Microtubule nucleation and anchoring at the centrosome are independent processes linked by ninein function

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Accepted 26 January 2005
Journal of Cell Science 118, 1565-1575 Published by The Company of Biologists 2005
doi:10.1242/jcs.02302

Summary

The centrosome organizes microtubules by controlling nucleation and anchoring processes. In mammalian cells, subdistal appendages of the mother centriole are major microtubule-anchoring structures of the centrosome. It is not known how newly nucleated microtubules are anchored to these appendages. We show here that ninein, a component of subdistal appendages, localizes to the centriole via its C-terminus and interacts with γ-tubulin-containing complexes via its N-terminus. Expression of a construct encoding the ninein C-terminus displaced endogenous ninein and the γ-tubulin ring complex (γ-TuRC) from the centrosome, leading to microtubule nucleation and anchoring defects. By contrast, expression of a fusion consisting of the N- and C-terminal domains (lacking the central coiled-coil region) displaced endogenous ninein without perturbing γ-TuRC localization. Accordingly, only anchoring defects were observed in this case. Therefore, expression of this fusion appeared to uncouple microtubule nucleation and anchorage activities at the centrosome. Our results suggest that ninein has a role not only in microtubule anchoring but also in promoting microtubule nucleation by docking the γ-TuRC at the centrosome. In addition, we show that the γ-TuRC might not be sufficient to anchor microtubules at the centrosome in the absence of ninein. We therefore propose that ninein constitutes a molecular link between microtubule-nucleation and -anchoring activities at the centrosome.

Key words: Centrosome, Microtubules, Nucleation, Anchoring, Ninein

Introduction

In vertebrate cells, the centrosome consists of a centriole pair associated with an amorphous matrix. Because of the mechanisms underlying the generation and maturation of the centrosome, the two centrioles are not equivalent. The older (mother) centriole is characterized by the presence of two distinct sets of nine appendages at distal and subdistal locations (Paintrand et al., 1992). The function of the distal appendages remains unclear. Based on electron microscopy, the subdistal appendages appear to be microtubule-anchoring structures allowing an aster of microtubules to be formed around the mother centriole during interphase (De Brabander et al., 1982; Gorgidze and Vorobjev, 1995; Piel et al., 2000). Centrosome-dependent organization of microtubules involves distinct processes such as nucleation, anchoring and release of microtubules. Protein complexes participating in microtubule nucleation have been extensively studied (Moritz and Agard, 2001). By contrast, microtubule-anchoring complexes have been less well characterized (Bornens, 2002; Dammermann and Merdes, 2002; Mogensen, 1999; Mogensen et al., 1997; Mogensen et al., 2000; Quintyne et al., 1999).

Nucleation of microtubules is initiated by the γ-tubulin ring complex (γ-TuRC), which contains γ-tubulin, Spc97p/GCP2, Spc98p/GCP3 and at least three additional proteins (GCP4, GCP5 and GCP6 in metazoans) (Fava et al., 1999; Murphy et al., 2001; Murphy et al., 1998; Tassin et al., 1998). Most of the γ-tubulin is cytoplasmic but is devoid of significant microtubule-nucleation activity until recruited to the centrosome (Moudjou et al., 1996; Stearns and Kirschner, 1994). Several centrosomal proteins might have γ-TuRC anchoring function at the centrosome, including pericentrin B (Dictenberg et al., 1998), CG-Nap (also known as AKAP350 and AKAP 450) (Takahashi et al., 2002), kendrin (Takahashi et al., 2002), CPAP (Hung et al., 2000) and Nlp (Casenghi et al., 2003).

Additional proteins including PCM-1, CEP135 or BBS4 are required for the formation and maintenance of a radial microtubule array anchored at the centrosome in interphase (Dammermann and Merdes, 2002; Kim et al., 2004; Ohta et al., 2002). The dynactin complex (composed of, among other proteins, p150^{Glued}, p50/dynamitin and p24) and EB1 have been implicated in anchoring function at the centrosome and might eventually prove to be components of the subdistal appendages (Askham et al., 2002; Clark and Meyer, 1999; Louie et al., 2004; Quintyne et al., 1999; Quintyne and Schroer, 2002). Other proteins, such as ninein (Mogensen et al., 2000), centriolin (Gromley et al., 2003), ε-tubulin (Chang et al., 2003) and cenexin/ODF2 (Lange and Gull, 1995; Nakagawa et al., 2001), have been identified by electron microscopy as true

components of the subdistal appendages. Among these, ninein has been shown to act as a microtubule-anchoring protein. Indeed, an overexpressed fusion of green-fluorescent protein (GFP) to ninein accumulates at the centrosome and decreases microtubule release to the cytoplasm (Abal et al., 2002). Moreover, reduction of ninein levels by RNA interference leads to microtubule-aster-organization defects (Dammermann and Merdes, 2002).

Functional studies suggest a close relationship between microtubule nucleation and anchoring. Knock-down of microtubule-anchoring proteins such as EB1, CEP135 and p50 leads to a delay in microtubule nucleation, as well as anchoring defects (Askham et al., 2002; Louie et al., 2004; Uetake et al., 2004). Similarly, overexpression of p50, p150^{Glued} and p24 interferes with endogenous p150^{Glued}, leading to both mislocalized γ-tubulin and anchoring defects (Quintyne et al., 1999). Thus, microtubule-anchoring defects at the centrosome might be accompanied by perturbation of the γ-TuRC [which caps the microtubule minus ends (Keating and Borisy, 2000; Moritz et al., 2000; Wiese and Zheng, 2000)]. However, in mammalian cells, γ-tubulin is localized to and nucleates microtubules at the two centrioles, whereas microtubule anchoring seems to be specific to the mother centriole (Piel et al., 2000). Furthermore, in polarized epithelial cells, microtubule-nucleation and -anchoring activities are taking place in distinct locations (Mogensen et al., 1997): γ-tubulin is concentrated at the centrosome, yet most microtubules are anchored at non-centrosomal apical sites, where, instead, ninein and centriolin accumulate. These results indicate that γ-tubulin might not be the sole factor dictating microtubule anchorage.

In order to dissect further the mechanisms underlying microtubule organization by the centrosome, we undertook structural-functional analysis of ninein in mammalian cells. In agreement with a recent study, we found that ninein might interact with γ-tubulin-containing complexes by its N-terminus. By contrast, the C-terminus can direct ninein to centrioles. A fusion of GFP to the C-terminal domain of ninein localized to the centrioles but competed out endogenous ninein with a concomitant reduction of γ-tubulin and nucleation at centrosomes. Finally, a fusion of the N-terminus with the C-terminus of ninein (lacking the coiled-coil region) still docked γ-tubulin at the centrosome, preserving nucleation, but disrupted microtubule anchoring. Our results point to separable contributions of γ-TuRC and ninein to the nucleation and anchoring processes.

Materials and Methods

Preparation of plasmid

A full-length ninein-encoding cDNA was constructed using overlapping clones obtained during the screening of a mouse thioglycolate-elicited peritoneal macrophages random-primed library (Bouckson-Castaing et al., 1996). The mouse ninein sequence was renumbered in order to start at the second methionine described by Bouckson-Castaing et al., because further sequencing analysis demonstrated that it was the only in-frame ATG to mark a translation start site. Moreover, a new splice variant diverging in the C-terminal domain of ninein was used in this study (GenBank/EMBL/DDBJ accession number AY515727). The insert was subcloned between the *Bgl*II and *Eco*RI sites of pEGFP-C1 (Clontech). Constructs encoding the different domains of ninein were engineered by PCR using the ninein-encoding cDNA as a template. The GFP N-terminal fusion construct (GFP/Nter-ninein) was generated using the oligonucleotides: 5′-CGGGATCCATGGATGAGGTGGAGGAGG-3′ (forward) and 5′-CGGAATTCCTAACAAGTGCCGGATCTCG-3′ (reverse), and the PCR product was cloned between the *Bgl*II and *Eco*RI sites of pEGFP-C1. The GFP C-terminal fusion construct (GFP/Cter-ninein) was generated using the oligonucleotides 5′-GGAATTCCCACCAAACACCAAAAACAACTC-3′ (forward) and 5′-CGGGATCCCTATGACCTCAGAGGAGGCATAG-3′ (reverse) and the PCR product was cloned between the *Eco*RI and *Bam*HI sites of pEGFP-C3. The GFP N-terminus/C-terminus fusion construct was engineered by subcloning Cter-ninein in the GFP/Nter-ninein construct.

Reagents and antibodies

Rat collagen type I, fibronectin and nocodazole were obtained from Sigma Chemical (St Louis, MO). The anti-ninein antibody was affinity purified against the polypeptide Pep3 (Bouckson-Castaing et al., 1996). GT335 is a monoclonal antibody specifically reacting with polyglutamylated α- and β-tubulin (Wolf and Spanel-Borowski, 1992). Anti-α-tubulin and anti-γ-tubulin antibodies were purchased from Amersham International (Little Chalfont, UK). Anti-Spc98 antibody was raised against the human homologue of Spc98p (Tassin et al., 1998). A24 is an affinity-purified polyclonal antibody raised against chicken A-kinase-anchoring protein AKAP450, kindly provided by Kemner. Nlp is an affinity-purified polyclonal antibody raised against ninein-like protein, kindly provided by E. A. Nigg (Casenghi et al., 2003). Anti-pericentrin antibody is a polyclonal antibody from Babco (Berkeley, CA). Polyclonal anti-C-Nap1 antibody was kindly provided by A. M. Fry. Polyclonal anti-GFP antibody was obtained from Molecular Probes (Eugene, OR) and polyclonal anti-myc antibody was obtained from Euromedex (Mundolsheim, France). Secondary antibodies used were Alexa-488, obtained from Molecular Probes (Eugene, OR) and Cy3, obtained from Jackson ImmunoResearch Laboratories.

Cell culture and transfection

L929, Hela and HCT116 cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL, Life Technologies) supplemented with 10% foetal calf serum. For transient transfections, cells were grown to 50-70% confluence, harvested with 0.05% trypsin-EDTA and then 5×10^6 cells were resuspended in 250 μl DMEM containing 150 mM Hepes, pH 7.2, 20 mM NaCl, and electroporated with 30 μg DNA at 288 mV and 750 μF. Cells were seeded on 12-mm^2 coated coverslips (collagen 20 μg ml^{-1}; fibronectin 10 μg ml^{-1}) and grown for 2 hours, 6 hours or 20 hours before being processed for immunofluorescence. Transfection efficiencies of 40-50% were routinely obtained.

Immunofluorescence

Cells were either rinsed with PBS and fixed in methanol –20°C for 4 minutes or, in cases of microtubule regrowth, rapidly extracted with 0.2% NP40 in BRB80 (80 mM KPIPES pH 6.8, 1 mM $MgCl_2$, 1 mM EGTA) for 30 seconds, fixed in 3% paraformaldehyde, 0.25% glutaraldehyde in BRB80 for 3 minutes and rinsed with 0.1% $NaBH_4$ in BRB80, to reduce aldehyde groups. Then, cells on coverslips were incubated for 1 hour in primary antibodies in PBS containing 0.1% Tween 20, 3% bovine serum albumin (BSA), washed in PBS containing 0.1% Tween 20, and incubated in secondary antibodies for 45 minutes in PBS containing 0.1% Tween 20, 3% BSA, all at room temperature. Samples were washed, dehydrated in ethanol and mounted on slides in CityFluor (City University, London, England). Images were usually taken using a Leica DMRB microscope with 100× magnification, 1.4-0.7 NA PL-APO objectives, a Coolsnap FX

camera (Princeton Instruments) and Metaview software (Universal Imaging). For microtubule-regrowth experiments and studies of colocalizations with centriolar markers, *z*-axis stacks were collected using a piezoelectric device mounted at the base of a 100× magnification, 1.4 NA PL-APO objective on a Leica DMRA2 microscope, a Coolsnap HQ camera controlled by Metamorph software (Universal Imaging) was used. The *z*-axis stacks were compiled as single two-dimensional projections using Metamorph software. All images were imported into Adobe Photoshop v5.0 for contrast manipulation and figure assembly.

Quantification of fluorescence intensity in digital images

Fluorescence intensity at the centrosome was determined on digital images on relevant immunofluorescence experiments. Maximum fluorescence-intensity values were measured in a constant region centred on the centrosome using Metamorph software. Images were acquired under conditions that ensured that saturation was not reached. The minimum intensity of fluorescence within the region was used for reference and subtracted from the maximal signal. For each protein analysed, the distribution of centrosomal intensity of fluorescence in individual cells (n) was expressed as intensity relative to the control mean value, set to 100 (arbitrary units). All distributions were compared using χ^2 analysis.

Cell extracts and immunoprecipitation

1×10^7 L929 cells transfected for 6 hours were resuspended in 250 μl lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.2, 1 mM $MgCl_2$, 1% NP40 and1 μg ml^{-1} each of the protease inhibitors aprotinin, leupeptin and pepstatin) and incubated on ice for 10 minutes. Lysates were clarified (12,000 ***g*** for 10 minutes at 4°C). Each immunoprecipitation was performed with 3×10^7 magnetic beads coated with affinity-purified sheep anti-mouse IgG (Dynal, Norway). Beads were incubated for 2 hours at 4°C with 6 μg anti-GFP antibody, or with 6 μg pre-immune serum as a control. Equal amounts of lysates were then added on beads and incubated for 2 hours at 4°C. Supernatants were mixed with 2× Laemmli buffer (62.5 mM Tris-HCl pH 6.8, 2% SDS, 25% glycerol, 0.01% Bromphenol Blue) and beads were extensively washed and eluted at 90°C for 5 minutes in 1× Laemmli buffer for SDS-PAGE.

Glutathione-*S*-transferase pull-downs

cDNA fragments encoding residues 1-246 and 1-496 of murine ninein were cloned into a glutathione-*S*-transferase (GST) expression vector and used to transform the *E. coli* bacterial strain ABV100 (Avidity). GST fusion proteins were expressed by inducing with 1 mM isopropyl-βD-thiogalactopyranoside (IPTG) and incubating the cultures at 25°C for 3 hours. Cells were harvested by centrifugation at 4000 ***g*** for 20 minutes at 4°C, lysed by sonicating in PBS containing protease inhibitors and 1% (final concentration) Triton X-100. Lysates were clarified by centrifugation and the GST fusions were bound to glutathione-Sepharose (Amersham Biosciences) by incubating overnight at 4°C and then washed extensively with PBS containing 400 mM NaCl. GST fusions were eluted with 10 mM reduced glutathione in 50 mM Tris-HCl, pH 8.0, dialysed into 100 mM $NaHCO_3$, 500 mM NaCl, pH 8.3, and approximately 1.5 mg of each protein coupled to 300 μl CNBr-activated Sepharose 4B beads according to the instructions provided (Amersham Biosciences). The GST fusions, coupled to Sepharose 4B, were packed into 10 ml columns (Bio-Rad) and washed sequentially with: 100 mM glycine, pH 2.5; 10 mM Tris-HCl, pH 7.5; and lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM Na_2HPO_4, pH 7.2, 1% Triton X-100). Clarified HeLa-cell extracts were prepared by lysing in the above buffer, added to the GST fusion proteins and incubated overnight at 4°C. After washing the columns extensively with lysis buffer (60 bed volumes), bound proteins were eluted with 100 mM glycine, pH 2.5, dialysed into PBS and concentrated using an Amicon centrifugal filter (MWCO 4,000, Millipore).

Western blotting was carried out using an anti-γ-tubulin monoclonal antibody (Sigma).

Microtubule regrowth assay

Cells were transfected, seeded on coverslips and grown for 5 hours as described above. Microtubules were depolymerized in 5 μM nocodazole in DMEM for 1 hour at 37°C and then cells were washed with DMEM and incubated at 37°C to allow regrowth. Cells were fixed at timed intervals in paraformaldehyde/glutaraldehyde for immunofluorescence as follows: 30-second extraction in BRB80 containing 3% paraformaldehyde, 0.25% glutaraldehyde in BRB80 for 3 minutes followed by rinsing with 0.1% $NaBH_4$ in BRB80, to reduce aldehyde groups. Quantifications shown correspond to a representative experiment from five repetitions.

Results

Ninein is targeted to both centrioles by its C-terminus and stabilized at the mother centriole by its N-terminus

To examine the cellular functions of ninein at the centrosome, we prepared a series of ninein deletion constructs that were transiently expressed in L929 cells. Three truncations of ninein were generated, encoding the N-terminal domain (amino acids 1-373), the coiled-coil region (amino acids 373-1874) and the C-terminal domain (amino acids 1874-2113). All fragments were fused to EGFP. Expression was monitored by immunoblotting (data not shown) and the subcellular localization of each fusion was examined by fluorescence microscopy (Fig. 1A). The N-terminal domain (GFP/Nter-ninein) was diffuse throughout the cytoplasm, whereas the coiled-coil domain (GFP/Coil-ninein) appeared as cytoplasmic dots. Only the C-terminal domain (GFP/Cter-ninein) localized to the centrosome. Co-staining with GT335, an antibody recognizing polyglutamylated tubulins of the centrioles (Bobinnec et al., 1998), revealed that both centrioles were labelled by the GFP/Cter-ninein. In addition to this centriolar localization, GFP/Cter-ninein accumulated in small dots over a diffuse cytoplasmic label (Fig. 3A). This dot-like staining eventually concentrated around the centrosome in a manner dependent on the level of expression and the time following transfection (data not shown).

To define the centriolar localization of the Cter-ninein, cells showing a low level of expression were immunolabelled for colocalization studies with several centriolar markers (Fig. 1B). The Cter-ninein did not colocalize with C-Nap1, a protein concentrated at the proximal end of both centrioles (Fry et al., 1998), but did colocalize with endogenous ninein on the mother centriole, where subdistal appendages are located (Mogensen et al., 2000). These results suggest that the Cter-ninein was first targeted to the distal part of both centrioles but then accumulated around the centrioles, whereas the endogenous protein is essentially localized to the subdistal appendages of the mother centriole. Localization of the Cter-ninein to the centriole was also confirmed using a myc-tagged construct (data not shown).

To test whether additional domains of ninein might be crucial

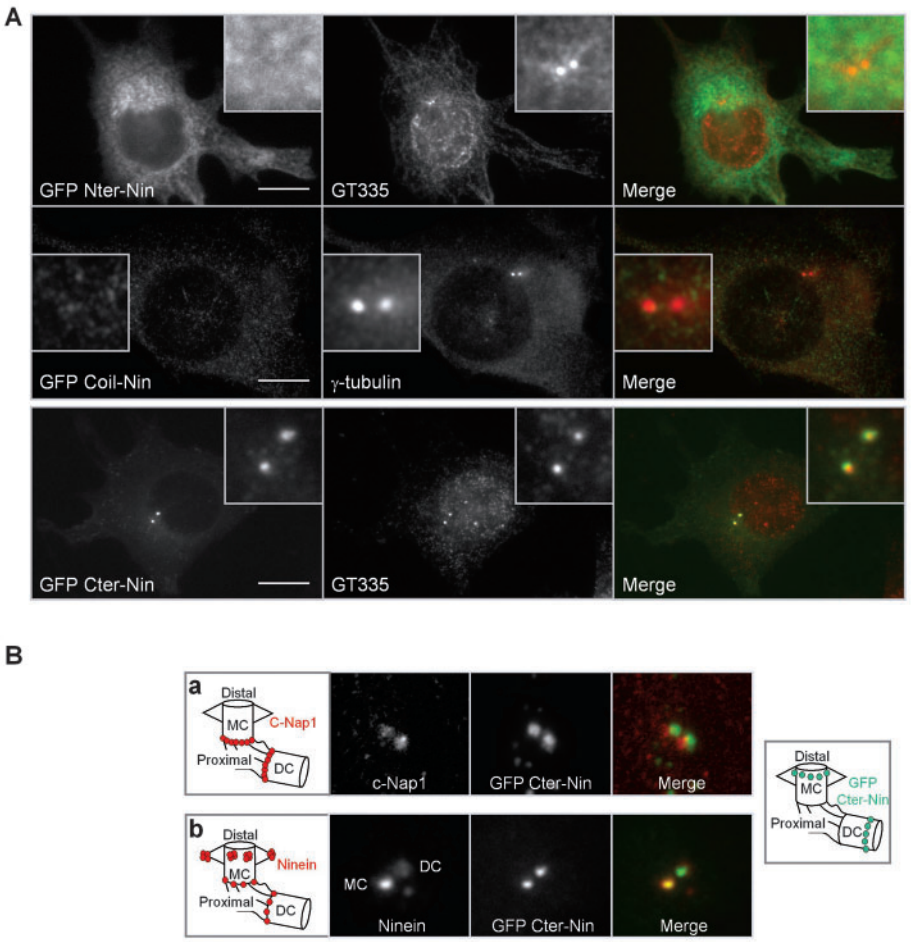


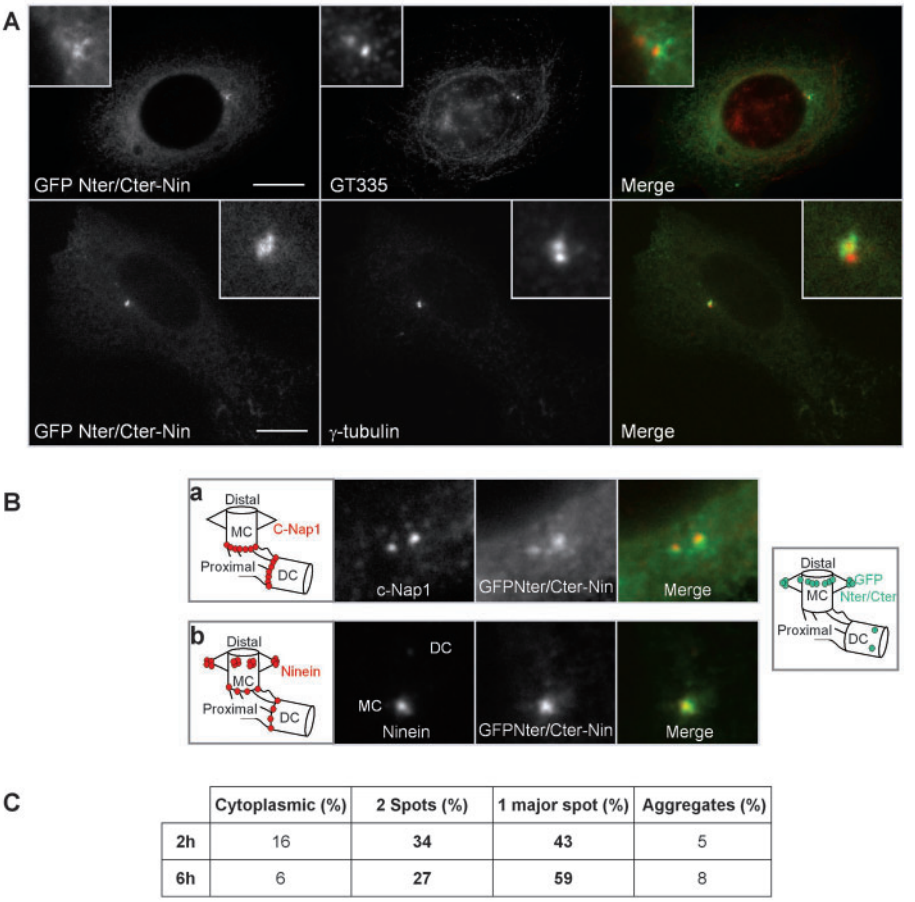
Fig. 1. The C-terminal domain of ninein associates with both centrioles. (A) L929 cells were transiently transfected with GFP-tagged ninein N-terminal domain, coiled-coil region and C-terminal domain (green). 2 hours after transfection, cells were fixed and centrioles labelled with either GT335 antibody or γ-tubulin antibody (red), demonstrating that only the C-terminal domain was targeted to centrioles. Bars, 10 μm (4× magnification in insets). (B) L929 cells expressing GFP/Cter-ninein for 2 hours were fixed and labelled with an antibody to C-Nap1 (a), which decorates the proximal ends of both centrioles (cartoon) or to ninein (b), which decorates essentially the distal end of the mother centriole (cartoon). The GFP signals did not colocalize with the C-Nap1 labelling but colocalized with ninein to the mother centriole, suggesting that GFP/Cter-ninein might be associated with the distal end of both centrioles (same magnification as insets in A).

for localization to the mother centriole, a construct lacking the coiled-coil domain (GFP/Nter/Cter-ninein) was used in transfection experiments. This GFP/Nter/Cter-ninein, in contrast to the C-terminal fusion, predominantly labelled a single centriole (43% of cells after 2 hours) and this localization became more prominent with time (59% of the cells after 6 hours) (Fig. 2). Cells showing a low level of Nter/Cter-ninein expression were co-stained for C-Nap1 and ninein (Fig. 2B). In contrast to Cter-ninein, the Nter/Cter-ninein was associated with the distal end of the mother centriole only and colocalized with the endogenous protein (Fig. 1B, Fig. 2B). The GFP/Nter/Cter-ninein was also localized diffusely in the cytoplasm in a manner similar to that of the C-terminus. These data indicate the importance of the ninein C-terminus for targeting to the centrosome, whereas the N-terminus was essential for conferring specificity of targeting to the mother centriole.

Expression of ninein C-terminal domain induces a delay in microtubule regrowth and prevents anchorage

We further analysed whether the two constructs described above (Cter-ninein and Nter/Cter-ninein) could have an effect on ninein localization or function. As early as 2 hours after Cter-ninein expression, immunostaining of endogenous ninein at the centrosome decreased by approximately 40% (Fig. 3). This effect was more pronounced at later times, consistent with the resulting increase in Cter-ninein around the centrioles. Indeed, 6 hours after transfection, we observed a decrease by approximately 60% of immunostaining of ninein at the centrosome, suggesting a dose-dependent effect. By displacing endogenous ninein, the Cter-ninein might act as a dominant inhibitor and could therefore be used to determine whether the displacement of ninein disrupted microtubule organization.

Microtubule organization at the centrosome depends on multiple processes such as nucleation, anchoring and release. Rather than analysing bulk microtubules at steady state, we performed microtubule-regrowth experiments to detect defects in microtubule nucleation (during short-term regrowth) or in microtubule anchoring (during long-term regrowth). Analysis was performed in HCT116 cells instead of L929 cells because the nucleated aster was more obvious (Abal et al., 2002). We analysed microtubule organization in cells transfected for 6 hours with GFP/Cter-ninein, at which time endogenous ninein at the centrosome decreased to less than 50% without excessive accumulation of Cter-ninein. Cells were fixed after a 1 minute or 10 minute regrowth following nocodazole washout to be immunostained for α-tubulin. Cells were scored for the presence of microtubules and for aster organization (Fig. 4). After a 1 minute regrowth, few if any microtubules were detected in cells expressing the Cter-ninein, whereas a typical aster of short microtubules was present in control cells (Fig. 4A). This pointed to delays or disruption of microtubule nucleation. When short microtubules were detected (in ~10% of the cells), they were concentrated close to the centrosome (Fig. 4Ab). This indicated that microtubule nucleation was only delayed and that microtubules regrew from the centrosome rather than in the cytoplasm. In 10-minute-regrowth experiments, microtubules were eventually detected in most of the expressing cells, showing that nucleation was only delayed (Fig. 4B). Nevertheless, microtubules were disorganized and unfocused in half of the expressing cells, in contrast to the radial array observed in control cells. In addition, a constant



	Cytoplasmic (%)	2 Spots (%)	1 major spot (%)	Aggregates (%)
2h	16	34	43	5
6h	6	27	59	8

Fig. 2. The ninein N-terminus/C-terminus chimerical fusion is concentrated on the mother centriole. (A) L929 cells were transiently transfected with a GFP-tagged construct encoding a ninein version lacking the coiled-coil domain (Nter/Cter-Nin, green). 2 hours after transfection, cells were fixed and centrioles labelled with either GT335 or anti-γ-tubulin antibody (red). Representative images show colocalization of centriole markers with the ninein fusion at one centriole. Bars, 10 μm (4× magnification in insets). (B) L929 cells expressing GFP/Nter/Cter-ninein for 2 hours were fixed and labelled with an antibody to C-Nap1 (a), which decorates the proximal ends of both centrioles (cartoon), or to ninein (b), which essentially decorates the distal end of the mother centriole (cartoon). The GFP signals did not colocalize with C-Nap1 labelling but colocalized with ninein to the mother centriole, showing that GFP/Nter/Cter-ninein is associated with the distal end of the mother centriole or of both centrioles, depending on the cell (C) (same magnification as insets in A). (C) Quantification of Nter/Cter-ninein localization in living cells at 2 hours and 6 hours after transfection. Most cells presented localization on one spot representing the mother centriole or on two spots representing both centrioles.

proportion of transfected cells (~10%) did not show any microtubules. These results indicated that displacement of ninein by Cter-ninein binding to the centrioles might disturb microtubule assembly and impair anchoring at the centrosome. The alternative possibility (that the effect of the Cter-ninein might have been indirect) has been ruled out (see below).

Functional interaction between ninein and the nucleation complex

To understand further the basis for microtubule-regrowth delay, cells transfected with GFP/Cter-ninein were labelled with antibodies directed against γ-tubulin and Spc98p, two components of the γ-TuRC (Fig. 5A). In most L929-transfected cells or in HCT116 cells (data not shown), as early as 2 hours after transfection, γ-tubulin and Spc98p levels at the centrosome were reduced. By contrast, proteins known to dock γ-TuRC components, such as Nlp (Casenghi et al., 2003), pericentrin (Dictenberg et al.,

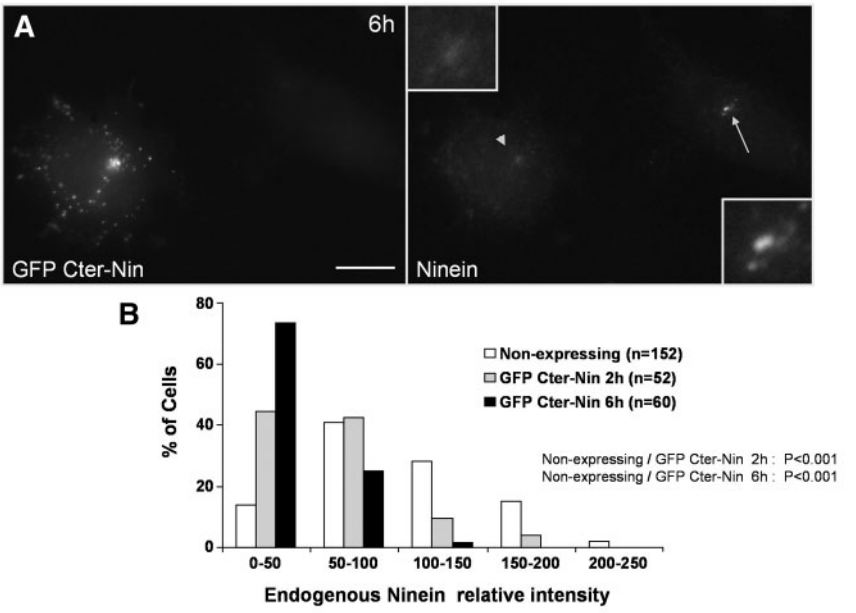
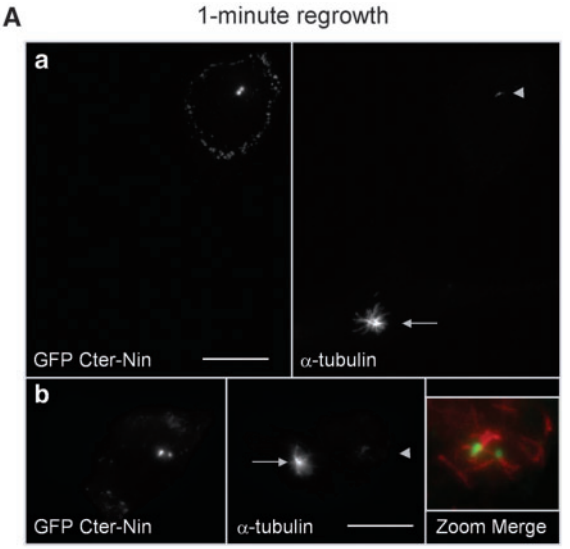
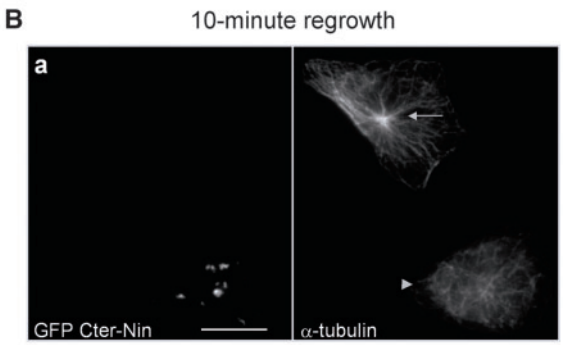


Fig. 3. The C-terminal domain of ninein displaces endogenous ninein from the centrosome. L929 cells transfected with the GFP/Cter-ninein construct for 6 hours (A) showing a decrease in endogenous ninein at centrioles (arrowhead) compared with control cells (arrow), as judged by staining with an anti-ninein antibody. (B) Distribution of ninein labelling relative intensity at centrosomes in non-expressing cells (white bars), cells 2 hours after transfection (grey bars) and 6 hours after transfection (black bars). Expression of Cter-ninein resulted in a reduction in the level of endogenous ninein at the centrosome, on average, decreases of 42% at 2 hours and of 62% at 6 hours were observed (P<0.001 in both cases). Bars, 10 μm (4× magnification in insets).



c

Transfection GFP Cter-Nin	No Regrowth (%)	No Aster (%)	Aster (%)
Non-expressing (n=150)	2	1	**97**
Expressing (n=116)	**82**	2	16



b

Transfection GFP Cter-Nin	No Regrowth (%)	No Aster (%)	Aster (%)	ND (%)
Non-expressing (n=160)	0	5	**89**	6
Expressing (n=114)	**17**	**51**	21	11

Fig. 4. Effect of the displacement of ninein from the centrosome on microtubule regrowth and anchorage. HCT116 cells transiently transfected with GFP/Cter-ninein for 5 hours and further treated with nocodazole for 1 hour were labelled using anti-α-tubulin antibodies to visualize microtubules upon regrowth. (A) One minute after nocodazole washout, expressing cells had no (a, arrowhead) or few (b, arrowhead) microtubules (compare with the control cells, arrow). When few microtubules were observed, as in b, they concentrated around the centrosome. (c) Quantification of microtubules and aster formation in expressing and non-expressing cells after 1-minute nocodazole washout, corresponding to the experiment illustrated in (a,b). (B) Ten minutes after nocodazole washout, microtubules polymerized in expressing cells but, in contrast to control cells (arrow), no asters were observed (a, arrowhead). (b) Quantification of microtubules and aster formation in expressing and non-expressing cells after 10 minutes nocodazole washout, corresponding to the experiment illustrated in a. ND (not determined) indicates cells in which microtubules were present but formation of an aster could not be discerned. Bars, 10 μm (2× magnification in insets).

1998) and AKAP450 (Takahashi et al., 2002), were not displaced from the centrosome (Fig. 5B,C). These results suggest that ninein might be important for the correct localization of the γ-TuRC at the centrosome. In support of an interaction between ninein and the γ-TuRC, we found that overexpressing full-length ninein increased the recruitment of the nucleation complex at the centrosome (Fig. 6A). Following microtubule depolymerization in cells overexpressing the full-length GFP ninein, the fluorescence signal became scattered in

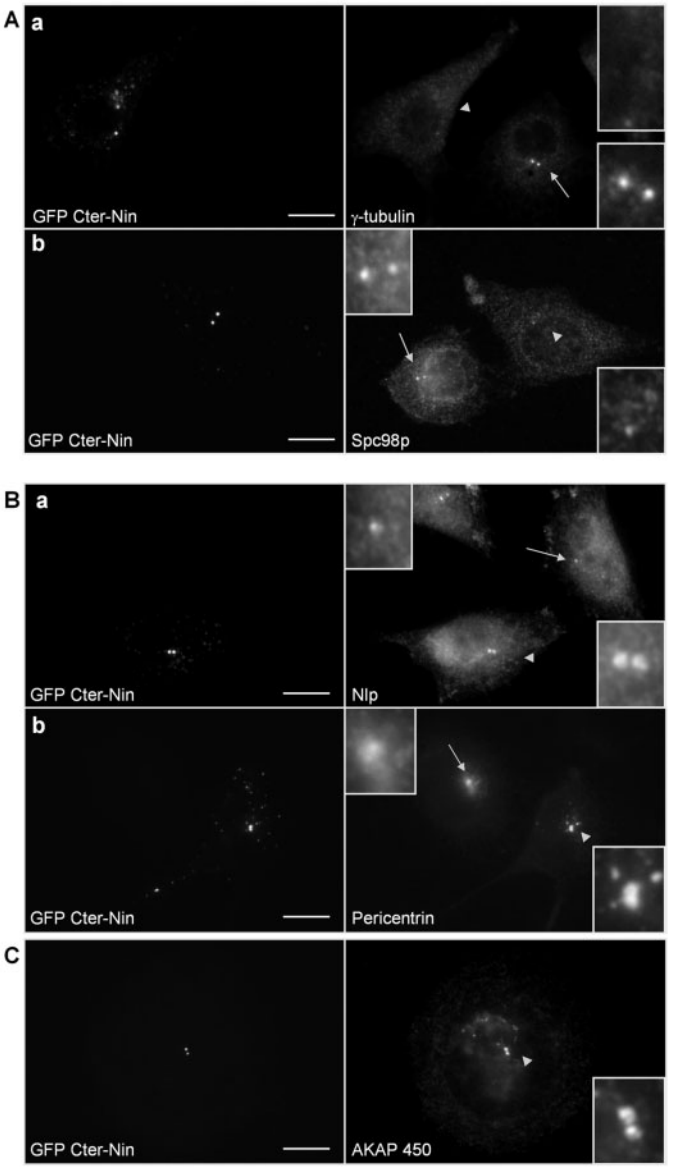


Fig. 5. Effect of the displacement of ninein on the localization of γ-TuRC components or γ-TuRC docking proteins. L929 (A,B) or Hela (C) cells transfected with GFP/Cter-ninein for 2 hours were fixed and labelled with different markers for γ-TuRC components (A) or for γ-TuRC docking proteins (B,C). (A) γ-Tubulin (a) and Spc98p (b) were displaced from the centrosome in expressing cells (arrowhead) compared with control cells (arrow). (B) Nlp (a) and pericentrin (b) were correctly localized to the centrosome in expressing cells (arrowhead) compared with control cells (arrow). (C) AKAP 450 was also present at the centrosome in expressing cells (arrowhead). Bars, 10 μm (the centrosome areas were magnified four times in insets).

the cytoplasm, appearing as dots that also contained γ-tubulin. However, in microtubule-regrowth experiments, microtubule nucleation was not observed on these dots until ninein reaccumulated at the centrosome (data not shown), in agreement with the results of Stillwell et al. (Stillwell et al., 2004).

Extracts from cells expressing the different ninein fusion constructs were assayed by co-immunoprecipitation to detect an interaction between ninein and the γ-TuRC, and to identify the domain involved. The coiled-coil domain and full-length overexpressed protein were totally insoluble and could not be included for analysis. Cells co-expressing the remaining GFP-ninein constructs and a myc-tagged γ-tubulin fusion were lysed 4 hours after transfection and immunoprecipitated with either a polyclonal anti-GFP antibody or a pre-immune serum. GFP/Nter-ninein and GFP/Nter/Cter-ninein co-precipitated with myc/γ-tubulin (Fig. 6B). The reciprocal co-immunoprecipitation using anti-myc antibodies confirmed this result (data not shown). These data indicated an interaction between γ-tubulin and the N-terminal domain of ninein. By contrast, GFP/Cter-ninein, which is less soluble than the other domains, co-precipitated with a small amount of γ-tubulin (the same as in a control immunoprecipitation using a pre-immune serum). Moreover, no GFP/Cter-ninein was detected by reciprocal co-immunoprecipitation using anti-myc antibodies (data not shown). Nevertheless, the Cter-ninein was not sufficient to retain γ-tubulin at the centrosome or to prevent nucleation delay in cells (Figs 4,5). Finally, pull-down experiments using two recombinant GST/Nter-ninein fusions, precipitated endogenous γ-tubulin from HeLa-cell extracts (Fig. 6C).

In conclusion, the N-terminal domain of ninein could dock, directly or indirectly, the γ-TuRC onto the centrosome. Accordingly, delocalization of the γ-TuRC induced by the binding of the Cter-ninein to the centriole could be a consequence of the displacement of endogenous ninein.

Ninein acts as a functional link between nucleation and anchoring of microtubules

Nter-ninein was able to interact with γ-tubulin without being targeted to the centrosome. By contrast, expression of Cter-ninein dispersed the γ-TuRC from the centrosome, presumably by displacing the endogenous ninein, leading to microtubule-nucleation and -anchoring defects. We therefore asked whether we could maintain γ-tubulin

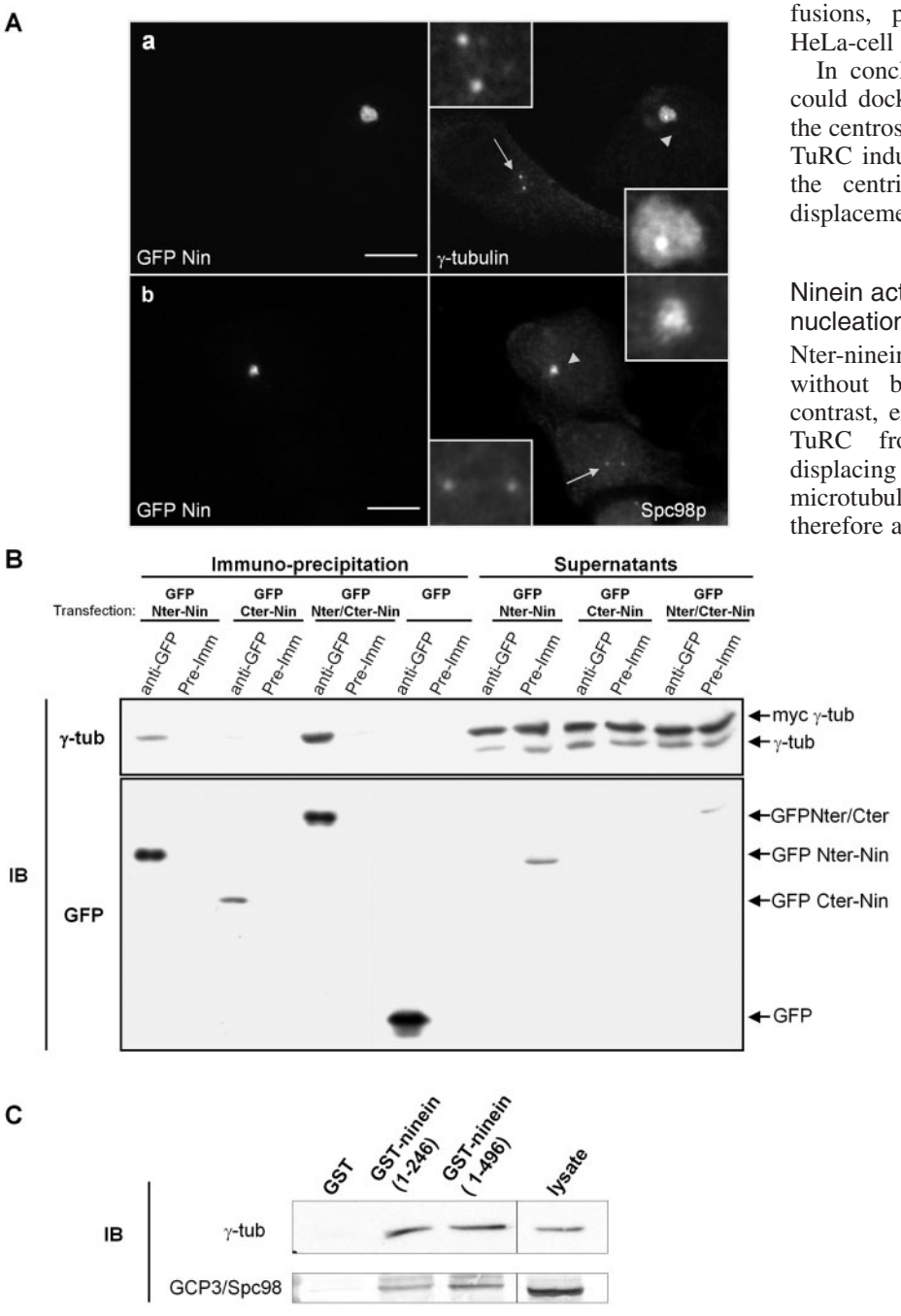


Fig. 6. Ninein interacts with γ-tubulin by its N-terminal domain. (A) GFP-ninein expressed for 2 hours in L929 cells recruited the microtubule-nucleation complex (arrowhead) as judged by γ-tubulin (a) or Spc98p (b) staining. The centrosome of a control cell is indicated by an arrow. Bars, 10 μm (the centrosome areas were magnified four times in insets). (B) Fusion constructs including the ninein N-terminal domain immunoprecipitated with myc/γ-tubulin. Extracts from L929 cells co-expressing myc/γ-tubulin and either one of the three different GFP-ninein constructs indicated were used for immunoprecipitation experiments with a polyclonal anti-GFP antibody. The weak band of γ-tubulin associated with immunoprecipitation of GFP/Cter-ninein is likely to be non-specific. (C) Two N-terminal domains (residues 1-246 and 1-496) of ninein were expressed as GST fusions and incubated with a lysate prepared from HeLa cells. γ-Tubulin was present in all of the ninein-GST pull-downs. A similar result was obtained for Spc98/GCP3.

at the centrosome and thus restore normal nucleating activity through expression of a construct fusing the N-terminus to the C-terminus (Nter/Cter-ninein). As shown before, this fusion localized to the centrosome (Fig. 2). Cells transfected with the GFP/Nter/Cter-ninein retained the nucleation complex at the centrosome (Fig. 7A). By contrast, endogenous ninein was still displaced from the centrosome, almost as efficiently as it was by the Cter-ninein (decreased by 50%, compare Fig. 7B with Fig. 3). We therefore examined the effect of this construct on microtubule anchoring and nucleation. Regrowth experiments following nocodazole-induced depolymerization revealed that, consistent with the observed retention of the γ-TuRC, Nter/Cter-ninein did not impair or delay microtubule nucleation (Fig. 8). Therefore, Nter-ninein, targeted to the centrosome through the Cter-ninein, restored γ-TuRC-dependent nucleation even though endogenous ninein was still displaced [compare with GFP/Cter-ninein (Fig. 4)]. However, microtubule anchoring was still impaired after a 10-minute regrowth in more than 40% of transfected cells. Thus, the Nter/Cter-ninein fusion was unable to compensate for displacement of endogenous ninein with regard to microtubule-anchorage function.

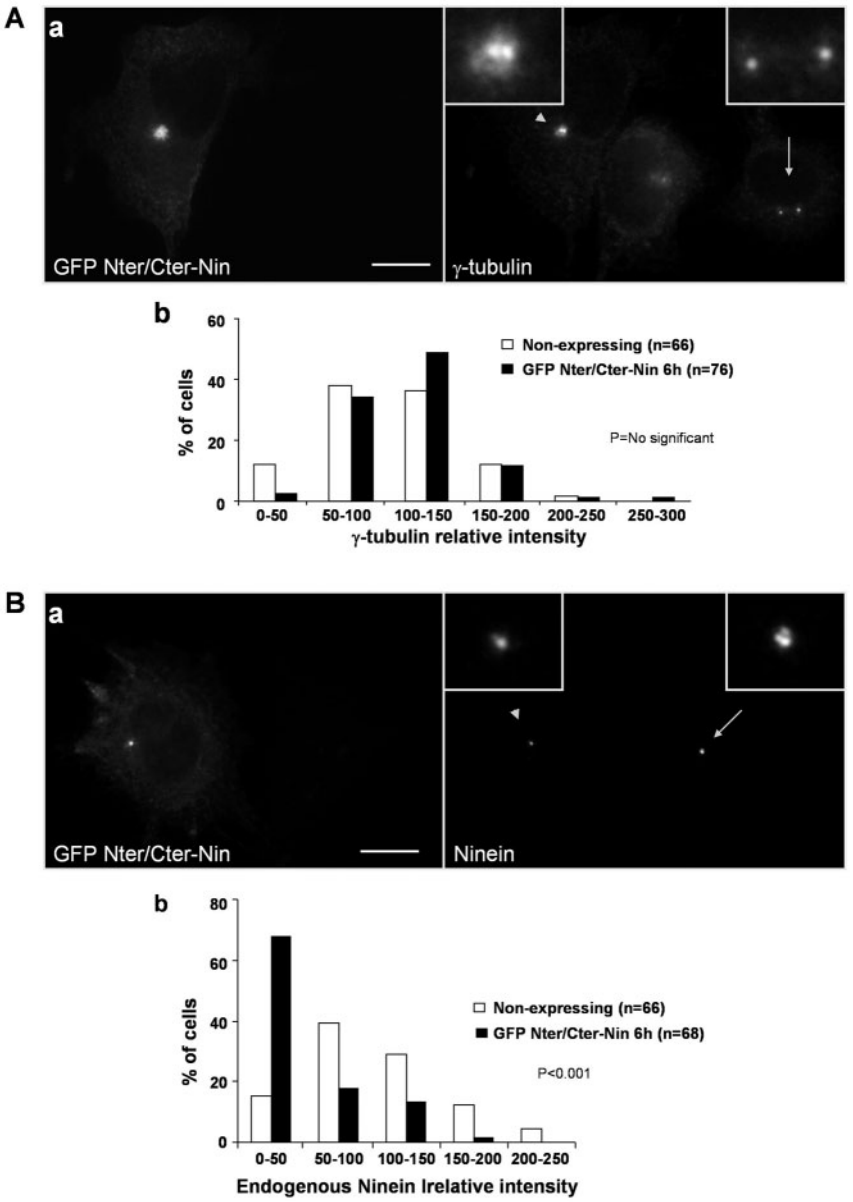


Fig. 7. The ninein N-terminus/C-terminus chimerical fusion displaces ninein but not γ-tubulin from the centrosome. (A) L929 cells expressing GFP Nter/Cter-ninein for 6 hours were fixed and immunolabelled with an anti-γ-tubulin antibody (a), showing that, in expressing cells (arrowhead), γ-tubulin was not displaced from the centrosome compared with control cells (arrow). (b) Quantification of endogenous γ-tubulin labelling relative intensity at the centrosome in expressing (GFP/Nter/Cter-ninein 6 hours, black bars) and non-expressing (white bars) cells, corresponding to the experiment illustrated in a. (B) L929 cells expressing GFP/Nter/Cter-ninein for 6 hours were fixed and immunolabelled with an anti-ninein antibody (a), showing that, in expressing cells (arrowhead), ninein was displaced from the centrosome compared with control cells (arrow). (b) Quantification of endogenous ninein labelling relative intensity at the centrosome, in expressing (GFP/Nter/Cter-ninein 6 hours, black bars) and non-expressing (white bars) cells, corresponding to the experiment illustrated in a. In expressing cells, the relative intensity of ninein label decreased on average to 49% (P<0.001). Bars, 10 μm (4× magnification in insets).

Discussion

Targeting Ninein to the mother centriole

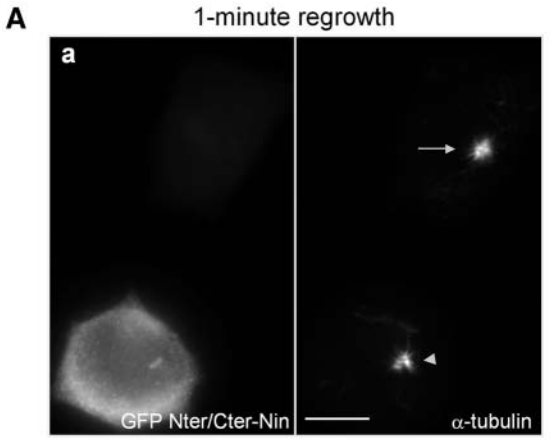
We have shown that the C-terminal domain (amino acids 1874-2113) of a mouse ninein isoform (GenBank/EMBL/DDBJ accession number AY515727) was targeted to both centrioles. We also showed that its N-terminal domain favoured the binding of an Nter/Cter-ninein fusion to the mother centriole.

At least five human ninein isoforms with divergent C-terminal domains (GenBank/EMBL/DDBJ accession numbers NP891989, NP065972, NP891990, NM020921, Q8N4C6) and two mouse isoforms (GenBank/EMBL/DDBJ accession numbers NM008697, AY515727) have been reported. The C-terminal domain used in the present work is highly divergent among the reported ninein isoforms (Bouckson-Castaing et al., 1996; Hong et al., 2000a; Hong et al., 2000b; Stillwell et al., 2004) but apparently contributes to the specific targeting of this isoform to the centrosome.

Two other centrosomal-targeting domains have been reported in two human isoforms [AF212162 (Chen et al., 2003) and AF223939 (Stillwell et al., 2004)]. These two stretches of amino acids (1617-1699 and 1291-1575, respectively) are highly conserved among the different ninein isoforms. They are also present in the longer Coil-ninein construct used in the present work (amino acids 1600-1682 and 1296-1582, with 85% and 71% similarity, respectively). However, they did not direct localization to the centrosome when expressed in Coil-ninein. This discrepancy could be

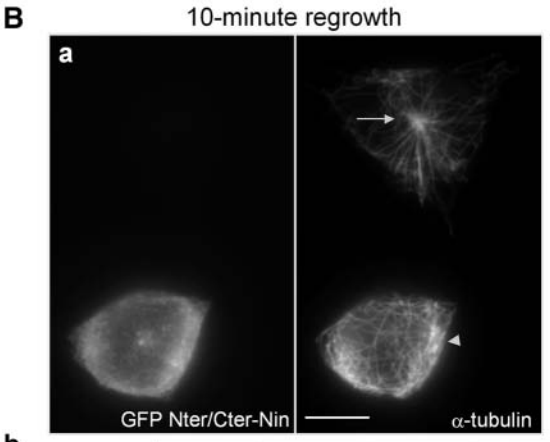
resolved if oligomerization of the longer coiled-coil domain (Chen et al., 2003) (data not shown) is fast and masks or inactivates these centrosomal targeting domains.

Our data indicate that the combined action of two domains (the C-terminal and N-terminal domains) might, in the absence of the coiled-coil region, be sufficient to localize the mouse ninein isoform described here to the mother centriole.



b

Transfection GFP Nter/Cter-Nin	No Regrowth (%)	No Aster (%)	Aster (%)
Non-expressing (n=150)	2	3	**95**
Expressing (n=116)	1	3	**96**



b

Transfection GFP Nter/Cter-Nin	No Regrowth (%)	No Aster (%)	Aster (%)	ND (%)
Non-expressing (n=160)	0	4	**93**	3
Expressing (n=114)	1	**42**	44	13

Fig. 8. The ninein N-terminus/C-terminus chimerical fusion impairs microtubule anchoring but not microtubule nucleation. HCT116 cells transfected with GFP/Nter/Cter-ninein for 5 hours were further treated with nocodazole for 1 hour and analysed, using immunolabelling with anti-α-tubulin antibody to visualize microtubules upon regrowth. (A) One minute after nocodazole washout, expressing cells (a, arrowhead) formed asters comparable to those in control cells (arrow). (b) Quantification of microtubules and aster formation 1 minute after nocodazole washout, corresponding to the experiment illustrated in a. (B) Ten minutes after nocodazole washout, expressing cells (a, arrowhead) had no asters compared with control cells (arrow). (b) Quantification of microtubules and aster formation 10 minutes after nocodazole washout, corresponding to the experiment depicted in a. Bars, 10 μm.

Ninein might dock γ-tubulin to the centrosome

We present four lines of evidence indicating that ninein might contribute to correct γ-TuRC localization to the centrosome. First, overexpression of full-length ninein resulted in a dramatic increase in γ-TuRC binding to the centrosome, as observed for a related human ninein isoform (Stillwell et al., 2004). Second, expression of the Cter-ninein, which acts in a dominant-negative manner by competing for localization with endogenous ninein, resulted in decreased association of the γ-TuRC and perturbation of microtubule nucleation at the centrosome. The displacement of the γ-TuRC resulted in a microtubule-nucleation delay rather than loss of nucleation. Similar delays have been observed after depletion of γ-tubulin (Hannak et al., 2002; Job et al., 2003; Sampaio et al., 2001; Strome et al., 2001). In these cases, nucleation might have been favoured by other proteins such as XMAP215 (Popov et al., 2002), p150^{Glued} (Ligon et al., 2003), EB1/APC (Nakamura et al., 2001) or dynein (Malikov et al., 2004). Third, the Nter/Cter-ninein restored γ-TuRC localization to the

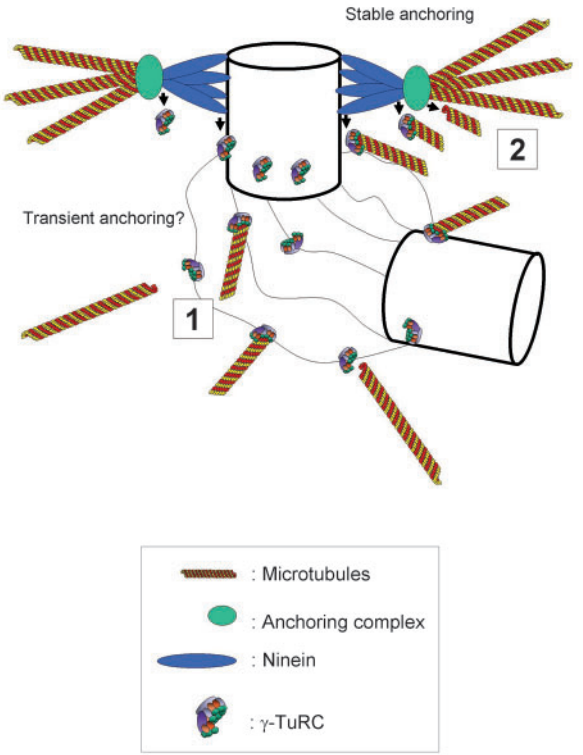


Fig. 9. Model for the role of ninein in microtubule organization by the centrosome. Microtubules are nucleated by the γ-TuRC, which is enriched at the centrosome. The newly nucleated microtubules could have different behaviours. (1) Microtubules are nucleated far from the subdistal appendages. They are transiently anchored by the γ-TuRC or released from the centrosome. (2) A proportion of the γ-TuRC is docked at the mother centriole by ninein. Microtubules nucleated by this γ-TuRC near or on the subdistal appendages would be quickly captured by the anchoring complex.

centrosome, suggesting a role for the N-terminal domain in γ-TuRC docking. Finally, co-immunoprecipitation and GST-pull-down experiments indicated that the ninein N-terminus interacts with γ-tubulin-containing complexes. Interestingly, Nter-ninein shares 37% identity with the N-terminal domain of Nlp, also implicated in γ-TuRC docking at the centrosome (Casenghi et al., 2003). Thus, both proteins could share the ability to dock the γ-TuRC.

Surprisingly, the presence of other docking proteins at the centrosome was not sufficient to retain the γ-TuRC in cells expressing Cter-ninein. Indeed, Nlp (Casenghi et al., 2003), pericentrin (Dictenberg et al., 1998) and AKAP450 (Takahashi et al., 2002) were not displaced from the centrosome in these expressing cells. It is possible that ninein perturbed the action of these docking proteins without displacing them, for example by disrupting a scaffold that is crucial for γ-tubulin localization. Alternatively, these results might show that ninein is more directly required for γ-TuRC docking.

Long-term expression (Stillwell et al., 2004) or microtubule depolymerization (data not shown) causes overexpressed ninein to form aggregates away from the centrosome. Consistent with a role for ninein in γ-TuRC docking, these ninein aggregates recruited γ-tubulin. Yet, while they are away from the centrosome, these cytoplasmic aggregates did not promote microtubule nucleation, owing either to an incorrect stoichiometry of the nucleating complex or to the absence of additional centrosomal components activating the γ-TuRC. In long-term regrowth experiments, as soon as overexpressed ninein reaccumulated around the centrosome, nucleation began (not shown). This pointed to a role for ninein in γ-TuRC anchoring at the centrosome but not in its activation, which remained dependent on centrosomal location only.

Because ninein is localized to the subdistal appendages of the mother centriole, our data might indicate that the interaction between ninein and the γ-TuRC is important to direct the nucleation of a subset of microtubules at the mother centriole.

Function of ninein in microtubule anchoring at the centrosome

Moderate overexpression of ninein reduces microtubule release from the centrosome (Abal et al., 2002). Conversely, ninein depletion in RNA-interference experiments leads to microtubule-aster-organization defects (Dammermann and Merdes, 2002). Ninein could anchor microtubules directly or via other anchoring proteins at the mother centriole, such as p150^{Glued} (Quintyne et al., 1999), EB1 (Askham et al., 2002; Louie et al., 2004) or ε-tubulin (Chang et al., 2003).

Here, we have showed that the γ-TuRC tethered to the centrosome by Nter/Cter-ninein was not sufficient to support anchorage. Therefore, the behaviour of cells expressing this fusion suggested that ninein's anchoring function could be separated from its ability to dock the γ-TuRC at the centrosome. Our results suggest that ninein, a component of the subdistal appendages, couples microtubule-nucleation and -anchoring processes at the centrosome of mammalian cells.

Ninein functionally links nucleation and anchoring processes at the mother centriole

The number of microtubules nucleated at the centrosome is probably much higher than the number of anchoring sites available at the tips of the nine appendages. One must therefore postulate a selective stabilization of microtubules at anchoring sites rather than a simplistic pathway of nucleation followed by anchoring. Interestingly, a proportion of the centrosomal γ-tubulin has been observed at the tips of subdistal appendages (Moudjou et al., 1996; Tassin et al., 1998). Moreover, proteins already implicated in microtubule anchoring such as p150^{Glued} or EB1, which are localized to the mother centriole, also have microtubule-nucleating properties (Ligon et al., 2003; Nakamura et al., 2001). These data suggest that a subset of microtubules could be nucleated and later anchored at the subdistal appendages. Based on our results, we propose that microtubules nucleated in a γ-TuRC-dependent manner at the centrosome would have different behaviours (see model in Fig. 9). Microtubules nucleated far from the subdistal appendages would be transiently anchored by the γ-TuRC but then released from the centrosome in a manner possibly dependent on specific factors. Indeed, many microtubule seeds are produced and released from the centrosome (Abal et al., 2002; Keating and Borisy, 1999). By contrast, microtubules nucleated at the vicinity or on the subdistal appendages would become anchored more readily at these structures on the mother centriole. Therefore, ninein, a component of these appendages, might favour microtubule nucleation (by docking the γ-TuRC), followed by anchoring. These newly formed microtubules might then be anchored directly, owing to ninein function, or might require additional elements for the transfer to anchorage sites. Interestingly, in ear pillar cells, microtubules are anchored at a considerable distance from the centrosome (Mogensen, 1999; Mogensen et al., 2000) and it is possible that similar modes of microtubule active transfer occur at the centrosome.

We thank E. A. Nigg for the anti-Nlp antibody, Kemner for the anti-AKAP450 antibody, A. M. Fry for the anti-C-Nap1 antibody, members of the UMR144 and Segal for critical reading of the manuscript and fruitful discussions. This work has been supported by the Centre National de la Recherche Scientifique (Unité Mixte Recherche 144) and Institut Curie, by a grant from Association pour la Recherche sur le Cancer (number 9407) to M.B., and by a fellowship from la Ligue National contre le Cancer to N.D.

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