Supporting Information

Hendershott and Vale 10.1073/pnas.1404133111

SI Materials and Methods

Homology Tree Generation. Homology trees were generated using the ENSEMBL tree-generating tool (www.ensembl.org/info/genome/compara/homology_method.html) (1, 2).

Plasmid Construction and Protein Expression. For protein expression, CKK domains were cloned into the NotI and KpnI sites of pET28a-GFP plasmid by using Gibson cloning (New England Biolabs) and expressed in BL-21 A1 *Escherichia coli* (Life Technologies). Briefly, overnight starter cultures were diluted 1:100, grown to log phase, and induced by using 1 mM IPTG for 6–8 h at 20 °C. Cells were harvested and lysed using an EmulsiFlex homogenizer, and proteins were purified by using NiNTA beads according to manufacturer instructions (Qiagen); following purification, proteins were dialyzed into 25 mM Hepes, pH 7.4, 150 mM KCl, 1 mM tris(2-carboxyethyl)phosphine (TCEP), and 10% (wt/vol) glycerol. For full-length calmodulin-regulated spectrin-associated protein (CAMSAP), Patronin, CAMSAP CC, and CC+CKK proteins, coding sequences of the genes were cloned into a pFasBac HTA+EGFP vector and expressed in Sf9 cells. EGFP was cloned into the pFasBac vector between BamHI and SacI by using conventional cloning; coding sequences of CAMSAP genes and truncations were cloned between SacI and XhoI sites by using Gibson cloning. Truncation constructs encode the following amino acids: Pat CC, 534–1457; Pat CC+CKK, 534–1689; Pat CKK, 1546–1689; CAMSAP2 CC, 707–1303; CAMSAP2 CC+CKK, 707–1484; CAMSAP2 CKK, 1342–1484; CAMSAP3 CC, 578–950; CAMSAP3 CC+CKK, 578–1239; and CAMSAP3 CKK, 1062–1239. Plasmids were transformed into DH10Bac cells (Life Technologies) and bacmids were prepared according to Bac-to-Bac Baculovirus Expression System manual (Life Technologies). Bacmids were transfected into Sf9 cells, and virus was harvested according to the manufacturer's instructions. Sf9 cells were used for protein expression and were cultured in suspension in Sf-900 II SFM (Invitrogen) supplemented with antibiotic/antimycotic. Expression was completed for 60–72 h for constructs with cells at a starting density of 2×10^6 cells per milliliter. After collection of infected cells, proteins were purified following cell lysis in 1% Triton X-100, using NiNTA beads as for CKK proteins.

Protein Preparation. Pig brain tubulin was prepared according to Castoldi and Popov (3). Microtubules (MTs) were prepared for each day of imaging by polymerizing ∼6 μM unlabeled tubulin in the presence of 1 mM guanylyl 5′-α,β methylenediphosphonate (GMPCPP) (Jena Biosciences). Labeled tubulin (Alexa-561, Cy5 or biotinylated, as specified in the text) was mixed into the reaction at a ratio of ∼1:10 and polymerized in a final volume of 20 μL for 30–60 min at 37 °C.

The kinesin motor domain, K420, was purified with an N-terminal 6×His tag. The dynein motor domain, VY268, was GST-dimerized and purified by using a ZZ-tag as in a previous work (4).

MT seeds were formed by incubating 6 μM tubulin plus Alexa-647 and biotinylated tubulin at a ratio of 1:10 with unlabeled tubulin, with 1 mM GMPCPP (Jena Biosciences) at 37 °C for 1 h.

Image Analysis: Quantification of Minus-End Intensities. Images were background-corrected where indicated by using the Fiji Background Subtraction plug-in. Similarly, where indicated, photobleaching correction was done by using the Fiji Bleach Correction plug-in.

To determine the approximate numbers of CAMSAPs bound to minus-end of GMPCPP-stabilized MTs, we used Fiji (5) to select particles on the surface of the coverslip and particles on MT minus-ends. We used MatLab code to determine the integrated intensity of the five closest GFP spots on the glass coverslip to each minus-end–bound GFP-CAMSAP spot and calculated the ratio of these intensities.

Patronin RNAi Experiments in *Drosophila* S2 Cells. *Drosophila* S2 cells lines were cultured in Schneider media supplemented with 10% (vol/vol) FBS (Invitrogen) and antibiotic/antimycotic mix. Cell lines stably expressing GFP-tubulin and mCherry–Patronin constructs were generated as previously described (6). GFP-tubulin was expressed under a constitutive actin promoter; mCherry–Patronin genes were expressed under an inducible metallothionein promoter. For RNAi experiments, dsRNA against the 3′ and 5′ UTRs of Patronin was synthesized by in vitro transcription with the Ambion T7 Megascript kit by using S2 cell genomic DNA template and primers amplifying 5′ and 3′ UTR sequences as follows:

5′ forward, TAATACGACTCACTATAGGGCACATGAAAATTTGTAAG; 5′ reverse, TAATACGACTCACTATAGGGGACTCCGGCTCTCCGACGCCCGCC; 3′ forward, TAATACGACTCACTATAGGGGGAAATGAAATCGTGTATGGGCCG; and 3′ reverse, TAATACGACTCACTATAGGGGTGTACATCCGCTGGCTCTCAC.

Cells were subjected to two rounds of RNAi over the course of 8 d. Cells were prepared for imaging by inducing expression of mCh-Patronin truncation with 100 μM Cu^{2+} overnight and plating onto concanavalin A for 1 h before imaging. For rescue experiments, cells expressing both GFP-tubulin and the mCh-Patronin construct were selected for time-lapse imaging. Cells were imaged on a Zeiss spinning-disk confocal microscope with a 100× 1.45 N.A. objective, by using an Andor EM CCD camera at 5–10-s intervals. Cells were scored for the Patronin phenotype by three blinded observers for the presence of more than five "treadmilling" MT fragments at the cell edge (scores were identical for all but five cells).

1. Vilella AJ, et al. (2009) EnsemblCompara GeneTrees: Complete, duplication-aware phylogenetic trees in vertebrates. *Genome Res* 19(2):327–335.
2. Hubbard TJ, et al. (2009) Ensembl 2009. *Nucleic Acids Res* 37(database issue): D690–D697.
3. Castoldi M, Popov AV (2003) Purification of brain tubulin through two cycles of polymerization-depolymerization in a high-molarity buffer. *Protein Expr Purif* 32(1):83–88.
4. Gennerich A, Reck-Peterson SL (2011) Probing the force generation and stepping behavior of cytoplasmic Dynein. *Methods Mol Biol* 783:63–80.
5. Schindelin J, et al. (2012) Fiji: An open-source platform for biological-image analysis. *Nat Methods* 9(7):676–682.
6. Goodwin SS, Vale RD (2010) Patronin regulates the microtubule network by protecting microtubule minus ends. *Cell* 143(2):263–274.

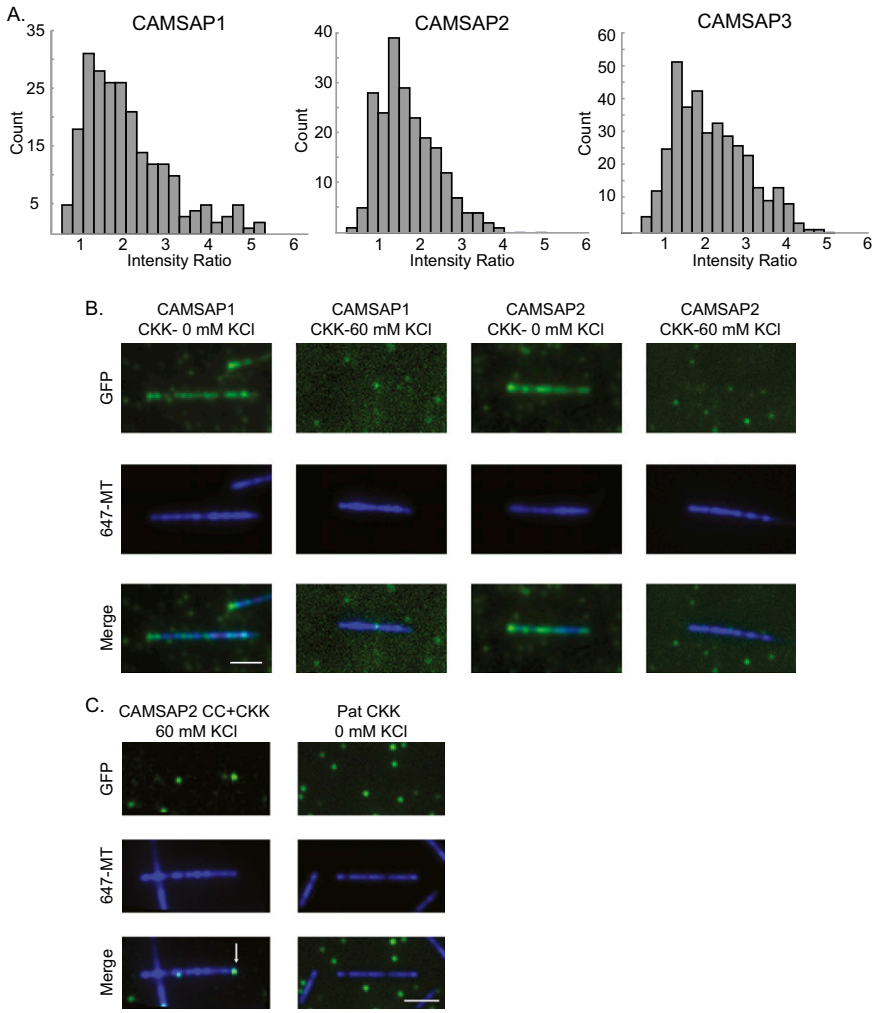


Fig. S1. All CAMSAP CKK domains exhibit similar MT binding behavior. (*A*) The intensity of GFP-CAMSAP bound at a MT minus-end was compared as a ratio to the average intensity of five nearby molecules bound on the glass surface and plotted for each CAMSAP family member (*SI Materials and Methods*). (*B*) Similar to CAMSAP3 (Fig. 3), the CAMSAP1 and CAMSAP2 CKK domains bind to MT length at 10–12 nM concentrations, with a slight preference for the MT minus-end, in 0 mM KCl (first and third columns). In the presence of 60 mM KCl, almost all MT binding is lost (second and fourth columns). (Scale bar: 2 μm.) (*C*) As for the CAMSAP3 CC+CKK (Fig. 3), the CAMSAP2 CC+CKK domain binds to MT minus-ends (white arrow, *Left*). In contrast to the mammalian CKK domains, the Pat CKK domain does not bind MT, even in 0 mM KCl (*Right*). (Scale bar: 2 μm.)

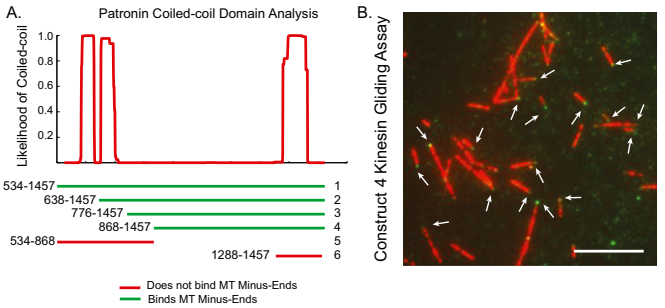
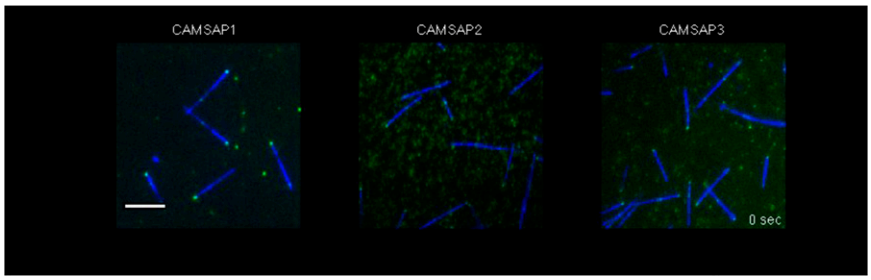
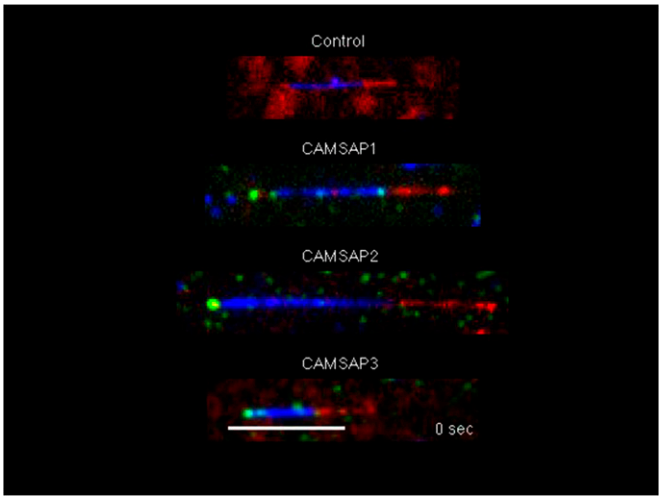


Fig. S2. A subdomain of the Patronin CC specifically binds MT minus-ends. (*A*) Indicated truncations of the Patronin CC were made based on structure predictions (truncations are denoted under CC prediction schematic). GFP-CC constructs that bound specifically to MT minus-ends in a kinesin gliding assay are shown in green and those that did not bind are shown in red (these constructs failed to bind to the minus-end or along the length). Numbered constructs correspond to the following amino acid regions: 1, 534–1457; 2, 638–1457; 3, 776–1457; 4, 868–1457; 5, 534–868; and 6, 1288–1457. Construct 4 (amino acids 868–1457) was the smallest CC region that could bind to MT; this construct contained a significant portion of a predicted unstructured region. (*B*) Gliding assay with Alexa-561–labeled MTs and the minimal CC domain (GFP-CC868-1457; construct 4); GFP-labeled minus-ends labeled with GFP-CC868-1457 are marked with white arrows. (Scale bar: 10 μm.)



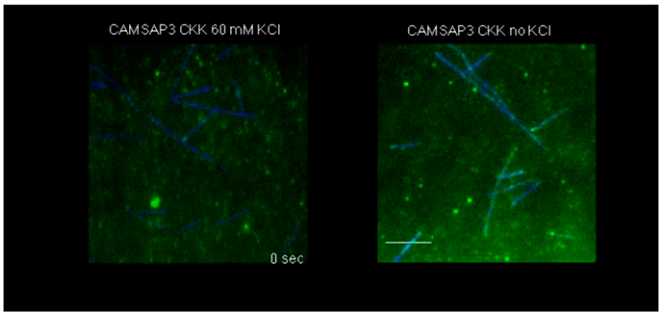
Movie S1. CAMSAPs bind to the minus-ends of GMPCPP-stabilized MTs. A kinesin gliding assay with Alexa-647 MTs (blue) and GFP-tagged full-length CAMSAP molecules. Minus-ends are leading in the direction of motion. (Scale bar: 5 μm.)

Movie S1



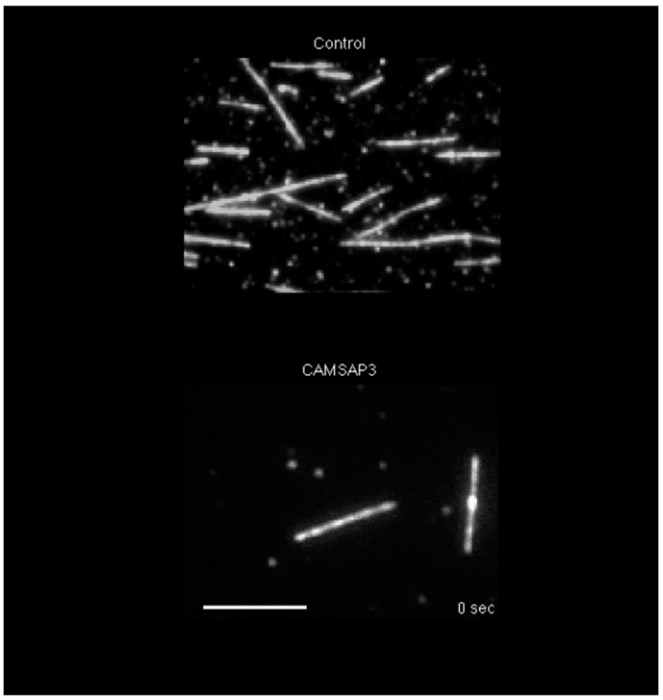
Movie S2. The effects of CAMSAPs on the dynamic growth of MT minus-ends. GMPCPP-stabilized MT seeds are shown in blue (Alexa-647–labeled tubulin), and new MT growth is marked in red (free tubulin labeled with Alexa-561). (*Top*) Control MT; the other three panels show dynamic assay in presence of 10–12 nM full-length CAMSAPs. Minus-ends are oriented to the left. (Scale bar: 5 μm.)

Movie S2



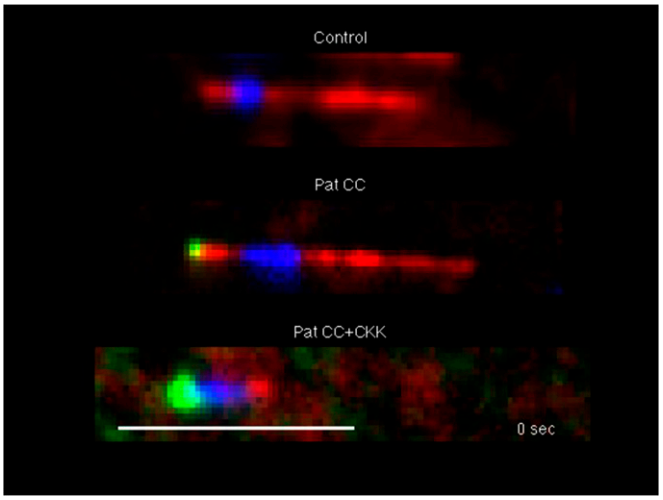
Movie S3. Binding of the GFP-CKK domain from CAMSAP3 to MTs with 0 and 60 mM KCl. Gliding assay with Alexa-647 MTs (blue) and GFP-tagged CKK domain of CAMSAP3. (*Left*) Assay buffer with 60 mM KCl. (*Right*) Buffer without added KCl. (Scale bar: 5 μm.)

Movie S3



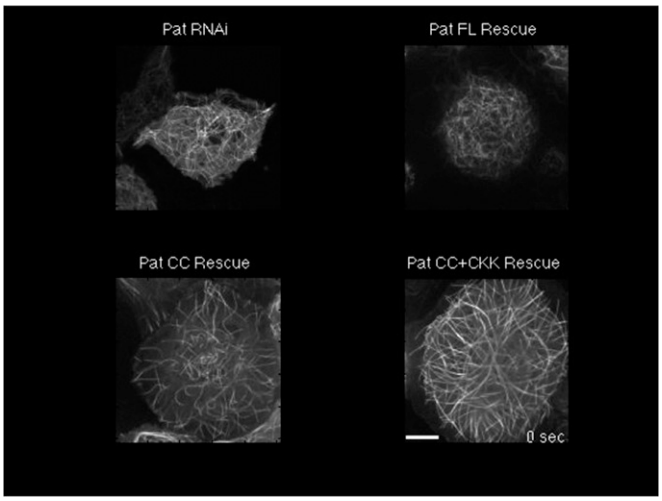
Movie S4. CAMSAPs protect MT minus-ends against kinesin-13 (6 nM of mitotic centromere-associated kinesin)-induced depolymerization. (*Upper*) Control conditions. (*Lower*) In the presence of 12 nM CAMSAP3. MTs in blue are stabilized with GMPCPP. (Scale bar: 5 μm.)

Movie S4



Movie S5. MT minus-end tracking by Patronin CC and Patronin CC+CKK. GMPCPP-stabilized MT seeds are shown in blue (Alexa-647–labeled tubulin), new MT growth is marked in red (free tubulin labeled with Alexa-561), and GFP-Patronin CC or Patronin CC+CKK is shown in green. Minus-ends are oriented to the left. (Scale bar: 5 μm.)

Movie S5



Movie S6. Patronin RNAi knockdown and rescue experiments with different Patronin constructs. Patronin knockdown produces short, treadmilling MTs in the periphery and a decrease in the overall numbers of MTs. This phenotype was rescued by expressing full-length Patronin and the CC+CKK domain but not by the CC alone. (*Inset*) Larger view of indicated region of cell periphery. (Scale bars: 10 μm; *Inset*, 1 μm.)

Movie S6