Supplemental Data

Kinetochore Dynein Is Required for Chromosome Motion and Congression Independent of the Spindle Checkpoint

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Supplemental Experimental Procedures

Cell Culture and Drug Treatments

U2OS cells expressing GFP-CENP-B (a gift from Dr. Kevin F. Sullivan, National University of Ireland, Galway) or Centrin-1-GFP (a gift from Dr. Alexey Khodjakov, Wadsworth Center, Albany, NY) were cultured with Dulbeco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). U2OS cells expressing photoactivatable-GFP-α-tubulin (a gift from Dr. Duane Compton, Dartmouth College, Hanover, NH) were cultured in McCoy's medium. LLC-PK1α cells were cultured as described [S1]. Cells were maintained in a humidified incubator at 37°C in a 5% CO2 atmosphere and were subcultured onto glass coverslips 24–48 hr prior to experimentation.

For experiments on the localization of ZW10 and/or dynein, cells were treated with 1 μM nocodazole (Sigma) for 1 hr before fixation. For those experiments on the spindle and kinetochore fibers, cells were treated with 5 μM MG132 (Calbiochem) for 2 hr before fixation. Cold treatment was performed by incubating cells in cold media (4°C) on ice for 10 min. For measurement of the distance between sister kinetochores in the absence of tension, cells were treated with 5 μM nocodazole for 3 hr.

siRNA Treatment and Transfection

Double-stranded siRNA to ZW10 (5′-UGAUCAAUGUGCUGUUCAA-3′; [S2]) was ordered from Dharmacon. Duplex was transfected at 200 nM with Oligofectamine (Invitrogen) for 96 hr. For cotransfecting with siRNA and GFP-CENP-B, 48 hr after transfecting with siRNA, U2OS Centrin-1-GFP cells were transfected with GFP-CENP-B plasmid (a gift from Dr. Kevin Sullivan) by Lipofectamine 2000 (Invitrogen) for another 48 hr before analysis.

Indirect Immunofluorescence Microscopy

Coverslip cultures of cells were rinsed briefly in Pipes + Hepes + EGTA + Magnesium (PHEM) (60 mM Pipes, 25 mM Hepes, 10 mM EGTA, and 4 mM MgSO4 [pH 6.9]) and permeabilized in 0.5% Triton-X/PHEM for 3 min. They were then fixed in 4% formaldehyde/PHEM for 20 min.

Antibodies were used at the following dilutions: rabbit ZW10 antibody (a gift from Dr. Michael Goldberg, Cornell University, Ithaca, NY) at 1:800, cytoplasmic dynein 70.1 intermediate chain (Sigma) at 1:1000, β-tubulin (Sigma) at 1:5000, rabbit anti-γ-tubulin (Sigma) at 1:200, rabbit anti-NuMA (gift from Dr. Duane Compton) at 1:500, and rabbit anti-Kif2a (a gift from Dr. Duane Compton) at 1:500. Goat anti-rabbit Alexa Fluor 546 (1:400) and goat anti-mouse IgM Alexa Fluor 546 (1:800) were purchased from Molecular Probes. Anti-mouse TRITC (1:100) was from Sigma. DNA was counterstained with 5 μg/ml Hoechst 33342 (Molecular Probes).

Immunofluorescence images were acquired on a Delta Vision deconvolution system (Applied Precision) equipped with a Photometrics CH350 CCD camera (Roper Scientific). Z section images were acquired with 0.3 μm steps with a 100 × 1.35 numerical aperture (NA) objective lens on an Olympus IX 70 microscope. Maximal-intensity projections were obtained from deconvolved 3D datasets and compiled with Photoshop CS2 (Adobe).

Live-Cell Imaging

The procedures used to image LLC-PK1α cells (Figure 1) have been described previously in detail [S1]. Coverslip cultures of mock- and ZW10-RNAi-transfected U2OS cells were assembled into Rose Chambers in phenol-free L-15 medium (Grand Island Biological Supply Company [GIBCO]) supplemented with 10% FBS. Cells were maintained at 37°C during filming.

For definition of the duration of mitosis, phase-contrast time-lapse images were acquired at 1 min intervals with a 40 × 0.75 NA PlanFluor objective lens mounted on a Nikon Eclipse TE2000-U microscope. This microscope was equipped with an ORCA-ER cooled-CCD camera (Hamamatsu, Japan) and fast Uni-Blitz shutters (Vincent Associates, Rochester, NY). The system was driven by Image-Pro Plus 5.1 (Media Cybernetics, Silver Spring, MD). For tracking kinetochores, Z series (1 μm steps, 9 μm total Z depth) fluorescence images were collected at 6 s (prometaphase) or 10 s

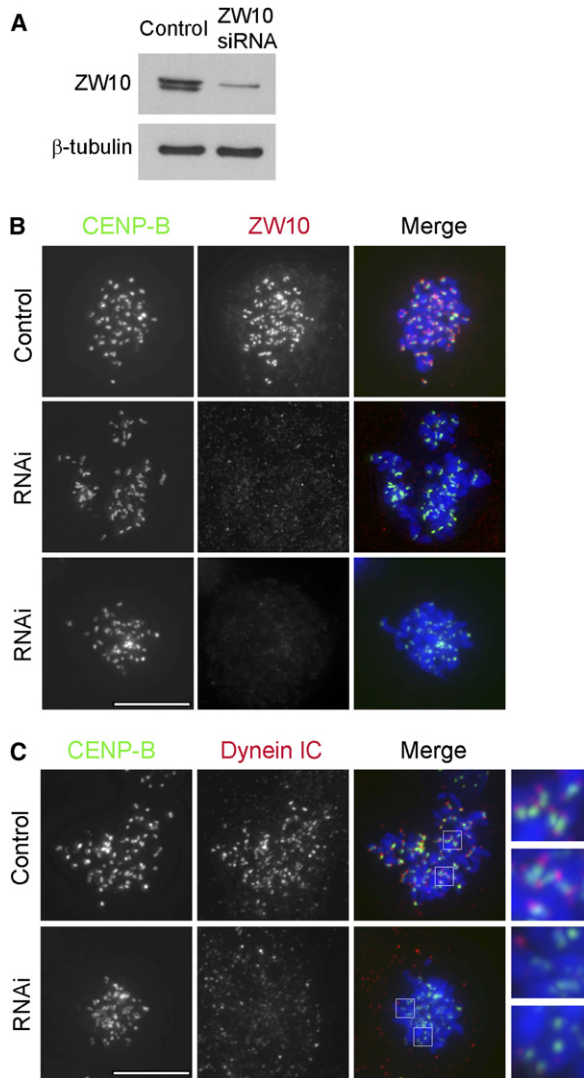

Figure S1. A 96 Hr Transfection with ZW10 siRNA Duplexes Significantly Reduces the Cytoplasmic-Dynein Content of Unattached U2OS Cell Kinetochores

(A) Western blot from U2OS cultures prior to and after a 96 hr treatment with ZW10 siRNA duplexes. This treatment reduces the ZW10 content of the cultures by ~85%.

(B) Maximum-intensity projections from deconvolved 3D data sets illustrating the reduced ZW10 content of kinetochores in nocodazole-treated CFP-CENP-B U2OS cells after RNAi treatment.

(C) Knocking down ZW10 significantly reduces the dynein content of kinetochores. Right-hand images represent high-magnification views of the boxes found in the merged channel. The scale bar represents 10 μm.

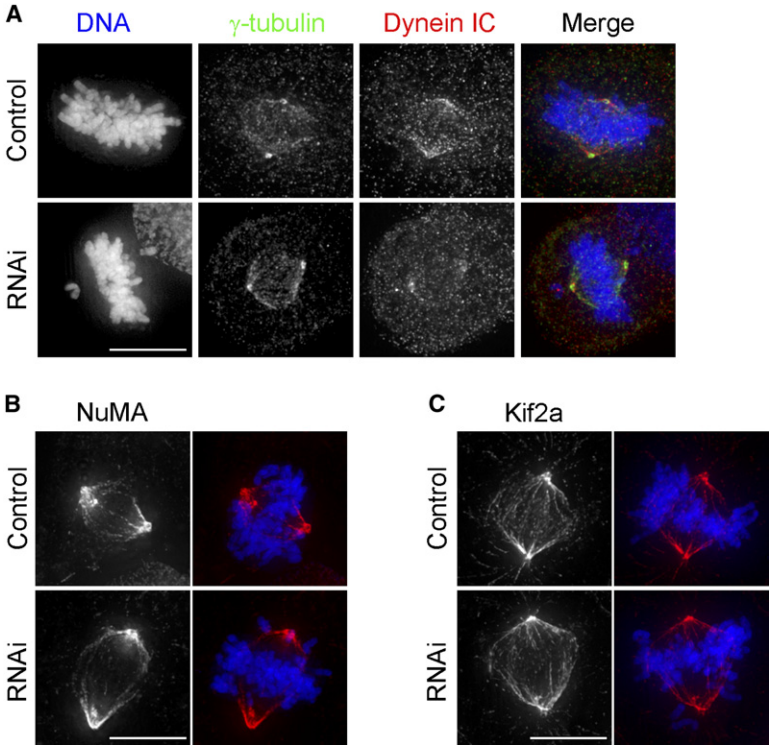

Figure S2. Depleting Kinetochore-Associated Dynein by Knocking Down ZW10 Does Not Prevent the Accumulation of γ-Tubulin, Dynein, NuMA, or Kif2a at Spindle Poles

Cultures depleted of ZW10 by RNAi were fixed as described (Supplemental Experimental Procedures) and stained for the IMF distribution and localization of γ-tubulin (A), dynein (A), NuMa (B), and Kif2a (C). Although selectively reducing dynein at kinetochores via RNAi for ZW10 leads to a diminished level of dynein in the region of the spindle poles, the amount and distribution of γ-tubulin, NuMA, and Kif2a are not noticeably affected. The scale bar represents 5 μm.

(anaphase) intervals with a 100 × 1.4 PlanApo objective lens on a Nikon Eclipse TE300 microscope equipped with a piezo Z positioner (Physik Instrumente, Germany) and a CoolSnap HQ (Photometrix, Tucson, AZ) cooled-CCD camera. This microscope was driven by Isee software (Isee Imaging, Raleigh, NC).

For photoactivation experiments, U2OS cells expressing photactivatable GFP-tubulin (a kind gift of Dr. Duane Compton) were exposed to 413 nm light (excitation filter D405/20, Chroma Tech) for 5–10 s from Exfo X-cite 120 epi-illuminator (EXFO America, Plano, TX). The area of photoactivation was selected by reducing the area of the field with slits (Lenox Laser) mounted in a Ludl filter wheel (Ludl Electronic Products) placed in a conjugate image plane in the light path. After photoactivation, images were recorded on a Nikon TE-300 microscope equipped with a 100 × 1.4 NA objective lens, a Perkin Elmer spinning-disc confocal scan head, and a Hamamatsu ORCA-ER interline transfer-cooled CCD camera (Hamamatsu, Bridgewater, NJ). MetaMorph Software (Molecular Devices, Downington, PA) was used to take image sequences of live cells.

Measurement and Statistics

All distance, velocity, and fluorescence-intensity measurements were conducted with Image J (National Institutes of Health [NIH]) software. Results and graphs were managed with Microsoft Excel software and presented as the mean ± standard deviation (SD).

Tension Measurements

Mock- or ZW10-RNAi-transfected GFP-CENP-B U2OS cells were fixed (see above) and stained with Hoechst 33342. 3D Z stacks of images were then acquired from metaphase and pseudometaphase (ZW10 RNAi) cells with an API DeltaVision deconvolution system. The distance between sister kinetochores, defined as the distance between the distal ends of sister GFP-CENP-B dots, was measured only for fully congressed sister kinetochore pairs that contained two sister CENP-B dots in the same optical section. The distance between sister-kinetochores in cells treated with 5 μM nocodazole for 1 hr defined a nontension control.

Quantization of Immunofluorescence

For quantification of the fluorescence intensity of cold-stable K-fibers, a single low magnification (20×) image of each cell was captured on a NIKON TE-2000-U, equipped with a Hamamatsu ORCA camera, and the same acquisition settings were used between control and experimental cells. A circular area containing the entire spindle was used to measure the fluorescence intensity of the whole spindle. Three small surrounding regions, external to the spindle, were selected to calculate the average background intensity.

Kinetochore Tracking

The motion of kinetochores was tracked with maximal-intensity projection of time-lapse Z series images by a manual tracking plugin of Image J. The trajectories of typical kinetochores were plotted.

Supplemental References

S1. Tulu, U.S., Fagerstrom, C., Ferenz, N.P., and Wadsworth, P. (2006). Molecular requirements for kinetochore-associated microtubule formation in mammalian cells. Curr. Biol. *16*, 536–541.

S2. Kops, G., Kim, Y., Weaver, B.A.A., Mao, Y., McLeod, I., Yates, J.R., Tagaya, M., and Cleveland, D.W. (2005). ZW10 links mitotic checkpoint signaling to the structural kinetochore. J. Cell Biol. *169*, 48–60.

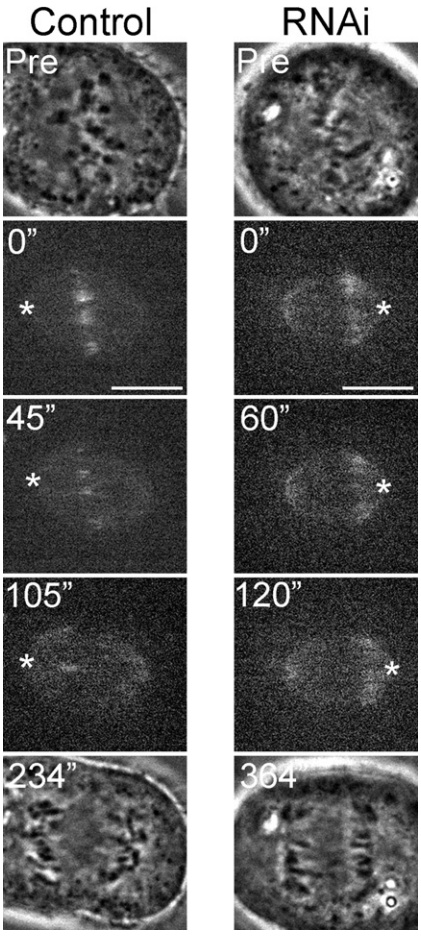

Figure S3. Depleting Kinetochore-Associated Dynein by Knocking Down ZW10 Does Not Diminish the Rate of Poleward Microtubule-Subunit Flux in Metaphase and Anaphase U20S Cells

U2OS cells expressing photoactivatable GFP-α-tubulin were irradiated to produce small fluorescently labeled regions on kinetochore fibers in metaphase cells that subsequently entered anaphase during the experiment. The rate of poleward flux was determined in control and ZW10 (RNAi)-depleted cells and found to be the same. See the main text for details. The time is given in seconds. The scale bars in 0 time represent 10 μm.