Figure S1. **Tandem MS analysis and biochemical characterization of T89 mutants.** (A) Fragmentation data from tandem MS analysis of mitotic ICs. The peptide sequence is presented with Y and B fragment data. Fragments containing T89 (T-101 D) are shifted by 80 D [phosphate]. (B) Serial dilution slot blots of wild-type (W.T.) IC-2C and T89D and T89E mutants were probed with recombinant p150Glued fragments (aa 1–811) by blot overlay analysis. A shift in half-maximal binding was observed in the T89 mutants, suggesting a decrease in affinity.
Figure S2. Characterization of PT89 antibody by IFM, Western blotting and immunoprecipitation, and sensitivity to in vitro phosphorylation by p38-MAPK. (A) IFM analysis with PT89 antibody demonstrates specificity for mitotic cells (yellow arrow) rather than interphase cells (yellow arrowhead) and kinetochores of mitotic cells when compared with DAPI staining. Bar, 5 µm. (B) Western blots of mitotic HeLa extracts probed with anti-dynein IC antibody and anti-PT89 antibody demonstrate binding to dynein ICs. Western blotting was performed without blocking agents to preserve phosphorylation. Immunoprecipitations from mitotic HeLa extracts with a pandynein antibody (74.1) and PT89 antibody were probed with rabbit anti-dynein IC antibody (V3) or mouse dynein IC antibody (74.1) to demonstrate that anti-PT89 antibody detects endogenous dynein ICs. (C) In vitro kinase assays using recombinant IC-2C fragments and purified p38-MAPK were analyzed by autoradiography. T89 mutants displayed reduced 32P incorporation similar to background in contrast to wild-type (W.T.) ICs, which displayed robust phosphorylation. Proteins phosphorylated by this approach were used for blot overlays in Fig. 5 A. (D) Western blot analysis of phosphorylated dynein ICs. Recombinant IC-2C was subjected to SDS-PAGE, transferred to polyvinylidene fluoride, and probed with PT89 antibody (IC Alone). The same protein was subjected to in vitro phosphorylation with p38-MAPK and analyzed in parallel (IC Kinase). The phosphorylated protein was subjected to phosphatase treatment after in vitro phosphorylation reactions and probed in parallel (IC Kinase/Phosphatase). The enhanced labeling of phosphorylated ICs and decreased labeling after phosphatase treatment are consistent with the phospho specificity of this antibody. CBB, Coomassie brilliant blue.
Figure S3. Detection of transfected dynein IC constructs by PT89 antibody. (A) IFM analysis of transfected ICs with anti-PT89 antibody. Panels 1 and 2 show transfection with a T89A mutant IC (aa 1–100) tagged with mCherry. Fluorescence from the mCherry channel (1) was compared with the signal from IFM with the PT89 antibody (2). Panels 3 and 4 show transfection with wild-type IC (aa 1–100). Fluorescence from the mCherry channel (3) was compared with the signal from IFM with the PT89 antibody (4). Intensity scales for panels 1 and 3 (0–800 intensity units) and 2 and 4 (0–1,000 intensity units) are matched and presented from raw data. Although the levels of transfected protein are similar in both sets (1 and 3; mean intensity of background-corrected images is ~140 intensity units), the signal from soluble wild-type IC in panel 4 is ~400 intensity units, whereas the soluble T89A signal in panel 2 is ~135 intensity units, reflecting phosphorylation of the wild-type protein but not the T89A mutant. Kinetochores in panel 4 have intensities of ~1,000 intensity units but were not included in the analysis of soluble protein pixel intensity. Insets provide higher magnification of cytoplasmic labeling. Bar, 5 µm. (B) Numerical comparison of signal from soluble IC protein in each sample demonstrating enhanced detection of the wild-type (W.T.) protein. The difference in mCherry signal between T89A and wild-type IC constructs is not statistically significant (P = 0.498), whereas the difference in PT89 antibody labeling is significant (P = 1.59 × 10⁹). Error bars represent SD.
Figure S4. Statistical analysis of PT89 intensities relative to kinetochore proteins, timing of mitotic progression, and interkinetochore distance measurements. (A) Signal ratios of kinetochore proteins demonstrate the effect of p50(dynamitin) transfection (P50Tx). Signal intensities from matched exposures were used to calculate ratios of PT89, BubR1, and p150Glued to an internal standard (ACA). Whereas the levels of PT89 do not change significantly after p50(dynamitin) transfection, levels of p150Glued on kinetochores are reduced significantly (P < 0.05) after p50(dynamitin) transfection. In contrast, p50(dynamitin) transfection induces a significant increase in the accumulation of BubR1 (P < 0.05). The latter is consistent with defective removal of BubR1 in the absence of dynactin. The numbers of kinetochores analyzed are presented in each bar. (B) Timing of anaphase onset after transfection with p50(dynamitin) (p50-Tx) in Fig. 3 C. In time-lapse sequences of cells expressing GFP-H2B, the time of progression from chromosome alignment to anaphase onset was measured. Control cells progress in ~8 min, whereas cells expressing p50(dynamitin) either enter anaphase ~17.5 min after alignment (*, P = 0.0054) or never enter anaphase during the course of the experiment. The numbers of cells analyzed are presented in each bar. (C) Interkinetochore distance measurements from experiments in Fig. 6. Individual kinetochore pairs from single z planes of deconvolved image stacks were used to measure peak-to-peak distances after treatments with nocodazole, taxol, and calyculin A or after transfection with H125A mutant PP1-γ. Bar graphs present means of these measurements and SD (error bars). Data for control cells were collected from 20 kinetochore pairs, nocodazole from 31 pairs (P = 5.49 \times 10^{-11}), taxol from 27 pairs (P = 1.41 \times 10^{-6}), calyculin A from 35 pairs (P = 5.44 \times 10^{-5}), and H125A from 36 pairs (P = 5.55 \times 10^{-11}).
Video 1. **Mitotic timing of NRK2 cell expressing GFP-H2B.** Time-lapse image sequence was collected at one frame per 20 s and played in video at six frames per second. Stills from the sequence are presented in Fig. 3 C. The white circle indicates time of alignment. The timestamp is given in minutes/seconds. Bar, 5 µm.

Video 2. **Mitotic timing of NRK2 cell coexpressing GFP-H2B and mCherry-p50(dynamitin).** Time-lapse image sequence was collected at one frame per 20 s and played in video at six frames per second. Stills from the sequence are presented in Fig. 3 C. The blue circle indicates time of alignment. The timestamp is given in minutes/seconds. GFP-H2B appears in green, and mCherry-p50(dynamitin) appears in red. Bar, 5 µm.

Video 3. **Poleward streaming of kinetochore-derived GFP-p50(dynamitin) in NRK2 cells.** Coarse particles of GFP-p50(dynamitin) move poleward from each kinetochore in this focal plane (red arrows). Time-lapse image sequence was collected at one frame per 1.4 s and played in video at six frames per second. Stills from the sequence are presented in Fig. 7 B. The white arrow indicates a moving particle. The timestamp is given in seconds/milliseconds. Bar, 5 µm.

Video 4. **Loss of poleward streaming of kinetochore-derived GFP-p50(dynamitin) in NRK2 cells after taxol treatment.** GFP-p50(dynamitin) fails to move poleward from each kinetochore in this focal plane (red arrow). Time-lapse image sequence was collected at one frame per 1.4 s and played in video at six frames per second. Stills from the sequence are presented in Fig. 7 B. The timestamp is given in seconds/milliseconds. Bar, 5 µm.