability of these two centrosomal proteins to affect the organization of the Golgi clearly depends on their capacity to associate with the cytoplasmic dynein-dynactin complex, a molecular motor complex primarly involved in the maintainance of Golgi architecture. We propose that the excess of Nlp and ninein could induce the disruption of the Golgi apparatus by sequestering the dynein-dynactin complexes.

Future investigations should be aimed at understanding whether the dissociation of the Golgi apparatus from the centrosome induced by the excess of Nlp and ninein could interfere with cell migration and cell polarization processes, which require a highly coordinated action of these two organelles. Cell migration and cell polarization represent critical events for immune responses as well as for embryonic development, invasive growth and metastasis. Thus, our findings raise the interesting possibility that an upregulation in the expression levels of structural centrosomal proteins could represent the molecular basis for developmental disorders and malfunctioning of the immune system and, on the other hand, modulate the acquisition of invasive properties by neoplastic cells.

AN OVERVIEW OF THE CENTROSOME

Centrosome organization

The term “centrosome” was coined by Theodor Boveri in 1888 to describe a “single extremely minute body, or more commonly a pair of bodies, staining intensely with haematoxylin […] and surrounded by a cytoplasmic radiating aster”. Because the poles that define the essential bipolar nature of the spindle each contain a centrosome, Boveri viewed this organelle as the “especial organ of cell division” (Wilson EB, 1911 The Cell in Development and Inheritance, the MacMillan Company), an opinion that as remained unchallenged until recently (see paragraph “The centrosome function”).

More than a century of research has revealed that the centrosome is a tiny (1-2μm^3) but complex non-membraneous organelle that is usually localized in close proximity to the nucleus and it functions as the major microtubule organizing center in animal cells (for reviews see Andersen, 1999; Hinchcliffe and Sluder, 2001; Doxsey, 2001; Bornens, 2002). Centrosomes are structurally conserved among all higher eukaryotes, exception made for higher plants. In animal cells, they are composed by a pair of centrioles, surrounded by a cloud of electrondense material known as the pericentriolar material or PCM (Figure 1; for reviews on centrosome ultrastructure see Bornens *et al*., 1987; Vorobjev and Nadhezhdina, 1987; Preble *et al.*, 2000)

The PCM consists of a fibrous meshwork, known as the centromatrix (Schackenberg and Palazzo 1999), that provides a scaffold for anchoring a variety of proteins, including functional components involved in MT nucleation such as the γ-tubulin ring complexes or γTuRCs (Dictenberg *et al*., 1998; for review see Wiese and Zheng, 1999; Zimmermann *et al*., 1999) as well as several coiled-coil proteins.

The centrioles are symmetrical, barrel-shaped structures formed by nine sets of triplet microtubules and characterized by the presence of several specific

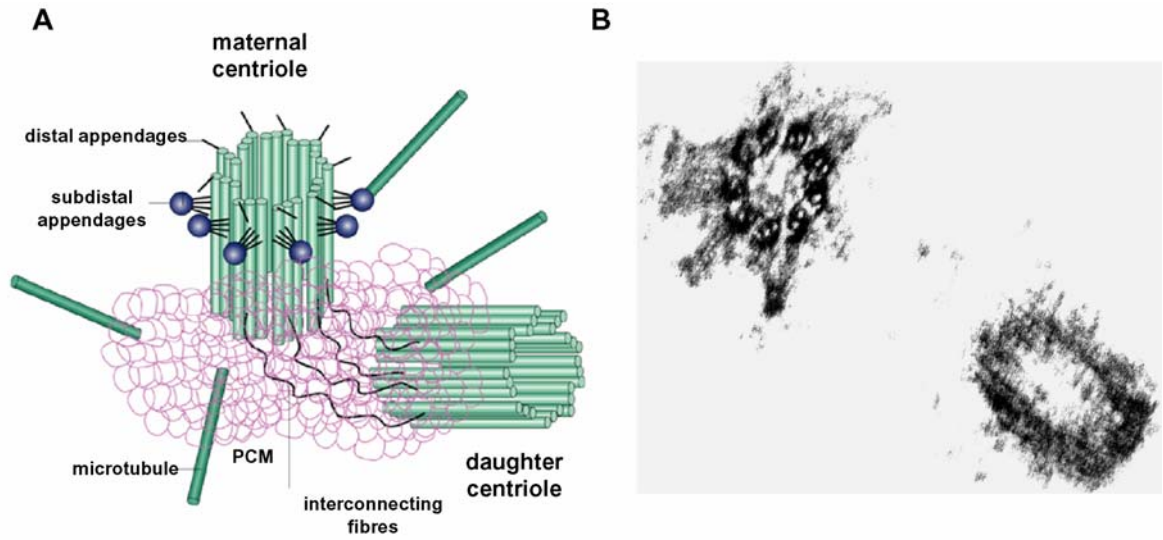

Figure 1. The centrosome structure
(**A**) Schematic drawing of the centrosome (Doxsey, 2002). (**B**) Electron microscopy picture showing the centrosome with the two centrioles. The microtubules triplets are visible in the crosscut centriole

proteins, including the tubulin subfamily members δ and ε (Dutcher, 2001), poly-glutamylated tubulin (Bobbinnec *et al*.,1998), centrin (Salisbury, 1995), cenexin/ODF2 (Lange and Gull, 1995), as well as tektin filaments and their associated structural proteins (Steffen *et al*., 1994; Hinchcliff and Linck, 1998). The two centrioles within a centrosome are positioned in an orthogonal arrangement with respect to each other and differ in their appearance: one centriole has two sets of nine appendages at the end distal to its partner and is called the mature centriole (also called the mother or maternal centriole) (Vorobjev and Chentsov, 1982).

Centrioles seem to play a major role in maintaining the organization of the pericentriolar material which surrounds them. Microinjection of antibodies raised against poly-glutamylated tubulin in HeLa cells, induces disruption of the centrioles structure and following disorganization and dispersion of the PCM components (Bobbinec *et al*., 1998). Interestingly, centrioles are structurally similar to basal bodies, which are found at the base of eukaryotic cilia and flagella. Primary cilia in somatic cells and flagella in sperm cells are generated directly from the centrosome (Hagiwara *et al*., 2000). *Viceversa,* during fertilization in

most animal cells, the basal bodies of the sperm become the centrioles of the first centrosome of the egg (Schatten, 1994).

In lower eukaryotes, such yeast, the functional equivalent of the centrosome is the spindle pole body (SPB) (reviewed in Kilmartin 1994; Snyder, 1994; Knop *et al*., 1999). Although the SPB appears differently structured and organized, many of its components are highly conserved in the centrosome of animal cell and important contributions in understanding the molecular organization and function of centrosomes came from the work on the spindle pole body.

The centrosome cycle

The morphology and composition of the centrosome changes during the cell cycle (Kuriyama and Borisy, 1981; Chretien *et al*.,1997). On the basis of morphological observations, the centrosome cycle can be subdivided in a series of defined structural events referred to as: **centrosome duplication**, **centrosome maturation**, **centrosome separation** and **centrosome disorientation** (for a schematic representation see Figure 2)**.**

The **centrosome duplication** is a semi-conservative process and is first seen at the beginning of S-phase or during S-phase by the appearance of procentrioles, or daughter centrioles, growing at right angles in a region close to the proximal end of each parental centriole (Kochanski and Borisy, 1990). **Elongation** of the procentrioles occurs throughout S and G2 phase, so that in late G2 the centrosome is composed by two pairs of centrioles. **Centrosome maturation** occurs at the G2/M transition and it is characterized by a dramatic increase in the centrosome size due to the recruitment of several proteins, in particular γ-tubulin ring complexes (γTuRCs) (Palazzo *et al*.,2000). At the onset of mitosis the amount of centrosomal γ-tubulin increases about three-to five fold (Khodjakov and Rieder, 1999) in mammalian cells, and it is accompanied by an increase in the MT nucleation potential of the centrosome. Concomintantly, the two centrosomes start to separate and migrate apart to form the poles of the

mitotic spindle. This **centrosome separation** process seems to occur in two step. In a first step, which is independent of MTs, cohesion between the two parental centrioles is lost (Fry *et al*.,1998b; Mayor *et al*.,2000). In a second step, the two centrosomes are separated through the action of MT-dependent molecular motor proteins (Vaisberg *et al*., 1993; Blangy *et al*., 1995; Boleti *et al*., 1996; Giet *et al*., 1999). Upon cell division each daughter cell inherits one centrosome. During late mitosis/early G1 phase the two centrioles move apart and thereby loose their orthogonal disposition. The physiological significance of this event, referred to as **centrosome disorientation**, remains uncertain. Although centrosome disorientation has been commonly considered to be the pre-requisite for centrosome duplication (Lacey *et al*., 1999; Freed *et al*., 1999), it has also been shown that centrioles can separate from each other in telophase in some cultured cells and this event has been proposed to be required for completion of cell division (Piel *et al*., 2000; Piel *et al*., 2001).

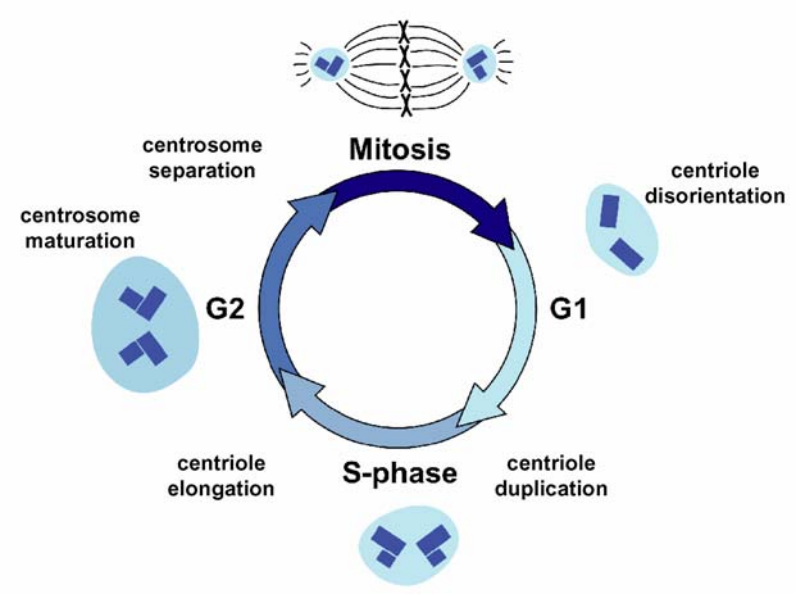

Figure 1. The centrosome cycle
Schematic representation of the changes occuring at the centrosome during cell cycle progression

Regulation of the centrosome cycle

Recently, several studies have identified both phosphorylation and proteolysis as major mechanisms for the regulation of the centrosome cycle (for review see Meraldi and Nigg, 2002; Fry *et al*., 2000; Hansen *et al*., 2002).

In particular, the identification of several centrosome-associated protein kinases and phosphatases has supported the concept that multiple regulatory phosphorylation/dephosphorylation pathways tightly control centrosome structure and function (summerized in Table 1). Intriguingly, it has recently been shown that at the centrosome kinases and phosphatases can associate in multiprotein complexes organized by scaffold centrosomal proteins such as C-Nap1 (Helps *et al.*, 2000) or the 453 kDa A-kinase anchoring protein (AKAP450, also referred to as AKAP350 or CG-NAP) (Takahashi *et al*., 1999). Kinases and phosphatases which can exert feedback inhibition on their reciprocal activities (Helps *et al*., 2000; Katayama *et al*., 2001) could therefore be located in close proximity to each other and to their substrates within these multiprotein complexes, allowing a fine modulation of the balance between kinase and phophatase activities at the centrosome and, as a consequence, a fine and rapid control of centrosome functions (Meraldi and Nigg, 2001).

In order to have a correct execution of cell division, the centrosome cycle must be coordinated with other cell cycle events. In particular, the centrosome must undergo regular rounds of duplication and separation in concert with the chromosomes themselves. An important link between the DNA replication and the **centrosome duplication** has emerged from studies demonstrating that cyclin-dipendent kinase 2 (Cdk2) is required for both of these key S-phase events in both embryonic and somatic cells (Hinchcliffe *et al*., 1999; Matsumoto *et al*., 1999; Meraldi *et al*., 1999). In *Xenopus* embryos the Cdk2 has been shown to act in a complex with cyclin E in controlling centrosome duplication (Hinchcliffe *et al*., 1999), while in somatic cells the Cdk2/cyclin A complex seems to play a major role (Meraldi *et al*., 1999). These observations raise the interesting possibility that different Cdk2-cyclin complexes could be involved in the regulation of centrosome duplication, depending on the developmental stage. However, regardless of

possible differences due to the developmental context, cell cycle progression into S phase and centrosome duplication appear to be linked through a rise in Cdk2 activity. Little is presently known concerning the targets of Cdk2 that could be relevant for centrosome duplication. Two proteins have been proposed as possible substrates: mMps1p (Fisk and Winey, 2001) and nucleophosmin NO/B23 (Okuda *et al*., 2000). Yet, the centrosomal localization of these proteins as well as their possible function at the centrosome are still subjects of scientific debate (Stucke *et al*., 2002; Okuwaki *et al*., 2002; Herrera *et al*., 1995). Therefore, their role in centrosome duplication awaits clarification. One of the most cryptic step in centrosome duplication is the generation of the pro-centrioles (or daughter centrioles). An important constituent of the molecular pathway regulating this process has been recently identified in the nematode *C.elegans*. The *C. elegans* ZYG-1 kinase has been shown to be essential for centrosome duplication but, remarkably, not for cell cycle progression (O'Connell *et al*., 2001). *Zyg-1* mutant embryos can not divide properly and they arrest with monopolar spindles containing single unpaired centrioles, indicating that ZYG-1 is required specifically for pro-centriole formation. The identity of possible ZYG-1 substrates and/or upstream regulators as well as the existance of functional homologues in other organisms are interesting open questions. Another kinase implicated in centrosome duplication is calcium-calmodulin kinase II (CAMKII). Inhibition of this kinase abolished centrosome duplication in assays performed in *Xenopus* egg extracts (Matsumoto and Maller, 2002). Calmodulin and the calcium binding protein centrin/Cdc31 have also been implicated in SPB and centrosome duplication in *S.cerevisiae* (Baum *et al*., 1986) and mammalian cells (Salisbury *et al*., 2002; Middendorp *et al*., 2000) respectively. Interestingly, these observations suggest the existance of a conserved mechanism for the regulation of MTOC duplication which depends on calcium-regulated protein.

Regulation of **centrosome maturation** undoubtedly involves networks of phosphorylation/dephosphorylation that we are just beginning to understand (see also Part I-Introduction). Both Polo-like kinases (Plks) and A-type Aurora kinases have been directly implicated in this process by genetic analysis, antibody injection and RNA-mediated interference (Berdnick and Knoblich, 2002;

Donaldson *et al*., 2001; Hannak *et al*., 2001; Lane and Nigg, 1996; Sunkel and Glover, 1988). Yet, little is known about the substrates through which these kinases can control centrosome maturation. Poorly characterized is also the role of the phosphatases that oppose these mitotic kinases. Protein phosphatases type 1 and 4 (PP1 and PP4) have been shown to localize to the centrosome (Andreassen *et al*., 1998; Helps *et al*., 1998). PP1 is present in a complex with Aurora A, in which the two enzymes can exert feedback control on the reciprocal activities (Katayama *et al*., 2001). *Drosophila* mutants characterized by a reduced levels of centrosomal PP4, also show a significant reduction of γ-tubulin centrosomal levels (Helps *et al*., 1998). Consistently, when PP4 function is suppressed by RNAi in *C. elegans*, both γ-tubulin and Polo-like kinase are mislocalized (Sumiyoshi *et al*., 2002), indicating a possible role for PP4 in centrosome maturation.

The protein kinase which play a major role in controlling **centrosome separation** is the NIMA-related kinase Nek2. Throughout most of the cell cycle, parental centrioles appear to be connected by a proteinaceous structure for which the centriole associated coiled-coil protein C-Nap1 may function as a docking site (Fry *et al*., 1998a; Mayor *et al*., 2000). C-Nap1 is a substrate of Nek2 (Fry *et al*., 1998a) and its association to the centrosome is regulated through phosphorylation (Fry *et al*., 1998a; Helps *et al*., 2000; Lutz *et al*., 2001). These two proteins cooperate in maintaining parental centriole cohesion as demonstrated by the induction of centrosome splitting observed upon overexpression of Nek2 and microinjection of antibodies against C-Nap1 (Fry *et al*., 1998a; Mayor *et al*., 2000). Intriguingly, Nek2 and C-Nap1 are present in a complex with PP1 and the two enzymes are interdependent in their activity *in vitro* (Helps *et al*., 2000). In particular, it has been suggested that PP1 activity controls Nek2 activity. A recent interesting model proposes that the inactivation of PP1 at the onset of mitosis would allow the Nek2 activity to prevail, leading to an increase in C-Nap1 phosphorylation levels and loss of centriole cohesion. Once the cohesion is lost, the two centrosome can be separated through the action of MT-dependent motor proteins. A major role in this event is played by the kinesin-related motor protein

Eg5 that is itself regulated by at least two kinases, Cdk1 and Aurora A (Blangy *et al*., 1997; Giet *et al*., 1999).

Table1. Protein kinases and phosphatases associated with the centrosome

Enzyme	Substrate	Reference
Centrosome duplication		
Cdk2/cyclin E or cyclin A	Mps1?, Nucleophosomin/B23?	Hinchcliff et al., 1999; Matsumoto et al., 1999; Meraldi et al., 1999 Lacey et al., 1999 Fisk and Winey 2001
Zyg-1	?	O' Connell et al., 2001
CaMkII	?	Matsumoto and Maller, 2002
Centrosome maturation		
Plk1	Asp?	Lane and Nigg, 1996 Sunkel and Glover, 1988 do Carmo et al., 2001
Aurora-A	D-TACC	Hannak et al., 2001 Giet et al., 2002, Lee et al., 2001
PP4	?	Helps et al., 1998 Sumiyoshi et al., 2002
Centrosome separation		
Nek2/PP1α	C-Nap1	Fry et al., 1998 Mayor et al., 2000 Helps et al., 2000
PKA?	centrin	Lutz et al., 2001
Aurora-A	Eg5	Giet et al., 1999
Cdk1	Eg5	Blangy et al., 1995 Sawin and Mitchison, 1995 Blangy et al., 1997
Centrosome positioning		
P160ROCK	Myosin light chain; Ezrin/radixin/ moesin family proteins	Chevrier et al., 2002 Amano et al., 1996 Matsui T et al., 1998
Pyk2	PSGAP	Ren et al., 2001 Sancho et al., 2000

In recent years it has become clear that reversible phosphorylation is not the sole mechanism responsible for regulating the centrosome cycle. Interesting evidence suggests that ubiquitin-dependent proteolysis also plays an important role (reviewed in Hansen *et al*., 2002). Components of both the E3/ubiquitin ligase complexes SCF (Skip1, Cullin, F-box) and APC (anaphase promoting complex) have been localized to the centrosome throughout the cell cycle in mammalian cells (Freed *et al*., 1999; Gstaiger *et al*., 1999; Tugendreich *et al*., 1995; Wigley *et al.*, 1999). A specific role for the SCF complex in regulating the centrosome cycle has been suggested by the observation that in *Xenopus* egg extract centriole disorientation could be blocked by antibodies against Skip1 and Cul1, two core components of the SCF complex which have been localized to the centrosome (Freed *et al*., 1999). In addition, proteasome inhibitors block centriole disorientation *in vitro* and centrosome duplication in *Xenopus* embryos (Freed *et al*., 1999), further suggesting a role for proteolysis at this stage of the centrosome cycle. Yet, in other organisms the perturbation of the SCF function has been shown to differently influence centrosome duplication. *Drosophila* mutants of the F-box protein Slimb contain abnormally high number of centrosome in larval neuroblast (Wojcik *et al*., 2000). Similarly, cells from knock-out mice lacking Skp2, an F-box adapter protein of the SCF complex, display abnormal centrosome amplification. However, these cells also show polyploidy and higher level of cyclin E (Nakayama *et al*., 2000), so that the mechanism which is causing the accumulation of extranumerary centrosome remains elusive. Thus, the precise role of controlled proteolysis in regulating centrosome duplication remains to be determined but an interesting possibility is that certain proteins need to be degraded in order to allow centrosome disorientation, which in turn may represent the prerequisite for centrosome duplication.

Evidence is also beginning to emerge for an involvement of the Ran GTPase network components in the control of centrosome structure and function. Ran has been recently shown to regulate both MT dynamics and MT nucleation from centrosomes in M-phase arrested *Xenopus* egg extracts (Carazo-Salas *et al*., 2001). In particular, sperm centrosomes pre-incubated in extract containing recombinant Ran-GTP aquired a higher capacity to nucleate microtubules than

centrosomes exposed to the Ran (T24N) mutant, which binds to RCC1 (the Ran guanidine exchange factor) and inhibits its exchange activity. This suggests a possible role for Ran-GTP in centrosome maturation. Furthermore, a subpopulation of both Ran and RanBP1, a cytosolic protein that binds Ran-GTP with high affinity, have been recently localized to the centrosome in mammalian cells (Di Fiore *et al*., 2003; Keryer *et al*., 2003). The mechanisms through which the Ran network components act locally in mitosis to influence centrosome function and structure still represent an interesting unresolved issue.

The centrosome function

It has long ben recognized that the centrosome is the primary microtubule organizing center (MTOC) of the cell. When disassembled, MTs rapidly and preferentially regrow form this organelle during recovery. The ability of the centrosome to nucleate MTs and to organize them in functional arrays throughout the cell cycle led to a general consensus that its essential function revolves around this feature, an opinion that has remained unchallenged until recently.

During interphase, the centrosome organizes a MT array that imparts shape and polarity to the cell and is essential for intracellular transport and positioning of organelles. In interphasic cells the position of the centrosome and its associated organelles may change in response to external stimuli. During cell migration, in many, but not all, cell types the centrosome reorients to a position in front of the nucleus toward the leading edge of the cell. The repositioning of the centrosome follows the extension of a leading edge rather than determining the direction of migration, and seems to be required for stabilizing cell movement through stabilization of the newly organized microtubule cytoskeleton (Yvon *et al*., 2002; for a review see Schliwa *et al*., 1999). Centrosome repositioning is also observed in T lymphocytes (T cells) in response to antigen specific loaded APCs (antigen presenting cells), as well as in natural killer (NK) cells and in cytotoxic T lymphocytes (CTLs) when they interact with target cells (Geiger *et al*., 1982; Kupfer *et al*., 1983; Kupfer *et al*., 1987; for review see Sancho *et al*., 2002). Thus,

a variety of immune functions operate through centrosome–directed polarization of the cytoplasm (see also below).

During mitosis, the centrosome was for a long time thought to be necessary for the formation of the bipolar spindle. However, higher plants as well as the female germline of many organisms in the animal kingdom, lack canonical centrosomes, as defined by the presence of a centriole pair, but can still organize normal spindle and undergo cell division (Heald *et al*., 1996; for reviews see Smirnova and Bajer, 1992; Compton, 2000). This observations suggested that there might be alternative mechanisms to ensure spindle bipolarity. Indeed, studies in female meiotic cells of many organisms, revealed that during meiosis functional bipolar spindle can be formed in the absence of centrosome through a pathway in which antagonistic motor molecules (including cytoplasmic dynein and members of the kinesin superfamily) can organize randomly nucleated MTs into bipolar structures (Hyman and Karsenti, 1996; Endow and Komma, 1997; Walczak *et al*., 1998). Surprisingly, when centrosomes are destroyed by laser-ablation or removed by a microneedle, mammalian cells can still form functional bipolar spindle (Hinchliffe *et al*., 2001; Khodjakov *et al*., 2000). Thus, a redundant centrosome-independent pathway for spindle assembly is also present in vertebrate somatic cells that, like oocytes, probably employ self organization of MTs and motor proteins to ensure spindle bipolarity (Compton, 1998; Wittmann *et al.*, 2001). It remains to be determined whether this pathway is activated only in the absence of centrosome function, or is always working but normally masked by centrosome activity. Indeed, it is important to emphasize that, when present, centrosomes act dominantly to organize spindle poles (Heald *et al*., 1997). Centrosome dominance in spindle pole formation might have important implications in cancer. The presence of abnormalities in structure and number of centrosomes has been frequently reported in cancer cells and tumour tissues (for reviews see Brinkley, 2001; Nigg, 2002). When present in more than two copies, centrosomes contribute to the assembly of multipolar spindles that can lead to chromosomes missegregation and aneuploidy. Whether the supranumerary centrosomes are cause or consequence of aberrant cell divisions, is presently a matter of debate.

Why animal cells have evolved a mechanism that involves centrosomes to form bipolar spindle when the spindle can assemble without centrosomes, represents an intriguing unresolved question. One possible answer is that being part of the spindle poles, centrosomes can themselves be inherited by each daughter cells during cell division, so that they can complete other essential cellular functions. For example, centrosomes are strictly necessary for the formation of primary cilia and flagella, cellular structures required for proper development and tissue function (Nonaka *et al*., 1998; Pazour *et al*., 2000). Moreover, despite being denied of their essential function in bipolar spindle assembly, centrosomes still play a key role in mitosis, ensuring the correct positioning of the spindle and therefore establishing the site of cell cleavage during cytokinesis. In both mammalian cells and yeast, molecules at the plus ends of centrosome-associated astral microtubules can interact with cytoplasmic dynein located at the cell cortex, contributing, at least in part, to the driving force for spindle positioning (Busson *et al*., 1998; O'Connell and Wang, 2000; Segal and Bloom, 2001; for review see Schuyler and Pellman, 2001). In mammalian cells depleted of centrosomes by laser ablation, no astral MTs are produced and spindles become mispositioned, causing subsequent problems during cytokinesis (Khodjakov and Rieder, 2001). Cytokinesis defects in acentrosomal cells have also been observed by Hinchcliffe et al. (Hinchcliffe *et al*., 2001), who used microsurgery to remove the centrosomes. Thus, the failure of cytokinesis in acentrosomal cells could be a consequence of a defective spindle positioning.

Alternatively, the centrosome could be directly involved in the completion of cytokinesis, as suggested by the elegant work of M. Bornens and coworkers (Piel *et al*., 2001). Using time-lapse microscopy, the authors examined the behaviour of centrosomes labelled with GFP-tagged centrin. They found that the final events of cell cleavage correlated with the movement of the maternal centriole to the intercellular bridge that connects dividing cells, and this localization in turn correlated with bridge narrowing and MT depolymerization. When the centriole moved away from the bridge, the cell proceded to cleave. These results indicate two mechanisms by which centrosomes might regulate the final stages of cytokinesis. Centrosomes could directly activate cytokinesis or could release cells

from arrest at a cytokinesis checkpoint. Recent studies in both budding and fission yeast favour the latter possibility (for a review see Pereira and Schiebel, 2001). In budding yeast, cells do not complete cytokinesis until the spindle pole body moves into the daughter cell (bud), bringing into contact the GDP-bound form of Tem1, localized at the spindle pole, and the guanine-nucleotide exchanging factor (GEF) Lte1 which is located in the bud. The consequent production of Tem1-GTP, together with microtubule depolymerization at the bud junction (neck) and loss of microtubule contact with the neck, triggers cytokinesis and exit from mitosis (Bardin *et al*., 2000; Bloecher *et al*., 2000; Gruneberg *et al*., 2000, Pereira *et al*., 2000). These observations indicate that there might be common elements in the pathways of yeast and mammals that mediate the completion of cytokinesis.

Upon centrosome removal, somatic mammalian cells have also been observed to arrest in G1 phase and to not initiate DNA replication (Hinchcliffe *et al*., 2001; Khodjakov and Rieder, 2001). One possible interpretation of this phenotype is that centrosomes directly activate or concentrate factors that are essential for the initiation of DNA synthesis. Consistent with this idea is the centrosomal localization of molecules that control entry into S phase (Hinchcliffe *et al*., 1999; Lacey *et al*., 1999). An alternative explanation is that animal cells monitor the presence of centrosomes and in their absence activate a checkpoint that prevents the onset of S-phase. The pathway that activates G1 arrest in acentrosomal cells has not yet been characterized. It is likely to be complicated by potential downstream effects of centrosome loss such as chromosome missegregation, cytokinesis defects and tetraploidy, which can also trigger G1 arrest (Andreassen *et al*., 2001). Regardless of the mechanism, one consequence of having a centrosome requirement for cell-cycle progression represents another way to ensure that dividing animal cells receive the appropriate number of centrosomes.

Another recently emerged role for centrosomes might be in monitoring DNA damage. In *Drosophila* embryos mutations in the DNA-replication checkpoint, as well as treatment with DNA-damaging agents or DNA-replication inhibitors, induce inactivation of centrosomes in mitosis (Sibon *et al*., 2000). Inactivation of the centrosomes might be part of a damage-control system that prevents

chromosome segregation if the checkpoint that monitors DNA replication or damage fails.

In conclusion, during the past recent years the perception of the role of the centrosomes has considerably changed. Their role in spindle assembly is in question as spindles form in the absence of centrosomes. Instead, new roles for centrosomes are emerging. Centrosomes seem to be involved in cytokinesis completion and in cell-cycle progression. This new glimpse into the complexity of these organelles suggests that we are only beginning to uncover their functions.

Centrosomes, MT minus ends and the Golgi apparatus

The centrosome and its associated MT network are important for the proper positioning of subcellular organelles. In particular, in most cell types the Golgi apparatus is closely associated with the centrosome in a juxtanuclear position, and relies on the presence of the MT network to maintain its structure and localization (for review see Rios and Bornens, 2003). These cellular organelles associate even under conditions where cellular architecture is undergoing major remodelling. As discussed above, this occurs, for example, during cell migration (Etienne-Manneville and Hall, 2001; Magdalena *et al*., 2003) or T cell polarization towards an antigen presenting cell (for reviews see Sancho *et al*., 2002; Serrador *et al*., 1999). Studies aimed to better understanding the basis of the Golgi localization and its relationship with the centrosome suggest that the Golgi complex preferentially associate with the slow-growing “minus” ends of microtubules, the majority of which are anchored at the MTOC in cell lines displaying a radial MT array. A relationship between the Golgi apparatus and the centrosomal MT minus-end activities is revealed during treatment of cells with taxol or during cell differentiation. In fibroblastic and epithelial cells, taxol treatment induces stable MTs to form bundles away from the centrosome. Under these conditions, Golgi elements seem to associate with the ends of MTs to which anchoring proteins and other centrosomal protein are recruited (Bornens 2002; Rios and Bornens, 2003). Moreover, during myogenesis the centrosome and the

Golgi apparatus are dramatically reorganized. In myotubes, the Golgi complex appears to disperse into elements that form a belt around the nuclei. This redistribution is accompanied by a similar reorganization of MTs and MT nucleation sites (Ralston *et al*., 1999; Ralston *et al*., 2001; Tassin *et al*., 1985) and the Golgi elements are found near the MT-nucleating centres (Lu *et al*., 2001; Ralston, 1993).

Why the Golgi membranes are anchored to the pericentriolar region in mammalian cells, is a question that remains unanswered. One possible explanation is that this association could ensure the coordination of centrosome and Golgi activities, therefore facilitating the control of cellular processes under conditions where cell architecture as a whole is undergoing remodelling. This occurs during cell migration, polarization or differentiation. Another interesting interpretation arises from a growing body of evidence indicating that the Golgi/centrosome region could play important roles in several physiological processes, such as intracellular signalling, mitosis and apoptosis. The notion that both the centrosome and the Golgi apparatus could function as signalling platforms has been fostered by the identification of a variety of signal transduction molecules in these organelles (for reviews see Donaldson and Lippincott-Schwartz, 2000; Lange, 2002).

In the previous paragraph we summarized recent evidence suggesting that the centrosome is involved in cell cycle checkpoints and the control of cell cycle progression. Fragmentation and dispersal of the Golgi, on the other hand, have been reported to be a pre-requisite for entry into mitosis in mammalian cells (Sutterlin *et al*., 2002). Furthermore, both the centrosome and the Golgi apparatus may also initiate apoptosis by specific stress sensors and release apoptosis-modulating signals to the rest of the cell (Ferri and Kroemer, 2001; Piekorz *et al*., 2002). Therefore, the close proximity of these two organelles could ensure the integration of different sensoring and signalling pathways, allowing the cell to efficiently check its status before any important decision on the life cycle is undertaken.

Goals of the project

The aim of this thesis has been to develop a better understanding of the function and regulation of the centrosome during the cell cycle, in particular through the characterization of the novel centrosomal protein Nlp (ninein-like protein). The present thesis has been structured in two parts. In the first part we describe the role played by Nlp in centrosomal MT nucleation and the regulation of its activity through the cell cycle by Polo-like kinase 1. In the second part we report an initial characterization of the molecular mechanisms underlying the ability of centrosomal components, such as Nlp and ninein, to perturb the positioning and shaping of the Golgi apparatus.

PART I: Nlp, a novel centrosomal substrate of Plk1 involved in MT nucleation

INTRODUCTION

The centrosome maturation process

In preparation for mitosis centrosomes undergo a striking structural reorganization termed maturation (Palazzo *et al*., 2000). This event is principally characterized by the recruitment of additional γTuRCs and a concomitant increase in microtubule nucleation activity (Khodjakov and Rieder, 1999) (see below). Although centrosome maturation is important for the formation of the mitotic spindle apparatus, the underlying mechanisms regulating this process remain largely unknown.

Two protein kinases, Polo-like kinase 1 (Lane and Nigg, 1996; Sunkel and Glover, 1988) and Aurora-A (Berdnik and Knoblich, 2002; Hannak *et al*., 2001) have been implicated in the regulation of centrosome maturation. Substrates of these enzymes remain to be characterized, but recent studies in *Drosophila* led to the identification of the first Aurora A and Polo kinase substrates possibly implicated in this process. Indeed, Aurora A mutants resulted unable to recruit to the centrosome the proteins D-TACC and Minispindles/XMAP215, which form a complex involved in MT growth and stabilization during mitosis (Giet *et al*.,2002; Lee *et al*., 2001). *Drosophila* Polo kinase has been shown to interact with and phosphorylate the *abnormal spindle* gene product Asp (do Carmo *et al*., 2001). This protein is associated with the centrosome throughout mitosis and plays a role in MT organization (discussed below). However, despite these recent advances, our knowledge of the events occuring at the centrosome at the onset of mitosis is still far from complete.

Centrosomes and MT nucleation activity

Nucleation of microtubules at the centrosome occurs with a distinct polarity: while the rapidly growing plus ends are distally disposed toward the periphery of

the cell, the slow-growing minus ends of the microtubule remain embedded into the pericentriolar material, the fraction of anchored minus ends varying extensively in different cell types. The discovery of γ-tubulin and γ-tubulin-containing multiprotein complexes has greatly advanced our understanding of the MT nucleation process. γ-tubulin is a conserved component of all MTOCs and its involvement in the microtubule nucleation reaction has been extensively documented in recent years (for review see Joshi, 1994; Pereira and Schiebel, 1997). Disruption of the γ-tubulin gene in several organisms prevents proper microtubule organization (Horio *et al*., 1991; Oakley *et al*., 1990; Sobel and Snyder, 1995; Sunkel *et al*., 1995) and microinjection of γ-tubulin antibodies disrupts MT nucleation by the centrosome in interphase cells and formation of the spindle in mitotic cells (Joshi *et al*., 1992).

In all organisms γ-tubulin is found as a component of a large protein complex (Knop *et al*., 1997; Oegema *et al*., 1999; Stearns and Kirschner, 1994; Zheng *et al*., 1995). The simplest known γ-tubulin complex is that of *S. cerevisiae*, which is composed of two molecules of γ-tubulin and one molecule each of Spc97 and Spc98, (Geissler *et al*., 1996; Knop and Schiebel, 1997). Orthologues of γ-tubulin, Spc97 and Spc98, named respectively GCP2 and GCP3, have been identified in flies, frogs, mammals and plants (Erhardt *et al*., 2002; Martin *et al*., 1998; Murphy *et al*., 1998; Oegema *et al*., 1999; Tassin *et al*., 1998) indicating that γ-tubulin complexes and the mechanism for MT nucleation is conserved among all eukaryotes. Nevertheless, the animal γTuRC complexes vary in size and complexity: all contain the orthologues of Spc97 and Spc98, as well as at least three additional proteins named GCP4, GCP5 and GCP6. All these GCPs are distantly related to each other forming an own protein family (Detraves *et al*., 1997; Fava *et al*., 1999; Murphy *et al*., 1998; Murphy *et al*., 2001; Oegema *et al*., 1999; Zheng *et al*., 1995).

In animal cells and fungi, most MTs nucleate from MTOCs, notably the centrosome and the SPB, 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, it is attractive to postulate that γ-tubulin complexes are activated only

after their recruitment to the MTOC, raising the question of how this recruitment is regulated (for review see Schiebel, 2000). The mechanism of attachment of the γ-tubulin complex to the MTOC has been uncovered in yeast, but is still mostly unclear in other organisms. Spc72 and Spc110, two spindle pole body components that interact with the γ-tubulin complex have been identified in *S. cerevisiae*, but so far no orthologues have been identified in other organisms. These γ-tubulin complex binding proteins (GTBPs) are located on the cytoplasmic side (Spc72) and on the nuclear side (Spc110) of the spindle pole body and act like receptors for the γ-tubulin complex (Knop and Schiebel, 1997; Knop and Schibel, 1998; Nguyen *et al*., 1998). In *Drosophila,* the *Asp* (*Abnormal spindle pole*) gene product displays properties suggesting that it could function as a GTBP. Interestingly, Asp is required to restore the ability of salt-stripped centrosomes to form MT asters (do Carmo and Glover, 1999). However, recent studies show that Asp does neither bind γ-tubulin nor is it involved in MT nucleation. Instead, it rather plays an important role for MT tethering at the spindle poles and the formation of the central spindle (Wakefield *et al*., 2001). In mammalian cells, members of the family of A-kinase anchoring proteins (AKAPs), notably kendrin/pericentrin/CG-NAP, may provide a structural scaffold for MT nucleating complexes and thus contribute to stimulate MT nucleation (Dictenberg *et al*., 1998; Doxsey *et al*., 1994; Takahashi *et al*., 2002), but the precise role of these proteins in centrosome organization and/or microtubule nucleation remains to be determined.

Centrosome mediated MT anchoring

A complete analysis of MT nucleation and dynamics at the centrosome needs to consider additional centrosome-associated activities, notably MT capping and MT anchoring. Of particular interest, recent evidence suggests that the centrosome harbors two distinct minus-end associated multiprotein complexes, one involved in MT nucleation, the other in MT anchoring (Bornens,

2002; Mogensen *et al*., 2000; Mogensen, 1999) . Whereas MT nucleation clearly depends on the γTuRC, the mechanisms underlying MT anchoring are only beginning to emerge. Interest in MT anchoring has been sparked by the identification of a 249 kD protein, termed ninein, whose properties appear to be most consistent with an anchoring function. Depletion or overexpression of ninein affects the ability of centrosome to organize microtubules without perturbing MT nucleation (Abal *et al*., 2002; Dammermann and Merdes, 2002). Moreover, immunocytological analyses of highly polarized cochlear epithelial cells have revealed that ninein localizes to the apical membrane, where the vast majority of MT minus ends are anchored to non-centrosomal sites. Intringuingly, these two centrosomal activities seem to be spatially separated within the centrosome structure. Indeed, while both centrioles can nucleate MTs, only the mother centriole via its subistal appendages, is able to anchor them (Gorgidze and Vorobjev, 1995; Piel *et al*. 2000). A number of centrosomal proteins, including cenexin/ODF2 (Nakagawa *et al*., 2001), centriolin/CEP110 (Gromley *et al*., 2003; Ou *et al*., 2002), ε-tubulin (Chang *et al*., 2003), and ninein (Mogensen *et al*., 2000), have been reported to associate almost exclusively with the mother centriole. Of these, however, only ninein has so far been shown to be required for microtubule anchoring at the centrosome.

Although a direct role in MT anchoring has been proposed for the subpopulation of dynactin localized to the mother centriole (Quintyne and Schroer, 1999; Quintyne and Schroer, 2002), this multisubunit complex is also indirectly involved in the maintenance of the centrosomal MT organization when it acts in association with the motor protein dynein (for a detailed desciption of the dynein/dynactin complex structure and function see Part II-Introduction). Indeed, the recruitment to the centrosome of anchoring factors by minus end-directed transport reinforces the radial microtubule organization established by localized nucleation and is essential to maintain robust centrosomal arrays (Dammermann and Merdes, 2002).

Interphasic centrosome versus mitotic centrosome

Besides the accumulation of γ-tubulin and the consequent increase in nucleating activity, a striking modification of the centrosomal anchoring activity also occurs at the onset of mitosis. The subdistal appendages, indeed, are no longer visible and are replaced by a halo, once centrosomes have migrated to opposite locations. This halo regresses in telophase, accompanied by a decrease in MT nucleation activity, and the anchoring subdistal appendages reappear at the parental centrioles in late telophase/early G1 phase (Chretien *et al*., 1997; Piel *et al*., 2001). While centrosomes become more efficient MTs nucleators, nuclear mitotic apparatus protein (NuMA), together with MT motor proteins, is ensuring binding and stabilization of MT minus ends, organizing the spindle poles (Compton, 2000). Thus, the striking switch in the major centrosomal activities at the onset of mitosis appears to be characterized by a drastic rearrengement of centrosome organization and is likely to require an exchange between factors involved in its interphasic and mitotic activities, respectively. A better understanding of the centrosome maturation process certainly requires the characterization of the PCM composition during interphase and mitosis as well as the identification of the regulatory activities which coordinate the exchange between interphasic and mitotic centrosomal components.

In this study, we report the characterization of a novel candidate GTBP. This 156 kD human protein was named Nlp (ninein-like protein) because it shows a 37% homology in its N-terminal half with the N-terminal domain of ninein. We show that Nlp is able to recruit γ-tubulin as well as hGCP4 *in vitro* and *in vivo*, suggesting that this protein is able to bind the whole γTuRC. Moreover, we show by overexpression studies and antibody microinjection that Nlp is involved in MT nucleation at the centrosome. We have also investigated the regulation of Nlp during the cell cycle. Our data indicate that Nlp is a novel 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 novel ninein-related centrosomal protein

To isolate potential interactors of Polo-like kinases, a yeast two-hybrid screen was performed by Dr. Peter Duncan using the catalytically inactive *Xenopus laevis* Polo-like kinase 1 ($Plx1^{N172A}$) as bait. Amongst the potential interactors one partial cDNA shared sequence similarity with the centrosomal protein ninein and 52% sequence identity, at the protein, level with the product encoded by a previously uncharacterized human full length cDNA (KIAA0980). 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 novel human protein was chosen for detailed analysis. Complete sequencing of KIAA0980 predicted a 156 kD protein whose N-terminal half showed 37% sequence identity to the corresponding domain of ninein. Therefore, this novel gene product was termed Nlp. Sequence analysis revealed that Nlp and ninein did not show significant sequence similarities in their C-terminal domains other than predicted coiled-coil regions, but their N-termini contained putative EF-hand Ca^{2+} binding domains (Figure. 3A). However, of the two motifs present in Nlp, the one constituting a closer match to the EF-hand consensus is not conserved in ninein. Instead, ninein contains a potential GTP binding site that is not present in Nlp (Bouckson-Castaing *et al*., 1996; Hong *et al*., 2000).

Upon overexpression in U2OS human osteosarcoma cells, myc epitope-tagged Nlp and ninein proteins both produced striking intracellular assemblies (Figure 3B). As shown by counter-staining with the GT335 antibody, a marker for centrioles (Bobinnec *et al*., 1998), these assemblies formed primarily around the centrosome. This localization did not appear to be dependent on the tag, since EGFP-tagged recombinant Nlp presented an identical subcellular distribution. However, with increasing protein expression levels, additional assemblies also formed at sites distant from the centrosome (Figure 3C, a/a'), as it has previously been seen upon overexpression of several centrosomal proteins containing

coiled-coil domains (e.g. C-Nap1, see Figure 3C, b/b' and Mayor *et al*., 2002; for other examples see Gergely *et al*., 2000; Ohta *et al*., 2002). Thus, these observations strongly suggested that Nlp is a centrosomal protein.

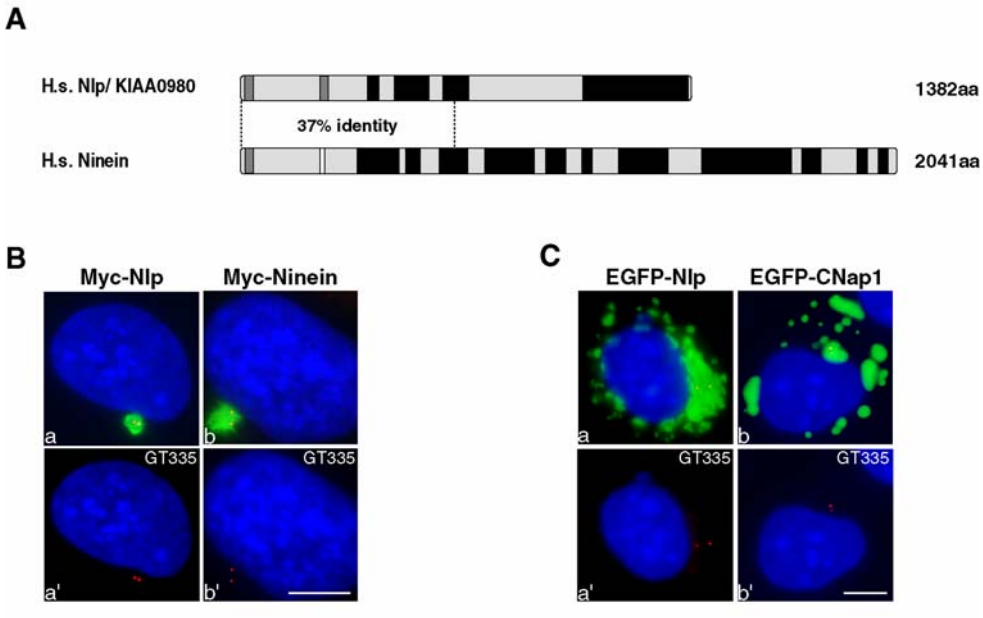

Figure 3. Nlp, a novel centrosomal protein

(**A**) Schematic sequence comparison between hNlp and hninein. Light bars indicate potential EF-hand Ca^{2+}-domains, dark bars indicate predicted coil-coiled domain and the white bar in ninein represents a potential GTP binding domain. The schematic sequence of human ninein depicted here refers to the most abundant isoform (ninein-Lm; Hong et al., 2000).

Myc-Nlp or myc-ninein (**B**) and EGFP-Nlp or EGFP-C-Nap1 (**C**) were expressed in U2OS cells and analyzed by immunofluorescence microcopy. Overexpressed proteins were detected with myc-antibodies (9E10) (green) or through EGFP fluorescence (green). Centrosomes were visualized with GT335 antibodies (red) and DNA stained with DAPI (blue). Scale bar represents 10μm. At high overexpression level both EGFP-Nlp and EGFP-C-Nap1 assemblies can form at sites distant from the centrosome (**C**, panel a and b).

To study endogenous Nlp, polyclonal antibodies were raised against both the N-terminal and C-terminal domains. Using these antibodies for immunoprecipitation-immunoblotting experiments, a 156 kD protein comigrating exactly with *in vitro* translated recombinant Nlp could be detected in human osteosarcoma (U2OS) cells (Figure 4A, compare lanes 1 and 3). A protein of the same size could also be seen in immunoblots performed on purified centrosomes, but no reliable signal could be obtained upon immunblotting of total

cell lysates (data not shown), suggesting that Nlp is probably a low abundance protein.

When used for immunofluorescence microscopy, affinity-purified anti-Nlp antibodies strongly labelled the centrosomes in U2OS and HeLa cells (Figure 4B, a and data not shown). This staining was independent of the fixation method (data not shown) and readily competed by antigen (Figure 4B, b). In contrast, no centrosomal staining was produced by pre-immune serum (data not shown). Although the staining patterns of Nlp and γ-tubulin overlapped substantially (Figure 4B, a,a'), a detailed comparison revealed that Nlp was distributed in distinct dots, preferentially surrounding one of the two centrioles (Figure. 4C). A similar localization pattern has previously been described for ninein, and in this case, 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 at the electron microscopic level. Thus, it is not clear whether Nlp and ninein localize to similar or distinct centrosomal substructures.

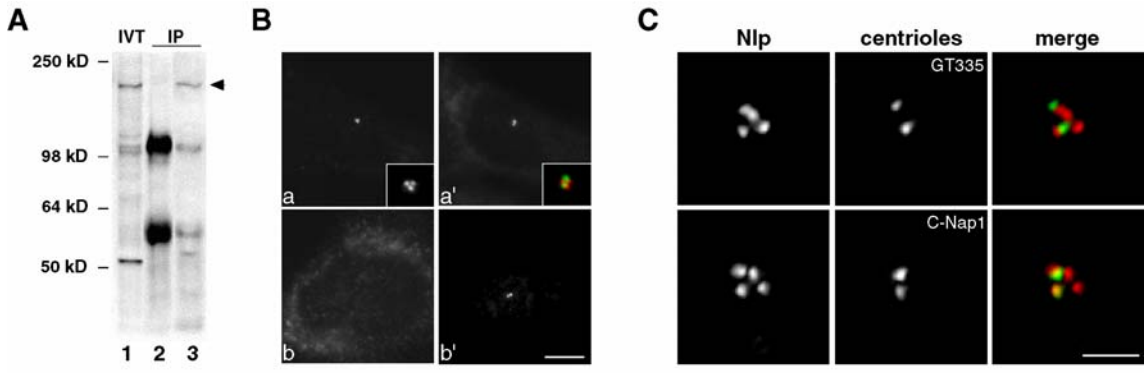

Figure 4. Characterization of Nlp antibodies and centrosome association of endogenpus Nlp

(**A**) Western blot with anti-Nlp antibodies against: in vitro translated Nlp (lane 1), immunoprecipitation with anti-Nlp preimmune (lane 2) and immunoprecipitation with affinity-purified anti-Nlp antibodies (lane 3) (**B**) Immunofluorescence microscopy on U2OS cells. Cells were stained with affinity-purified Nlp antibodies (a) and affinity-purified antibodies competed with antigen (b). Centrosomes were visualized with antibodies against γ-tubulin. The staining with the affinity-purified antibodies is shown in an enlarged view in panel a. Scale bar represents 10μm. (**C**) Analysis, by Deltavision microscopy, of centrosomes stained with anti-Nlp antibodies and the centriole marker GT335 (upper panels) or antibodies against Nlp and C-Nap1 (Transduction laboratories) (lower panels). As can be seen from the merged images, anti-Nlp antibodies (red) usually stained three dots associated with one of the two centrioles (green), and a fourth dot with the other. Scale bar represents 2 μm.

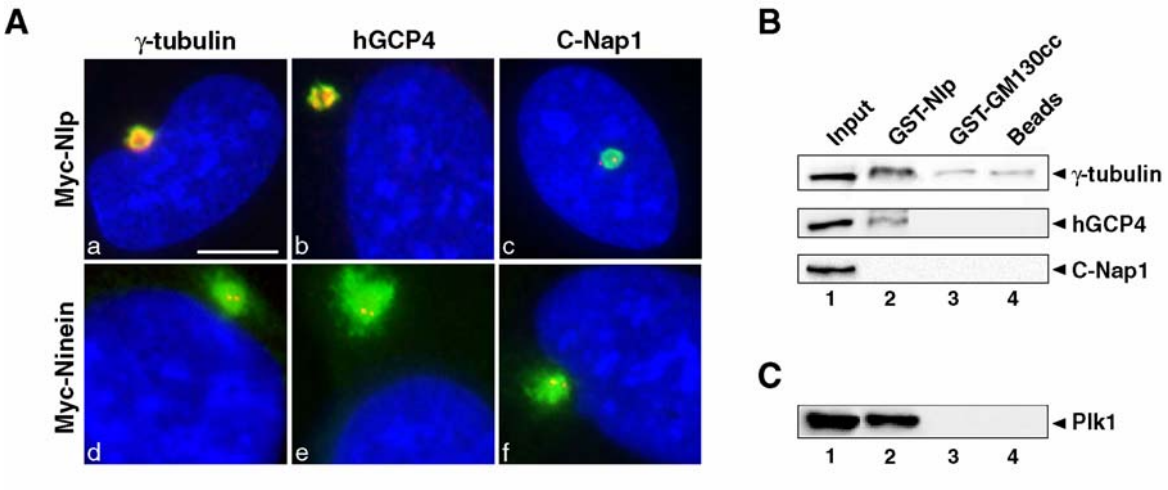

Figure 5. Nlp recruits the γTuRC

(**A**) Myc-Nlp and myc-ninein were expressed in U2OS cells and cells analyzed by immunofluorescence microscopy. Cells were stained with myc-antibodies to detect the overexpressed protein (green) and counterstained with antibodies against γ-tubulin (a,d), hGCP4 (b,e) and C-Nap1 (c,f) (red). Scale bar represents 10μm. (**B**) Total cell extracts (lane 1) were prepared as described in material and methods and incubated in the presence of GST-Nlp (lane 2), GST-GM130cc (lane 3) or glutathione-sepharose beads alone (lane 4). The pull-downs were then analyzed by immunoblotting with antibodies against γ-tubulin, hGCP4 and C-Nap1. (**C**) In order to verify the interaction with Plk1, total cell extract were prepared from U2OS synchronized in G2/M phase and incubated in the presence of GST-Nlp (lane 2), GST-GM130cc (lane 3) or glutathione-sepharose beads alone (lane 4). The pull-downs were then analyzed by immunoblotting with antibodies against Plk1. The amount of total protein extract loaded with the input (lane1) in (**B**) and (**C**) corresponds to 1/20 of the amount loaded for pull-downs (lane 2, 3 and 4).

Nlp recruits the γTuRC

To investigate the functional consequences of Nlp overexpression, Nlp assemblies were stained with antibodies against known centrosomal proteins. Interestingly, we observed a striking recruitment of γ-tubulin to Nlp assemblies (Figure 5A, a). A similar recruitment was also seen for a second subunit of the γTuRC, hGCP4 (Fava *et al*., 1999; Figure 5A, b), but not for the unrelated centrosomal protein C-Nap1 (Fry *et al*., 1998a; Figure 5A, c). This suggested that Nlp assemblies were able to recruit the entire γTuRC. In contrast, the staining

patterns of γ-tubulin, hGCP4 or C-Nap1 were not significantly altered by the formation of myc-ninein assemblies (Figure 5A, d-f), indicating that ninein was not causing a major redistribution of either γTuRC components or C-Nap1. To further demonstrate the ability of Nlp to bind to γTuRC components, pull-down experiments were performed, using recombinant GST-Nlp bound to glutathione-beads (Figure 5B). A GST-fusion to the coiled-coil domain of the Golgi protein GM130 (Barr *et al*., 1998) and glutathione beads alone were used for control (Figure 5B). Following incubation with total cell extract, GST-Nlp specifically brought down both γ-tubulin and hGCP4, but not the centrosomal protein C-Nap1. Interestingly, similar experiments performed with extract form U2OS cells synchronized in G2 phase, revealed that Plk1 also specifically interacted with GST-Nlp, confirming the yeast two hybrid data (Figure 5C). Thus, Nlp was able to specifically interact *in vitro* and *in vivo* with two distinct components of the γTuRC, implying that it most likely bound the entire complex.

Nlp affects MT regrowth in cells

The ability of Nlp to recruit components of the γTuRC suggested that this protein might influence MT nucleation. To examine this possibility, MT regrowth assays were performed in U2OS cells expressing assemblies of myc-Nlp. Cells transfected with myc-ninein were analysed in parallel. MTs were completely depolymerised through cold treatment for 30 min and then allowed to regrow for a short period of 45 sec. A pronounced formation of MT asters was observed in cells overexpressing myc-Nlp (Figure 6 a/a'). In contrast, MT nucleation was reduced in cells overexpressing myc-ninein, possibly due to steric hindrance (Figure 6 b/b'). A similar inhibitory effect on MT regrowth has previously been observed upon overexpression of the centrosomal protein C-Nap1 (Mayor *et al*., 2002).

Compared to MT asters formed from centrosomes of non-transfected cells, the asters formed by Nlp assemblies were poorly focused, suggesting that MT

nucleation occurred throughout these assemblies (Figure 6 a/a'). The most straightforward interpretation of these results is that Nlp assemblies acted as platforms for the recruitment of γTuRCs, thereby stimulating MT nucleation.

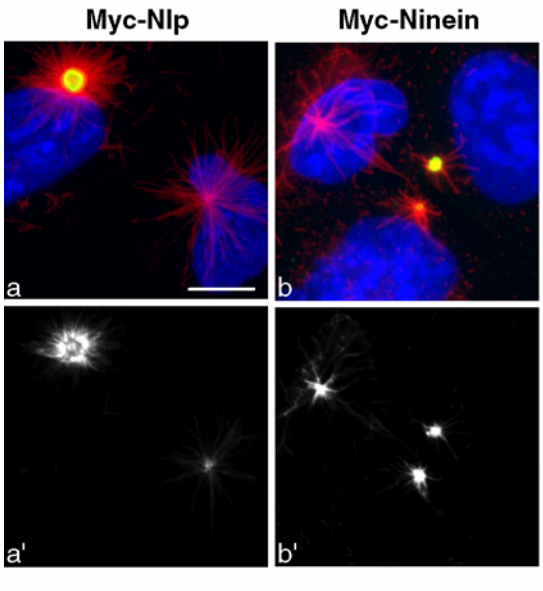

Figure 6. Nlp affetcs MT nucleation

Myc-Nlp (a,a') and myc-ninein (b,b') were expressed in U2OS cells and the cells subjected to a MT regrowth assay as described in material and methods. Overexpressed protein was detected with myc-antibodies (green) and MTs visualized with anti-α-tubulin antibodies (red). Scale bar represents 10μm.

Nlp binds γTuRCs through its N-terminal half

To characterize the binding of the γTuRCs to Nlp in more detail, N- and C-terminal halves of Nlp were expressed separately in cells, and their ability to recruit γ-tubulin and affect MT nucleation was examined by immunofluorescence microscopy. The N-terminal part of Nlp formed many small aggregates throughout the cytoplasm. These were able to recruit γ-tubulin, so that the typical γ-tubulin staining indicative of a centrosome could no longer be observed (Figure 7A, d). In contrast, the C-terminal half of Nlp formed large assemblies in the cytoplasm, but these produced no effect on the centrosomal γ-tubulin staining (Figure 7A, c). To assess the consequences of

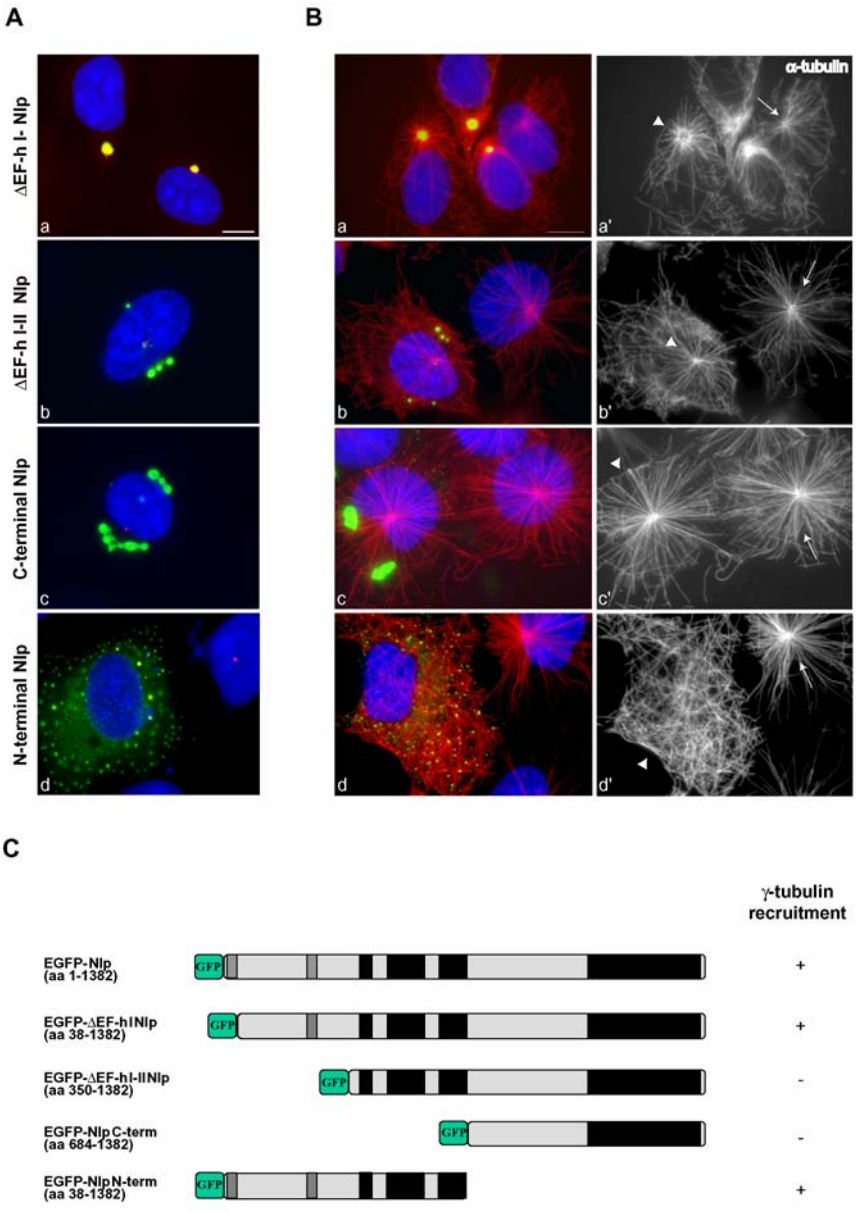

Figure 7. Nlp binds the γ-TuRC through its N-terminal domain

(**A**) U2OS cells were transfected with EGFP-Nlp ΔEF-h I (Δ1-38aa), EGFP-Nlp ΔEF-h I-II (Δ1-350aa), EGFP-Nlp C-terminal half, EGFP-Nlp Nterminal half and analyzed by immunofluorescence microscopy. Transfected cells were detected with EGFP-fluorescence (green), the γ-TuRC was detected with antibodies against γ-tubulin (red) and DNA stained with DAPI (blue). (**B**) Transfected cells were subjected to a MT regrowth assay and stained with antibodies against α-tubulin to visualize the MT (red). Arrows point to MT asters formed by untransfected cells; arrow-heads mark transfected cells. Scale bar represents 10µm. (**C**) Schematic representation of the different EGFP-Nlp deletion mutants.

the γ-tubulin displacement induced by the N-terminal part of Nlp, MT regrowth assays were performed on cells expressing either half of Nlp. Whereas cells overexpressing the C-terminal part of Nlp formed typical MT arrays emanating from the centrosome, as expected, those expressing the N-terminal half no longer formed any distinct MT asters, although they still showed extensive MT nucleation throughout the cytoplasm (Figure 7B, c/c' and d/d'). It appears likely

that much of this cytoplasmic nucleation originated from γTuRCs associated with Nlp aggregates, but due to the complexity of the MT network and the large number of Nlp aggregates, this was difficult to establish. In contrast, the overexpression of the N-terminal region of ninein sharing sequence similarity with the N-terminal domain of Nlp, did not perturb either γ-tubulin localization or MT nucleation (data not shown), confirming that Nlp has a higher binding affinity for γTuRCs than ninein.

To better define the γTuRC interaction domain within Nlp, we used the same assay to examine the behaviour of two additional N-terminal deletion mutants of Nlp that lacked the first 38 (Nlp ΔEF-h I) or 350 (ΔEF-h I-II) residues, respectively. While the Nlp ΔEF-h I mutant behaved identically to Nlp wild-type, the properties of the ΔEF-h I-II mutant were indistinguishable from those of the C-terminal half, resulting unable to recruit γ-tubulin or stimulate MT nucleation (Figure 7A, a and b and 7B, a/a' and b/b'). These results, schematically summerized in figure 7C, indicated that the recruitment of γTuRCs required the Nlp region encoded by the residues 38-350, containing the second putative EF-hand domain (aa 237-265). In order to test whether the EF-h domains were essential for the γTuRC binding activity of Nlp, we generated a EGFP-tagged Nlp mutant bearing a single point mutation in the second EF-hand domain, the one of the two which present a closer match to the EF-hand motif consensus sequence (Nlp EF-h II mutant; Figure 8C). Moreover, in order to exclude a possible cooperative interaction between the two EF-hand domains, a second mutant was also generated which carried point mutations in both the EF-hand domains (Nlp EF-h double mutant; Figure 8C) . The mutagenesis was performed substituting the conserved glutamate residue in the -Z position of the EF-hand loops with a lysine residue (Pottgiesser *et al*., 1994; Wilkie *et al*., 2001). When overexpressed in U2OS cells, both Nlp EF-hand mutants were able to localize to the centrosome, recruit γ-tubulin and affect MT nucleation as efficiently as the wild-type protein (Figure 8A and 8B), suggesting that the EF-h domains are not essential for the interaction with the γTuRCs.

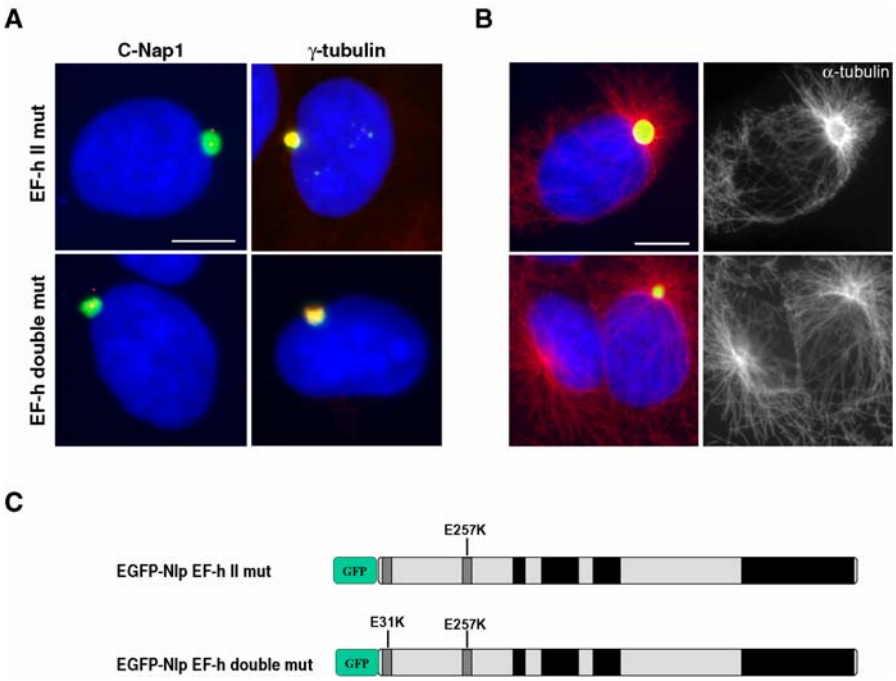

Figure 8. Mutagenesis of the EF-hand domains does not interfere with the ability of Nlp to recruit γTuRCs and affect MT nucleation.

(**A**) EGFP-tagged Nlp EF-hand mutants (green) were expressed in U2OS and their localization and ability to interact with γTuRCs were analyzed by immunofluorescence microscopy using antibodies against the centriolar marker C-Nap1 (left panels in red) and the γTuRC component γ-tubulin (right panels in red), respectively. DNA was stained with DAPI (blue). Scale bar represents 10μm. (**B**) Transfected cells were subjected to a MT regrowth assay and stained with antibodies against α-tubulin to visualize the MT (red). Scale bar represents 10μm. (**C**) Schematic representation of the EGFP-Nlp EF-hand mutants.

Nlp assemblies trigger aster formation in Xenopus egg extracts

To provide further evidence for a role of Nlp in MT nucleation, assemblies of recombinant EGFP-Nlp were purified from U2OS cells (Blomberg-Wirschell and Doxsey, 1998) and tested for their ability to trigger the formation of MT asters in M-phase extracts prepared from *Xenopus laevis* eggs. Because EGFP-ninein assemblies proved unstable during purification, they could not be tested in this assay. Instead, EGFP-C-Nap1 assemblies, purified under identical conditions, were used as controls. Upon incubation in *Xenopus* egg extracts, 82% of the purified Nlp assemblies, but only 25% of the C-Nap1 assemblies, were able to

trigger aster formation (Figure 9A and B). In parallel experiments, EGFP-Nlp and EGFP-C-Nap1 assemblies were examined carefully for the presence or absence of the centrosomal marker C-Nap1 or γ-tubulin, respectively. This analysis revealed that 25% of both the Nlp and the C-Nap1 assemblies were stained by the centrosomal markers C-Nap1 and γ-tubulin respectively, indicating that they had formed around U2OS centrosomes; the remainder lacked the centrosome, indicating that they had formed at sites distant from the centrosome in U2OS cells or been produced by fragmentation of assemblies during purification (Figure 9C, and data not shown; see also Figure 1C). Thus, whereas aster formation by C-Nap1 assemblies depended on the presence of centrosomes, the majority of the Nlp assemblies were able to trigger MT aster formation in the absence of centrosomes. These latter assemblies all stained positive for γ-tubulin (data not shown), indicating that MT nucleation was promoted through γTuRCs. To exclude the possibility that the induction of aster formation could be due to mere elongation of copurifying microtubules, the assemblies were pre-treated with nocodazole before incubation in *Xenopus* egg extract. The ability of Nlp assemblies to induce aster formation was not detectably reduced under these conditions (data not shown). Taken together, the above data demonstrate that Nlp assemblies could stimulate MT nucleation in both mammalian cells and *Xenopus* egg extracts.

Microinjection of anti-Nlp antibody inhibits centrosomal MT nucleation

In order to study the function of endogenous Nlp, we attempted to deplete the protein from human cells through the use of small interfering RNAs (siRNA), but three different siRNA duplex oligonucleotides failed to significantly reduce centrosomal Nlp levels, thus precluding this type of analysis. We then asked whether the inhibition of endogenous Nlp by antibody microinjection would interfere with MT nucleation. To this end, affinity-purified anti-Nlp antibodies were

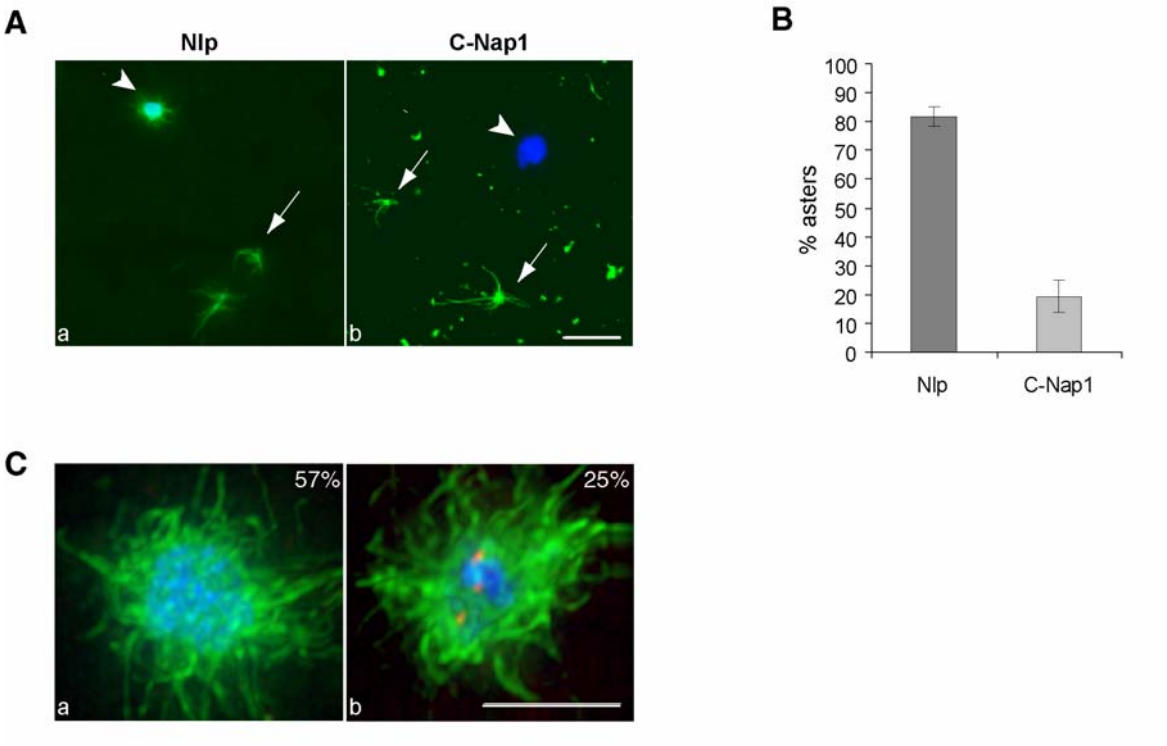

Figure 9. Nlp triggers MT nucleation in Xenopus egg extracts independently of centrosome.
(**A**) EGFP-Nlp and EGFP-C-Nap1 assemblies were purified along with centrosomes from transfected U2OS cells as described in Material and Methods and methods and incubated in *Xenopus* egg extracts. The MT asters were spun down on coverslips and analyzed by immunofluorescence microscopy. The assemblies (arrow-heads) were detected from the EGFP-fluorescence (blue) and the MTs stained with anti-α-tubulin antibodies (green). Arrows point to MT asters triggered by copurifying centrosomes. Scale bar represents 10μm. (**B**) EGFP-Nlp and EGFP-C-Nap1 assemblies were counted as either being able to trigger MT asters or not. Histogram shows results from 3 independent experiments (total of 300 aggregates). Error bars indicate standard deviations. (**C**) EGFP-Nlp assemblies triggering MT aster formation were analyzed for the presence of centrosomes by immunofluorescence microscopy. Aggregates were detected from the EGFP-fluorescence, MT stained with anti-α-tubulin antibodies (green) and centrosomes visualized with anti-C-Nap1 antibodies (red). Specifically, individual assemblies were analysed with a Deltavision microscope, taking optical sections in 0.2 μm steps. Data sets were then subjected to a deconvolution algorithm and projected onto a single plane. Shown is one Nlp assembly with centrosomes and one without. Scale bar represents 5μm.

microinjected into asynchronously growing U2OS cells. Six hours later, MT regrowth assays were performed. As shown by immunofluorescence microscopy, nearly 50% of the cells injected with anti-Nlp antibodies were devoid of detectable MT asters, whilst only 14% of cells injected with control rabbit immunoglobulins lacked obvious asters (Figure 10A and B). The inhibition produced by anti-Nlp antibodies was only partial, but this is likely to reflect limitations inherent in the microinjection assay. In fact, a quantitatively very similar, partial inhibition of MT nucleation was also observed upon microinjection of antibodies against γ-tubulin and the γTuRC components GCP3/HsSpc98 (data not shown and Tassin *et al*., 1998). Moreover, anti-Nlp microinjection did not affect either progression into mitosis or mitotic MT nucleation (Figure 10C), consistently with the observed absence of Nlp from the spindle poles (see below). Thus, the anti-Nlp antibody injection data strengthen the view that Nlp is involved in MT nucleation *in vivo*.

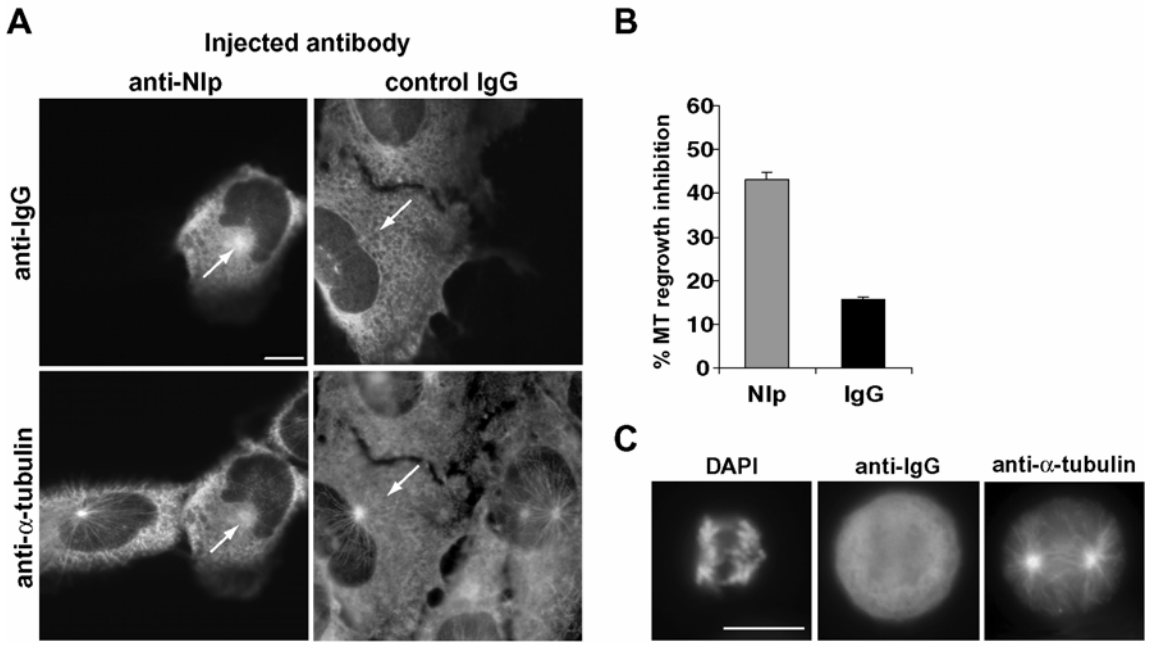

Figure 10. Anti-Nlp antibody microinjection inhibits MT nucleation.

(**A**) U2OS were microinjected with affinity-purified Nlp-antibodies or rabbit anti-IgG antibodies, subjected 6 hours later to a MT regrowth assay and analyzed by immunofluorescence microscopy. Cells were stained with anti-rabbit antibodies to detect the microinjected antibodies and anti-α-tubulin antibodies to stain the MTs. Arrows indicate the injected cells (**B**) Injected cells were counted as either having a normal growing MT array or an inhibited MT regrowth. Histogram shows results from 3 independent experiments (total of 400 cells). Error bars indicate standard deviations. (**C**) Nlp antibody microinjection did not inhibit MT nucleation in mitotic cells. Scale bar represents 10μm.

Plk1 phosphorylates Nlp and regulates its centrosomal localization

The observed interaction between Polo like kinase 1 and Nlp suggested that Nlp could be a substrate of this kinase. Indeed, wild-type recombinant Plk1, but not the catalytically inactive K82R Plk1 mutant, was able to readily phosphorylate *in vitro* translated myc-tagged Nlp (Figure11A, lane 2 and 3). In comparison, *in vitro* translated myc-tagged ninein was barely phosphorylated by Plk1 (Figure 11A, lane 5 and 6), although immunoprecipitated in similar amounts (not shown). To determine whether Nlp could be a substrate of Plk1 also *in vivo,* EGFP-tagged Nlp was transfected into 293 cells, alone or together with either myc-tagged $Plk1^{T210D}$ or myc-tagged $Plk1^{K82R}$. $Plk1^{T210D}$ represents an activated Plk1 mutant, which mimics the phosphorylated and activated form of Plk1 present in mitosis, bearing a 5 times higher activity than wild-type Plk1 (Kelm *et al*., 2002; Qian *et al*., 1999; Smits et al., 2000). Twentyfour hours after transfection cells were directly lysed in sample buffer and examined by immunoblotting with anti-GFP antibodies. EGFP-Nlp showed a reduced gel electrophoretic mobility, suggestive of phosphorylation, when cotransfected with $Plk1^{T210D}$, but not with $Plk1^{K82R}$ or when expressed alone (Figure 11B). In contrast, in the same experiment EGFP-ninein showed no reduction in mobility when coexpressed together with $Plk1^{T210D}$ (data not shown).

To study the functional consequences of Nlp phosphorylation, U2OS cells were cotransfected with EGFP-Nlp together with either $Plk1^{T210D}$ or $Plk1^{K82R}$ and analysed by immunofluorescence microscopy. Whereas EGFP-Nlp localized at the centrosome forming the typical assemblies when coexpress together with catalytically inactive $Plk1^{K82R}$ (Figure 11C, upper panel), cotransfection with $Plk1^{T210D}$ strikingly induced displacement of Nlp assemblies from the centrosome and dispersion throughout the cytoplasm (Figure 11C, lower panel). Quantitative analysis revealed that Nlp was displaced from centrosomes in 65% of cells coexpressing $Plk1^{T210D}$, but only in 5% of cells coexpressing $Plk1^{K82R}$ (Figure 11D). Wild-type Plk1 had only a very moderate effect on the localization of Nlp (Figure 11D), suggesting that only a fully activated form of this kinase is able to disrupt the centrosomal localization of Nlp. In contrast, coexpression of active

Plk1^{T210D} did not have any effect on the localization of recombinant EGFP tagged ninein (data not shown). Taken together, these results suggest that Nlp is a novel substrate for Plk1 and that Plk1 activity can modulate the Nlp association with the centrosome.

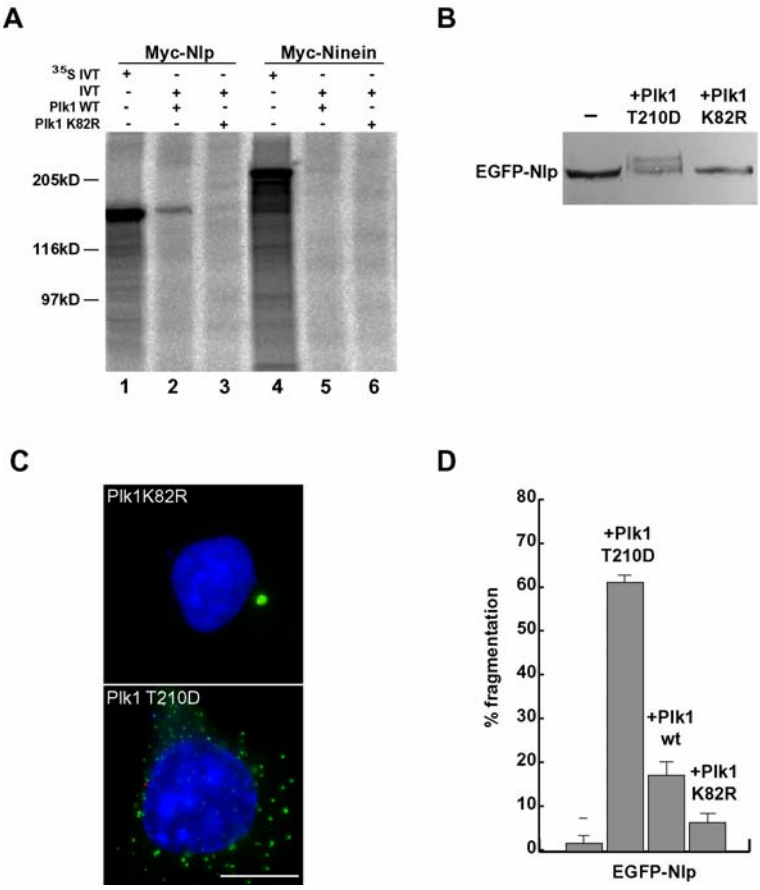

Figure 11. Plk1 can phosphorylate Nlp and regulate its centrosomal localization

(**A**) myc-Nlp and myc-ninein were in vitro translated in the presence (lanes 1 and 4) or absence of ^{35}S-methionine (lanes 2,3 and 5,6), immunoprecipitated with anti-myc antibodies, subjected to in vitro phosphorylation assay in the presence of wild type Plk1 (lane 2 and 5) or catalytically inactive Plk1^{K82R} (lane 3 and 6) and analyzed by phosphorimager. (**B**) EGFP-Nlp was express in 293 cells alone or in combination with either Plk1^{T210D} or Plk1^{K82R}. Cell lysates were analyzed by immunoblotting with anti-GFP antibodies. (**C**) U2OS cells were transfected with EGFP-Nlp (green) together with Plk1^{K82R} (upper panel) or Plk1^{T210D} (lower panel). Centrosomes were visualized with GT335 antibodies (red). Scale bar represents 10μm (**D**) U2OS cells transfected with EGFP-Nlp alone or co-transfected with either Plk1wt, Plk1^{T210D} or Plk1^{K82R} were analyzed for the appearance of EGFP-Nlp assemblies.The histogram shows the percentage of transfected cells exhibiting a fragmented phenotype (defined as multiple Nlp fragments distinct from the centrosomes) determined for three independent experiments counting 400-600 cells). Error bars indicate standard

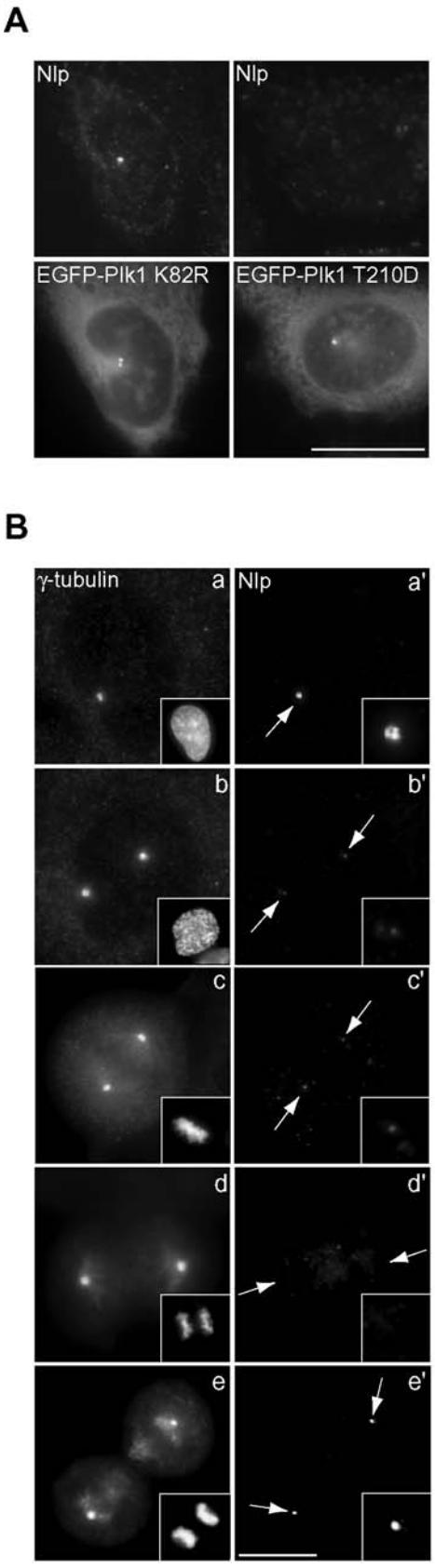

Figure 12. Nlp is displaced from the centrosome during centrosome maturation

(**A**) U2OS overexpressing EGFP-Plk1^{K82R} (left panels) or Plk1^{T210D} (right panels) were analyzed by immunoflluo-rescence microscopy staining with antibodies against Nlp. Scale bar represent 10μm. (**B**) Cell cycle analysis of Nlp centrosomal localization was performed by immunofluo-rescence microscopy. Cells in (from top to bottom) interphase, prophase, metaphase, anaphase and telophase were stained with anti-γ-tubulin (left panels) and anti-Nlp antibodies (right panels; arrows indicate the position of the centrosomes as revealed by γ-tubulin staining). Insets show DNA stained with DAPI (left panels) and an enlargement of Nlp staining (right panels). Scale bar represent 10μm

Nlp is removed from the centrosome during centrosome maturation

We next investigated whether Plk1 could also regulate the centrosomal localization of the endogenous Nlp during the cell cycle. Interestingly, the overexpression of EGFP-tagged Plk1^{T210D} in U2OS cells completely abolished the Nlp staining at the centrosome, whereas Plk1^{K82R} had no effect on Nlp centrosomal localization and wild-type Plk1 induced only a partial reduction (Figure 12A and data not shown). These results strengthened the hypothesis that Nlp is a novel substrate of Plk1, and that phosphorylation by Plk1 regulates its centrosomal localization. They also suggested that a mitotic activation of Plk1 is required to efficiently target Nlp.

The analysis of endogenous Nlp localization revealed that Nlp associated with the centrosome in a cell-cycle dependent manner, consistently with the expectation that Plk1 could regulate Nlp *in vivo* (Figure 12B). Indeed, Nlp could be readily localized at the centrosome throughout interphase, but the centrosomal staining produced by Nlp antibodies appeared strongly reduced at the onset of mitosis, concomitant with the increase of centrosomal γ-tubulin levels, an event indicative for centrosome maturation (Figure 12B, b/b'). This corresponds to the time of maximal Plk1 activation (Golsteyn *et al*., 1995). Nlp staining at the centrosome resulted undetectable throughout metaphase and anaphase, before reappearing during telophase (Figure 12B, c/c', d/d' and e/e'). These data confirmed that the localization of Nlp at the centrosome is regulated in a cell cycle dependent manner and fell in line with a potential regulation of Nlp through Plk1 during centrosome maturation.

We next asked whether the loss of Nlp centrosomal staining during mitosis could be a consequence of controlled proteolysis. Due to the low abundance of Nlp, we could not directly analyse the levels of endogenous protein in synchronized cell extracts, but we were forced to perform immunoprecipitation-Western blotting analysis. U2OS cells were synchronized either in G1/S phase by aphidicolin treatment or in mitosis by nocodazole treatment (see Material and Methods), and the efficiency of synchronization was tested by immunoblotting analysis of cell lysates using antibodies against cyclin B1 (Figure 13A). We next

compared the levels of endogenous Nlp immunoprecipitated from interphasic and mitotic U2OS cell extracts, choosing as an internal control Hec1, an unrelated protein known to be present at comparable levels during G1/S and M phase (Chen *et al*., 1997). Equivalent amounts of G1/S and M phase cell lysates were incubated with protein A beads coupled either to anti-Nlp antibodies and anti-Hec1 antibodies or to non-specific rabbit IgG and anti-Hec1 antibodies. We first confirmed that similar amounts of Hec1 were immunoprecipitated from G1/S and M phase cell extracts by immunoblotting the immunoprecipitated sample with antibodies against Hec1 (Figure 13B, lane 3 and 6). Immunoblotting analysis of the same samples with antibodies against Nlp revealed that, similarly to Hec1, comparable amounts of the protein were brought down from G1/S and M phase cell extracts (Figure 13B lane 3 and 6). This result suggests that Nlp is present in the cell throughout mitosis and the loss of its centrosomal staining is not due to protein degradation.

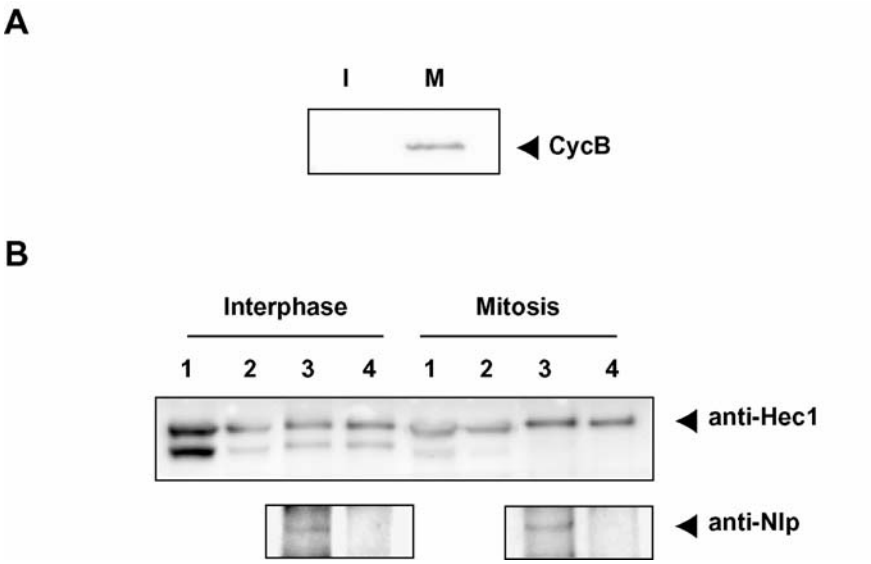

Figure 13. Nlp is present throughout mitosis

(**A**) U2OS cells were synchronized in G1/S or in M phase as described in Material and Methods. To check the efficiency synchronization, cell lysates were analyzed by immunoblotting using antibodies against cyclin B1 (**B**) Immunoprecipitation was performed incubating the cell lystates with either a combination of anti-Hec1 and anti Nlp antibodies, or a combination of anti-Hec1 antibodies and non-immune rabbit IgG as control. Immunoprecipitated samples were analyzed by immunoblotting with antibodies against Hec1 and Nlp. Lane 1:input; lane 2: supernatant of the samples immunoprecipitated with anti-Hec1/anti-Nlp; lane 3: IP anti-Hec1/anti-Nlp; lane 4: IP anti Hec1/rb IgG.

Expression of the Nlp phosphorylation mutant leads to aberrant mitotic spindle

To assess whether Nlp displacement from the centrosome at the onset of mitosis is required for proper spindle assembly, we proceded to map the phosphorylation sites recognized by Plk1 and analyse the functional consequences of overexpressing the corrisponding non-phosphorylatable Nlp mutants. Sequence analysis suggested the presence of 8 potential *in vitro* Plk1 phosphorylation sites (Kelm *et al*., 2002) within the N-terminal half of Nlp but not ninein (Figure 14A). Of these predicted sites, three (S87 or 88, T161 and S686) could be positively identified by mass spectrometry (data not shown). Thus, a mutant (GST-NlpΔ8) with all 8 candidate serine/threonine Plk1 phosphorylation sites altered to alanines was constructed and tested for its ability to serve as a substrate for Plk1. Compared to wild-type Nlp, the mutant protein was barely phosphorylated and did not show any significant change in gel electrophoretic mobility (Figure 14B), indicating that most Plk1 phosphorylation sites had indeed been made inaccessible.

To determine the relevance of these phosphorylation sites in vivo, three different mutants of EGFP-Nlp were generated. Two mutants were characterized by different sets of four serine/threonine to alanine mutations (EGFP-NlpΔ4A and Δ4B) and the third one carried a combination of all 8 mutations (EGFP-Nlp Δ8) (Figure 14C). When tested in MT regrowth assays, the Δ8 mutant was still able to recruit γ-tubulin and stimulate MT-nucleation, arguing against severe misfolding (data not shown) The three mutants were overexpressed in U2OS cells together with Plk1^{T210D} or Plk1^{K82R}. Whereas Plk1^{T210D} was able to efficiently disperse assemblies formed by Nlp wild-type, assemblies formed by the Δ8 mutant showed an almost complete resistance to fragmentation and the Δ4A and Δ4B mutants were partially resistant (Figure 14D and E). These results provide strong evidence for direct phosphorylation of the N-terminal half of Nlp by Plk1. They further show that this phosphorylation disrupts the association of Nlp with the

centrosome and that complete disassembly of Nlp requires phosphorylation at multiple sites.

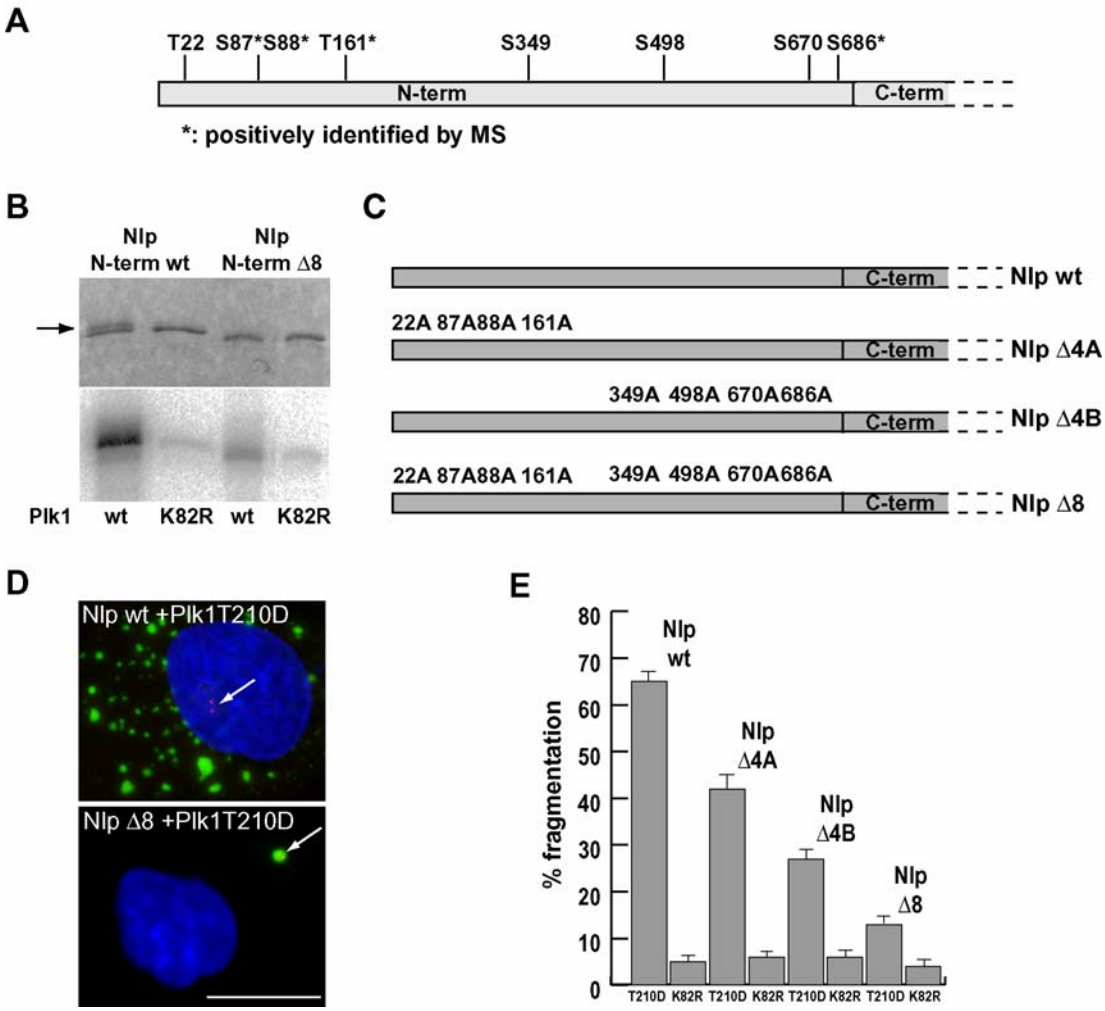

Figure 14. Nlp phosphorylation mutant

(**A**) Schematic representation of the potential Plk1 phosphorylation sites within the N-terminal domain of Nlp, comforming to a E/DxS/T consensus. The asteriks mark the the residues contained in the phosphopeptide identified by mass spectrometry. (**B**) GST-Nlp N-terminal domain wild type or Δ8 mutant were subjected to *in vitro* phosphorylation assay with either Plk1 wt or the kinase dead mutant $Plk1^{K82R}$ and analyzed by phosphorimager (lower panel); protein abundance was determined by Coomassie staining. The arrow indicate the slower migrating, phosphorylated form of wt Nlp (N-terminal domain) (**C**) Schematic representation of serine/threonine to alanine mutants of Nlp. (**D**) and (**E**) U2OS cells were transfected with the indicated EGFP-Nlp mutants together with either $Plk1^{T210D}$ or $Plk1^{K82R}$ and analyzed 24 hr later by immunofluorescence microscopy. Centrosomes were stained with antibodies against GT335 (red) and transfected cells identified by EGFP-fluorescence. Cells were analyzed for either having a single EGFP-Nlp assembly at the centrosome or displaying multiple Nlp fragments distinct from the centrosome. Histogram shows the percentage of cell displaying a fragmented Nlp distribution. determined from three independent experiments counting 400-600 cells. Bars indicate standard deviations. Scale bars represent 10μm.

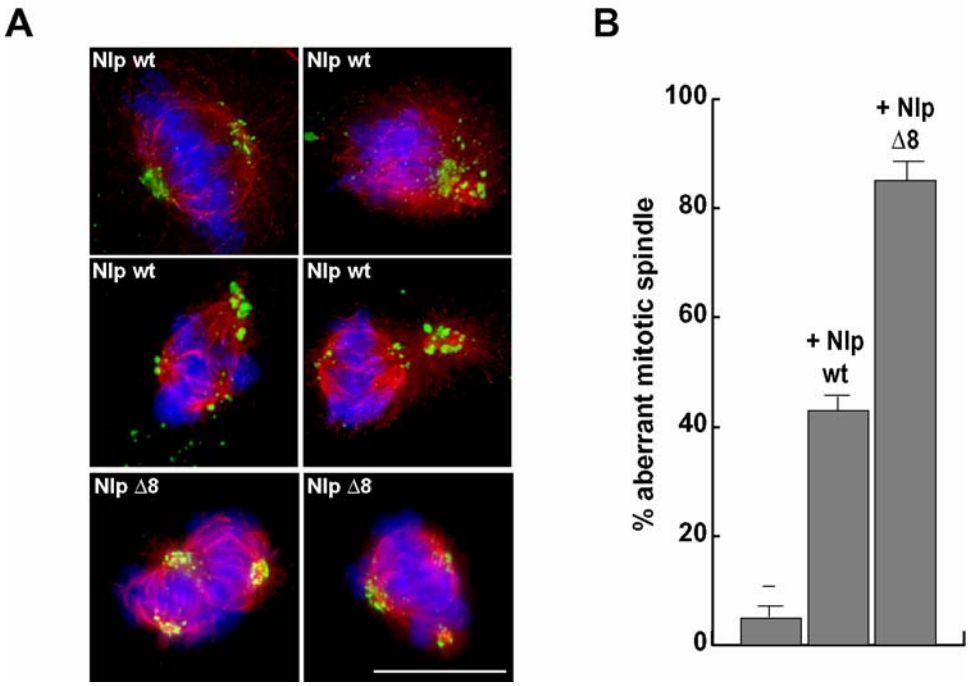

Figure 15. Overexpression of Nlp induces formation of aberrant mitotic spindles

(**A**) U2OS cells transfected with either EGFP-Nlp wild type or EGFP Δ8 (green) were analyzed 48 hr later by immunofluorescence microscopy staining with antibodies against α-tubulin (red) to reveal the mitotic spindle. DNA was stained with DAPI (blue). Scale bars represent 10μm. (**B**) Transfected mitotic cells were counted as having either normal or aberrant mitotic spindle. Histogram shows result from three independent experiments (total of 300 cells) and bars indicate standard deviations.

We next asked whether the overexpression of wild-type or Δ8 mutant Nlp could affect mitotic progression. U2OS cells were transiently transfected with both EGFP-tagged Nlp wild-type or Δ8 mutant and analyzed 48 hours later by immunofluorescence microscopy. Interestingly, overexpression of wild-type Nlp led to formation of aberrant mitotic spindles in about 40% of the mitotic cells, while the remaining mitotic cells showed normal bipolar spindles (Figure 15A and B). The observed defects comprised monopolar spindles, tri- and tetrapolar spindles as well as acentrosomal MT asters (Figure 15A). These extra spindle poles and MT asters were invariably associated with overexpressed Nlp, suggesting that Nlp was directly responsible for their formation. Strikingly, the overexpression of the Δ8 mutant induced qualitatively similar mitotic defects but it affected a higher percentage of cells, since nearly 85% of mitotic cells displayed

aberrant spindles (Figure 15A and 15B). This results suggest that the presence of an excess of Nlp at the onset of mitosis induces defects in mitotic spindle formation. Interestingly, the observed phenotype becomes more pronounced upon overexpressing the non-phosphorylatable mutant form, which can not respond to Plk1 and thereby can not be displaced from the centrosome. (The experiments on the phosphorylation of Nlp by Plk1 have been performed in collaboration with P. Meraldi, post-doctoral fellow in the laboratory of Erich Nigg).

DISCUSSION

MT nucleation represents one of the main function of the centrosome and has been recently shown to depend on γTuRCs. However, the mechanisms underlying the recruitment of these complexes to the centrosome remain poorly understood in higher eukaryotes. Here we have described a novel 156 kD centrosomal protein, termed Nlp, whose properties suggest that it functions as a docking protein for γTuRCs, therefore being involved in centrosomal MT nucleation activity. Nlp shares significant sequence similarity to ninein, a protein that has been implicated in the capping and anchoring of MT minus-ends at both centrosomal and non-centrosomal sites. Thus, the mammalian ninein family comprises products of at least two distinct genes, both of which appear to play important roles in the organization of MT arrays. Furthermore, we have shown that Nlp association with the centrosome is regulated during the cell cycle. In particular, Nlp is not localized at the centrosome during mitosis. We have also demonstrated that Nlp is a substrate for Plk1 and it dissociates from the centrosome upon phosphorylation by Plk1. This suggets that Plk1 phosphorylation could trigger an exchange of γ-tubulin complex binding proteins (GTBPs) at the centrosome. Such a modification in the composition of the PCM could represent a critical step for the centrosome maturation process occurring at the G2/M transition.

Nlp is a candidate GTBP

γ-tubulin complex binding proteins have been identified and characterized in *Saccharomyces cerevisiae,* but the identification of GTBPs in other organisms has proven difficult. In *S. cerevisiae*, the spindle pole body binds the γ-tubulin complex via Spc72p on its cytoplasmic side and via Spc 110p on its nuclear side (Knop and Schiebel, 1997; Knop and Schiebel, 1998; Nguyen *et al*., 1998). In

mammals, a protein immunologically related to yeast Spc110 has been described, but the molecular identity of this protein remains unknown (Tassin *et al*., 1997). Some sequence similarity has been noted between Spc110 and kendrin/pericentrin-B, but this similarity is largely restricted to a putative calmodulin-binding domain (Flory *et al*., 2000, Li *et al*., 2001). Furthermore, other mammalian proteins including members of the kendrin/pericentrin/GC-Nap family, Cep135 and CPAP (centrosomal P4.1-associated protein), have been proposed to bind γ-tubulin, but the precise contribution of these proteins to MT organization remains to be clarified (Dictenberg *et al*., 1998; Hung *et al*., 2000; Ohta *et al*., 2002; Li *et al*., 2001; Takahashi *et al*., 2002). Thus, although several centrosomal proteins have been implicated in the binding of γ-tubulin, it remains to be determined, which, if any, of these proteins function as *bonafide* GTBPs.

Our present study identifies Nlp (the product of cDNA KIAA0980) as a novel candidate GTBP in human cells. Nlp was able to recruit both γ-tubulin and hGCP4, both *in vitro* and *in vivo*. Although we could not test all known γTuRC components in these binding assays, it appears reasonable to conclude that Nlp binds the entire complex. In support of this conclusion, we found that Nlp assemblies promoted MT nucleation both in mammalian cells and in *Xenopus* egg extracts. This was particularly striking in egg extracts, where Nlp assemblies triggered the formation of MT asters even in the absence of centrosomes. Regrowth assays performed in mammalian cells harboring Nlp assemblies confirmed that Nlp was able to recruit and organize all the components required for MT nucleation. Conversely, microinjection of antibodies against Nlp severely suppressed MT nucleation.

Analysis of Nlp functional domains

Analysis of Nlp deletion mutants revealed that the N-terminal region of Nlp including the residues 38-350 was required for the binding of γTuRCs. This region contains a putative EF-hand motif (EF-hand domain II, aa 237-265), raising the

possibility that the Nlp interaction with the γTuRC could be regulated by calcium. Interestingly, the EF-hand domain II present in Nlp is not conserved in the N-terminal domain of ninein. This structural difference could account for the observed different ability of the two proteins to interact with the γTuRCs. To examine this hypothesis, Nlp mutants carrying single point mutations in the EF-hand domains were generated. When tested for their ability to recruit γ-tubulin and affect MT regrowth, Nlp mutants behaved as the wild-type protein, suggesting that the EF-hand domains are not essential for the interaction with γ-tubulin. However, mutagenesis of additional EF-hand loop residues important for calcium chelation could be required to efficiently interfere with the functions of EF-hand domains (Middendorp *et al*., 2000). Thus, our data do not allow to unequivocally exclude a role for these domains in the binding of γTuRCs.

Further biochemical work will be required for a more detailed characterization of the interaction between Nlp and the γTuRC. We do not presently know which of the known γTuRC components makes direct contact with Nlp. It is also possible that multiple γTuRC components contribute to create an Nlp-binding domain, or that the interaction between the γTuRC and Nlp requires as yet unknown proteins. Definitive structural information on the interaction between Nlp and γ-tubulin may have to await the *in vitro* reconstitution of the complete γTuRC from recombinant proteins.

Nlp, a novel 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. Human Nlp maps to 20p11.22-p11.1, whereas ninein is located at 14q21.3; thus the two proteins clearly represent the products of two distinct genes. Detailed analyses of the subcellular localization of ninein in both cultured fibroblasts and cochlear supporting epithelial cells have lead to the proposal that ninein is part of

a multiprotein complex that functions in MT anchoring rather than MT nucleation (Mogensen *et al*., 2000). As it is difficult to rigorously distinguish various MT minus end-associated activities, it would be premature to exclude that Nlp may also contribute to MT anchoring. In fact, our immunolocalization data suggesting a preferential association of Nlp with one of the two centrioles would be consistent with such a function. On the other hand, however, our data strongly indicate that Nlp plays an important role in the recruitment of γTuRCs to the centrosome. This identifies Nlp as a novel candidate GTBP, and implies a role for this protein in MT nucleation.

The available evidence indicates that Nlp and ninein perform at least partially distinct functions. Although the N-terminal domains of the two proteins display 37% sequence identity, only Nlp interacted efficiently with the γTuRC, in line with the view that Nlp, but not ninein, plays a role in MT nucleation. Thus, whilst both Nlp and ninein clearly play important roles in the organization of MT networks in mammalian cells, the two ninein family members appear to have functionally diverged during evolution.

Nlp is a novel centrosomal substrate for Plk1

Yeast two-hybrid screen and biochemical data strongly indicated an interaction between Plk1 and Nlp, therefore suggesting that Nlp could be a physiological substrate for Plk1. Our results showed that Plk1 can phosphorylate Nlp, but not ninein, indicating that these two proteins are diffferently regulated. We could further demonstrate that Nlp dissociates from the centrosome as a functional consequence of Plk1 phosphorylation. Interestingly, in all *in vivo* experiments only $Plk1^{T210D}$, which mimics the activated mitotic form of Plk1, strongly affected Nlp. This is likely to reflect the higher specific activity of $Plk1^{T210D}$ but also raises the interesting possibility that only the mitotic form of Plk1 is able to efficiently recognize and phosphorylate Nlp. Moreover, *in vitro* Plk1 phosphorylation sites were identified. Our analysis of the Nlp mutant carrying

serine to alanine substitution in the identified Plk1 phosphorylation sites revealed that Nlp is a direct substrate of Plk1 *in vitro* and *in vivo*. These results also confirmed the proposed consensus for Polo-like kinases (E/DxS/T) (Kelm *et al*., 2002). Although still awaiting for further confirmation, the identification of the putative consensus sequence could in the future facilitate the identification and characterization of additional Polo-like kinase substrates.

Nlp is displaced from the centrosome during centrosome maturation

The analysis of Nlp subcellular localization during cell cycle progression revealed that Nlp is displaced from the centrosome at the onset of mitosis. Several lines of evidence suggest that activation of Plk1 at the G2/M transition results in the displacement of Nlp from the maturing centrosome. Indeed, a Plk1 mutant mimicking the activated mitotic form of the kinase, but not the wild-type form, is able to efficiently displace Nlp from the centrosome. In line with these results, the activated mitotic form of Plk1 did not affect the centrosomal localization of the unphosphorylatable Nlp mutant. Furthermore, we showed that the persistence of Nlp at the centrosome during mitotic progression induced mitotic spindle aberrations. Thus, we propose that Nlp is involved in docking γTuRCs at the centrosome during interphase but not during mitosis. At the onset of mitosis Nlp needs to be displaced from the centrosome in order to ensure correct spindle formation.

The fate of Nlp during mitosis still represents an unresolved question which requires further investigation. Our preliminary data suggest that Nlp protein is present throughout mitosis in human cells. Initial characterization of the *Xenupus laevis* Nlp homologue has also revealed that the protein is stable during the cell cycle (A. Fry, personal communication). Although still preliminary, these observations indicate that the absence of Nlp from mitotic centrosome is not due to protein degradation. As an alternative explanation to degradation we propose that, upon phosphorylation by Plk1 at the onset of mitosis, Nlp dissociates from the centrosome and becomes dispersed in a cytoplasmic pool. Nlp is then

targeted again to the centrosome in telophase, when Plk1 activity starts to decrease.

A model for the role of Plk1 in centrosome maturation

At the onset of mitosis the centrosome undergoes a striking structural and functional reorganization, known as centrosome maturation, which is essential to ensure an efficient switching from the interphasic to the mitotic centrosomal activities. This process is principally characterized by an abrupt increase of the MT nucleation capacity, due to a massive recruitment of γ-tubulin containing complexes to the centrosome (Dictenberg *et al*., 1998; Khodjakov and Rieder, 1999). Electron-microscopy has also shown that centrosome maturation is accompanied by ultrastructural changes, with the disappearance of the subdistal appendages and the appearance of a optical dense halo around the centrosome (Chretien *et al*., 1997; Rieder and Borisy, 1982). Thus, the centrosome maturation process is expected to require substantial changes in PCM composition. These changes appear to be regulated 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). Here we have characterized a novel centrosomal protein, Nlp, whose properties suggest that it plays a role in centrosomal MT nucleation, acting as a tethering protein for γTuRCs during interphase but not during mitosis. This implies the existence of distinct GTBPs which function as interphasic or mitotic docking factors for γTuRCs at the centrosome. We hypothesize that the removal of the interphasic GTBP Nlp from the centrosome at the onset of mitosis represents a prerequisite for the recruitment of mitotic GTBPs, required to ensure an enhanced MT nucleation capacity (Figure 16). According to this model, Plk1 could have the important role of regulating and coordinating this exchange in PCM components. Remarkably, the finding that in *Drosophila* the recruitment to the centrosome of Asp, a protein involved in MT

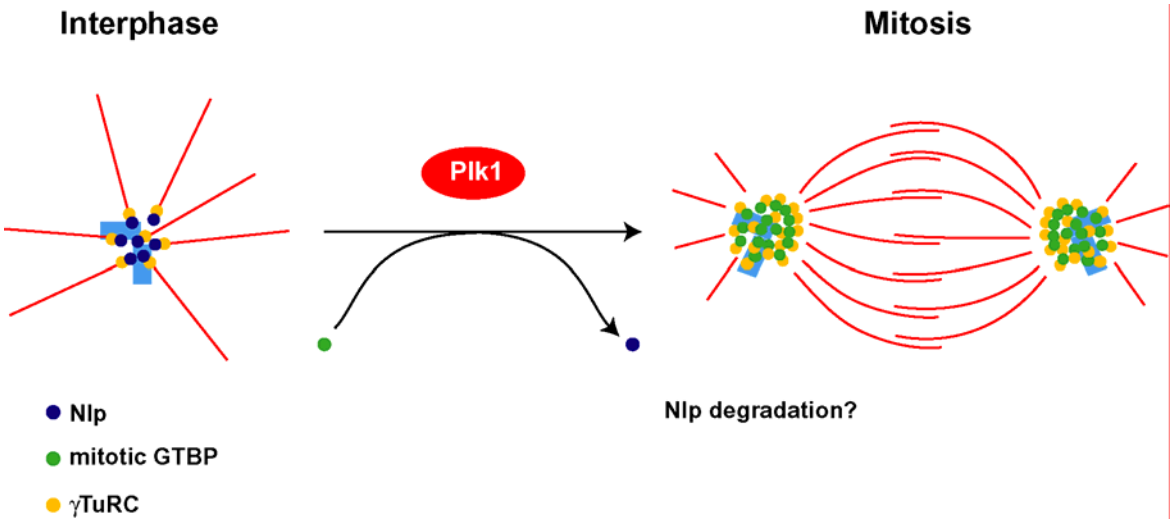

Figure 16. Model for the regulation of Nlp during centrosome maturation

The model proposes that Nlp is a novel candidate GTBP involved in docking the γTuRC complexes to the centrosome during interphase. At the onset of mitosis Nlp needs to be displaced from the centrosome and substituted by an hypothetical mitotic GTBP, in order to allow proper mitotic spindle assembly. Our results show that the removal of Nlp from the centrosome is regulated by Plk1. Yet, additional mechanisms of regulation (i.e. protein degradation, phosphorylation by other kinases) could be involved and their identification certainly represents an interesting subject for future investigation

tethering at the spindle pole (Riparbelli *et al*., 2002; Wakefield *et al*., 2001), depends on phosphorylation by Plk1 falls in line with our model.

Validation of this model will require to identify the hypothetical mitotic GTBP(s) which is recruited to the centrosome upon Nlp removal and to verify whether it is subjected to regulation by Plk1. Further investigations will be also required to identify possible additional enzymes involved in the regulation of Nlp (i.e. other protein kinases, protein phosphatases). These clearly represent intriguing tasks for future work.

PART II: the overexpression of the centrosomal proteins Nlp and ninein induces fragmentation of the Golgi apparatus

INTRODUCTION

Cytoplasmic dynein: one major motor for a vast breadth of functions

The high degree of spatial/temporal organization within a cell is made possible by the presence of several motor proteins that transport components to various destinations within the cytoplasm (for reviews see Berg *et al*, 2001; Goldstein, 2001; Hirokawa *et al*., 1998; Schliwa and Woehlke, 2003; Vale, 2003). Three classes of cytoskeletal molecular motor proteins exist, which have been retained and widely used throughout eukaryotic evolution. These are represented by kinesins, which are mostly plus-end-directed microtubule motors, although a few family members power minus-end-directed transport; dyneins, which are minus end directed microtubule motors; and myosins, which are actin–dependent motors.

Vertebrate cytoplasmic dynein is a multisubunit protein (1-2 MDa) composed of 2 identical heavy chains (DHC, ≈ 500 kDa each) which contain a large motor domain and a MT-binding domain, the intermediate chains (DIC, 70-74 kDa), multiple light intermediate chains (DLIC, 53-54 kDa) and light chains (DLC, 8-22 kDa) including members of the Tctex-1/rp3, roadblock and LC8 families (the structure of the dynein motor protein is reviewed in King, 2000). The growing list of activities attributed to this motor includes mRNA localization, nuclear migration, nuclear envelope break-down, virus transport, mitotic spindle assembly, kinetochore function and movement of spindle checkpoint proteins, retrograde axonal transport, vescicular trafficking and transport of centrosomal proteins (for reviews see Gonczy, 2002; Karki and Holzbaur, 1999; Morris, 2003; Tekotte and Davis, 2002; Zimmerman and Doxsey, 2000).

Unlike the kinesin superfamily, whose members have evolved various primary structure differences in their heavy chains in order to perform a variety of different functions (Hirokawa *et al*., 1998), only a few isoforms of cytoplasmic dynein heavy chains have been described to date (Criswell *et al*., 1996; Criswell and Asai, 1998; Gibbons *et al*., 1994; Tanaka *et al*., 1995; Vaisberg *et al*., 1996).

Thus, the wide range of dynein's functions described above and the specificity of motor-cargo interactions are not the result of motor gene expansion. Rather, the versatility and ability of this motor protein to recognize specific cargoes are mainly due to mechanisms of functional specialization as well as temporal and spatial regulation. These include: isoform diversity of intermediate or light chains, use of functional adaptors like dynactin and post-translation modifications such as phosphorylation.

Different dynein subunits have been reported to interact with distinct protein partners, potentially facilitating the association of dynein with several different cargoes. While dynein light chain LC8 and dynein intermediate chains seem to be responsible for the binding of viral proteins (Jacob *et al*., 2000; Raux *et al*., 2000; Ye *et al*., 2000), light chains of the Tctex-1 family have been shown to directly interact with the visual pigment rhodopsin (Tai *et al*., 1999) and the neurotrophin receptor TrkA (Yano *et al*., 2001), suggesting that the specificity of the dynein-cargo interaction strongly depends on dynein's subunit composition.

Although not a constitutively associated subunit, the dynactin multiprotein complex also binds to cytoplasmic dynein (Gill *et al*., 1991; Holleran *et al*., 1998; Schroer and Sheetz, 1991; Vaughan and Vallee 1995; for a schematic representation of the dynein-dynactin complex see Figure 17). The disruption of the dynactin complex by overexpression of its dynamitin subunit interferes with most, if not all, cytoplasmic dynein mediated transport processes, suggesting that dynactin plays a general regulatory role on dynein motor activity (Burkhardt *et al*., 1997). Enhancing processive movement of cytoplasmic dynein along MTs could represent such a general regulatory action of the dynactin complex (King and Schroer, 2000). The binding of dynactin to MT through its $p150^{Glued}$ subunit (Waterman-Storer *et al*., 1995) may, indeed, stabilize the dynein-microtubule interaction and allow the dynein motor to move more processively. However, the dynactin complex can also act as an "adaptor" that links dynein to different cargoes. The Arp1 and dynamitin subunits of dynactin have been implicated in providing a link between the microtubule motor complex and membraneous organelle (see below). Dynamitin also interacts with the kinetochore component

ZW10 (first characterized in *Drosophila*), mediating the targeting to the kinetochores of the dynein/dynactin complex (Starr *et al*., 1998).

Interaction of motor proteins with their protein partners is important not only for the specificity of cargo binding but is also key to the regulation of transport itself. Indeed, the movement of cargoes might be controlled at the stage of binding to the motor proteins. Even if the mechanisms of regulation of cargo-motor binding are still poorly understood, it is becoming apparent that phosphorylation of the dynein intermediate and light chains plays a role in regulating dynein binding to organelle membranes (Addinal *et al*., 2001; Dell *et al*., 2000; Vaughan *et al*., 2001).

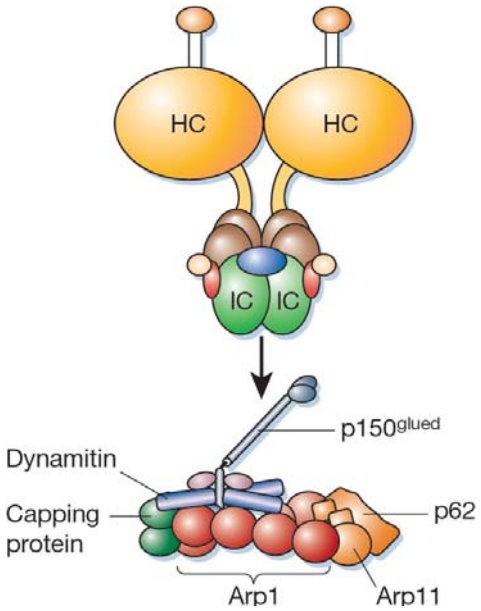

Figure 17. Schematic representation of the dynein-dynactin complex

Dynein is a complex of heavy (DHC), intermediate (DIC) and lights chains (DLIC and DLC). Dynactin is also a multisubunit complex which consists of two structural domains: an actin like backbone, composed by Arp1, Arp11, p62, p27, capping proteins α and β, and a projecting shoulder/sidearm composed of dynamitin, p24 and $p150^{Glued}$. Dynein interacts with the $p150^{Glued}$ subunit of dynactin through its intermediate chain (arrow), although the precise mode of interaction is not known (Schliwa and Woehlke, 2003).

Role for dynein/dynactin dependent transport in the assembly of the centrosome

The role played by microtubule-dependent transport of centrosomal components in regulating and determining the centrosome composition throughout the cell cycle is just beginning to be elucidated (for reviews see Blagden and Glover, 2003; Zimmerman and Doxsey, 2000). The presence of a

functional MT network is required for the correct assembly of new centrioles. Centrosome duplication assays performed in the presence of the MT depolymerizing drugs nocodazole or colcemid have revealed that the centrosome duplication process does not occur efficiently in the absence of MTs (Balczon *et al*.,1999). Furthermore, *de novo* formation of centrioles upon removal of centrosomes by laser-ablation, is prevented when cells are treated with nocodazole, although γ-tubulin foci still form under these conditions (Khodjakov *et al*., 2002). However, unlike in cells containing microtubules, which form a single large PCM focus, in the absence of microtubules several individual foci form in the cytoplasm. This suggests that both MT-dependent and MT-independent mechanisms contribute to the assembly of the centrosome. Centrosomal proteins seem able to self-assemble even in the absence of microtubules, but microtubules may be necessary to coordinate the assembly into a unique organelle.

The recruitment to the centrosomes and spindle poles of NuMA, pericentrin and PCM-1 has been shown to depend on dynein-mediated transport in *Xenopus* egg extracts and mammalian cells (Dammerman and Merdes, 2002; Dictenberg *et al*., 1998; Gaglio T *et al*., 1996; Kubo *et al*., 1999; Merdes *et al*.,1996; Merdes *et al*., 2000; Young *et al*., 2000). While NuMA has been proposed to directly interact with the Arp1 subunit of dynactin (Clark and Meyer, 1999), pericentrin can directly bind to dynein through the dynein light intermediate chain 1 subunit (DLIC1) (Purohit *et al*.,1999; Tynan *et al*., 2000). In contrast, the molecular basis for the interaction between PCM1 and the dynein/dynactin complex remains to be clarified. Pericentrin and PCM-1 have been shown to mediate the assembly onto the centrosome of other centrosomal components. For example, γ-tubulin appears to undergo dynein-mediated transport to centrosomes through its association with pericentrin (Dictenberg *et al*., 1998; Young *et al*., 2000), although it should be noted that a microtubule-independent mechanism for recruitment of γ-tubulin to the centrosomes has also been demonstrated (Khodjakov and Rieder, 1999; Moritz *et al*., 1998; Schnackenberg *et al*., 1998; Stearns and Kirschner, 1994). Moreover, inhibition of PCM-1 function using a variety of approaches leads to the reduced targeting of centrin, pericentrin and ninein to the centrosome

(Dammermann and Merdes, 2002). When overexpressed as GFP-chimerae pericentrin and PCM-1 form small granules which move along microtubules toward their minus ends (Kubo *et al*., 1999; Young *et al*., 2000). Thus, the current model proposes that pericentrin and PCM-1 may function as carriers which mediate the dynein/dynactin-dependent transport of centrosomal components and their assembly onto centrosomes (Zimmerman and Doxsey, 2000). Both endogenous pericentrin and PCM-1 associate with the pericentriolar satellites, non membranous particles (or granules) localized to the pericentriolar region, (Dictenberg *et al*., 1998; Doxsey *et al*., 1994; Kubo and Tsukita, 2003). However, they have been recently shown to localize to distinct subset of satellites, suggesting the presence of at least two different classes of centrosome particles. Yet, pericentrin granules and PCM-1 granules are frequently associated with each other. This indicates a possible dynamic interaction between the different classes of particles which would also explain the ability of PCM-1 to affect pericentrin localization (Kubo and Tsukita, 2003).

In conclusion, centrosome assembly can occur by MT-dependent (e.g. dynein-mediated transport) and independent mechanism (e.g. passive diffusion; see fig 18). One possible advantage of a dynein-mediated transport system over a simple diffusion-based mechanism is that it has the potential to increase the local concentration of transported molecules and facilitate their assembly onto centrosomes and spindle poles. The presence of large protein particles containing centrosomal proteins suggests that packaging and transport of large assemblies of preassembled subunits may be a mechanism for the construction and the maintenance of a large intracellular organelles such as the centrosome. The mechanisms by which the proteins discussed above become organized into large particles, as well as the complete molecular composition of these particles, still await further investigation.

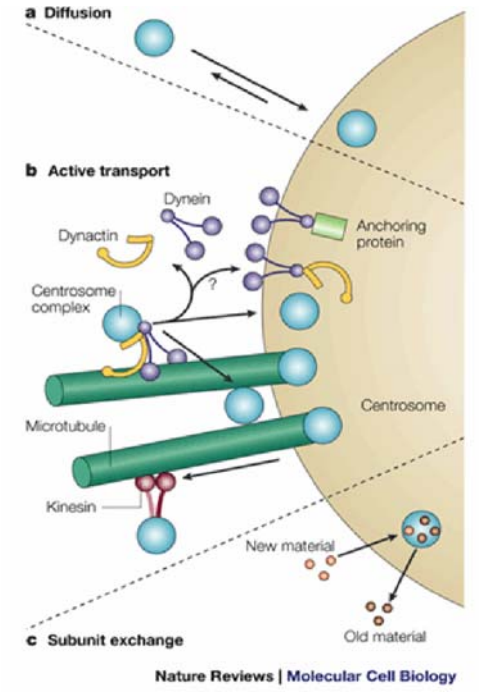

Figure 18. Model for centrosome protein assembly
(a) Centrosome complexes can diffuse to centrosomes in a microtubule- and dynein-independent manner. (b) Centrosome complexes, in the form of large particles, are transported to centrosomes by cytoplasmic dynein motors. Particles are delivered to centrioles or PCM. Dynein motors may be anchored at centrosomes by dynactin or other anchoring proteins or released after cargo delivery. Whereas dynactin is at the centrosome during all cell cycle stages, dynein only accumulates there during S and G2. (c) Exchange between the cytopasmic and the centrosomal pool of proteins which have been assembled onto centrosomes either in a MT-independent (a) or MT-dependent (b) way. This exchange could occur continuously or in a cell-cycle-dependent manner (Doxsey, 2001).

Positioning and shaping the Golgi complex

In most cell types the Golgi apparatus is closely linked to the MTOC (see also General Introduction) but there are exceptions to this rule and the mechanisms responsible for maintaining the position and morphology of the Golgi are still poorly characterized. It has long been clear that in animal cells the Golgi apparatus depends on microtubules for its positioning and organization. In the absence of microtubules, the Golgi apparatus becomes fragmented and progressively dispersed throghout the cytoplasm (Thyberg and Moskalewsky, 1985; Thyberg and Moskalewsky, 1999). Each Golgi fragment seems to consist of stacked cisternae and is functional in terms of imparting post-translational modifications to transiting proteins and delivering them to the cell surface (Cole *et al*., 1996; Rogalski *et al*., 1984; for review see Bloom and Goldstein, 1998). If microtubules are allowed to repolymerize, the scattered Golgi mini-stacks move along microtubules toward the MTOC, where, in most cell type, the minus end of microtubules are located (Ho *et al*., 1989).

Although stable MTs enriched in detyrosinated and/or acetylated tubulin have been proposed to function as a framework to which Golgi elements maybe anchored (Burgess *et al*., 1991; Mizuno and Singer, 1994; Skoufias *et al*., 1990; Thyberg and Moskalewsky, 1993), the major role played by MTs in the maintenance of Golgi structure and position depends on the action of microtubule motor proteins belonging to the kinesin and dynein families (for reviews see Allan *et al*., 2002; Burkhardt, 1998; Lippincott-Schwartz, 1998). The major player seems to be the cytoplasmic dynein motor complex. Indeed, the Golgi apparatus appears clearly disrupted in blastocysts derived from DHC1$^{-/-}$ mice lacking the most abundant isoform of cytoplasmic dynein heavy chain (Harada *et al*., 1998). Disruption of the Golgi structure is also observed upon inhibition of cytoplasmic dynein function by overexpression of the dynamitin subunit of dynactin or microinjection of antibodies against the DIC (Burkhardt *et al*., 1997; Presley *et al*., 1997). The molecular mechanisms underlying the interactions of cytoskeletal motor proteins with organelle membrane are just beginning to be elucidated. An interaction between dynactin and spectrin has been described, which may mediate the attachment of the dynein/dynactin complex to the surface of vesicles undergoing transport to the Golgi (Holleran *et al*., 1996; Muresan *et al*., 2001). This interaction seems to be mediated by the Arp1 subunit of dynactin which has been demonstrated to bind in a two hybrid screen spectrin βIII, a Golgi-associated spectrin (Holleran *et al*., 2001). Yet, Fath *et al*. found that, upon incubation with cytosol, the Arp1 and p150^{Glued} subunits of dynactin could re-bind to pre-extracted Golgi membranes lacking the spectrin matrix (Fath *et al*., 1997). This result suggest that interaction of dynein/dynactin with the Golgi can occur independently of the spectrin matrix.

Recently, the mammalian homologues of *Drosophila* Bicaudal-D, termed BICD1 and BICD2, and the Golgi-associated small GTPase Rab6 have been shown to play a role in the recruitment of cytoplasmic dynein-dynactin complexes to Golgi membranes (Hoogenraad *et al*., 2001; Matanis *et al*., 2002; Short *et al*., 2002). BICD1 and BICD2 colocalize with the dynactin complex on the Golgi membranes and at the plus-end of MTs (Hoogenraad *et al*., 2001; Matanis *et al*., 2002). Biochemical data show that the C-terminal domain of BICD2 binds directly

the dynamitin subunit of dynactin while the N-terminal domain can associate with cytoplasmic dynein (Hoogenraad *et al*., 2001). The association of BICD1 and BICD2 with the Golgi membranes appears to be mediated by Rab6 since both proteins interact with the GTP-bound form of Rab6 in *in vitro* binding assays (Matanis *et al*., 2002; Short *et al*., 2002). Rab6 can also directly bind to dynactin through the $p150^{Glued}$ subunit (Short *et al*., 2002). The network of interactions involved in the targeting of the cytoplasmic dynein/dynactin complex at the Golgi apparatus is summarised in Figure 19.

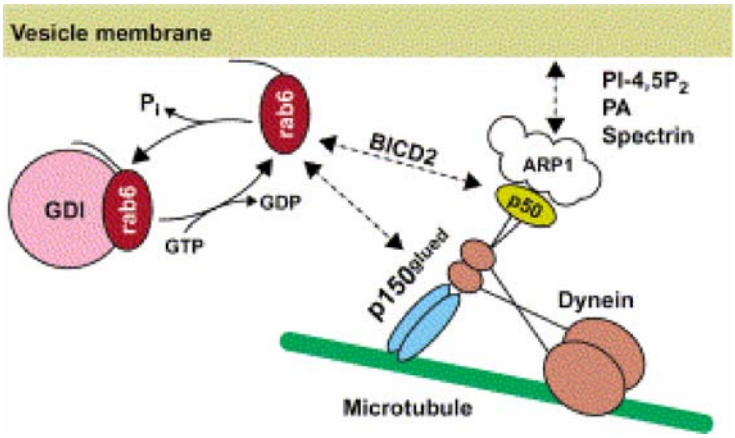

Figure 19. Recruiting the dynein-dynactin complex to specific sites on vescicle membranes
Acidic phospholipids such as phosphatidyl-4,5 biphosphate (PI-4,5P_2) and phosphatidic acid (PA) act in conjunction with some isoforms of spectrin to mediate attachament of dynactin to axonal vesicles. Rab6 recruitment to Golgi membranes is governed by a cycle of GTP binding and hydrolysis. When in the GTP form and associated with the membrane surface, Rab6 recruits dynactin via interactions with BICD2 and p150Glued (dotted lines) (Short et al., 2002).

Another isoform of cytoplasmic dynein heavy chain, named DHC2, and a dynein light intermediate chain which interacts specifically with it (D2LIC), have been found to localize predominantly at the Golgi apparatus in a variety of mammalian cell lines (Grissom *et al*., 2002; Vaisberg *et al*., 1996). Microinjection of DHC2 antibodies resulted in the fragmentation and dispersal of the Golgi apparatus in a fraction of cells, indicating that more than one type of cytoplasmic dynein may be involved in the organization of this organelle (Vaisberg *et al*., 1996). Which of the dynein heavy chain isoforms (DHC1 or DHC2) plays a

dominant role in Golgi organization is still an open question. Further complications in resolving this issue arise from the fact that it is not known to which degree the different dynein chains co-assemble and to which extent dynein complexes containing distinct heavy chains subunits can still share intermediate, light intermediate and light chains subunits.

Dyneins are not the only family of microtubule motors that are active at the Golgi apparatus. Members of the kinesin family of microtubule motors are also present. Conventional kinesin heavy chain (Johnson *et al*., 1996; Marks *et al*., 1994) and a specific kinesin light chain isoform KLC1D/E (Gyoeva *et al*., 2000), have been localized at the Golgi complex. Furthermore, when kinesin heavy chain (KIF5B) expression was reduced in cultured astrocytes using an anti-sense approach, the Golgi apparatus switched from an extended ribbon to a much more compact structure (Feiguin *et al*., 1994). This suggest that an ongoing “tug-of-war” between cytoplasmic dynein and kinesin occurs at the level of the Golgi ribbon, perhaps determining its overall morphology. It is necessary to point out here that in addition to maintaining the structure and position of the Golgi apparatus, motor proteins are also necessary for the multiple membrane trafficking events that originate from the Golgi.

Non-motor MT-binding proteins are also involved in maintaining Golgi morphology and localization. Two recently described examples are GMAP 210 (Golgi microtubule associated protein 210; Infante *et al*., 1999; Pernet-Gallay *et al*., 2000) and hHk3 (human hook protein 3; Walenta *et al*., 2001). Both these proteins have been localized to the cis-Golgi region in mammalian cells and were shown to bind microtubules and Golgi membranes in vitro (Infante *et al*., 1999; Rios *et al*., 1994; Walenta *et al*., 2001). Overexpression of GMAP 210 and hHk 3 causes perturbations of the MT network and induces fragmentation of the Golgi complex (Infante *et al*., 1999; Walenta *et al*., 2001). In the case of hHk3, it has been demonstrated that the perturbation of the MT network was not sufficient to cause the dispersal of the Golgi complex, suggesting that hHK3 plays a direct role in the positioning of the Golgi complex (Walenta *et al*., 2001). In the current model (Infante *et al*., 1999; Walenta *et al*., 2001), GMAP 210 and hHK3 are proposed to function as CLIPs (cytoplasmic linker proteins) which may link Golgi membrane

compartments to microtubules and therefore participate in defining the architecture and localization of the Golgi complex.

In the present work we have investigated the functional consequences of the overexpression of the two centrosomal proteins Nlp and ninein on the structure and positioning of the Golgi apparatus. Overexpression of Nlp or ninein in human cells does not perturb the structural integrity of the MT network. Yet the morphology and localization of the Golgi complex is dramatically affected in Nlp or ninein overexpressing cells, resulting in the fragmentation and dispersal of the Golgi apparatus throughout the cytoplasm. Here we report an initial characterization of the molecular basis of the Nlp and ninein overexpression phenotype.

RESULTS

Overexpression of Nlp and ninein induces Golgi fragmentation

To study the functional consequences of the overexpression of the centrosomal proteins Nlp, ninein, and C-Nap1 on the positioning of the Golgi apparatus, U2OS stable cell lines expressing EGFP-tagged C-Nap1 or Myc-tagged Nlp and ninein fusion proteins under the "tet-on" inducible promoter were stained with the cis-Golgi marker GM130 and analyzed by immunofluorescence microscopy. As shown in Figure 20A, a dramatic disassembly of the Golgi stacks was specifically observed in most cells overexpressing Nlp and ninein (Nlp: 77% *n*=300; ninein: 81.4% *n*=300) whereas the morphology of the Golgi apparatus was not affected by the overexpression of the centrosomal protein C-Nap1 in the majority of cells (C-nap1: 8.6% *n*=300; Figure 20A, lower pannel). To assess whether the fragmentation of the Golgi observed upon the overexpression of Nlp and ninein was comparable to that induced by depolymerization of MTs (Thyberg and Moskalewsky, 1985; Thyberg and Moskalewsky, 1999), myc-Nlp and myc-ninein stable cell lines were grown in the absence of tetracycline, treated with nocodazole (200ng/ml) 1hr before fixation and analyzed by immunofluorescence microscopy using antibodies against GM130. The disruption of the Golgi complex observed upon expressing either Nlp or ninein indeed resulted very similar to that induced by nocodazole treatment (Figure 20B), suggesting that the phenotype observed could result from impared microtubule integrity. However, the staining of Nlp and ninein overexpressing cells using anti α-tubulin antibodies revealed that the structural integrity of the microtubule network was not affected in those cells (Figure 20C).

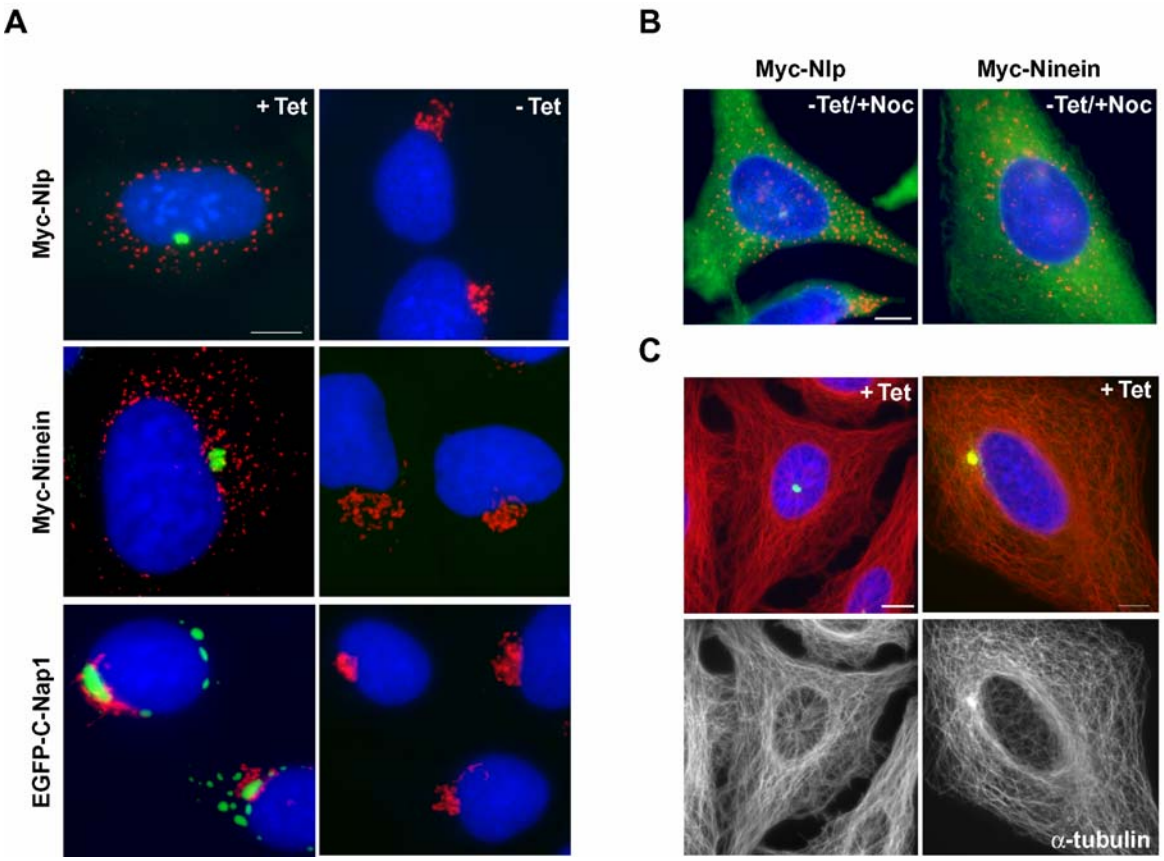

Figure 20. Effect of Nlp and ninein overexpression on Golgi complex organization

(**A**) Tetracycline inducible U2OS stable cell lines expressing myc-Nlp, myc-ninein or EGFP-C-Nap1 were either treated (left panels) or not (right panels) with tetracycline and analyzed by immunofluorescence microscopy. Overexpressed proteins were detected with anti-myc antibodies or through EGFP fluorescence (green). The Golgi apparatus was detected using antibodies against the Golgi marker GM130 (red). DNA was stained with DAPI (Blue). (**B**) Tet-on U2OS stable cell line expressing myc-Nlp or myc-ninein were grown in the absence of tetracycline and treated with nocodazole (200ng/ml) 1 hour before fixation. Cells were analyzed by immunofluorescence microscopy using antibodies against α-tubulin (green) to verify the MT-depolymerization. The Golgi apparatus was deteced with antibodies against GM130 (red) and DNA was stained with DAPI (blue) (**C**) Myc-Nlp and myc-ninein were expressed in U2OS cells and cells analyzed by immunofluorescence microscopy. Cells were stained with myc-antibodies to detect the overexpressed protein (green) and counterstained with antibodies against α-tubulin (red). Scale bar represents 10μm.

Nlp and ninein associate with the dynein-dynactin complex

Dispersal of the Golgi apparatus has also been observed upon inactivation of the minus-end directed motor dynein either genetically (Harada *et al*., 1998) or by

overexpression of the dynamitin subunit of dynactin (Burkahardt *et al*., 1997). In order to test whether the overexpression of Nlp or ninein could perturb the localization of the dynein-dynactin complex, thereby interfering with its activity, we stained Nlp- or ninein-overexpressing cells using antibodies directed against the dynactin subunit p150^{Glued} and the dynein intermediate chain (DIC). Interestingly, Nlp and ninein assemblies recruited p150^{Glued} and DIC (Figure 21A and B, panels a/a' and b/b'), whilst similar assemblies formed by EGFP-C-Nap1 did not alter the staining patterns of the dynein and dynactin subunits (Figure 21A and B, d-d'). This suggested that Nlp and ninein assemblies were able to associate with the dynein-dynactin complex. The ability of Nlp and ninein to bind the complex could also be demonstrated by pull-down experiments with GST-tagged recombinant proteins. Since the purification of the recombinant full length ninein had proven difficult, the N-terminal region of ninein was tested in the pull down experiments in order to assess whether the conserved domains of the two proteins shared similar functions. Following incubation with total cell extracts, GST-tagged Nlp full length and the N-terminal half of the protein specifically brought down the p150^{Glued} and the p50 subunits of dynactin (Figure 21C, lane 2 and 3), whereas the presence of the motor protein dynein could not be detected. The N-terminal domain of ninein also associated with the dynactin complex, albeit slightly less efficiently then the N-terminal half of Nlp (Figure 21C, lane 5). In contrast, the GST-tagged C-terminal domain of Nlp, as well as a GST-fusion to the coiled-coil domain of the Golgi protein GM130 (Barr *et al*., 1998) which served as negative control, did not bring down any of the dynactin complex subunits (Figure 21C, lane 4 and 6). These results indicate that the N-terminal domain of Nlp and ninein are responsible for the interaction with the dynactin complex.

As Nlp associates with the dynactin complex, we next asked to what extent the intracellular distribution of the endogenous proteins overlapped. Immunofluorecence analysis of U2OS cells stained with anti-Nlp and anti-p150Glued antibodies revealed that the centrosomal staining pattern of the two proteins overlapped significantly (Figure 21D), indicating that the observed interaction could be relevant also under physiological conditions.

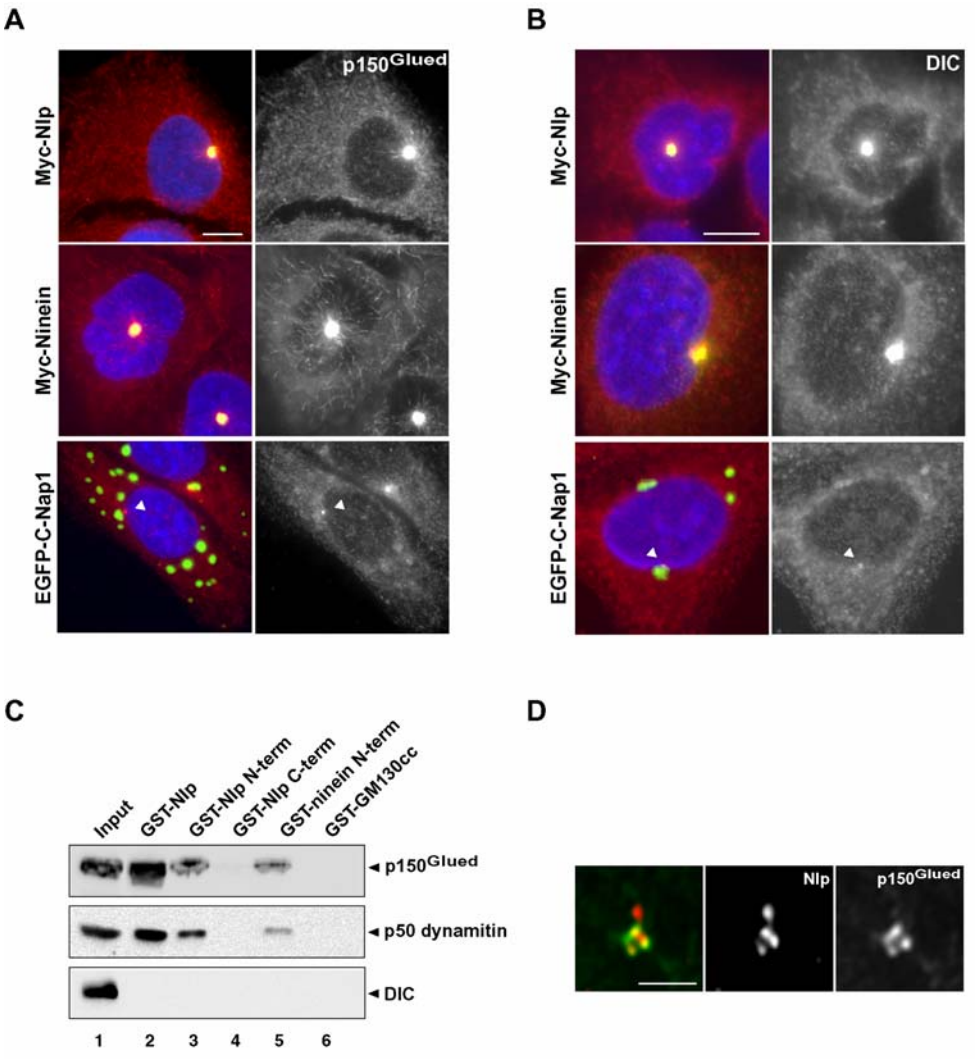

Figure 21. Nlp and ninein associate with the dynein-dynactin complex

(**A**) and (**B**) U2OS cells overexpressing myc-Nlp, myc-ninein or EGFP-C-Nap1 were analyzed by immunofluorescence microscopy. Overexpressed proteins were detected with anti-myc antibodies or through EGFP fluorescence (green). Cells were counterstained either with anti-p150^{Glued} antibodies (**A**) or with antibodies against the dynein intermediate chain (DIC) (**B**) (red). DNA was stained with DAPI (blue). Arrow-heads point to centrosomes (**C**) Total cell extracts (lane 1) were prepared as described in material and methods and incubated in the presence of GST-Nlp full length (lane 2), GST-Nlp N-terminal (lane 3) and C-terminal half (lane 4), GST-ninein N-terminal domain (lane 5) or GST-GM130cc (lane 6). The pull-downs were then analyzed by immunoblotting with antibodies against the dynactin subunits p150^{Glued} and p50-dynamitin. For the detection of dynein we used antibodies against the dynein intemediate chain (DIC) subunit**.** The amount of total protein extract loaded with the input (lane1) corresponds to 1/20 of the amount loaded for pull-downs (lanes 2-6). (**D**) Analysis, by Deltavision microscopy, of centrosomes stained with anti-Nlp (red) antibodies and p150^{Glued} antibodies (green). p150^{Glued} has been shown to localize to the mother centriole (Quyntine et al., 2000). As can be seen from the merged images, anti-Nlp antibodies staining overlapped significantly with the staining produced by anti-p150^{Glued} antibodies at the mother centriole. Anti-Nlp antibodies also stained the daughter centriole. Scale bar represents 2 μm.

The ability of Nlp and ninein to induce Golgi fragmentation depends on their ability to affect p150^{Glued} localization

To understand in more detail whether the Golgi fragmentation induced by the overexpression of Nlp and ninein depends on the ability of the two proteins to interact with the dynein-dynactin complex, we expressed in U2OS cells deletion mutants of the two proteins as EGFP- or myc-fusions. Since the pull down experiments indicated that the N-terminal half of Nlp was responsible for the association with the dynactin complex, deletion mutants lacking portions of the N-terminal domain were expressed in U2OS cells and tested for their ability to recruit dynactin and induce Golgi fragmentation. As shown in Figure 22A, a/a' and B, a/a', the removal of the first 38 aa of Nlp, containing the first EF-hand domain (EF-h I), did not affect Nlp centrosomal localisation nor its ability to disperse the Golgi and recruit p150^{Glued}. In contrast, deletion mutants lacking the of larger portions of the N-terminal domain (EGFP-Nlp ΔEF-h I-II and EGFP-Nlp C-terminal), formed large assemblies in the cytoplasm which did not have any effect on either p150^{Glued} localization or Golgi apparatus morphology (Figure 22A, panels b/b' and c/c'; Figure 22B panels b/b' and c/c'). Conversely, the N-terminal domain of the two proteins, both showing a cytoplasmic localization, profoundly affected the localization of p150^{Glued} and induced fragmention of the Golgi apparatus (Figure 22A, panels d/d' and e/e'; Figure 22B, panels d/d' and e/e'). Indeed, the overexpression of the N-terminal domain of Nlp or ninein caused the displacement of p150^{Glued} from the centrosome, without perturbing the localization at the plus-ends of MTs. Overexpressing cells were positively stained by antibodies against the centrosomal marker C-Nap1 (data not shown), excluding the possibility that the absence of p150Glued signal at the centrosome could be merely due to disruption of the centrosome structure. These results, schematically summarized in Figure 22C, indicate that the association of Nlp and ninein with the dynein-dynactin complex occurs through their N-terminal domains, confirming the biochemical data (Figure 21C). Furthermore, they suggest that the fragmentation of the Golgi induced by Nlp and ninein is a consequence of their ability to alter the intracellular distribution of dynein-dynactin complexes.

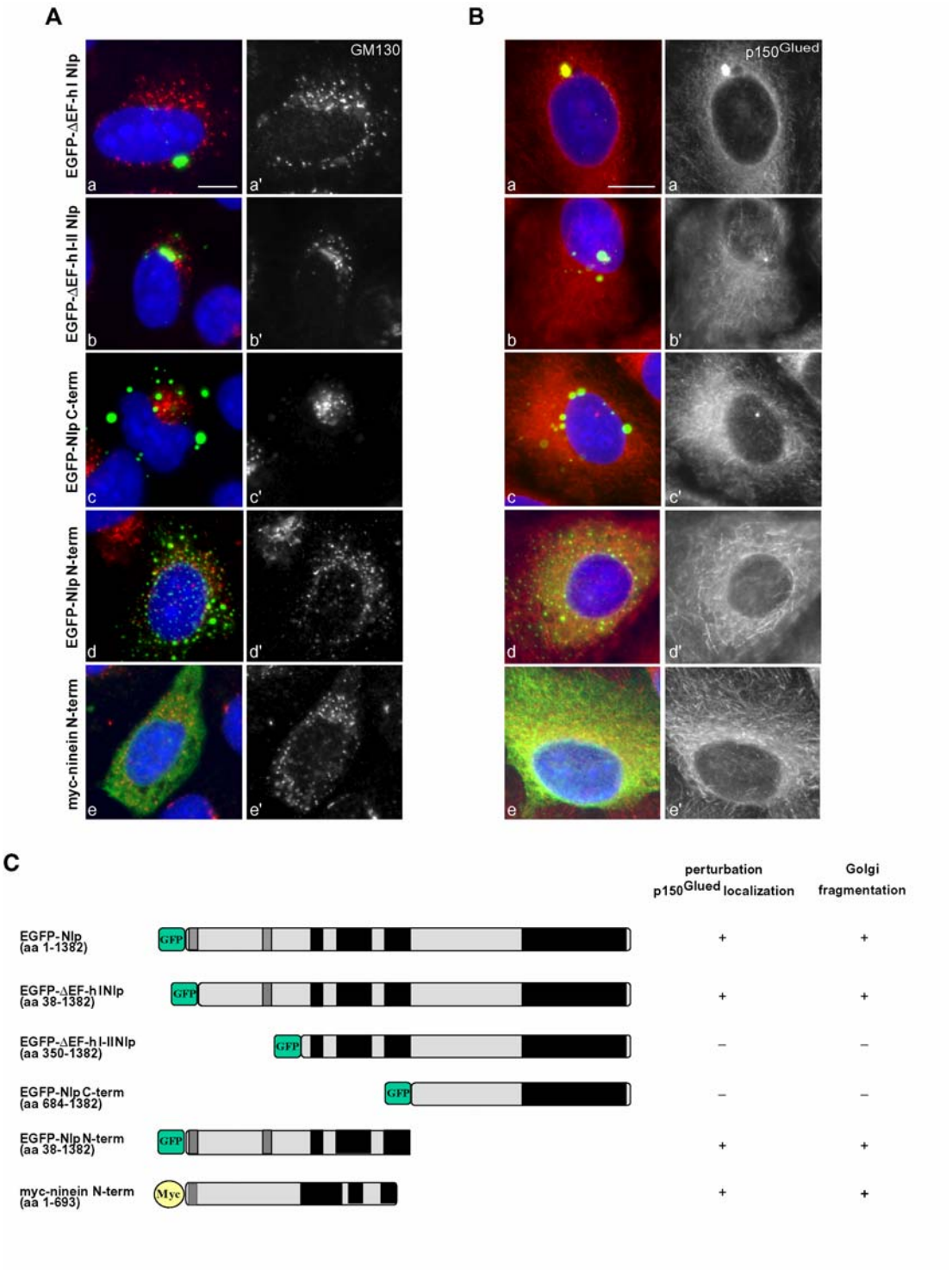

Figure 22. **The ability of Nlp and ninein to disrupt the Golgi complex correlates with their ability to associate with the dynein-dynactin complex**

(**A**) and (**B**) U2OS cells were transfected with EGFP-Nlp ΔEF-h I (Δ1-38aa), EGFP-Nlp ΔEF-h I-II (Δ1-350aa), EGFP-Nlp C-terminal half, EGFP-Nlp N-terminal half, or myc-ninein N-terminal domain and analyzed by immunofluorescence microscopy. Transfected cells were detected through EGFP-fluorescence or anti-myc antibodies (green). The Golgi apparatus was stained with antibodies against GM130 (red) (**A**) and the dynactin complex was stained with antibodies against $p150^{Glued}$ (**B**). DNA was stained with DAPI (blue). Scale bar represents 10µm. (**C**) Schematic representation of Nlp and ninein deletion mutants.

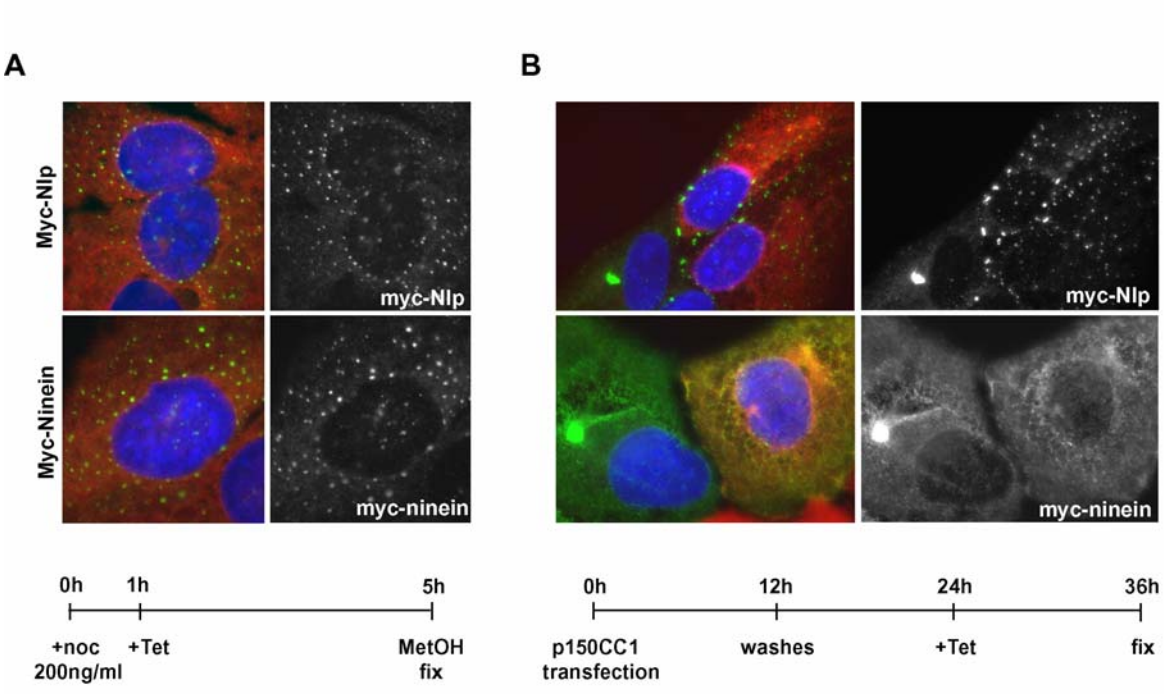

Figure 23. Targeting to the centrosome of Nlp and ninein depends on dynein-mediated transport
(**A**) U2OS stable cell lines expressing myc-Nlp or myc-ninein under the control of a "tet-on" inducible promoter were treated with nocodazole (200 ng/ml) for 1 hour before the expression of the recombinant protein was induced by adding tetracycline to the culture medium. Cells were grown for 5 hours in the presence of tetracycline, fixed and analyzed by immunofluorescence microscopy. Cells were stained with myc-antibodies to detect the overexpressed protein (green) and counterstained with antibodies against α-tubulin (red). (**B**) Tet-on U2OS stable cell lines expressing myc-Nlp or myc-ninein were transfected with DsRed-p150 CC1. 12 hours after transfection the expression of myc-Nlp or myc-ninein was induced by adding tetracycline to the culture medium. Cells were grown for 12 hours in the presence of tetracycline, fixed and analyzed by immunofluorescence microscopy. Cells transfected with p150 CC1 were detected by DsRed–fluorescence. Cells were counterstained with anti-myc antibodies and DNA was stained with DAPI (blue). Scale bar represents 10μm. Schematic representations of the experimental procedures are depicted below the image panels.

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

To investigate the functional consequences of Nlp and ninein association with the dynein-dynactin complex, we asked whether the localisation to the centrosome of the two proteins was microtubule-dependent and mediated by the transport machinery. Immunofluorescence analysis of nocodazole-treated cells

(6μg/ml, 4hr) showed that MT depolymerization caused the displacement of a small fraction of endogenous and overexpressed proteins from the centrosome (data not shown). This suggests that only a small subpopulation of Nlp and ninein localises to the centrosome in a MT-dependent manner, whilst the bulk of the two proteins is stably associated with the organelle. Yet, when the expression of Nlp and ninein was induced in Tet-on U2OS myc-Nlp and myc-ninein stable cell lines, pre-treated with nocodazole, the overexpressed proteins did not form the typical assemblies around the centrosome but many small assemblies throughout the cytoplasm (Figure 23A). To determine whether the targeting of Nlp and ninein to the centrosome was dependent on dynein-dynactin mediated transport, 12 hr before inducing the expression of myc-Nlp and myc-ninein, cells were transfected with DsRed-tagged p150 CC1, a deletion mutant of $p150^{Glued}$ which has been reported to inhibit dynein based motility (Quintyne *et al*., 1999). While Nlp and ninein localized to the centrosome in cells transfected with DsRed alone, no centrosomal assemblies of the two proteins were observed in p150 CC1-expressing cells (Figure 23B). Thus, a functional MT network and the activity of the dynein motor protein are required for the targeting to the centrosome of the newly synthesized Nlp and ninein proteins.

DISCUSSION

In most cell types the Golgi apparatus is found in close association with the MTOC, but the mechanisms responsible for maintaining its dynamic structure and positioning remain poorly understood. It has long been clear that microtubules play a major role in ensuring the proper organization and localization of the Golgi apparatus, mainly through the action of molecular motor complexes. Here we show that the overexpression of the centrosomal protein Nlp and ninein induces the fragmentation of the Golgi apparatus. Remarkably, the ability of Nlp and ninein to affect the Golgi morphology and localization clearly depends on the ability of these two proteins to interact with dynein-dynactin complexes and perturb their intracellular distribution. We also show that the targeting of Nlp and ninein to the centrosome is MT–dependent and requires the activity of the dynein motor complex. Our data suggest that the Golgi vesicles and the centrosomal proteins Nlp and ninein depend on the same components of the transport machinery for their movement toward the minus end of MTs. We therefore propose a model whereby the excess of Nlp and ninein leads to the sequestration of dynein-dynactin complexes, causing the malfunctioning of the dynein-based transport and the loss of the Golgi pericentrosomal localization.

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

Both MT-dependent and MT-independent mechanisms have been implicated in the process of centrosome assembly. With PCM-1, NuMA and pericentrin three examples of centrosomal proteins have been described which rely on MTs and on the activity of the molecular motor cytoplasmic dynein for their recruitment to centrosomes and spindle poles (Dammerman and Merdes, 2002; Dictenberg et al., 1998; Kubo et al., 1999; Merdes et al., 1996; Merdes et al., 2000; Young et al., 2000). Here we show that the targeting to the centrosome of Nlp and ninein

requires a functional MT network and the activity of the molecular motor dynein. Our data also indicate that once Nlp has been assembled onto the centrosome, the bulk of the protein remains stably associated with it. However we can not exclude that a minor fraction of the protein keeps shuttling between a cytoplasmic and a centrosomal pool. Similarly to γ-tubulin (Khodjakov and Rieder, 1999), two distinct population of Nlp and ninein could be present at the centrosome: one that exchanges with the cytoplasmic pool and one that is more stably associated with the organelle. This hypothesis could be verified in future by applying the FRAP (Fluorescence Recovery After Photobleaching) methodology.

Consistent with the requirement of dynein activity for their transport to the centrosome, Nlp and ninein were found to interact with the dynein-dynactin complex in vivo. Our biochemical data suggest that the mechanism of interaction of Nlp and ninein with the dynein-dynactin complex differs from that described for pericentrin. While pericentrin was shown to interact with the light intermediate chain 1 subunit of the motor protein dynein (Purohit et al., 1999; Tynan et al., 2000), Nlp and ninein associate with the dynactin but not the dynein complex in *in vitro* binding assays, as expected if dynactin were to function as an adaptor mediating the interaction of the two centrosomal cargoes with the motor complex. The loss of binding between dynein and dynactin in our pull-down experiments was not surprising since this interaction has been reported to be unstable (Schroer and Sheetz, 1991). Further confirming the interaction of Nlp with the dynactin complex is the finding that Nlp colocalises with the subpopulation of dynactin associated with the mother centriole (Quintyne and Schroer, 2002). Importantly, this result also implies that Nlp, similarly to ninein (Mogensen et al., 2000), primarily localises to the mother centriole.

Because PCM-1 and pericentrin are part of centrosomal particles or granules which probably represent assemblies of large protein complexes (Dammermann and Merdes 2002; Dictenberg et al., 1998; Kubo and Tsukita, 2003; Young et al., 2000), the current model proposes that these proteins could function as carriers which mediate dynein/dynactin-dependent transport of centrosomal components and their assembly onto centrosomes (Zimmerman and Doxsey, 2000). Inhibition of PCM-1 function has been shown to interfere with the

recruitment of ninein, centrin and pericentrin to the centrosome (Dammermann and Merdes, 2002). However, the effect of PCM-1 inhibition on ninein localization appears rather indirect since the depletion of centrin also results in the loss of ninein centrosomal localization (Dammermann and Merdes, 2002). Our data leave open the question of whether the loss of Nlp and ninein centrosomal localization observed upon MT-depolymerization or inhibition of the dynein motor activity is the direct effect of an impaired transport of these centrosomal components. We can not exclude that dynein mediates the transport of other centrosomal factors necessary for the recruitment to the centrosome of Nlp and ninein which would then move towards the centrosome by a different mechanism (e.g. passive diffusion). A kinetic analysis would be required to visualize the direct movement of these two proteins along the MTs towards the centrosome. Furthermore, a more detailed biochemical analysis of the interaction between Nlp or ninein and the dynactin complex would be necessary to verify whether this interaction is direct and to identify which subunit(s) of dynactin mediate the binding of the two centrosomal proteins. Alternatively, it is possible that Nlp or ninein do not interact directly with dynactin, but are transported to the centrosome as part of larger assemblies of centrosomal proteins (Dammermann and Merdes, 2002; Young et al., 2000; Zimmerman and Doxsey, 2000). A third possibility is that the binding of Nlp or ninein to the dynein-dynactin complex would require an additional adaptor (see below).

Molecular basis for the Nlp and ninein overexpression phenotype

Two distinct isoforms of dynein heavy chains, DHC1 and DHC2 have been implicated in controlling the shaping and positioning of the Golgi apparatus (Grissom *et al*., 2002; Harada *et al*.,1998; Vaisberg *et al*., 1996), but which of these isoforms is primarily involved in Golgi organization remains an open question. A clear understanding of the role played by the dynein-dynactin complexes in regulating Golgi structure and localization is complicated by the fact that the exact subunit composition of the distinct dynein complexes acting at the

Golgi apparatus is still largely unknown. Furthermore, the molecular mechanisms underlying the interactions of the dynein motor protein with membrane organelles are just starting to be elucidated (Fath *et al*., 1997; Holleran *et al*., 2001; Hoogenraad *et al*., 2001; Matanis *et al*., 2002; Muresan *et al*., 2001; Short *et al*., 2002). Despite this complicated picture, our results allow some simple conclusions to be drawn. We have shown that the ability of Nlp or ninein to induce the fragmentation of the Golgi apparatus clearly correlates with their ability to affect the intracellular distribution of the dynein-dynactin complexes (Figure 22). Therefore, we propose a sequestration model to explain the Nlp and ninein overexpression phenotype (Figure 24). Two possible mechanisms have to be taken into consideration. One possibility is that Nlp or ninein could directly interact with dynactin and titrate out the dynein-dynactin complexes, therefore having a general inhibitory effect on dynein-mediated transport (Figure 24A). However, the observation that the N-terminal domain of Nlp and ninein displaced dynactin from the centrosome without altering its localization at the plus end of MTs, suggests that Nlp and ninein can associate only with a proportion of the total population of dynactin complexes. The second plausible mechanim would predict that Nlp and ninein could specifically interact with a subpopulation of dynein-dynactin complexes also responsible for mediating the transport of Golgi vesicles toward the centrosomal region (Figure 24B). Recently BICD1 and BICD2 have been proposed to function as novel components of the dynein-dynactin transport machinery (Hoogenraad *et al*., 2001; Matanis *et al*., 2002; Short *et al*., 2002) and to be primarly involved in targeting the cytoplasmic dynein-dynactin complexes to Golgi membranes (Hoogenraad *et al*., 2002; Short *et al*., 2002). This suggests that the binding of the dynein-dynactin complex to particular cargoes could require the presence of additional adaptors. This could represents another mechanism for regulating the motor-cargo interaction and for conferring to the motor protein the ability to specifically recognize a wider range of cargo. We therefore envision that a hypothetical additional adaptor could be required for the interaction of the dynein-dynactin complex with both centrosomal proteins and Golgi vesicles, identifying a subpopulation of complexes responsible for the transport of these two classes of cargoes. The validation of this model certainly awaits the

identification of the hypothetical additional adaptor(s). Possible candidates are the BICD proteins, even if the fact that *in vivo* they are found mainly associated with Golgi membranes and vesicle-like structures does not particularly support this hypothesis. The identification of such adaptor(s) would provide new insights in understanding the mechanisms regulating motor-cargo interactions.

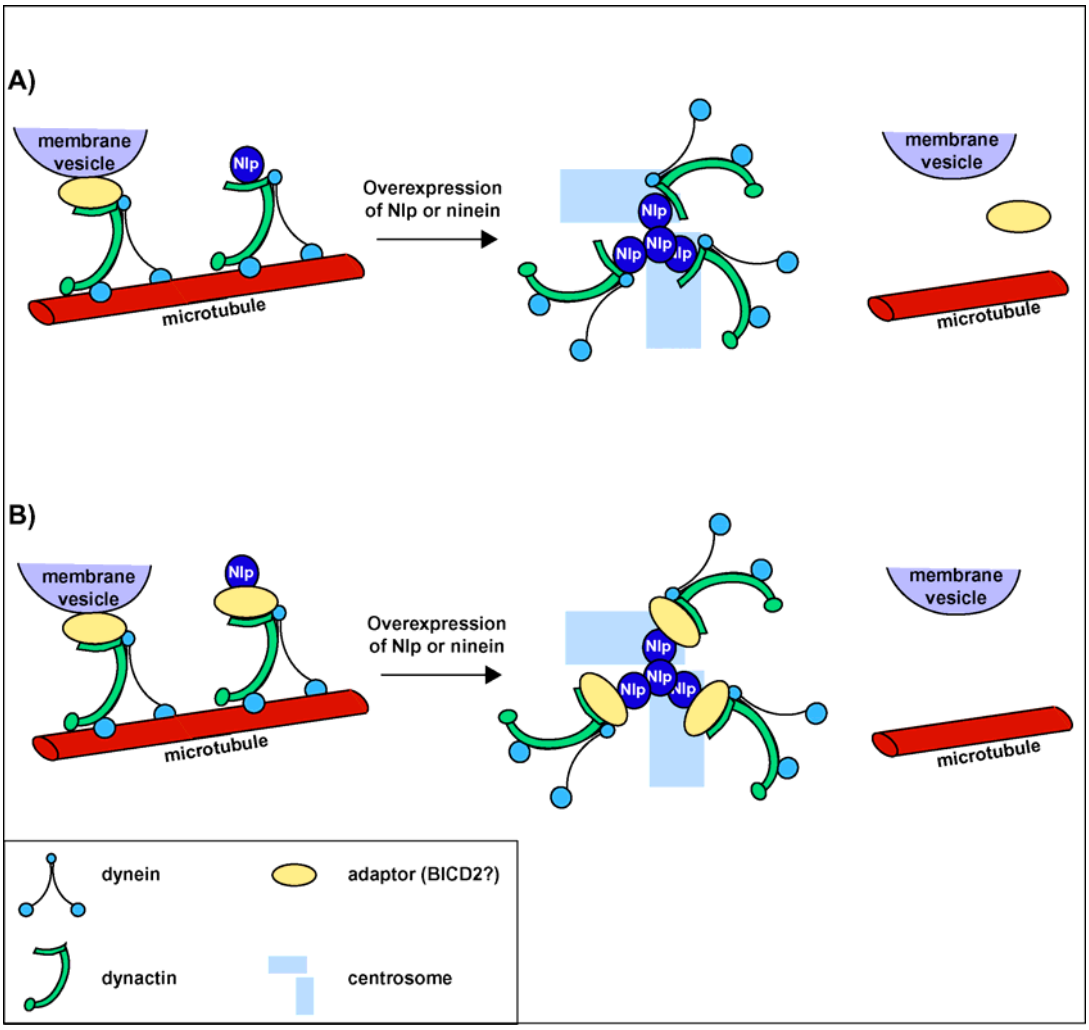

Figure 24. A sequestration model to explain Nlp and ninein overexpression phenotypes

The model proposes two possible molecular mechanisms underlying the Golgi fragmentation phenotype induced by the overexpression of Nlp or ninein. If Nlp and ninein associate directly with the dynein-dynactin complex, the excess of these two centrosomal proteins could sequester the complex having a general inhibitory role on dynein-mediated transport (**A**). Alternatively, the association of the dynein-dynactin complex to Golgi vescicles and centrosomal proteins such as Nlp and ninein could be mediated by the same protein "adaptor" (**B**). In this case, the excess of Nlp and ninein would specifically sequester a supopulation of dynein-dynactin complexes responsible for the transport of Golgi vescicles and the maintanance of the Golgi organization.

Functional consequences for the loss of the Golgi apparatus pericentrosomal localization

Why the Golgi apparatus is closely associated with centrosomes in most cell types remains a question of considerable interest. In fact, the pericentrosomal localization of the Golgi apparatus is not crucial for its function, in particular secretion, as clearly demonstrated by the fact that in some polarized cell types the Golgi is not localized in the proximity of centrosomes or MTOCs (Achler *et al*., 1989; Bacallao *et al*., 1989). Furthermore, it has been shown that the fragmentation of the Golgi apparatus induced by nocodazole treatment does not interfere with the functionality of this organelle (Cole *et al*., 1996; Rogalski *et al*., 1984).

A possible explanation is that the tight association between the centrosome/MTOC and the Golgi apparatus could be crucial to allow the cell to rapidly modify its internal organization under conditions where cell architecture as a whole must undergo remodelling in response to external stimuli. This occurs, for example, during cell migration or cell polarisation. Indeed, fibroblasts and endothelial cells at the edge of a wounded monolayer, as well as migrating macrophages, localize their MTOC and the associated Golgi apparatus in a position between the nucleus and the leading edge of the cell (Gotlieb *et al*., 1983; Magdalena *et al*., 2003; Nemere *et al*., 1985). A similar reorientation of the MTOC and the Golgi apparatus occurs in T lymphocytes in response to antigen specific loaded APCs, as well as in natural killer (NK) cells and in cytotoxic T lymphocytes (CTLs) when they interact with target cells (Geiger *et al*., 1982; Kupfer *et al*., 1983; Kupfer *et al*., 1987). Whereas the repositioning of the centrosome in migrating cells is required for stabilizing the cell movement (Schliwa *et al*., 1999), in T cells the polarization of the Golgi apparatus/MTOC ensures the restricted delivery of secreted lymphokines to the appropriate APC but not to nearby cells (Kupfer *et al*., 1987; Kupfer *et al*., 1994) and contributes to the maturation of the immunological synapse (Davis and van der Merwe 2001; Krummel *et al*., 2000).

Thus, by inducing the dissociation of the Golgi apparatus from the centrosome, the overexpression of the centrosomal proteins Nlp and ninein could dramatically interfere with cell migration and polarization processes which normally require a highly coordinated action of these two organelles. Consistent with this idea is the finding that the overexpression of ninein inhibits cell movement (Abal *et al*., 2002). Bornens and coworkers have shown that this inhibitory effect is primarily due to the suppression of microtubule release from the centrosomes of ninein-overexpressing cells. The data we have presented here suggest that the fragmentation of the Golgi induced by ninein overexpression could also contribute to the observed inhibitory effect on cell movement.

To investigate the possible consequences of Nlp and ninein overexpression on crucial cellular processes such as cell migration and polarization certainly represents an intriguing task for future work.

CONCLUSIONS

Although the centrosome was described for the first time more then a century ago, our understanding of its molecular composition is still limited. A full appreciation of how centrosomes contribute to cellular functions requires the isolation and characterization of unknown centrosome-associated molecules. This project aimed at characterizing the novel human centrosomal component Nlp (ninein-like protein). The observation that Nlp shares 37% identity with the centrosomal protein ninein over its N-terminal domain, led us to identify Nlp as a possible second member of the Ninein protein familiy. A comparative analysis was therefore performed in order to verify to which extent the two proteins shared similar functions. Both protein have been shown to primarily associate with the mother centriole and to play a major role in MT organization (Mogensen et al., 2000; cfr PartI–results and Part II-results). Whereas ninein has been proposed to function in MT anchoring (Mogensen *et al*., 2000), the data presented here strongly indicate that Nlp is rather involved in the MT nucleation activity, playing an important role in the recruitment of the γTuRC to the centrosome. However, no proper assays are presently available to rigorously distinguish various MT minus-end associated activities. Therefore, it would be premature to exclude that Nlp may also contribute to MT anchoring. Immunofluorescence analysis of Nlp localization in cochlear epithelial cells characterized by the presence of a MT-anchoring site spatially separated from the MT-nucleating site (Mogensen *et al*., 1997) would certainly contribute to resolve this issue.

We have also shown that both Nlp and ninein can associate with the dynein-dynactin complex and they depend on dynein-mediated transport for their targeting to the centrosome. Inhibition of the dynein motor protein activity has been shown to perturb microtubule organization in interphasic and in mitotic cells (Gaglio *et al*., 1997; Heald *et al*., 1997; Quintyne *et al*., 1999). Indeed the dynein-dynactin complex plays a direct role in maintaining the MT organization through its focusing and anchoring activities (Gaglio *et al*., 1997; Heald *et al*., 1997; Quntyne *et al*.,1999) but can also indirectly influence centrosomal functions by mediating

the transport to the centrosome of nucleating and anchoring activities (Dammerman and Merdes, 2002; Young *et al.* 2000). This raises the question of whether the ability of Nlp and ninein to affect MT organization is a mere consequence of their ability to recruit the dynein-dynactin complex. Our data show that whereas both proteins can associate with the dynein-dynactin complexes, only Nlp specifically displays the ability to interact with the γTuRC and affect MT nucleation, strongly supporting a direct involvement of Nlp in regulating the centrosomal MT nucleation activity. An interesting open question is whether the sequestration of the dynein-dynactin complexes represents the molecular basis explaining the ability of Nlp to induce aberrant spindles when expressed in mitotic cells. This is consistent with the finding that the overexpression of the centrosomal protein pericentrin induces similar mitotic defects by affecting dynein localization (Purohit *et al*., 1999; Tynan *et al*., 2000)

The sequestration of dynein-dynactin complexes is possibly the molecular mechanism explaining the ability of Nlp and ninein to induce the disruption of the Golgi apparatus. The dissociation of the Golgi apparatus from the centrosome induced by the overexpression of these centrosomal proteins could interfere with cell migration and polarization processes which instead require a highly coordinated action of these two organelles. Cell migration and cell polarization represent critical events for for immune responses as well as for embryonic development, invasive growth and metastasis. This suggests the intriguing possibility that an upregulation in the expression levels of structural centrosomal proteins could represent the molecular basis for developmental disorders and malfunctioning of the immune system and, on the other hand, modulate the acquisition of invasive properties by neoplastic cells.

MATERIALS AND METHODS

Preparation of plasmids

hNlp gene

The KIAA0980/Nlp cDNA was obtained from the Kazusa DNA Research Institute in pBluescript II SK (PM72). It was completely sequenced, excised SalI/NotI and inserted into the pEGFP-C1 mammalian expression vector (PM74). This construct contained a stretch of 91 bp before the start codon (GTCGACCCACGCGTCCGGCGGTACGGGGGTGGTGCCGCGCTCCTGGCCC CGCGCGGGCGGACGGCGGAGGCGCCTCCCAGCCTGCTATGGGATGGATGA AGAA). In order to remove the 91 bp strech, PCR was performed to introduce a SalI-NdeI site at the start codon amplifing the SalI-NdeI/AflII fragment and re-inserting it in PM72 (PM 87). The Nlp gene was then excised NdeI (blunted with Klenow)/NotI from PM 87 and inserted in pBluescript II KS-myc vector. The myc-tagged Nlp was further introduced into the pCDNA4/TO vector for the generation of tetracycline-inducible stable cell lines. To generate Glutathione-S-transferase (GST)-tagged Nlp (MC14), the gene was excised SalI blunt/NotI from PM87 and inserted into the pGEX-6P-3 bacterial expression vector.

EGFP-, GST- and polyhistidine (His)-tagged Nlp deletion mutants were produced and they are listed in Table 2.

To generate the Nlp EF-h mutants, mutagenesis was performed following the Quickchange site-directed mutagenesis method (12 to 18 PCR cycles with Pfu Turbo Polymerase, using to complementary oligos with the codon to be mutated in the middle; addition of DpnI to the PCR reaction and 1h incubation at 37°C, to specificallly digest the template methylated DNA; 10 μl of the resulting reaction were used to transform 100 μl of chemically competent XL1Blue bacteria, which were then plated on the appropriate selectve media and eventually processed to isolate the DNA).

The pBluescript II KS-Nlp N-N-terminal (PM 121) construct was used as a template. Mutagenic oligos were designed to introduce the point mutations E31K and E257K in the first (EF-h I) and in the second (EF-h II) EF-h domain respectively.

mNinein gene

The cDNA encoding for the mouse ninein gene was kindly provided by M. Bornens in the pCB6 vector (PM89). It was excised Eco47III/Bam HI and introduced into the pEGFP-C1 and pBluescript II KS-myc vectors The myc-tagged ninein was further introduced into the pCDNA4/TO vector for the generation of tetracycline-inducible stable cell lines.

GST-, EGFP- and Myc-tagged deletion mutant encoding for the N-terminal domain of ninein were genarated and they are listed in Table 2 .

Table 2. Summary of Nlp and ninein deletion mutants

Deletion mutants	*Insert*	*Tag*	*Vector*
∆EF-h I Nlp (MC36)	hNlp (aa 38-1382)	EGFP	pEGFP-C1
∆EF-h I-II Nlp(PM96)	hNlp (aa 350-1382)	EGFP	pEGFP-C1
N-terminal Nlp (MC9)	hNlp (aa 1-702)	EGFP	pEGFP-C1
N-terminal Nlp (MC15)	hNlp (aa 1-702)	GST	pGEX-6P-3
C-terminal Nlp (MC10)	hNlp (aa 694-1382)	EGFP	pEGFP-C1
C-terminal Nlp (MC16)	hNlp (aa 694-1382)	GST	pGEX-6P-3
C-terminal Nlp (PM76)	hNlp (aa 909-1382)	His_6	pQE-30
N-terminal ninein (MC44)	mNinein (aa 1-750)	GST	pDEST15
N-terminal ninein (MC35)	mNinein (aa 1-693)	EGFP	pEGFP
N-terminal ninein (MC34)	mNinein (aa 1-693)	Myc	pBK-CMV

Wild type and mutant (T210D and K82R) Plk1 plasmids have been described (Meraldi *et al*., 2002; Smits *et al*., 2000). The EGFP-C-Nap1 plasmid has been published in Mayor et al., 2002. The DsRed p150 CC1 constructs was a kind gift of T. Schroer (Quintyne and Schroer, 2002).

Antibody production

Rabbit antibodies were raised against the N-terminal and the C-terminal domains of Nlp, both expressed in *E.coli*. Data shown in this study were obtained primarily with the antibody directed against the N-terminus, but we emphasize that both antibodies produced qualitatively similar results. GST-N-term Nlp (residues 1-702) (MC15) was isolated as inclusion bodies, whilst the His_6-C-term Nlp (residues 909-1382) (PM76) was purified over a Ni^{2+}-agarose column under denaturing conditions. The purified fragments were injected into New-Zealand white rabbits at 4-week intervals (500 µg for each injection). Antibodies against the N-terminal half of Nlp were affinity-purified on recombinant antigen, from which the GST-moiety had been cleaved by Prescission Protease (Amersham Pharmacia Biotech), bound to an AffiGel 15 (Biorad) column. Antibodies against the C-terminal fragment of Nlp were affinity-purified by elution from nitrocellulose strips bearing EGFP-Nlp expressed in 293 cells.

Cell culture, transfection and generation of stable cell lines

Human U2OS osteosarcoma, 293 epithelial kidney and KE 37 T-lymphoblastoid cells were grown at 37°C in a 5% CO_2 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 100µg/ml, respectively, all Gibco-BRL). For synchronization in G1/S phase U2OS cells were treated for 14hr with 1.6 µg/ml aphidicolin (Sigma, A-6781), washed 3 times with pre-warmed medium (during the last wash cells were incubated 5 min at 37°C), reincubated in normal medium for 12hr, treated again with 1.6 µg/ml aphidicolin for 14hr and collected. For G2/M synchronization U2OS were treated with aphidicolin as described above, released in normal medium for 9hr and collected. For synchronization in mitosis U2OS cells were treted with 0.5 µg/ml for 16hr and collected by mechanical shake-off.

Transient transfection of U2OS and 293 cells was performed using calcium phosphate precipitates as described previously (Seelos, 1997).

To generate the Tet-on U2OS myc-Nlp and myc-ninein stable cell lines, $5x10^5$ Tet-On U20S cells (Invitrogen) were transfected in a 10cm dish with 20µg of plasmid DNA, using the calcium phosphate precipitation method (Krek and Nigg, 1991). 24 hours after transfection cells were placed under Zeocin (Invitrogen, R250-05) selection for 14 days and diluted for single cell colony formation. The stable cell lines were grown at 37°C in a 5% CO_2 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 100µg/ml, respectively, all Gibco-BRL) under selection of 200µg/ml Zeocin and 50µg/ml Hygromycin. Expression of myc-Nlp or myc-ninein fusion proteins was induced adding 1µg /ml tetracycline (Invitrogen, Q100-19) to the medium.

The Tet-on U2OS EGFP-C-Nap1 stable cell line has been described (Mayor *et al*., 2002).

Cell extracts and pull-down experiments

For pull-down experiments performed to verify the interactions with the γTuRCs components (adapted from Murphy *et al*., 2001), cells were washed once with cold HBS (50mM Hepes, pH 7.4, 150mM NaCl) and collected into cold HBS +1mM PMSF at 4°C. Cells were lysed in 50mM Hepes, pH 7.4, 150mM NaCl, 2mM EDTA, 1mM DTT, 0.5% Triton X-100, 0.25mM GTP, 1mM$MgCl_2$, 1mM PMSF, and aprotinin, leupeptin, pepstatin at 1µg/ml each and incubated for 10 min on ice. Lysates were clarified (10,000 rpm, 4°C, 20min) and incubated with glutathione beads carrying recombinant GST-fusion proteins for 3hr at 4°C. The glutathione beads were then washed once in lysis buffer, once in 50mM Hepes, pH 7.4, 250mM NaCl, once in HBS, and finally resuspended in gel sample buffer.

For pull-down experiments performed to verify interactions with dynactin complexes cells were washed once in cold PBS, collected in PBS+1mM PMSF at

4°C and lysed in 25mM Tris, pH 8, 50mM NaCl, 0.5% Triton X-100, 1mM PMSF, 1mM NaF, and aprotinin, leupeptin, pepstatin at 1μg/ml each, and incubated for 30 min on ice. Lysates were clarified and incubated with glutathione beads carrying the recombinant proteins as described above. The glutathione beads were then washed once in lysis buffer, once in 50mM Tris, pH 8, 250mM NaCl, once again in lysis buffer and resuspended in gel sample buffer.

Immunoprecipitation and immunoblotting experiments

For immunoprecipitation experiments, cells were washed once in PBS + 1mM PMSF and resuspended in RIPA extraction buffer (100mM Tris-HCl, pH 8, 150mM NaCl, 1% Igepal, 1mM EGTA, 1mM EDTA, 0.5% Na deoxycholate, 0.1% SDS, 1mM PMSF, aprotinin, leupeptin, pepstatin at 1μg/ml each, 1mM Na_3VO_4, 1mM NaF). Extracts were left on ice for 30 min, clarified (12,000rpm, 4°C, 20min) and the protein concentration of the cell lysates was determined using the Bradford assay. Cell lysates were then pre-absorbed on protein A beads (Affiprep, Biorad) for 45min at 4°C and incubated with beads bearing anti-Nlp antibodies, pre-immune sera, affinity-purified anti-hHec1 rabbit polyclonal antibodies (Martin-Lluesma *et al*., 2002) or non-immune rabbit IgG (Sigma-Aldrich) at 4°C for 2 hours. Immunoprecipitates were washed three times in RIPA buffer and resuspended in gel sample buffer. Proteins samples were then separated on SDS-PAGE and electrophoretically transferred to nitrocellulose membranes using a semy-dry transfer system. For immunoblotting with anti-Nlp antibodies (1μg/ml) proteins were transferred to PVDF membranes using a tank transfer system. Membranes were incubated for 1h in blocking buffer (5% low-fat dry milk in PBS/0.1% Tween-20). All antibody incubations were carried out in blocking buffer for 1h at room temperature or overnight at 4°C (anti-myc monoclonal antibodies 1:2 dilution; anti-GFP monoclonal antibodies 1:2 dilution; anti-GST monoclonal antibodies 1:2 dilution; anti γ-tubulin monoclonal antibodies, clone GTU-88, Sigma 1:1000 dilution; anti-hHec1 rabbit polyclonal antibodies, 1:500 dilution from serum;

anti-cyclin B1, Upstate Biotechnology cat # 05-373, 1:1000 dilution; anti-DIC monoclonal antibodies, Sigma D-5167, 1:200 dilution; anti-p150^{Glued} monoclonal antibodies Translab laboartories cat # 610474, 1:150 dilution; anti-dynactin p50 monoclonal antibodies, Translab laboratories, cat # 611002, 1:100 dilution). Immunoblots were revealed using enhanced chemiluminescence detection system (Pierce).

Purification of recombinant proteins

GST full length, N- and C-terminal halves of Nlp and GST-N-term ninein were expressed in *E. Coli* BL21RIL. For the expression of the recombinant proteins, the overnight bacterial cultures were diluted 1:100 and bacteria were grown at 30°C in LB medium under Ampicillin selection until the OD_{600} was approximately 0.5. The expression of the recombinant proteins was induced adding IPTG to a final concentration of 1mM and the cultures were grown for additional 15 hr at 20°C. Cells were then collected, resuspended in RIPA buffer + protease inhibitors (for the purification of GST-Nlp full length) or in 50mM Tris, pH8, 300mM NaCl, 0.5% Igepal + protease inhibitors (for the purification of the N- and C-terminal halves of Nlp and GST-N-term ninein) and mechanically disrupted using a French Press. The lysates were clarified (15,000rpm, 4°C, 45min) and then incubated with glutathione beads overnight at 4°C an a rotating wheel. Glutathione beads carrying the recombinants proteins were washed 3 times in lysis buffer, twice in 100mM Tris, pH 8, 120mM NaCl. Elution was performed in 100mM Tris, pH8, 120mM NaCl, 20mM Glutathione (3 batch elutions for 45 mins each on a rotating wheel).

Recombinant EGFP-Nlp and EGFP-C-Nap1 assemblies were purified from transfected U2OS cells, using a rapid centrosome purification method developed for adherent cells (Blomberg-Wirschell and Doxsey, 1998). U2OS cells were transfected with EGFP-Nlp or EGFP-C-Nap1 and the aggregates purfied 24 hours later. 1 hour before purification cells were treated with 60ng/ml nocodazole and 1μg/ml Cytochalasin D. Cells were washed at 4°C once in PBS, once in 1/10 PBS

+ 8 % Sucrose, once in H_2O + 8 % Sucrose and then incubated for 10 minutes in lysis buffer (1mM TrisHCl, pH 8.0, 0.1% β-Mercaptoethanol, 0.5% Triton-X 100, 1mM PMSF, 1μg/ml Aprotinin, 1μg/ml Leupeptin, 1 μg/ml Pepstatin). Hepes pH 7.2 and EDTA were added to the lysate to a final concentration of 10 mM and 1mM respectively before loading it on 20% Ficoll cushion (20% w/v Ficoll 400.000, 10mM Hepes, pH 7.2, 1mM EDTA, 0.1% Triton-X 100). Samples were centrifuged for 20 minutes at 12500 rpm in a HB-6 rotor at 4°C, the supernatant was removed and the interface region of the Ficoll cushion containing the aggregates collected.

Aster formation assays in Xenopus egg extracts

Cytostatic factor (CSF)-arrested *Xenopus* egg extracts (M phase) were prepared as described (Murray, 1991). For the aster assembly assay, purified recombinant EGFP-Nlp and EGFP-C-Nap1 assemblies, as well as co-purifying centrosomes, were added to 20μl of extract. The reactions were incubated for 30 min at 20°C and diluted with 1 ml BRB80 (80 mM K-PIPES, pH 6.8, 1 mM EGTA, and 1 mM MgCl2) containing 10% glycerol, 0.25% glutaraldehyde, 1 mM GTP, and 0.1% Triton X-100 and subsequently centrifuged (12,000 rpm, 12 min, 16°C) through a 25% glycerol cushion in BRB80 onto coverslips as described (Sawin and Mitchison, 1991). The coverslips were fixed in -20°C methanol, incubated twice for 10 min in 0.1% $NaBH_4$ in PBS and processed for immunofluorescence.

Antibody microinjection

For microinjection experiments U2OS cells were seeded onto HCl-treated coverslips and injected when they reached 50-60% of confluence. Microinjections were performed using the micromanipulator 5171 coupled to the FemtoJet (Eppendorf). Antibody were injected into the cytoplasm. Both anti-N-term 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) to yeld a concentration of 2mg/ml for injections. Before injection, the antibody were centrifuged at 55,000 rpm for 30 min at 4°C in a TLA55 rotor using polyallomer microfuge tubes in a Optima centrifuge (Beckman).

Immunofluorescence microscopy

Cells were grown on HCl-treated coverslips and fixed with methanol at –20°C for 6 min or longer. For immunostaining of microtubules and microtubule-associated proteins ($p150^{Glued}$, DIC), cells were fixed for 5 min with methanol at –20°C. Coverslips were washed three times in PBS and incubated for 30 min at room temperature in blocking solution (PBS-0.05% Tween20, 3% bovine serum albumin, BSA). All subsequent antibody incubations were carried out in blocking solution. Primary antibodies were anti-Nlp antibodies (affinity-purified 1µg/ml), GT335 monoclonal antibodies (1:10,000 dilution from ascites, gift of B. Eddé), rabbit anti-C-Nap1 antibodies (affinity-purified; 1µg/ml) (Fry *et al*., 1998a), rabbit anti-myc antibodies (1:800 dilution, SantaCruz Biotechnology, sc-789), α-tubulin monoclonal antibodies (1:2000 dilution from ascites, clone B-5-1-2, Sigma), rabbit anti-γ-tubulin antibodies (purified IgG 5µg/ml) (Fry *et al*., 1998b) anti-γ-tubulin monoclonal antibodies (1:1000 dilution from ascites, clone GTU-88, Sigma), anti-$p150^{Glued}$ monoclonal antibodies (1:150 dilution, Translab laboratories cat # 610474), anti-DIC monoclonal antibodies (1:100 dilution, Sigma D5167), sheep anti-GM130 antibodies (1:2000 dilution from serum, gift from F. Barr). Incubations with primary antibodies were for 1 hour at room temperature in a humidified chamber, followed by three washes with PBS-0.05% Tween20. Secondary antibodies were biotinylated donkey anti-rabbit or sheep anti-mouse (1:200, Amersham) followed by Texas red-conjugated streptavidin (1:100, Amersham), Alexa Fluor 488 conjugated goat anti-mouse or anti-rabbit IgG (1:1000, Molecular Probes), Cy5-coniugated donkey anti-rabbit (1:100, Jackson ImmunoResearch laboratories), Cy2-coniugated donkey anti-sheep (1:1000, Jackson

ImmunoResearch laboratories). DAPI (2 µg/ml) was included in the last incubation to stain DNA. Following three washes with PBS and two final washes in water, coverslips were mounted in 80% glycerol, 3% DABCO (in PBS) mounting medium. Immunofluorescence microscopy was performed using a Zeiss Axioplan II microscope and 63X oil immersion objectives. Photograph were taken using Micromax (Princeton Instruments) CCD cameras and Metaview (Universal Imaging) softwares. For high resolution images a Deltavision microscope on a Nikon TE200 base (Applied Precision), equipped with an APOPLAN 100x 1.4 oil immersion objective, was used for collecting optical sections at distances of 0.2µm in the Z-axis. Pictures at individual focal planes were processed with a deconvolution algorhythm and then projected into one picture using Softworx (Applied Precision).

Mass spectrometry

Coomassie Blue stained protein bands were in-gel digested (Shevchenko *et al*., 1996) by trypsin (Promega, sequencing grade) or LysC (Roche, sequencing grade). Matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) mass spectra were acquired on a Reflex III instrument (Bruker Daltonik) in positive or negative ion mode. As a matrix, 2,5 dihydroxybenzoic acid (Bruker Daltonik) or α-cyano-4-hydroxycinnamic acid (Bruker Daltonik) were used. Mass spectra were searched for signals showing a mass difference of 80 mass units (phosphate group) by a self-developed software script. Such phosphopeptide-candidates were submitted to post-source decay (PSD) fragment ion analysis (Hoffmann *et al*., 1999). Peptides showing the typical losses of 98 mass units (phosphoric acid) and 80 mass units (phosphate group) were considered as phosphopeptides.

Miscellaneous Techniques

Human centrosomes were purified from the T-lymphoblastic cell line KE37 according to Moudjou and Bornens (1994). MT regrowth assays in asynchronously growing U2OS cells were performed by placing tissue culture dishes for 30 min on ice, before MT regrowth was induced by adding prewarmed medium (37°C) and placing the dishes for 45s at 37°C. Then, cells were pre-extracted for 40 sec in 1X BRB80 (80 mM K-PIPES, pH 6.8, 1 mM EGTA, and 1 mM MgCl2) containing 0.1% Triton X-100, fixed with cold methanol and processed for immunofluorescence microscopy.

In vitro translated proteins were synthesized from plasmid DNA templates using the TNT coupled reticulocyte system following manufacturer instruction (Promega).

To induce Golgi fragmentation, cells were treated with 200ng/ml nocodazole for 1h, fixed in methanol and processed for immunofluorescence.

ACKNOWLEDGMENTS

First, I would like to thank Prof. Erich Nigg for having given me the opportunity to work in his laboratory. This has represented an important experience, which allowed me to improve a lot my scientific background.

I am sincerely grateful to Dr. Luigina Renzi, for the support and the precious advises that she gave me, and to Prof. Maurizio Gatti. Without their help all this would have never happened.

My thanks are owed to the people who contributed to this work: Dr. Peter Duncan, who performed the two hybrid screen that led to the isolation of Nlp, and Dr. Patrick Meraldi who characterized the phosphorylation of Nlp by Plk1. I sincerely thank Dr. Roman Körner for performing the Mass Spectrometry analysis and Ulrike Hacker for her precious help in raising and screening the anti-Nlp antibodies and generating the stable cell lines.

I would like to thank all the past and present members of the lab.

In particular, I thank Patrick Meraldi, for being my supervisor and guiding me through the secrets of the centrosome, and Francis Barr for the countless helpful advises and stimulating discussions.

My sincere thanks are owed to my “lab-mates” (Peter, Olaf and Silvia) for the really enjoyable, friendly and stimulating atmosphere that they were able to create in the lab. I hope that Peter one day will forgive me for having to repeat his best jocks at least twice before I could get them! I thank “Olaf-ClonePower, ja man” for his generosity and Silvia for all our nice discussions.

I am grateful to the people who have seen the beginning of this experience: Catherine, Thibault, Olaf, Peter, Silvia, George, Clemens, Paolo, Giulia…their presence made everything a bit more shining. In particular, I want to thank Paolo for having revealed me the “Principle of the Bulb” and for the strawberry-chocolate-flavored discussions. I will never thank Giulia enough for having chosen to share so much!!! I am really really glad that Ben and Björn “shared” all the way through.

I thank Guido for giving the nicest meaning to an end…Grazie!

Ringrazio i miei genitori, per avermi dato ed insegnato l’essenziale e per essere sempre stati cosi’ piacevolmente presenti nonostante la distanza.

Ringrazio Chiara e Matteo per la loro colorata compagnia.

Ringrazio la mia Gente a Roma (Antonio, Marco, Francesca, FrancescaBella, Filippo, Daniele , Enrico, Carlo) e quella sparsa per l’Italia (Filo, Mazzo, Chicco) e per l’Europa (Chiara, Francesco e Simone). Grazie perche’ quello che c’era e’ rimasto…e nonostante la lontananza si continua a crescere (o a rifiutarsi di crescere!) insieme.

Un Grazie di cuore a Chiara e Arianna per essere da sempre un punto di riferimento cosi’ caro e prezioso per ritrovare il senso del Vero quando tutto sembra scivolare via impalpabile tra le dita.

ABBREVIATIONS

AA	amino acid
APC	antigen-presenting cell
BSA	bovine serum albumin
Cdk	cyclin-dependent kinase
CMV	cytomegalovirus
CTL	cytotoxic lymphocyte
DAPI	4',6-diamidino-2-phenylindole
DHC	dynein heavy chain
DIC	dynein intermediate chain
DLC	dynein light chain
DLIC	dynein light intermediate chain
DNA	deoxyribonucleic acid
DTT	dithiothreitol
ECL	enhanced chemiluminescensce
EDTA	ethylenedinitrilotetraacetic acid
EGFP	enhanced green fluorescent protein
GST	glutathione-S transferase
GTBP	γ-tubulin binding protein
GTP	guanosine triphosphate
HCl	hydrochloric acid
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethane sulfonic acid
IgG	immunoglobulin G
IP	immunoprecipitation
MTs	microtubules
MTOC	microtubule organizing centre
NK	natural killer (cells)
PBS	phosphate-buffered saline
PCM	pericentriolar material
PCR	polymerase chain reaction

PIPES	1,4-Piperazinediethanesulfonic acid
PMSF	phenylmethylsulfonyl fluoride
SPB	spindle pole body
γTuRC	γ-tubulin ring complexes
WT	wild-type

REFERENCES

Abal,M., Piel,M., Bouckson-Castaing,V., Mogensen,M., Sibarita,J.B., and Bornens,M. (2002). Microtubule release from the centrosome in migrating cells. J. Cell Biol. *159*, 731-737.

Achler,C., Filmer,D., Merte,C., and Drenckhahn,D. (1989). Role of microtubules in polarized delivery of apical membrane proteins to the brush border of the intestinal epithelium. J Cell Biol. *109*, 179-189.

Addinall,S.G., Mayr,P.S., Doyle,S., Sheehan,J.K., Woodman,P.G., and Allan,V.J. (2001). Phosphorylation by cdc2-CyclinB1 kinase releases cytoplasmic dynein from membranes. J Biol. Chem. *276*, 15939-15944.

Allan,V.J., Thompson,H.M., and McNiven,M.A. (2002). Motoring around the Golgi. Nat. Cell Biol. *4*, E236-E242.

Amano,M., Ito,M., Kimura,K., Fukata,Y., Chihara,K., Nakano,T., Matsuura,Y., and Kaibuchi,K. (1996). Phosphorylation and activation of myosin by Rho-associated kinase (Rho-kinase). J. Biol. Chem. *271*, 20246-20249.

Andersen,S.S. (1999). Molecular characteristics of the centrosome. Int. Rev. Cytol. *187*, 51-109.

Andreassen,P.R., Lohez,O.D., Lacroix,F.B., and Margolis,R.L. (2001). Tetraploid State Induces p53-dependent Arrest of Nontransformed Mammalian Cells in G1. Mol. Biol. Cell *12*, 1315-1328.

Bacallao,R., Antony,C., Dotti,C., Karsenti,E., Stelzer,E.H., and Simons,K. (1989). The subcellular organization of Madin-Darby canine kidney cells during the formation of a polarized epithelium. J Cell Biol. *109*, 2817-2832.

Balczon,R., Varden,C.E., and Schroer,T.A. (1999). Role for microtubules in centrosome doubling in Chinese hamster ovary cells. Cell Motil. Cytoskeleton *42*, 60-72.

Bardin,A.J., Visintin,R., and Amon,A. (2000). A mechanism for coupling exit from mitosis to partitioning of the nucleus. Cell *102*, 21-31.

Barr,F.A., Nakamura,N., and Warren,G. (1998). Mapping the interaction between GRASP65 and GM130, components of a protein complex involved in the stacking of Golgi cisternae. EMBO J. *17*, 3258-3268.

Baum,P., Furlong,C., and Byers,B. (1986). Yeast gene required for spindle pole body duplication: homology of its product with Ca2+-binding proteins. Proc. Natl. Acad. Sci. U. S. A *83*, 5512-5516.

Berdnik,D. and Knoblich,J.A. (2002). Drosophila Aurora-A is required for centrosome maturation and actin-dependent asymmetric protein localization during mitosis. Curr. Biol. *12*, 640-647.

Berg,J.S., Powell,B.C., and Cheney,R.E. (2001). A millennial myosin census. Mol. Biol. Cell *12*, 780-794.

Blagden,S.P. and Glover,D.M. (2003). Polar expeditions--provisioning the centrosome for mitosis. Nat. Cell Biol. *5*, 505-511.

Blangy,A., Arnaud,L., and Nigg,E.A. (1997). Phosphorylation by p34cdc2 protein kinase regulates binding of the kinesin-related motor HsEg5 to the dynactin subunit p150. J. Biol. Chem. *272*, 19418-19424.

Blangy,A., Lane,H.A., d'Herin,P., Harper,M., Kress,M., and Nigg,E.A. (1995). Phosphorylation by p34cdc2 regulates spindle association of human Eg5, a kinesin-related motor essential for bipolar spindle formation in vivo. Cell *83*, 1159-1169.

Bloecher,A., Venturi,G.M., and Tatchell,K. (2000). Anaphase spindle position is monitored by the BUB2 checkpoint. Nat. Cell Biol. *2*, 556-558.

Blomberg-Wirschell,M. and Doxsey,S.J. (1998). Rapid isolation of centrosomes. Methods Enzymol. *298:228-38.*, 228-238.

Bloom,G.S. and Goldstein,L.S. (1998). Cruising along microtubule highways: how membranes move through the secretory pathway. J Cell Biol. *140*, 1277-1280.

Bobinnec,Y., Moudjou,M., Fouquet,J.P., Desbruyeres,E., Edde,B., and Bornens,M. (1998). Glutamylation of centriole and cytoplasmic tubulin in proliferating non-neuronal cells. Cell Motil. Cytoskeleton *39*, 223-232.

Boleti,H., Karsenti,E., and Vernos,I. (1996). Xklp2, a novel Xenopus centrosomal kinesin-like protein required for centrosome separation during mitosis. Cell *84*, 49-59.

Bornens,M. (2002). Centrosome composition and microtubule anchoring mechanisms. Curr. Opin. Cell Biol. *14*, 25-34.

Bornens,M., Paintrand,M., Berges,J., Marty,M.C., and Karsenti,E. (1987). Structural and chemical characterization of isolated centrosomes. Cell Motil. Cytoskeleton *8*, 238-249.

Bouckson-Castaing,V., Moudjou,M., Ferguson,D.J., Mucklow,S., Belkaid,Y., Milon,G., and Crocker,P.R. (1996). Molecular characterisation of ninein, a new coiled-coil protein of the centrosome. J. Cell Sci. *109*, 179-190.

Brinkley,B.R. (2001). Managing the centrosome numbers game: from chaos to stability in cancer cell division. Trends Cell Biol. *11*, 18-21.

Burgess,T.L., Skoufias,D.A., and Wilson,L. (1991). Disruption of the Golgi apparatus with brefeldin A does not destabilize the associated detyrosinated microtubule network. Cell Motil. Cytoskeleton *20*, 289-300.

Burkhardt,J.K. (1998). The role of microtubule-based motor proteins in maintaining the structure and function of the Golgi complex. Biochim. Biophys. Acta *1404*, 113-126.

Burkhardt,J.K., Echeverri,C.J., Nilsson,T., and Vallee,R.B. (1997). Overexpression of the dynamitin (p50) subunit of the dynactin complex disrupts dynein-dependent maintenance of membrane organelle distribution. J Cell Biol. *139*, 469-484.

Busson,S., Dujardin,D., Moreau,A., Dompierre,J., and De Mey,J.R. (1998). Dynein and dynactin are localized to astral microtubules and at cortical sites in mitotic epithelial cells. Curr. Biol. *8*, 541-544.

Carazo-Salas,R.E., Gruss,O.J., Mattaj,I.W., and Karsenti,E. (2001). Ran-GTP coordinates regulation of microtubule nucleation and dynamics during mitotic-spindle assembly. Nat. Cell Biol. *3*, 228-234.

Chang,P., Giddings,T.H., Jr., Winey,M., and Stearns,T. (2003). Epsilon-tubulin is required for centriole duplication and microtubule organization. Nat. Cell Biol. *5*, 71-76.

Chen,Y., Riley,D.J., Chen,P.L., and Lee,W.H. (1997). HEC, a novel nuclear protein rich in leucine heptad repeats specifically involved in mitosis. Mol. Cell Biol. *17*, 6049-6056.

Chevrier,V., Piel,M., Collomb,N., Saoudi,Y., Frank,R., Paintrand,M., Narumiya,S., Bornens,M., and Job,D. (2002). The Rho-associated protein kinase p160ROCK is required for centrosome positioning. J. Cell Biol. *157*, 807-817.

Chretien,D., Buendia,B., Fuller,S.D., and Karsenti,E. (1997). Reconstruction of the centrosome cycle from cryoelectron micrographs. J. Struct. Biol. *120*, 117-133.

Clark,I.B. and Meyer,D.I. (1999). Overexpression of normal and mutant Arp1alpha (centractin) differentially affects microtubule organization during mitosis and interphase. J Cell Sci *112*, 3507-3518.

Cole,N.B., Sciaky,N., Marotta,A., Song,J., and Lippincott-Schwartz,J. (1996). Golgi dispersal during microtubule disruption: regeneration of Golgi stacks at peripheral endoplasmic reticulum exit sites. Mol. Biol. Cell *7*, 631-650.

Compton,D.A. (1998). Focusing on spindle poles. J. Cell Sci. *111*, 1477-1481.

Compton,D.A. (2000). Spindle assembly in animal cells. Annu. Rev. Biochem. *69:95-114.*, 95-114.

Criswell,P.S. and Asai,D.J. (1998). Evidence for four cytoplasmic dynein heavy chain isoforms in rat testis. Mol. Biol. Cell *9*, 237-247.

Criswell,P.S., Ostrowski,L.E., and Asai,D.J. (1996). A novel cytoplasmic dynein heavy chain: expression of DHC1b in mammalian ciliated epithelial cells. J Cell Sci *109*, 1891-1898.

Dammermann,A. and Merdes,A. (2002). Assembly of centrosomal proteins and microtubule organization depends on PCM-1. J. Cell Biol. *159*, 255-266.

Davis,S.J. and van der Merwe,P.A. (2001). The immunological synapse: required for T cell receptor signalling or directing T cell effector function? Curr. Biol. *11*, R289-R291.

Dell,K.R., Turck,C.W., and Vale,R.D. (2000). Mitotic phosphorylation of the dynein light intermediate chain is mediated by cdc2 kinase. Traffic. *1*, 38-44.

Detraves,C., Mazarguil,H., Lajoie-Mazenc,I., Julian,M., Raynaud-Messina,B., and Wright,M. (1997). Protein complexes containing gamma-tubulin are present in mammalian brain microtubule protein preparations. Cell Motil. Cytoskeleton *36*, 179-189.

Di Fiore,B., Ciciarello,M., Mangiacasale,R., Palena,A., Tassin,A.M., Cundari,E., and Lavia,P. (2003). Mammalian RanBP1 regulates centrosome cohesion during mitosis. J. Cell Sci. *116*, 3399-3411.

Dictenberg,J.B., Zimmerman,W., Sparks,C.A., Young,A., Vidair,C., Zheng,Y., Carrington,W., Fay,F.S., and Doxsey,S.J. (1998). Pericentrin and gamma-tubulin form a protein complex and are organized into a novel lattice at the centrosome. J. Cell Biol. *141*, 163-174.

do Carmo,A.M. and Glover,D.M. (1999). Abnormal spindle protein, Asp, and the integrity of mitotic centrosomal microtubule organizing centers. Science *283*, 1733-1735.

do Carmo,A.M., Tavares,A., and Glover,D.M. (2001). Polo kinase and Asp are needed to promote the mitotic organizing activity of centrosomes. Nat. Cell Biol. *3*, 421-424.

Donaldson,J.G. and Lippincott-Schwartz,J. (2000). Sorting and signaling at the Golgi complex. Cell *101*, 693-696.

Donaldson,M.M., Tavares,A.A., Hagan,I.M., Nigg,E.A., and Glover,D.M. (2001). The mitotic roles of Polo-like kinase. J. Cell Sci. *114*, 2357-2358.

Doxsey,S. (2001). Re-evaluating centrosome function. Nat. Rev. Mol. Cell Biol. *2*, 688-698.

Doxsey,S.J., Stein,P., Evans,L., Calarco,P.D., and Kirschner,M. (1994). Pericentrin, a highly conserved centrosome protein involved in microtubule organization. Cell *76*, 639-650.

Dutcher,S.K. (2001). Motile organelles: the importance of specific tubulin isoforms. Curr. Biol. *11*, R419-R422.

Endow,S.A. and Komma,D.J. (1997). Spindle dynamics during meiosis in Drosophila oocytes. J. Cell Biol. *137*, 1321-1336.

Erhardt,M., Stoppin-Mellet,V., Campagne,S., Canaday,J., Mutterer,J., Fabian,T., Sauter,M., Muller,T., Peter,C., Lambert,A.M., and Schmit,A.C. (2002). The plant Spc98p homologue colocalizes with gamma-tubulin at microtubule nucleation sites and is required for microtubule nucleation. J. Cell Sci. *115*, 2423-2431.

Etienne-Manneville,S. and Hall,A. (2001). Integrin-mediated activation of Cdc42 controls cell polarity in migrating astrocytes through PKCzeta. Cell *106*, 489-498.

Fath,K.R., Trimbur,G.M., and Burgess,D.R. (1997). Molecular motors and a spectrin matrix associate with Golgi membranes in vitro. J Cell Biol. *139*, 1169-1181.

Fava,F., Raynaud-Messina,B., Leung-Tack,J., Mazzolini,L., Li,M., Guillemot,J.C., Cachot,D., Tollon,Y., Ferrara,P., and Wright,M. (1999). Human 76p: A new member of the gamma-tubulin-associated protein family. J. Cell Biol. *147*, 857-868.

Feiguin,F., Ferreira,A., Kosik,K.S., and Caceres,A. (1994). Kinesin-mediated organelle translocation revealed by specific cellular manipulations. J Cell Biol. *127*, 1021-1039.

Ferri,K.F. and Kroemer,G. (2001). Organelle-specific initiation of cell death pathways. Nat. Cell Biol. *3*, E255-E263.

Fisk,H.A. and Winey,M. (2001). The mouse mps1p-like kinase regulates centrosome duplication. Cell *106*, 95-104.

Flory,M.R., Moser,M.J., Monnat,R.J., Jr., and Davis,T.N. (2000). Identification of a human centrosomal calmodulin-binding protein that shares homology with pericentrin. Proc. Natl. Acad. Sci. U. S. A *97*, 5919-5923.

Freed,E., Lacey,K.R., Huie,P., Lyapina,S.A., Deshaies,R.J., Stearns,T., and Jackson,P.K. (1999). Components of an SCF ubiquitin ligase localize to the centrosome and regulate the centrosome duplication cycle. Genes Dev. *13*, 2242-2257.

Fry,A.M., Mayor,T., Meraldi,P., Stierhof,Y.D., Tanaka,K., and Nigg,E.A. (1998a). C-Nap1, a novel centrosomal coiled-coil protein and candidate substrate of the cell cycle-regulated protein kinase Nek2. J. Cell Biol. *141*, 1563-1574.

Fry,A.M., Mayor,T., and Nigg,E.A. (2000). Regulating centrosomes by protein phosphorylation. Curr. Top. Dev. Biol. *49:291-312.*, 291-312.

Fry,A.M., Meraldi,P., and Nigg,E.A. (1998b). A centrosomal function for the human Nek2 protein kinase, a member of the NIMA family of cell cycle regulators. EMBO J. *17*, 470-481.

Gaglio,T., Dionne,M.A., and Compton,D.A. (1997). Mitotic spindle poles are organized by structural and motor proteins in addition to centrosomes. J Cell Biol. *138*, 1055-1066.

Gaglio,T., Saredi,A., Bingham,J.B., Hasbani,M.J., Gill,S.R., Schroer,T.A., and Compton,D.A. (1996). Opposing motor activities are required for the organization of the mammalian mitotic spindle pole. J Cell Biol. *135*, 399-414.

Geiger,B., Rosen,D., and Berke,G. (1982). Spatial relationships of microtubule-organizing centers and the contact area of cytotoxic T lymphocytes and target cells. J. Cell Biol. *95*, 137-143.

Geissler,S., Pereira,G., Spang,A., Knop,M., Soues,S., Kilmartin,J., and Schiebel,E. (1996). The spindle pole body component Spc98p interacts with the gamma-tubulin-like Tub4p of Saccharomyces cerevisiae at the sites of microtubule attachment. EMBO J. *15*, 3899-3911.

Gergely,F., Karlsson,C., Still,I., Cowell,J., Kilmartin,J., and Raff,J.W. (2000). The TACC domain identifies a family of centrosomal proteins that can interact with microtubules. Proc. Natl. Acad. Sci. U. S. A *97*, 14352-14357.

Gibbons,B.H., Asai,D.J., Tang,W.J., Hays,T.S., and Gibbons,I.R. (1994). Phylogeny and expression of axonemal and cytoplasmic dynein genes in sea urchins. Mol. Biol. Cell *5*, 57-70.

Giet,R., McLean,D., Descamps,S., Lee,M.J., Raff,J.W., Prigent,C., and Glover,D.M. (2002). Drosophila Aurora A kinase is required to localize D-TACC to centrosomes and to regulate astral microtubules. J. Cell Biol. *156*, 437-451.

Giet,R., Uzbekov,R., Cubizolles,F., Le Guellec,K., and Prigent,C. (1999). The Xenopus laevis aurora-related protein kinase pEg2 associates with and phosphorylates the kinesin-related protein XlEg5. J. Biol. Chem. *274*, 15005-15013.

Gill,S.R., Schroer,T.A., Szilak,I., Steuer,E.R., Sheetz,M.P., and Cleveland,D.W. (1991). Dynactin, a conserved, ubiquitously expressed component of an activator of vesicle motility mediated by cytoplasmic dynein. J Cell Biol. *115*, 1639-1650.

Goldstein,L.S. (2001). Molecular motors: from one motor many tails to one motor many tales. Trends Cell Biol. *11*, 477-482.

Golsteyn,R.M., Mundt,K.E., Fry,A.M., and Nigg,E.A. (1995). Cell cycle regulation of the activity and subcellular localization of Plk1, a human protein kinase implicated in mitotic spindle function. J. Cell Biol. *129*, 1617-1628.

Gonczy,P. (2002). Nuclear envelope: torn apart at mitosis. Curr. Biol. *12*, R242-R244.

Gorgidze,L.A. and Vorobjev,I.A. (1995). Centrosome and microtubules behavior in the cytoplasts. J. Submicrosc. Cytol. Pathol. *27*, 381-389.

Gotlieb,A.I., Subrahmanyan,L., and Kalnins,V.I. (1983). Microtubule-organizing centers and cell migration: effect of inhibition of migration and microtubule disruption in endothelial cells. J Cell Biol. *96*, 1266-1272.

Grissom,P.M., Vaisberg,E.A., and McIntosh,J.R. (2002). Identification of a novel light intermediate chain (D2LIC) for mammalian cytoplasmic dynein 2. Mol. Biol. Cell *13*, 817-829.

Gromley,A., Jurczyk,A., Sillibourne,J., Halilovic,E., Mogensen,M., Groisman,I., Blomberg,M., and Doxsey,S. (2003). A novel human protein of the maternal centriole is required for the final stages of cytokinesis and entry into S phase. J. Cell Biol. *161*, 535-545.

Gruneberg,U., Campbell,K., Simpson,C., Grindlay,J., and Schiebel,E. (2000). Nud1p links astral microtubule organization and the control of exit from mitosis. EMBO J. *19*, 6475-6488.

Gstaiger,M., Marti,A., and Krek,W. (1999). Association of human SCF(SKP2) subunit p19(SKP1) with interphase centrosomes and mitotic spindle poles. Exp. Cell Res. *247*, 554-562.

Gyoeva,F.K., Bybikova,E.M., and Minin,A.A. (2000). An isoform of kinesin light chain specific for the Golgi complex. J Cell Sci *113*, 2047-2054.

Hagiwara,H., Ohwada,N., Aoki,T., and Takata,K. (2000). Ciliogenesis and ciliary abnormalities. Med. Electron Microsc. *33*, 109-114.

Hannak,E., Kirkham,M., Hyman,A.A., and Oegema,K. (2001). Aurora-A kinase is required for centrosome maturation in Caenorhabditis elegans. J. Cell Biol. *155*, 1109-1116.

Hansen,D.V., Hsu,J.Y., Kaiser,B.K., Jackson,P.K., and Eldridge,A.G. (2002). Control of the centriole and centrosome cycles by ubiquitination enzymes. Oncogene *21*, 6209-6221.

Harada,A., Takei,Y., Kanai,Y., Tanaka,Y., Nonaka,S., and Hirokawa,N. (1998). Golgi vesiculation and lysosome dispersion in cells lacking cytoplasmic dynein. J Cell Biol. *141*, 51-59.

Heald,R., Tournebize,R., Blank,T., Sandaltzopoulos,R., Becker,P., Hyman,A., and Karsenti,E. (1996). Self-organization of microtubules into bipolar spindles around artificial chromosomes in Xenopus egg extracts. Nature *382*, 420-425.

Heald,R., Tournebize,R., Habermann,A., Karsenti,E., and Hyman,A. (1997). Spindle assembly in Xenopus egg extracts: respective roles of centrosomes and microtubule self-organization 120. J. Cell Biol. *138*, 615-628.

Helps,N.R., Brewis,N.D., Lineruth,K., Davis,T., Kaiser,K., and Cohen,P.T. (1998). Protein phosphatase 4 is an essential enzyme required for organisation of microtubules at centrosomes in Drosophila embryos. J. Cell Sci. *111*, 1331-1340.

Helps,N.R., Luo,X., Barker,H.M., and Cohen,P.T. (2000). NIMA-related kinase 2 (Nek2), a cell-cycle-regulated protein kinase localized to centrosomes, is complexed to protein phosphatase 1. Biochem. J. *349* , 509-518.

Herrera,J.E., Savkur,R., and Olson,M.O. (1995). The ribonuclease activity of nucleolar protein B23. Nucleic Acids Res. *23*, 3974-3979.

Hinchcliffe,E.H., Li,C., Thompson,E.A., Maller,J.L., and Sluder,G. (1999). Requirement of Cdk2-cyclin E activity for repeated centrosome reproduction in Xenopus egg extracts [see comments]. Science *283*, 851-854.

Hinchcliffe,E.H. and Linck,R.W. (1998). Two proteins isolated from sea urchin sperm flagella: structural components common to the stable microtubules of axonemes and centrioles. J. Cell Sci. *111*, 585-595.

Hinchcliffe,E.H., Miller,F.J., Cham,M., Khodjakov,A., and Sluder,G. (2001). Requirement of a centrosomal activity for cell cycle progression through G1 into S phase. Science *291*, 1547-1550.

Hinchcliffe,E.H. and Sluder,G. (2001). "It takes two to tango": understanding how centrosome duplication is regulated throughout the cell cycle. Genes Dev. *15*, 1167-1181.

Hirokawa,N., Noda,Y., and Okada,Y. (1998). Kinesin and dynein superfamily proteins in organelle transport and cell division. Curr. Opin. Cell Biol. *10*, 60-73.

Ho,W.C., Allan,V.J., van Meer,G., Berger,E.G., and Kreis,T.E. (1989). Reclustering of scattered Golgi elements occurs along microtubules. Eur. J Cell Biol. *48*, 250-263.

Hoffmann,R., Metzger,S., Spengler,B., and Otvos,L., Jr. (1999). Sequencing of peptides phosphorylated on serines and threonines by post-source decay in matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. J. Mass Spectrom. *34*, 1195-1204.

Holleran,E.A., Karki,S., and Holzbaur,E.L. (1998). The role of the dynactin complex in intracellular motility. Int. Rev. Cytol. *182:69-109.*, 69-109.

Holleran,E.A., Ligon,L.A., Tokito,M., Stankewich,M.C., Morrow,J.S., and Holzbaur,E.L. (2001). beta III spectrin binds to the Arp1 subunit of dynactin. J Biol. Chem. *276*, 36598-36605.

Holleran,E.A., Tokito,M.K., Karki,S., and Holzbaur,E.L. (1996). Centractin (ARP1) associates with spectrin revealing a potential mechanism to link dynactin to intracellular organelles. J Cell Biol. *135*, 1815-1829.

Hong,Y.R., Chen,C.H., Chang,J.H., Wang,S., Sy,W.D., Chou,C.K., and Howng,S.L. (2000). Cloning and characterization of a novel human ninein protein that interacts with the glycogen synthase kinase 3beta. Biochim. Biophys. Acta *1492*, 513-516.

Hoogenraad,C.C., Akhmanova,A., Howell,S.A., Dortland,B.R., De Zeeuw,C.I., Willemsen,R., Visser,P., Grosveld,F., and Galjart,N. (2001). Mammalian Golgi-associated Bicaudal-D2 functions in the dynein-dynactin pathway by interacting with these complexes. EMBO J *20*, 4041-4054.

Horio,T., Uzawa,S., Jung,M.K., Oakley,B.R., Tanaka,K., and Yanagida,M. (1991). The fission yeast gamma-tubulin is essential for mitosis and is localized at microtubule organizing centers. J. Cell Sci. *99*, 693-700.

Hung,L.Y., Tang,C.J., and Tang,T.K. (2000). Protein 4.1 R-135 interacts with a novel centrosomal protein (CPAP) which is associated with the gamma-tubulin complex. Mol. Cell Biol. *20*, 7813-7825.

Hyman,A.A. and Karsenti,E. (1996). Morphogenetic properties of microtubules and mitotic spindle assembly. Cell *84*, 401-410.

Infante,C., Ramos-Morales,F., Fedriani,C., Bornens,M., and Rios,R.M. (1999). GMAP-210, A cis-Golgi network-associated protein, is a minus end microtubule-binding protein. J Cell Biol. *145*, 83-98.

Jacob,Y., Badrane,H., Ceccaldi,P.E., and Tordo,N. (2000). Cytoplasmic dynein LC8 interacts with lyssavirus phosphoprotein. J Virol. *74*, 10217-10222.

Johnson,K.J., Hall,E.S., and Boekelheide,K. (1996). Kinesin localizes to the trans-Golgi network regardless of microtubule organization. Eur. J Cell Biol. *69*, 276-287.

Joshi,H.C. (1994). Microtubule organizing centers and gamma-tubulin. Curr. Opin. Cell Biol. *6*, 54-62.

Karki,S. and Holzbaur,E.L. (1999). Cytoplasmic dynein and dynactin in cell division and intracellular transport. Curr. Opin. Cell Biol. *11*, 45-53.

Katayama,H., Zhou,H., Li,Q., Tatsuka,M., and Sen,S. (2001). Interaction and feedback regulation between STK15/BTAK/Aurora-A kinase and protein phosphatase 1 through mitotic cell division cycle. J. Biol. Chem. *276*, 46219-46224.

Kelm,O., Wind,M., Lehmann,W.D., and Nigg,E.A. (2002). Cell cycle-regulated phosphorylation of the Xenopus polo-like kinase Plx1. J. Biol. Chem. *277*, 25247-25256.

Keryer,G., Di Fiore,B., Celati,C., Lechtreck,K.F., Mogensen,M., Delouvee,A., Lavia,P., Bornens,M., and Tassin,A.M. (2003). Part of Ran is associated with AKAP450 at the centrosome: involvement in microtubule-organizing activity. Mol. Biol. Cell *14*, 4260-4271.

Khodjakov,A., Cole,R.W., Oakley,B.R., and Rieder,C.L. (2000). Centrosome-independent mitotic spindle formation in vertebrates. Curr. Biol. *10*, 59-67.

Khodjakov,A. and Rieder,C.L. (1999). The sudden recruitment of gamma-tubulin to the centrosome at the onset of mitosis and its dynamic exchange throughout the cell cycle, do not require microtubules. J. Cell Biol. *146*, 585-596.

Khodjakov,A. and Rieder,C.L. (2001). Centrosomes enhance the fidelity of cytokinesis in vertebrates and are required for cell cycle progression. J. Cell Biol. *153*, 237-242.

Khodjakov,A., Rieder,C.L., Sluder,G., Cassels,G., Sibon,O., and Wang,C.L. (2002). De novo formation of centrosomes in vertebrate cells arrested during S phase. J Cell Biol. *158*, 1171-1181.

Kilmartin,J.V. (1994). Genetic and biochemical approaches to spindle function and chromosome segregation in eukaryotic microorganisms. Curr. Opin. Cell Biol. *6*, 50-54.

King,S.J. and Schroer,T.A. (2000). Dynactin increases the processivity of the cytoplasmic dynein motor. Nat. Cell Biol. *2*, 20-24.

King,S.M. (2000). The dynein microtubule motor. Biochim. Biophys. Acta *1496*, 60-75.

Knop,M., Pereira,G., Geissler,S., Grein,K., and Schiebel,E. (1997). The spindle pole body component Spc97p interacts with the gamma-tubulin of Saccharomyces cerevisiae and functions in microtubule organization and spindle pole body duplication. EMBO J. *16*, 1550-1564.

Knop,M., Pereira,G., and Schiebel,E. (1999). Microtubule organization by the budding yeast spindle pole body. Biol. Cell *91*, 291-304.

Knop,M. and Schiebel,E. (1997). Spc98p and Spc97p of the yeast gamma-tubulin complex mediate binding to the spindle pole body via their interaction with Spc110p. EMBO J. *16*, 6985-6995.

Knop,M. and Schiebel,E. (1998). Receptors determine the cellular localization of a gamma-tubulin complex and thereby the site of microtubule formation. EMBO J. *17*, 3952-3967.

Kochanski,R.S. and Borisy,G.G. (1990). Mode of centriole duplication and distribution. J Cell Biol *110*, 1599-605.

Krek,W. and Nigg,E.A. (1991). Mutations of p34cdc2 phosphorylation sites induce premature mitotic events in HeLa cells: evidence for a double block to p34cdc2 kinase activation in vertebrates. EMBO J. *10*, 3331-3341.

Krummel,M.F., Sjaastad,M.D., Wulfing,C., and Davis,M.M. (2000). Differential clustering of CD4 and CD3zeta during T cell recognition. Science *289*, 1349-1352.

Kubo,A., Sasaki,H., Yuba-Kubo,A., Tsukita,S., and Shiina,N. (1999). Centriolar satellites: molecular characterization, ATP-dependent movement toward centrioles and possible involvement in ciliogenesis. J Cell Biol. *147*, 969-980.

Kubo,A. and Tsukita,S. (2003). Non-membranous granular organelle consisting of PCM-1: subcellular distribution and cell-cycle-dependent assembly/disassembly. J Cell Sci *116*, 919-928.

Kupfer,A., Dennert,G., and Singer,S.J. (1983). Polarization of the Golgi apparatus and the microtubule-organizing center within cloned natural killer cells bound to their targets. Proc. Natl. Acad. Sci. U. S. A *80*, 7224-7228.

Kupfer,A., Swain,S.L., and Singer,S.J. (1987). The specific direct interaction of helper T cells and antigen-presenting B cells. II. Reorientation of the microtubule organizing center and reorganization of the membrane-associated cytoskeleton inside the bound helper T cells. J. Exp. Med. *165*, 1565-1580.

Kupfer,H., Monks,C.R., and Kupfer,A. (1994). Small splenic B cells that bind to antigen-specific T helper (Th) cells and face the site of cytokine production in the Th cells selectively proliferate: immunofluorescence microscopic studies of Th-B antigen-presenting cell interactions. J Exp. Med. *179*, 1507-1515.

Kuriyama,R. and Borisy,G.G. (1981). Centriole cycle in Chinese hamster ovary cells as determined by whole- mount electron microscopy. J Cell Biol *91*, 814-21.

Lacey,K.R., Jackson,P.K., and Stearns,T. (1999). Cyclin-dependent kinase control of centrosome duplication. Proc. Natl. Acad. Sci. U. S. A *96*, 2817-2822.

Lane,H.A. and Nigg,E.A. (1996). Antibody microinjection reveals an essential role for human polo-like kinase 1 (Plk1) in the functional maturation of mitotic centrosomes. J. Cell Biol. *135*, 1701-1713.

Lange,B.M. (2002). Integration of the centrosome in cell cycle control, stress response and signal transduction pathways. Curr. Opin. Cell Biol. *14*, 35-43.

Lange,B.M. and Gull,K. (1995). A molecular marker for centriole maturation in the mammalian cell cycle. J. Cell Biol. *130*, 919-927.

Lee,M.J., Gergely,F., Jeffers,K., Peak-Chew,S.Y., and Raff,J.W. (2001). Msps/XMAP215 interacts with the centrosomal protein D-TACC to regulate microtubule behaviour. Nat. Cell Biol. *3*, 643-649.

Li,Q., Hansen,D., Killilea,A., Joshi,H.C., Palazzo,R.E., and Balczon,R. (2001). Kendrin/pericentrin-B, a centrosome protein with homology to pericentrin that complexes with PCM-1. J. Cell Sci. *114*, 797-809.

Lippincott-Schwartz,J. (1998). Cytoskeletal proteins and Golgi dynamics. Curr. Opin. Cell Biol. *10*, 52-59.

Lu,Z., Joseph,D., Bugnard,E., Zaal,K.J., and Ralston,E. (2001). Golgi complex reorganization during muscle differentiation: visualization in living cells and mechanism. Mol. Biol. Cell *12*, 795-808.

Lutz,W., Lingle,W.L., McCormick,D., Greenwood,T.M., and Salisbury,J.L. (2001). Phosphorylation of centrin during the cell cycle and its role in centriole separation preceding centrosome duplication. J. Biol. Chem. *276*, 20774-20780.

Magdalena,J., Millard,T.H., Etienne-Manneville,S., Launay,S., Warwick,H.K., and Machesky,L.M. (2003). Involvement of the Arp2/3 complex and Scar2 in Golgi polarity in scratch wound models. Mol. Biol. Cell *14*, 670-684.

Marks,D.L., Larkin,J.M., and McNiven,M.A. (1994). Association of kinesin with the Golgi apparatus in rat hepatocytes. J Cell Sci *107*, 2417-2426.

Martin-Lluesma,S., Stucke,V.M., and Nigg,E.A. (2002). Role of Hec1 in spindle checkpoint signaling and kinetochore recruitment of Mad1/Mad2. Science *297*, 2267-2270.

Martin,O.C., Gunawardane,R.N., Iwamatsu,A., and Zheng,Y. (1998). Xgrip109: a gamma tubulin-associated protein with an essential role in gamma tubulin ring complex (gammaTuRC) assembly and centrosome function. J. Cell Biol. *141*, 675-687.

Matanis,T., Akhmanova,A., Wulf,P., Del Nery,E., Weide,T., Stepanova,T., Galjart,N., Grosveld,F., Goud,B., De Zeeuw,C.I., Barnekow,A., and Hoogenraad,C.C. (2002). Bicaudal-D regulates COPI-independent Golgi-ER transport by recruiting the dynein-dynactin motor complex. Nat. Cell Biol. *4*, 986-992.

Matsui,T., Maeda,M., Doi,Y., Yonemura,S., Amano,M., Kaibuchi,K., Tsukita,S., and Tsukita,S. (1998). Rho-kinase phosphorylates COOH-terminal threonines of ezrin/radixin/moesin (ERM) proteins and regulates their head-to-tail association. J. Cell Biol. *140*, 647-657.

Matsumoto,Y., Hayashi,K., and Nishida,E. (1999). Cyclin-dependent kinase 2 (Cdk2) is required for centrosome duplication in mammalian cells. Curr. Biol. *9*, 429-432.

Matsumoto,Y. and Maller,J.L. (2002). Calcium, calmodulin, and CaMKII requirement for initiation of centrosome duplication in Xenopus egg extracts. Science *295*, 499-502.

Mayor,T., Hacker,U., Stierhof,Y.D., and Nigg,E.A. (2002). The mechanism regulating the dissociation of the centrosomal protein C-Nap1 from mitotic spindle poles. J. Cell Sci. *115*, 3275-3284.

Mayor,T., Stierhof,Y.D., Tanaka,K., Fry,A.M., and Nigg,E.A. (2000). The centrosomal protein C-Nap1 is required for cell cycle-regulated centrosome cohesion. J. Cell Biol. *151*, 837-846.

Meraldi,P., Honda,R., and Nigg,E.A. (2002). Aurora-A overexpression reveals tetraploidization as a major route to centrosome amplification in p53(--/--) cells. EMBO J. *21*, 483-492.

Meraldi,P., Lukas,J., Fry,A.M., Bartek,J., and Nigg,E.A. (1999). Centrosome duplication in mammalian somatic cells requires E2F and Cdk2- cyclin A. Nat. Cell Biol. *1*, 88-93.

Meraldi,P. and Nigg,E.A. (2001). Centrosome cohesion is regulated by a balance of kinase and phosphatase activities. J. Cell Sci. *114*, 3749-3757.

Meraldi,P. and Nigg,E.A. (2002). The centrosome cycle. FEBS Lett. *521*, 9-13.

Merdes,A., Heald,R., Samejima,K., Earnshaw,W.C., and Cleveland,D.W. (2000). Formation of spindle poles by dynein/dynactin-dependent transport of NuMA. J Cell Biol. *149*, 851-862.

Merdes,A., Ramyar,K., Vechio,J.D., and Cleveland,D.W. (1996). A complex of NuMA and cytoplasmic dynein is essential for mitotic spindle assembly. Cell *87*, 447-458.

Middendorp,S., Kuntziger,T., Abraham,Y., Holmes,S., Bordes,N., Paintrand,M., Paoletti,A., and Bornens,M. (2000). A role for centrin 3 in centrosome reproduction. J. Cell Biol. *148*, 405-416.

Mizuno,M. and Singer,S.J. (1994). A possible role for stable microtubules in intracellular transport from the endoplasmic reticulum to the Golgi apparatus. J Cell Sci *107*, 1321-1331.

Mogensen,M.M., Malik,A., Piel,M., Bouckson-Castaing,V., and Bornens,M. (2000). Microtubule minus-end anchorage at centrosomal and non-centrosomal sites: the role of ninein. J. Cell Sci. *113*, 3013-3023.

Moritz,M., Zheng,Y., Alberts,B.M., and Oegema,K. (1998). Recruitment of the gamma-tubulin ring complex to Drosophila salt-stripped centrosome scaffolds. J Cell Biol. *142*, 775-786.

Morris,N.R. (2003). Nuclear positioning: the means is at the ends. Curr. Opin. Cell Biol. *15*, 54-59.

Moudjou,M., and Bornens,M. (1994). Isolation of centrosomes from cultured animal cells. In Cell Biology: A Laboratory Handbook. Vol. 1. J.E. Celis,editor. Academic Press, Inc., London, pp. 595-604.

Moudjou,M., Bordes,N., Paintrand,M., and Bornens,M. (1996). gamma-Tubulin in mammalian cells: the centrosomal and the cytosolic forms. J. Cell Sci. *109*, 875-887.

Muresan,V., Stankewich,M.C., Steffen,W., Morrow,J.S., Holzbaur,E.L., and Schnapp,B.J. (2001). Dynactin-dependent, dynein-driven vesicle transport in the absence of membrane proteins: a role for spectrin and acidic phospholipids. Mol. Cell *7*, 173-183.

Murphy,S.M., Preble,A.M., Patel,U.K., O'Connell,K.L., Dias,D.P., Moritz,M., Agard,D., Stults,J.T., and Stearns,T. (2001). GCP5 and GCP6: two new members of the human gamma-tubulin complex. Mol. Biol. Cell *12*, 3340-3352.

Murphy,S.M., Urbani,L., and Stearns,T. (1998). The mammalian gamma-tubulin complex contains homologues of the yeast spindle pole body components spc97p and spc98p. J. Cell Biol. *141*, 663-674.

Murray,A.W. (1991). Cell cycle extracts. Methods Cell Biol. *36:581-605.*, 581-605.

Nakagawa,Y., Yamane,Y., Okanoue,T., Tsukita,S., and Tsukita,S. (2001). Outer dense fiber 2 is a widespread centrosome scaffold component preferentially associated with mother centrioles: its identification from isolated centrosomes. Mol. Biol. Cell *12*, 1687-1697.

Nakayama,K., Nagahama,H., Minamishima,Y.A., Matsumoto,M., Nakamichi,I., Kitagawa,K., Shirane,M., Tsunematsu,R., Tsukiyama,T., Ishida,N., Kitagawa,M., Nakayama,K., and Hatakeyama,S. (2000). Targeted disruption of Skp2 results in accumulation of cyclin E and p27(Kip1), polyploidy and centrosome overduplication. EMBO J. *19*, 2069-2081.

Nemere,I., Kupfer,A., and Singer,S.J. (1985). Reorientation of the Golgi apparatus and the microtubule-organizing center inside macrophages subjected to a chemotactic gradient. Cell Motil. *5*, 17-29.

Nguyen,T., Vinh,D.B., Crawford,D.K., and Davis,T.N. (1998). A genetic analysis of interactions with Spc110p reveals distinct functions of Spc97p and Spc98p, components of the yeast gamma-tubulin complex. Mol. Biol. Cell *9*, 2201-2216.

Nigg,E.A. (2002). Centrosome aberrations: cause or consequence of cancer progression? Nat. Rev. Cancer *2*, 815-825.

Nonaka,S., Tanaka,Y., Okada,Y., Takeda,S., Harada,A., Kanai,Y., Kido,M., and Hirokawa,N. (1998). Randomization of left-right asymmetry due to loss of nodal cilia generating leftward flow of extraembryonic fluid in mice lacking KIF3B motor protein. Cell *95*, 829-837.

O'Connell,C.B. and Wang,Y.L. (2000). Mammalian spindle orientation and position respond to changes in cell shape in a dynein-dependent fashion. Mol. Biol. Cell *11*, 1765-1774.

O'Connell,K.F., Caron,C., Kopish,K.R., Hurd,D.D., Kemphues,K.J., Li,Y., and White,J.G. (2001). The C. elegans zyg-1 gene encodes a regulator of centrosome duplication with distinct maternal and paternal roles in the embryo. Cell *105*, 547-558.

Oakley,B.R., Oakley,C.E., Yoon,Y., and Jung,M.K. (1990). Gamma-tubulin is a component of the spindle pole body that is essential for microtubule function in Aspergillus nidulans. Cell *61*, 1289-1301.

Oegema,K., Wiese,C., Martin,O.C., Milligan,R.A., Iwamatsu,A., Mitchison,T.J., and Zheng,Y. (1999). Characterization of two related Drosophila gamma-tubulin complexes that differ in their ability to nucleate microtubules. J. Cell Biol. *144*, 721-733.

Ohta,T., Essner,R., Ryu,J.H., Palazzo,R.E., Uetake,Y., and Kuriyama,R. (2002). Characterization of Cep135, a novel coiled-coil centrosomal protein involved in microtubule organization in mammalian cells. J. Cell Biol. *156*, 87-99.

Okuda,M., Horn,H.F., Tarapore,P., Tokuyama,Y., Smulian,A.G., Chan,P.K., Knudsen,E.S., Hofmann,I.A., Snyder,J.D., Bove,K.E., and Fukasawa,K. (2000). Nucleophosmin/B23 is a target of CDK2/cyclin E in centrosome duplication. Cell *103*, 127-140.

Okuwaki,M., Tsujimoto,M., and Nagata,K. (2002). The RNA binding activity of a ribosome biogenesis factor, nucleophosmin/B23, is modulated by phosphorylation with a cell cycle-dependent kinase and by association with its subtype. Mol. Biol. Cell *13*, 2016-2030.

Ou,Y.Y., Mack,G.J., Zhang,M., and Rattner,J.B. (2002). CEP110 and ninein are located in a specific domain of the centrosome associated with centrosome maturation. J. Cell Sci. *115*, 1825-1835.

Palazzo,R.E., Vogel,J.M., Schnackenberg,B.J., Hull,D.R., and Wu,X. (2000). Centrosome maturation. Curr. Top. Dev. Biol. *49:449-70.*, 449-470.

Pazour,G.J., Dickert,B.L., Vucica,Y., Seeley,E.S., Rosenbaum,J.L., Witman,G.B., and Cole,D.G. (2000). Chlamydomonas IFT88 and its mouse homologue, polycystic kidney disease gene tg737, are required for assembly of cilia and flagella. J. Cell Biol. *151*, 709-718.

Pereira,G., Hofken,T., Grindlay,J., Manson,C., and Schiebel,E. (2000). The Bub2p spindle checkpoint links nuclear migration with mitotic exit. Mol. Cell *6*, 1-10.

Pereira,G. and Schiebel,E. (1997). Centrosome-microtubule nucleation. J. Cell Sci. *110*, 295-300.

Pereira,G. and Schiebel,E. (2001). The role of the yeast spindle pole body and the mammalian centrosome in regulating late mitotic events. Curr. Opin. Cell Biol. *13*, 762-769.

Pernet-Gallay,K., Antony,C., Johannes,L., Bornens,M., Goud,B., and Rios,R.M. (2002). The overexpression of GMAP-210 blocks anterograde and retrograde transport between the ER and the Golgi apparatus. Traffic. *3*, 822-832.

Piekorz,R.P., Hoffmeyer,A., Duntsch,C.D., McKay,C., Nakajima,H., Sexl,V., Snyder,L., Rehg,J., and Ihle,J.N. (2002). The centrosomal protein TACC3 is essential for hematopoietic stem cell function and genetically interfaces with p53-regulated apoptosis. EMBO J. *21*, 653-664.

Piel,M., Meyer,P., Khodjakov,A., Rieder,C.L., and Bornens,M. (2000). The respective contributions of the mother and daughter centrioles to centrosome activity and behavior in vertebrate cells. J. Cell Biol. *149*, 317-330.

Piel,M., Nordberg,J., Euteneuer,U., and Bornens,M. (2001). Centrosome-dependent exit of cytokinesis in animal cells. Science *291*, 1550-1553.

Pottgiesser,J., Maurer,P., Mayer,U., Nischt,R., Mann,K., Timpl,R., Krieg,T., and Engel,J. (1994). Changes in calcium and collagen IV binding caused by mutations in the EF hand and other domains of extracellular matrix protein BM-40 (SPARC, osteonectin). J. Mol. Biol. *238*, 563-574.

Preble,A.M., Giddings,T.M., Jr., and Dutcher,S.K. (2000). Basal bodies and centrioles: their function and structure. Curr. Top. Dev. Biol. *49:207-33.*, 207-233.

Presley,J.F., Cole,N.B., Schroer,T.A., Hirschberg,K., Zaal,K.J., and Lippincott-Schwartz,J. (1997). ER-to-Golgi transport visualized in living cells. Nature *389*, 81-85.

Purohit,A., Tynan,S.H., Vallee,R., and Doxsey,S.J. (1999). Direct interaction of pericentrin with cytoplasmic dynein light intermediate chain contributes to mitotic spindle organization. J Cell Biol. *147*, 481-492.

Qian,Y.W., Erikson,E., and Maller,J.L. (1999). Mitotic effects of a constitutively active mutant of the Xenopus polo-like kinase Plx1. Mol. Cell Biol. *19*, 8625-8632.

Quintyne,N.J., Gill,S.R., Eckley,D.M., Crego,C.L., Compton,D.A., and Schroer,T.A. (1999). Dynactin is required for microtubule anchoring at centrosomes. J. Cell Biol. *147*, 321-334.

Quintyne,N.J. and Schroer,T.A. (2002). Distinct cell cycle-dependent roles for dynactin and dynein at centrosomes. J. Cell Biol. *159*, 245-254.

Ralston,E. (1993). Changes in architecture of the Golgi complex and other subcellular organelles during myogenesis. J. Cell Biol. *120*, 399-409.

Ralston,E., Lu,Z., and Ploug,T. (1999). The organization of the Golgi complex and microtubules in skeletal muscle is fiber type-dependent. J. Neurosci. *19*, 10694-10705.

Ralston,E., Ploug,T., Kalhovde,J., and Lomo,T. (2001). Golgi complex, endoplasmic reticulum exit sites, and microtubules in skeletal muscle fibers are organized by patterned activity. J. Neurosci. *21*, 875-883.

Raux,H., Flamand,A., and Blondel,D. (2000). Interaction of the rabies virus P protein with the LC8 dynein light chain. J Virol. *74*, 10212-10216.

Ren,X.R., Du,Q.S., Huang,Y.Z., Ao,S.Z., Mei,L., and Xiong,W.C. (2001). Regulation of CDC42 GTPase by proline-rich tyrosine kinase 2 interacting with PSGAP, a novel pleckstrin homology and Src homology 3 domain containing rhoGAP protein. J. Cell Biol. *152*, 971-984.

Rieder,C.L. and Borisy,G.G. (1982). The centrosome cycle in Ptk2 cells. Asymmetric distribution and structural changes in the pericentriolar material. Biology of the Cell *44*, 117-132.

Rios,R.M. and Bornens,M. (2003). The Golgi apparatus at the cell centre. Curr. Opin. Cell Biol. *15*, 60-66.

Rios,R.M., Tassin,A.M., Celati,C., Antony,C., Boissier,M.C., Homberg,J.C., and Bornens,M. (1994). A peripheral protein associated with the cis-Golgi network redistributes in the intermediate compartment upon brefeldin A treatment. J Cell Biol. *125*, 997-1013.

Riparbelli,M.G., Callaini,G., Glover,D.M., and Avides Md,M.C. (2002). A requirement for the Abnormal Spindle protein to organise microtubules of the central spindle for cytokinesis in Drosophila. J. Cell Sci. *115*, 913-922.

Rogalski,A.A., Bergmann,J.E., and Singer,S.J. (1984). Effect of microtubule assembly status on the intracellular processing and surface expression of an integral protein of the plasma membrane. J Cell Biol. *99*, 1101-1109.

Salisbury,J.L. (1995). Centrin, centrosomes, and mitotic spindle poles. Curr. Opin. Cell Biol. *7*, 39-45.

Salisbury,J.L., Suino,K.M., Busby,R., and Springett,M. (2002). Centrin-2 is required for centriole duplication in mammalian cells. Curr. Biol. *12*, 1287-1292.

Sancho,D., Nieto,M., Llano,M., Rodriguez-Fernandez,J.L., Tejedor,R., Avraham,S., Cabanas,C., Lopez-Botet,M., and Sanchez-Madrid,F. (2000). The tyrosine kinase PYK-2/RAFTK regulates natural killer (NK) cell cytotoxic response, and is translocated and activated upon specific target cell recognition and killing. J. Cell Biol. *149*, 1249-1262.

Sancho,D., Vicente-Manzanares,M., Mittelbrunn,M., Montoya,M.C., Gordon-Alonso,M., Serrador,J.M., and Sanchez-Madrid,F. (2002). Regulation of microtubule-organizing center orientation and actomyosin cytoskeleton rearrangement during immune interactions. Immunol. Rev. *189:84-97.*, 84-97.

Sawin,K.E. and Mitchison,T.J. (1991). Mitotic spindle assembly by two different pathways in vitro. J. Cell Biol. *112*, 925-940.

Sawin,K.E. and Mitchison,T.J. (1995). Mutations in the kinesin-like protein Eg5 disrupting localization to the mitotic spindle. Proc. Natl. Acad. Sci. U. S. A *92*, 4289-4293.

Schatten,G. (1994). The centrosome and its mode of inheritance: the reduction of the centrosome during gametogenesis and its restoration during fertilization. Dev. Biol. *165*, 299-335.

Schiebel,E. (2000). gamma-tubulin complexes: binding to the centrosome, regulation and microtubule nucleation. Curr. Opin. Cell Biol. *12*, 113-118.

Schliwa,M., Euteneuer,U., Graf,R., and Ueda,M. (1999). Centrosomes, microtubules and cell migration. Biochem. Soc. Symp. *65:223-31.*, 223-231.

Schliwa,M. and Woehlke,G. (2003). Molecular motors. Nature *422*, 759-765.

Schnackenberg,B.J., Khodjakov,A., Rieder,C.L., and Palazzo,R.E. (1998). The disassembly and reassembly of functional centrosomes in vitro. Proc. Natl. Acad. Sci U. S. A *95*, 9295-9300.

Schnackenberg,B.J. and Palazzo,R.E. (1999). Identification and function of the centrosome centromatrix. Biol. Cell *91*, 429-438.

Schroer,T.A. and Sheetz,M.P. (1991). Two activators of microtubule-based vesicle transport. J Cell Biol. *115*, 1309-1318.

Schuyler,S.C. and Pellman,D. (2001). Search, capture and signal: games microtubules and centrosomes play. J. Cell Sci. *114*, 247-255.

Seelos,C. (1997). A critical parameter determining the aging of DNA-calcium-phosphate precipitates. Anal. Biochem. *245*, 109-111.

Segal,M. and Bloom,K. (2001). Control of spindle polarity and orientation in Saccharomyces cerevisiae. Trends Cell Biol. *11*, 160-166.

Serrador,J.M., Nieto,M., and Sanchez-Madrid,F. (1999). Cytoskeletal rearrangement during migration and activation of T lymphocytes. Trends Cell Biol. *9*, 228-233.

Shevchenko,A., Wilm,M., Vorm,O., and Mann,M. (1996). Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. Anal. Chem. *68*, 850-858.

Short,B., Preisinger,C., Schaletzky,J., Kopajtich,R., and Barr,F.A. (2002). The Rab6 GTPase regulates recruitment of the dynactin complex to Golgi membranes. Curr. Biol. *12*, 1792-1795.

Sibon,O.C., Kelkar,A., Lemstra,W., and Theurkauf,W.E. (2000). DNA-replication/DNA-damage-dependent centrosome inactivation in Drosophila embryos. Nat. Cell Biol. *2*, 90-95.

Skoufias,D.A., Burgess,T.L., and Wilson,L. (1990). Spatial and temporal colocalization of the Golgi apparatus and microtubules rich in detyrosinated tubulin. J Cell Biol. *111*, 1929-1937.

Smirnova,E.A. and Bajer,A.S. (1992). Spindle poles in higher plant mitosis. Cell Motil. Cytoskeleton *23*, 1-7.

Smits,V.A., Klompmaker,R., Arnaud,L., Rijksen,G., Nigg,E.A., and Medema,R.H. (2000). Polo-like kinase-1 is a target of the DNA damage checkpoint. Nat. Cell Biol. *2*, 672-676.

Snyder,M. (1994). The spindle pole body of yeast. Chromosoma *103*, 369-380.

Sobel,S.G. and Snyder,M. (1995). A highly divergent gamma-tubulin gene is essential for cell growth and proper microtubule organization in Saccharomyces cerevisiae. J. Cell Biol. *131*, 1775-1788.

Starr,D.A., Williams,B.C., Hays,T.S., and Goldberg,M.L. (1998). ZW10 helps recruit dynactin and dynein to the kinetochore. J Cell Biol. *142*, 763-774.

Stearns,T. and Kirschner,M. (1994). In vitro reconstitution of centrosome assembly and function: the central role of gamma-tubulin. Cell *76*, 623-637.

Steffen,W., Fajer,E.A., and Linck,R.W. (1994). Centrosomal components immunologically related to tektins from ciliary and flagellar microtubules. J. Cell Sci. *107*, 2095-2105.

Stucke,V.M., Sillje,H.H., Arnaud,L., and Nigg,E.A. (2002). Human Mps1 kinase is required for the spindle assembly checkpoint but not for centrosome duplication. EMBO J. *21*, 1723-1732.

Sumiyoshi,E., Sugimoto,A., and Yamamoto,M. (2002). Protein phosphatase 4 is required for centrosome maturation in mitosis and sperm meiosis in C. elegans. J. Cell Sci. *115*, 1403-1410.

Sunkel,C.E. and Glover,D.M. (1988). polo, a mitotic mutant of Drosophila displaying abnormal spindle poles. J. Cell Sci. *89*, 25-38.

Sunkel,C.E., Gomes,R., Sampaio,P., Perdigao,J., and Gonzalez,C. (1995). Gamma-tubulin is required for the structure and function of the microtubule organizing centre in Drosophila neuroblasts. EMBO J. *14*, 28-36.

Sutterlin,C., Hsu,P., Mallabiabarrena,A., and Malhotra,V. (2002). Fragmentation and dispersal of the pericentriolar Golgi complex is required for entry into mitosis in mammalian cells. Cell *109*, 359-369.

Tai,A.W., Chuang,J.Z., Bode,C., Wolfrum,U., and Sung,C.H. (1999). Rhodopsin's carboxy-terminal cytoplasmic tail acts as a membrane receptor for cytoplasmic dynein by binding to the dynein light chain Tctex-1. Cell *97*, 877-887.

Takahashi,M., Shibata,H., Shimakawa,M., Miyamoto,M., Mukai,H., and Ono,Y. (1999). Characterization of a novel giant scaffolding protein, CG-NAP, that anchors multiple signaling enzymes to centrosome and the golgi apparatus. J. Biol. Chem. *274*, 17267-17274.

Takahashi,M., Yamagiwa,A., Nishimura,T., Mukai,H., and Ono,Y. (2002). Centrosomal Proteins CG-NAP and Kendrin Provide Microtubule Nucleation Sites by Anchoring gamma-Tubulin Ring Complex. Mol. Biol. Cell *13*, 3235-3245.

Tanaka,Y., Zhang,Z., and Hirokawa,N. (1995). Identification and molecular evolution of new dynein-like protein sequences in rat brain. J Cell Sci *108*, 1883-1893.

Tassin,A.M., Celati,C., Moudjou,M., and Bornens,M. (1998). Characterization of the human homologue of the yeast spc98p and its association with gamma-tubulin. J. Cell Biol. *141*, 689-701.

Tassin,A.M., Celati,C., Paintrand,M., and Bornens,M. (1997). Identification of an Spc110p-related protein in vertebrates. J. Cell Sci. *110*, 2533-2545.

Tassin,A.M., Paintrand,M., Berger,E.G., and Bornens,M. (1985). The Golgi apparatus remains associated with microtubule organizing centers during myogenesis. J. Cell Biol. *101*, 630-638.

Tekotte,H. and Davis,I. (2002). Intracellular mRNA localization: motors move messages. Trends Genet. *18*, 636-642.

Thyberg,J. and Moskalewski,S. (1985). Microtubules and the organization of the Golgi complex. Exp. Cell Res *159*, 1-16.

Thyberg,J. and Moskalewski,S. (1993). Relationship between the Golgi complex and microtubules enriched in detyrosinated or acetylated alpha-tubulin: studies on cells recovering from nocodazole and cells in the terminal phase of cytokinesis. Cell Tissue Res *273*, 457-466.

Thyberg,J. and Moskalewski,S. (1999). Role of microtubules in the organization of the Golgi complex. Exp. Cell Res *246*, 263-279.

Tugendreich,S., Tomkiel,J., Earnshaw,W., and Hieter,P. (1995). CDC27Hs colocalizes with CDC16Hs to the centrosome and mitotic spindle and is essential for the metaphase to anaphase transition. Cell *81*, 261-268.

Tynan,S.H., Purohit,A., Doxsey,S.J., and Vallee,R.B. (2000). Light intermediate chain 1 defines a functional subfraction of cytoplasmic dynein which binds to pericentrin. J Biol. Chem. *275*, 32763-32768.

Vaisberg,E.A., Grissom,P.M., and McIntosh,J.R. (1996). Mammalian cells express three distinct dynein heavy chains that are localized to different cytoplasmic organelles. J Cell Biol. *133*, 831-842.

Vaisberg,E.A., Koonce,M.P., and McIntosh,J.R. (1993). Cytoplasmic dynein plays a role in mammalian mitotic spindle formation. J. Cell Biol. *123*, 849-858.

Vale,R.D. (2003). The molecular motor toolbox for intracellular transport. Cell *112*, 467-480.

Vaughan,K.T. and Vallee,R.B. (1995). Cytoplasmic dynein binds dynactin through a direct interaction between the intermediate chains and p150Glued. J Cell Biol. *131*, 1507-1516.

Vaughan,P.S., Leszyk,J.D., and Vaughan,K.T. (2001). Cytoplasmic dynein intermediate chain phosphorylation regulates binding to dynactin. J Biol. Chem. *276*, 26171-26179.

Vorobjev,I.A. and Chentsov,Y. (1982). Centrioles in the cell cycle. I. Epithelial cells. J. Cell Biol. *93*, 938-949.

Vorobjev,I.A. and Nadezhdina,E.S. (1987). The centrosome and its role in the organization of microtubules. Int. Rev. Cytol. *106:227-93.*, 227-293.

Wakefield,J.G., Bonaccorsi,S., and Gatti,M. (2001). The drosophila protein asp is involved in microtubule organization during spindle formation and cytokinesis. J. Cell Biol. *153*, 637-648.

Walczak,C.E., Vernos,I., Mitchison,T.J., Karsenti,E., and Heald,R. (1998). A model for the proposed roles of different microtubule-based motor proteins in establishing spindle bipolarity. Curr. Biol. *8*, 903-913.

Walenta,J.H., Didier,A.J., Liu,X., and Kramer,H. (2001). The Golgi-associated hook3 protein is a member of a novel family of microtubule-binding proteins. J Cell Biol. *152*, 923-934.

Waterman-Storer,C.M., Karki,S., and Holzbaur,E.L. (1995). The p150Glued component of the dynactin complex binds to both microtubules and the actin-related protein centractin (Arp-1). Proc. Natl. Acad. Sci U. S. A *92*, 1634-1638.

Wiese,C. and Zheng,Y. (1999). Gamma-tubulin complexes and their interaction with microtubule-organizing centers. Curr. Opin. Struct. Biol. *9*, 250-259.

Wigley,W.C., Fabunmi,R.P., Lee,M.G., Marino,C.R., Muallem,S., DeMartino,G.N., and Thomas,P.J. (1999). Dynamic association of proteasomal machinery with the centrosome. J. Cell Biol. *145*, 481-490.

Wilkie,S.E., Li,Y., Deery,E.C., Newbold,R.J., Garibaldi,D., Bateman,J.B., Zhang,H., Lin,W., Zack,D.J., Bhattacharya,S.S., Warren,M.J., Hunt,D.M., and Zhang,K. (2001). Identification and functional consequences of a new mutation (E155G) in the gene for GCAP1 that causes autosomal dominant cone dystrophy. Am. J. Hum. Genet. *69*, 471-480.

Wilson E.B. (1911). The Cell in Development and Inheritance, The MacMillan Company.

Wittmann,T., Hyman,A., and Desai,A. (2001). The spindle: a dynamic assembly of microtubules and motors. Nat. Cell Biol. *3*, E28-E34.

Wojcik,E.J., Glover,D.M., and Hays,T.S. (2000). The SCF ubiquitin ligase protein slimb regulates centrosome duplication in Drosophila. Curr. Biol. *10*, 1131-1134.

Yano,H., Lee,F.S., Kong,H., Chuang,J., Arevalo,J., Perez,P., Sung,C., and Chao,M.V. (2001). Association of Trk neurotrophin receptors with components of the cytoplasmic dynein motor. J Neurosci. *21*, RC125.

Ye,G.J., Vaughan,K.T., Vallee,R.B., and Roizman,B. (2000). The herpes simplex virus 1 U(L)34 protein interacts with a cytoplasmic dynein intermediate chain and targets nuclear membrane. J Virol. *74*, 1355-1363.

Young,A., Dictenberg,J.B., Purohit,A., Tuft,R., and Doxsey,S.J. (2000). Cytoplasmic dynein-mediated assembly of pericentrin and gamma tubulin onto centrosomes. Mol. Biol. Cell *11*, 2047-2056.

Yvon,A.M., Walker,J.W., Danowski,B., Fagerstrom,C., Khodjakov,A., and Wadsworth,P. (2002). Centrosome reorientation in wound-edge cells is cell type specific. Mol. Biol. Cell *13*, 1871-1880.

Zheng,Y., Wong,M.L., Alberts,B., and Mitchison,T. (1995). Nucleation of microtubule assembly by a gamma-tubulin-containing ring complex. Nature *378*, 578-583.

Zimmerman,W. and Doxsey,S.J. (2000). Construction of centrosomes and spindle poles by molecular motor-driven assembly of protein particles. Traffic. *1*, 927-934.

Zimmerman,W., Sparks,C.A., and Doxsey,S.J. (1999). Amorphous no longer: the centrosome comes into focus. Curr. Opin. Cell Biol. *11*, 122-128.

APPENDIX A: LIST OF PLASMIDS

name	tag	gene	insert	species	vector
MC 1	HA	Fhos	full lenght	human	pCMV5
MC 2		Fhos	full lenght	human	VH127 (pUni 10-BlnI)
MC 3		Fhos	N-term fragment (1-320aa)	human	VH127 (pUni 10-BlnI)
MC 4		Fhos	C-term fragment	human	VH127 (pUni 10-BlnI)
MC 5	Myc	Fhos	full lenght	human	pHM200-Myc3
MC 6	His6	Fhos	full lenght	human	pHB3-His6
MC 7	His6	Fhos	N-term fragment (1-320aa)	human	pHB3-His6
MC 8	His6	Fhos	C-term fragment	human	pHB3-His6
MC 9	GFP	Nlp	N-term fragment (aa 1-702) from PM74	human	pEGFP-C1
MC10	GFP	Nlp	C-term fragment (aa 694-1382) from PM74	human	pEGFP-C1
MC11	GFP	Nlp	N-term Ef-h domain	human	pEGFP-C1
MC12	GFP	Nlp	N-term coiled-coil domain	human	pEGFP-C1
MC13	His6	Nlp	full lenght	human	pcDNA3.1/HisC
MC14	GST	Nlp	full lenght	human	pGEX-6P-3
MC15	GST	Nlp	N-term fragment (aa 1-702) from MC14	human	pGEX-6P-3
MC16	GST	Nlp	C-term fragment (aa 694-1382)	human	pGEX-6P-3
MC17		Nlp	full lenght	human	pUNI 10
MC18		Nlp	N-term fragment	human	pUNI 10
MC19		Nlp	C-term fragment	human	pUNI 10
MC20		Ninein	full lenght	mouse	pUNI 10
MC21		hGCP2	full lenght	human	pBS-SK
MC22		hGCP3	full lenght	human	pBS-SK
MC23		h76p	full lenght	human	pBS-SK
MC24		hGCP2-NdeI	full lenght	human	pBS-SK
MC25		hGCP3	full lenght	Human	pUNI10
MC26		hGCP3-NcoI	full lenght	Human	pUni10
MC27		h76p	full lenght	Human	pUni10
MC28		hGCP2-NdeI	full length	human	pUNI 10
MC29	HA	hGCP3	full length	human	pHM200-HA3
MC30		Fhos	C-term (EcoRI)	human	VH127 (pUni 10-BlnI)
MC31		Fhos	C-term (EcoRI)	human	pHB3-His6
MC32	HA	h76p	full length	Human	pHM200 HA3
MC32	HA	h76p	full length	Human	pHM200 HA3
MC33	Myc	ninein	N-term	mouse	pCRIITopo
MC34	Myc	ninein	N-term	mouse	pBKdeltaLac-CMV
MC35	EGFP	ninein	N-term	mouse	pT7EGFP-C1
MC36	EGFP	hNlp	Nlp delta EF-h I (aa 35-1382)	human	pEGFP-C1
MC37		hNlp	Nlp N-N-term EF-h I mutant (from PM121)	human	pBS KS
MC38		hNlp	Nlp N-N-term EF-h II mutant	human	pBS KS
MC39		hNlp	Nlp N-N-term EF-h double mutant	human	pBS KS
MC40		hNlp	Nlp N-N-term EF-h double mut Sal I - ATG	human	pCRII-Topo
MC41	EGFP	hNlp	Nlp EF-h II mutant	human	pT7EGFP-C1
MC42	EGFP	hNlp	Nlp EF-h double mutant	human	pEGFP-C1
MC43	EGFP	mPericentrin	pericentrin	mouse	pEGFP-C2
MC44	GST	ninein	N-terminal fregment (aa 1-750)	mouse	pDEST15

APPENDIX B

The work in this Thesis has been carried out at the Max-Planck Institute of Biochemistry (Martinsried), Department of Cell Biology, under the supervision of Prof.Dr. Erich A. Nigg.

Part of this Thesis has been published in the following paper:

Casenghi M, Meraldi P, Weinhart U, Duncan PI, Korner R, Nigg EA (2003). *Polo-like kinase 1 regulates Nlp, a centrosome protein involved in microtubule nucleation*. Dev Cell. 5(1):113-25.

A copy of this paper is presented in the following pages.

Developmental Cell, Vol. 5, 113–125, July, 2003, Copyright ©2003 by Cell Press

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

Martina Casenghi,[1,2] Patrick Meraldi,[1,2,3] Ulrike Weinhart,[1] Peter I. Duncan,[1,4] Roman Körner,[1] and Erich A. Nigg[1,*]
[1]Department of Cell Biology
Max Planck Institute of Biochemistry
Am Klopferspitz 18a
D-82152 Martinsried
Germany

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 Spc 98, 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

*Correspondence: nigg@biochem.mpg.de
[2]These authors contributed equally to this work.
[3]Present address: Department of Biology, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, Massachusetts 02139.
[4]Present address: Nutrition Department, Nestlé Research Center, P.O. Box 44, CH-1000 Lausanne 26, Switzerland.

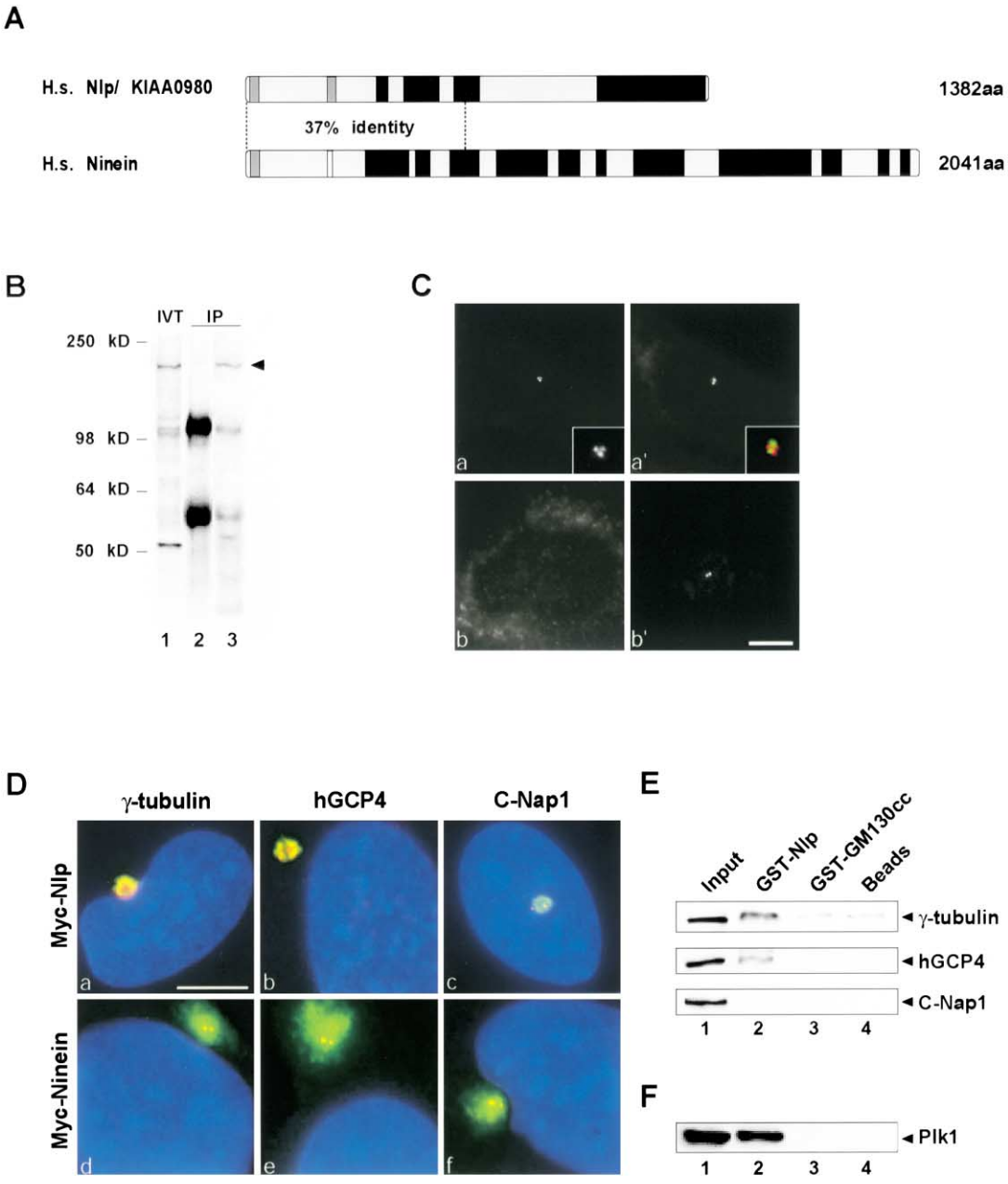

Figure 1. Nlp, a Centrosomal Protein Able to Recruit γ-TuRCs

(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

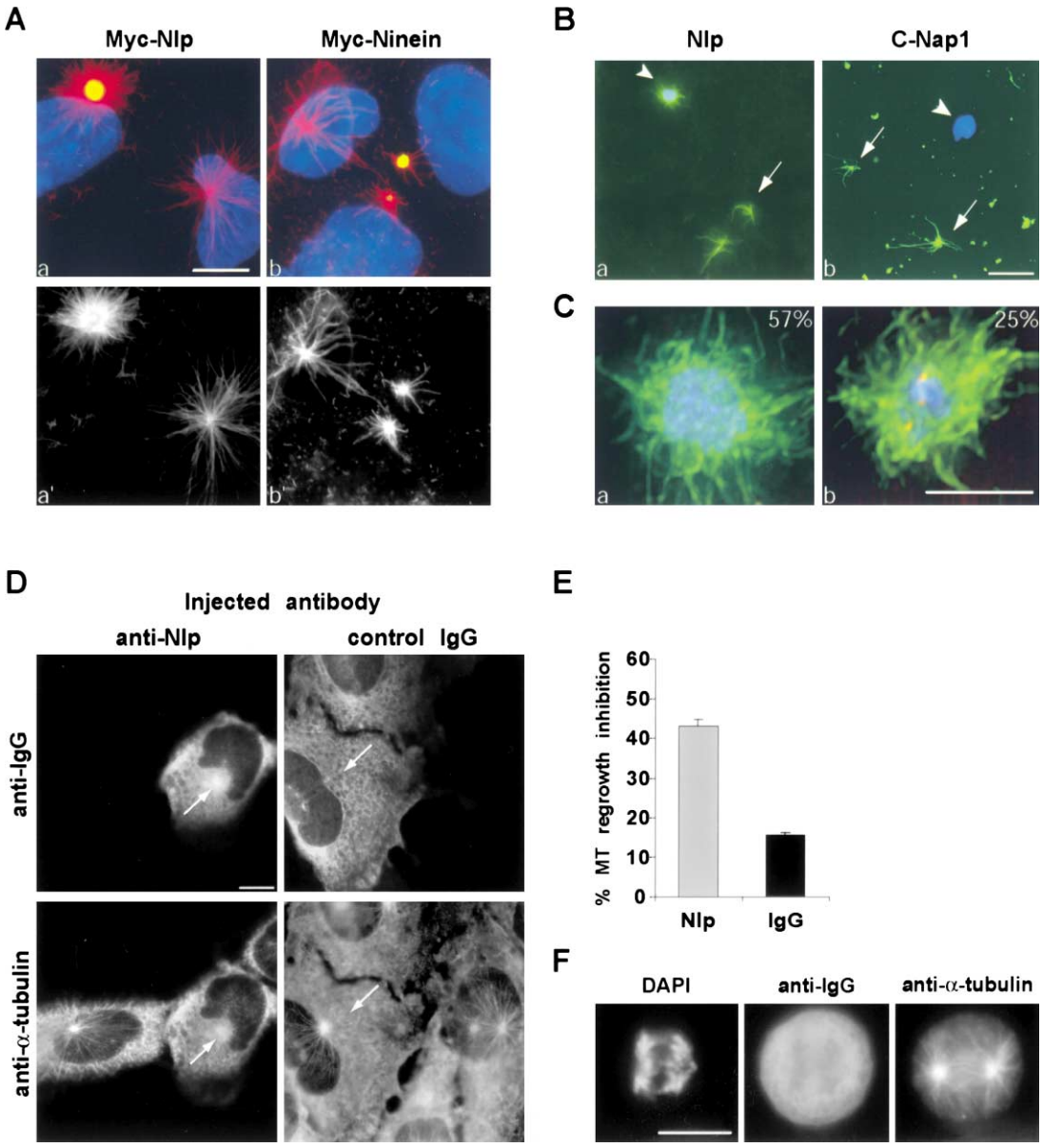

Figure 2. Nlp Stimulates MT Nucleation

(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 μm in (A), (D), and (F), and 5 μm 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 (*n*inein-*l*ike-*p*rotein) 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 (*n*inein-*l*ike *p*rotein). 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 Ca^{2+} 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

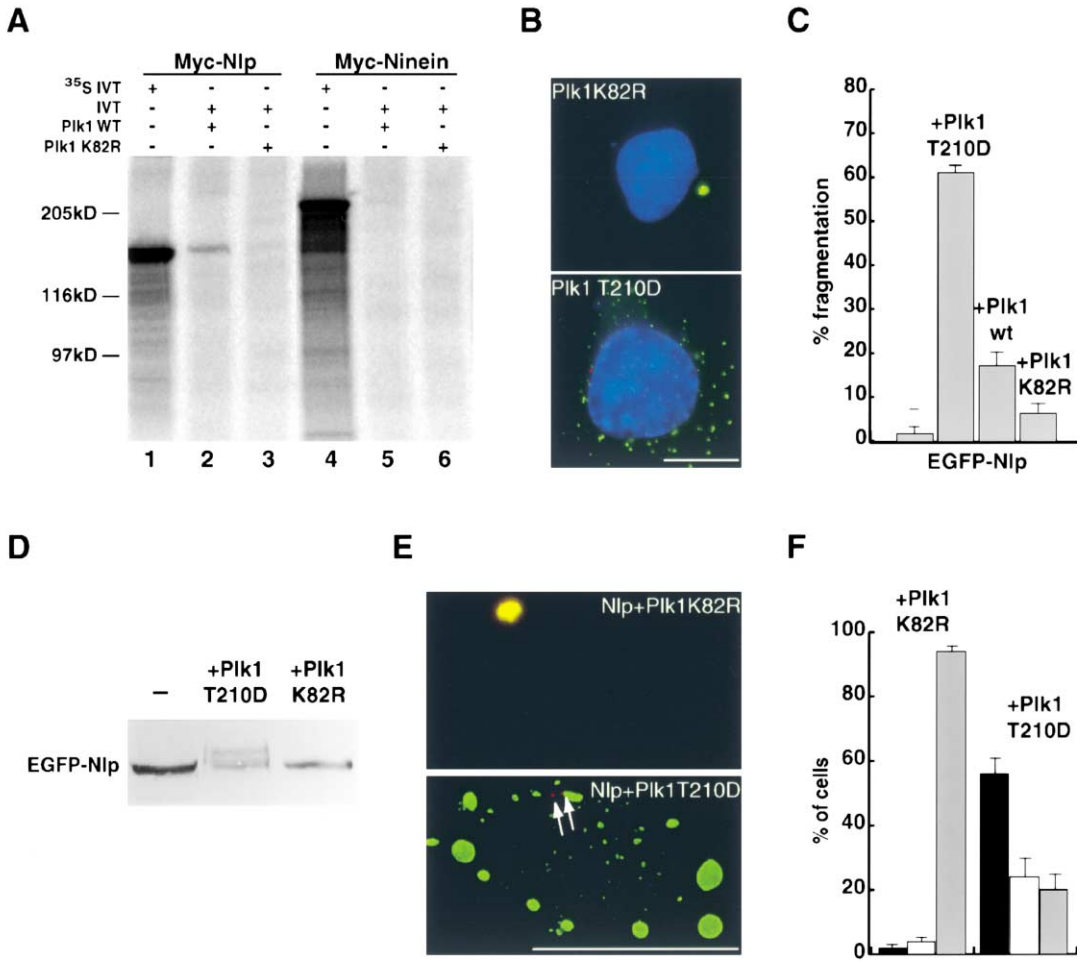

Figure 3. Plk1 Regulates Centrosome Association and γ-Tubulin Interaction of Nlp

(A) Myc-Nlp and myc-ninein were in vitro translated with (lanes 1 and 4) or without (lanes 2, 3, 5, and 6) [^{35}S]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 μm.

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

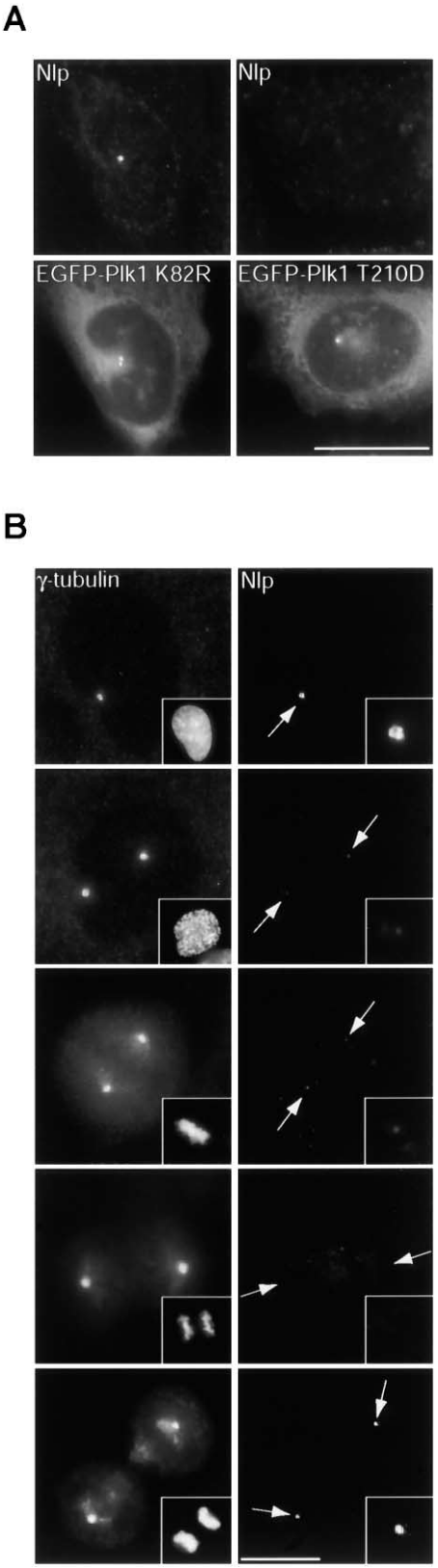

Figure 4. Nlp Is Removed from the Centrosomes during Centrosome Maturation

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

(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 μm.

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

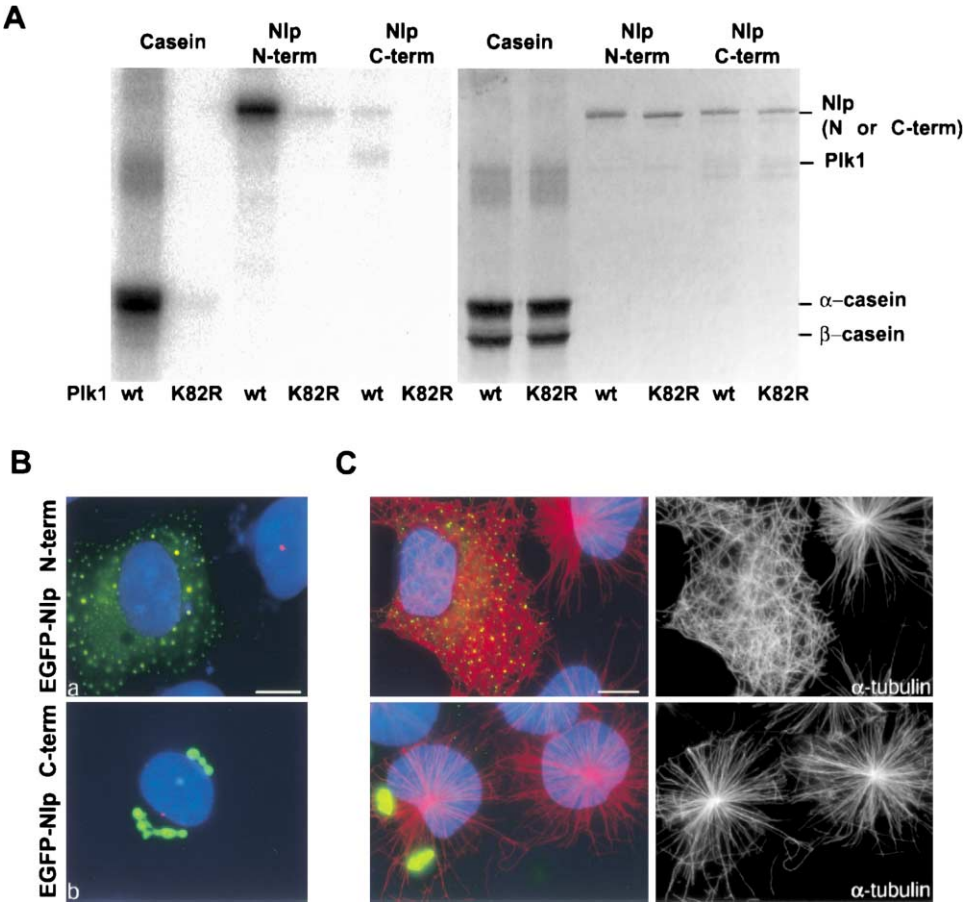

Figure 5. Plk1 Regulates Interaction between the N Terminus of Nlp and γ-TuRCs

(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 μm.

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 *P*4.1-*a*ssociated *p*rotein), have been proposed to bind to γ-tubulin, but their precise contributions to MT organization remain to be clarified (Dictenberg 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 pBluescriptKSII-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 His_6-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-NlpΔ4A), in positions 349, 498, 670, and 686 (pEGFP-NlpΔ4B), or in all eight positions (pEGFP-NlpΔ8). N-terminal NlpΔ8A (residues 1–702) was further subcloned into pGEX-6P-3. Wild-type and mutant (K82R, T210D) Plk 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 His_6-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% CO_2 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 μg/ml, respectively, all GIBCO-BRL). The Tet-On U2OS myc-Nlp and myc-ninein stable cell lines were grown additionally under selection of 200 μg/ml zeocin and 50 μg/ml hygromycin. These lines were obtained by transfection of Tet-On U2OS cells (Invitrogen) with 20 μg 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 $MgCl_2$, 1 mM PMSF, and aprotinin, leupeptin, and pepstatin at 1 μg/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 NlpΔ8 were expressed in *E. coli* BL21RIL and purified by glutathione affinity

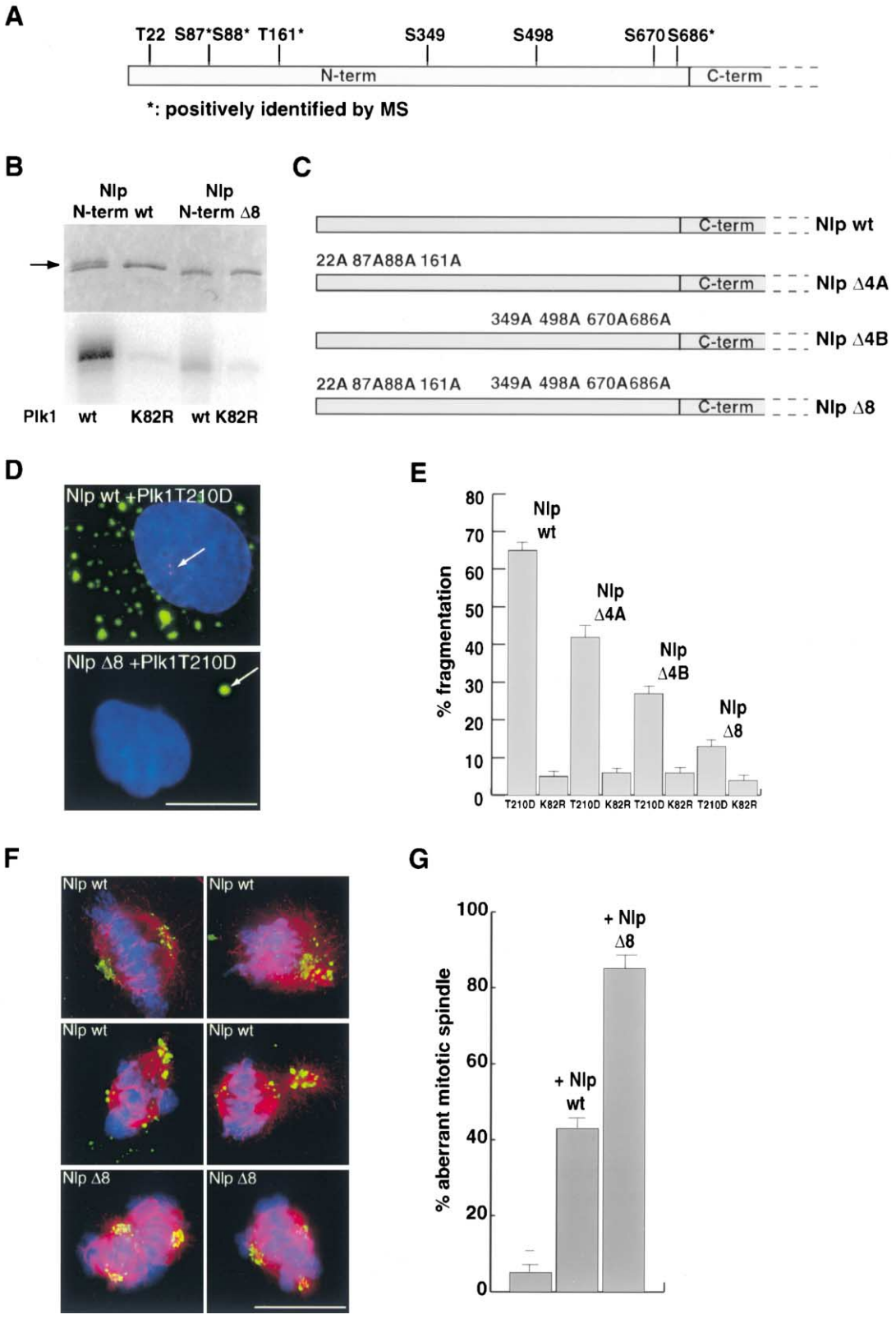

Figure 6. Overexpression of Phosphorylation Site Mutant Nlp Causes Aberrant Mitotic Spindle Formation

(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

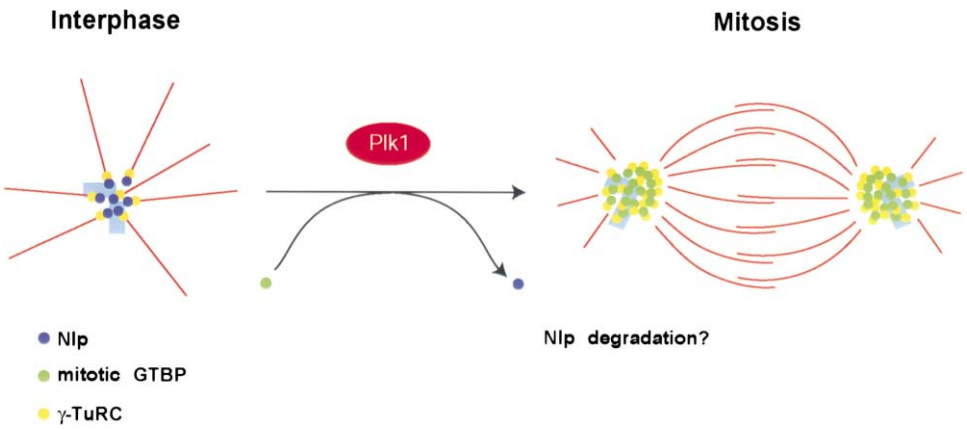

Figure 7. Model for the Regulation of Nlp during Centrosome Maturation

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 μl 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 nonimmune 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 μg/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 μg/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 μg/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× 1.4 oil immersion objective, was used for collecting optical sections at distances of 0.2 μm 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 His_6-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 $MgCl_2$, 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.

Acknowledgments

We thank the Kazusa DNA Research Institute (Japan) for the KIAA0980 cDNA, M. Bornens (Institut Curie, Paris), B. Eddé (Collège de France, Paris), M. Wright (CNRS, Toulouse), and F. Barr (MPI, Martinsried) for antibodies and cDNAs. We also thank A. Fry (University of Leicester), G. Guarguaglini, T. Mayer, and C. Wilkinson for helpful discussions. M.C. and P.M. were supported by the SFB413 from the Deutsche Forschungsgemeinschaft, and the Swiss National Science Foundation, respectively. E.A.N. thanks the Max-Planck-Society and the "Fonds der Chemischen Industrie" for support.

(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-NlpΔ8 (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 μm.

Received: December 6, 2002
Revised: March 17, 2003
Accepted: May 9, 2003
Published: July 7, 2003

References

Barr, F.A., Nakamura, N., and Warren, G. (1998). Mapping the interaction between GRASP65 and GM130, components of a protein complex involved in the stacking of Golgi cisternae. EMBO J. *17*, 3258–3268.

Berdnik, D., and Knoblich, J.A. (2002). *Drosophila* Aurora-A is required for centrosome maturation and actin-dependent asymmetric protein localization during mitosis. Curr. Biol. *12*, 640–647.

Blomberg-Wirschell, M. and Doxsey, S.J. (1998). Rapid isolation of centrosomes. Methods Enzymol. *298*, 228–238.

Bobinnec, Y., Moudjou, M., Fouquet, J.P., Desbruyeres, E., Edde, B., and Bornens, M. (1998). Glutamylation of centriole and cytoplasmic tubulin in proliferating non-neuronal cells. Cell Motil. Cytoskeleton *39*, 223–232.

Bornens, M. (2002). Centrosome composition and microtubule anchoring mechanisms. Curr. Opin. Cell Biol. *14*, 25–34.

Bouckson-Castaing, V., Moudjou, M., Ferguson, D.J., Mucklow, S., Belkaid, Y., Milon, G., and Crocker, P.R. (1996). Molecular characterisation of ninein, a new coiled-coil protein of the centrosome. J. Cell Sci. *109*, 179–190.

Brinkley, B.R. (2001). Managing the centrosome numbers game: from chaos to stability in cancer cell division. Trends Cell Biol. *11*, 18–21.

Dictenberg, J.B., Zimmerman, W., Sparks, C.A., Young, A., Vidair, C., Zheng, Y., Carrington, W., Fay, F.S., and Doxsey, S.J. (1998). Pericentrin and γ-tubulin form a protein complex and are organized into a novel lattice at the centrosome. J. Cell Biol. *141*, 163–174.

do Carmo, A.M., and Glover, D.M. (1999). Abnormal spindle protein, Asp, and the integrity of mitotic centrosomal microtubule organizing centers. Science *283*, 1733–1735.

Doxsey, S. (2001). Re-evaluating centrosome function. Nat. Rev. Mol. Cell Biol. *2*, 688–698.

Doxsey, S.J., Stein, P., Evans, L., Calarco, P.D., and Kirschner, M. (1994). Pericentrin, a highly conserved centrosome protein involved in microtubule organization. Cell *76*, 639–650.

Fava, F., Raynaud-Messina, B., Leung-Tack, J., Mazzolini, L., Li, M., Guillemot, J.C., Cachot, D., Tollon, Y., Ferrara, P., and Wright, M. (1999). Human 76p: a new member of the γ-tubulin-associated protein family. J. Cell Biol. *147*, 857–868.

Flory, M.R., Moser, M.J., Monnat, R.J., Jr., and Davis, T.N. (2000). Identification of a human centrosomal calmodulin-binding protein that shares homology with pericentrin. Proc. Natl. Acad. Sci. USA *97*, 5919–5923.

Francis, S.E., and Davis, T.N. (2000). The spindle pole body of *Saccharomyces cerevisiae*: architecture and assembly of the core components. Curr. Top. Dev. Biol. *49*, 105–132.

Fry, A.M., Mayor, T., Meraldi, P., Stierhof, Y.D., Tanaka, K., and Nigg, E.A. (1998a). C-Nap1, a novel centrosomal coiled-coil protein and candidate substrate of the cell cycle-regulated proteinkinase Nek2. J. Cell Biol. *141*, 1563–1574.

Fry, A.M., Meraldi, P., and Nigg, E.A. (1998b). A centrosomal function for the human Nek2 protein kinase, a member of the NIMA family of cell cycle regulators. EMBO J. *17*, 470–481.

Golsteyn, R.M., Mundt, K.E., Fry, A.M., and Nigg, E.A. (1995). Cell cycle regulation of the activity and subcellular localization of Plk1, a human protein kinase implicated in mitotic spindle function. J. Cell Biol. *129*, 1617–1628.

Hannak, E., Kirkham, M., Hyman, A.A., and Oegema, K. (2001). Aurora-A kinase is required for centrosome maturation in *Caenorhabditis elegans*. J. Cell Biol. *155*, 1109–1116.

Helps, N.R., Brewis, N.D., Lineruth, K., Davis, T., Kaiser, K., and Cohen, P.T. (1998). Protein phosphatase 4 is an essential enzyme required for organisation of microtubules at centrosomes in *Drosophila* embryos. J. Cell Sci. *111*, 1331–1340.

Helps, N.R., Luo, X., Barker, H.M., and Cohen, P.T. (2000). NIMA-related kinase 2 (Nek2), a cell-cycle-regulated protein kinase localized to centrosomes, is complexed to protein phosphatase 1. Biochem. J. *349*, 509–518.

Hinchcliffe, E.H., and Sluder, G. (2001). "It takes two to tango": understanding how centrosome duplication is regulated throughout the cell cycle. Genes Dev. *15*, 1167–1181.

Hong, Y.R., Chen, C.H., Chang, J.H., Wang, S., Sy, W.D., Chou, C.K., and Howng, S.L. (2000). Cloning and characterization of a novel human ninein protein that interacts with the glycogen synthase kinase 3β. Biochim. Biophys. Acta *1492*, 513–516.

Hung, L.Y., Tang, C.J., and Tang, T.K. (2000). Protein 4.1 R-135 interacts with a novel centrosomal protein (CPAP) which is associated with the γ-tubulin complex. Mol. Cell. Biol. *20*, 7813–7825.

Kelm, O., Wind, M., Lehmann, W.D., and Nigg, E.A. (2002). Cell cycle-regulated phosphorylation of the *Xenopus* polo-like kinase Plx1. J. Biol. Chem. *277*, 25247–25256.

Khodjakov, A., and Rieder, C.L. (1999). The sudden recruitment of γ-tubulin to the centrosome at the onset of mitosis and its dynamic exchange throughout the cell cycle, do not require microtubules. J. Cell Biol. *146*, 585–596.

Knop, M., and Schiebel, E. (1998). Receptors determine the cellular localization of a γ-tubulin complex and thereby the site of microtubule formation. EMBO J. *17*, 3952–3967.

Krek, W., and Nigg, E.A. (1991). Mutations of p34cdc2 phosphorylation sites induce premature mitotic events in HeLa cells: evidence for a double block to p34cdc2 kinase activation in vertebrates. EMBO J. *10*, 3331–3341.

Lane, H.A., and Nigg, E.A. (1996). Antibody microinjection reveals an essential role for human polo-like kinase 1 (Plk1) in the functional maturation of mitotic centrosomes. J. Cell Biol. *135*, 1701–1713.

Li, Q., Hansen, D., Killilea, A., Joshi, H.C., Palazzo, R.E., and Balczon, R. (2001). Kendrin/pericentrin-B, a centrosome protein with homology to pericentrin that complexes with PCM-1. J. Cell Sci. *114*, 797–809.

Mayor, T., Stierhof, Y.D., Tanaka, K., Fry, A.M., and Nigg, E.A. (2000). The centrosomal protein C-Nap1 is required for cell cycle-regulated centrosome cohesion. J. Cell Biol. *151*, 837–846.

Mayor, T., Hacker, U., Stierhof, Y.D., and Nigg, E.A. (2002). The mechanism regulating the dissociation of the centrosomal protein C-Nap1 from mitotic spindle poles. J. Cell Sci. *115*, 3275–3284.

Meraldi, P., Honda, R., and Nigg, E.A. (2002). Aurora-A overexpression reveals tetraploidization as a major route to centrosome amplification in p53(–/–) cells. EMBO J. *21*, 483–492.

Mogensen, M.M. (1999). Microtubule release and capture in epithelial cells. Biol. Cell *91*, 331–341.

Mogensen, M.M., Malik, A., Piel, M., Bouckson-Castaing, V., and Bornens, M. (2000). Microtubule minus-end anchorage at centrosomal and non-centrosomal sites: the role of ninein. J. Cell Sci. *113*, 3013–3023.

Moritz, M., and Agard, D.A. (2001). γ-tubulin complexes and microtubule nucleation. Curr. Opin. Struct. Biol. *11*, 174–181.

Moudjou, M., and Bornens, M. (1994). Isolation of centrosomes from cultured animal cells. In Cell Biology: A Laboratory Handbook, J.E. Celis, ed., Volume 1 (London: Academic Press), pp. 595–604.

Moudjou, M., Bordes, N., Paintrand, M., and Bornens, M. (1996). γ-Tubulin in mammalian cells: the centrosomal and the cytosolic forms. J. Cell Sci. *109*, 875–887.

Murphy, S.M., Preble, A.M., Patel, U.K., O'Connell, K.L., Dias, D.P., Moritz, M., Agard, D., Stults, J.T., and Stearns, T. (2001). GCP5 and GCP6: two new members of the human γ-tubulin complex. Mol. Biol. Cell *12*, 3340–3352.

Murray, A.W. (1991). Cell cycle extracts. Methods Cell Biol. *36*, 581–605.

Nigg, E.A. (2002). Centrosome aberrations: cause or consequence of cancer progression? Nat. Rev. Cancer *2*, 815–825.

Oakley, B.R. (2000). γ-Tubulin. Curr. Top. Dev. Biol. *49*, 27–54.

Ohta, T., Essner, R., Ryu, J.H., Palazzo, R.E., Uetake, Y., and Kuriyama, R. (2002). Characterization of Cep135, a novel coiled-coil centrosomal protein involved in microtubule organization in mammalian cells. J. Cell Biol. *156*, 87–99.

Palazzo, R.E., Vogel, J.M., Schnackenberg, B.J., Hull, D.R., and Wu, X. (2000). Centrosome maturation. Curr. Top. Dev. Biol. *49*, 449–470.

Riparbelli, M.G., Callaini, G., Glover, D.M., and Avides Md, M.C. (2002). A requirement for the Abnormal Spindle protein to organise microtubules of the central spindle for cytokinesis in *Drosophila*. J. Cell Sci. *115*, 913–922.

Sawin, K.E., and Mitchison, T.J. (1991). Mitotic spindle assembly by two different pathways in vitro. J. Cell Biol. *112*, 925–940.

Schiebel, E. (2000). γ-tubulin complexes: binding to the centrosome, regulation and microtubule nucleation. Curr. Opin. Cell Biol. *12*, 113–118.

Seelos, C. (1997). A critical parameter determining the aging of DNA-calcium-phosphate precipitates. Anal. Biochem. *245*, 109–111.

Smits, V.A., Klompmaker, R., Arnaud, L., Rijksen, G., Nigg, E.A., and Medema, R.H. (2000). Polo-like kinase-1 is a target of the DNA damage checkpoint. Nat. Cell Biol. *2*, 672–676.

Sumiyoshi, E., Sugimoto, A., and Yamamoto, M. (2002). Protein phosphatase 4 is required for centrosome maturation in mitosis and sperm meiosis in *C. elegans*. J. Cell Sci. *115*, 1403–1410.

Sunkel, C.E., and Glover, D.M. (1988). polo, a mitotic mutant of *Drosophila* displaying abnormal spindle poles. J. Cell Sci. *89*, 25–38.

Takahashi, M., Yamagiwa, A., Nishimura, T., Mukai, H., and Ono, Y. (2002). Centrosomal proteins CG-NAP and kendrin provide microtubule nucleation sites by anchoring γ-tubulin ring complex. Mol. Biol. Cell *13*, 3235–3245.

Tassin, A.M., Celati, C., Moudjou, M., and Bornens, M. (1998). Characterization of the human homologue of the yeast spc98p and its association with γ-tubulin. J. Cell Biol. *141*, 689–701.

Wakefield, J.G., Bonaccorsi, S., and Gatti, M. (2001). The *Drosophila* protein asp is involved in microtubule organization during spindle formation and cytokinesis. J. Cell Biol. *153*, 637–648.

CURRICULUM VITAE ET STUDIORUM

Personal data

Name:	Martina Casenghi
Date of Birth:	25 June 1973
Place of Birh:	Rome

Education

-Jan 2000-Dec 2003:	Ph.D student at the Max-Planck Institute of Biochemistry Dept. of Cell Biology Prof. Erich A. Nigg
-Sept 1999-Dec 1999:	fellow at the CNR Center of Evolutionary Genetics. Dr. Patrizia Lavia
-September 1998-1999:	Post-graduate training (“Tirocinio”) at the CNR National Research Council Center of Evolutionary Genetics c/o University of Rome “La Sapienza”, in fulfillment of the requirements of the National Board of Biologists
-July 1998:	Graduation Degree (“Laurea”) at the University “La Sapienza” (Rome) with highest honors (110/110 cum laude) Supervisors: Dr E. Cundari, Prof. M. Gatti
-Sept 1996-July 1998:	undergraduate student at the CNR Center of Evolutionary Genetics c/o Dept. of Genetics and Molecular Biology University of Rome “La Sapienza”
-Sept 1992-July 1998:	Faculty of Biology, University of Rome “La Sapienza”
-July 1992:	High school Degree