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Supplemental Information

Graded Control of Microtubule Severing by Tubulin Glutamylation

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SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Spastin purification and fluorescent labeling

Drosophila melanogaster full-length spastin, with an engineered N-terminal KKCK motif for fluorescent labeling, was expressed in *Escherichia coli* as a N-terminal glutathione S-transferase (GST) fusion and purified by affinity chromatography. Following cleavage with Prescission protease, spastin was further purified by ion exchange and size exclusion chromatography as described (Ziolkowska and Roll-Mecak, 2013). For fluorescent labeling, spastin was incubated with DyLight 488 Maleimide (Thermo Scientific) on ice at a 5:1 dye to protein molar ratio for one hour. The reaction was quenched with 14.3 mM β-mercaptoethanol and labeled protein was recovered using a Fluorescent Dye Removal Column (Thermo Scientific). The microtubule severing activity of the labeled spastin construct is similar to that of the unlabeled protein (Figure S3E).

Mass spectrometric analysis of differentially modified tubulin

The number of glutamates added to α- and β-tubulin was determined by liquid chromatography-electrospray mass spectrometry (LC-MS) using a C18 column (Zorbax 300SB) in line with a 6224 ESI-TOF mass spectrometer (Agilent). The spectra display the characteristic distribution of masses with peaks separated by 129 Da corresponding to one glutamate (Figure 1). The extent of tubulin glutamylation on α or β-tubulin was determined by calculating the weighted average of peak intensities for each tubulin species present. Experiments were performed on different days with at least two independent preparations. The mass spectrometric analyses are reproducible within 0.1 $\langle n^E \rangle$ between at least three independent measurements. The number of glutamates

detected using this procedure is robust over on order magnitude of injected material (Figure S1F). While it is possible that we are not detecting all the lower abundance highly glutamylated species in our samples because of different ionization efficiencies, we note that each added glutamate leads to a small incremental difference in the overall charge of the tubulin protomer (pI changes from 4.78 to 4.59 to 4.4 for unmodified, +10 Glu and +25 Glu β-tubulin, respectively).

We used in-gel digest coupled with tandem mass spectrometry to verify that TTLL7 did not modify sites on the tubulin body in our reactions. Four microtubule samples modified with a range of glutamate numbers (6.5, 11.9, 19.8 and 21.7; spectra shown in Figure S1D) were subjected to proteolytic digests coupled with MS/MS analysis. We used ^{13}C Glu in our enzymatic reactions to make assignment of the enzymatically added Glu unambiguous from the glutamates present in the tubulin sequence. MS/MS analysis of digested samples with trypsin (85% peptide coverage), chymotrypsin (81% peptide coverage) and Asp-N (78% peptide coverage) provided complete coverage of the entire β-tubulin sequence and detected no glutamylated tubulin body peptides. We also incubated subtilisin treated microtubules missing their C-terminal tails with TTLL7 for 5 hours (at 1:5 TTLL7:tubulin ratios as opposed to 1:10 used for generating the glutamylated microtubule series) and then subjected them to LC-MS analysis. This also showed no modification of the microtubules missing the C-terminal tails, but robust glutamylation in the control microtubule sample (Figure S1H), consistent with the fact that TTLL7 binding to microtubules is mediated largely through the C-terminal tubulin tails (Garnham et al., 2015).

High-resolution SDS-PAGE of glutamylated tubulin

High-resolution separation of glutamylated α- and β-tubulin samples by SDS-PAGE was performed as described previously (Banerjee et al., 2010) using freshly cast gels with a final acrylamide concentration of 6.5% in the resolving layer. Gels were run at 120V for 2.5 hours at 4° C. Each lane contains 2 μg of sample.

Generation of acetylated microtubule substrates

100% acetylated microtubules were prepared by incubation with recombinant *Danio rerio* tubulin acetyltransferase (TAT) (Kormendi et al., 2012) at a 1:1 molar ratio of TAT to tubulin in BRB80, 250 mM KCl, 1 mM DTT, 100 μM acetyl-coA, 10 μM taxol at room temperature for 24 hours. Completion of the acetylation reaction was confirmed by mass spectrometric analysis. Mass spectra displayed a 42 Da shift in the α-tubulin mass, corresponding to the addition of a single acetate group. No unacetylated α-tubulin species was detected (Figure 4).

Generation of recombinant tailless and tyrosinated/detyrosinated human tubulin

Genes coding for human α1A tubulin (NP_001257328) with a His-tag inserted in the acetylation loop and a C-terminally flag-tagged βIII tubulin (NM_006077) were custom synthesized by Integrated DNA Technologies and cloned in a pFastBac™-Dual vector.. The internal His-tag was used to produce an α-tubulin construct with a native C-terminus to investigate the effects of tyrosination/detyrosination. The internal His-tag was

previously used for the affinity purification of *S. cerevisiae* α-tubulin (Sirajuddin et al., 2014). Human tubulin variants lacking either the α- or β-tubulin tails or the α-tubulin C-terminal tyrosine were obtained by Quikchange mutagenesis. The Bac-to-Bac System (Life Technologies) was used to generate recombinant baculoviruses (Minoura et al., 2013). SF9 or HighFive cells were infected with the recombinant viruses and cells were harvested after 48 hours. Collected cell pellets were washed in PBS and flash frozen. Cells were lysed by gentle sonication in BRB80 buffer (80 mM PIPES pH 6.8, 1 mM $MgCl_2$, 1mM EGTA) with addition of 0.5 mM ATP, 0.5 mM GTP and 1mM PMSF. The lysate was cleared by centrifugation and the crude supernatant was loaded on an anti-flag G1 affinity resin (Gen Script) column. Flag-tagged tubulin was eluted with BRB80 containing 0.25 mg/ml Flag peptide (Gen Script). The eluate was further purified on a Ni-NTA column (Qiagen). His-tagged tubulin was eluted with BRB80 buffer containing 200 mM imidazole. The purified tubulin dimer was subjected to buffer exchange on a PD10 desalting column (GE Healthcare) equilibrated with BRB80, 20 μM GTP. Aliquots were frozen in liquid nitrogen and stored at −80 °C until use. The presence of only the engineered unmodified α1A and βIII tubulin isoforms was confirmed by mass spectrometry (Figure S3A).

Generation of microtubules missing β-tubulin tails by limited proteolysis

Taxol-stabilized unmodified human microtubules were prepared as described above. Microtubules missing β-tubulin tails to various extents were obtained by digesting microtubules with subtilisin at a 1:200 subtilisin:tubulin mass ratio for 30, 60 or 90 minutes. Reactions were performed at room temperature and quenched with 5 mM

phenylmethylsulfonyl fluoride. Microtubules were recovered through a glycerol cushion. Digests were subjected to mass spectrometric analysis as described above. Experiments with partially proteolyzed native microtubules were performed to complement the experiments with engineered human microtubules missing the C-terminal tails to ensure that the results are the same regardless of the presence of the additional residues introduced by the affinity tag on the C-terminus of β-tubulin.

Microscopy based severing assays with modified and engineered microtubules

Flow chambers were assembled from clean, silanized coverglass and slides as described previously (Ziolkowska and Roll-Mecak, 2013). Chambers were first perfused with 0.1 g/L NeutrAvidin (Thermo Scientific), washed with BRB80 containing 2 g/L casein and then incubated with microtubules for 10 minutes, and washed again. The chamber was then perfused with BRB80 containing an oxygen scavenger mix to remove free oxygen from solution (3.25 U/μL catalase, 0.3 U/μL glucose oxidase, 20 mM glucose). Severing reactions were started by perfusing spastin into the chamber in 50 mM KCl, 2.5 mM $MgCl_2$, 1 mM ATP, 1% Pluronic F-127 (Life Technologies), 10 μM taxol and the oxygen scavenger mix. Images were acquired at 1 Hz with an inverted epifluorescence microscope (Nikon Ti-E) equipped with a CoolSNAP HQ2 CCD camera (Photometrics) except for the severing reactions on unmodified microtubules at 400, 570 and 800 nM spastin concentration, and on the $<n^{E}>\sim 5.6$ microtubules at 200, 400 and 800 nM spastin concentrations (Figure 5C, first two panels from the left) where the frame rate was 3.3 Hz. Microtubule severing progress was monitored by counting the number of observed microtubule severing sites after perfusion of spastin. For the severing assays in Figures 1,

2, 3A, 4, 6, 7, S1A, S3C, S3E and S4B, 50 nM spastin was perfused into the chamber in severing reaction buffer and images were recorded every 1 s with a 200 ms exposure. The 50 nM spastin concentration was chosen as it gave a good dynamic range for assaying the entire array of glutamylated microtubules. For the experiments in Figure 3B, 20 nM spastin was perfused into the chamber and imaged as above. Experiments at 50 nM spastin also did not reveal any significant differences between tyrosinated and detyrosinated microtubules. For the severing assays in Figure S3D, 1 μM spastin was perfused into the chamber in severing reaction buffer and imaged as above. Microtubule severing rates were determined as the time required to observe one severing event per 10 μm of microtubule. Initial rates in Figure 5C were determined from the fit to the initial linear portion of the reaction profiles after the lag phase. The same cooperativity dependence was obtained regardless of the scoring method. Microtubule severing assays in Figure 5D were performed at constant number of spastin molecules bound per tubulin dimer. Specifically, spastin was perfused in the chamber at concentrations of 570, 230, 100, 63 and 50 nM for unmodified microtubules and microtubules with $\langle n^E \rangle$ of 5.6, 12.8, 19.6 and 23.7, respectively. Microtubule severing rates were determined from the fit to the initial linear portion of the reaction profiles after the lag phase.

Microtubule severing assays in the presence of poly-L-glutamic acid

Spastin (50 nM) was incubated with either 0.75-5 kDa poly-L-glutamic acid (Sigma P1943) or 3-15 kDa poly-L-glutamic acid (Sigma P4636) for one minute prior to perfusion into the chamber. Severing rates were measured as described above.

TIRF microscopy based microtubule-binding assays

Images were acquired using an inverted TIRF microscope (Nikon Ti-E with TIRF attachment). The excitation light was provided by a 488 nm Coherent CUBE and a 532 nm Coherent Sapphire CUBE for DyLight 488 and TMR, respectively, operating at 20 mW before being coupled into an optical fiber and delivered into the microscope. Light was delivered to the sample through a 100X 1.49 NA TIRF objective (Nikon CFI Apo TIRF 100x). The emitted light was collected through the same objective and split using an Andor TuCAM equipped with a dichroic mirror (Semrock Di02-R594). The emitted fluorescence was imaged on two separate EMCCD cameras (Andor iXON3-897) equipped with the appropriate filters (Semrock-FF01-514/30 for DyLight 488 and FF01-593/LP for TMR). The TuCAM introduces an additional 2x magnification factor yielding a final pixel size of 89 nm. Images were acquired at an exposure of 100 ms for both the TMR and DyLight 488 channels. Multiple fields of view were imaged. Background corrected line scan intensities were measured using ImageJ (NIH; http://imagej.nih.gov/ij/) and normalized to microtubule length (Schneider et al., 2012).

ATPase assays

ATPase assays were performed at room temperature in the same buffer as the microtubule severing assays (BRB80, 50 mM KCl, 2.5 mM $MgCl_2$, 1 mM ATP, 10 μM taxol) and at spastin concentrations as those used in the severing assays (50 nM). Assays at 200 nM spastin concentrations gave similar results (not shown). ATPase activities were measured using an EnzChek Phosphate Assay (Life Technologies). Initial rates were calculated from the linear portion of the reaction profiles after addition of 1 mM

ATP. ATPase rates were adjusted by subtraction of the measured release of phosphate in the absence of ATP. ATPase assays in the presence of poly-glutamate were performed at room temperature in BRB80 supplemented with 50 mM KCl, 10 mM $MgCl_2$ and 1 mM ATP.

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