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NuMA タンパク質とキネシン Eg5 との相互作用：
その双極性紡錘体の形成と染色体整列における役割

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SUMMARY

Bipolar spindle assembly in mitotic cells is a prerequisite to ensure correct alignment of chromosomes for their segregation to each daughter cell; spindle microtubules are tethered at plus ends to chromosomes and focused at minus ends to either of the two spindle poles. The nuclear mitotic apparatus protein (NuMA) is present solely in the nucleus in interphase cells, but relocalizes during mitosis to the spindle poles to play a crucial role in spindle assembly via focusing spindle microtubules to each pole. Here I show that the kinesin-5 family motor Eg5 is a protein that directly interacts with NuMA, using a proteomics approach and various binding assays both *in vivo* and *in vitro*. During mitosis Eg5 appears to interact with NuMA in the vicinity of the spindle poles, whereas the interaction does not occur in interphase cells, where Eg5 is distributed throughout the cytoplasm but NuMA exclusively localizes to the nucleus. Slight but significant depletion of Eg5 in HeLa cells by RNA interference results in formation of less focused spindle poles with misaligned chromosomes in metaphase; these phenotypes are similar to those induced by depletion of NuMA. Since NuMA is less accumulated at the spindle poles in Eg5-depleted cells, Eg5 likely contributes to spindle assembly via regulating NuMA localization. Furthermore, depletion of cytoplasmic dynein induces mislocalization of NuMA and phenotypes similar to those observed in NuMA-depleted cells, without affecting Eg5 localization to the spindles. Thus dynein appears to control NuMA function in conjunction with Eg5.

INTRODUCION

Upon cell cycle transition from interphase to mitosis, the microtubule network is extensively reorganized: the mono-focused interphase microtubule array becomes transformed into a bipolar mitotic spindle, which is required for accurate alignment of chromosomes at the equatorial metaphase plate and their subsequent segregation [1,2]. The poles of the spindle serve as major focal points for the minus ends of spindle microtubules and as the final destination for segregated chromosomes. During mitosis of animal somatic cells, spindle poles are coincident with centrosomes, known as major cellular microtubule organizer. The centrosomes that have been duplicated before mitosis become separated at the onset of mitosis. The separation requires Eg5, a plus-end-directed motor protein of the kinesin-5 family, as a crucial molecular engine [3–6]. Although Eg5 is distributed along the mitotic spindles, it is enriched near the spindle poles, which may be unexpected from its plus-end-directed motor activity [7,8]. This kinesin forms a homo-tetrameric motor that is thought to slide antiparallel microtubules emanating from each centrosome to push centrosomes apart, thereby establishing spindle bipolarity [9]. Although Eg5 also appears to contribute to maintenance of spindle bipolarity [10,11], the role of Eg5 except centrosome separation has remained unclear.

Centrosome separation is followed by bipolar spindle assembly, a process in which the nuclear mitotic apparatus protein (NuMA) plays a crucial role [12–14]. Although NuMA is exclusively distributed in the nucleus during interphase, it relocalizes in mitotic cells to the spindle poles in a crescent-shaped pattern [12,13]. NuMA participates in focusing microtubules toward the mitotic spindle poles and tethering spindle microtubules to centrosomes, as initially indicated by experiments using *Xenopus laevis* egg extracts [15,16]. Depletion of NuMA in human cells by RNA interference (RNAi) and knockout strategies in mice have recently revealed that this protein is required for proper spindle assembly and chromosome alignment in intact mammalian cells [17,18]. Recruitment of the microtubule-

associating protein NuMA toward the spindle poles is considered to involve cytoplasmic dynein, a minus end-directed motor that is known to transport various proteins to spindle poles [19]. This is because, in a cell-free system using frog egg extracts, dynein and its activator dynactin associate with NuMA, and thereby participate in NuMA targeting to spindle poles [15,20]. However, the role of dynein in NuMA localization in intact cells is presently obscure. Thus the detailed mechanism for localization and function of NuMA still has remained to be elucidated.

In the present study, I have identified Eg5 as a novel NuMA-interacting protein by the use of a proteomics approach, followed by various binding assays both *in vivo* and *in vitro*. During mitosis of HeLa cells, Eg5 appears to directly interact with NuMA in the vicinity of the spindle poles, although the interaction does not occur in interphase cells. This kinesin is required for proper accumulation of NuMA at the spindle poles, and accumulated NuMA likely plays a crucial role in bipolar spindle assembly and chromosome alignment. I also show that dynein functions in NuMA recruitment to the spindle poles in intact HeLa cells probably in conjunction with Eg5, and thus appears to allow NuMA to regulate spindle assembly.

EXPERIMENTAL PROCEDURES

Materials

Nocodazole, MG132, and *S*-trityl-L-cysteine (STLC) were purchased from Sigma–Aldrich. Staurosporine was obtained from Wako Pure Chemical Industries. Anti-NuMA rabbit polyclonal antibodies were purchased from Cell Signaling Technology; the anti-NuMA mouse monoclonal antibody (Ab-2) that recognizes the central coiled-coil region of NuMA from Calbiochem; the anti-Eg5 mouse monoclonal antibody (20/EG5) and the anti-$p150^{Glued}$ mouse monoclonal antibody (12/P150GLUED) from BD Transduction Laboratories; the anti-β-tubulin mouse monoclonal antibody (TUB 2.1) and the anti-FLAG mouse monoclonal antibody (M2) from Sigma–Aldrich; the anti-HA mouse monoclonal antibody (16B12) from Covance; the anti-dynein intermediate chain 1 mouse monoclonal antibody (70.1/ab6304), anti-β-tubulin rabbit polyclonal antibodies, and anti-γ-tubulin rabbit polyclonal antibodies from Abcam; antibodies isolated from human auto-antiserum available as anti-centromeric antibodies from Immunovision; and Alexa Fluor 488-labeled antibodies against rabbit IgG, mouse IgG, or human IgG, and Alexa Fluor 594-labeled antibodies against mouse IgG, mouse IgM, or rabbit IgG, from Invitrogen.

Plasmid construction

The cDNA encoding human NuMA of 2,101 amino acid residues [12] is a generous gift from Prof. Duane A. Compton (Dartmouth Medical School). The cDNA for human Eg5 comprising 1,056 amino acid residues [5] was obtained from Open Biosystems (clone ID: 9020463). The cDNA for human LGN was prepared as described previously [21,22]. The DNA fragments encoding NuMA-N (amino acids 1–213) and NuMA-C (amino acids 1818–2001) were prepared by PCR using the cDNA for full-length NuMA as a template. The DNA fragments encoding Eg5-N (amino acids 1–366), Eg5-S (amino acids 367–797), Eg5-C (amino acids 798–1056), and Eg5-SC (amino acids 367–1056) were prepared by PCR using

the cDNA for full-length Eg5 as a template. The cDNAs were ligated to the following expression vectors: pGEX-6P (GE Healthcare) for expression of proteins fused to glutathione *S*-transferase (GST) in *Escherichia coli*; pGBT (Invitrogen) and pGADGH (Invitrogen) for expression in yeast HF7c cells; pcDNA3.0 (Invitrogen) for expression of HA–tagged proteins in the TnT® T7 Quick-coupled *in vitro* transcription/translation system (Promega); pEF-BOS for expression of FLAG– or HA–tagged proteins in mammalian cells [23,24]. All the constructs were sequenced for confirmation of their identities.

Cell culture and synchronization

FreeStyle HEK-293F cells (Invitrogen) were cultured in FreeStyle 293 Expression Medium (Invitrogen) in an incubator set at 8% CO_2 at 37˚C. HeLa and COS-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum in an incubator set at 5% CO_2 at 37˚C.

FreeStyle HEK-293F cells synchronized at mitotic phase were obtained by culture for 16 h in the presence of nocodazole (50 ng/ml). HeLa cells were synchronized by the double thymidine block method [7,25]. Briefly, cells were cultured for 16 h in the presence of 2 mM thymidine and released for 9 h in the absence of the reagent, followed by culture for 16 h with 2 mM thymidine. Synchronized mitotic cells were obtained by further culture for 9.5 h in the absence of thymidine. Alternatively, HeLa cells arrested in early prophase were obtained by culture for 20 h in the presence of 5 μM STLC, followed by release for 20 min [26].

Immunoprecipitation for identification of proteins complexed with NuMA

FreeStyle HEK-293F cells were transfected using 293fectin (Invitrogen) with pEF-BOS-FLAG–NuMA. After culture for 8 h, transfected cells were further cultured for 16 h with 50 ng/ml of nocodazole, and lysed with a lysis buffer (150 mM NaCl, 0.2 mM EDTA, 1 mM

dithiothreitol (DTT), 10% glycerol, 0.5% Triton X-100, and 50 mM Tris-Cl, pH 7.5) containing Protease Inhibitor Cocktail (Sigma–Aldrich). Cell lysates were centrifuged for 10 min at 20,000 x g, and the resultant supernatant were precipitated with the anti-FLAG M2 antibody-conjugated agarose (Sigma–Aldrich). After washing three times with a Tris-buffered saline (150 mM NaCl, 0.05% Tween-20, and 50 mM Tris-Cl, pH 7.4) containing Protease Inhibitor Cocktail, the proteins were subjected to 10% SDS-PAGE, followed by silver staining.

Protein identification by LC–MS/MS analysis

Protein identification using LC–MS/MS was performed at the Laboratory for Technical Support, Medical Institute of Bioregulation, Kyushu University. Bands separated on an SDS-PAGE gel, as described above, were excised, and the proteins in the gel were subjected to reduction with DTT, *S*-carbamidomethylation with iodoacetamide, and digestion with trypsin, as described by the method of Matsuzaki *et al.* [27]. Fragmented peptides were applied to a nanoflow LC system (Paradigm MS4; Michrom BioResources, Auburn, CA) equipped with an L-column (C_{18}; Chemical Evaluation and Research Institute, Tokyo, Japan). Eluted peptides were sprayed directly into a Finnigan LTQ mass spectrometer (Thermo Fisher Scientific, San Jose, CA). MS/MS spectra were obtained automatically in a data-dependent scan mode with a dynamic exclusion option, and compared with those in the International Protein Index (IPI; European Bioinformatics Institute) version 3.16 with the use of the MASCOT search engine (Matrix Science, Boston, MA); tolerance of MS/MS ions was ±2.0 Da. Assigned high-scoring peptide sequences were manually confirmed by comparison with the corresponding spectra.

An *in vivo* binding assay using immunoprecipitation and immunoblot analysis

Synchronized HeLa cells were lysed by sonication in the lysis buffer containing Protease Inhibitor Cocktail. COS-7 cells were transfected using LipofectAMINE and Plus Reagent (Invitrogen) with indicated cDNAs and cultured for 24 h in DMEM with 10% fetal calf serum; transfected cells were lysed at 4˚C with the lysis buffer containing Protease Inhibitor Cocktail. Proteins were precipitated with the anti-NuMA antibody (Ab-2) or the anti-FLAG antibody (M2) in the presence of protein G-Sepharose (GE Healthcare), as previously described [21]. The precipitants were analyzed by immunoblot with the indicated antibodies. The blots were developed using ECL-plus (GE Healthcare) for visualization of the antibodies.

Two-hybrid experiments

For analysis of two-hybrid interaction between NuMA and Eg5, yeast HF7c cells were transformed with pGADGH–Eg5-SC and pGBT–NuMA. After selection for the Trp^+ and Leu^+ phenotypes, transformed cells were tested for their ability to grow on plates lacking histidine in the presence of 100 μM 3-aminotriazole, as previously described [28].

An *in vitro* GST pull-down binding assay using purified proteins

GST–fused NuMA-N (amino acids 1–213) and NuMA-C (amino acids 1818–2001) were expressed in the *E. coli* BL21 strain and purified with glutathione-Sepharose 4B (GE Healthcare), as previously described [21]. HA–tagged Eg5-S (amino acids 367–797) and Eg5-SC (amino acids 367–1056) were synthesized *in vitro* with the cloned pcDNA3.0 vector encoding either of these proteins, instead of PCR products, using TnT® T7 Quick for PCR DNA (Promega), according to the manufacturer's protocol. HA–Eg5-S or HA–Eg5-SC was mixed with GST–NuMA-N, GST–NuMA-C, or GST alone in 500 μl of a binding buffer (100 mM NaCl and 20 mM Tris-HCl, pH 7.6) containing 0.01% Triton X-100 and 1 mM DTT. A slurry of glutathione-Sepharose 4B beads was subsequently added, followed by further

incubation for 60 min at 4˚C. After washing three times with the buffer containing 0.05% Triton X-100, proteins were eluted from the beads with 20 mM glutathione in 200 mM NaCl and 150 mM Tris-HCl, pH 8.0. The eluate was subjected to SDS-PAGE, followed by staining with *Coomassie Brilliant Blue* (CBB) or by immunoblot analysis using the anti-HA monoclonal antibody.

RNA interference (RNAi)-mediated knockdown of NuMA, Eg5, and dynein

As double strand small interfering RNAs (siRNAs) targeting human NuMA, Eg5, and the dynein heavy chain (DHC), 25-nucleotide modified synthetic RNAs (Stealth^{TM} RNAi; Invitrogen) were used:

NuMA-#1, 5'-CAUGGCACUGAAGAGGGACAGCAAA-3' (sense),

5'-UUUGCUGUCCCUCUUCAGUGCCAUG-3' (antisense);

NuMA-#2, 5'-CCUCACCGAGAAGGAUGCACAGAUA-3' (sense),

5'-UAUCUGUGCAUCCUUCUCGGUGAGG-3' (antisense);

Eg5-#1, 5'-UAUGGUGUUUGGAGCAUCUACUAAA-3' (sense),

5'-UUUAGUAGAUGCUCCAAACACCAUA-3' (antisense);

Eg5-#2, 5'-GAGAGCUCGGGAAGCUGGAAAUAUA-3' (sense),

5'-UAUAUUUCCAGCUUCCCGAGCUCUC-3' (antisense);

DHC-#1, 5'-AGAAAAGCCAAAAGUUACAGACUUU-3' (sense),

5'-AAAGUCUGUAACUUUUGGCUUUUCU-3' (antisense);

DHC-#2, 5'-GAUGCUAGGAUCAAACAUGACGGAA-3' (sense),

5'-UUCCGUCAUGUUUGAUCCUAGCAUC-3' (antisense).

Medium GC Duplex of Stealth^{TM} RNAi Negative Control Duplexes (Invitrogen) was used as a negative control.

For knockdown of Eg5, HeLa cells at 1.3 x 10^4/cm^2 were cultured for 16 h in the presence of 2 mM thymidine, released for 9 h in the absence of thymidine, and subsequently

transfected with Eg5 siRNAs (40 nM or 0.3 nM) using LipofectAMINE 2000 (Invitrogen); transfected cells were cultured for 16 h with 2 mM thymidine and then for 9.5 h in the absence of thymidine to obtain synchronized mitotic cells. In some cases, HeLa cells transfected with Eg5 siRNAs using LipofectAMINE 2000 were cultured for 22 h, followed by treatment for 2 h with 5 μM MG132 for cell synchronization. For knockdown of NuMA or DHC, HeLa cells were transfected with NuMA siRNAs (120 nM) or DHC siRNAs (40 nM) using LipofectAMINE 2000, and cultured for 46 h or 70 h, respectively; cells were subsequently treated for 2 h with 5 μM MG132. This is because sufficient depletion of NuMA or dynein required a longer culture than that for depletion of Eg5 (data not shown).

Immunofluorescence staining and confocal imaging

HeLa cells grown on glass coverslips were fixed for 5 min in ice-cold methanol, and blocked for 30 min with phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, and 1.5 mM KH_2PO_4, pH 7.4) containing 3% bovine serum albumin (BSA), as previously described [20]. The samples were incubated overnight at 4˚C with primary antibodies in PBS containing 3% BSA, and subsequently incubated for 60 min at room temperature with secondary antibodies in PBS containing 3% BSA. DNA was visualized by staining with Hoechst 33342 (Invitrogen). Images of fixed cells were acquired using a 63x/1.4 NA objective lens on a confocal microscope LSM510 META (Carl Zeiss).

For estimation of chromosome alignment errors, siRNA-transfected HeLa cells were treated for 2 h with 5 μM MG132 for block of the cell cycle at metaphase, as described by Vanneste *et al.* [7]. Chromosome alignment errors were estimated according to previous methods [29,30] with minor modifications. After centromere staining, cells with all chromosomes aligned at the equatorial metaphase plate were counted as having normal chromosome alignment, whereas those with one or more unaligned chromosomes were counted as having chromosome alignment errors.

For estimation of spindle pole to cytoplasmic ratio of NuMA intensity, images were obtained in the plane in which one spindle pole was in focus, using a microscope AxioVert 200 (Carl Zeiss) coupled to AxioCam HRm (Carl Zeiss). Fluorescence intensity of NuMA in the cytoplasm was measured in the region in which chromosomes were aligned in metaphase-arrested cells.

RESULTS

Identification of Eg5 as a novel NuMA-binding protein in mitotic cells

Although NuMA exclusively localizes to the nucleus in interphase cells, it associates with the spindle poles during mitosis to play a crucial role in spindle assembly and chromosome alignment. To identify proteins that interact with NuMA and function together in mitosis, I adopted a proteomics approach. Human NuMA tagged with FLAG at the N-terminus was expressed in FreeStyle HEK-293F cells. After cells were arrested at mitosis, FLAG–NuMA and its binding proteins were precipitated from cell lysates with anti-FLAG-agarose resins. Precipitated proteins were separated with SDS-PAGE, followed by silver staining (Figure 1A). The stained gel was sliced, and proteins in the gel pieces were subsequently digested with trypsin. The produced peptides were analyzed by liquid chromatography and tandem mass spectrometry (LC–MS/MS). The analysis identified Eg5 as a candidate of novel NuMA-binding proteins (Figure 2A). To confirm the interaction between NuMA and Eg5, I ectopically expressed these proteins in COS-7 cells and performed a co-immnunoprecipitation assay. As shown in Figure 2B, FLAG–NuMA indeed interacted with HA–Eg5 as well as with HA–tagged LGN, a well-characterized NuMA-binding protein [21,31].

To further investigate the interaction between NuMA and Eg5, I next compared the subcellular localization of the endogenous proteins in HeLa cells. During interphase, NuMA localized exclusively to the nucleus, whereas Eg5 was distributed throughout the cytoplasm (Figure 1B). In mitotic cells, NuMA associated with the spindle poles in a crescent-shape; Eg5 localized along microtubules with enrichment toward the spindle poles (Figure 1B). Thus the distributions of these two proteins overlap in the vicinity of the spindle poles. Consistent with this finding, endogenous Eg5 was effectively coprecipitated with the anti-NuMA antibodies during mitosis in HeLa cells, which are synchronized by the double thymidine block method (Figure 1C). On the other hand, endogenous Eg5 did not interact with endogenous NuMA in unsynchronized cells. Similarly, the interaction between these

endogenous proteins occurred in cells arrested in early mitosis by treatment with STLC, an Eg5-specific inhibitor [26], but not in untreated HeLa cells (Figure 1D). Treatment with STLC resulted in facilitated interaction of exogenously-expressed FLAG–NuMA with Eg5 (Figure 1E). On the other hand, a weak but significant interaction between FLAG–NuMA and Eg5 was observed even in untreated cells; this may be due to the presence of mitotic cells at a substantial level and enhanced expression of NuMA, which is consistent with the observation shown in Figure 2B. Taken together with the present findings, endogenous NuMA appears to interact with endogenous Eg5 in a mitosis-dependent manner.

The dependence of NuMA–Eg5 interaction on mitosis may be largely explicable by distinct intracellular distribution of these proteins in interphase cells: NuMA and Eg5 exclusively localize to the nucleus and cytoplasm, respectively (Figure 1B). On the other hand, it is known that NuMA undergoes mitosis-specific phosphorylation [32, 33]. As shown in Figure 2C, treatment of mitotic cells with staurosporine, a broad-range protein kinase inhibitor, led to a slightly higher mobilization of NuMA on SDS-PAGE, indicative of a less phosphorylated form. Nevertheless, NuMA interacted with Eg5 in the presence of staurosporine (Figure 2C), suggesting that the interaction does not require phosphorylation of NuMA. In addition, it has been also reported that Eg5 as well as NuMA is phosphorylated by the cyclin-dependent protein kinase Cdc2 [5, 34], an enzyme that is efficiently inhibited by staurosporine [35]. The finding that the inhibitor does not block Eg5 binding to NuMA (Figure 2C) also suggests that phosphorylation of Eg5 is not involved. Thus phosphorylation does not seem to play a major role in mitosis-dependent interaction between NuMA and Eg5.

Direct interaction of Eg5 with NuMA via the stalk region

To know the Eg5 region responsible for interaction with NuMA, I coexpressed FLAG–tagged, full-length NuMA and HA–fused Eg5 with various lengths in COS-7 cells (Figure 3A).

Immunoprecipitation analysis with an anti-FLAG antibody revealed that the central stalk region of Eg5 (Eg5-S; amino acid residues 367–797) associates with NuMA (Figure 3B). On the other hand, NuMA interacted neither with the N-terminal motor domain (Eg5-N; amino acid residues 1–366) nor with the C-terminal tail region (Eg5-C; amino acid residues 798–1056). Thus the stalk region of Eg5 is responsible for binding to NuMA. The involvement of the stalk region is confirmed by the yeast two-hybrid experiment: a protein comprising the stalk and tail regions (Eg5-SC; amino acid residues 367–1056) interacted with full-length NuMA (Figure 3C). A protein with solely the stalk or tail region was unavailable in the experiments, because these proteins gave false-positive signals under the present conditions (data not shown).

I next examined whether Eg5 directly binds to NuMA or not, by a GST pull-down assay using bacterially-expressed NuMA proteins and in vitro translated Eg5 proteins. As shown in Figure 3D, Eg5-SC directly interacted with the tail domain of NuMA (NuMA-C; amino acid residues 1818–2001), and also with the globular head (NuMA-N; amino acid residues 1–213), but to a lesser extent. In addition, the stalk domain of Eg5 made a direct contact with NuMA-N and NuMA-C as well (Figure 3E). These findings indicate that Eg5 directly interacts with NuMA mainly via the stalk region.

Dispensable role for NuMA in localization of Eg5

Since NuMA and Eg5 colocalize in the vicinity of the spindle poles during mitosis (Figure 1B), it seems possible that the interaction between both proteins affects their localization. To test the possibility, I first depleted NuMA from HeLa cells by RNAi using two distinct NuMA siRNAs (Figure 4A), which did not affect the protein level of Eg5 and $p150^{Glued}$, a subunit of the dynactin complex that is known to regulate Eg5 localization [34]. Consistent with the observation by Haren *et al.* [17] using NuMA siRNAs, which are targeted to regions different from those of the siRNAs used in the present study, the spindle poles in NuMA-

depleted cells were less focused than those in cells transfected with control RNA: the depletion induced mitotic abnormalities including pole-defocused spindles and disorganized microtubule structures (Figure 4B). Furthermore, as clearly visualized by centromere staining, a high number of these mitotic cells contained chromosomes that were not aligned at the equatorial metaphase plate (Figures 4C and 4D), confirming the involvement of NuMA in correct chromosome alignment. In NuMA-depleted cells, Eg5 remained on the microtubules with strong staining near spindle poles (Figures 4E and 4F). It is thus likely that NuMA does not participate in Eg5 localization.

Role for Eg5 in localization of NuMA

To further investigate the role for the interaction between NuMA and Eg5, I next tested the effect of inhibition or depletion of Eg5 on NuMA localization. The plus-end-directed kinesin motor Eg5 plays a crucial role in centrosome separation during bipolar microtubule assembly; suppression of Eg5 leads to monopolar spindle formation and mitotic arrest [5,9]. Although STLC, a specific inhibitor for the motor activity of Eg5 [26], did not affect the protein level of Eg5 in HeLa cells (Figure 5A), the inhibitor blocked centrosome separation but not centrosome duplication *per se*, as indicated by staining with the antibody against the centrosomal protein γ-tubulin (Figure 5B). Thus, STLC induced formation of monopolar spindles that appeared exclusively as chromosome rosettes surrounding a central γ-tubulin-positive pole (Figure 5B), which observation is consistent with that in previous reports [18,26]. Under these conditions, NuMA was concentrated at the center of monopolar spindles with duplicated centrosomes (Figure 5B), indicating that Eg5 activity is dispensable for NuMA localization to the spindle pole.

Monopolar spindles were also formed in HeLa cells (Figure 5B), when Eg5 was strongly knocked down by transfection of cells with two distinct Eg5-specific siRNAs (Figure 5A). In cells severely depleted of Eg5, the accumulation of NuMA to the monopolar spindle was

markedly reduced (Figure 5B), indicating a role of Eg5 protein on NuMA localization. Concomitantly with less nuclear distribution, NuMA was more distributed to the cytoplasm in Eg5-depleted cells; this was revealed when images were obtained at increased detector gain (Figure 5C). Thus the plus end-directed motor protein Eg5 regulates localization of NuMA to spindle poles, which does not require its motor activity. It has been indicated that the minus end-directed motor cytoplasmic dynein is involved in NuMA localization [15,20]; the protein level of the dynein intermediate chain (DIC), a component of the dynein complex, was not affected even in cells that were almost completely depleted of Eg5 (Figure 5A). This finding suggests that dynein is not sufficient for targeting of NuMA to the spindle poles.

Role for Eg5 in function of NuMA

Since NuMA localization depends on Eg5 (Figure 5), it is possible that Eg5 plays a role in regulating NuMA function. It is well established that NuMA is crucial for spindle assembly, a process that follows separation of the duplicated centrosomes, which is Eg5 activity dependent [5,9]. NuMA is therefore considered to function in a stage after spindle pole separation, implying that NuMA function cannot be tested under the conditions where Eg5 is almost completely depleted. To avoid this, I knocked down Eg5 to a lesser extent by treating HeLa cells with a much lower amount of Eg5-specific siRNAs (Figures 6A and 6B). As expected, these Eg5-depleted cells were able to form bipolar spindles (Figure 6C). However, slight depletion of Eg5 resulted in formation of less focused spindle poles (Figure 6C) with misaligned chromosomes in metaphase (Figures 6C and 6D); these phenotypes were similar to those of NuMA-depleted cells (Figures 4C and 4D). Taken together with the role for Eg5 in NuMA localization (Figure 5B), Eg5 likely regulates chromosome alignment by recruitment of NuMA to spindle poles. Indeed, in cells slightly depleted of Eg5, NuMA was less concentrated at the spindle poles (Figures 6E and 6F). Thus Eg5 is crucial for

centrosome separation and also functions in a subsequent step, *i.e.*, spindle assembly for chromosome alignment, via controlling NuMA recruitment to spindle poles.

Role for dynein in localization and function of NuMA

Reconstitution using mitotic frog egg extracts and demembranated frog sperm has revealed that minus-end-directed motor dynein and its activator dynactin associate with NuMA and thereby play an important role in NuMA targeting to spindle poles [15,20]. However, it has remained to be clarified whether dynein regulates NuMA localization in intact cells. To investigate the role for the dynein complex in HeLa cells, I knocked down dynein by treatment with siRNAs targeting the dynein heavy chain (DHC), which resulted in a significant loss of the dynein intermediate chain (DIC) (Figures 7A and 7C), as reported in human osteosarcoma cells [10]. On the other hand, the treatment did not affect Eg5 or NuMA at the protein level (Figure 7A). In dynein-depleted cells, NuMA was not restricted to the spindle poles but expanded along spindle fibers (Figures 7C, 8A, and 8B). Furthermore, dynein-depleted cells displayed aberrant spindle morphology (Figures 7D, 8A and 8B) and chromosome alignment defect (Figures 7B and 8C), both of which were also observed in NuMA-depleted cells (Figures 4C and 4D). These findings indicate that dynein participates in NuMA recruitment to the spindle poles along the spindle fibers, thereby functioning in bipolar spindle assembly for correct chromosome alignment.

In contrast to NuMA, which is almost exclusively present in the vicinity of the spindle poles, Eg5 localized along spindle microtubules with enrichment near the spindle poles (Figure 7D). Although the dynein–dynactin complex has been also reported to participate in localization of Eg5 to spindle poles on the basis of the results obtained in reconstitution experiments using frog egg extracts [35,36], cytoplasmic dynein appears to be dispensable for Eg5 accumulation at the spindle poles in intact mammalian cells [8,10]. I thus tested the role for dynein in Eg5 localization in HeLa cells. As shown in Figures 7D and 8B, in

dynein-depleted cells, Eg5 appeared to remain accumulated at spindle poles, suggesting that dynein does not play a major role in localization of Eg5 in HeLa cells. Taken together with the crucial role for dynein in NuMA localization (Figure 7C), it seems likely that Eg5 localizes to spindle poles in a dynein-independent manner and regulates NuMA recruitment to spindle poles in conjunction with dynein.

DISCUSSION

In the present study, I have identified the kinesin-5 family motor protein Eg5 as a novel NuMA-binding protein, using a proteomics approach and several *in vivo* and *in vitro* binding assays (Figures 1, 2, and 3). Eg5 directly binds to NuMA via the stalk region that exists between the N-terminal motor domain and the C-terminal tail region (Figure 3). During mitosis Eg5 appears to interact with NuMA in the vicinity of the spindle poles (Figure 1). The interaction does not occur in interphase cells, where Eg5 is distributed throughout the cytoplasm but NuMA exclusively localizes to the nucleus (Figure 1). The presence of Eg5, but not its motor activity, is required for proper accumulation of NuMA at the spindle poles (Figures 5 and 6), which likely enables NuMA to function in bipolar spindle assembly and chromosome alignment. In contrast to this study, Merdes *et al.* has reported that Eg5 did not co-immunoprecipitate with NuMA from metaphase-arrested frog egg extracts using antibodies raised against a NuMA C-terminal peptide [20]. The reason for this discrepancy is presently unknown. A possible explanation might be due to inhibition of NuMA–Eg5 interaction by antibodies used. Since NuMA interacts with Eg5 mainly via the C-terminal domain, as shown here, the interaction might be blocked by antibodies that bind to the NuMA C-terminus; endogenous Eg5 can be co-precipitated with the monoclonal antibody that recognizes the central coiled-coil region of NuMA (Figure 1).

This study thus provides a novel role of Eg5 in mitosis, *i.e.*, recruitment of NuMA for spindle assembly. It is well established that the motor activity of Eg5 plays an essential role in formation of spindle bipolarity in mammalian cells: Eg5 provides outward forces necessary for centrosome separation [3–9]. Indeed the duplicated centrosomes are not separated in HeLa cells treated with the Eg5 inhibitor STLC as well as in cells almost fully depleted of Eg5 (Figure 5). Because centrosome separation is a prerequisite for subsequent mitotic processes such as bipolar spindle assembly and chromosome alignment, the role of Eg5 in these later stages of mitosis has not been well investigated. As shown in the present study,

slight depletion of Eg5 allows centrosome separation in HeLa cells but prevents accumulation of NuMA to spindle poles (Figure 6). In these Eg5-depleted cells, both spindle assembly and chromosome alignment are perturbed (Figure 6), which phenotypes are similar to those observed in NuMA-depleted cells (Figure 4). Thus, besides centrosome separation, Eg5 also appears to function in spindle assembly via interacting with NuMA but not via its motor activity.

Furthermore, the present study demonstrates that targeting of NuMA to the spindle poles is crucial for its function, *i.e.*, bipolar spindle assembly and chromosome alignment, in intact cells. Depletion of Eg5 and dynein each induces mislocalization of NuMA and causes phenotypes similar to those of NuMA-knockdown cells (Figures 6 and 7). The significance of dynein in NuMA localization has been indicated by previous studies using a system reconstituted with mitotic *Xenopus laevis* egg extracts and demembranated *Xenopus* sperm [15,20]. In the reconstitution system, NuMA is essential for spindle assembly and is transported along the mitotic spindle by the minus end-directed motor cytoplasmic dynein, in a complex with the activator dynactin; inhibition of dynein blocks poleward transport of NuMA and spindle assembly. Consistent with these *in vitro* data, depletion of dynein in HeLa cells results in distribution of NuMA along spindle microtubules with a much less accumulation at spindle poles (Figure 7C).

As shown in the present study, recruitment of NuMA to the spindle poles (the minus ends of spindle microtubules) involves two microtubule motor proteins, which are intrinsically directed toward the opposite end: the plus-end-directed kinesin Eg5 and the cytoplasmic dynein directed toward the minus end. Similarly, both Eg5 and dynein are required for enrichment at spindle poles of the microtubule-associated protein TPX2 [37], although the motor activity of Eg5 appears to participate in transport of TPX2 [37] but not in that of NuMA (Figure 5). It is presently unclear about the mechanism by which these distinct motor proteins function in recruitment of NuMA to the spindle poles. It may be possible

that the plus-end-directed motor Eg5 is transported to spindle poles by the minus-end-directed motor dynein, and thus contributes to NuMA recruitment via direct interaction. This is because Eg5 is shown to move toward microtubule minus ends in the half spindle of *Xenopus laevis* egg extract spindles in a dynein-dependent manner [36,38]. It should be noted, however, that dynein does not seem to play a major role in Eg5 targeting to the spindle poles in intact mammalian cells [8,10] (Figure 7). Another possible scenario is that NuMA is transported poleward by the function of cytoplasmic dynein and subsequently tethered to the spindle poles via its direct interaction with Eg5. In either case, poleward microtubule flux [39] may be involved. The precise mechanism for NuMA localization by Eg5 and dynein should be clarified in future studies.

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FIGURE LEGENDS

Figure 1. Identification of Eg5 as a NuMA-binding protein. (**A**) Silver staining of proteins coprecipitated with FLAG–NuMA. HEK-293F cells transfected with an expression vector encoding FLAG–tagged NuMA (FLAG–NuMA) or the empty vector (Vector) were synchronized at mitotic phase by nocodazole treatment. Proteins of the cell lysates were immunoprecipitated with anti-FLAG affinity beads, subjected to 10% SDS-PAGE, and visualized by silver staining. Positions for marker proteins are indicated in kDa. (**B**) Localization of endogenous NuMA and Eg5 in interphase (upper panels) and mitotic phase (lower panels). Asynchronous HeLa cells were fixed in methanol, and subjected to indirect immunostaining with the anti-NuMA and anti-Eg5 antibodies. DNA was stained with Hoechst 33342. Scale bars, 5 μm. (**C** and **D**) Interaction between endogenous NuMA and Eg5 in mitotic cells. HeLa cells were synchronized using the double thymidine block method (**C**) or arrested at prometaphase by treatment with the Eg5-specific inhibitor STLC (**D**), as described under "EXPERIMENTAL PROCEDURES". Proteins of the cell lysates were immunoprecipitated (IP) with an anti-NuMA monoclonal antibody and then analyzed by immunoblot (Blot) with indicated antibodies. (**E**) Interaction between FLAG–NuMA and Eg5. HeLa cells were transfected with pEF-BOS-FLAG–NuMA, and arrested at prometaphase by treatment with STLC. Proteins of the cell lysates (Lysate) were immunoprecipitated (IP) with an anti-FLAG monoclonal antibody, and then analyzed by immunoblot (Blot) using the indicated antibodies.

Figure 2. Identification of Eg5 as an interacting partner of NuMA. (**A**) Amino acid sequences of Eg5 and peptides derived from a coimmunoprecipitant with NuMA. The full-length amino acid sequence of human Eg5 is shown in capital letters. A protein coimmunoprecipitated with NuMA is digested with trypsin, followed by LC–MS/MS analysis. The amino acid sequences of peptides detected by LC–MS/MS are shown in red. (**B**)

Interaction between FLAG–NuMA and HA–Eg5. COS-7 cells were transfected with a pair of pEF-BOS-FLAG–NuMA and pEF-BOS-HA encoding Eg5 or LGN. Proteins of the cell lysates (Lysate) were immunoprecipitated (IP) and then analyzed by immunoblot (Blot) using the indicated antibodies. (**C**) Effect of staurosporine on interaction between endogenous NuMA and Eg5 in mitotic cells. HeLa cells were synchronized by the double thymidine block method, followed by treatment with 20 nM staurosporine for 45 min. Proteins of the cell lysates (Lysate) were immunoprecipitated (IP) and then analyzed by immunoblot (Blot) using the indicated antibodies.

Figure 3. Interaction between NuMA and Eg5. (**A**) Schematic representation of the structures of the full-length (FL) and various lengths of human Eg5 and those of human NuMA. CH, calponin-homology domain. (**B**) Interaction of NuMA with the Eg5 stalk region (Eg5-S). FLAG–NuMA-FL was coexpressed in COS-7 cells with HA–Eg5-N, HA–-Eg5-S, or HA–Eg5-C. Proteins of the cell lysates (Lysate) were immunoprecipitated (IP) with the anti-FLAG antibody and analyzed by immunoblot (Blot) with indicated antibodies. (**C**) Two-hybrid interaction between NuMA and Eg5. The yeast HF7c cells were co-transformed with a pair of pGBT encoding NuMA-FL and pGADGH encoding both the stalk and tail regions of Eg5 (Eg5-SC). Following the selection for Trp^+ and Leu^+ phenotype, its histidine-dependent growth was tested. (**D** and **E**) Direct interaction between NuMA and Eg5. HA–Eg5-SC (**D**) or HA–Eg5-S (**E**) was synthesized *in vitro* by reticulocyte lysate and then incubated with GST–NuMA-N, GST–NuMA-C, or GST alone. The proteins were pulled down with glutathione-Sepharose beads and subjected to SDS-PAGE, followed by staining with CBB (*bottom panel*) or by immunoblot analysis with the anti-HA antibody (*upper and middle panels*). Positions for marker proteins are indicated in kDa. The data are representative of results from four independent experiments.

Figure 4. Abnormal spindle formation and chromosome misalignment in NuMA-depleted cells. (**A**) Effects of NuMA-specific siRNAs in protein levels of NuMA and Eg5. HeLa cells were transfected with NuMA-specific siRNAs (#1 and #2) or control RNAs. After culture for 48 h, the amounts of endogenous NuMA, Eg5, $p150^{Glued}$, and β-tubulin (as a loading control) were estimated by immunoblot analysis with indicated antibodies. (**B**, **D**, **E, and F**) Immunostaining of NuMA-depleted cells. HeLa cells were treated as in (**A**) and incubated with 5 μM MG132 for 2 h before fixation, followed by costaining with antibodies against β-tubulin (*red*) (**B**, **D**, and **E**), NuMA (*red*) (**F**), (*green*) (**B**), centromere (*green*) (**D**), or Eg5 (*green*) (**E** and **F**). DNA was stained with Hoechst 33342 (*blue*). Scale bar, 5 μm. (**C**) Chromosome alignment errors in NuMA-depleted cells in comparison with undepleted cells. Cells with chromosome alignment errors were counted ($n \geq 50$ cells/experiment) as described under "EXPERIMENTAL PROCEDURES". Error bars indicate ± S.D. from three independent experiments. ***$p < 0.005$, compared with cells transfected with control RNA.

Figure 5. Effect of almost complete depletion of Eg5 on NuMA localization. (**A**) Effect of a high concentration of Eg5-specific siRNAs on the protein level of Eg5 and NuMA. HeLa cells were synchronized by the double thymidine block method, and transfected with 40 nM Eg5-specific siRNAs (#1 and #2) or control RNAs, as described under "EXPERIMENTAL PROCEDURES." Cells transfected with control RNAs were treated with (+) or without (–) 5 μM STLC. The amounts of Eg5, NuMA, the dynein intermediate chain (DIC), and β-tubulin (as a loading control) were estimated by immunoblot analysis with indicated antibodies. (**B** and **C**) Localization of NuMA in STLC-treated or Eg5-knockdown cells. HeLa cells were treated as in (**A**), followed by fixation and costaining with the anti-NuMA and anti-γ-tubulin antibodies. Images in (**C)** were obtained at increased detector gain,

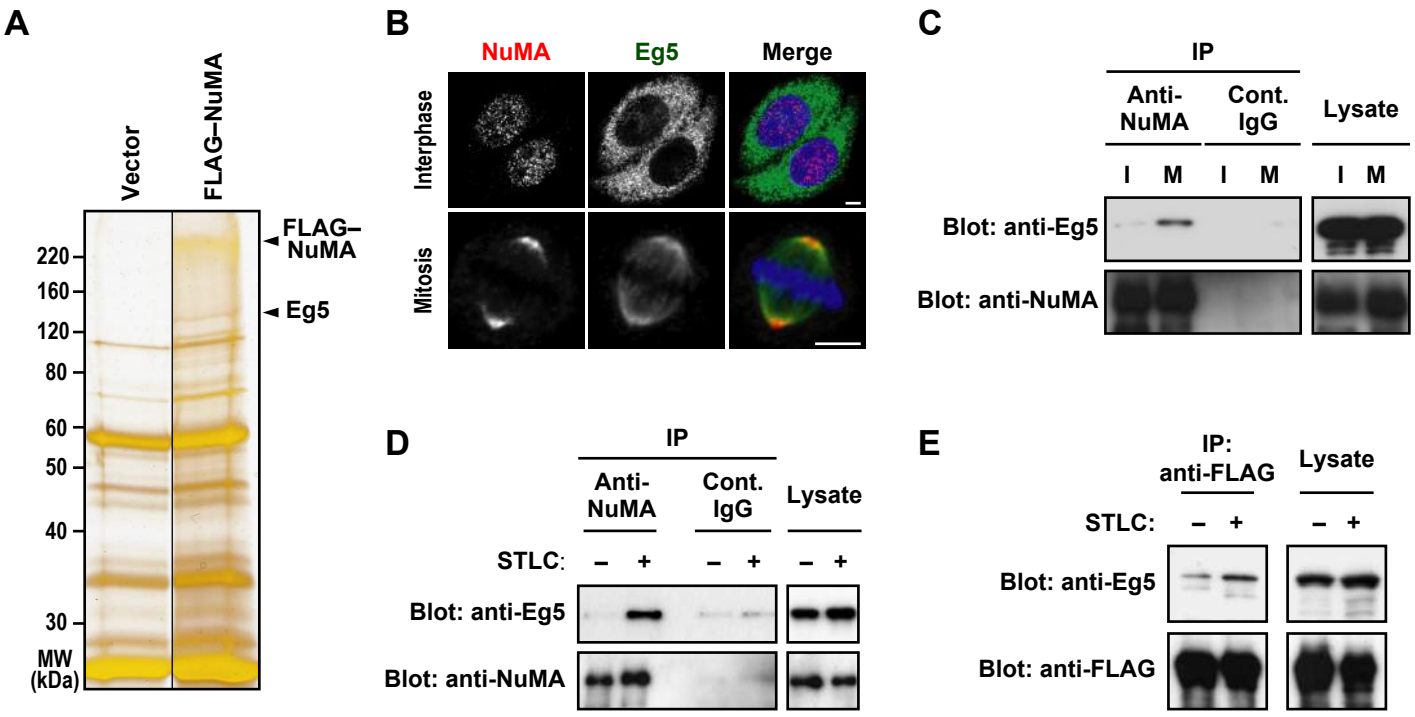
compared with those in (**B)**, for better visualization of the NuMA signal from the cytoplasm. DNA was stained with Hoechst 33342. Scale bar, 5 μm.

Figure 6. Effect of slight depletion of Eg5 on localization and function of NuMA. (**A** and **B**) Effect of a low concentration of Eg5-specific siRNAs in Eg5 protein level. HeLa cells were synchronized by the double thymidine block method, and transfected with 0.3 nM Eg5-specific siRNAs (#1 and #2) or control RNAs, as described under "EXPERIMENTAL PROCEDURES." The amounts of Eg5 and β-tubulin (as a loading control) were estimated by immunoblot analysis with indicated antibodies (**A**). The band intensity of Eg5 protein was normalized by that of β-tubulin and represented as histograms (**B**). Error bars indicate ± S.D. from three independent experiments. $*p < 0.05$, $**p < 0.01$, compared with cells transfected with control RNAs. (**C)** Chromosome alignment errors in cells slightly depleted of Eg5. HeLa cells were treated as in (**A**) and incubated for 2 h with 5 μM MG132, followed by costaining with antibodies against centromere (*green*) and β-tubulin (*red*). DNA was stained with Hoechst 33342 (*blue*). Scale bar, 5 μm. (**D)** Chromosome alignment errors in Eg5-depleted cells in comparison with undepleted cells. Cells with chromosome alignment errors were counted ($n \geq 50$ cells/experiment) as described under "EXPERIMENTAL PROCEDURES". Error bars indicate ± S.D. from three independent experiments. $***p < 0.005$, compared with cells transfected with control RNA. (**E** and **F)** Localization of NuMA in Eg5-depleted cells. HeLa cells were treated with as in (**C**), followed by co-staining with the anti-NuMA (*green*) and the anti-β-tubulin (*red*) antibodies (**E**). DNA was stained with Hoechst 33342 (*blue*). Scale bar, 5 μm. The amount of NuMA at the spindle pole, relative to that in the cytoplasm, was quantified ($n \geq 30$ cells/experiment). Error bars indicate ± S.D. from five independent experiments, $*p < 0.05$ compared with cells transfected with control RNAs.

Figure 7. Role for cytoplasmic dynein in localization of NuMA and Eg5. (**A**) Effect of the dynein heavy chain (DHC)-specific siRNAs. HeLa cells were transfected with dynein heavy chain (DHC)-specific siRNAs (#1 and #2) or control RNAs, as described under "EXPERIMENTAL PROCEDURES." The amounts of the dynein intermediate chain (DIC), Eg5, NuMA, and β-tubulin (as a loading control) were estimated by immunoblot analysis with antibodies against the respective protein. (**B**) Chromosome alignment errors in dynein-depleted cells in comparison with undepleted cells. Cells with chromosome alignment errors were counted ($n \geq 50$ cells/experiment) as described under "EXPERIMENTAL PROCEDURES." Error bars indicate ± S.D. from three independent experiments. ***$p < 0.005$, compared with cells transfected with control RNAs. (**C** and **D**) Localization of NuMA and Eg5 in dynein-depleted cells. HeLa cells were treated as in (**A**) and incubated for 2 h with 5 μM MG132, followed by costaining with antibodies against NuMA (*green*) and DIC (*red*) (**C**), or Eg5 (*green*) and β-tubulin (*red*) (**D**). DNA was stained with Hoechst33342 (*blue*). Scale bar, 5 μm.

Figure 8. Abnormal spindle formation and chromosome misalignment in dynein-depleted cells. HeLa cells were transfected with DHC-specific siRNA (#1 and #2) or control RNAs, and cultured for 72 h. The cells were incubated with 5 μM MG132 for 2 h before fixation, followed by staining with the anti-β-tubulin (*red*) and anti-NuMA (*green*) antibodies (**A**); the anti-Eg5 (*red*) and anti-NuMA (*green*) antibodies (**B**); or the anti-β-tubulin (*red*) and anti-centromeric (*green*) antibodies (**C**). DNA was stained with Hoechst 33342 (*blue*). Merged images are on the right. Scale bars, 5 μm.

Figure 1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MW (kDa)
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B
NuMA
Eg5
Merge
Interphase
Mitosis
C
IP
Anti-NuMA
Cont. IgG
Lysate
I M I M I M
Blot: anti-Eg5
Blot: anti-NuMA
D
IP
Anti-NuMA
Cont. IgG
Lysate
STLC: – + – + – +
Blot: anti-Eg5
Blot: anti-NuMA
E
IP: anti-FLAG
Lysate
STLC: – + – +
Blot: anti-Eg5
Blot: anti-FLAG

Figure 2

A

```
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  51 SVRTGGLADK SSRKTYTFDM VFGASTKQID VYRSVVCPIL DEVIMGYNCT
 101 IFAYGQTGTG KTFTMEGERS PNEEYTWEED PLAGIIPRTL HQIFEKLTDN
 151 GTEFSVKVSL LEIYNEELFD LLNPSSDVSE RLQMFDDPRN KRGVIIKGLE
 201 EITVHNKDEV YQILEKGAAK RTTAATLMNA YSSRSHSVFS VTIHMKETTI
 251 DGEELVKIGK LNLVDLAGSE NIGRSGAVDK RAREAGNINQ SLLTLGRVIT
 301 ALVERTPHVP YRESKLTRIL QDSLGGRTRT SIIATISPAS LNLEETLSTL
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 401 SEENFRVMSG KLTVQEEQIV ELIEKIGAVE EELNRVTELF MDNKNELDQC
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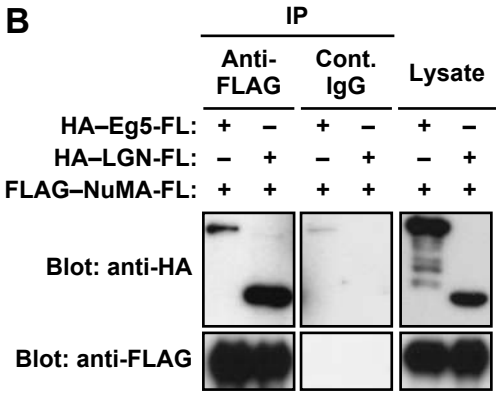
B



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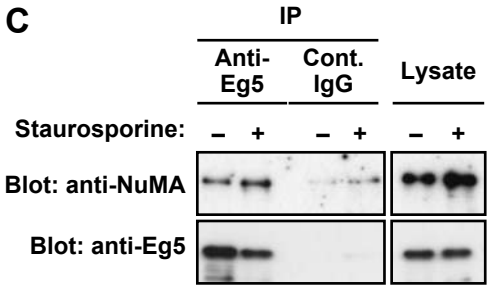
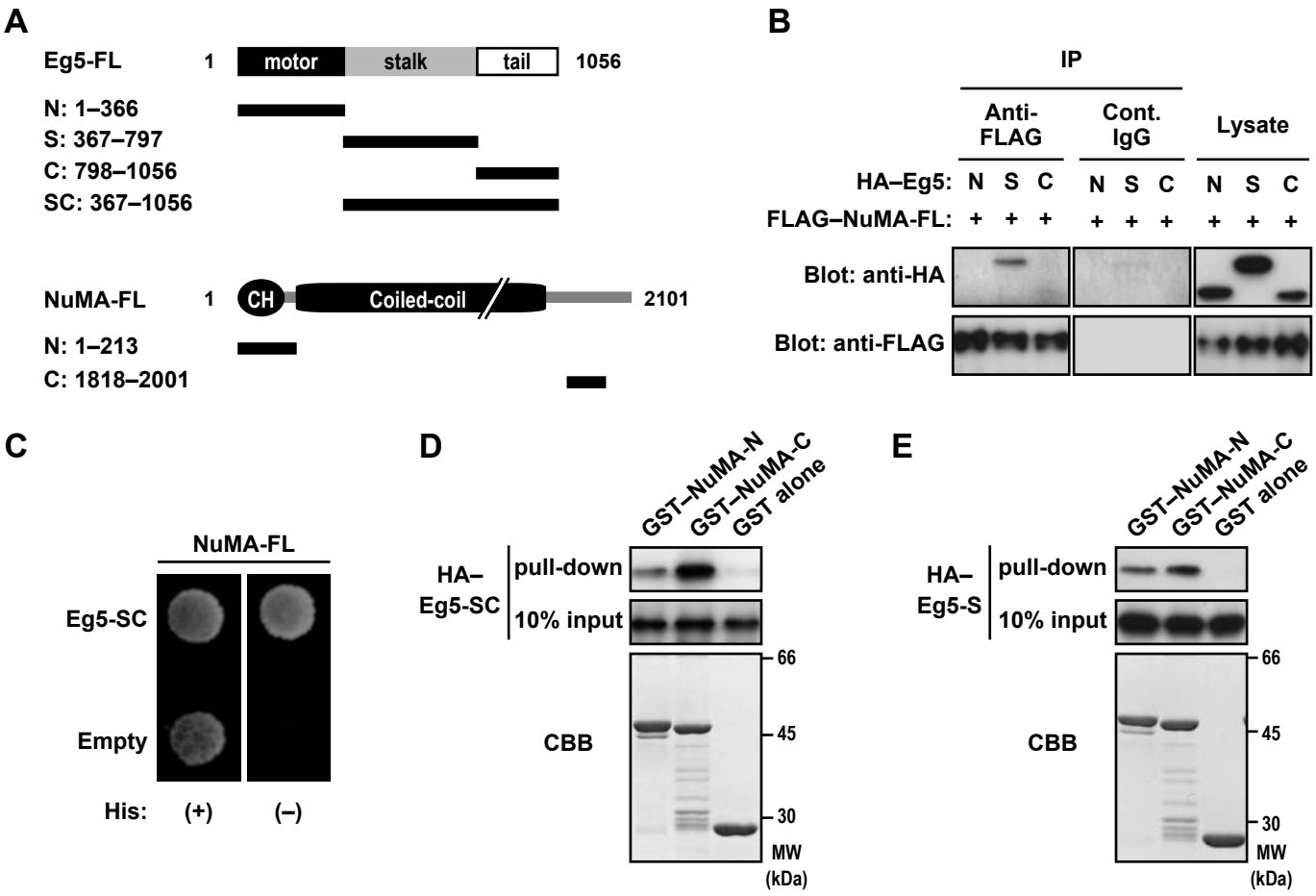


Figure 3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motor
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1
CH
Coiled-coil
2101
N: 1–213
C: 1818–2001
B
IP
Anti-FLAG
Cont. IgG
Lysate
HA–Eg5:
N S C N S C N S C
FLAG–NuMA-FL:
+ + + + + + + + +
Blot: anti-HA
Blot: anti-FLAG
C
NuMA-FL
Eg5-SC
Empty
His:
(+)
(–)
D
GST–NuMA-N
GST–NuMA-C
GST alone
HA–Eg5-SC
pull-down
10% input
CBB
66
45
30
MW (kDa)
E
GST–NuMA-N
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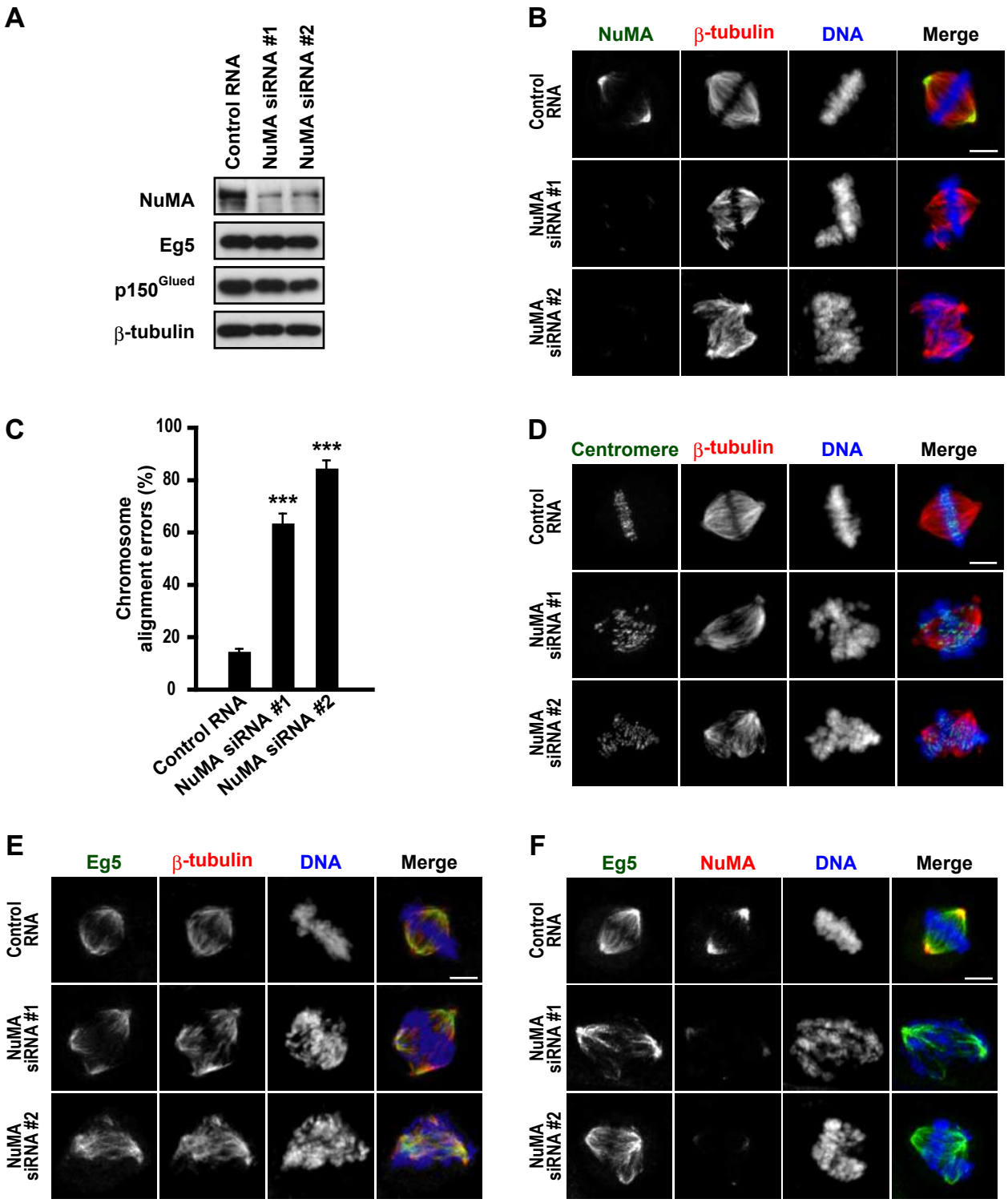
pull-down
10% input
CBB
66
45
30
MW (kDa)

Figure 4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NuMA siRNA #2
NuMA
Eg5
p150Glued
β-tubulin
B
NuMA
β-tubulin
DNA
Merge
Control RNA
NuMA siRNA #1
NuMA siRNA #2
C
Chromosome alignment errors (%)
100
80
60
40
20
0
***
***
Control RNA
NuMA siRNA #1
NuMA siRNA #2
D
Centromere
β-tubulin
DNA
Merge
Control RNA
NuMA siRNA #1
NuMA siRNA #2
E
Eg5
β-tubulin
DNA
Merge
Control RNA
NuMA siRNA #1
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Eg5
NuMA
DNA
Merge
Control RNA
NuMA siRNA #1
NuMA siRNA #2

Figure 5

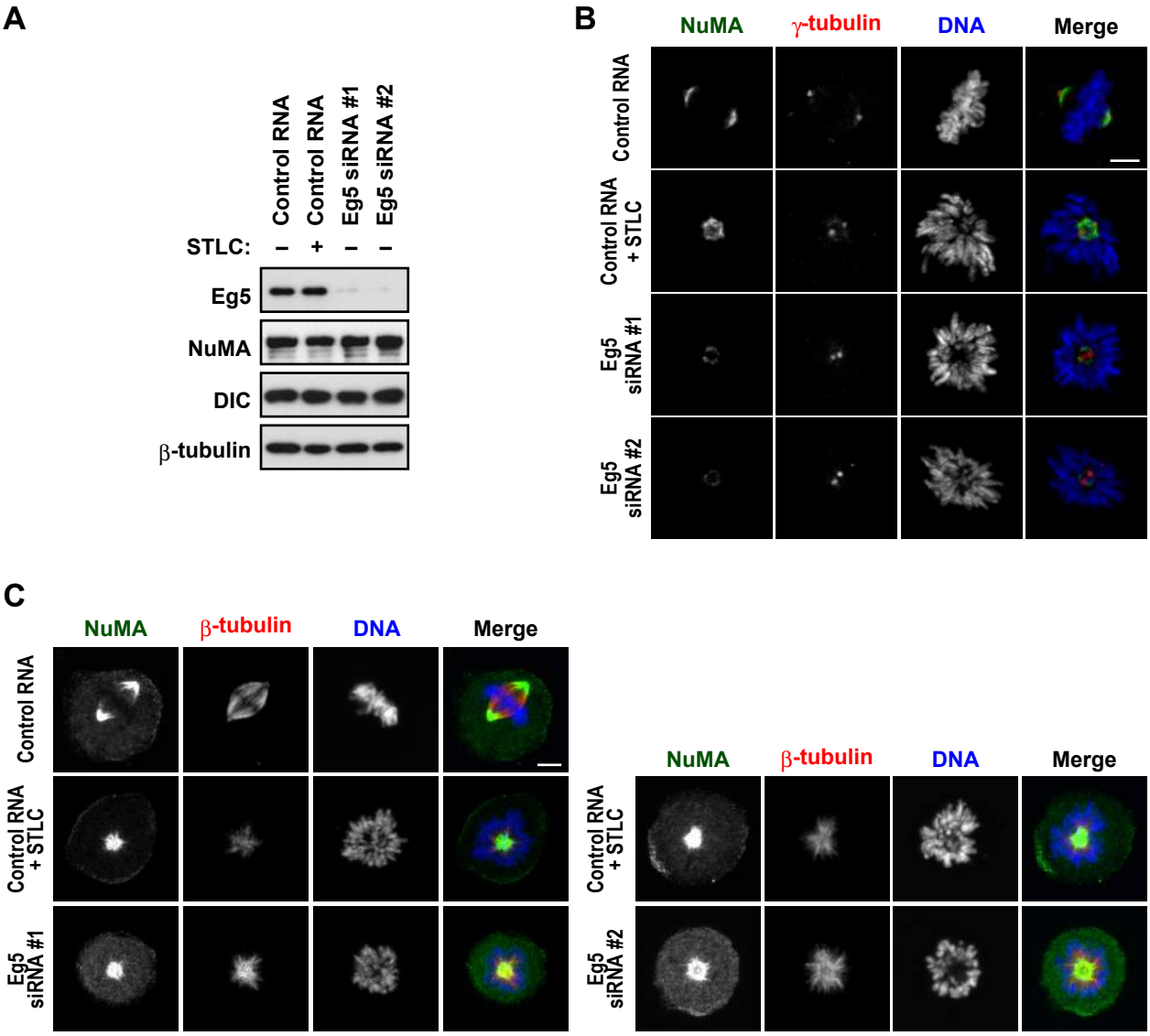

A
Control RNA
Control RNA
Eg5 siRNA #1
Eg5 siRNA #2
STLC: – + – –
Eg5
NuMA
DIC
β-tubulin
B
NuMA
γ-tubulin
DNA
Merge
Control RNA
Control RNA + STLC
Eg5 siRNA #1
Eg5 siRNA #2
C
NuMA
β-tubulin
DNA
Merge
Control RNA
Control RNA + STLC
Eg5 siRNA #1
NuMA
β-tubulin
DNA
Merge
Control RNA + STLC
Eg5 siRNA #2

Figure 6

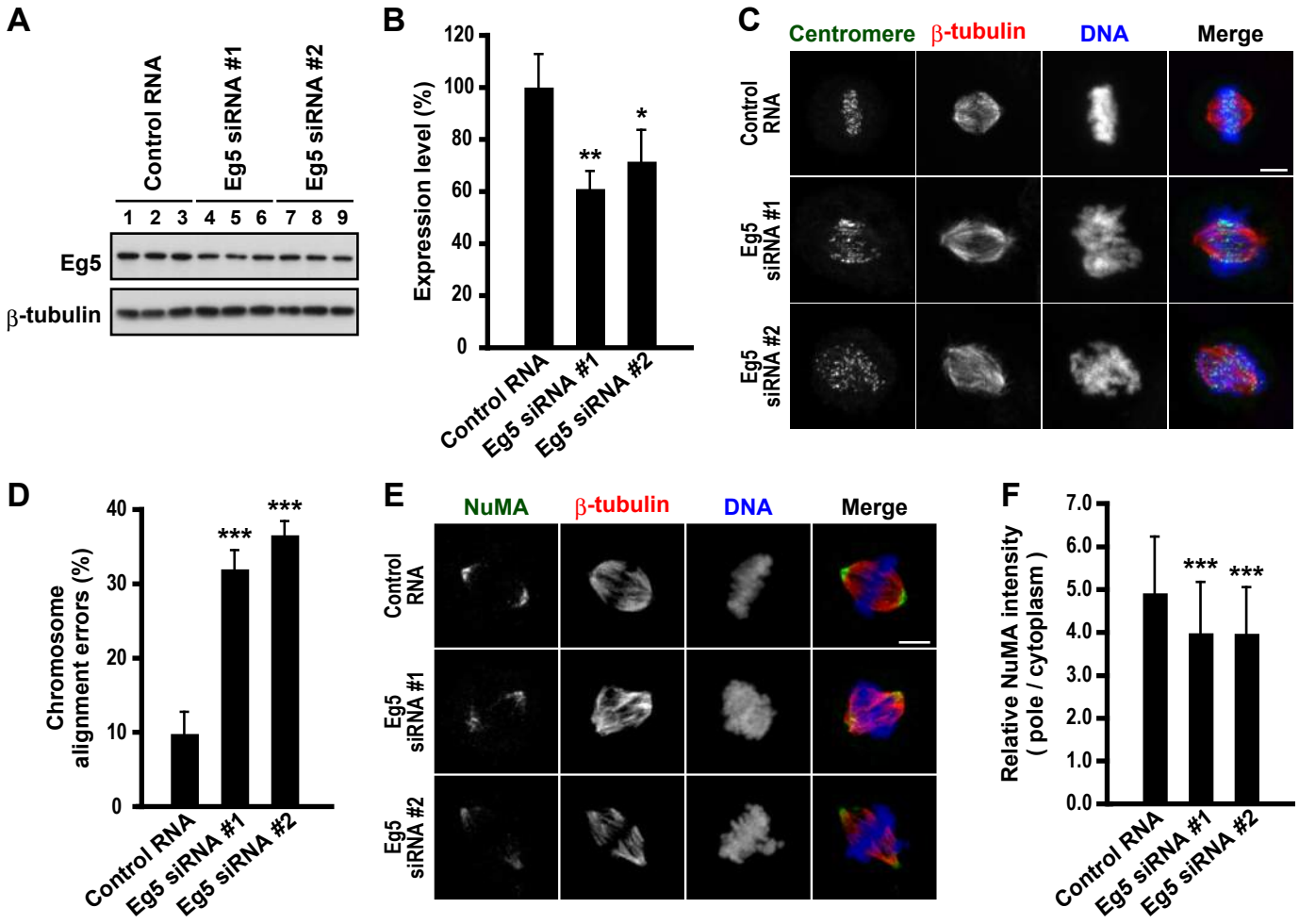

A
Control RNA
Eg5 siRNA #1
Eg5 siRNA #2
1 2 3 4 5 6 7 8 9
Eg5
β-tubulin
B
Expression level (%)
120
100
80
60
40
20
0
**
*
Control RNA
Eg5 siRNA #1
Eg5 siRNA #2
C
Centromere
β-tubulin
DNA
Merge
Control RNA
Eg5 siRNA #1
Eg5 siRNA #2
D
Chromosome alignment errors (%)
40
30
20
10
0
***
***
Control RNA
Eg5 siRNA #1
Eg5 siRNA #2
E
NuMA
β-tubulin
DNA
Merge
Control RNA
Eg5 siRNA #1
Eg5 siRNA #2
F
Relative NuMA intensity (pole / cytoplasm)
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
***
***
Control RNA
Eg5 siRNA #1
Eg5 siRNA #2

Figure 7

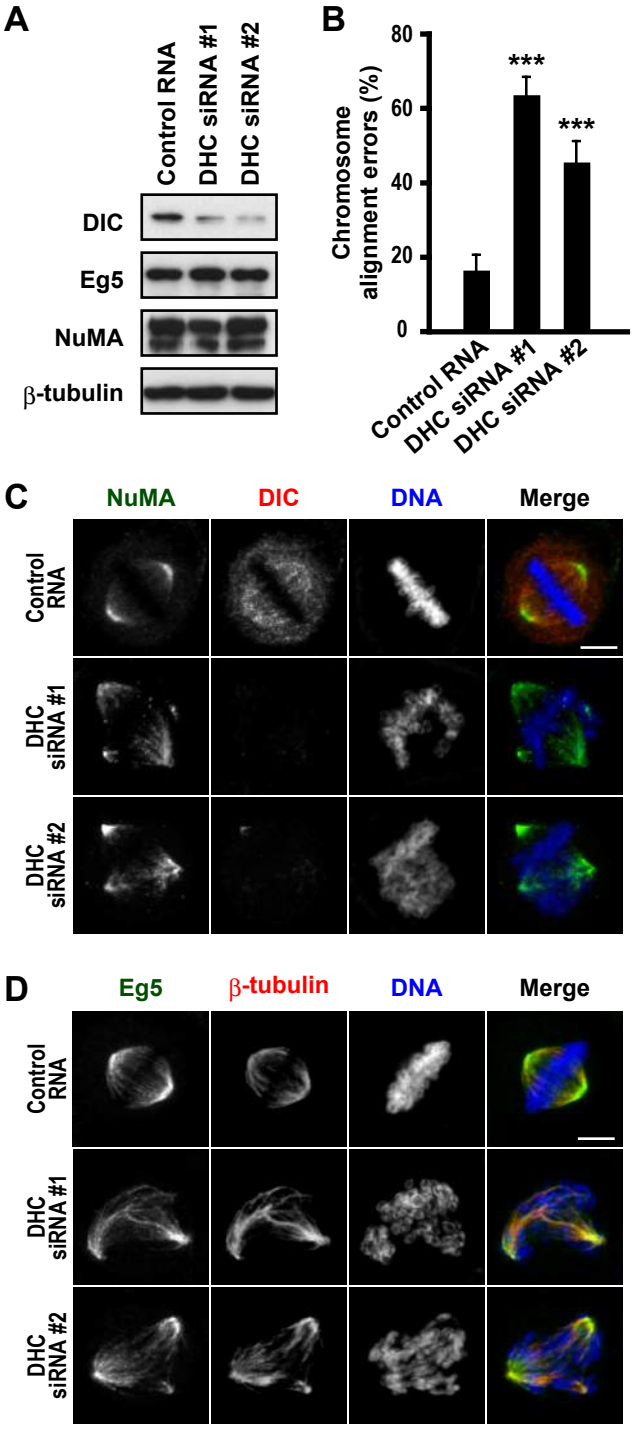
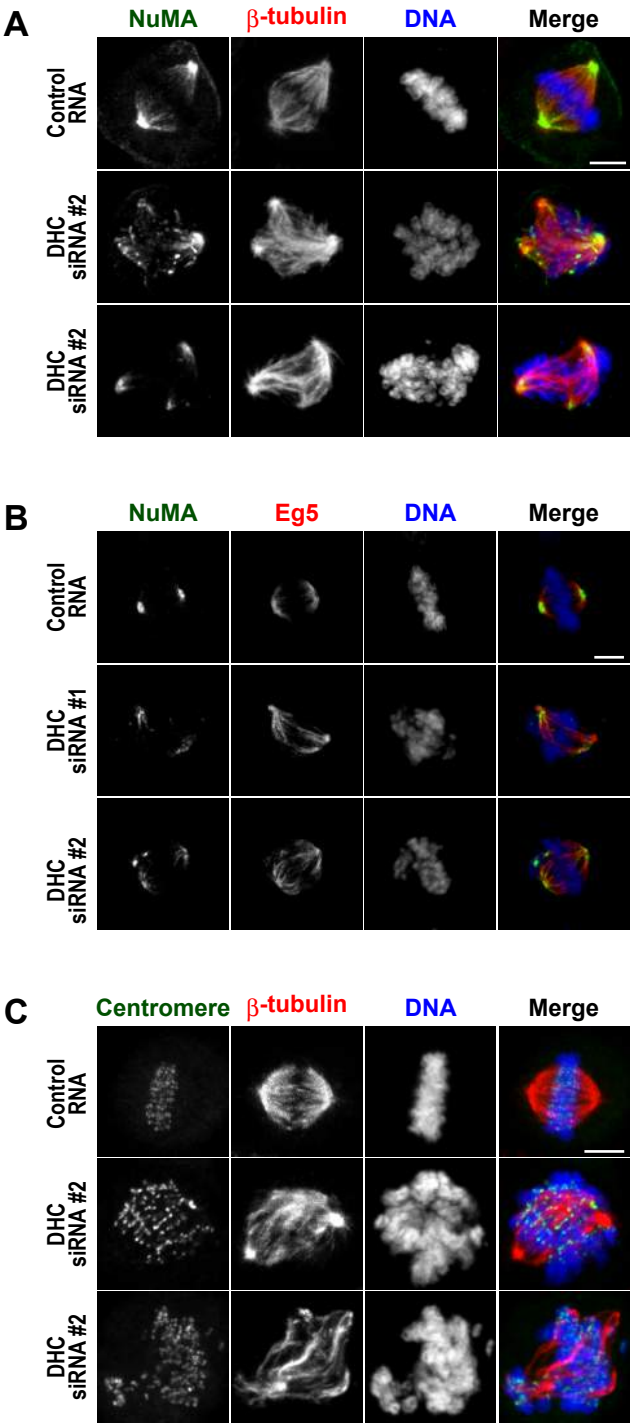

A
Control RNA
DHC siRNA #1
DHC siRNA #2
DIC
Eg5
NuMA
β-tubulin
B
Chromosome alignment errors (%)
80
60
40
20
0
***
***
Control RNA
DHC siRNA #1
DHC siRNA #2
C
NuMA
DIC
DNA
Merge
Control RNA
DHC siRNA #1
DHC siRNA #2
D
Eg5
β-tubulin
DNA
Merge
Control RNA
DHC siRNA #1
DHC siRNA #2

Figure 8


A
NuMA
β-tubulin
DNA
Merge
Control RNA
DHC siRNA #2
DHC siRNA #2
B
NuMA
Eg5
DNA
Merge
Control RNA
DHC siRNA #1
DHC siRNA #2
C
Centromere
β-tubulin
DNA
Merge
Control RNA
DHC siRNA #2
DHC siRNA #2