Supporting Information

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SI Materials and Methods

FP Assay for Secondary and Tertiary Screens. FP experiments for the purpose of secondary screening were performed in duplicate in black nonbinding 96-well plates with flat-bottomed wells (655900; Corning). Fifty compounds identified from the initial TR-FRET assay were supplied prealiquoted on plates in a 1-μL volume at concentrations of 0.25 mM–4 mM, and tested in a twofold dilution series, giving final reaction concentrations ranging from 3.5–57 μM. Seventy microliters of a master mix solution containing 8 μM nonlabeled aiKLC2^{TPR} produced as described above, 150 nM TAMRA conjugated *SKIP*^{WD} peptide (11), 25 mM Hepes (pH 7.5), 150 mM NaCl, and 5 mM β-ME were added to each well (giving a final DMSO concentration of 1.4%) per reaction and mixed by gentle pipetting. Millipolarization (mP) values for control reactions were at ∼60% of those obtained with a saturating amount of protein (25). Plates were incubated at room temperature for 30 min and subsequently analyzed using a POLARstar Omega plate reader (BMG Labtech), using the Omega software at an excitation wavelength of 540 nm and an emission wavelength of 590 nm. For each independent 96-well plate reading the gain of the parallel and perpendicular channel was adjusted according to a 150 nM SKIP-TAMRA peptide-only control [25 mM Hepes (pH 7.5), 150 mM NaCl, 5 mM β-ME, 1.5% (vol/vol) DMSO] to target 100 mP. Compounds identified as causing a concentration dependent decrease in FP (eight compounds, 0.28% of initial library) were selected for further analysis by GST-pull down analysis and optimized FP conditions (as above with 100-μL total volume). These data were used to inform a small-scale targeted screen of compounds selected from the Hit2Lead library from Chembridge, which were analyzed in the same way. Kinesore was identified as 3,5-dibromo-N′-{[2,5-dimethyl-1-(3-nitrophenyl)-1H-pyrrol-3-yl]methylene}-4-hydroxybenzohydrazide, compound number 6233307. The concentration-dependent decrease in FP signal due to the addition of compound was fitted using a four-parameter dose–response curve without constraints to derive IC_{50} and apparent K_i was determined as previously described by Nikolovska-Coleska et al. (30). Data were analyzed using GraphPad Prism v7.00 (GraphPad Software).

Kinesore Treatment of Cells. Kinesore stocks were prepared by solubilization of dry powder at a concentration of 50 mM in 100% DMSO and stored at −20 °C before use. All treatments were carried out in Ringer's buffer [155 mM Nacl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 2 mM NaH_2PO_4, 10 mM glucose, 10 mM Hepes (pH 7.4)]. Final concentration of DMSO in 50-μM treatment was therefore 0.1%. Vehicle control experiments refer to cells treated with 0.1% DMSO in Ringers buffer. For lower-concentration treatments, stocks were first diluted in DMSO to yield a 1,000× solution that was subsequently added to Ringers buffer.

Plasmids, Antibodies, and Cells. All bacterial and mammalian expression plasmids used in this study have been described previously (11, 25). FRET biosensor plasmids are described in ref. 25. The SU.K.4 monoclonal antibody was supplied by the Developmental Studies Hybridoma Bank. Anti-HA monoclonal antibody (HA-7) used for Western blot, anti-HA polyclonal antibody (H6908) used for immunofluoresence, and anti–β-tubulin (B-5-1-2) were supplied by Sigma-Aldrich. HAP1 cells were supplied by Horizon Discovery.

GST-Pull Down from Cell Extracts. GST-SKIP (1–310) was expressed and purified from *E. coli* as described previously (28). HEK 293T cells expressing transiently transfected HA-KLC2 were harvested in 1 mL lysis buffer [25 mM Hepes pH 7.5, 150 mM NaCl, 0.1% Nonidet P-40, 0.1% Triton-X 100 containing a protease inhibitor mixture (Roche)]. Lysates were incubated on ice for 10 min before centrifugation at 13,000 × *g* for 10 min at 4 °C. The resulting supernatant was preincubated with the corresponding concentration of kinesore for 30 min in 0.1% DMSO (from 1,000× stocks, in 100% DMSO) or without kinesore with 0.1% DMSO (lane 5), and subsequently incubated for 1.5 h with the indicated GST-fusion protein (0.5 μmol of protein per reaction, unless otherwise indicated) bound to 20-μL glutathione Sepharose beads. Beads were washed four times and boiled in 60 μL SDS loading buffer. Twenty-microliter samples were separated on SDS/PAGE gels, transferred onto PVDF membrane, blocked in 5% milk in TBS-T (20 mM Tris, 0.25 M NaCl, 0.1% Tween-20, pH 7.5 with HCl), and probed with the indicated primary antibodies followed by detection with HRP-conjugated secondary antibodies. Blots were developed with an ECL kit (Bio-Rad) and chemiluminescent signal detected and quantified using a Bio-Rad XR system and ImageLab software.

Immunofluoresence and Live-Cell Imaging. For immunofluorescence and live-cell imaging, 2×10^5 cells were plated onto fibronectin-coated coverslips in six-well plates and transfected, where indicated the plasmid DNA 16 h before analysis. Cells treated with kinesore as indicated, and fixed for 5 min with −20 °C methanol before blocking and probing with primary and secondary antibodies (Alexa 488-, 568-, and 633-conjugated anti-mouse or anti-rabbit secondary antibodies; Thermoscientific). Confocal images were collected using a Nikon A1 system with a 40× or 60× objective running NIS Elements and are presented as maximum-intensity projections, where indicated. Superresolution imaging was performed using an N-SIM Superresolution system with a 100× objective lens and images presented are maximum-intensity projections. For live-cell imaging, 1×10^5 of the indicated cells were plated and transfected in fibronectin coated 35-mm Mattek dishes, and were imaged at a either using an inverted Nikon A1 confocal system with a 100× objective lens (GFP-tubulin) or an inverted CSU-X1 Spinning Disk Confocal system (EB3-RFP) with an Andor Ixon3 EM-CCD camera and a 60× objective lens, both equipped with temperature and CO_2 control and running NIS Elements. Movies were processed using NIS elements and ImageJ. Figures were assembled using ImageJ in conjunction with Adobe Photoshop and Illustrator packages (Adobe). Spectrum projection images in Fig. 2*E* were generated using the time-lapse color coder ImageJ plugin.

FRET/Fluorescence Lifetime Imaging Microscopy Sample Preparation and Data Acquisition. FRET biosensor studies were carried out as previously described, using an updated microscope set-up (25). Briefly, 1×10^4 HeLa cells were seeded onto 13-mm fibronectin-coated coverslips in 24-well plates. Cells were transfected with plasmids expressing GFP-KLC2-HaloTag and HA-KHC, with or without myc-CSTN1 (869–971). At 6 h posttransfection, the medium was replaced with fresh medium containing HaloTag TMRDirect ligand (Promega) at a 1:1,000 dilution according to the manufacturer's instructions. After an overnight incubation, cells were fixed in 4% paraformaldehyde and permeabilized in 0.2% (wt/vol) Triton X-100 in PBS. After quenching with 1 mg/mL sodium borohydride in PBS for 10 min at room temperature,

cells were washed in PBS and mounted in Mowiol containing 2.5% (wt/vol) Dabco (Sigma-Aldrich). Time domain fluorescence lifetime imaging microscopy was performed with a multiphoton microscope system (Ti Eclipse microscope; Nikon). Fluorescence lifetime imaging capability was provided by time-correlated single-photon counting electronics (SPC-830) on DCC-100 control (both Becker & Hickl). A 40× objective was used throughout (Plan Fluor N.A. 1.3; DIC H, WD 0.2; Nikon), and data were collected at 515 ± 20 nm through a bandpass filter. Acquisition times of the order of 250 s at a low 900-nm excitation laser power (MaiTai, DeepSee; Spectra-Physics) were used to achieve sufficient photon statistics for fitting, while avoiding either pulse pile-up or significant photobleaching. Corresponding widefield fluorescent images were acquired for donor (GFP) and acceptor (RFP) channels (DS-Qi1Mc camera; Nikon). Lifetime raw data were analyzed with TRI2 software and histogram data are plotted as mean FRET efficiency from specified numbers of cells per sample over two experiments. Lifetime images of example cells are presented using a pseudocolor scale, whereby blue depicts normal GFP lifetime (no FRET) and red depicts lower GFP lifetime (areas of high FRET).

Microtubule Polymerization Assay. In vitro microtubule polymerization assay was carried out using the Tubulin Polymerization Assay Kit (Cytoskeleton) according to the manufacturer's protocol using a POLARstar Omega (BMG Labtech) plate reader, with kinesore at 10 μM or a 0.1% DMSO control. Data are presented as background subtracted from $t = 0$ timepoint and are mean of two independent experiments.

Cellular Microtubule Sedimentation Assay. This assay was adapted from the Cytoskeleton Microtubule/Tubulin In Vivo Assay Biochem Kit and was previously described (28). Briefly, 2×10^6 HeLa-Kyoto cells expressing GFP-Kif5B were plated onto a 10-cm dish. Cells were lysed at 24 h postplating, and 1 h postkinesore treatment, in 3 mL of microtubule stabilization buffer [100 mM Pipes pH 6.9, 5 mM $MgCl_2$, 1 mM EGTA, 30% (vol/vol) glycerol, 0.1% (vol/vol) Nonidet P-40, 0.1% (vol/vol) Triton X-100, 0.1% (vol/vol) Tween-20, 0.1% (vol/vol) β-mercaptoethanol, 100 μM GTP] supplemented with protease inhibitors. One-milliliter of lysate was subjected to ultracentrifugation to pellet intact microtubules (100,000 × g for 30 min at 37 °C). Supernatant containing the soluble tubulin fraction was removed into a microfuge tube with 5× SDS sample buffer; 50 μM 1× sample buffer was added to the pellet fraction. Twenty microliters of each fraction were separated by SDS/PAGE electrophoresis, immunoblotted and analyzed using Bio-Rad Imagelab software.

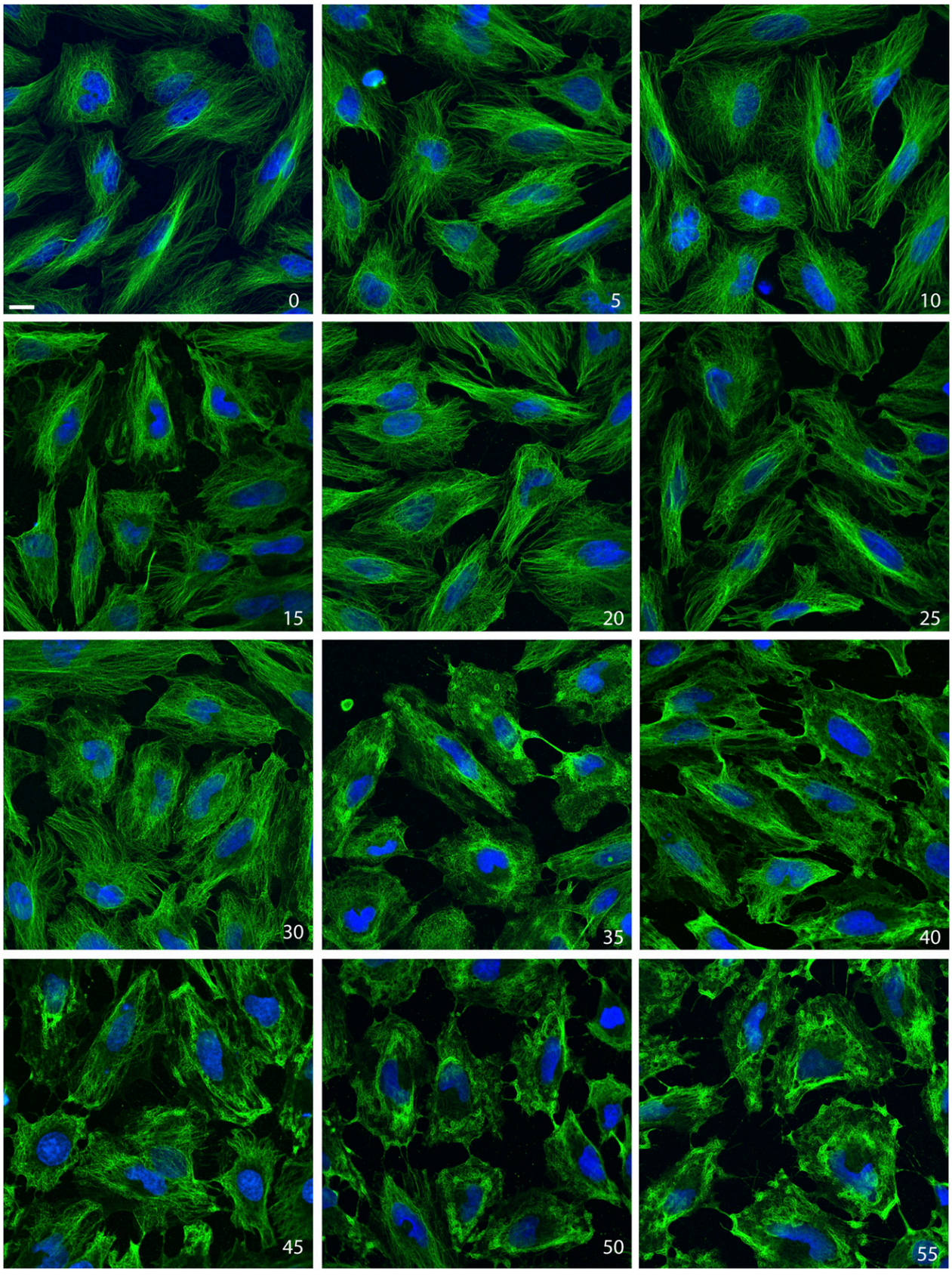


Fig. S1. Time course of kinesore-induced microtubule reorganization in fixed HeLa cells. Time of treatment of kinesore at 50 μM (in minutes) is indicated in the lower righthand corner of each panel. Nuclei are labeled with Hoescht (blue); microtubules (green) are immunostained. Images are representative of three independent experiments. (Scale bar, 10 μm.)

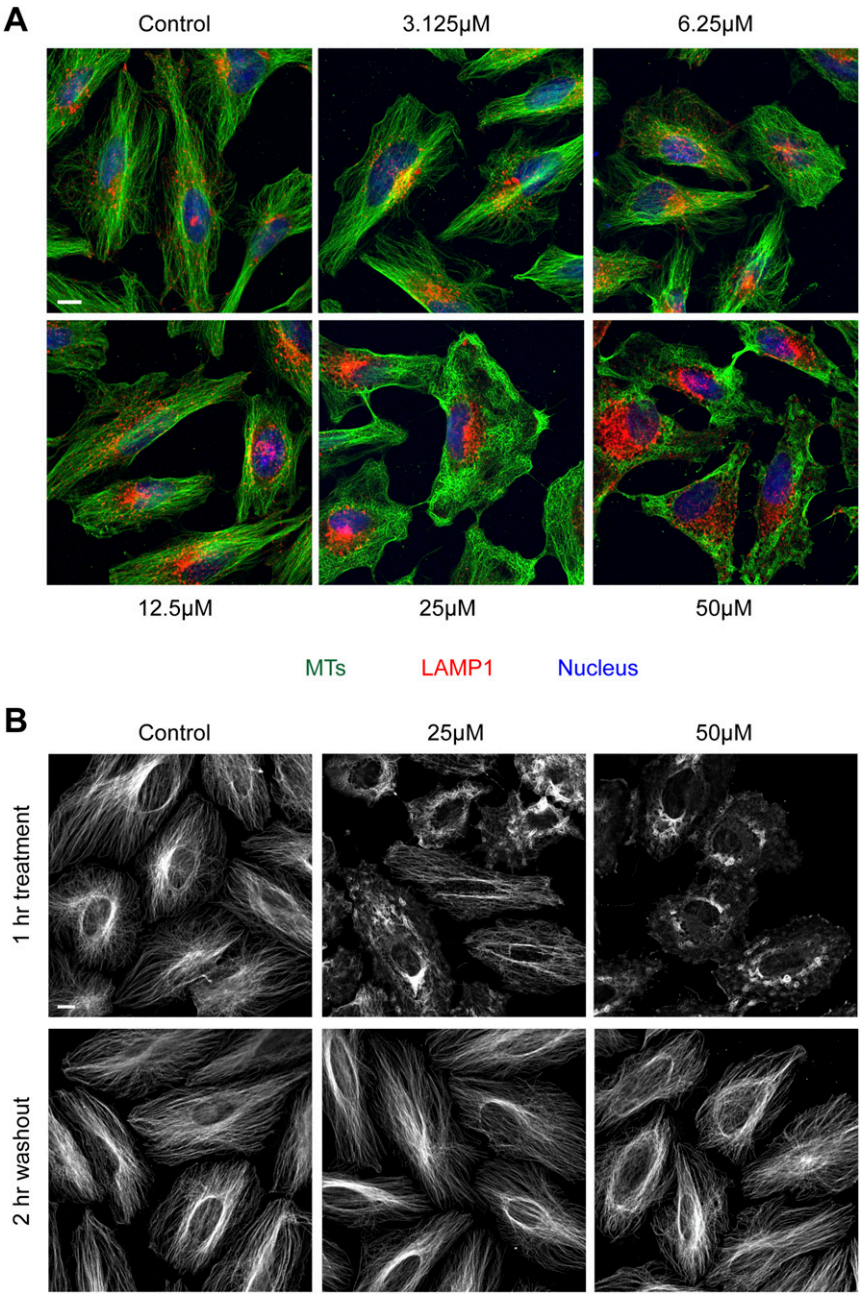


Fig. S2. Concentration dependence and effect of washout on kinesore phenotype. (*A*) Concentration dependence of kinesore phenotype. HeLa cells were treated for 1 h with the indicated concentration of compound, fixed and immunostained for tubulin (green) and LAMP1 (red). Nuclei are stained with Hoechst (blue). (*B*) Two-hour washout experiment for cells treated for 1 h with the indicated concentration of kinesore. The radial microtubule array is reestablished. (Scale bars, 10 μm.)

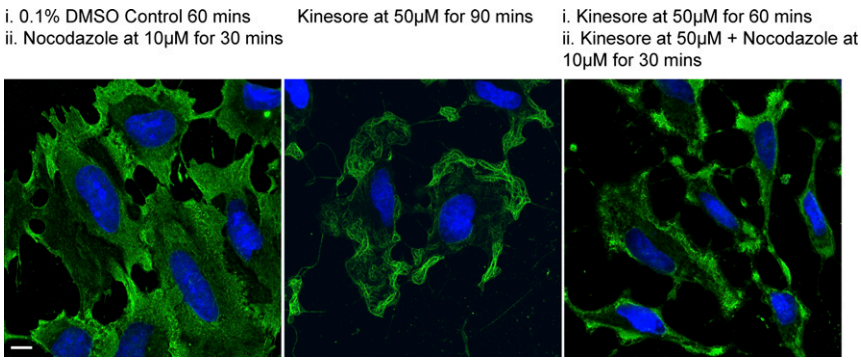


Fig. S3. Nocodazole disrupts kinesore induced loops and bundles. HeLa cells were treated with (*Left*) DMSO control for 60 min, followed by 10 μM nocodazole, (*Center*) 50 μM kinesore for 90 min, or (*Right*) 50 μM kinesore for 60 min followed by 10 μM nocodazole + 50 μM kinesore for 30 min. (Scale bar, 10 μm.)

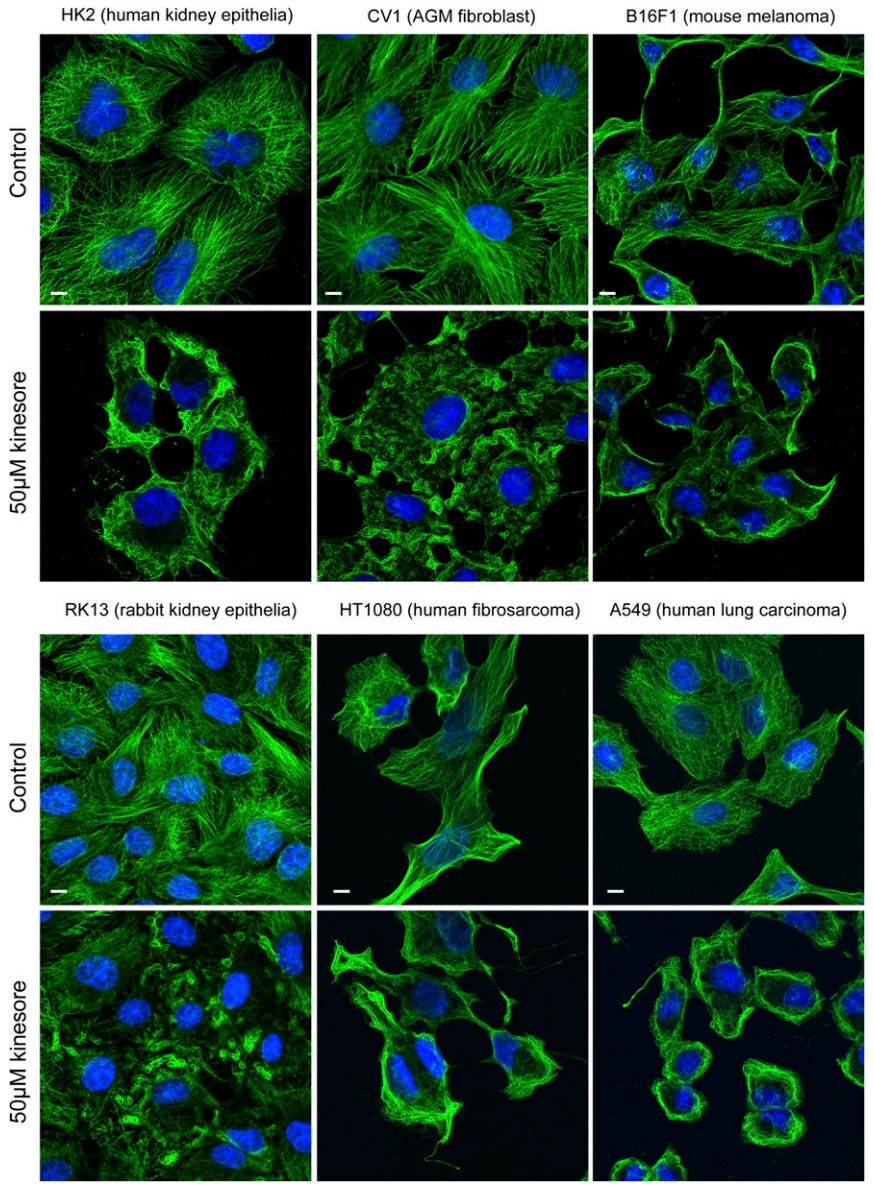


Fig. S4. Effect of kinesore on the microtubule network in a panel of normal and cancer cell lines. The indicated cells were treated with vehicle or kinesore for 1 h, methanol-fixed, and immunostained for tubulin (green). Nuclei are labeled with Hoescht (blue). (Scale bars, 10 μm.)

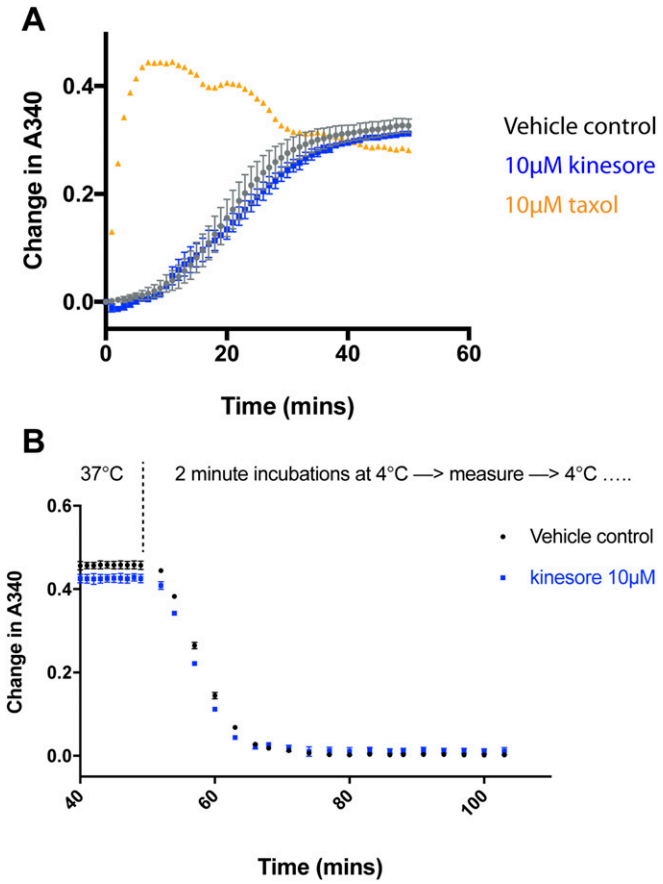


Fig. S5. Effect of kinesore on the in vitro polymerization and depolymerization kinetics of tubulin. (*A*) In vitro tubulin polymerization assays performed with 10 μM kinesore or vehicle control (0.1% DMSO) and extent of tubulin assembly was monitored by absorbance at 340 nm. Data are derived from two independent experiments and error bars show ± SEM. A control curve from assays performed in the presence of 10 μM taxol is shown for comparison. (*B*) Results from a separate experiment where microtubules were polymerized in the presence of DMSO control or kinesore until maximum absorbance was reached. Plates were then incubated at 4 °C for 2-min intervals and absorbance measured. This was repeated until absorbance decreased to a plateau.

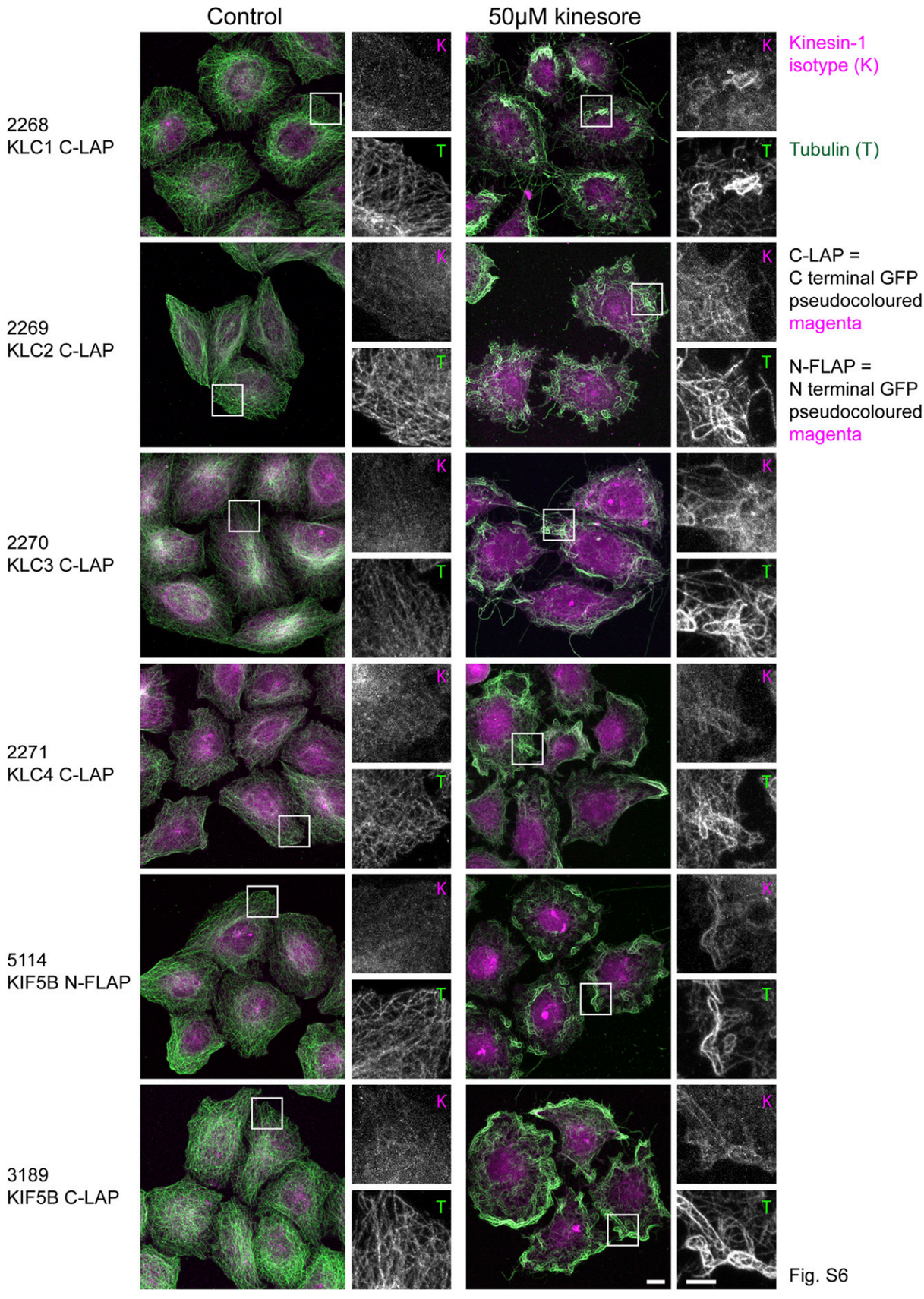


Fig. S6. Effect of kinesore on the localization of kinesin-1 subunits, fused to GFP, and stably expressed under control of their endogenous promoter. The indicated cells were treated with vehicle or kinesore for 1 h, methanol-fixed, and immunostained for tubulin (green). GFP (C-LAP/N-FLAP) kinesin-1 subunits are shown in magenta. (Scale bar, 10 μm; 5 μm in enlargement.)

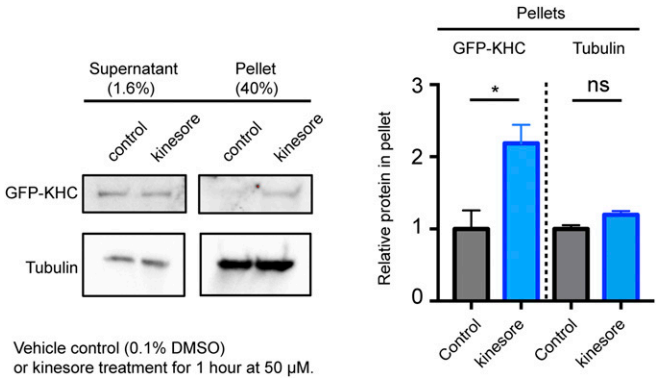
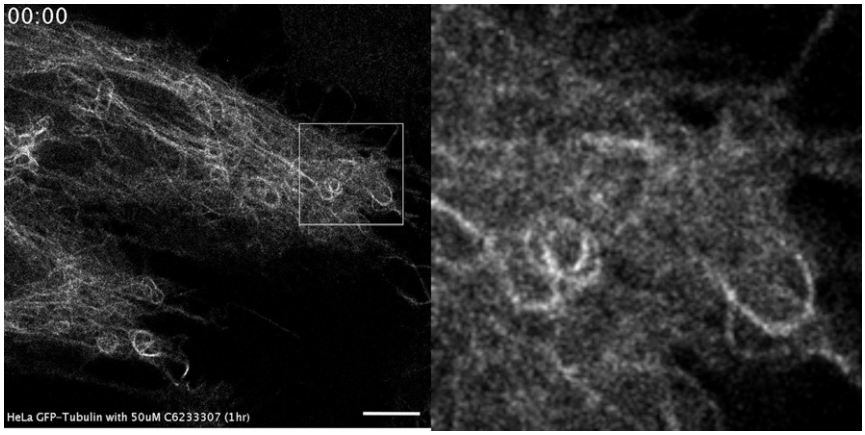
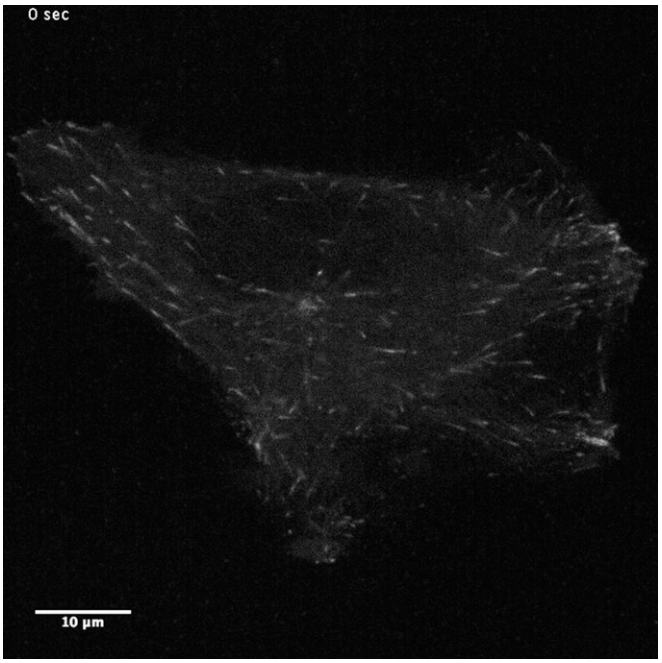


Fig. S7. Effect of kinesore on association of GFP-KIf5B (N-FLAP) association with microtubules. Cells were lysed in a microtubule stabilizing buffer following 1-h treatment with kinesore at 50 μM and lysates were subjected to centrifugation to pellet polymerized microtubules and associated proteins. Pellets and supernatant fractions were analyzed by Western blot for GFP (Kif5B/KHC) and tubulin. Graph shows quantification of protein in pellet fractions from three independent experiments, following normalization to control. Error bars show ± SEM, $*P < 0.05$ obtained using two-tailed *t* test; ns, not significant.



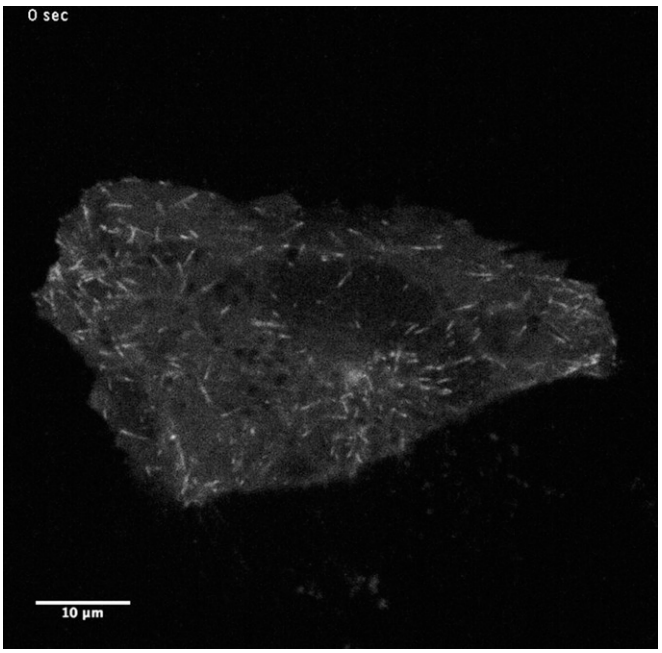
Movie S1. Confocal image sequence at 5-s intervals for 9 min of HeLa cells stably expressing GFP-tubulin and treated with 50 μM kinesore for 1 h. Image sequence begins after 1 h of treatment. (Scale bar, 10 μm.)

Movie S1



Movie S2. Spinning disk confocal image sequences at 5-s intervals for 2 min of HeLa cells stably expressing EB3-RFP in control conditions.

Movie S2



Movie S3. Spinning disk confocal image sequences at 5-s intervals for 2 min of HeLa cells stably expressing EB3-RFP in conditions treated with 50 µM kinesore for 1 h.

Movie S3