Supporting Information

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SI Materials and Methods

Affinity Purification and Mass Spectrometry. For protein A (PtA) purifications, ~10^7 cells were harvested by centrifugation and frozen in liquid nitrogen. The cell pellet was then resuspended in 5 mL extraction buffer [50 mM Hapes at pH 7.5, 100 mM KAc, 50 mM KCl, 2 mM MgCl2, 2 mM EGTA, 0.1% (vol/vol) Nonidet P-40, 5 mM DTT, 5% (vol/vol) glycerol, and Roche Complete Protease Inhibitors] and homogenized using a high-performance disperser (Fisher). Homogenates were agitated at 4°C for 30 min and clarified by centrifugation at 6,000 rpm in an SS34 rotor (Sorvall, Thermo Scientific). Next, 200 µL Dynabeads (Invitrogen) conjugated to rabbit IgG (Sigma) were added to the supernatants and incubated for 4 h under continuous agitation at 4°C. Beads were then washed five times for 10 min in 10 mL extraction buffer. Proteins were then eluted from beads with 0.5 M Na2HPO4 and 0.5 mM EDTA, concentrated, and analyzed by liquid chromatography-mass spectrometry (LC-MS/MS). The MS/MS fragmentation data achieved were used to search the National Center for Biotechnology Information and Flybase databases, using the MASCOT search engine (www.matrixscience.com). Probability-based MASCOT scores were used to evaluate identifications. Only matches with P < 0.05 for random occurrence were considered significant.

In Vivo Pull-Down Assays. Six million cells stably expressing either PtA:StiCC1 (the N-terminal region of the Sti coiled-coil domain is dubbed “StiCC1”) or PtA alone were transfected with plasmids encoding GFP::Neb [Neb stands for Nebblish, the fly counter-part of human kinesin family member 14 (KIF14)] and Pav::Myc [Myc stands for Mycubl, Pav stands for Pavartoti, the Drosophila ortholog of human mitotic kinesin-like protein 1 (MKLP1)]. and Myc stands for myelocytomatosis viral oncogene homolog] transgenes, using the FuGene HD transfection reagent (Promega) and incubated at 25°C for 48 h. The proteasome inhibitor MG132 (Sigma) was added to the medium at a concentration of 25 µM 5 h before harvesting the cells, whereas the cyclin-dependent kinase 1 (Cdck1) inhibitor RO-3306 (Calbiochem) was added 2 h before collection at a final concentration of 10 µM. Cells were then collected, washed in PBS, and stored at −80°C. The cell pellet was resuspended in 0.5 mL of extraction buffer [50 mM Hapes at pH 7.5, 100 mM KAc, 50 mM KCl, 2 mM MgCl2, 2 mM EGTA, 0.1% (vol/vol) Nonidet P-40, 5 mM DTT, 5% (vol/vol) glycerol, and Roche Complete Protease Inhibitors] and homogenized using a high-performance disperser (Fisher). Homogenates were centrifuged at 2,000 rpm at 4°C in an Eppendorf 5417R centrifuge for 15 min, and supernatants were transferred into new tubes. Next, 30 µL Dynabeads (Invitrogen) conjugated to rabbit IgG (Sigma) were added to the supernatants and incubated for 2 h on a rotating wheel at 4°C. Beads were then washed five times for 5 min in 1 mL extraction buffer, resuspended in 30 µL Laemmli buffer, boiled for 10 min, and stored at −20°C. Proteins were separated on a SDS PAGE gel, transferred onto PVDF membrane, and probed to detect the antigens shown in Fig. S1.

Molecular Biology, Cell Culture, DNA, dsRNA, and siRNA Transfections. Gateway technology (Invitrogen) was used in all cloning procedures as described (1). The destination vectors used for expression in Drosophila cultured cells were described (1–3). The pDEST15 vector (Invitrogen) was used for bacterial expression of GST-tagged proteins. Drosophila cDNAs were obtained from the Drosophila Genomics Resource Center. The MKLP1 cDNA was a gift of M. Mishima (University of Warwick, Warwick, United Kingdom). The CIT-K region corresponding to StiCC1, CIT-K420-785, was amplified by PCR, using total RNA from HeLa Kyoto cells, the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen), and the following primers: forward, TACAGAAAGCACTGGGGATT, and reverse, CTCCAGTGTCCTCCTGTGACGC.

The Dmel strain of Schneider 2 (S2) cells (Invitrogen) was grown in serum-free medium supplemented with antibiotics. dsRNA production and RNAi treatments were performed as described previously (1). Primers used to generate sti dsRNAs were described (4); the primers used to generate the nub dsRNA were, forward, GACGCCGTATTGTACTGGATGGT, and reverse, ATCTTCTATGCTTACCCGAGTTG. DNA transfections and generation of fly stable cell lines were described (3).

HeLa Kyoto cells were maintained in DMEM (Invitrogen) supplemented with 10% FCS and antibiotics. CIT-K RNAi knockdown was performed using the siRNAs described in Gruneberg et al. (4) and Lipofectamine RNAiMAX (Invitrogen), following the manufacturer’s instructions.

In Vitro Binding Assay. DNA fragments coding for Sti, Neb, Pav, and MKLP1 were generated by PCR and cloned into pDEST15 (Invitrogen) to express N-terminal GST-tagged polyptides in Escherichia coli. The GST-tagged products were then purified using Glutathione Sepharose 4B (GE Healthcare), according to the manufacturer’s instructions. [35S]Methionine-labeled Sti, Neb, Pav, Fascetto, and CIT-K fragments were prepared from corresponding PCR products amplified using primers harboring a T7 promoter, and then transcribed and translated in vitro, using the Tnt T7 Quick Coupled Transcription/Translation System (Promega) in the presence of [35S]methionine (Perkin-Elmer). GST pull-down assays were carried out as described (5).

Antibodies and Fluorescence Microscopy. The following antibodies were used in this study: mouse monoclonal α- tubulin (clone DM1A, Sigma), chicken polyclonal α-tubulin (Abcam, ab89984), mouse monoclonal α-Myc (clone 9E10, Santa Cruz Biotechnology), rabbit polyclonal α-Myc (Abcam, ab9106), rabbit polyclonal α-Sticky (6), rabbit polyclonal α-Pav (7), rabbit polyclonal α-Neb (a kind gift of D. M. Glover and H. Okhura, University of Cambridge, Cambridge, United Kingdom), mouse monoclonal α-CIT-K (BD Laboratories), rabbit polyclonal α-KIF14 (Bethyl Laboratories), rabbit polyclonal α-MKLP1 (clone sc-867, Santa Cruz Biotechnology), rabbit polyclonal α-PRC1 (a kind gift of F. Barr, University of Oxford, Oxford, United Kingdom) (4). Peroxidase and Alexa-fluor conjugated secondary antibodies were purchased from Jackson Laboratories and Invitrogen.

Dmel cells were grown on 22 × 22-mm coverslips (Menzel-Glaser) and fixed in PHEM [1,4 piperazinediethanesulfonic acid (PIPES), Hepes, EGTA, and MgCl2] buffer (60 mM PIPES, 25 mM Hepes at pH 7, 10 mM EGTA, 4 mM MgCl2, 3.7% formaldehyde) for 12 min. HeLa cells were also plated on coverslips but fixed in ice-cold methanol for 10 min. All cell types were then processed and visualized as described (5). ImageJ software was used to generate RGB fluorescence profiles.

Transmission Electron Microscopy. Cells were plated on coverslips and fixed overnight at 4°C in 2.5% glutaraldehyde in PBS, postfixed for 1 h in 1% OsO4 in PBS, dehydrated in a graded series of alcohol embedded in Epon, and polymerized for 2 d at 60°C. Glass slides were separated from the resin after a short
immersion in liquid nitrogen. Sections were obtained with a LKB ultratome, stained with uranyl acetate and lead citrate, and observed and photographed with a Philips CM10 electron microscope at 80 kV.


Fig. S1. StiCC1 forms a complex with Neb and Pav in vivo. S2 cells stably expressing either the StiCC1 fragment tagged with PtA or PtA alone were co-transfected with plasmids expressing GFP::Neb and Pav::Myc for 48 h and treated with the Cdk1 inhibitor RO-3306 2 h before collection, and then proteins were extracted and used in PtA pull-down assay. The extracts and pull downs were analyzed by Western blot to detect GFP, Myc, and PtA. The numbers on the left indicate the sizes, in kilodaltons, of the molecular mass marker.

Fig. S2. StiCC1 localization to the midzone requires Neb. Drosophila S2 cells stably expressing GFP::StiCC1 were treated with dsRNAs directed against kanamycin (control) or neb for 96 h and then fixed and stained to detect GFP, tubulin, and DNA (blue). Arrowhead marks GFP::StiCC1 localization to the spindle midzone. (Scale bars, 10 μm.)
Fig. S3.  Analysis of Neb depletion in Drosophila S2 cells. (A) Western blot analysis of Neb protein levels in Drosophila S2 cells treated for 96 h with either kanamycin (kana, control) or neb dsRNAs. The arrows mark the bands corresponding to Neb and a nonspecific band used as loading control. The numbers on the left indicate the sizes in kilodaltons of the molecular mass marker. (B) Drosophila S2 cells were treated with dsRNAs directed against kanamycin (control) or neb for 96 h and then fixed and stained to detect Neb, tubulin, and DNA (blue). (Scale bars, 10 μm.)

Table S1. Identification of Neb and Pav by affinity purification and mass spectrometry

<table>
<thead>
<tr>
<th>Bait and interactors</th>
<th>Score</th>
<th>Peptides</th>
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<tbody>
<tr>
<td>StiFL::PtA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pav</td>
<td>364</td>
<td>7</td>
</tr>
<tr>
<td>Tumbleweed/RacGAP50C</td>
<td>122</td>
<td>3</td>
</tr>
<tr>
<td>PtA::StiCC1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neb</td>
<td>143</td>
<td>4</td>
</tr>
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The two kinesins, Pav and Neb, identified from the PtA affinity purification assays are listed along with their respective MASCOT scores and number of peptides. Tumbleweed/RacGAP50C is the RacGAP centralspindlin component in Drosophila. The baits are indicated in bold font.

Fig. S4.  Localization patterns of CIT-K, KIF14, and MKLP1 during cytokinesis in HeLa cells. (A) HeLa (Kyoto) cells were fixed and stained to detect CIT-K, KIF14, and tubulin. (B) HeLa (Kyoto) cells were fixed and stained to detect CIT-K, MKLP1, and tubulin. The insets show a 2.5x magnification of the midbody. (Scale bars, 10 μm.)