NuMA Interacts with and Regulates Astrin at the Mitotic Spindle

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ABSTRACT
The large nuclear mitotic apparatus (NuMA) protein is an essential player in mitotic spindle assembly and maintenance. We report here the identification of Astrin, the spindle and kinetochore associated protein, as a novel interactor of NuMA. We show that the C-terminal tail of NuMA can directly bind to the C terminus of Astrin, and this interaction helps to recruit Astrin to microtubules. Knockdown of NuMA by RNA interference dramatically impaired Astrin recruitment to the mitotic spindle. Overexpression of the N terminus of LGN, which blocks the microtubule binding of NuMA and competes with Astrin for NuMA binding, also led to similar results. Furthermore, we found that cytoplasmic dynein is required for the spindle pole accumulation of Astrin, and dynein-mediated transport is important for balanced distribution of Astrin between spindle poles and kinetochores. On the other hand, if Astrin levels are reduced, NuMA could not efficiently concentrate at the spindle poles. Our findings reveal a direct physical link between two important regulators of mitotic progression and demonstrate the critical role of the NuMA-Astrin interaction for accurate cell division.

INTRODUCTION
Mitosis is the essential process through which chromosomes are accurately segregated into two daughter cells (1,2). Chromosome segregation is mediated by a complex called mitotic spindle that pulls the sister chromatids apart and moves a complete set of chromosomes to each pole of the cell, where they are packaged into daughter nuclei (1,3). The mitotic spindle is based on a bipolar array of microtubules (MTs), which are organized with their less dynamic minus ends tightly focused into two spindle poles (4,5). The sister chromatids are active participants in spindle assembly and function. Each chromatid carries a kinetochore, a multi-protein complex that attaches the chromatid to MTs connected to a spindle pole. Kinetochores are responsible for maintaining attachment to growing and shrinking microtubules resulting in chromosome movements that lead to alignment during metaphase and segregation during anaphase. A proper kinetochore-microtubule association is one in which each sister kinetochore is appropriately attached to a microtubule bundle associated with an opposite spindle pole (6,7).

The organization of MTs into bipolar spindles with focused poles during mitosis requires numerous microtubule-associated proteins including both motor and non-motor proteins (8-10). Microtubule-associated motor proteins include cytoplasmic dynein and kinesins that can travel along microtubules and have important roles in the assembly and stability of the microtubule array and the movement of chromosomes on the spindle (11,12). Multiple non-motor proteins also contribute to the formation of mitotic spindles through diverse mechanisms including microtubule nucleation and organization, influence on motor function, and cell cycle regulation (13,14). Non-motor microtubule associated proteins show diverse localization on mitotic spindles including...
spindle poles, centrosomes, spindle body, central spindle, and kinetochores (13). Several important non-motor proteins bind to the plus ends of MTs and control plus-end dynamics or link the plus end to other cellular structures. Another important group of microtubule-regulatory proteins stabilizes and cross-links microtubule minus ends to form focused spindle poles, often with the assistance of motor proteins that travel along MTs in the direction of the minus ends. Complicating the understanding of these functions, many of these proteins cooperate or interact with each other in some activities (13,14).

Nuclear mitotic apparatus (NuMA) is a 220kDa nonmotor protein with a discontinuous coiled-coil domain between N- and C-terminal globular domains (15). NuMA is distributed in the nucleus during interphase. During mitosis, NuMA translocates to the spindle poles, and functions in focusing MTs to the mitotic spindle poles and bundling spindle MTs to centrosomes (16-19). Depletion of NuMA in mammalian cells by RNA interference and knockout strategies in mice have revealed that NuMA is required for proper spindle assembly and chromosome alignment (20,21). NuMA interacts with a number of essential mitotic components, including MTs (15,22,23), dynein/dynactin (18) and mammalian homologue of Drosophila Pins (LGN) (24). We reported previously that LGN functions as a conformational switch that links NuMA and Gαi protein and the Gαi/LGN/NuMA complex can exert forces on astral MTs in cultured mammalian cells (22,24,25). Recent studies have indicated that the Gαi/LGN/NuMA complex regulates mitotic spindle orientation during epithelial morphogenesis and asymmetric cell division (26-32). In addition to regulating spindle orientation, our studies also found cortical NuMA and dynein contribute to efficient chromosome separation during cell division (31,32).

Although previous studies indicate that NuMA is essential for the spindle assembly and mitotic progression, the precise molecular mechanisms remain less well characterized. We carried out a new search for proteins that interact with NuMA using yeast two-hybrid assay. We report here the identification of the spindle and kinetochore associated protein Astrin as a novel interactor of NuMA. By using yeast two-hybrid assays, biochemistry, and immunocytochemistry, we found that NuMA directly interacts with Astrin in mitotic spindle. This interaction is critical for the assembly and stabilization of mitotic spindle and alignment of chromosome in mammalian cells.

**RESULTS**

**Identification of Astrin as a novel interacting partner of NuMA**—To identify new interacting proteins for NuMA, we carried out a yeast two-hybrid screening using the NuMA C-terminal tail fragment (amino acid 1717-2101) as the bait. Our two-hybrid screen was proved to be successful by the isolation of several previously identified NuMA interacting proteins, including protein 4.1 and LGN (data not shown). Among the positive clones sequenced, we focused on one clone encodes the C-terminal region of Astrin. The interaction between NuMA and Astrin was verified by β-galactosidase (β-gal) assay using yeast co-transformed with NuMA bait vector and Astrin prey plasmid. To further verify the specificity of the interaction between NuMA and Astrin in yeast, we switched the bait and prey vectors by subcloning NuMA in prey vector and Astrin in bait vector, the β-gal activities remained positive after vector swapping (data not shown).

To confirm the interactions between NuMA and Astrin observed in our yeast two-hybrid assay, a co-immunoprecipitation assay was carried out in COS7 cells. As shown in Fig.1A, NuMA bound to full length and the C-terminus of Astrin, suggesting that Astrin and NuMA could form a cognate complex in mammalian cells. To test whether NuMA binds directly to Astrin, we performed a pull-down assay using purified recombinant proteins. As shown in Fig. 1B, GST-Astrin901-1193 could efficiently pull-down His-NuMA1858-2101. The GST protein, used as a control, did not bind His-NuMA1858-2101 in this experiment. These results suggest that the interaction between Astrin and NuMA is direct. To determine whether endogenous NuMA and Astrin can form a complex, we performed immunoprecipitation analysis using anti-NuMA antibody. We could reproducibly detect a portion of Astrin in the NuMA immunoprecipitates (Fig. 1C).

To further examine the binding of Astrin to NuMA, the binding domains in these two proteins were mapped using both yeast two-hybrid and co-IP assays. A series of bait vectors (pGBK7) and prey vectors (pVP16) containing NuMA or Astrin
fragments were constructed and co-transformed into *Saccharomyces cerevisiae* Y190. Yeasts grew on Trp/Leu dropout plates were subjected to β-gal assay. Our data revealed that the C-terminal region of Astrin, comprising amino acids 901-1193 is sufficient to bind NuMA (Fig.1D). The Astrin-interacting region in NuMA was mapped to amino acids 1878-2101, containing the C-terminal microtubule and LGN binding domains (Fig.1E). The mapped interacting regions were then tested in mammalian cells using co-immunoprecipitation experiment. In support of the conclusion from the yeast system, our co-immunoprecipitation experiment showed the similar results (Fig. 1F).

**NuMA and Astrin co-localize at spindle during mitosis** —Given the interaction between NuMA and Astrin established above, we sought to examine their spatio-temporal distribution profiles and inter-relationship during cell cycle. Astrin is a MT-associated protein. It colocalized with MTs around centrosomes in prophase and localized to spindle MTs throughout prophase, prometaphase, metaphase, anaphase, and telophase. From late prometaphase to anaphase, another pool of Astrin was localized at kinetochores (33,34). To visualize and localize the interaction of NuMA and Astrin, a double indirect immunofluorescence microscopy was performed. GFP tagged NuMA and RFP tagged Astrin were co-transfected into HeLa cells (Fig. 2A). In interphase, Astrin was diffused in the cytoplasm while NuMA localized in the nuclear. As the nuclear envelope disassembled in early mitosis, Astrin colocalized with NuMA at mitotic spindle. During the metaphase and anaphase, Astrin and NuMA accumulated in the spindle pole region (Fig. 2A). Similar localization pattern was observed for stably expressed GFP-NuMA and endogenous Astrin in MDCK cells (Fig.2B). The spatial vicinity provides the physical foundation for the functional relationship between NuMA and Astrin.

**Astrin is essential for efficient spindle pole organization and proper chromosome alignment** —To assess the functional relevance of the interaction between Astrin and NuMA, specific siRNA was used to effectively deplete endogenous Astrin. The diminution of Astrin signal on immunoblots and staining in cells verifies efficient Astrin knockdown (Fig.3A-B). Consistent with the observation from previous study (34,35), the depletion of Astrin resulted in an increase of the mitotic index, suggesting mitotic arrest (Fig.3C). Staining of MTs showed that the depletion of Astrin resulted in the formation of less-focused spindle poles and the occurrence of multipolar spindles. (Fig.3A, 3D). Spindle disruption was accompanied by chromosome alignment defect as previously reported (Fig. 3D-F) (34-36). These phenotypes are similar to those of NuMA-depleted cells (20). We also examined the effect of repressing Astrin on the localization of NuMA to the spindle. In cells in which Astrin had been suppressed, the intensity of NuMA at spindle poles decreased and more NuMA localized along spindle MTs (Fig.3D, 3G). Our data suggest that except for its well-studied role at kinetochores, Astrin is also required for the accumulation of NuMA to spindle poles, which is important for efficient spindle pole organization and chromosome alignment.

**NuMA can recruit Astrin to microtubules** —Although Astrin is proposed to be a MT-associated protein, it only localizes to spindle MTs during mitosis and is diffuse in the cytoplasm of interphase cells (33,37), suggesting that either unknown linker protein(s) or specific modifications are needed for Astrin’s localization to MTs. NuMA is a well-known microtubule binding protein (22,23), and the Astrin binding region of NuMA contains its microtubule binding domain, raising the possibility that NuMA may link Astrin to MTs. To test this hypothesis, we co-transfected different fragments of NuMA and Astrin into COS7 cells. In consistent with our previous observation (22), the fragments of NuMA-C (aa 1818-2101, 1818-C) associated with MTs when ectopically expressed in COS7 cells (Fig.4A). However, the association with MTs was never seen when either the C-terminal of Astrin (aa 901-1193, 901-C) or the N-terminal of Astrin (aa 1-608) was expressed alone or with vector control (Fig.4B, 4D). Interestingly, GFP-Astrin901-1193 (901-C), the NuMA-binding fragment, but not GFP-Astrin1-608, was recruited to MTs when co-expressed with RFP-NuMA1818-C (1818-2101) (Fig.4C-D). To prove the specificity of this effect, we also co-expressed a truncated fragment of NuMA (aa 1818-2060), which contains the microtubules binding domain but could not interact with Astrin from our co-immunoprecipitation and yeast two-hybrid results (Fig.1). As shown in Fig.4E-F, NuMA1818-2060 itself could bind MTs but failed to bring Astrin to the MTs, indicating that the MTs recruitment of Astrin relies on its interaction with NuMA. Our
results suggest that NuMA could be one of the important linker proteins to assist Astrin localization to MTs.

**The NuMA-Astrin interaction contributes to the localization of Astrin at spindle poles**—To examine the function of the NuMA and Astrin interaction in mitosis, we tested whether NuMA is required for the localization of Astrin on the spindle poles. A previously published small interfering RNA (siRNA) was used to knockdown endogenous NuMA (20,38), and western blot analysis of total extracts from HeLa cells showed that treatment with NuMA siRNA oligonucleotide for 72 hours led to efficient depletion of NuMA protein. Immunostaining also confirmed the knockdown efficiency (Fig.5A-B, 5D). Consistent with previous observation (20), NuMA depletion induced mitotic abnormalities including disorganized spindle structures and improper chromosome alignment (Fig.5A, 5C). We then compared the localization of Astrin in control and NuMA knockdown cells. Remarkably, the spindle pole association of Astrin were decreased in NuMA depleted cells (Fig.5C, 5E).

To further confirm the role of NuMA in the localization of Astrin during mitosis, we perturbed NuMA function using the NuMA-binding protein LGN. By virtue of competitive binding between MTs and LGN for NuMA, LGN acts as a negative regulator of NuMA function in spindle (16,24,39). In control HeLa cells, the endogenous NuMA is associated predominantly with the spindle poles during metaphase. However, in cells that express Venus-tagged LGN1-481, NuMA was aberrantly organized into round spots at the centrosomes (Fig.6A), which were often seen to be displaced from the splayed spindle MTs (Fig.6B). Similar to the NuMA siRNA knockdown results, Astrin was dispersed and no longer localized to the spindle poles in Venus-LGN expressing cells (Fig.6C-D). Our co-immunoprecipitation analysis indicated that both GFP-tagged Astrin and Venus-tagged LGN1-481 were able to associate with HA-tagged NuMA. However, once GFP-tagged Astrin and Venus-tagged LGN1-481 were co-expressed, the association of Astrin with NuMA was decreased (Fig.6E), suggesting that LGN1-481 competes with Astrin for NuMA binding. These data corroborate our siRNA depletion results, supporting the idea that NuMA contributes to the spindle pole localization of Astrin.

**The accumulation of Astrin at spindle poles is dynein dependent**—Previous work has shown that the spindle pole localization of NuMA is mediated by cytoplasmic dynein, the minus end-directed motor protein (18,40). The requirement of NuMA for the spindle pole localization of Astrin led us to speculate that dynein may also regulate Astrin’s transport in intact cells. To test this hypothesis, we perturbed dynein function using a dynein heavy chain (DYNC1H1) specific short hairpin RNA (shRNA)-knockdown approach (32). Western blot analysis of total extracts from HeLa cells confirmed the efficiency of DYNC1H1 knockdown (Fig.7A, 7E). Consistent with the previous observation, knockdown of DYNC1H1 led to the disorganization of spindle and the improper chromosome alignment (Fig.7A). Disruption of dynein function strongly inhibits NuMA translocation and accumulation at the spindle poles (Fig.7B). In DYNC1H1 depleted cells, NuMA was not restricted to the spindle poles, but localized on the spindle fibers (Fig.7B). As expected, substantial amounts of Astrin were still bound along the surface of MTs and the accumulation of Astrin at spindle poles was dramatically reduced in DYNC1H1 depleted cells (Fig.7C, 7F), suggesting that dynein-mediated transport may be responsible for the accumulation of Astrin at spindle pole. Previous work has shown that Astrin preferentially localized to fully attached and aligned chromosomes (33). Surprisingly, in DYNC1H1 depleted cells, kinetochore bound Astrin could be seen in most of the mitotic cells with unaligned chromosomes, and the kinetochore bound Astrin appeared obviously enhanced when compared with that of control cells (Fig.7D, 7G). These results suggest that dynein, likely through NuMA, is required for the spindle pole localization of Astrin; and that dynein may also play an important role in balancing the distribution of Astrin between the spindle poles and kinetochores.

**NuMA balances the localization of Astrin between spindle poles and kinetochores**—To further investigate the potential role of NuMA in Astrin’s dynamic localization during mitosis, we treated HeLa cells with nocodazole, a MT depolymerizing drug which can abolish the mitotic spindle apparatus by binding to tubulin monomers and preventing MT formation. Treatment with MT inhibitor resulted in dissolve of both of spindle MT
and polar crescent of NuMA and Astrin. This effect is reversible and upon nocodazole washout, MTs polymerize de novo from centrosomes. Microtubule regrowth assay showed that recovery of MTs following wash out quickly re-established their localization. Astrin was first observed at the centrosome similar as the localization of NuMA and subsequently, as the spindle reformed, it became more evident at the spindle and at the attached KTs (Fig.8). This result supports the idea that Astrin is not a constitutive KT component and localizes to spindle pole and kinetochore in a temporarily and spatially specific manner.

Next, we examined whether enhanced spindle association of NuMA could affect the distribution of Astrin. We overexpressed RFP-tagged NuMA C terminus (1818-C), which could accumulate at spindle poles in mitotic cells (Fig.9A-B). The disturbed spindle architecture and disorganized chromatin structure observed in NuMA1818-C overexpressing cells was very similar to the Astrin depletion phenotype (Fig.9B). As a consequence, more Astrin seemed to be attracted to the spindle poles, while the kinetochore-bound Astrin were dramatically decreased (Fig.9A, 9B). To prove the specificity of this effect, we also expressed a truncated fragment of NuMA 1818-2060, which could not bind Astrin from our interaction analysis. Although this fragment also accumulated at spindle poles, it did not affect the normal localization of Astrin at spindle poles and kinetochores (Fig.9C). We could reproduce these results using Flag-tagged NuMA C termini (Fig.9D-G). Taken together, these data show that enhancing the spindle pole accumulation of NuMA could impair the kinetochore localization of Astrin, probably by affecting the transport of Astrin between spindle poles and kinetochores.

**DISCUSSION**

In this study we identified the microtubule and kinetochore associated protein Astrin as a novel interactor of NuMA. By using yeast two-hybrid assays, biochemistry, and immunocytochemistry, we demonstrated that NuMA physically interact with Astrin in vivo and in vitro. Both NuMA and Astrin are essential players in mitotic spindle assembly and maintenance. NuMA a non-motor protein that is sequestered in the nucleus during interphase and then is transported to spindle poles by dynein during mitosis (40). The mitotic function of NuMA appears to be involved in the nucleation, stabilization and organization of the MTs. Inhibition of NuMA leads to unfocused, splayed spindle poles, indicating that NuMA is required to focus and stabilize MTs at spindle poles during mitosis (41). Astrin is another mitotic MT-associated protein, which can oligomerize to form an aster-like structure in vitro (34). Various evidence has indicated that Astrin play multiple roles in mitosis. Highly reminiscent of the NuMA inhibition phenotype, the depletion of Astrin by RNAi in HeLa cells also resulted in unfocused spindle pole, disorganized spindle and the failure of chromosome alignment (Fig.3) (34). Importantly, cells deficient in Astrin or NuMA are delayed in progression through mitosis. Both NuMA and Astrin have been implicated in the formation of MT-kinetochore attachments, and SAC silencing (20,42). We propose that Astrin may functionally cooperate with NuMA in spindle organization, chromosome alignment and mitosis regulation.

As a MTs associated protein, Astrin shows a cell cycle specific localization on spindle and kinetochore during mitosis. In interphase cells, however, neither the full-length Astrin, nor the isolated N- or C-terminus of Astrin localizes to MTs, suggesting that mitotic specific modifications or binding to other microtubule associated interactor are responsible for Astrin’s association with the spindle. Until now, however, no clear candidate protein has emerged to establish a direct link between Astrin and the spindle MTs. In this study, we observed that NuMA and Astrin display cooperativity in their association with microtubules, thus providing the functional relevance of the NuMA-Astrin interaction. We show that the C-terminus of NuMA binds directly to the C-terminus of Astrin and recruits Astrin to MTs in interphase cells. Indeed, the C-terminal region of Astrin is the primary determinant for targeting Astrin to the spindles (33). We also performed several experiments to demonstrate that NuMA is required for the spindle association of Astrin in mitotic cells. First, knockdown of NuMA resulted in significantly reduced association of Astrin with the mitotic spindle. Second, overexpression of the N terminus of LGN, which blocks the microtubule binding of NuMA and competes with Astrin for NuMA binding, also led to the similar results. All these evidence suggest that NuMA is one of the good
candidates to facilitate the mitosis-specific recruitment of Astrin to spindle MTs.

Immunofluorescence analysis of Astrin’s localization showed a centrosomal pool and an outer-kinetochore-associated pool of Astrin. Astrin is recruited to the two pools in a temporarily and spatially specific manner during mitosis. In prophase and early prometaphase, Astrin does not localize to kinetochores. It only localize to kinetochores that are attached to the bipolar spindle and under tension, concomitant with stronger kinetochore and reduced spindle staining in late prometaphase and metaphase (33,43). The correct localization of Astrin appears to be critical for the timely progression through mitosis and normal entry into anaphase (35,36). How is the dynamic distribution of Astrin at spindle poles and kinetochores achieved? Our data showed that increasing the spindle association of NuMA led to enhanced spindle pole and reduced kinetochore distribution of Astrin, while depletion of cytoplasmic dynein resulted in the opposite, suggesting that NuMA and dynein are required for the balanced distribution of Astrin between spindle poles and kinetochores. Moreover, upon inhibition of dynein function, substantial amounts of Astrin were still bound along the surface of spindle MTs, suggesting that the binding of Astrin to the spindle and transport towards the pole and kinetochore are two distinct events. The redistribution of Astrin from spindle pole to kinetochore could be the consequence of Astrin’s transport by other kinetochore associated motor and non-motor proteins. Recently, several factors including SKAP and LC8 have been identified to be required for the kinetochore targeting of Astrin (43-45). SKAP also binds to the mitotic kinesin CENP-E, which is required for SKAP targeting to microtubule plus ends (44). Recent studies also found minimal cytoplasmic dynein associated with Astrin and SKAP (36,43). Importantly, the pool of dynein at the metaphase kinetochores would only transiently interact with the Astrin-SKAP complex before moving toward the spindle pole via kinetochore MTs (43). Our study is in excellent agreement with this report suggesting that Astrin-SKAP complex in kinetochore promotes dynein mediated transport from MT plus ends toward spindle poles of factors, such as NuMA and spindly.

In summary, we have identified a direct physical link between NuMA and Astrin, two critical regulators of the mitotic progression. The NuMA-Astrin interaction underlies the interdependent spindle pole localization of Astrin and NuMA; and NuMA and cytoplasmic dynein may balance the localization of Astrin between spindle poles and kinetochores (Fig.10). Our results suggest that NuMA and Astrin cooperate to support the assembly of the mitotic spindle and the alignment of chromosomes during mitosis.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Synchronization**—HeLa cells and COS7 cells were maintained as subconfluent monolayers in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum (HyClone) and 100 units/ml penicillin plus 100 μg/ml streptomycin (Invitrogen) at 37°C in 5% CO2. HeLa cells were synchronized at G1/S with 5 mM thymidine for 16 h, and then washed with phosphate-buffered saline (PBS) three times, and cultured in thymidine-free medium for 12 h to release. After another round of thymidine treatment for 12 h, cells were released for 9-10 h to initiate prometaphase.

**Plasmids, Transfection and Co-immunoprecipitation**—Human NuMA and Astrin cDNA were kindly provided by Dr. Duane Compton (Dartmouth Medical School). Compared with NuMA1 (Gene Bank NP_006176), the cDNA we used has a 14-aa deletion between amino acid 1535 and 1536. Plasmids expressing NuMA or Astrin fragments were PCR amplified and cloned in pK-Venus or pK-monomeric red fluorescent protein (mRFP) as previously described (25,31). For immunofluorescence microscopy and co-immunoprecipitation, cells were transfected using an electroporation device (Lonza, Basel, Switzerland) following the manufacturers’ instructions. Co-Immunoprecipitation was performed as described previously (27). In brief, COS 7 cells were lysed in cell lysis buffer and equal amounts of cell lysate were incubated with 2 μg anti-flag antibody at 4°C for 1 h, GammaBind-Plus Sepharose (GE Healthcare), blocked with 5% BSA (Sigma-Aldrich), were added, and the mixture was incubated for 45 min at 4°C. Immunoprecipitates were washed four times with cell lysis buffer and separated by SDS-PAGE and transferred onto nitrocellulose membrane to perform western blotting.
Yeast Two-hybrid Screen—Yeast two-hybrid assays were performed as described (24). Briefly, NuMA baits containing amino acid 1717-2101 were inserted into the BamHI and EcoRI sites of pGBK7 (clontech) individually to create a fusion with amino acids 1-147 of the Gal4 DNA-binding domain. The resultant pGBK7/NuMA C termini were transformed into strain HF7C along with the GAL4 reporter plasmid pVP16 (clontech). Protein expression was validated by Western blotting. Transformants did not activate the HIS3 reporter gene and were transformed with the cDNA library. Transformants were selected on SD Trp-/Leu-/His- plates plus 10 mM 3-amino-1, 2, 4-triazole, and positive clones were streaked Trp-/Leu-/His- 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) - positive synthetic dropout plates. Almost 300 colonies were picked out on Trp-/Leu-/His/-X-gal-positive synthetic dropout plates. Minipreparation plasmids were isolated from Escherichia coli DH5, and PCR products were digested with several restriction enzymes and grouped according to their digestion patterns. Specificity of the interaction was independently verified by re-transforming the candidate prey plasmid back into strain HF7c along with pGBK7-NuMA C termini. Those cDNAs that could form colonies on Trp-/Leu-/His- synthetic dropout plates were sent for sequencing.

Expression and Purification of Recombinant Proteins and In Vitro Binding Assays—GST, GST-Astrin901-1193 were expressed from pGEX vectors in Escherichia coli BL21 (DE3). His- and S-tagged NuMA1858-2101 were expressed from the pET30a vector in Escherichia coli BL21 (DE3) and purified using Ni-NTA agarose beads (Qiagen). GST or various GST fusion proteins were loaded on glutathione-sepharose beads and incubated with equal amounts of S-tagged NuMA1858-2101 for 1 h in binding buffer (50 mM HEPES pH 7.6, 150 mM NaCl, 5 mM MgCl2, 0.1 mM DTT and 0.01% Tween-20). After washing, bound proteins were separated by SDS-PAGE and detected by WB. Binding assays were performed using glutathione-sepharose 4b (GE Healthcare) or S protein agarose (Novagen) beads in binding buffer (50 mM HEPES [pH 7.5], 150 mM NaCl, 5 mM MgCl2, 1 mM DTT, and 0.01% Tween-20) as described (24). After washing, bound proteins were separated by SDS-PAGE, and detected by immunoblotting using anti-GST (Santa Cruz Biotechnology, 1:2,000), anti-6x-

His (Pierce, 1:1,000).

Indirect Immunofluorescence and Imaging—HeLa cells were grown on glass coverslips and fixed using either 4% paraformaldehyde in PBS or cold methanol. Fixed cells were blocked with 1% BSA/10% NGS in PBS for 1 hour, and then incubated for 1 hour with the primary antibodies: rabbit anti-NuMA (1:1,000) and rabbit anti-Astrin (1:1,000) (kind gifts from Dr. Duane Compton, Dartmouth Medical School, Hanover, NH); human anti-centromere 1:3,000 (Immunovision); and mouse anti-α-tubulin 1:1,000 (Sigma). Cells were then washed and incubated for 1 hour with the DNA stain Hoechst and goat anti-rabbit, anti-mouse or anti-human secondary antibodies coupled to Alexa 488 or Alexa 594 (Invitrogen). A SlowFade AntiFade kit (Invitrogen) was used to inhibit photobleaching. Cells were imaged using an oil objective on a Nikon TE2000 inverted microscope (Nikon Instruments). Images were collected using Metamorph software (Molecular Device) and processed for publication with Adobe Photoshop. For comparison of fluorescent intensities, Quantification of the level of spindle pole and kinetochore-associated protein was conducted as described (27). In brief, the average pixel intensities from at least 20 spindle poles and 100 kinetochores pairs from ten cells were measured, and background pixel intensities were subtracted. The pixel intensities at each kinetochore pair were then normalized against ACA pixel values to account for any variations in staining or image acquisition. The values of specific siRNA-treated cells were then plotted as a percentage of the values obtained from cells transfected with a control siRNA duplex.

Small Interfering RNA (siRNA) and short hairpin RNA (shRNA) mediated Knockdown—for the siRNA studies, the 21-mers of the siRNA duplexes against NuMA (38), Astrin (46) and control were synthesized by Dharmacon Research Inc. (Lafayette, CO). The two single-stranded RNAs (20 μM) were annealed to one another by incubation in annealing buffer for 1 min at 90 ℃, followed by 1 h at 37 ℃. Transfections of HeLa cells were carried out with siRNA in a 24-well plate using Oligofectamine reagent or Lipofectamine 2000 (Invitrogen), respectively, according to the manufacturer’s recommendations. After trial experiments using a series of concentrations and time course assays, treatment at 100 nM for 72 h.
was finally selected as the most efficient conditions for repressing target proteins.

For transient shRNA-mediated Knockdown, pRNAi-neo-based vectors were used for the knockdown of Dynein heavy chains (DYNC1H1) in HeLa cells (32). Briefly, long oligos containing target sequences were cloned downstream of the H1 promoter of pRNAi-neo. After sequencing verification, individual plasmids were electroporated into HeLa cells using Nucleofector (Lonza) to test knockdown efficiency by Western blot and immunofluorescence analysis. Cells were harvested 48 h later for analysis.

\textit{Microtubule Regrowth Assay—}Microtubule regrowth assay was carried out as reported previously (47). Briefly, HeLa cells were treated with 100 nM nocodazole in DMEM for 4 hours at 37°C and then nocodazole was removed by washing with PBS and cells were incubated with DMEM at 37°C. Cells were fixed at different time points with PBS containing 4% paraformaldehyde and 0.05% Triton X-100, and then stained with antibodies.

Statistical Analysis—Prism software was used to plot data and determine statistical significance using a Student’s t test. Data are presented as mean ± S.E. as indicated. A \( p \) value of <0.05 was considered to be statistically significant.

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Author contributions: QD conceived and designed the study. XGC, XYC, QW and ZZ performed the experiments and data analysis. XGC and QD wrote the paper.

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**FIGURE LEGENDS**

**Figure1. Astrin interacts with NuMA**

(A) Over-expressed Astrin co-immunoprecipitates with NuMA. COS 7 cells were transiently transfected with Flag tagged NuMA1818-C and GFP, GFP tagged Astrin N terminus (1-608), GFP tagged Astrin C terminus (609-C) or GFP tagged Astrin full length (Fl), respectively. Cells were pre-synchronized with thymidine, released and then arrested in mitosis with 100 nM nocodazole. Whole cell lysates were immunoprecipitated with anti-Flag M2 antibody followed by immunoblotting with anti-GFP antibody.

(b) *In vitro* GST pulldown assays. GST and GST-tagged Astrin901-C prepared from E. coli BL21 (DE3) were bound to glutathione-Sepharose beads and incubated with His-tagged NuMA1858-C. Proteins on the beads were analyzed by immunoblot (IB) analysis with anti-His or anti-GST antibody.

(c) Co-immunoprecipitation of endogenous Astrin and NuMA. HeLa cells were synchronized by double thymidine block, released and arrested in mitosis with 100 nM nocodazole. Cell lysates were subjected to immunoprecipitation using rabbit anti-NuMA antibody or rabbit IgG. The immunoprecipitates were separated by SDS-PAGE and blotted with anti-NuMA and anti-Astrin antibodies.

(d, e) Mapping the binding region of Astrin and NuMA in yeast two-hybrid system. + indicates positive interaction, and - indicates negative interaction. Numbers indicate the position of the amino acid residues in Astrin and NuMA. Domain structure shows predicted secondary structure and domain organization of Astrin and NuMA. A schematic diagram of Astrin or NuMA and its deletion used in the yeast two-hybrid system is shown.

(f) Mapping the binding region of Astrin and NuMA by co-IP. GFP tagged Astrin C terminal (901-C) and various Flag-tagged NuMA C terminus (1818-C, 1878-C, 1981-C or 1818-2060) were co-expressed in COS7 cells. Co-immunoprecipitation and Western blot (WB) were performed as described above.

**Figure2. Cell cycle dependent colocalization of NuMA and Astrin**

(A) HeLa cells were co-transfected with pKmRFP Astrin-Fl and pkVenus NuMA-Fl. 24 hours after transfection, cells were fixed and stained with DNA dye (blue). Representative images of cells at different stages of the cell cycle were shown. Scale bar: 5 μm.

(b) MDCK cells stably expressing GFP-NuMA were fixed and stained with anti-Astrin antibody (red) and DNA dye (blue). Representative images of cells at different stages of mitosis were shown. Scale bar: 5 μm.

**Figure3. Disruption of Astrin function in mitosis demonstrates a role in spindle Assembly and**
chromosomal congression

(A) HeLa cells were transfected with control or Astrin-specific siRNA duplexes. 72 hours after the transfection, cells were fixed and stained for Astrin, α-tubulin and DNA. Scale bar: 5 μm.

(B) HeLa cells were transfected with 100 nM control or Astrin siRNA for 72 h. Cell lysates were subjected to SDS-PAGE and immunoblotting using anti-Astrin and anti-α-tubulin antibodies.

(C) A graph showing the mitotic index of control or Astrin siRNA transfected HeLa cells. Values represent the means ± SEM (error bars) from three independent experiments.

(D, E) HeLa cells were transfected with control or Astrin-specific siRNA duplexes. 72 hours after the transfection, cells were fixed and stained for NuMA, DNA, and α-tubulin (D) or CREST (E). Scale bar: 5 μm.

(F) Quantification of misaligned chromosomes following control or Astrin siRNA treatment in HeLa cells. n>70 mitotic cells per experiment. Results were pooled from three independent experiments.

(G) Quantitation of the fluorescence intensity of spindle pole NuMA from images acquired in D, *** p<0.001. Error bars represent standard error of the mean (SEM). n>20 cells per group.

Figure 4. NuMA recruits the C terminus of Astrin to microtubules

Representative fluorescent images of COS7 cells transfected with (A) pKmRFP-NuMA1818-C; (B) pKmRFP and pKGFP-Astrin901-C; (C) pKmRFP-NuMA1818-C and pKGFP-Astrin901-C; (D) pKmRFP-NuMA1818-C and pKGFP-Astrin1-608; (E) pKGFP-NuMA1818-2060; (F) pKGFP-NuMA1818-2060 and pKmRFP-Astrin901-C. 24 hours after transfection, cells were fixed and stained with DNA dye (blue). Cells in A and E were also stained with anti-α-tubulin antibody (green). Scale bar: 5 μm.

Figure 5. The NuMA-Astrin interaction contributes to the localization of Astrin to spindle poles

(A) HeLa cells were transfected with control or NuMA-specific siRNA duplexes. 72 hours after the transfection, cells were fixed and stained for NuMA, α-tubulin, and DNA. Scale bar: 5 μm.

(B) Equal amounts of HeLa cells were transfected with 100 nM control or NuMA siRNA for 72 h and subjected to western blot analysis using anti-NuMA and anti-α-tubulin antibodies.

(C) HeLa cells were transfected with control or NuMA-specific siRNA duplexes. 72 hours after the transfection, cells were fixed and stained for Astrin, α-tubulin and DNA. Scale bar: 5 μm.

(D) Quantitation of the fluorescence intensity of spindle pole NuMA from images acquired in A, *** p<0.001. Error bars represent standard error of the mean (SEM).

(E) Quantitation of the fluorescence intensity of spindle pole Astrin from images acquired in C, *** p<0.001. Error bars represent standard error of the mean (SEM).

Figure 6. Inhibition of NuMA function by LGN affects the localization of Astrin

(A-C) HeLa cells were transfected with pKvenus control or pKvenus-LGN1-481, then fixed, permeabilized, and stained with specific antibodies against NuMA (A, red), α-tubulin (B, red) and Astrin (C, red). DNA was stained with DNA dye (blue). Merged images are shown on the right. Scale bar: 5 μm.

(D) Quantitation of the fluorescence intensity of spindle pole Astrin from images acquired in C, *** p<0.001.

(E) LGN competitively inhibits the association of NuMA and Astrin. COS7 cells were transiently transfected with pKH3-NuMA Fl and pkGFP-Astrin Fl with or without pkVenus-LGN 1-481. An empty pKH3 vector was used as a control. Cells were pre-synchronized with thymidine, released and then arrested in mitosis with nocodazole. Whole cell lysates were immunoprecipitated with anti-HA antibody followed by immunoblotting with anti-GFP and anti-HA antibodies.

Figure 7. NuMA and Astrin localization to the spindle poles is dynein-dependent.

(A-D) HeLa cells were transfected with plasmids expressing control or DYNC1H1 shRNA. 48h after transfection, cells were fixed and stained with anti-α-tubulin and anti-DYNC1H1 (A), anti-α-tubulin and anti-NuMA (B), anti-α-tubulin and anti-Astrin (C), or anti-Astrin and anti-CREST (D) antibodies.
Representative images are shown. In D, enlargements of selected regions (square) were shown at the upper left corner of each image. Scale bar: 5 μm.

(E) HeLa cells were transfected with plasmids expressing control or DYNC1H1 shRNA. Cell lysates were harvested 48h later and subjected to Western blotting using anti-DYNC1H1 and anti-α-tubulin antibodies. (F, G) Quantitation of the fluorescence intensity of spindle pole (F) and kinetochore (G) Astrin from images acquired in C and D, *** p<0.001.

**Figure 8. Dynamic localization of Astrin in spindle poles and kinetochores upon microtubule regrowth.** HeLa cells were treated with nocodazole for 14 hours and released for indicated period of time. Cells were fixed and stained with antibodies for NuMA, Astrin and α-tubulin. DNA was stained with DNA dye. Scale bar: 5 μm.

**Figure 9. NuMA balances the dynamic localization of Astrin between spindle poles and kinetochores**

(A-C) HeLa cells were transfected with plasmids expressing RFP or RFP-tagged NuMA C terminus (pKmRFP-NuMA1818-2101(1818-C) or pKmRFP-NuMA1818-2060). Cells were fixed, permeabilized, and stained with anti-Astrin antibody and DNA dye. Scale bar: 5 μm.

(D-F) HeLa cells were transfected with plasmids expressing Flag or Flag-tagged NuMA C terminus (pKFlag-NuMA1818-2101(1818-C) or pKFlag-NuMA1818-2060). Cells were fixed, permeabilized, and stained with anti-Astrin, anti-CREST antibodies and DNA dye. Enlargements of selected regions (square) were shown at the upper left corner of each image. Scale bar: 5 μm. (G) Quantitation of the fluorescence intensity of kinetochore Astrin from images acquired in A-C, *** p<0.001.

**Figure 10. Working model**

Astrin associates with NuMA at the spindle pole upon mitotic spindle formation. NuMA may balance the localization of Astrin between spindle poles and kinetochores. Astrin functionally cooperate with NuMA in spindle organization, chromosome alignment and mitosis regulation. Disruption of NuMA-Astrin interaction will lead to mitotic defects such as unfocused spindle poles, uncongressed chromosomes, unattached kinetochores, multipolar spindle, and increase of mitotic index.
Chu et al. Figure 9

A  
RFP  Astrin  DNA

B  
RFP-NuMA1818-C  Astrin  DNA

C  
RFP-NuMA1818-2060  Astrin  DNA

D  
Astrin  CREST  DNA

E  
Flag control

F  
Flag-NuMA1818-C

G  
Flag-NuMA1818-2060

Relative Astrin intensity in Kinetochore

- N.S.
- ***p<0.001
- ***p<0.01
Chu et al. Figure 10

Prophase  Prometaphase  Metaphase  Anaphase

- Unfocused Poles
- Uncongressed Chromosomes
- Unattached kinetochores
- Multipolar Spindle
- Mitotic Arrest

- NuMA
- Astrin
- Kinetochores
NuMA Interacts with and Regulates Astrin at the Mitotic Spindle
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