Current Biology, Volume *18*

Supplemental Data

Kinetochore-Microtubule Attachment Relies on the Disordered N-Terminal Tail Domain of Hec1

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Supplemental Experimental Procedures

PtK1 Hec1 Sequence Identification

The sequence for PtK1 *HEC1* was determined using degenerate primers and reverse transcriptase PCR (RT-PCR) as previously described [1]. Briefly, Hec1 nucleotide sequences from *Xenopus laevis, Gallus gallus, Homo sapiens, and Mus musculus* were aligned using the Clustal W program (http://www.ebi.ac.uk/Tools/clustalw2/index.html). Both 5' and 3' degenerate primers were designed to flank a region of approximately 1000 base pairs of Hec1 nucleotide sequence. RNA was isolated from PtK1 cells (Mo Bio RNA isolation kit) and RT-PCR was carried out using the 5' and 3' degenerate primers and PtK1 RNA as a template. The amplified PCR fragment was sequenced and aligned with the human Hec1 sequence. A PtK1-specific small, interfering RNA (siRNA) that differed in nucleotide sequence from the human Hec1 sequence was designed so that PtK1-specific siRNA-resistant human Hec1 could be introduced into PtK1 cells. We determined the remaining PtK1 Hec1 sequence by designing additional primer pairs in which one primer was degenerate and the other non-degenerate, based on our initial PtK1 Hec1 sequence. Hec1 siRNA (UGAGCCGAAUCGUCUAAUAU) was ordered from Qiagen.

Cell Culture and Drug Treatment

Photo-activatable GFP-tubulin PtK1 cells (a gift from Dr. Alexey Khodjakov, Wadsworth Center, New York Department of Health) were cultured in HAMS F-12 medium (Invitrogen) supplemented with 10% fetal bovine serum (Atlanta Biologics), 1% antibiotic/antimycotics (Invitrogen) and grown at 37°C in a humidified incubator with 5% CO_2. For immunofluorescence experiments, cells were grown on acid washed, sterile coverslips. For live cell imaging experiments, cells were grown in 35mm glass bottom dishes (MatTek Corp.) and imaged in

Leibovitz's L-15 medium supplemented with 10% fetal bovine serum, 1% antibiotic/mycotics, and 4.5g/L glucose. For nocodazole (Sigma) experiments, cells were treated with 20μM of the drug for 1hr at 37°C and then either fixed for immunostaining or time-lapse imaged. For cold-induced microtubule depolymerization assays, cells were lysed for 5 min at 20ºC with 0.5% Triton X-100 in PHEM buffer (60 mM PIPES, 25 mM HEPES, 10 mM EGTA, 4 mM $MgSO_4$, pH 6.9) plus 10 mM ATP. Cells were subsequently incubated at 4ºC in a calcium-containing buffer (60 mM PIPES, 25 mM HEPES, 4 mM $MgCl_2$, and 10 mM $CaCl_2$, pH 7.0) for 60 min. Cells were then processed for immunofluorescence, described below.

Immunofluorescence

Cells were rinsed rapidly in PHEM buffer followed by a 5 sec pre-fixation in 4% paraformaldehyde. Cells were lysed for 5 min in freshly prepared lysis buffer (PHEM buffer + 0.5% Triton X-100). Following lysis, cells were fixed for 20 min at room temperature in freshly prepared PHEM plus 4% formaldehyde (Ted Pella Inc.). Cells were rinsed in PHEM + 0.1% Triton-X-100 (3 x 5 min) and blocked in 5% boiled donkey serum in PHEM for 1 hr at room temperature. Antibodies were used at the following dilutions: Hec1 9G3, 1:1000 (GeneTex); DM1α anti-tubulin, 1:200 (Sigma); ACA (anti-centromere antibody), 1:300 (Antibodies, Inc.); rat tubulin, 1:300 (Sigma); and Mad2 (a gift from Dr. Ted Salmon), 1:1000. Secondary antibodies conjugated to Cy5, Alexa488, or Rhodamine RedX (Jackson ImmunoResearch) were used at a dilution of 1:300. All antibodies were diluted in 5% boiled donkey serum in 1xPHEM buffer. Cells adhered to coverslips were incubated in primary antibodies overnight at 4°C, rinsed in PHEM-T (3 x 5 min), incubated in the secondary antibody for 45 min at room temperature, rinsed in PHEM-T (3 x 5 min), quick-rinsed in 1X PHEM, counterstained with DAPI, and mounted in an antifade solution containing 90% glycerol and 0.5% *N*-propyl gallate. For silence and rescue experiments, cells were analyzed if they were both Cy5 positive (indicating transfection with the labeled Hec1 siRNA) and GFP-positive (indicating transfection with Hec1-GFP fusion protein). GFP positive cells were chosen for analysis based on average kinetochore fluorescence intensity. Raw intensity values of GFP fluorescence at kinetochores of cells transfected with full-length Hec1-GFP ranged from 200,000 counts (total integrated intensity) to 800,000 counts. For mutant Hec1-GFP constructs, cells with kinetochore GFP intensity levels within this range were analyzed.

Microscopy and Image Acquisition

All microscopy was performed using a DeltaVision PersonalDV Imaging System (Applied Precision) equipped with a Photometrics CoolSnap HQ^2 camera and a 100x/1.30NA Phase Planapochromat oil immersion lens (Olympus). For immunofluorescence experiments, Z-stacks at 0.2 μm intervals were acquired through each cell. For live-cell imaging, stage temperature was maintained at 37°C with an environmental chamber. For live-cell imaging of silence/rescue experiments, phase contrast time-lapse images were acquired every 3 minutes using a 100x/1.30NA Phase Planapochromat oil immersion lens. At the beginning of time-lapse imaging, a single fluorescence image was acquired for each cell to confirm GFP-fusion protein expression and Cy-5-labeled siRNA transfection. A 20X/0.5NA Phase Planapochromat objective was used for low magnification time-lapse imaging (Olympus). For these experiments, transmitted light and fluorescence images were collected every 3-5 min for 10 hr.

Image Analysis

Kinetochore fluorescence intensity measurements were carried out as described previously [2] using Metamorph software (Molecular Devices). The intensity of a test protein was calculated by determining the ratio of its calculated intensity to the corresponding intensity of ACA fluorescence. Microtubule end-on attachment was measured using SoftWorx software (Applied Precision) by analyzing Z-stacks of deconvolved images.

RNAi and Recombinant Protein Expression

Cells were transfected with either Hec1 siRNAs using Oligofectamine (Invitrogen) or mock-transfected with Oligofectamine alone. For coverslips, 4 μL of Oligofectamine were incubated at room temperature with 46 μL of OptiMem (Invitrogen) for 5 min with periodic flicking of the tube to mix. After incubation, 8 μL of 20 μM siRNA and 142 μL of OptiMem were added to the tube and incubated for 20-30 min at room temperature with periodic flicking to mix. Following incubation, the solution was added to 800 μL of Optimem + 10% FBS and added to a coverslip in a 6-well dish or 35 mm Petri dish. Cells were supplemented with 1 mL of OptiMem + 10% FBS 24 hr post-transfection, and assayed at 48 hr post-transfection. For silence/rescue experiments, GFP-fusion constructs were co-transfected into cells at the time of siRNA transfection. For coverslips, 7 μL Oligofectamine were incubated at room temperature with 143 μL of OptiMem (Invitrogen) for 5 min with periodic flicking. After incubation, 8 μL of 20 μM siRNA, 1 μL of plasmid DNA (1 mg/mL), and 142 μL of OptiMem were added to the tube and

allowed to incubate with periodic flicking for 20-30 min at room temperature. Following incubation, the solution was added to 2 mL of Optimem + 10% FBS and added to a coverslip in a 6-well dish or 35 mm Petri dish. Cells were supplemented with 1 mL of OptiMem + 10% FBS 24 hr post-transfection and assayed at 48 hr post-transfection.

Electron Microscopy

Cells were grown on coverslips etched with a finder grid (Electron Microscopy Sciences, Hatfield, PA). Transfected cells were identified by fluorescence and by the phenotype as determined by time-lapse imaging. Coordinates on the grid were noted and the filmed cells followed through EM preparation using standard same cell correlative light and electron microscopy [3]. Briefly, cells were fixed for 30 minutes in 2.5% EM grade glutaraldehyde (Sigma, St Louis, MO), rinsed, incubated for 15 minutes in 0.15% tannic acid, rinsed, post-fixed for 60 minutes in 2.0% osmium tetroxide, rinsed, enblock stained for 60 minutes with 1% uranyl acetate, and rinsed. After the final rinse the cells were dehydrated in a graded ethanol series, followed by propylene oxide, and embedded in Epon. Cells were relocated, marked with on the Epon side with a diamond objective scribe, and the coverslip removed with hydrofluoric acid. After remounting and trimming the specimen, plastic sections 80-100 nm thick were cut and imaged on a Phillips 420 or a Zeiss 910 electron microscope. Kinetochore structure and number of microtubules bound were determined directly on film negatives. All calculations were performed in Microsoft Excel.

Supplemental Figures 1-4

A

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                   **  *     *                                  *        *    *
Human              MKRSSVSSGGAGRLSMQELRSQDVNKQGLYTPQTKEKPTFGKLSIN-----KPTSERKVS
PtK1               MKCSSVSSGAHGRQSMQSLRSQDFNKQGLYTPQTKERPAFWKLSTSRLTPGTSTSERKIS  60

                        *     *
Human              LFGKRTSGHGSRNSQLGIFSSSEKIKDPRPLNDKAFIQQCIRQLCEFLTENGYAHNVSMK
PtK1               LFGKGASGPGSRNSLLGVFGGNEKIKDPRPLNDKAFIQQYIRQLYEFLAENGYACGISMK 120

Human              SLQAPSVKDFLKIFTFLYGFLCPSYELPDTKFEEEVPRIFKDLGYPFALSKSSMYTVGAP
PtK1               SLQSPSVKDFLKIFTFIFAFLSPSYELPDSKFEEEIPRILKDLGYPFTLPKSSMYTVGAP 180

Human              HTWPHIVAALVWLIDCIKIHTAMKESSPLFDDGQPWGEETEDGIMHNKLFLDYTIKCYES
PtK1               HTWPHVLASLNWLIDCFKLIFVGKQSSPSFDDGQPWGGESEDGIMHNKLFLDYTVKCYEN 240

Human              FMSGADSFDEMNAELQSKLKDLFNVDAFKLESLEAKNRALNEQIARLEQEREKEPNRLES
PtK1               FMTGADSFEDLDTELYSKLKDLFNVDDSKLESLASENKRLTEEIARVEREKENEPNRLIS 300

Human              LRKLKASLQGDVQKYQAYMSNLESHSAILDQKLNGLNEEIARVELECETIKQENTRLQNI
PtK1               LRKVKASLKADVQKYQAYMNNLESHSSILDQKLSGFNEEVPAVELELEAVKQENARLQSI 360

Human              IDNQKYSVADIERINHERNELQQTINKLTKDLEAEQQKLWNEELKYARGKEAIETQLAEY
PtK1               MDNQKYSIADIERIHHERNEIQQTVKKLTTELATEQKQLWNEELKYARSKEAIEAQLEEY 420

Human              HKLARKLKLIPKGAENSKGYDFEIKFNPEAGANCLVKYRAQVYVPLKELLNETEEEINKA
PtK1               HKLARKLKLIPKSAENSKGYDFEVKFNPEVGTNCLVKYRTQVYIPLKELLNQYEERISSV 480

Human              LNKKMGLEDTLEQLNAMITESKRSVRTLKEEVQKLDDLYQQKIKEAEEEDEKCASELESL
PtK1               QHKKMGSEETLEQVNTMVREVKRSTKMLNNEVQKLKDFIEQKIKEAEEKDRKCTAEVESL 540

Human              EKHKHLLESTVNQGLSEAMNELDAVQREYQLVVQTTTEERRKVGNNLQRLLEMVATHVGS
PtK1               DNHKHLLESGVNEGPSEAISELDAIQQQYQLVLQMTTEERRKASSYLQKTLEMVATHIGS 600

Human              VEKHLEEQIAKVDREYEECMSEDLSENIKEIRDKYEKKATLIKSSEE
PtK1               VEKYLEDQIGRVDRECEESITEDFLENIREMGDKY------------ 635
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B

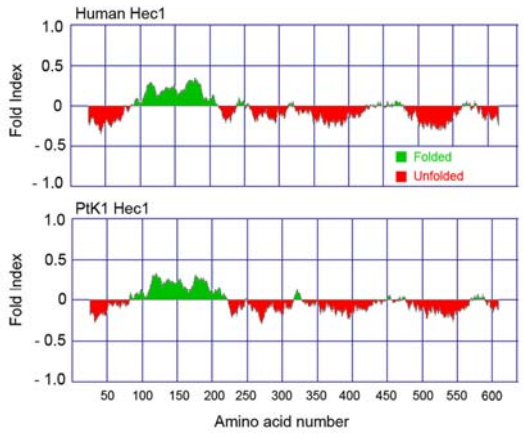


Figure S1. PtK1 Hec1 sequence analysis.

(A) Amino acid alignment of PtK1 and human Hec1. Aurora B phosphorylation target sites are denoted with an asterisk [4, 5]. We were unable to obtain sequence for the extreme 3' end of PtK1 Hec1 (approximately 36 nucleotides).

(B) Intrinsic disorder plots for human Hec1 (top) and PtK1 Hec1 (bottom) based on the "Fold Index" algorithm [6]. Red regions are predicted to be intrinsically disordered and green regions are predicted to maintain a stably folded conformation based on parameters including sequence charge and average residue hydrophobicity [6]. The 80 amino acid N-terminal regions of both human and PtK1 Hec1 are largely intrinsically disordered.

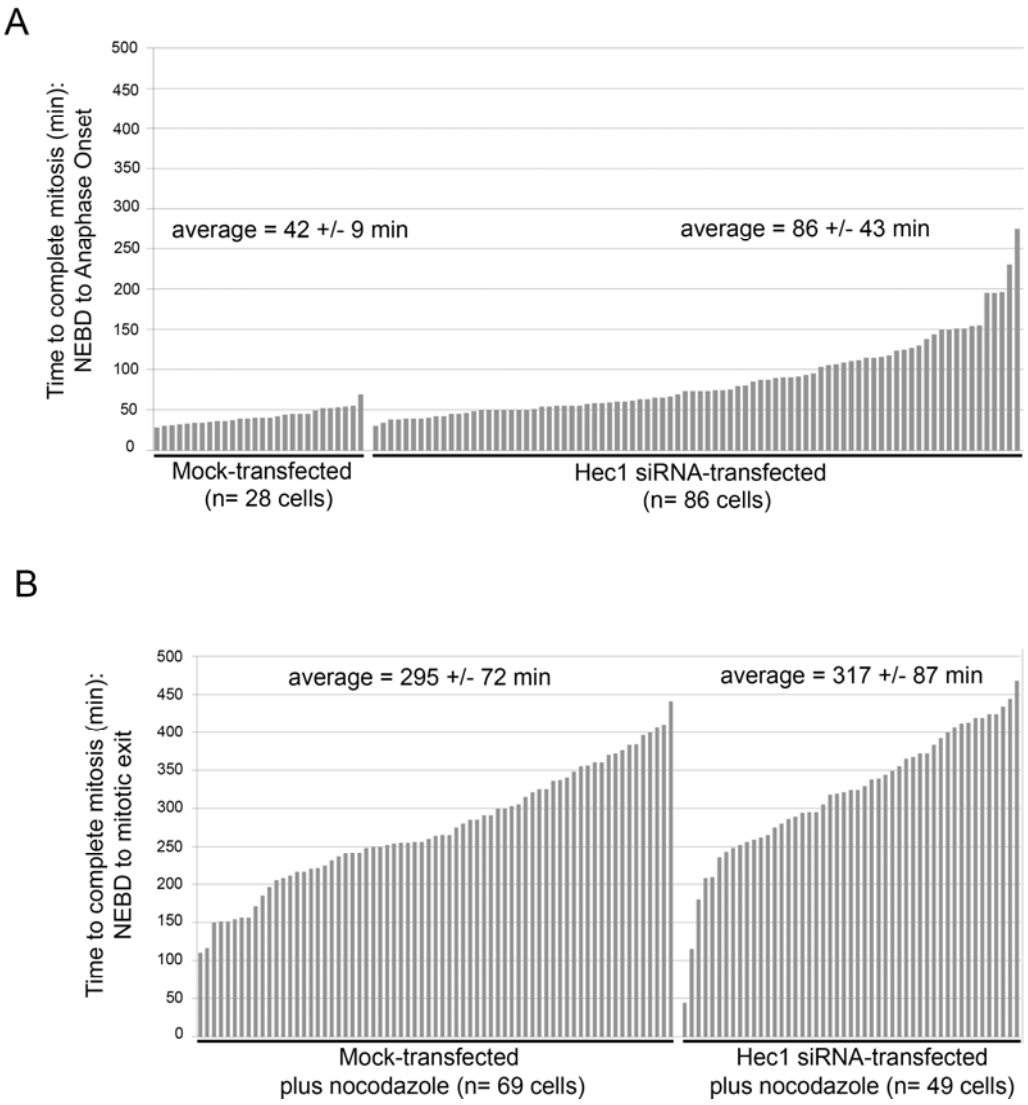


Figure S2. Timing through mitosis: mock-transfected and Hec1-siRNA transfected PtK1 cells in the absence and presence of nocodazole. Cells were mock-transfected or transfected with Cy5-labeled Hec1 siRNA. Forty-eight h post-transfection or mock-transfection, cells were incubated with filming media or filming media plus 20 μM nocodazole and time-lapse imaged. Phase-contrast and Cy5 fluorescence images were captured every 5 min for 10 h. Time-lapse movies were used to determine timing through mitosis. Each bar represents an individual cell, and the cells are displayed in ascending order of timing through mitosis.

(A) No drug treatment: timing through mitosis was scored from nuclear envelope breakdown (NEBD) to anaphase onset, as determined by sister chromatid separation.

(B) Nocodazole treatment: timing through mitosis was scored from NEBD to mitotic exit, as determined by chromosome decondensation and cell re-spreading. Mitotic exit was scored here as opposed to anaphase onset due to difficulty discerning anaphase onset in nocodazole-treated cells.

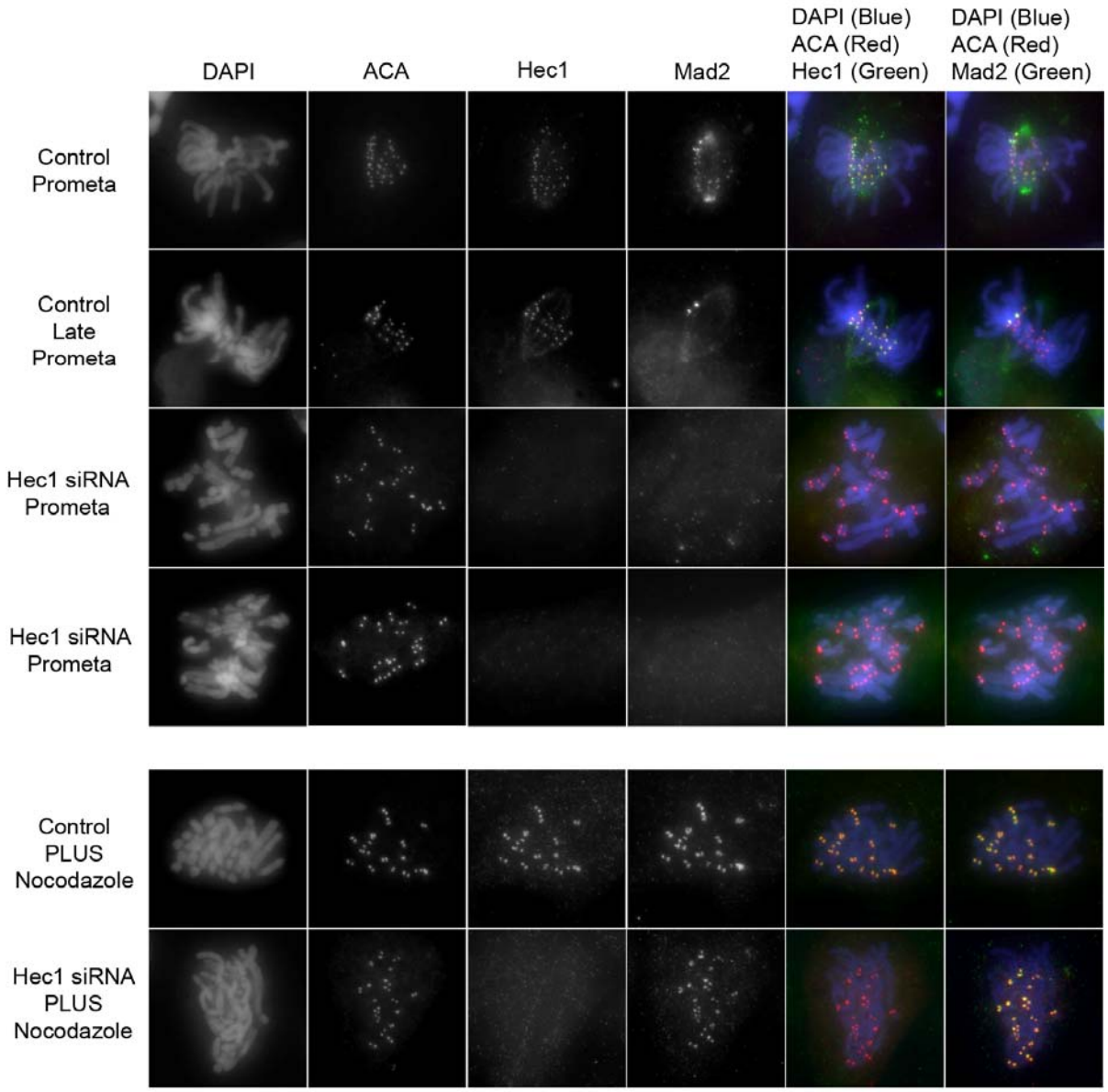
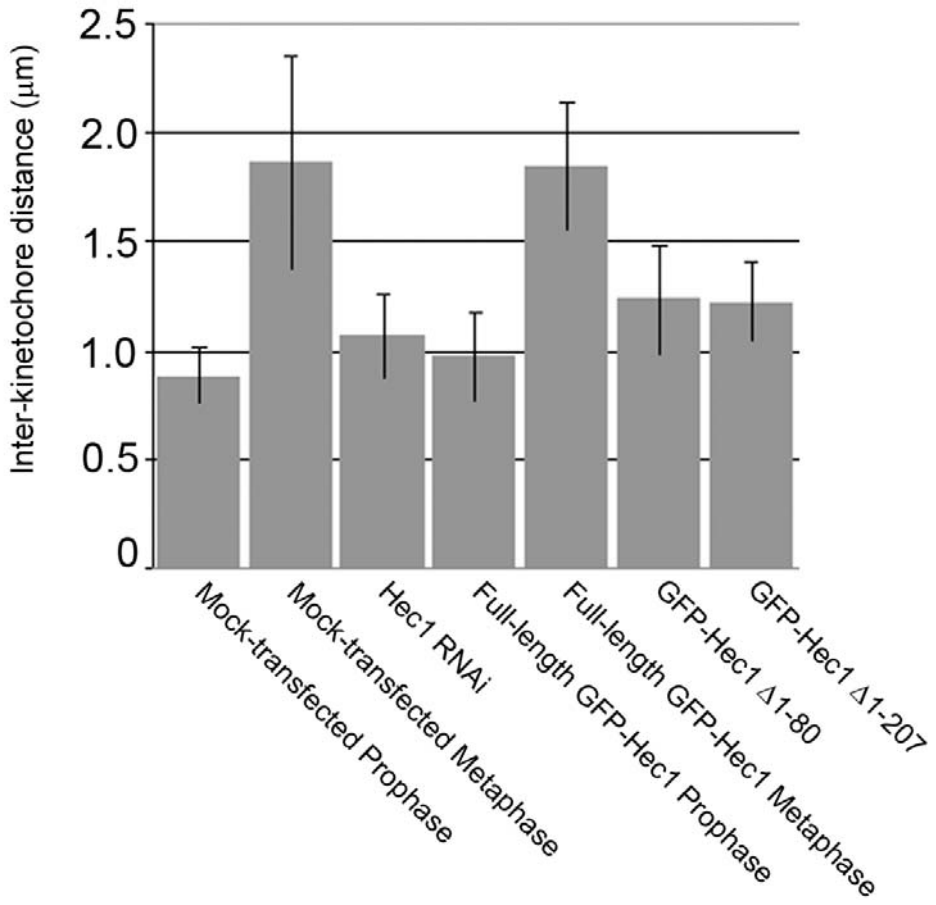


Figure S3. Mad2 is prematurely depleted from unattached kinetochores in Hec1-depleted PtK1 cells. Mock-transfected or Hec1 siRNA-transfected PtK1 cells were fixed and immunostained with the indicated antibodies. For nocodazole experiments, the drug was added at 20 μM for 1 h prior to fixation. (Top) Kinetochores in mock-transfected cells bind high levels of Mad2 at early mitosis, and levels decrease as chromosomes align. Kinetochores in Hec1 siRNA-transfected cells do not bind high levels of Mad2 in the presence of unaligned chromosomes. (Bottom) Kinetochores in both mock-transfected and Hec1 siRNA-transfected cells bind high levels of Mad2 in the absence of microtubules

A



B

	distance (μm)	standard deviation	kinetochore n value	cell n value
Mock-transfected Prophase	0.88	0.13	20	5
Mock-transfected Metaphase	1.87	0.49	61	14
Hec1 RNAi	1.07	0.20	307	42
Full-length GFP-Hec1 Prophase	0.97	0.20	8	1
Full-length GFP-Hec1 Metaphase	1.84	0.30	69	10
GFP-Hec1 Δ1-80	1.23	0.25	64	17
GFP-Hec1 Δ1-207	1.22	0.19	88	21

Figure S4. Summary of inter-kinetochore distance data for N-terminal GFP fusion proteins. In addition to the N-terminal fusion protein data, data for mock-transfected and Hec1 siRNA-transfected cells (without expression of exogenous GFP fusion proteins) are included. For mock-transfected and Hec1 siRNA-transfected, distances were measured from ACA centroid to ACA centroid. For silence and rescue experiments, distances were measured from GFP centroid to GFP centroid. Vertical line in the bar graphs indicates standard deviation.

Supplemental References

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