Figures and figure supplements

Dynein–Dynactin–NuMA clusters generate cortical spindle-pulling forces as a multi-arm ensemble

Masako Okumura et al
Figure 1. Optogenetic targeting of NuMA to the mitotic cell cortex is sufficient for dynein-dynactin recruitment and spindle pulling. (A) Diagram summarizing cortical complexes in the indicated conditions. (B) Live fluorescent images of NuMA-RFP-Nano (upper) and DHC-SNAP (lower) in control metaphase cells (left), and LGN-depleted cells arrested with MG132. (C) Quantification of cortical NuMA-RFP-Nano and DHC-SNAP signals around the light illuminated region (n = 5). Error bars indicate SEM. (D) Quantification of the pole-to-cortex distance (NuMA-RFP-Nano, n = 10; RFP-Nano, n = 6). Error bars indicate SEM. (E) Kymographs obtained from image sequences in Figure 1—figure supplement 2A. Asterisk indicates the duration in which one of the spindle poles moves away from the focal plane. (F) When NuMA-RFP-Nano (upper) was optogenetically repositioned at multiple adjacent cortical regions around the cell membrane by sequential illumination (from 1 to 9), the spindle (lower) rotated about 90° in a directed manner coupled with the changes in cortical NuMA enrichment in 55% (n = 11) of cells, but not by repositioning RFP-Nano alone (Figure 1—figure supplement 2D, n = 6). Dashed lines indicate the spindle axis. Scale bars = 10 μm.

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Figure 1—figure supplement 1. Generation of cell lines for light-induced targeting of endogenous NuMA. (A) Diagram summarizing the iLID system (Guntas et al., 2015). Following blue light illumination, AsLOV2 domain of membrane-targeted iLID induces a conformational change and exposes SsrA peptide, which forms hetero-dimer with Nano-fusion. Upon termination of light illumination, Nano-fusion dissociates from the SsrA with a half-life of <30 s (Guntas et al., 2015). (B) Time-lapse images of RFP-Nano from a single z-section showing different patterns of cortical RFP-Nano recruitment in response to light illumination. A metaphase HeLa cell transiently expressing RFP-Nano (Addgene #60415) and Venus-iLID-caax (Addgene #60411) was illuminated with a single 488 nm laser pulse (250-msec exposure, 25 mW) at the indicated regions (circles with 1.95 μm diameter). See Video 1. (C) Genomic PCR showing clone genotype after Puromycin (Puro) selection. The clone No.2 was used as a parent in the second, and third selection. (D) Live images of Mem-BFP-iLID, DNA, and DIC in the clone No.2 selected in (C). (E) Genomic PCR showing clone genotype after Neomycin (Neo) selection. All clones displayed a single 4.4 kb band, indicating that the RFP-Nano (Neo) cassette was inserted in both NuMA1 gene loci. The clone No.2 was used as a parent in the second, and third selection. (F) Genomic PCR showing clone genotype after Puromycin (Puro) selection. The clone No.2 was used as a parent in the second, and third selection. (G) Genomic PCR showing clone genotype after Neomycin (Neo) selection. All clones displayed a single 4.4 kb band, indicating that the RFP-Nano (Neo) cassette was inserted in both NuMA1 gene loci. The clone No.2 was used as a parent in the second, and third selection. (H) Live images of Mem-BFP-iLID, DNA, and DIC in the clone No.2 selected in (C). (E) Genomic PCR showing clone genotype after Neomycin (Neo) selection. All clones displayed a single 4.4 kb band, indicating that the RFP-Nano (Neo) cassette was inserted in both NuMA1 gene loci. The clone No.2 was used as a parent in the second, and third selection. (F) Genomic PCR showing clone genotype after Neomycin (Neo) selection. All clones displayed a single 4.4 kb band, indicating that the RFP-Nano (Neo) cassette was inserted in both NuMA1 gene loci. The clone No.2 was used as a parent in the second, and third selection.
four was used as a parent in the third selection. (F) Genomic PCR showing clone genotype after Hygromycin (Hygro) selection. DHC-SNAP (No. 8, and 9) and p150-SNAP (No. 15) display a single band, as expected, indicating that the SNAP (Hygro) cassette was inserted in both gene loci. The clone DHC-SNAP (No.8) and p150-SNAP (No.15) were used in this study. (G) Western blot probing for anti-NuMA, anti-DHC, anti-p150, anti-SNAP, and anti-α-tubulin (TUB, loading control) showing the bi-allelic insertion of the indicated tags. Protein levels were not significantly affected by tagging with RFP-Nano and SNAP. (H) Western blot showing the efficiency of the RNAi-based depletion for LGN. Tubulin was used as a loading control. (I) Live fluorescent images of NuMA-RFP-Nano and DHC-SNAP. NuMA and DHC accumulate at the cell cortex during anaphase (Kiyomitsu and Cheeseman, 2013). (J) Quantification of cortical NuMA-RFP-Nano and DHC-SNAP signals around the polar cell cortex or light-illuminated region (n = 5). Error bars indicate SEM. Scale bars = 10 μm.

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Figure 1—figure supplement 2. Light-induced cortical targeting of NuMA is sufficient for dynein-dynactin recruitment and spindle pulling. (A) Live fluorescent images of NuMA-RFP-Nano (upper) and DHC-SNAP (lower) in the indicated conditions. Both NuMA-RFP-Nano and DHC-SNAP signals dissociated from the cell cortex following the termination of light illumination (t = 6:00), supporting that light-induced NuMA recruits dynein at the cell cortex. Unexpectedly, the displaced spindle gradually returned toward the center of the cell despite the fact that dynein was unable to accumulate at the distal cell cortex to generate opposing cortical pulling forces to center the spindle (t = 20:00), suggesting that additional mechanisms exist independently of cortical dynein to center the spindle, and explain why the spindle is roughly positioned in the center of the cell in LGN depleted cells (t = 0:00) (Kiyomitsu and Cheeseman, 2012). (B) Left: live fluorescent images of NuMA-RFP-Nano (upper) and DHC-SNAP (lower). Images on the right show a higher magnification of the indicated area. DHC-SNAP signals were initially observed along the cell cortex similarly to NuMA-RFP-Nano (t = 1:30), but were selectively diminished from the cell cortex in proximity to the spindle (t = 4:30), supporting our model that spindle-pole derived signals negatively regulate the cortical dynein-NuMA interaction in a distance dependent manner (Kiyomitsu and Cheeseman, 2012). Right: line scan showing the relative fluorescence intensity of cortical NuMA-RFP-Nano (upper) and DHC-SNAP (lower) around the cell cortex on the left at 4:30. Arrow indicates a decrease in DHC-SNAP signals near the spindle pole. (C) Live fluorescent images of NuMA-RFP-Nano (upper) and p150-SNAP (lower). Similarly to dynein, p150-SNAP was also recruited to the light illuminated region by NuMA-RFP-Nano (t = 2:00), but was subsequently excluded by the spindle proximity (t = 4:00). (D) Live fluorescent images of RFP-Nano (upper) and DHC-SNAP (lower) in LGN-depleted cells arrested with MG132. RFP-Nano was expressed from the Rosa 26 locus following Dox treatment (see Figure 4—figure supplement 1A–B and Figure 5—figure supplement 1B). (E) Left: live fluorescent images of NuMA-RFP-Nano (upper) and DHC-SNAP (lower) in a Gαi1 (1 + 2 + 3) depleted cell. Right: kymograph obtained from image sequences on the left. The spindle was displaced toward the light-illuminated region. (F) Western blot showing the efficiency of the RNAi-based treatment.
Figure 1—figure supplement 2 continued

depletions for Gαi-1. Tubulin was used as a loading control. An asterisk indicates non-specific bands recognized by the anti-Gαi-1 antibody. Scale bars = 10 µm.

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Figure 2. Light-induced cortical NuMA-dynein complex pulls on taxol-stabilized astral microtubules. (A) Fluorescent images of astral microtubules in fixed HCT116 cells treated with drugs as indicated. Cells were arrested at metaphase with MG132 for 1 hr, and DMSO/nocodazole or taxol were then added to cells. (B) Time-lapse images of NuMA-RFP-Nano and DHC-SNAP showing the pull of the NuMA-dynein complex on taxol-stabilized microtubules. (C) Time-lapse images of NuMA-RFP-Nano and DHC-SNAP showing the pull of the NuMA-dynein complex on taxol-stabilized microtubules in nocodazole-treated cells. (D) Time-lapse images of NuMA-RFP-Nano and DHC-SNAP showing the pull of the NuMA-dynein complex on taxol-stabilized microtubules in nocodazole-treated cells. (E) Graph showing the time-course of pole-cortex distance in control and nocodazole-treated cells. (F) Time-lapse images of NuMA-RFP-Nano and SIR-TUB,SIR-DNA showing the pull of the NuMA-dynein complex on taxol-stabilized microtubules in nocodazole-treated cells. (G) Time-lapse images of NuMA-RFP-Nano and SIR-TUB,SIR-DNA showing the pull of the NuMA-dynein complex on taxol-stabilized microtubules in nocodazole-treated cells. (H) Time-lapse images of NuMA-RFP-Nano and DHC-SNAP showing the pull of the NuMA-dynein complex on taxol-stabilized microtubules in nocodazole-treated cells. (I) Graph showing the time-course of pole-cortex distance in control and taxol-treated cells.
Figure 2 continued

added for 30 or 1 min, respectively. Images are maximally projected from 15 z-sections acquired using 0.2 μm spacing. (B and C) Live fluorescent images of NuMA-RFP-Nano (upper) and DHC-SNAP (lower) treated with DMSO (B) or nocodazole (C). (D) Kymographs obtained from image sequences in (B) and (C) showing the movement of the spindle at 30 s intervals. (E) Pole-to-cortex distance for control (black, n = 5), and nocodazole-treated cells (blue or light-blue). Blue and light-blue graphs indicate immobile (n = 5/9) and partially mobile pools (n = 4/9), respectively. Error bars indicate SEM. (F and G) Live fluorescent images of NuMA-RFP-Nano (upper), and SiR-tubulin and SiR-DNA (Lukinavičius et al., 2015) (lower), treated with DMSO (F) or taxol (G). DMSO and Taxol were added at −1:00, and light illumination began at 0:00, when SiR-tubulin images were selectively abolished by taxol treatment. (H) Kymographs obtained from image sequences in (F) and (G) at 30 s intervals. In taxol-treated cells, the spindle did not attach to the cell cortex as indicated with an asterisk, likely due to stabilized astral microtubules. (I) Pole-to-cortex distance for control (black, n = 4), and taxol-treated cells (red, n = 5). Error bars indicate SEM. Scale bars = 10 μm.

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Figure 3. Dynein activity is required for spindle pulling, but light-induced cortical dynein targeting is not sufficient to pull on the spindle. (A) Schematic of experimental procedures. The FBS concentration in the culture medium was changed from 10 to 0.5% at the 68 hr mark. DMSO or ciliobrevin D was added at the 69.5 hr mark and the cells were analyzed for 1 hr. (B and C) Live fluorescent images of NuMA-RFP-Nano (upper) and DHC-SNAP (lower) treated with DMSO (B) or ciliobrevin D (C). (D) Kymographs obtained from image sequences in (B) and (C) showing the movement of the spindle at 30 s intervals. (E) Cortical complexes formed by light-induced targeting of Nano-mCherry-DHC. (F) Left: live fluorescent images of Nano-mCherry-DHC treated with LGN RNAi + MG132. Right: Kymographs showing the movement of the spindle at 30 s intervals.

Figure 3 continued on next page
Figure 3 continued

(upper) and p150-SNAP (lower). Right: kymograph obtained from image sequences on the left. (G) Quantification of cortical Nano-mCherry-DHC and p150-SNAP signals around the light illuminated region (n = 6). Error bars indicate SEM. (H) Live fluorescent images of Nano-mCherry-DHC (upper) and NuMA-SNAP (lower). (I) Measurement of the pole-to-cortex distance (n = 10). Error bars indicate SEM. Scale bars = 10 μm.

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Figure 3—figure supplement 1. Generation of knock-in cell lines for the DHC and mitotic phenotypes caused by ciliobrevin D treatment. (A) Genomic PCR showing clone genotype after Neomycin (Neo) selection. Both clones display a single 5.4 kb band, which indicates that the mAID-mClover-3FLAG DHC-mAID-mClover-FLAG(mACF) gene is not successfully integrated into the genome.

(B) Procedures

- **Procedures**
  - Plate cells
  - +RO-3306
  - +Hoechst
  - +SIR-TUB
  - Wash & release in MG312
  - +DMSO or +Ciliobrevin D
  - Analysis

- **Wash & release in MG312**
  - 0
  - 24
  - 42
  - 44
  - 46 (hr)

- **Analysis**
  - G2
  - Mitosis

- **10% FBS**
- **0.5% FBS**

(C) Chromosome misalignment

- Normal
- Mild (5x)
- Severe (5x)

(D) Frequency (%)

- n=53
- 58
- 68
- 54

- FBS (%)
  - 0.5
  - 0.5
  - 0.5
  - 10

(E) The 2nd knock-in selection (BSD)

- Parent: Mem-BFP-ILID No.2
- Nano-mCh-DHC

(F) Procedures

- +M312 in 0.5% FBS
- +DMSO (t=0)
- +Ciliobrevin D (75 μM, t=0)

- SIR-TUB
- mCh

- DNA

(G) Frequency (%)

- Bipolar spindle
- Abnormal spindle

- Ciliobrevin D (μM)
  - 0
  - 75
  - 75
  - 75

- Time (min)
  - 60
  - 30
  - 60

(H) The 2nd knock-in selection (BSD)

- Parent: Mem-BFP-ILID No.2
- Nano-mCh-DHC

(I) The 3rd knock-in selection (Hygro)

- Parent: Mem-BFP-ILID+Nano-mCh-DHC No.1
- p150-SNAP
- NuMA-SNAP

**PCl products**

- Target
  - (p150) (NuMA)

- 3.1 kb
- 4.0 kb
(mACF) (Neo) cassette is inserted at both the DHC1 gene loci. Here, clone No.1 was used. (B) Schematic of the experimental procedures. Cells were treated with RO-3306 and MG132 to synchronize at G2 and to arrest cells at metaphase, respectively. The FBS concentration in the culture medium was changed from 10 to 0.5% at the 44 hr mark. Cells were treated with DMSO or ciliobrevin D at the 44 hr mark and analyzed for 1 hr from the 45 to the 46 hr mark. (C) Live fluorescent images of DNA (upper) and DHC-mACF (middle) in ciliobrevin D-treated cells showing the mitotic chromosome-misalignment phenotype. Cells labeled with the light- or dark-blue squares indicate mild or severe chromosome misalignment phenotypes, respectively. (D) Histogram showing the quantification of the chromosome misalignment phenotype. Note that ciliobrevin D worked under the 0.5%, but not the 10% FBS condition, and induced severe phenotypes in a dose-dependent manner. (E) Schematic of the experimental procedures. Metaphase-arrested cells were treated with DMSO or ciliobrevin D at the 45.5 hr mark under 0.5% FBS culture condition and analyzed for 1 hr from the 45.5 to the 46.5 hr mark. (F) Live fluorescent images of DNA (upper, magenta), SiR-TUB (upper and middle, green), and DHC-mACF (bottom) in DMSO (left)- and ciliobrevin D (right)-treated cells. (G) Histogram showing abnormal spindle quantification following ciliobrevin D treatment. Note that the bipolar spindle structure was maintained for ~30 min even in the presence of ciliobrevin D and disrupted during the subsequent 30–60 min. (H) Genomic PCR showing the genotype of clones after Blasticidin (BSD) selection. Both clones display a single 2.2 kb band, indicating that the Nano-mCherry (BSD) cassette was inserted at both DHC1 gene loci. The clone No.1 was used as a parent in the third selection. (I) Genomic PCR showing the genotype of clones after Hygromycin (Hygro) selection. P150-SNAP (No.10) and NuMA-SNAP (No.7, 8) display a single band, as expected, indicating that the SNAP (Hygro) cassette was inserted at both gene loci. The clone p150-SNAP (No.10) and NuMA-SNAP (No.8) were used in this study.

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Figure 4. A Spindly-like motif in NuMA is required for cortical dynein recruitment, but not sufficient for spindle pulling. (A) Cortical complexes formed by light-induced targeting of NuMA fragments fused with RFP-Nano. (B) Full-length NuMA and the tested NuMA truncation fragments. Globular domains at N- and C-terminal regions of NuMA are indicated in light-gray and gray, respectively. (C) A summary of the frequency of cortical dynein recruitment and spindle displacement by targeted constructs. See Figure 4—figure supplement 1I–J for details. (D) Amino acid sequence alignment Figure 4 continued on next page
of the Spindly-motif like region of NuMA proteins in *H. Sapiens* (NP_006176), *R. norvegicus* (NP_001094161), *M. musculus* (NP_598708), *G. gallus* (NP_001177854), *X. laevis* (NP_001081559), *D. rerio* (NP_001316910), and human Spindly (NP_001316568) and Hook3 (NP_115786) aligned by ClustalWS. The conserved L and E substituted by alanine are indicated with red triangles. (E) Lupas coils prediction (window 21). Spindly motif (purple) is commonly located at the C-terminal region of the coiled-coil, with 200 – 280 residues. (F) Live fluorescent images of NuMA (1-705) WT (upper) and SpM mutant (lower). DHC-SNAP images are shown to the right. (G–I) Left: live fluorescent images of NuMA constructs (upper) and DHC-SNAP (lower). Right: kymographs obtained from image sequences of DHC-SNAP on the left at 30 s intervals. Scale bars = 10 μm.

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**Figure 4—figure supplement 1.** The N-terminal region of NuMA is required for cortical dynein recruitment. (A) A schematic illustration of exogenous gene expression of Mem-BFP-iLID and NuMA-Nano fusions from the AAVS1 and Rosa 26 loci, respectively. Whereas Mem-BFP-iLID is stably expressed, NuMA-Nano is transiently expressed. (B) Procedures for transfection and siRNA knockdown. (C) Globular head and tail of NuMA. (D) LGN RNAi + MG132 + NuMA(214-705)-RFP-Nano (5). (E) LGN RNAi + MG132 + NuMA(1-505)-RFP-Nano (6). (F) LGN RNAi + MG132 + NuMA(1-413)-RFP-Nano (7). (G) LGN RNAi + MG132 + NuMA(1-213)-RFP-Nano (8). (H) NuMA(1-705)-RFP-Nano. (I) Definition of the spindle displacement distance. (J) The endogenous NuMA-RFP-Nano fragments. (K) The 2nd knock-in selection (BSD). (L) The 3rd knock-in selection of NuMA-RFP-Nano (Hygro).
Figure 4—figure supplement 1 continued

NuMA-Nano fusions are conditionally expressed following the treatment with Doxycycline (Dox). The SNAP-tag was also inserted at the DHC1 gene loci. (B) Schematic of experimental procedures. LGN siRNA, Dox, and SiR-647 were used to deplete endogenous LGN, to induce expression of NuMA-Nano fusions, and to label endogenous DHC-SNAP, respectively. Cells were treated with RO-3306 and MG132 to synchronize at G2 and to arrest cells at metaphase, respectively. Cells were observed by microscopy 1 hr after the release from G2 arrest, and analyzed for 3 hr. (C) Left: the tested NuMA truncation fragments. Right: a summary of the cortical dynein recruitment. (D–G) Live fluorescent images of indicated NuMA constructs (upper) and DHC-SNAP (lower). NuMA (1-505) was sufficient to recruit dynein to the cell cortex, and both its N-terminal globular domain (aa: 1–213) and short coiled-coil region (aa: 414–505) were required for cortical dynein recruitment. Similar to NuMA (1-705) WT, NuMA (1-505), but not other truncated fragments, accumulated around spindle pole adjacent to light-illuminated region following cortical recruitment. (H) Merged images of NuMA(1-705)-RFP-Nano (magenta) and DHC-SNAP (green) from Figure 4F. NuMA(1-705)-RFP-Nano WT, but not 4A mutant, asymmetrically accumulates around the spindle pole, indicating that NuMA N-terminal fragments containing the Spindly motif are sufficient to recruit dynein-dynactin to the mitotic cell cortex, and likely to activate dynein’s motility, which in turn transports these NuMA fragments toward the spindle pole. (I) The definition of spindle displacement. For cases where $D_{\text{max}}$ (maximal spindle displacement distance) is longer than $D_{t=0}$ (starting pole-to-cortex distance) $\times 1/2$, the spindle is assessed as displaced. (J) Scatterplots of $D_{\text{max}}$ as a percentage of $D_{t=0}$ for each condition. Cells with displaced spindles are plotted to the right side of the red line, which indicates 50% of the spindle displacement distance. For NuMA fragments, all samples (except #2 and #11) show statistical difference (p<0.05) when compared to control (#1) using the Mann-Whitney test. Purple lines indicate mean ± SD. See Figure 4C and Figure 5B for the number of cells. (K) Genomic PCR showing the genotype of clones after Blasticidin (BSD) selection. Both clones display a single 4.6 kb band, indicating that the SNAP (BSD) cassette was inserted in both DHC1 gene loci. The clone No.11 was used as a parent in the 3rd selection. (L) Genomic PCR showing the genotype of clones after Hygromycin (Hygro) selection. A 1.6 kb band confirms the insertion of NuMA-RFP-Nano (Hygro) cassettes with different NuMA fragments at the Rosa 26 locus. The following clones were used; ΔNLS (#1: No.7), 1–1700 (#2: No.28), 1–705 (#3: No.5), 1–705 4A (#4: No.18), 214–705 (#5: No.2), 1–505 (#6: No.21), 1–413 (#7: No.23), and 1–213 (#8: No.8); Scale bars = 10 μm.

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Figure 5. NuMA's C-terminal microtubule-binding domains and central coiled-coil are required for spindle pulling. (A) Full-length NuMA and the tested NuMA truncation fragments. Microtubule binding domains (MTBDs) are indicated in green. (B) Summary of the frequency of cortical dynein recruitment, punctate signals, and spindle displacement.

#1 NLS + (19/19) + (19/19) 35.7% (9/14)
#2 1-1700 + (16/16) - (0/16) 0.0% (0/14)
#9 1-1895 + (24/25) + (25/25) 5.6% (1/18)
#10 1-1985 + (11/14) + (14/14) 0.0% (0/10)
#11 ex24 + (14/14) + (14/14) 37.5% (3/8)
#12 N+C NLS + (10/13) + (13/13) 0.0% (0/10)
#13 NuMA-C (1700-2115) - (0/7) + (7/7) 0.0% (0/6)
#14 NLS (5A-3) + (20/20) - (0/20) 7.7% (1/13)

Figure 5 continued on next page...
recruitment, dot signal formation and spindle displacement by targeted constructs. Figure 4—figure supplement 1I–J for details. (C–E) Left: live fluorescent images of indicated NuMA constructs (upper) and DHC-SNAP (lower). Right: kymographs obtained from image sequences of DHC-SNAP on the left at 30 s intervals. (F) Live fluorescent images of NuMA Δex24-RFP-Nano (upper) and DHC-SNAP (lower). Expression level of NuMA Δex24-RFP-Nano was lower than that in (E), but the spindle was still displaced. (G) Left: live fluorescent images of NuMA (N + C ΔNLS)-RFP-Nano (upper) and DHC-SNAP (lower). Right: kymographs obtained from image sequences of DHC-SNAP on the left at 30 s intervals. (H) Enlarged images of NuMA (N + C ΔNLS)-RFP-Nano (upper) and DHC-SNAP (lower) at indicated times. (I) Live fluorescent images of NuMA-C-RFP-Nano (upper) and DHC-SNAP (lower). Scale bars = 10 μm.
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A. NuMA’s C-terminal domains

- 4.1-BD: 1802-1824 (Mattagajasingh et al., 2009)
- LGN-BD: 1900-1926 (Zhu et al., 2011)
- MTBD (1): 1914-1985 (Du et al., 2002)
- MTBD (2): 2002-2115 (Gallini et al., 2016)
- NuMA-TIP: 1811-1985 (Seidlin et al., 2016)
- Exon 24: 1944-2003 (Gallini et al., 2016)
- NLS: 1984-1989 (Tang et al., 1994)

Region required for oligomerization: 1700-2003

CDK phosphorylation sites: T2015, T2055, S2087

MTBD (1): 1914-1985 (Du et al., 2002)

MTBD (2): 2002-2115 (Gallini et al., 2016)

PIPs BD (1): 1699-1876 (Kotak et al., 2014)

PIPs BD (2): 1996-2074 (Zheng et al., 2014)

B. The 3rd knock-in selection of NuMA-RFP-Nano (Hygro)

Parent: Mem-BFP-ILID + DHC-SNAP No.11

NuMA-C (#13)

NuMA-TIP (SA-3) (#14)

RFP-Nano

C. NuMA(1-1985)-RFP-Nano (#10)

DHC (+): 78.6% (11/14)

DHC (-): 21.4% (3/14)

D. NuMA (N+C NLS)-RFP-Nano (#12)

DHC (+): 76.9% (10/13)

DHC (-): 23.1% (3/13)

Figure 5—figure supplement 1. Light-induced targeting of exogenously expressed NuMA fragments lacking C-terminal MTBDs and central coiled-coil. (A) NuMA C-terminal fragment with known domains. 4.1-binding domain (4.1-BD) and microtubule binding domains (MTBDs) are indicated in light-

Figure 5—figure supplement 1 continued on next page
blue and green, respectively. The amino acid numbers of NuMA conform to isoform 1 (aa: 1–2115; NP_006176). LGN binding domain (BD) (Zhu et al., 2011) is indicated in red. NuMA C-terminal fragment containing MTBD1 (aa: 1811–1985, called NuMA-TIP) accumulates at microtubule tips, and remains associated with stalled and/or depolymerizing microtubules (Seldin et al., 2016). (B) Genomic PCR showing the genotype of clones after Hygromycin (Hygro) selection. Arrows indicate a 1.6- or 3.2 kb band, which confirms the insertion of NuMA-RFP-Nano (Hygro) cassettes with different NuMA fragments at the Rosa 26 locus. The following clones were used; 1–1895 (#9: No.4), 1–1985 (#10: No.19), Δex24 (#11: No.17), N + C ΔNLS (#12: No.4), NuMA-C (#13: No.5), ΔNLS(5A-3) (#14: No.4), and RFP-Nano (No.18). (C) Live fluorescent images of NuMA(1–1985)-RFP-Nano (upper) and DHC-SNAP (lower). Whereas DHC was recruited to the cell cortex following light-induced NuMA(1–1985)-RFP-Nano targeting in 78.6% of cells (n = 14), DHC was not detectable in the remaining 21.4% of cells, in which NuMA(1–1985)-RFP-Nano fusion apparently aggregates at the cell cortex, suggesting that NuMA C-terminal fragment containing MTBD2 is also required to prevent hyper-clustering of NuMA. (D) Live fluorescent images of NuMA(N + C ΔNLS)-RFP-Nano (upper) and DHC-SNAP (lower). In 77% of cells (n = 13), DHC was recruited to the cell cortex following light-induced targeting of NuMA (N + C ΔNLS)-RFP-Nano, but not in the remaining 23% of the cells. Scale bars = 10 μm.
Figure 6. Clustering of the DDN complex by NuMA is critical for spindle pulling. (A) GFP-tagged NuMA C-terminal fragment and the tested NuMA mutant fragments. (B) Live fluorescent images of nocodazole-arrested HeLa cells expressing GFP-tagged NuMA-C 3A fragments. (C) Amino acid sequence alignment of the clustering domain of NuMA proteins aligned by ClustalW. Accession numbers are indicated in Figure 4D. (D–E) Left: live fluorescent images of indicated NuMA constructs (upper) and DHC-SNAP (lower). Right: kymographs obtained from image sequences of DHC-SNAP on the left. Asterisk in (D) indicates the duration in which one of the spindle poles moves away from the focal plane. Scale bars = 10 μm.

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Figure 6—figure supplement 1. Identification of a clustering domain on NuMA’s C-terminal region. (A) Live fluorescence images of nocodazole-arrested HeLa cells showing mCherry-NuMA-C 3A and PLCδ-PH-GFP, an indicator of PtdIns(4,5)P$_2$ (Kotak et al., 2014). NuMA-C 3A, but not PLCδ-PH, displays punctate signals. The localization of PLCδ-PH was not affected by the expression of mCherry-NuMA-C 3A. DNA was visualized with SiR-DNA. (B) Live fluorescence images of endogenous NuMA-mACF and 4.1G-mCherry in HCT116 cells. NuMA-mACF, but not 4.1G-mCherry, shows punctate signals during prometaphase. (C) Live fluorescent images of HeLa cells showing GFP-NuMA-C 3A (upper) and SiR-DNA/SiR-actin (middle/left) following cytochalasin-D treatment (right). The NuMA foci preferentially accumulated at actin-poor cortical regions (left), and still localized following the disruption of actin polymerization (right). (D) GFP-tagged NuMA C-terminal fragment and the tested NuMA mutant fragments. 5A mutation sites are indicated in red. 3A mutation sites for CDK phosphorylation are shown in black AAA. (E) Amino acid sequence alignment of the 1700–1828 aa region of NuMA proteins aligned by ClustalWS. Highly conserved 5A mutation sites (5A-1 to 5A-4) are indicated by red triangles. (F) Live fluorescent images of nocodazole-arrested HeLa cells expressing GFP-tagged NuMA-C 3A fragments. NuMA C3 fragment (aa: 1990–2115) is sufficient for cortical localization. NuMA-C 5A-1 and 5A-4 mutants still displayed punctate foci. (G) Live fluorescent images of nocodazole-arrested HeLa cells expressing GFP-tagged NuMA-C 3A fragments. Whereas NuMA (1700–1895: C6) diffused in the cytoplasm, this fragment was required for the NuMA (1990–2115: C3) fragment to display dots-like signals, which was abolished by 5A-2 mutation (C5). (H) Left: NuMA (1–2115 ΔNLS) RFP-Nano (5A-3) (#14). Right: a kymograph obtained from image sequences of DHC-SNAP on the left at 30 s intervals. The metaphase spindle was not fully displaced regardless of cortical dynein recruitment. Scale bars = 10 μm.

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Figure 7. Clustering activity of NuMA is required for spindle positioning, but not for spindle pole focusing. (A) Diagram summarizing auxin inducible degradation (AID) system (Natsume et al., 2016). In the presence of OsTIR1 and auxin (IAA), mAID fusion proteins are rapidly degraded upon poly-ubiquitylation by proteasome. Because RNAi-mediated depletion of NuMA is insufficient to completely deplete NuMA proteins even after 72 hr (Kiyomitsu and Cheeseman, 2013), we sought to degrade endogenous NuMA using the auxin-induced degron technology. (B) Left: metaphase NuMA-mACF cell lines showing live fluorescent images of NuMA-mACF, mCherry-NuMA WT, or 5A3 mutant, and SiR-tubulin (SiR-TUB) after 24 hr following the treatment of Dox and IAA. The degradation of endogenous NuMA-mACF was induced by the treatment with Dox and IAA. The expression of mCherry-NuMA WT or 5A-3 was also induced by the Dox treatment. Right: histogram showing frequency of the focused bipolar spindle in each condition. * indicates statistical significance according to a Student’s t-test (p<0.0001). Error bars indicate SEM; n > 25, from three independent experiments. (C) Left: orthogonal views of the metaphase spindle on the x-y (top) and x-z (bottom) plane. In each case, endogenous NuMA was replaced with either mCherry-NuMA WT or 5A-3. Right: scatterplots of the spindle orientation on the x-z plane. Red lines indicate mean ± SD; n > 27, from three independent experiments. (D) Model showing multiple-arm capture and pulling of an astral microtubule by the cortical DDN cluster. Scale bars = 10 μm.

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Auxin-inducible degradation of endogenous NuMA and its replacement with NuMA 5A-3 mutant. (A) Left: genomic PCR showing the genotype of clones after Neomycin (Neo) selection. All clones displayed a single 4.3 kb band, indicating that the mAID-mClover-3xFLAG construct had been successfully integrated. (B) Immunoblot analysis showing the absence of endogenous NuMA and the expression of NuMA-mACF in clones No.1 and No.2, and the expression of NuMA-5A-3 in clones No.3 and No.4. (C) Western blot analysis showing the absence of endogenous NuMA and the expression of NuMA-mACF in clones No.1 and No.2, and the expression of NuMA-5A-3 in clones No.3 and No.4. (D) Immunohistochemistry showing the localization of NuMA and NuMA-5A-3 in the cell cortex. (E) Confocal microscopy images showing the localization of NuMA and NuMA-5A-3 in the cell cortex. (F) Replaced with mCh-NuMA in the cell cortex. (G) Recruitment of DDN complex to the cell cortex. (H) Assembly of DDN clusters at the cell cortex. (I) Astral microtubule capture and pulling. Large pulling forces lead to the formation of Dynein-based motility and/or Microtubule depolymerization. Figure 7—figure supplement 1 continued on next page
3FLAG (mACF) (Neo) cassette was inserted at both NuMA1 gene loci. The clone No.1 was used as a parent in the third selection. Middle: genomic PCR showing the genotype of clones after Blasticidin (BSD) selection. Both clones displayed a single 4.6 kb band, indicating that the SNAP (BSD) cassette was inserted in both DHC1 gene loci. The clone No.18 was used as a parent in the fourth selection. Right: genomic PCR showing the genotype of clones after Hygromycin (Hygro) selection. Arrows indicate a 2.0 kb band, which confirms the insertion of mCherry-NuMA WT or 5A-3 mutant cassette (Hygro) at the Rosa 26 locus. The clone WT (No.7) and 5A-3 (No.4) were used in this study. (B and C) Live fluorescent images of endogenous NuMA-mACF and DHC-SNAP (B) or SNAP-LGN (C) showing punctate signals at mitotic cell cortex. These images were single z-sections, and the images in (B) are captured with camera binning 1. DNA was visualized with Hoechst 33342. (D) Western blot probing for anti-NuMA, anti-OsTIR1, anti-α-tubulin (TUB, loading control), and anti-histone H3S10P (a mitotic marker) at 24 hr following treatment. Band shifts in NuMA-mACF indicate bi-allelic insertion of mAID tag. Treatment with both Dox and IAA caused degradation of NuMA-mACF and accumulation of phosphorylated Histone H3S10, indicating mitotic arrest or delay. (E) Live fluorescent images of NuMA-mACF, SiR-tubulin (TUB) and DNA showing the spindle un-focusing phenotype. Intensity of SiR-TUB images was enhanced compared to Figure 7B to improve clarity of the phenotypes. DNA was visualized with Hoechst 33342. (F) Live fluorescent images of endogenous NuMA-mACF, ectopically expressed mCherry-NuMA WT or 5A-3, and SiR-tubulin (TUB) showing spindle mis-orientation in the NuMA 5A-3 mutant cells. Single z-section images on the x-y plane are shown. (G) Model showing recruitment (#1) and assembly (#2) of the DDN cluster at the mitotic cell cortex, and multiple-arm capture and pulling of a single astral microtubule by the cortical DDN cluster (#3). The ring-like structure for a NuMA cluster is an imaginary structure based on Harborth et al., (Harborth et al., 1999). See Discussion for details. Scale bars = 10 μm.

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