## SUPPLEMENTAL METHODS

### *cDNA construction*

Full-length human Hec1 was generated by PCR from a human Testis cDNA library (Clontech). For mammalian expression of the full-length of Hec1 and its mutants, Hec1 cDNA was digested with *Kpn* I and *Sma* I and cloned into pEGFP-N2 vector (Clontech, Palo Alto, CA), while the full-length of CENP-H cDNA was digested with *Bgl* II and *EcoR* I, and cloned into pEGFP-C1 or DsRed-C1 vector (Clontech, Palo Alto, CA). GFP-Hec1 mutations S165A and S165E were created by standard PCR methods. All constructs were sequenced in full.

### *Pull-down assay*

The GST-Spc24/25 affinity pull-down was previously described (Ciferri *et al,* 2005). In brief, GST-Spc24/25 complex immobilized on the glutathione agarose beads (Sigma) was used as an affinity matrix to incubate with purified Nuf2-Hec1$^{S165A}$ and Nuf2-Hec1$^{S165E}$, respectively. The agarose beads were washed with PBS five times before being boiled in SDS-PAGE sample buffer. The proteins were then fractionated on SDS-PAGE and the gel was stained with Coomassie brilliant blue.

### *Transient transfection and immunoprecipitation*

293T cells were grown to ~50% confluency in DMEM with 10% FBS at 37 °C in 10% $CO_2$, and were transfected with GFP–Hec1 by Fugene 6 (Roche, CA), according to manufacturer's protocol. Cells were collected 24~36 h after transfection, and proteins were solubilized in lysis buffer (50 mM HEPES pH 7.4, 150 mM NaCl, 2 mM EGTA, 0.1% Triton X-100, 1 mM PMSF, 10 μg/ml leupeptin and 10 μg/ml pepstatin A). Lysates were clarified by centrifugation at 16,000 *g* for 10 min at 4 °C. GFP-Hec1 proteins were precipitated using a rabbit anti-GFP antibody bound to protein-A/G beads (Pierce Chemical, IL). Beads were washed five times with lysis buffer and then boiled in protein sample buffer for 2 min. After SDS–PAGE, proteins were transferred to nitrocellulose membrane. The membrane was divided into three strips and probed with antibodies against Hec1, and phospho-Ser165 (Chen *et al,* 2002),

respectively. Immunoreactive signals were detected with ECL kit (Pierce Chemical, IL) and visualized by autoradiography on Kodak BioMAX film.

***Immunofluorescence microscopy***

For immunofluorescence, cells were seeded onto sterile, acid-treated 18-mm coverslips in 6-well plates (Corning Glass Works, Corning, New York). Double thymidine blocked and released HeLa cells were transfected with 2 μg/ml lipofectamine 2000 pre-mixed with various Hec1 constructs as described above. In general, 36 hours post-transfection, HeLa cells were rinsed for 1 min with PHEM buffer (100 mM PIPES, 20 mM HEPES pH 6.9, 5 mM EGTA, 2 mM $MgCl_2$ and 4 M glycerol) and were permeabilized for 2 min with PHEM plus 0.2% Triton X-100 as described (Yao *et al,* 1997). Extracted cells were then fixed and blocked prior to incubation with various primary antibodies. Monoclonal antibody (9G3, Novus Biologicals; Littleton, CO) bound to Hec1 was visualized using fluorescein-conjugated goat anti-mouse IgG, respectively, while binding of anti-centromere antibody (ACA) was visualized using Texas Red-conjugated goat anti-human IgG+IgM. DNA was stained with DAPI (Sigma Chemical Co.). CENP-H staining was achieved using a rabbit peptide antibody reacting with its C-terminal 47 amino acids (Bethyl Laboratories, Montgomery TX). Slides were examined with a Zeiss axiovert-200 fluorescence microscope and images were collected and analyzed with Image-5 (Carl Zeiss, Germany). All images were acquired as Z-stacks with 0.2-μm spacing using a 100 X 1.35 NA objective on a Zeiss LSM510-NLO (Carl Zeiss, Germany. Maximal intensity projections of the entire Z-stack are shown, and optical sections show individual kinetochores more clearly (insets). Image analysis was performed using either Metamorph (Universal Imaging, Downington, PA) software. Quantification of the level of kinetochore-associated protein was conducted as described (Johnson *et al,* 2005; Liu *et al,* 2007)

***Cell Culture, Transfection and Synchronization***

HeLa and 293T cells, from American Type Culture Collection (Rockville, MD), were maintained as subconfluent monolayers in DMEM (Invitrogen; Carlsbad, CA) with 10% FCS (Hyclone, Logan, UT) and

100 U/ml penicillin plus 100 μg/ml streptomycin (Invitrogen; Carlsbad, CA).

The siRNA-mediated NEK2A protein knock-down was performed as described (e.g., Lou *et al,* 2004). In brief, HeLa cells were transfected with 21-mer siRNA oligonucleotides or control scramble oligonucleotide synthesized by Dharmacon Research, Inc. (Boulder, CO). To examine whether the phosphorylation of Ser165 is essential for the localization of Hec1 to the kinetochore, we introduced exogenous GFP-Hec1 (wild type and mutant) into HeLa cells 24 hours after siRNA treatment. Trial experiments revealed that exogenous GFP-Hec1 protein expression reaches a level comparable to that of endogenous at 36 hours after the plasmid transfection.

To synchronize transfected HeLa cells in prometaphase/metaphase, 10 μM MG132 and 10 ng/ml nocodazole (Sigma, St. Louis, MO) were included in cell culture 24 hours post-transfection for additional 18 hrs before harvested for Western blotting and flow cytometric analyses. MG132 was included in the nocodazole treatment to prevent sister chromatid separation and APC/C-mediated NEK2A degradation (e.g., Hames et al., 2001; Hayes *et al.*, 2006). Western blotting analysis of HeLa cells synchronized by nocodazole using anti-phospho-Ser10 of histone 3 (Upstates Biotechnology; New York) confirmed that nocodazole treatment arrested cell in prometaphase/metaphase (Fig. S2B).

To collect naturally cycled mitotic cells, mitotic shake-off was carried out as described previously (e.g., Yao *et al.*, 1997).

***Flow Cytometry***

Flow cytomertic analysis of cell cycle distribution was carried out as described (e.g. Yuan *et al.*, 2007). Briefly, transfected and nocodazole-treated adherent HeLa cells were trypsinized and pooled with the floating cells. Cell suspension of 1 x $10^6$ cells was washed with PBS, fixed in ice-cold 70% ethanol at -20 °C, and stained with a 20 μg/ml propidium iodide, 0.1% Triton X-100, 200 μg/ml RNase A solution. Stained cells were analyzed using a FACScan (BD Biosciences, CA) and Modfit 2.0.

***Analysis of kinetochore position along the spindle***

Kinetochore positions were determined from maximal intensity projections of confocal images, using an automated analysis. Based on ACA staining, objects (representing kinetochores) were defined as regions of contiguous pixels with intensity above a threshold. For each object, an internal threshold of 75% of the maximum for that object was used to separate overlapping objects. An average of 30 kinetochore pairs per cell was selected by this method. The position of each kinetochore was recorded as the object centroid, as well as the positions of the two spindle poles, determined from $\gamma$-tubulin staining. For each kinetochore, the nearest point on the pole-pole line was determined, and the distance from that point to the nearest pole was calculated and normalized by the pole-pole distance. Misaligned kinetochores were defined as those with normalized distance <0.2. More than 10 cells (>300 kinetochores) were analyzed for each experiment expressing either wild type Hec1, Hec1$^{S165A}$, and Hec1$^{S165E}$, respectively.

***Western blot***

Samples were subjected to SDS-PAGE on 6~16% gradient gel and transferred onto nitrocellulose membrane. Proteins were probed by appropriate primary and secondary antibodies and detected using ECL (Pierce, IL). The band intensity was then scanned using a Phosphoimager (Amersham Bioscience, NJ).

**References:**

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Liu D, Ding X, Du J, Cai X, Huang Y, Ward T, Shaw A, Yang Y, Hu R, Jin C, Yao X. (2007). Human NUF2 interacts with CENP-E and is essential for a stable spindle microtubule-kinetochore attachment. *J Biol. Chem.* **282:** 21415-21424

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# Fig S1

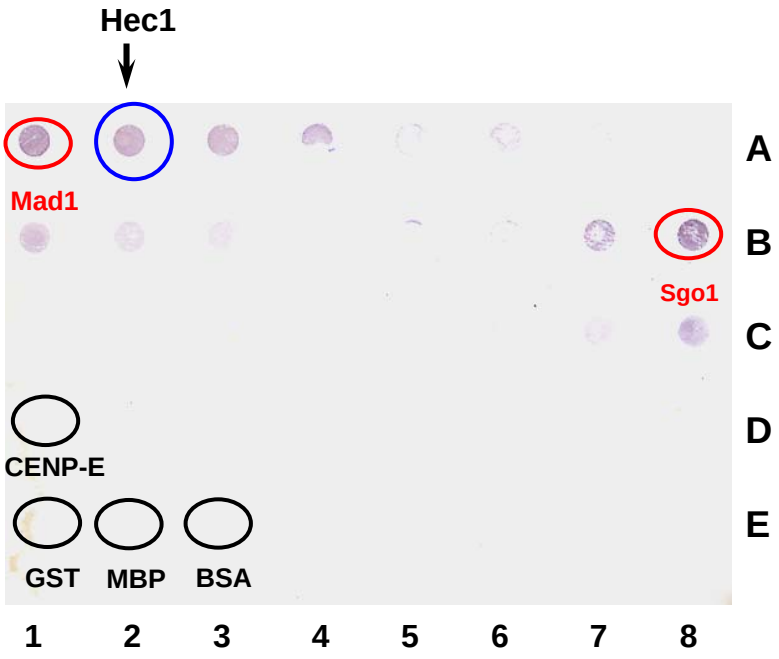


**Fig S1. A "high-content" far-western blotting screen for identification of NEK2A interacting proteins.**
Aliquots (0.5 µg per sample) of GST-tagged kinetochore proteins such as CENP-E, Hec1 and Sgo1 were dipped onto a nitrocellulose membrane. The membrane was then blocked and incubated with histidine-tagged NEK2A protein (1 µg/ml) for 2 h as described under "Methods". After washing, the blot was then incubated with an anti-NEK2A antibody and developed by using alkaline phosphatase substrates. Note that NEK2A did not react with CENP-E, GST, MBP and BSA, verifying the specificity of Hec1-NEK2A interaction. Sgo1 and MAD1 serve as positive control for this far-western blotting screen.

# Fig S2

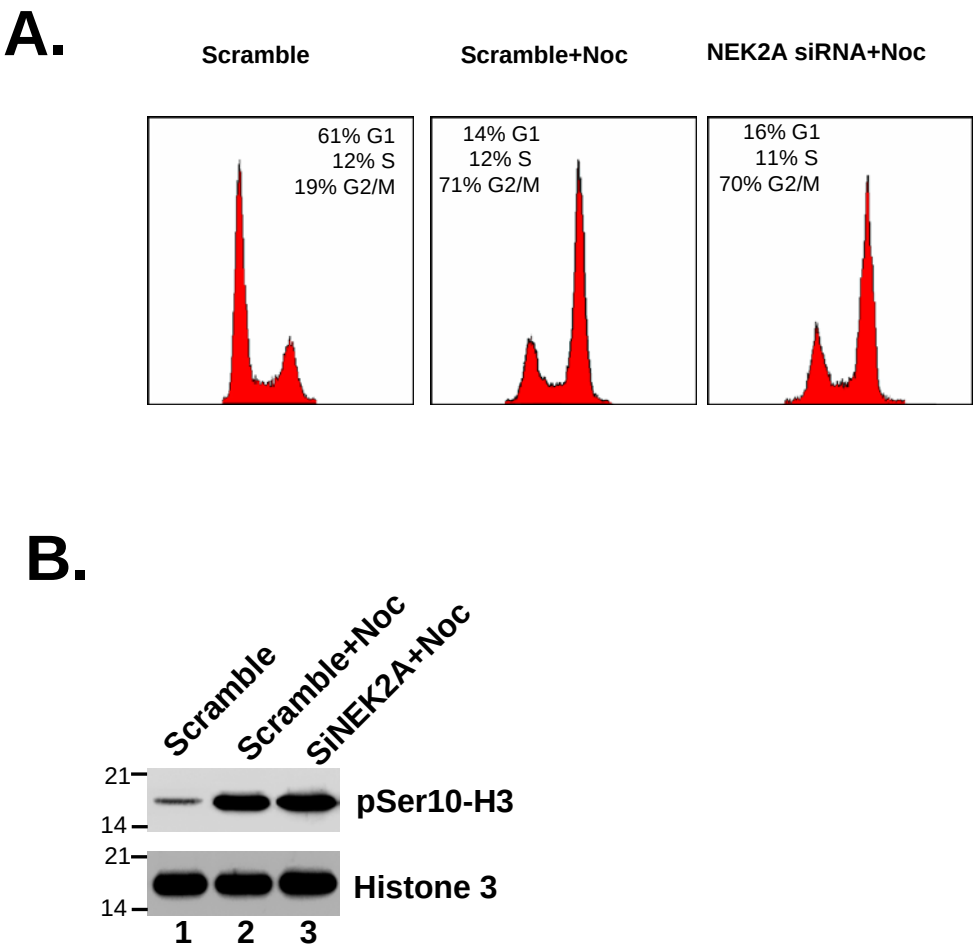


**Fig. S2. Cell cycle analysis of HeLa cells synchronized by nocodazole**

A. Cell cycle analyses of HeLa cells treated with NEK2A siRNA and synchronized with 10 ng/ml nocodazole (Noc). Twenty-four hours after the siRNA transfection, these cells were exposed to 10 ng/ml nocodazole and 10 μM MG132 for 18 hours. At the end of incubation, these cells were then stained with PI and assessed for cell cycle distribution.

B. Aliquots of treated cells as described in **A** were subjected to Western blotting analysis using anti-phospho-serine 10 (histone 3; pSer10-H3) antibody. Equal amount of total proteins (35 μg) was applied from each preparations.

# Fig S3

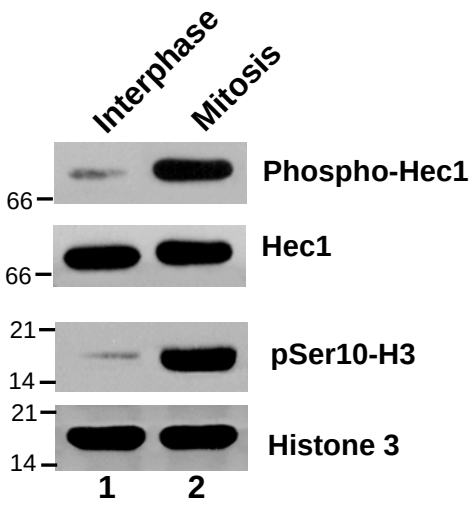


**Fig. S3. Hec1$^{S165}$ is phosphorylated in mitosis**
To test if Hec1S165 is phosphorylated in naturally cycled cells, mitotic HeLa cells were harvested by shake-off followed by sedimentation and solubilization SDS-PAGE sample buffer. The extracts were then sonicated and centrifuged to remove the residual insoluble materials. Equal amount of mitotic and interphase cell lysates were applied for Western blotting analyses using anti-histone 3 phospho-serine 10 antibody (pSer10-H3) and phospho-Hec1$^{S165}$ antibody (Phospho-Hec1), respectively.

**Supplemental Table 1. Inhibition of Hec1 phosphorylation releases the tension between sister kinetochores**

| Treatment | Distance (μm)* |
|---|---|
| Wild type Hec1 | 1.7±0.15 |
| $Hec1^{S165A}$ | |
| *Aligned chromosomes* | 1.5±0.13 |
| *Lagging chromosomes* | 1.1±0.21 |
| $Hec1^{S165E}$ | 1.8±0.19 |

*Distance between ACA-labeled sister-kinetochores. Data obtained from >300 kinetochore pairs in which both sister kinetochores were in the same focal plane (see Methods).