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LGN Blocks the Ability of NuMA to Bind and Stabilize Microtubules: A Mechanism for Mitotic Spindle Assembly Regulation

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Summary

LGN is closely related to a *Drosophila* protein, Partner of inscuteable (Pins), which is required for polarity establishment and asymmetric cell divisions during embryonic development [1–3]. In mammalian cells, LGN binds with high affinity to the C-terminal tail of NuMA, a large nuclear protein that is required for spindle organization, and accumulates at the spindle poles during mitosis [4–9]. LGN also regulates spindle organization, possibly through inhibition of NuMA function [10], but the mechanism of this effect has not yet been understood. Using mammalian cells, frog egg extracts, and in vitro assays, we now show that a small domain within the C terminus of NuMA stabilizes microtubules (MTs), and that LGN blocks stabilization. The nuclear localization signal adjacent to this domain is not involved in stabilization. NuMA can interact directly with MTs, and the MT binding domain on NuMA overlaps by ten amino acid residues with the LGN binding domain. We therefore propose that a simple steric exclusion model can explain the inhibitory effect of LGN on NuMA-dependent mitotic spindle organization.

Results and Discussion

The mammalian Pins homolog, LGN, plays a key role in spindle pole organization during mitosis of vertebrate cells [10]. Either the overexpression of LGN or a reduction in LGN expression causes profound disruption of bipolar spindles and consequently leads to chromosome missegregation and cell death. We have proposed that these effects of LGN are mediated by NuMA, a large nuclear protein that is required in vertebrates for spindle pole organization. LGN binds to and inhibits the function of NuMA, but the mechanism of this regulation has not yet been understood.

The C Terminus of NuMA Contains an MT-Stabilizing Domain

When expressed in COS cells, the isolated C-terminal tail of NuMA(1818–2001) caused microtubule (MT) bundling in interphase cells (Figure 1A, upper panels) and the aberrant formation of multiple asters in mitotic cells (Figure 1A, lower panels). Moreover, if the cells were briefly permeabilized with digitonin prior to fixation, so as to remove excess cytoplasmic NuMA, the remaining tagged NuMA showed substantial colocalization with α-tubulin staining. The MT bundling and aster formation are reminiscent of the effects of taxol, a drug that binds to and stabilizes MTs. We reasoned, therefore, that NuMA might possess an MT-stabilizing function. To test this possibility, we treated the transfected cells with nocodazole, which disrupts MTs. As shown in Figure 1B, untransfected cells lost their tubulin filaments, but the bundled MTs and asters in the transfected cells were retained, strongly suggesting that the C-terminal tail of NuMA prevents the dissociation of MTs into tubulin subunits.

The multiple asters that are induced in mitotic cells by the C-terminal tail of NuMA could arise from inappropriate centrosome duplication or by acentrosomal organization of the MTs. To distinguish between these possibilities, we stained transfected cells for γ-tubulin. In all cases, only 1–2 centrosomes were detectable (Figure 1C), showing that NuMA(1818–2001) expression does not trigger abnormal centrosome duplication.

To identify the MT-stabilizing domain of NuMA, a series of truncation mutants were tested. The smallest fragment with stabilizing function was found to be NuMA(1900–1971) (Figure 1D). Again, clear colocalization of the tagged NuMA fragment with MTs was observed, and a GFP fusion of the same fragment also colocalized with MTs, even when fixed prior to permeabilization (Figure 1E). Similar results were recently described by Haren and Merdes [11] for a fragment spanning residues 1868–1967. Note that a slightly shorter fragment, NuMA(1910–1971), had no effect on MT organization (Figure 1D).

NuMA Possesses Overlapping Binding Sites for LGN and MTs

We have reported previously that the binding site on NuMA for LGN lies between residues 1818 and 1930 [10]. We used a series of truncation mutants to map this domain more precisely, and we found that residues 1878–1910 are essential for the interaction with LGN (Figures 2A and 2B). Interestingly, NuMA(1818–1900) did not bind LGN, suggesting that the amino acid residues between 1900 and 1910 are critical for this interaction. These residues are also essential for the MT-stabilizing function of NuMA (Figure 1D). Therefore, these two domains overlap (see the schematic in Figure 2B).

We next tested whether LGN could inhibit the effect of NuMA(1818–2001) on MTs in transfected COS cells. When either full-length LGN or the NuMA binding domain of LGN(1–373) was cotransfected with NuMA(1818–2001), MT bundling was inhibited and tubulin organization within the cells appeared normal (Figure 2C, upper two panels). Note that overexpressed LGN aggregates into ring-like structures, which sequester the NuMA (see Figure S1 in the Supplementary Material available with this

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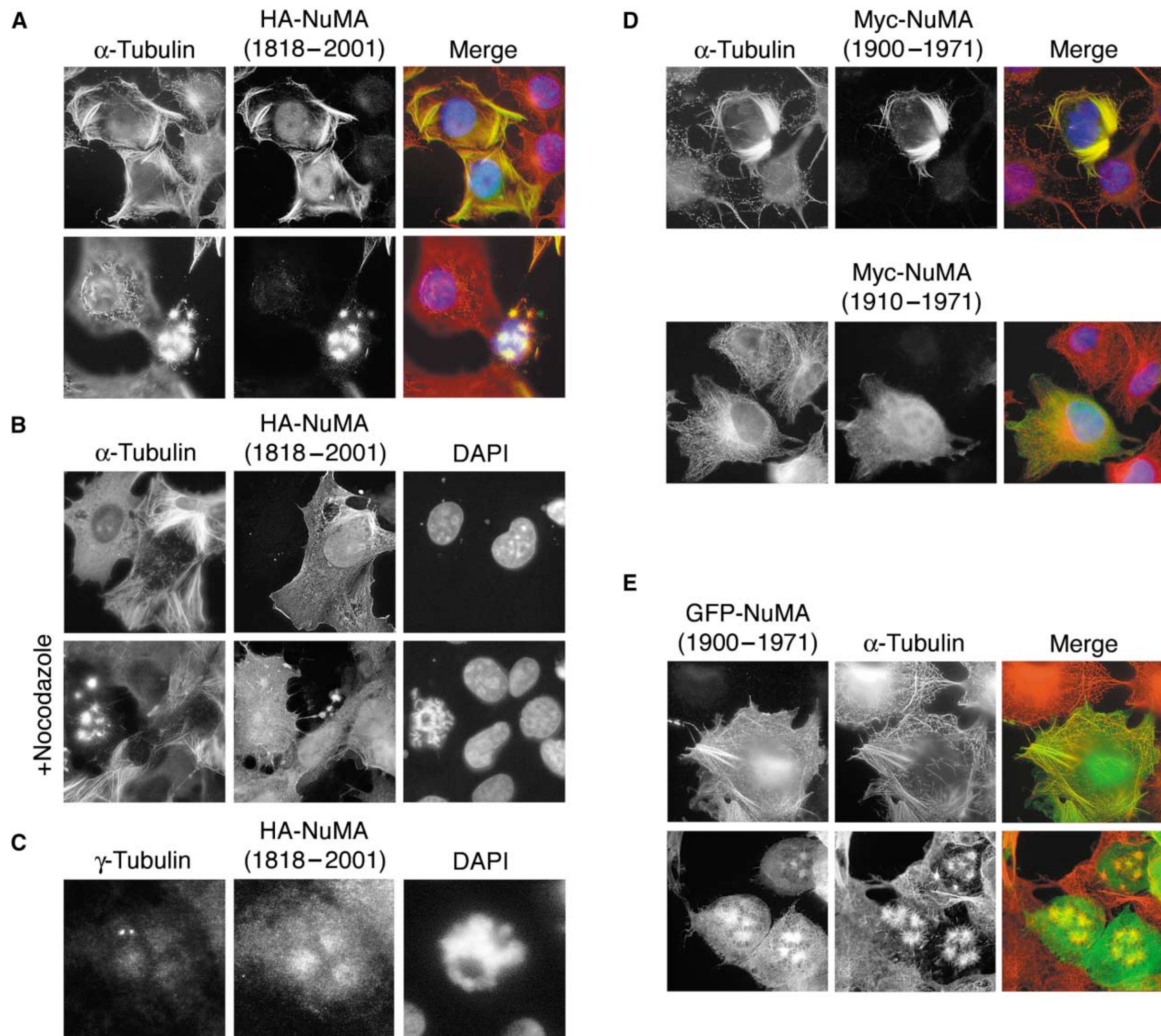

Figure 1. Overexpression of a Small NuMA Fragment Stabilizes Microtubules in Transfected Cells

(A) Immunofluorescence images of COS-7 cells transfected with HA-NuMA(1818–2001). Cells were permeabilized for 5 min with 0.008% digitonin, then fixed and stained for HA-NuMA (green in merged images), α-tubulin (red), and DNA (blue). Note that NuMA residue numbers are based on Compton et al. [6]. A splice variant of NuMA described elsewhere contains an additional 14 residues [20].

(B) COS-7 cells were transfected with HA-NuMA(1818–2001) for 36 hr and were treated with nocodazole (80 ng/ml) for 2 hr before fixation. Cells were stained as in (A).

(C) Transfected cells as in (A). γ-tubulin was stained with monoclonal anti-γ-tubulin antibody, and HA-NuMA and DNA were stained as in (A).

(D) Cos-7 cells were transfected with Myc-NuMA(1900–1971) or Myc-NuMA(1910–1971) (green in merged images). Cells were stained as in (A).

(E) COS-7 cells were transfected with GFP-NuMA(1900–1971) (green) and fixed, then stained for α-tubulin (red) as in (A). Merged images show colocalization.

article online). However, when an N-terminal deletion mutant of LGN, LGN(128–677), which does not bind to NuMA, was used, bundled tubulin, which is characteristic of stabilized MTs, was seen (Figure 2C). Importantly, when a smaller fragment of NuMA, NuMA(1900–2001), which cannot bind LGN, was introduced into the cells, cotransfected LGN was unable to block MT stabilization (Figure 2C, bottom panel). These results suggest that LGN inhibits the MT stabilization function of NuMA by binding to the overlapping site in the C-terminal tail and by blocking MT binding.

The effects of NuMA on MTs, and of LGN on NuMA, might each be either direct or indirect. We first approached this question by asking whether we could detect binding of NuMA to MTs produced from purified tubulin, and by asking whether LGN would inhibit binding. Tubulin was assembled into MTs stabilized by taxol and was incubated with ^{35}S-labeled NuMA(1566–2001) produced by coupled transcription-translation. This larger fragment was chosen because it is expressed robustly by the in vitro translation system. MTs were sedimented by ultracentrifugation and were assayed for

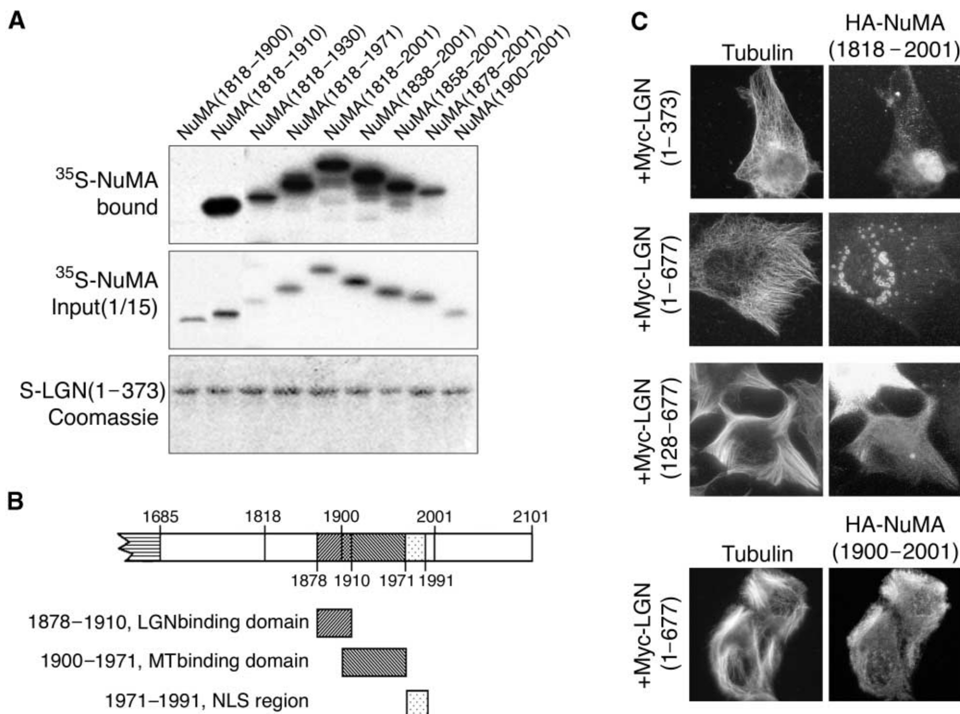

Figure 2. LGN Inhibits the Stabilization of MTs by NuMA

(A) Mapping of LGN binding region in NuMA. Equal amounts of S-tagged LGN(1–373) were bound to S-agarose beads and incubated with ^{35}S-labeled NuMA fragments translated in vitro. Bound proteins were detected by fluorography.

(B) A schematic of domains in the C-terminal region of NuMA.

(C) Immunofluorescence images of COS-7 cells cotransfected with HA-NuMA and Myc-LGN fragments. HA-NuMA was stained with rabbit anti-HA antibodies, and tubulin was stained with monoclonal anti-α-tubulin antibody. Note that the different localization of HA-NuMA in the upper two panels is due to the colocalization with Myc-LGN and Myc-LGN-N(1–373) (see Figure S1).

associated NuMA by fluorography. As shown in Figure 3A, the NuMA coprecipitated efficiently with the MTs. Addition of GST-LGN(1–373), which can bind NuMA, inhibited the coprecipitation. However, addition of GST alone had no effect. Therefore, LGN can block the association of the NuMA C-terminal tail with MTs.

The MT bundling effects of NuMA fragments described above could result either from direct MT binding and stabilization or from the association of NuMA with other MT regulatory factors. To test whether NuMA has a direct effect on tubulin polymerization, we employed a solution MT formation assay. Tubulin labeled with rhodamine was incubated with an energy-regenerating system in buffer alone, with glycerol, which is known to induce MT polymerization, with GST-NuMA fusion proteins, or with GST alone (Figure 3B). Those NuMA fragments that we had found to stabilize MTs when transfected into cells also efficiently generated MTs in the MT formation assay. NuMA(1910–1971), which did not stabilize MTs, was also inactive in this assay (Figures 3B–3D). Therefore, the effect of NuMA on MTs is direct and does not require additional cofactors. Moreover, when the N terminus of LGN was added to the reaction together with GST-NuMA(1878–2001) fusion protein, MT formation was blocked, but LGN did not inhibit MT formation induced by glycerol or by GST-NuMA(1900–1971) (Figure 3D). Therefore, the inhibitory effect of LGN on NuMA is also direct and does not require additional cofactors.

Regulation of Aster Formation by NuMA and LGN

Finally, we tested the effects of the NuMA MT stabilization domain in a *Xenopus* egg extract. Even at low concentrations (1 μM), GST fusions of NuMA(1900–1971) and NuMA(1878–2001) each efficiently induced aster formation in the extracts (Figure 4), and, as we had expected, the coaddition of the N terminus of LGN prevented aster formation. However, it did not block the formation of MTs induced by the shorter LGN-insensitive fragment, NuMA(1900–1971). Interestingly, though, in this experiment, the stabilized MTs did not assemble into asters (Figure 4, bottom left panel). We interpret this result to mean that aster assembly requires endogenous NuMA and that this endogenous NuMA is inhibited by the addition of LGN [10].

The MT-stabilizing domain of NuMA is just N-terminal to a nuclear localization signal (NLS; residues 1971–1991; see Figure 2B). In studies on the role of the Ran GTPase in mitotic spindle formation, it has been proposed that the association of nuclear transport factors importin-α and -β with NuMA can inhibit the ability of NuMA to focus MTs into asters, and that RanGTP relieves this effect by binding to importin-β and triggering its release [12, 13]. However, we found that aster abundance was similar for both of the GST-NuMA fragments tested (7–10 asters/field), even though the larger one contains the NLS and the other does not. This result is inconsistent with the proposed mechanism for Ran-induced aster formation in egg extracts, by which im-

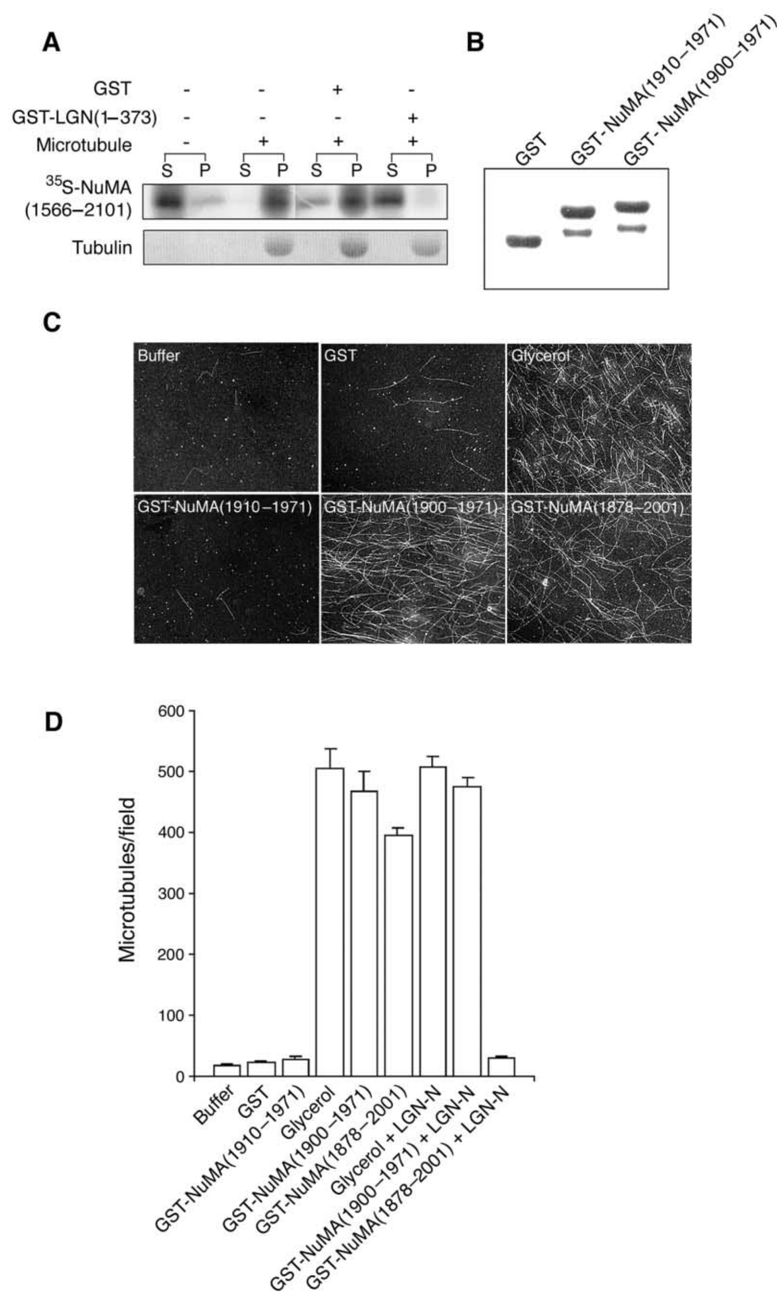

Figure 3. LGN Inhibits the Microtubule Binding and Stabilization Activity of NuMA In Vitro

(A) Microtubule cosedimentation assay. In vitro-translated ^{35}S-labeled NuMA(1566–2101) was incubated, at room temperature for 20 min, with buffer alone, with taxol-stabilized MTs, or with taxol-stabilized MTs plus GST or GST-LGN(1–373), then centrifuged at 10,000 $\times$ g for 1 hr. The supernatants (S) and pellets (P) were separated by SDS-PAGE, and ^{35}S-labeled NuMA was detected by fluorography. Coomassie-stained tubulin is shown in the lower panel.

(B) A Coomassie-stained gel showing recombinant GST (left), GST-NuMA(1910–1971) (middle), and GST-NuMA(1900–1971) (right).

(C) Representative fields from solution MT formation assays.

(D) Quantification of solution MT formation assays. MTs in ten microscope fields were counted, and the average number of MTs/field is plotted $\pm$ 1 SD.

portin-α and/or -β bind and inhibit aster-promoting activities such as NuMA [12, 13]. According to this model, the NuMA C-terminal tail induces aster formation by sequestering endogenous importin-α and/or -β, so the shorter GST-NuMA fragment (1900–1971) that lacks the NLS should not induce aster formation. (We note, however, that we have been unable to detect specific binding of importin-α or -β to the C-terminal tail of NuMA). We propose instead that any agent capable of generating stable MTs will produce asters in *Xenopus* egg extract that contains functional NuMA and other cofactors that provide MT-focusing activity. To validate this model, we asked if an unrelated MT-associated protein would induce asters in egg extracts, and, as shown in Figure 4 (bottom right panel), a low concentration (1 μM) of Tau efficiently produced asters in the extract. Tau contains a conserved MT binding domain that is completely unrelated to that of NuMA.

Taken together, our data define a simple steric exclusion model that explains the role of LGN in controlling mitotic spindle organization. We have shown that the binding site on NuMA for LGN overlaps by ten amino acid residues with the binding site on NuMA for MTs. The interaction of NuMA with LGN and MTs is therefore mutually exclusive. We have measured the equilibrium dissociation constant of the LGN/NuMA complex to be $\sim$25 nM (data not shown), which is about 10-fold higher affinity than that of NuMA for MTs [11]. We have also estimated the abundance of LGN in the cell to be about 2-fold higher than that of NuMA (which is $\sim 2 \times 10^5$

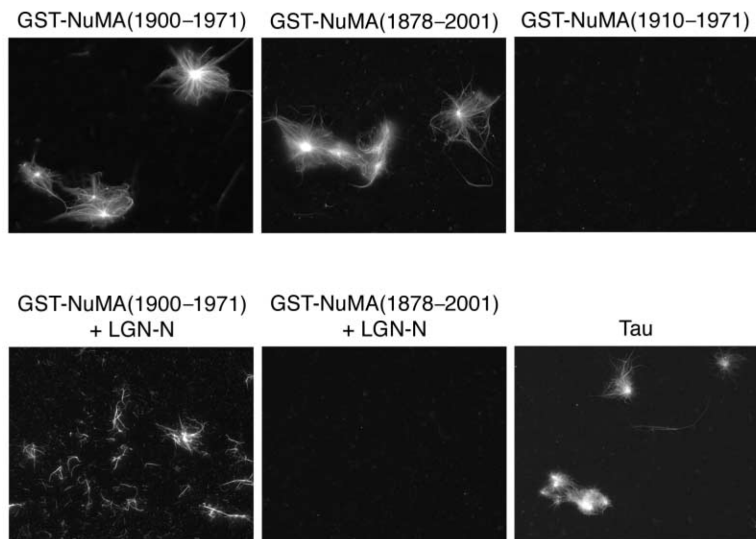

Figure 4. Effect of LGN and NuMA Fragments on Aster Formation in *Xenopus* Egg Extracts

Representative fields from MT polymerization assays in *Xenopus* egg extracts. Mitotic extracts were incubated with GST-NuMA(1900–1971), GST-NuMA(1878–2001), GST-NuMA(1910–1971), GST-NuMA(1900–1971) plus His-LGN(1–373), GST-NuMA(1878–2001) plus His-LGN(1–373), or Tau for 20 min at 20°C. Reactions were then fixed and spun onto coverslips.

copies/cell; [14]) (data not shown). Therefore, ample LGN is present in the cell to inhibit the majority of the endogenous NuMA from binding MTs. Such inhibition may be essential for normal spindle pole assembly, because the minus ends of spindle MTs are dynamic and lose subunits as a consequence of poleward MT flux [15]. Stabilization by NuMA binding must necessarily, therefore, be transient. How LGN is itself localized to the poles and regulated remains to be established.

Experimental Procedures

Cell Transfection and Indirect Immunofluorescence

COS-7 cells were grown on the Lab-Tek Chamber (Nalge Nunc International) in DMEM culture medium supplemented with 10% fetal bovine serum (GIBCO-BRL). Cells were transfected by using the Effectene transfection reagent kit (Qiagen). For cotransfection of HA-NuMA(1818–2001) and Myc-LGN fragments, a molecular ratio of 1 (HA-NuMA):2 (Myc-LGN) was kept throughout the experiments. Under these conditions, almost 100% of cells expressing HA-NuMA coexpress Myc-LGN fragments. A total of 40 hr after transfection, cells were either fixed and stained as described [10] or were permeabilized with 0.008% digitonin in PHEM buffer (60 mM PIPES·Na [pH 6.9], 25 mM HEPES·Na [pH 7.4], 10 mM EGTA, and 5 mM $MgSO_4$) for 5 min prior to fixation.

In Vitro Binding Assays

^{35}S-labeled NuMA fragments were produced by using an in vitro transcription and translation kit (Promega). Equal amounts (3 μg) of His- and S-tagged LGN-N(1–373) were loaded on S-agarose beads (Novagen) and were incubated with ^{35}S-labeled NuMA fragments for 1 hr. After washing, bound proteins were separated by SDS-PAGE and were detected by fluorography.

MT Cosedimentation Assays

^{35}S-labeled NuMA(1566–2101) was produced by using an in vitro transcription and translation kit (Promega). Equal amounts of ^{35}S-labeled NuMA were incubated with buffer, GST (2 μg), or GST-LGN-N(1–373) (2 μg) on ice for 30 min. MT cosedimentation assays were performed by using a MT spin-down assay kit (Cytoskeleton).

Solution MT Formation Assays

Solution MT formation assays were performed as described [16]. Briefly, 5 μl reactions of identical final buffer composition (0.5× BRB80, 0.5× PBS, 500 μM GTP) containing 4 mg/ml rhodamine-labeled tubulin (Cytoskeleton) and 25 μM GST, GST-NuMA(1910–1971), GST-NuMA(1900–1971), GST-NuMA(1878–2001), and glycerol (10%, v/v), with or without 25 μM His-LGN-N(1–373), were incubated at 37°C for 4 min and were fixed at room temperature for 3 min by adding 45 μl 1% glutaraldehyde in BRB80. Samples were then diluted with 200 μl BRB80 + 50% glycerol, and 3 μl of the sample was squashed, sealed, and visualized with a 60× water-immersion objective lens (NA = 1.2) on a Nikon inverted microscope equipped with a Hamamatsu Orca CCD camera. To quantitate MTs, after fixation, 10 μl was removed and diluted in 1 ml BRB80, and MTs were spun onto coverslips. Ten random fields were photographed, and the MTs were counted.

MT-Stabilization Assays in *Xenopus* Egg Extracts

Cytoplasmic *Xenopus* egg extracts were prepared and driven into mitosis by the addition of nondegradable cyclin B1 as described [17, 18]. Rhodamine-labeled tubulin (Cytoskeleton) was added to a final concentration of 0.2 mg/ml. A total of 20 μl of extract was used for each reaction. GST-NuMA(1910–1971), GST-NuMA(1900–1971), GST-NuMA(1878–2001), and Tau (Cytoskeleton) were added to a final concentration of 1 μM. His-LGN-N(1–373) was added to a final concentration of 5 μM. Reactions were carried out at 20°C for 30 min and were fixed and spun onto coverslips as described [19].

Supplementary Material

Supplementary Material showing the colocalization of overexpressed NuMA(1818–2001) with LGN in ring-like structures is available at http://images.cellpress.com/supmat/supmatin.htm.

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References

1. Schaefer, M., Shevchenko, A., and Knoblich, J.A. (2000). A protein complex containing Inscuteable and the Gα-binding protein Pins orients asymmetric cell divisions in *Drosophila*. Curr. Biol. *10*, 353–362.
2. Schaefer, M., Petronczki, M., Dorner, D., Forte, M., and

Knoblich, J.A. (2001). Heterotrimeric G proteins direct two modes of asymmetric cell division in the *Drosophila* nervous system. Cell *107*, 183–194.

3. Yu, F., Morin, X., Cai, Y., Yang, X., and Chia, W. (2000). Analysis of partner of inscuteable, a novel player of *Drosophila* asymmetric divisions, reveals two distinct steps in inscuteable apical localization. Cell *100*, 399–409.
4. 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.
5. Gordon, M., Howard, L., and Compton, D. (2001). Chromosome movement in mitosis requires microtubule anchorage at spindle poles. J. Cell Biol. *152*, 425–434.
6. Compton, D.A., and Cleveland, D.W. (1993). NuMA is required for the proper completion of mitosis. J. Cell Biol. *120*, 947–957.
7. Compton, D.A., and Cleveland, D.W. (1994). NuMA, a nuclear protein involved in mitosis and nuclear reformation. Curr. Opin. Cell Biol. *6*, 343–346.
8. 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.
9. Zeng, C. (2000). NuMA: a nuclear protein involved in mitotic centrosome function. Microsc. Res. Tech. *49*, 467–477.
10. Du, Q., Stukenberg, P.T., and Macara, I.G. (2001). A mammalian Partner of inscuteable binds NuMA and regulates mitotic spindle organization. Nat. Cell Biol. *3*, 1069–1075.
11. Haren, L., and Merdes, A. (2002). Direct binding of NuMA to tubulin is mediated by a novel sequence motif in the tail domain that bundles and stabilizes microtubules. J. Cell Sci. *115*, 1815–1824.
12. Nachury, M.V., Maresca, T.J., Salmon, W.C., Waterman-Storer, C.M., Heald, R., and Weis, K. (2001). Importin-β is a mitotic target of the small GTPase Ran in spindle assembly. Cell *104*, 95–106.
13. Wiese, C., Wilde, A., Moore, M.S., Adam, S.A., Merdes, A., and Zheng, Y. (2001). Role of importin-β in coupling Ran to downstream targets in microtubule assembly. Science *291*, 653–656.
14. Compton, D.A., Szilak, I., and Cleveland, D.W. (1992). Primary structure of NuMA, an intranuclear protein that defines a novel pathway for segregation of proteins at mitosis. J. Cell Biol. *116*, 1395–1408.
15. Mitchison, T.J., and Sawin, K.E. (1990). Tubulin flux in the mitotic spindle: where does it come from, where is it going? Cell Motil. Cytoskeleton *16*, 93–98.
16. Oegema, K., Wiese, C., Martin, O.C., Milligan, R.A., Iwamatsu, A., Mitchison, T.J., and Zheng, Y. (1999). Characterization of two related *Drosophila* γ-tubulin complexes that differ in their ability to nucleate microtubules. J. Cell Biol. *144*, 721–733.
17. Stukenberg, P.T., Lustig, K.D., McGarry, T.J., King, R.W., Kuang, J., and Kirschner, M.W. (1997). Systematic identification of mitotic phosphoproteins. Curr. Biol. *7*, 338–348.
18. Murray, A.W. (1991). Cell cycle extracts. Methods Cell Biol. *36*, 581–605.
19. Desai, A., Murray, A., Mitchison, T.J., and Walczak, C.E. (1999). The use of *Xenopus* egg extracts to study mitotic spindle assembly and function in vitro. Methods Cell Biol. *61*, 385–412.
20. Yang, C.H., Lambie, E.J., and Snyder, M. (1992). NuMA: an unusually long coiled-coil related protein in the mammalian nucleus. J. Cell Biol. *116*, 1303–1317.