ONLINE METHODS

Plasmids

Human (Hs-spastin) and D. melanogaster spastin (Dm-spastin) vectors. Hs-spastin can be expressed as at least four isoforms (M1 with exon 4, M1 without exon 4, M87 with exon 4, and M87 without exon 4)[41]. Vectors for mutant constructs were generated by site-directed mutagenesis (QuikChange XL Kit, Agilent, cat# 200517). Incorporation of the desired mutations was verified by Sanger sequencing.

The wildtype (WT, isoform M87 with exon 4, with N-terminal HA- and EGFP-tags) spastin plasmid used to generate cell lines was a gift from C. Campsteijn (Oslo University Hospital). WT and mutant spastin M87e4 ORFs were sub-cloned into the pcDNA^{TM}5.0/FRT/TO vector (ThermoFisher) using HindIII and NotI restriction sites. The cDNA for sequence for Hs-spastin (aa 229-616, numbered according to NCBI RefSeq: NP_055761.2) used for protein expression was PCR amplified from the ORFeome collection (Dharmacon), and cloned into a pGEX-6P1 vector using BamHI and NotI restriction sites.

The vector (pDEST15-spastin-*D.melanogaster*) used for Dm-spastin expression was a gift Dr. Antonina Roll-Mecak (NIH) and had been previously used in Ref. 8. We note that in this construct, comprised of aa 209-758, there is a deletion at aa 311-372 (corresponding to exon 3 of Drosophila spastin). However, the overall residue numbering used is that for full-length Dm-spastin isoform A (Uniprot: Q8I0P1), as per earlier publications by others.

Xenopus laevis katanin vector. MAL-c5x-*X.laevis* p60 (full-length, NCBI RefSeq NP_001084226.1) was a gift form Dr. R. Heald (UC Berkeley).

Mus musculus VCP vector. pQE9-His-p97 (100% identical to *Homo sapiens* VCP protein, full-length, NCBI RefSeq NP_009057.1, Ile206Val variant) was obtained from Addgene (G. Warren, plasmid #14666).

Homo sapiens PCH2 vector. The cDNA for Hs-PCH2 (full length, NCBI RefSeq NP004228) was PCR amplified from the ORFeome collection and cloned using the ApaI and AscI restriction sites into a pDEST15 vector.

Homo sapiens FIGL1 vector. The cDNA for Hs-FIGL1 (aa 288 to 674, NCBI RefSeq NP_001036227.1) was PCR amplified from the ORFeome collection and cloned into a pDEST15 vector using the ApaI site and InFusion technology (Clontech).

Homo sapiens VPS4B vector. The cDNA for the sequence Hs-VPS4B (full-length, NCBI RefSeq. BC039574.1) was PCR amplified from the ORFeome collection and cloned using the NdeI and EcoRI restriction sites into a pET-SUMO vector.

Cell lines, cell culture and viability analyses

Cells expressing wildtype or mutant tagged-spastin M87e4 were generated using the T-REx™ HeLa Flp-In cell line as per the vendor's protocols (ThermoFisher). Genomic DNA was extracted from cells using the DNeasy Blood and Tissue kit (Qiagen), insertions were PCR amplified and sequenced. Cells were grown in DMEM (ThermoFisher) supplemented with 10% (v/v) fetal bovine serum (Sigma Aldrich), L-glutamine (2 mM; ThermoFisher), Hygromycin B (250 µg/mL) and Blasticidin (20 µg/mL) at 37ºC and 5% CO_2. Cells were confirmed to be mycoplasma free using a PCR-based method[42].

For western blotting cells were cultured with or without doxycycline (1 µg/mL for 24 hours) prior to lysis at 4 °C. Antibodies: mouse monoclonal anti-spastin antibody (1:750; Sp6C6; Abcam), and goat polyclonal anti-GAPDH (control, 1:1000; V-18; Santa Cruz). Membranes were imaged using a LI-COR Odyssey Infrared Imager. Cell viability assays were conducted using a CellTiter-Glo® Luminescent Cell Viability Assay (Promega) according to the manufacturer recommendations. The luminescence signal quantified using a Synergy NEO Microplate Reader.

Microscopy

Live-cell imaging. HeLa-WT and -N386C cells were grown on 22x22 mm coverslips and mounted in a custom Rose chamber in 10% FBS in L-15 media without phenol red (Invitrogen) and maintained at 35–37 °C. For inhibitor treatment, cells were incubated with spastazoline for 40 min, before mounting and imaging.

Confocal GFP fluorescence micrographs were acquired using a Nikon TE2000 microscope (Morrell Instruments), with a 100x objective (Plan Apo, 1.45 NA), a Yokogawa CSU10 confocal head, and an EMCCD Photometrics Cascade II 512B camera (Roper Scientific). For the detection of GFP-spastin dots, the FIJI[43] ComDet v.0.3.8 plugin was used (https://github.com/ekatrukha/ComDet), which detects particles by applying a 2D Gaussian mexican hat filter to the original image, followed by thresholding, finding of maxima, segmentation, and centroid calculation. The quality of the automated detection was visually checked. This plugin was used to detect particles >2 pixels with a signal-to-noise ratio of ranging from 1.1 to 3, depending on the signal intensity over background.

Immunofluorescence analyses. Cells were plated on acid-washed coverslips (#1.5; Fisherbrand cat#12-545-81) coated with poly-D-lysine hydrobromide (Sigma cat#P6407) and cultured for 24-48 hours prior to fixation.

For intercellular bridge analyses, spastin expression was induced (1 µg/mL doxycycline for ~24 hours), cells were treated with 10 µM spastazoline or 0.1% DMSO (solvent control) for 4.5 hours, and then fixed at 37ºC, using 0.5% (w/v) glutaraldehyde in PBS, for 10 min. Coverslips were washed with PBS, incubated with a solution of 0.1% $NaBH_4$ for 10 min, and blocked (blocking buffer: 2% bovine serum albumin, 0.1% (w/v) sodium azide, and 0.1% Triton™ X-100 in Tris-buffered saline) for 60 min. Cells were stained with mouse monoclonal anti-acetylated tubulin antibody (Sigma cat#T7451; 1:1000 dilution) for 2 hrs, and with Texas-Red conjugated anti-mouse

secondary antibody (Jackson Immunoresearch; 0.1 µg/mL) for 60 min. DNA was stained with Hoechst 33342 (ThermoFisher cat#H1399).

For intercellular bridge quantification, 5x5 fields of view were acquired using a 40x objective (Plan Fluor 0.6 NA) and stitched together into ~905 μm^2 images using NIS-Elements. The number of cells and intercellular bridges were detected manually, with over 10000 cells analyzed.

For 3D imaging of fixed cells, we used a DeltaVision Image Restoration Microscope (Applied Precision) with an Olympus IX-70 base and Resolve 3D softWoRx-Acquire acquisition software (v: 6.5.2, Release RC1). Z-stacks were collected with a 0.25 µm step size and images were deconvolved using sofWoRx.

For microtubule staining, cells were treated with 10 µM spastazoline or 0.1% DMSO (solvent control) for 4 hours, and then fixed at 37ºC using 4% formaldehyde in 100 mM PIPES, 10 mM EGTA, 1 mM $MgCl_2$ and 0.2% Triton™ X-100, pH 6.9 for 10 min. Coverslips were blocked (blocking buffer) for 45 min before staining with anti-alpha-tubulin-FITC antibody (DM1A, Sigma cat#F2168; 1:2000). Confocal fluorescence images were acquired as Z-stacks with 0.3 µm step size using a Nikon TE2000 microscope with a 100x objective (Plan Apo, 1.45 NA).

Biochemical assays

Recombinant protein expression

Recombinant proteins were expressed in *Escherichia coli* Rosetta (DE3) pLysS cells (Merck, Cat#70954) grown in Miller's LB medium (LMM, Formedium, cat#LMM105). For all constructs protein expression was induced at OD_{600} = 0.6-0.8 with 0.5 mM IPTG (Goldbio). The cultures were grown at 18 ºC for 12-16 h, pelleted and resuspended in lysis buffer (Buffer A, see below). All subsequent purification steps were performed at 4ºC. Cell lysis was carried out using a Emulsiflex-C5 homogenizer (Avestin, 5-6 cycles at 10,000-15,000 psi). The homogenized lysate was centrifuged at 40,000 rpm for 45 to 60 min using a Ti45 rotor in a Beckman Coulter Optima LE-80K ultracentrifuge. All recombinant proteins were purified using multi-step strategies, which involved combinations of affinity, ion exchange and size exclusion chromatography. Specific buffers and purification conditions, optimized for yield and specific activity, are summarized:

Dm-spastin wildtype and mutants.

Buffer A: PBS, 10 mM $MgCl_2$, 1 mM PMSF, 20 U/mL benzonase, and cOmplete™ EDTA-Free Protease Inhibitor Cocktail. *Buffer B1:* 50 mM Tris-HCl, 500 mM KCl, 10 mM $MgCl_2$, 5 mM DTT, pH 7.5. *Buffer B2:* 50 mM Tris-HCl, 300 mM NaCl, 10 mM $MgCl_2$, 5 mM DTT, 20 mM reduced glutathione, pH 8.0. *Buffer C*: 50 mM Na-MES, 10% (w/v) glycerol, 10 mM $MgCl_2$, 5 mM DTT, pH 6.5. *Buffer D*: 50 mM Na-MES, 2 M NaCl, 10% (w/v) glycerol, 10 mM $MgCl_2$, 5 mM DTT, pH 6.5. *Buffer E*: 20 mM K-HEPES, 300 mM NaCl, 10 mM $MgCl_2$, 5 mM DTT, 15% (w/v) glycerol, pH 7.5.

Conditions. Briefly, the clarified lysate containing GST-Dm-spastin was incubated with a GSTrap 4B matrix (GE Healthcare) and eluted in Buffer B supplemented with 20 mM reduced glutathione. The protein solution was incubated with PreScission protease (0.1 mg/mL) for 8-12 h, diluted 1:2.5 with Buffer C and loaded into a CaptoS cation exchange column (GE Healthcare) equilibrated in

95% Buffer C and 5% Buffer D. Fractions eluted from the ion exchange chromatography column were pooled, concentrated 10-fold with an Amicon Ultra 30K MWCO centrifugal filter, and further purified over a 16/60 Superdex 200 column (GE Healthcare) in Buffer E. The eluate containing purified spastin was pooled, concentrated using an Amicon Ultra 30K MWCO centrifugal filter to at least 1 mg/mL and stored at −80 °C. PreScission cleavage left six nonnative residues at the N terminus (GPQGSK).

Hs-spastin wildtype and N386C mutant.

Buffer A: PBS, 10 mM $MgCl_2$, 10 mM DTT, 1 mM PMSF, 5 U/mL benzonase, and cOmplete™ EDTA-Free Protease Inhibitor Cocktail, pH 7.4. *Buffer B*: 50 mM K-HEPES, 250 mM KCl, 5 mM $MgCl_2$, 5 mM DTT, 0.1 mM PMSF, 0.005% (v/v) Triton™ X-100, pH 7.5. *Buffer C*: 50 mM Na-MES, 10% (w/v) glycerol, 5 mM $MgCl_2$, 5 mM DTT, pH 6.5. *Buffer D*: 50 mM Na-MES, 2 M NaCl, 10% (w/v) glycerol, 5 mM $MgCl_2$, 5 DTT, pH 6.5.

Conditions. Briefly, the clarified lysate containing GST-Hs-spastin was incubated with a GSTrap 4B matrix (GE Healthcare) and eluted in Buffer B supplemented with 20 mM reduced glutathione. The protein solution was incubated with PreScission protease (0.1 mg/mL) for 10-12 h, diluted 1:2 with Buffer C and loaded into a CaptoS cation exchange column (GE Healthcare) equilibrated in 93% Buffer C and 7% Buffer D. Fractions eluted from the ion exchange chromatography column were pooled, concentrated 10-fold with an Amicon Ultra 50K MWCO centrifugal filter. The protein solution was centrifuged at 20,000*g* for 15 min and the soluble fraction was stored at −80 °C. PreScission cleavage left five nonnative residues at the N terminus (GPLGS).

GST-FIGL1 (Hs-FIGL1).

Hs-FIGL1 protein used for measurement of kinetic parameters was obtained by the following procedure (Method 1):

Buffer A: 25 mM Tris-HCl, 300 mM NaCl, 5 mM $MgCl_2$, 5 mM DTT, 1 mM PMSF, 0.01% (v/v) Triton™ X-100, and cOmplete™ EDTA-Free Protease Inhibitor Cocktail, pH 8.0. *Buffer B1*: 25 mM Tris-HCl, 300 mM NaCl, 5 mM $MgCl_2$, 5 mM DTT, 0.4 mM PMSF, 0.01% (v/v) Triton™ X-100, pH 8.0. *Buffer B2*: 25 mM Tris-HCl, 150 mM NaCl, 5 mM $MgCl_2$, 5 mM DTT, 10 mM reduced glutathione, pH 8. *Buffer C*: 25 mM Tris-HCl, 75 mM NaCl, 5 mM $MgCl_2$, 5 mM DTT, pH 8.5. *Buffer D*: 25 mM Tris-HCl, 500 mM NaCl, 5 mM $MgCl_2$, 5 mM DTT, pH 8.5. *Buffer E*: 25 mM Tris-HCl, 150 mM NaCl, 5 mM $MgCl_2$, 5 mM DTT, 5% (w/v) glycerol, pH 7.5

Conditions 1. The clarified lysate from cells expressing GST-FIGL1 was loaded onto GSTrap4B (GE Healthcare), washed with Buffer B1, then Buffer D and finally eluted with Buffer B2. Fractions containing the proteins were pooled, diluted with 1 volume of Buffer C and loaded on a MonoQ 5/50 GL column (GE Healthcare), equilibrated in 95% Buffer C and 5% Buffer D. The protein fractions were pooled, concentrated 10-fold using an Amicon® Ultra 50K MWCO centrifugal filter, and further purified over a 10/300 Superdex 200 column (GE Healthcare) in Buffer E. Fractions from size-exclusion column were concentrated to a minimum of 1 mg/mL using an Amicon® Ultra 30K MWCO centrifugal filter, and stored at -80 ºC

Hs-FIGL1 protein used for testing inhibitors was obtained with method 1 or method 2. We note that protein purity and ATPase activity were comparable between the methods (data not shown).

Method 2:

Buffer A: 50 mM Tris-HCl, 150 mM NaCl, 5 mM $MgCl_2$, 10 mM DTT, 0.1 mM PMSF, 0.01% (v/v) Triton™ X-100, 5 U/mL benzonase, and cOmplete™ EDTA-Free Protease Inhibitor Cocktail, pH 8.0. *Buffer B1*: 25 mM Tris-HCl, 300 mM NaCl, 5 mM $MgCl_2$, 5 mM DTT, 0.1 mM PMSF, 0.01% (v/v) Triton™ X-100, pH 8.0. *Buffer B2*: 25 mM Tris-HCl, 150 mM NaCl, 5 mM $MgCl_2$, 5 mM DTT, 10 mM reduced glutathione, pH 8.0. *Buffer C*: 25 mM Tris-HCl, 75 mM NaCl, 5 mM $MgCl_2$, 5 mM DTT, pH 8.5. *Buffer D*: 25 mM Tris-HCl, 1 M NaCl, 5 mM $MgCl_2$, 5 mM DTT, pH 8.5. *Buffer E*: 25 mM Tris-HCl, 150 mM NaCl, 5 mM $MgCl_2$, 5 mM DTT, 5% (w/v) glycerol, pH 7.5.

Conditions. The clarified lysate from cells expressing GST-FIGL1 was incubated with sepharose glutathione beads (GE Healthcare) for 1h, washed with Buffer B1 and eluted with Buffer B2. Fractions containing the protein were pooled, diluted with 3 volumes of Buffer C and loaded on a MonoQ 5/50 GL column (GE Healthcare), equilibrated in 95% Buffer C and 5% Buffer D. The protein fractions were pooled, concentrated 10-fold using an Amicon® Ultra 50K MWCO centrifugal filter, and further purified over a 10/300 Superdex 200 column (GE Healthcare) in Buffer E. Fractions from size-exclusion column were concentrated to a minimum of 1 mg/mL using an Amicon® Ultra 30K MWCO centrifugal filter, and stored at -80 ºC.

MBP-katanin (Xl-katanin).

Buffer A: 20 mM Tris-HCl, 250 mM NaCl, 5 mM $MgCl_2$, 100 µM ATP, 5 mM DTT, 10% (w/v) glycerol, 1 mM PMSF and cOmplete™ EDTA-Free Protease Inhibitor Cocktail, pH 7.5. *Buffer B*: 20 mM Tris-HCl, 250 mM NaCl, 5 mM $MgCl_2$, 100 µM ATP, 5 mM DTT, 10% (w/v) glycerol, pH 7.5. *Buffer C*: 20 mM Tris-HCl, 50 mM NaCl, 1 mM $MgCl_2$, 5 mM DTT, 100 µM ATP, 10% (w/v) glycerol, pH 7.5. *Buffer D*: 20 mM Tris-HCl, 500 mM NaCl, 5 mM $MgCl_2$, 5 mM DTT, 100 µM ATP, 10% (w/v) glycerol, pH 7.5. *Buffer E*: 20 mM Na-HEPES, 250 mM NaCl, 5 mM $MgCl_2$, 5 mM DTT, 10% (w/v) glycerol, pH 7.5.

Conditions. The clarified lysate from cells expressing MBP-katanin was loaded onto MBP trap column (GE Healthcare). The column was washed with 30 column volumes of Buffer B and eluted with a Buffer B supplemented with 10 mM maltose. The eluate was diluted 1:2 with Buffer C and loaded on a 16/60 MonoQ GL ion exchange column and eluted with a gradient of Buffer D. Combined MonoQ fractions were further purified on a 10/300 Superdex 200 gel filtration column in Buffer E. The fractions containing purified MBP-katanin protein were frozen in liquid nitrogen and stored at -80°C.

VCP (Mm-VCP).

Mm-VCP protein used for measurement of kinetic parameters was obtained by the following procedure (Method 1):

Buffer A: 25 mM K-HEPES, 500 mM KCl, 20 mM imidazole, 2 mM DTT, 5 U/mL benzonase, and cOmplete™ EDTA-Free Protease Inhibitor Cocktail, pH 8.0. *Buffer B*: 25 mM K-HEPES, 500 mM KCl, 20 mM imidazole, 2 mM DTT, pH 8.0. *Buffer C*: 25 mM K-HEPES, 500 KCl, 1 mM $MgCl_2$, 2 mM DTT, 500 mM imidazole, pH 7.5. *Buffer D*: 25 mM K-HEPES, 250 mM KCl, 1 mM $MgCl_2$, 2 mM DTT, pH 7.5.

Conditions. The clarified lysate from cells expressing His6-VCP was incubated with Ni-NTA beads (Qiagen) for 40 min, and the beads were extensively washed using Buffer B. The protein was eluted with Buffer C. Fractions containing the protein were concentrated using an Amicon® Ultra 50K MWCO centrifugal filter, the concentrated protein sample was filtered though a 0.22-µm Millex-GP PES membrane and loaded on a 10/300 Superose 6 column (GE Healthcare) in Buffer D. Fractions from size-exclusion column were concentrated using an Amicon® Ultra 50K MWCO centrifugal filter and concentrated protein sample was stored at −80 °C.

Mm-VCP protein used for testing inhibitors was obtained with method 1 or method 2. We note that protein purity and ATPase activity were comparable between the methods (data not shown).

Method 2:

Buffer A: 50 mM Tris-HCl, 400 mM NaCl, 2 mM $MgCl_2$, 20 mM imidazole, 1 mM ATP, 2 mM TCEP, 0.025% (v/v) Triton™ X-100, and cOmplete™ EDTA-Free Protease Inhibitor Cocktail, pH 7.5 (at 25°C). *Buffer B*: 50 mM Tris-HCl, 300 mM NaCl, 40 mM imidazole, 5 mM $MgCl_2$, 1 mM TCEP, 0.01% (v/v) Triton™ X-100, pH 7.5 (at 25°C). *Buffer C*: 50 mM Na-HEPES, 100 NaCl, 5 mM $MgCl_2$, 1 mM TCEP, pH 7.5 (at 25°C). *Buffer D*: 50 mM Na-HEPES, 1 M NaCl, 5 mM $MgCl_2$, 1 mM TCEP, pH 7.5 (at 25°C). *Buffer E*: 50 mM Na-HEPES, 300 mM NaCl, 5 mM $MgCl_2$, 1 mM TCEP, pH 7.5 (at 25°C).

The clarified lysate in buffer A from cells expressing His6-VCP was incubated with Ni-NTA beads (Qiagen) for 40 min, and the beads were extensively washed with Buffer B. The protein was eluted with Buffer B supplemented with 500 mM imidazole. Eluted fractions were pooled and dialysed in Buffer C. After dialysis the protein was loaded onto a MonoQ column 5/50 GL (GE Healthcare) and fractioned over a gradient with Buffer D. Protein was eluted at approximately 350 mM NaCl. Combined MonoQ fractions were concentrated using an Amicon Ultra 50K MWCO centrifugal filter, and the concentrated protein sample was filtered though a 0.22-µm Millex-GP PES membrane before loading onto a 10/300 Superdex 200 column (GE Healthcare) equilibrated in Buffer E. Fractions from size-exclusion column were concentrated using an Amicon® Ultra 50K MWCO centrifugal filter and concentrated protein sample was mixed with 15% (w/v) glycerol and stored at −80 °C.

PCH2 (Hs-PCH2).

Buffer A: 50 mM Tris-HCl, 250 mM NaCl, 10 mM $MgCl_2$, 5 mM DTT, 1.2 mM PMSF, 0.02% (v/v) Triton™ X-100, 0.5 mM MgATP, 1 mM EGTA, 5 U/mL benzonase, and cOmplete™ EDTA-Free Protease Inhibitor Cocktail, pH 7.5. *Buffer B1*: 25 mM Tris-HCl, 350 mM NaCl, 10 mM $MgCl_2$, 5 mM DTT, 0.2 mM MgATP, 0.02% (v/v) Triton™ X-100, 1 mM EGTA, pH 8.0. *Buffer B2*: 25 mM Tris-HCl, 150 mM NaCl, 10 mM $MgCl_2$, 5 mM DTT, 20 µM reduced glutathione, 0.5 mM EGTA, pH 8.0. *Buffer E*: 25 mM Tris-HCl, 150 mM NaCl, 5 mM $MgCl_2$, 5 mM DTT, 10 % (w/v) glycerol, pH 8.0.

Conditions. The clarified lysate from cells expressing GST-PCH2 was incubated with sepharose glutathione beads (GE Healthcare) for 1 h and the beads were washed with 50 volumes of Buffer B1 and then with 10 volumes of Buffer B2. PreScission protease (0.1 mg/mL) was added and the beads were incubated for 12 h. The protein-containing solution was recovered by filtration and incubated with fresh sepharose glutathione beads for 1 h. Beads were removed by centrifugation

at 400*g*, and the solution was concentrated 20-fold using an Amicon® Ultra 30K MWCO centrifugal filter, filtered through a 0.33-µm Millex-GP PES membrane and further purified over a 10/300 Superdex 75 column (GE Healthcare) in Buffer E. Fractions containing purified protein from the size-exclusion column were concentrated to a minimum of 1 mg/mL using an Amicon® Ultra 30K MWCO centrifugal filter and stored at -80 ºC.

6XHis-SUMO-VPS4B (Hs-VPS4B)

Buffer A: 20 mM Na-HEPES, 500 mM NaCl, 2 mM $MgCl_2$, 10% (w/v) glycerol, 0.5 mM DTT, 1 mM PMSF, 1 mM ATP, and cOmplete™ EDTA-Free Protease Inhibitor Cocktail, pH 7.6. *Buffer B1*: 20 mM Na-HEPES, 500 mM NaCl, 2 mM $MgCl_2$, 0.5 mM DTT, 1 mM PMSF, 1 mM ATP, 15 mM Imidazole, pH 7.6. *Buffer B2*: 20 mM Na-HEPES, 500 mM NaCl, 2 mM $MgCl_2$, 0.5 mM DTT, 1 mM PMSF, 1 mM ATP, 400 mM Imidazole, pH 7.6. *Buffer C*: 25 mM Na-HEPES, 2 mM $MgCl_2$, 1 mM DTT, pH 7.6. *Buffer D*: 25 mM Na-HEPES, 1M NaCl, 2 mM $MgCl_2$, 1 mM DTT, pH 7.6. *Buffer E*: 50 mM Na-HEPES, 150 mM NaCl, 3 mM $MgCl_2$, 2 mM DTT, pH 7.6.

Conditions. The clarified lysate from cells expressing VPS4B-6xHIS was incubated with Ni-NTA beads for 1h and the beads were washed with 10 volumes of Buffer A, then 10 volumes of Buffer B1, and eluted with 10 volumes of Buffer B2. The protein-containing solution was diluted with 2.5 volumes of diluted Buffer A (1:1 in H_2O), loaded onto a MonoQ column 5/50 GL (GE Healthcare) equilibrated in Buffer C, and fractioned over a gradient with Buffer D. Combined MonoQ fractions were concentrated using an Amicon Ultra 30K MWCO centrifugal filter, and the concentrated protein sample was filtered though a 0.22-µm Millex-GP PES membrane before loading onto a 10/200 Superdex 200 column (GE Healthcare) equilibrated in Buffer E. Fractions from size-exclusion column were concentrated using an Amicon® Ultra 30K MWCO centrifugal filter and concentrated protein sample was mixed with 10% (w/v) glycerol and stored at −80 °C.

Analyses of ATPase activity. Steady-state ATPase activity of AAA proteins was determined using the NADH-coupled assay. For all analyses, the time course of fluorescence decrease was measured using a Synergy NEO Microplate Reader (λ_{ex} = 340 nm, 440 nm emission filter). The rate from a control reaction with no ATP (background rate of fluorescence decrease) was subtracted from all rates. The assay buffers were based on literature precedents, as available[3,25,44].

Assay conditions for $K_{1/2}$ and k_{cat} analyses (enzyme concentrations):

Dm-spastin wildtype and mutants (85 nM): 25 mM K-HEPES pH 7.5, 225 mM KCl, 25 mM K_2HPO_4, 5 mM $MgCl_2$, 2.5 mM DTT, 175 µM NADH.

Hs-spastin wildtype and mutants (100 nM): 25 mM K-HEPES pH 7.5, 225 mM KCl, 25 mM K_2HPO_4, 5 mM $MgCl_2$, 2.5 mM DTT, 200 µM NADH.

Xl-katanin (80 nM): 25 mM K-HEPES pH 7.5, 70 mM KCl, 25 mM K_2HPO_4, 5 mM $MgCl_2$, 2.5 mM DTT, 175 µM NADH.

Hs-FIGL1 (50 nM): 25 mM Na-MES pH 6.5, 70 mM KOAc, 25 mM K_2HPO_4, 5 mM $Mg(OAc)_2$, 1 mM TCEP, 175 µM NADH.

Mm-VCP (450 nM): 25 mM K-HEPES pH 7.5, 25 mM KCl, 25 mM K_2HPO_4, 15 mM $MgCl_2$, 1 mM TCEP, 100-125 µM NADH.
Hs-PCH2 (340 nM): 25 mM TRIS-HCl pH 8.5, 150 mM KCl, 25 mM K_2HPO_4, 5 mM $MgCl_2$, 2.5 mM DTT, 0.025% (v/v) Triton™ X-100, 125 µM NADH.
Each buffer also contained: 0.1 mg/mL BSA, 1 mM PEP, 40 U/mL LDH, 40 U/mL PK.

Assay conditions for analysis of chemical inhibitors:
While testing compounds we observed some precipitation, which we found to be dependent on buffer conditions. Replacing K_2HPO_4 with $(NH_4)_2SO_4$ addressed this issue. As Dm-spastin is more active than Hs-spastin, 0.5 mM MgATP was used rather than 1mM MgATP. For each assay we included Triton™ X-100 to prevent non-specific aggregation[45].

Dm-spastin wildtype and mutants: protein 70-100 nM, 25 mM K-HEPES pH 7.5, 200 mM KCl, 20 mM $(NH4)_2SO_4$, 5 mM $MgCl_2$, 2.5 mM DTT, 175 µM NADH.
Hs-spastin type and mutants: protein 50 nM, 25 mM K-HEPES pH 7.5, 225 mM KCl, 2.5 mM $(NH4)_2SO_4$, 5 mM $MgCl_2$, 2.5 mM DTT, 0.005% w/v Triton™ X-100, 175 µM NADH.
Xl-katanin: protein 70 nM, 25 mM K-HEPES pH 7.5, 70 mM KCl, 20 mM $(NH_4)_2SO_4$, 5 mM $MgCl_2$, 2.5 mM DTT, 0.005% w/v Triton™ X-100, 150 µM NADH.
Hs-FIGL1: protein 50-70 nM, 25 mM Na-MES pH 6.5, 70 mM KOAc, 20 mM $(NH_4)_2SO_4$, 5 mM $Mg(OAc)_2$, 1 mM TCEP, 0.005% w/v Triton™ X-100, 150 µM NADH.
Mm-VCP: protein 300 nM, 50 mM K-HEPES pH 7.5, 25 mM KCl, 20 mM $(NH_4)_2SO_4$, 15 mM $MgCl_2$, 1 mM TCEP, pH 7.5, 100 µM NADH.
Hs-PCH2: protein 270 nM, 25 mM Tris-HCl pH 8.5, 150 mM KCl, 20 mM $(NH_4)_2SO_4$, 5 mM $MgCl_2$, 2.5 mM DTT, 0.025% (v/v) Triton™ X-100, 125 µM NADH.
Hs-VPS4B: protein 80 nM, 25 mM Na-HEPES pH 7.5, 25 mM KOAc, 2.5 mM $(NH_4)_2SO_4$, 2 mM $MgCl_2$, 1 mM TCEP, 0.01% (v/v) Triton™ X-100, 150 µM NADH.
Each buffer also contained: 0.1 mg/mL BSA, 1 mM PEP, 30 U/mL LDH, 30 U/mL PK.

Equations used for data fitting. Enzyme parameters $K_{1/2}$, k_{cat} and Hill coefficients (h) for the recombinant enzymes were determined by fitting the rates to the Hill equation (1) using Prism v. 6.0 (GraphPad Software Inc) at different ATP concentrations (x):

$$V = ATPase\ rate = \frac{V_{max}\, x^h}{{K_{1/2}}^h + x^h} \qquad (1)$$

For each experiment the measured activity versus concentration of compound were plotted and data were fit using a sigmoidal dose-response curve equation (2) in Prism to determine the IC_{50}.

$$Y = \%\ ATPase\ rate\ relative\ to\ DMSO\ control = (Y_{min}) + \frac{(Y_{max} + Y_{min})}{1 + 10^{\log IC50 - x}} \qquad (2)$$

Differential Scanning Fluorimetry. These experiments were carried out on a C1000 Touch Thermal cycler CFX-96 instrument (GE Healthcare). Purified Hs-spastin wildtype or Hs-spastin N386C mutant were diluted to 16 µM in a buffer containing 50 mM K_2HPO_4, 200 mM KCl, 2 mM $MgCl_2$, and 2 mM DTT, pH 7.4. Spastazoline was diluted in this buffer supplemented with 4% (v/v) DMSO and SYPRO® Orange (1:250 dilution), and an equal volume was added to the Hs-spastin solution (assay concentrations: compound, 6.25 µM to 200 µM; Hs-spastin, 8 µM; DMSO, 2%). Assays were conducted in a 96-well plate (Hard-shell® HSP9665 Bio-Rad). The temperature was linearly increased with a step of 0.5 ºC for 55 min, from 25 ºC to 95 ºC and fluorescence readings were taken at each interval (excitation 490 nm, emission 590 nm). Melting temperatures were recorded as the minimum value of the first derivative of the fluorescence vs. temperature curves.

In vitro microtubule-severing assays. A fluorescence-based assay was adapted from similar assays reported previously[46]. Briefly, X-rhodamine labeled, taxol (Sigma cat#T7402)-stabilized microtubules (bovine brain) were diluted in buffer A (20 mM K-HEPES, 30 mM KCl, 5 mM $MgCl_2$, 2 mM TCEP, 0.2 mg/mL BSA, 0.01% Triton™ X-100). Compound **5** (2 µM, 1% DMSO final) and MgATP (0.5 mM) in buffer A were added and the mixture was incubated for 5 min. Dm-spastin was diluted to 150 nM in buffer B (50 mM K-HEPES, 300 mM KCl, 10 mM $MgCl_2$, 1 mM TCEP, 10% glycerol, 0.01% Triton™ X-100, pH 7.5) and then added to the mixture (final concentration: 15nM). Aliquots were removed at selected time points, fixed, and imaged using a Zeiss Axiovert 200M wide-field microscope equipped with a Zeiss 100x/1.45 NA α-Plan-Fluar objective and an EMCCD camera (iXon DU-897, Andor Technology).

Computational methods

Analysis of nucleotide-binding sites of AAA proteins. We employed a 3D structural alignment to compare the residue composition of the ATP binding site in selected AAA proteins (Dm-spastin, Xl-katanin, Hs-FIGL1, Mm-VCP, and Hs-PCH2). We used available structural models for Dm-spastin (PDB: 3B9P), Hs-FIGL1 (PDB: 3D8B, chain A), the D1-AAA domain (PDB: 5FTK, chain A, residues 200-477), and the D2-AAA domain (residues 471-761) of Mm-VCP. Since no structural models for katanin or human PCH2 were available at the beginning of these studies, homology models were generated for the ATPase domains of these proteins using Bioluminate® (version 2.3, Schrodinger, LLC, New York, 2016). Hs-PCH2 (aa 132-432, Uniprot reference sequence Q15645) was modeled using the structure of *C. elegans* PCH2 (PDB:4XGU) as a template. Xl-katanin (aa 200-486, Uniprot reference sequence Q9PUL2) was modeled using 3D8B_A, 3VFD_A, and 3B9P_A as templates.

To identify the amino acid residues lining the adenine-binding pocket in these proteins the atom coordinates from the structural or homology models were aligned to the coordinates of ADP-bound Hs-FIGL1 using UCSF Chimera[47]. Hs-FIGL1 residues within 6 Å of the adenine were selected and the corresponding residues were identified in the ATPase domains of Dm-spastin, Xl-katanin, Mm-VCP, and Hs-PCH2 or in an extended set of 24 AAA protein. These amino acid

residues, along with the corresponding secondary structure motifs, are shown in **Supplementary Figures 2d-2e.**

Inhibitor docking. The molecular dynamics (MD) system was set up for Dm-spastin (PDB: 3B9P) using the Protein Preparation Wizard in Maestro® (Schrödinger Release 2016.1, New York). First, all non-protein atoms in the PDB file were kept, and the protein loops with no coordinates were modeled using Uniprot: Q8I0P1 as reference sequence. Hydrogen atoms were added to the protein structure, and the protonation states of ionizable residues were assigned for pH 7.0. This structure was neutralized with KCl ions, additional KCl ions were placed to reproduce a salt concentration of 0.15 M, and the system was solvated with an orthorhombic box of simple point charge (SPC) water molecules using the Desmond package in Maestro® (Desmond Molecular Dynamics System, version 4.6, D. E. Shaw Research).

To generate the MD atomic trajectory, the standard system relaxation protocol provided in Maestro® was used, followed by a 100 ns of NPT molecular dynamics (300 K, 1.01325 bar), during which atomic positions were recorded every 50 ps. To maintain the pressure and temperature of the system, isotropic position scaling and the Nosè-Hoover chain thermostat methods were used with relaxation times of 2 ps and 1 ps, respectively. A RESPA integrator scheme was employed (step: 2.0 fs for bonded interactions; 2 fs for van der Waals and short-range electrostatic interactions; 6 fs for long-range electrostatic interactions). Short-range electrostatic interactions were cut off at 9.0 Å.

For the ensemble docking procedure all non-protein atoms were removed from the 5000 MD frames and the spastin structures from each frame were aligned to remove translational and rotational movement of the macromolecule. 100 representative frames were selected using the MD-trajectory clustering methods in Schrödinger, using the coordinates of the nucleotide-binding site residues. To remove overlap of the atoms' van der Waals radii, representative structures were minimized using 25 iterations of the Polak-Ribière conjugated gradient method or until the root mean square of the gradient of the energies was < 0.05 kJ mol^{-1} $Å^{-1}$, using OPLS2003e force field and a cut-off of 20 Å for the electrostatic interactions and 8 Å for the van der Waals interactions. Molecular docking was performed on the resulting 100 structures using the XGlide script with the OPLS2003e forcefield and Schrödinger's GlideScore multi-ligand scoring function[48]. A box of 22 Å was placed, centered approximately at the P-loop residues of Dm-spastin. Grids for docking calculations were obtained with the grid generation module in Schrödinger. A scaling factor of 0.8 was applied to the atom radii for every atom with assigned partial charge <0.25. The AROMATIC_H-BONDS option was set true. The major tautomer at pH 7.0 of compound **4** [1-methyl-4-(4-((5-phenyl-1*H*-pyrazol-3-yl)amino)quinazolin-2-yl)piperazin-1-ium] was calculated using the LigPrep panel in Maestro®. For docking the REWARD_INTRA_HBONDS, SAMPLE_RINGS, HBOND_DONOR_AROMH, FORCEPLANAR, and AROMATIC_H-BONDS options was set true, and a scaling factor of 0.75 was applied to atom radii for every atom with assigned partial charge <0.15. Compound **5** ((*R*)-4-(4-((5-(*tert*-butyl)-1*H*-pyrazol-3-yl)amino)quinazolin-2-yl)-3-methylpiperazin-1-ium) and Compound **6** ((*R*)-4-(4-((5-(*tert*-butyl)-1*H*-

pyrazol-3-yl)amino)-7*H*-pyrrolo[2,3-*d*]pyrimidin-2-yl)-3-methylpiperazin-1-ium) were docked using an analogous procedure.

Chemical inhibitors

Compound syntheses and characterizations are reported in the Supplementary Note: “Chemical Synthesis Information”.

Data Availability and Code Availability Statements.

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