Supplementary material

NuMA forms condensates through phase separation to drive spindle pole assembly

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Running title: NuMA phase separation in spindle pole assembly

Supplementary material contains Materials and methods and six figures.

Materials and Methods

Antibodies, chemicals, siRNAs, and plasmids

Anti-NuMA (Abcam and Proteintech), anti-α-tubulin (Abcam), anti-γ-tubulin (Abcam), anti-β-actin (Santa Cruz Biotechnology), and IgG (Santa Cruz Biotechnology) were purchased from the indicated sources. Horseradish peroxidase-conjugated secondary antibodies and Alexa Fluor-488, -568, or -647-conjugated secondary antibodies were obtained from Jackson ImmunoResearch Laboratories or Thermo Fisher Scientific. Paclitaxel was obtained from MedChemExpress, and GTP was from Roche. 4'-6'-Diamidino-2-phenylindole (DAPI) was obtained from Sigma-Aldrich. Control siRNA (5'- CGUACGCGGAAUACUUCGA-3') and NuMA siRNAs (#1: 5'-CTAGCTGAGCTCCATGCCA-3'; #2: 5'-CCGGCCTTGAAGAGAAGAA-3') were synthesized by GenePharma. The pEGFP-C1-NuMA plasmid was from Addgene, and the plasmids expressing truncated or site-directed mutants of NuMA were constructed by PCR.

Cell culture and transfection

HeLa cells were obtained from the American Type Culture Collection and cultured in DMEM supplemented with 10% fetal bovine serum at 37 ℃ in 5% CO_2 humidified atmosphere. Plasmids were transfected into cells with Lipofectamine 3000 (Thermo Fisher Scientific), and siRNAs were transfected with Lipofectamine RNAiMAX (Thermo Fisher Scientific).

Protein expression and purification

Bacterial expression plasmids for GFP-NuMA-C wild-type and mutants were constructed by using the pET28a vector. The recombinant proteins were expressed in the BL21(DE3) strain and purified with Ni-NTA agarose beads (QIAGEN) for 1 h at 4 ℃ and then washed with washing buffer (300 mM NaCl, 10 mM Tris-HCl pH 8.0, 10/20/30/50 mM imidazole pH 7.9). Purified proteins were concentrated by using centrifugal filter units (Millipore) according to the manufacturer's protocols. Protein concentration was determined by the Bradford assay (Thermo Fisher Scientific).

In vitro phase separation assay

Proteins were diluted in the dilution buffer containing 25 mM HEPES pH 7.5, 0.15 M NaCl, 1 mM DTT, and PEI to the desired concentrations. NuMA droplets were formed by adding the concentrated protein to the dilution buffer containing different concentrations of PEG-8000 (Sigma). For imaging, droplets were formed in a glass-bottomed 35-mm dish, and images were taken using the TCS SP8 confocal microscope (Leica). The size of droplets was measured with the ImageJ software (National Institutes of Health).

Droplet fusion and FRAP analysis

The droplet fusion *in vitro* was recorded with the TCS SP8 confocal microscope with 60 × oil immersion objective. For FRAP experiments, the droplets were bleached with 488-nm diode four times until the region of interest had almost no fluorescence. The recovery of fluorescence after photobleaching was then recorded.

Protein sequence analysis

The Porter 5.0 software (distilldeep.ucd.ie/porter/) was used to analyze the protein sequence and predict the secondary structure.

Immunofluorescence microscopy

Cells were fixed in 4% paraformaldehyde for 15 minutes and then permeabilized with 0.3% Triton X-100 for 15 minutes. After blocking in 4% bovine serum albumin for 1 hour, cells were stained with primary antibodies overnight at 4℃ followed by secondary antibodies at room temperature for 1 hour. Cells were stained with DAPI for 5 minutes and then examined with the TCS SP8 confocal microscope.

Immunoblotting

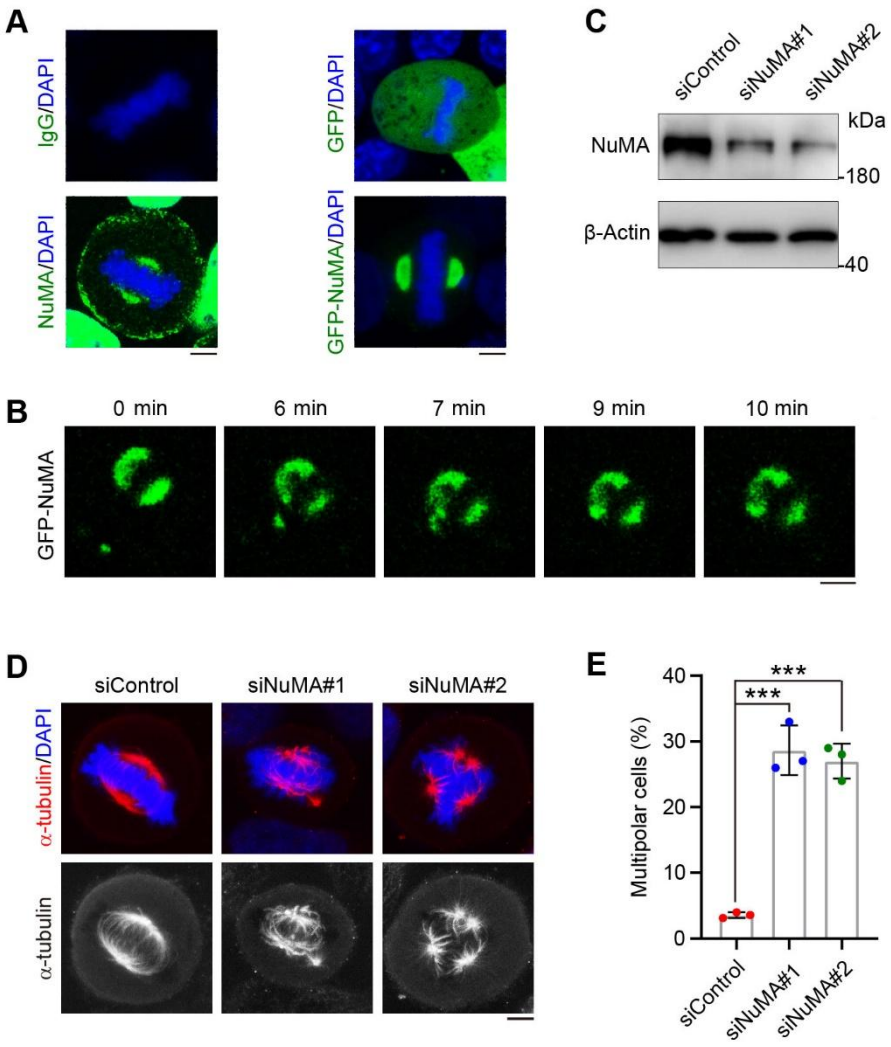
SDS-PAGE and immunoblotting were carried out using standard procedures. Cell lysates were prepared in standard SDS loading buffer for immunoblotting. Signals were detected using corresponding primary and secondary antibodies.

Microtubule co-sedimentation assay

To examine the NuMA-microtubule interaction, HeLa cells expressing GFP-NuMA-C-WT or GFP-NuMA-C-P/A were added to a buffer containing 40 mM PIPES, 5 mM EDTA, 5 mM $MgCl_2$, 500 mM KOH PH 6.8, and centrifuged at 4 ℃ for 1 hour at 33,000 g. The supernatant was added with 40 M paclitaxel and 1 mM GTP and incubated at 37 ℃ for 1 hour. The mixture was then transferred to a tube containing 4% sucrose and centrifuged at 37 ℃ for 30 minutes at 33,000 g. The pellet and supernatant fractions were then collected respectively and examined with immunoblotting.

Statistical analysis

All experiments were repeated at least three times, and data were presented as mean ± SD. Statistical analyses were performed with two-tailed unpaired Student's *t* test in GraphPad Prism 6. Multiple comparisons were performed with two-way repeated-measures ANOVA.



Supplementary Figure S1 NuMA is required for spindle pole assembly.

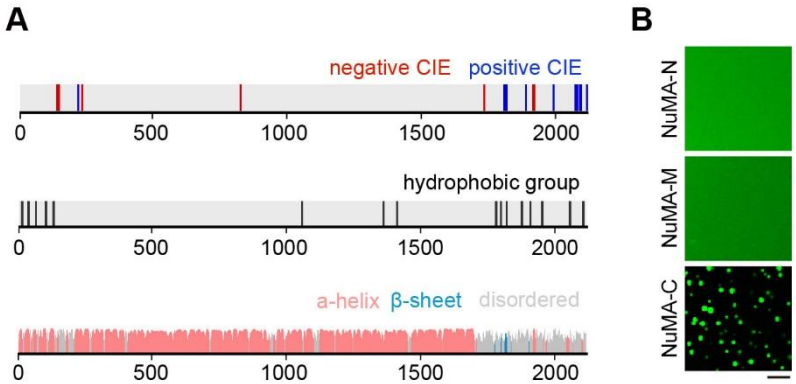
(**A**) Fluorescence images showing the localization of endogenous and exogeneous NuMA in HeLa cells. Scale bars, 5 μm.

(**B**) Time-lapse images showing the fusion of GFP-NuMA condensates into the spindle pole in a metaphase HeLa cell. Scale bar, 5 μm.

(**C**) Immunoblot analysis of NuMA in cells transfected with control or NuMA siRNAs.

(**D** and **E**) Immunofluorescence images of mitotic spindles (**D**) and quantification of mitotic cells with multipolar spindles (**E**, n = 3 independent experiments) in HeLa cells transfected with control or NuMA siRNAs. For each experiment, 60 mitotic cells were quantified. Scale bar, 5 μm.

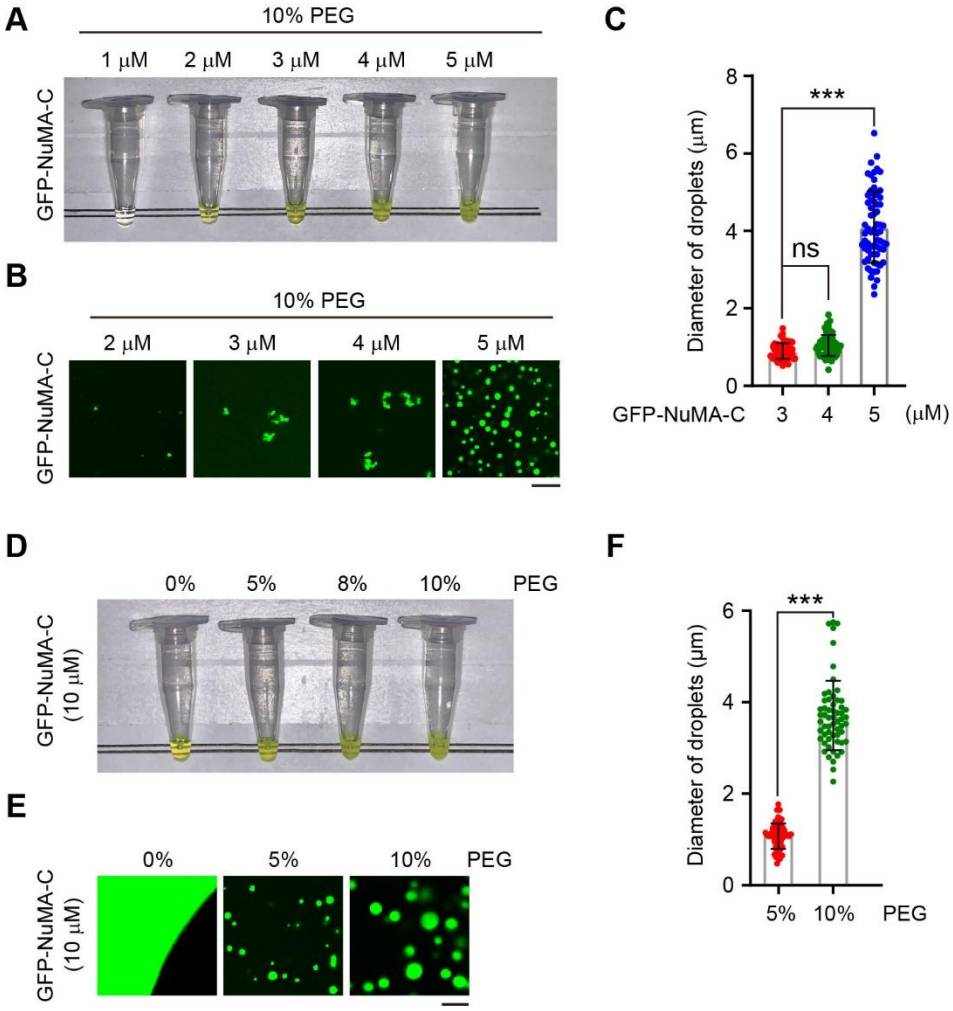
Data are shown as mean ± SD. ***$P < 0.001$.



Supplementary Figure S2 The carboxyl terminus of NuMA is intrinsically disordered, contains charged and hydrophobic segments, and forms droplets *in vitro*.

(**A**) Analysis of the electrical charge (top panel), hydrophobicity (middle panel), and secondary structure (bottom panel) of NuMA. CIE, charge-interacting element.

(**B**) Fluorescence images of droplets formed by 20 μM purified GFP-NuMA-N, GFP-NuMA-M, or GFP-NuMA-C. Scale bars, 10 μm.



Supplementary Figure S3 The carboxyl terminus of NuMA undergoes phase separation *in vitro*.

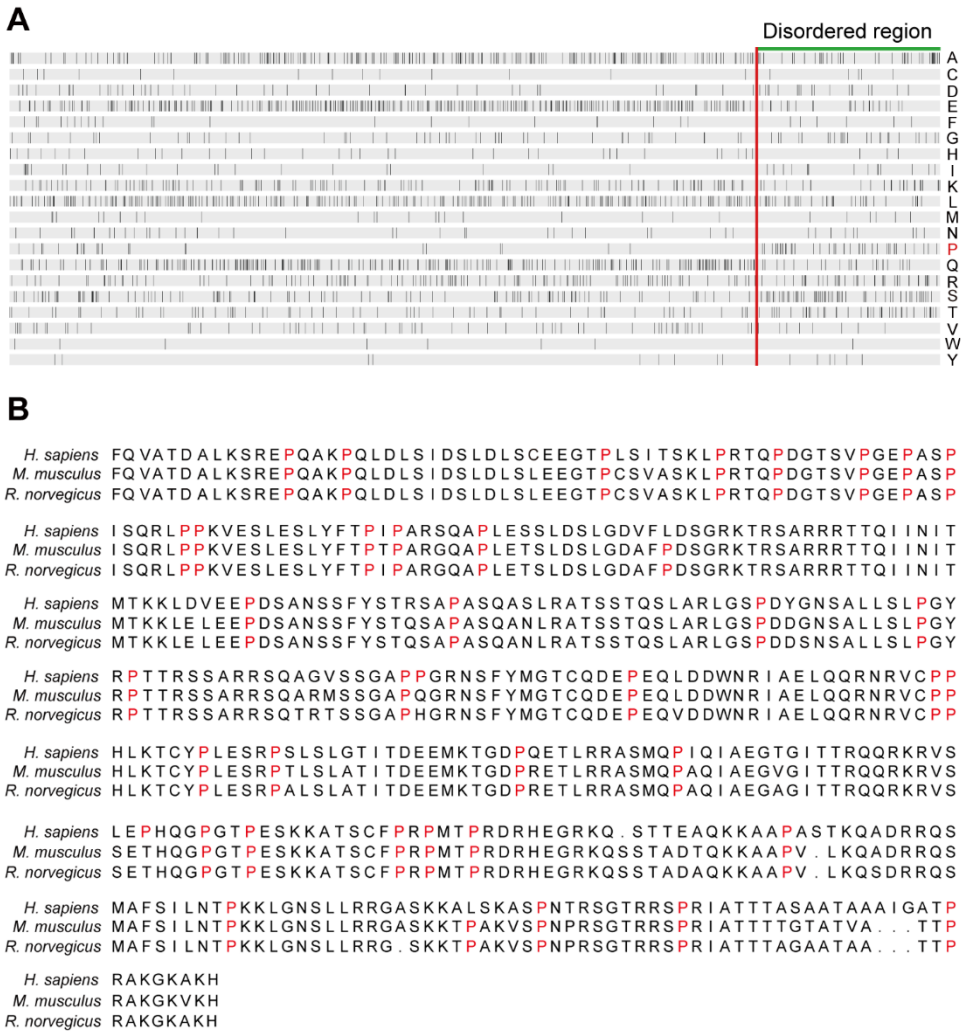
(**A**) Spin-down assay of GFP-NuMA-C to visualize the formation of condensates at increasing protein concentrations.

(**B** and **C**) Fluorescence images (**B**) and quantification of the diameter (**C**, *n* = 60 droplets from three independent experiments) of GFP-NuMA-C droplets formed at the indicated concentrations. Scale bar, 10 μm.

(**D**) Phase separation of GFP-NuMA-C in different PEG-8000 concentrations.

(**E** and **F**) Fluorescence images (**E**) and quantification of the diameter (**F**, *n* = 60 droplets from three independent experiments) of the GFP-NuMA-C droplets formed in different PEG-8000 concentrations. Scale bar, 5 μm.

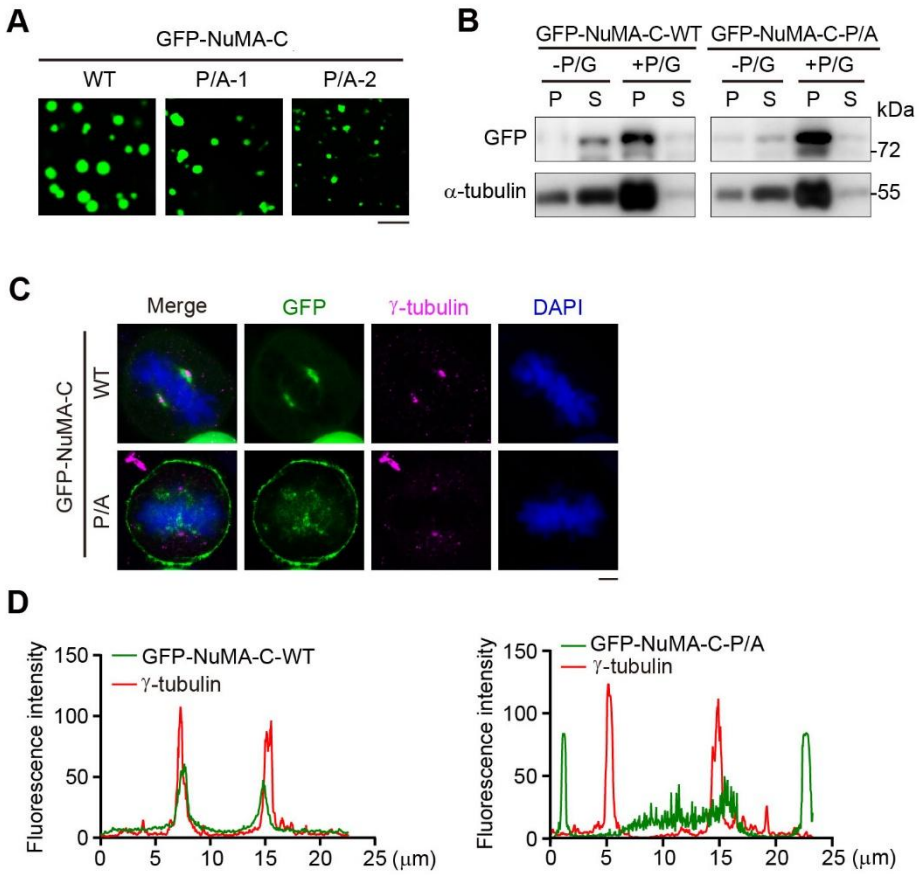
Data are shown as mean ± SD. ****P* < 0.001, ns, not significant.



Supplementary Figure S4 Prolines in the carboxyl terminus of NuMA are conserved in different species.

(**A**) Analysis of the amino acid composition of full-length NuMA. The black strings represent the positions of individual amino acids indicated at the right.

(**B**) Conservation of prolines in the carboxyl terminus of NuMA in different species.

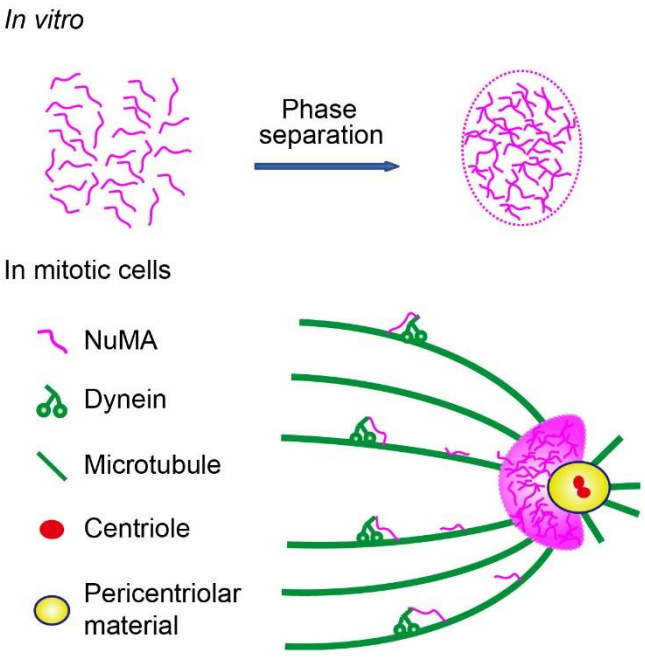


Supplementary Figure S5 Prolines in the carboxyl terminus of NuMA are important for its phase separation and localization at the spindle pole.

(**A**) Fluorescence images of droplets formed by purified GFP-NuMA-C wild-type (WT) or proline-to-alanine (P/A) mutants. In the P/A-1 mutant, the first 20 prolines of NuMA-C were mutated to alanines. In the P/A-2 mutant, the last 18 prolines of NuMA-C were mutated to alanines. Scale bar, 5 μm.

(**B**) Microtubule co-sedimentation assays performed in the absence or presence of paclitaxel and GTP (P/G), using cells transfected with GFP-NuMA-C wild-type or the P/A mutant. Proteins present in the pellet (P) and supernatant (S) fractions were detected by immunoblotting. In the P/A mutant, all the 36 prolines in NuMA-C were mutated to alanines.

(**C** and **D**) Fluorescence images (**C**) and fluorescence intensity profiles (**D**) showing the colocalization of GFP-NuMA-C wild-type, but not the P/A mutant, with γ-tubulin in metaphase cells. Scale bar, 5 μm.



Supplementary Figure S6 A model depicting the role of NuMA in promoting spindle pole assembly through its phase separation property.