**SUPPLEMENTAL INFORMATION**

**SUPPLEMENTAL TABLE**

**Table S1 (related to Figure 1)**

**Crystallographic Data and Refinement statistics**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | TTLL7 (EMP)a | TTLL7 (SeMet) a | TTLL7:ADPa | TTLL7:AMPPNPa |
| **Data collection** |  |  |  |  |
| Space group | C2221 | C2221 | C2221 | C2221 |
| Cell dimensions  |  |  |  |  |
|  *a*, *b*, *c* (Å) | 78.8, 121.4, 130.0 | 78.8, 122.7, 129.3 | 78.5, 121.8, 129.9 | 79.1, 122.3, 129.4 |
|  α, β, γ (°) | 90, 90, 90 | 90, 90, 90 | 90, 90, 90 | 90, 90, 90 |
|  | *Inflection* | *Peak* |  |  |
| Wavelength | 1.009 | 0.9792 | 0.9774 | 0.9774 |
| Resolution (Å) | 46.3-2.51 (2.65–2.51) | 46.2-3.24 (3.41–3.24) | 50.0-2.55 (2.59–2.55) | 50.0-2.6 (2.64–2.6) |
| *R*sym  | 10.2 (92.5) | 17.3 (108.6) | 12.4 (48.2) | 14.4 (52.8) |
| *I* / σ*I* | 33.1 (2.9) | 20.9 (2.4) | 17.5 (2.3) | 24.7 (3.1) |
| Completeness (%) | 99.9 (99.7) | 99.9 (99.8) | 93.0 (79.4) | 97.8 (84.6) |
| Redundancy | 18 (10.9) | 19.1 (11.2) | 5.5 (3.9) | 10.1 (9.7) |
| Phasing FOM | 0.33 | 0.25 |  |  |
| **Refinement** |  |  |  |  |
| Resolution (Å) |  |  | 50.0 – 2.55 | 50.0 - 2.60 |
| No. reflections |  |  | 19556 | 19214 |
| *R*work / *R*free |  |  | 22.2/26.9 | 21.0/24.6 |
| No. atoms |  |  |  |  |
|  Protein |  |  | 2565 | 2581 |
|  Ligand/ion |  |  | 27 | 27 |
|  Water |  |  | 15 | 20 |
| *B*-factors |  |  |  |  |
|  Protein |  |  | 66.4 | 73.1 |
|  Ligand |  |  | 105.4 | 100.9 |
|  Water |  |  | 55.3 | 56.1 |
| R.m.s deviations |  |  |  |  |
| Bond lengths (Å) |  |  | 0.002 | 0.002 |
| Bond angles (°) |  |  | 0.56 | 0.59 |
| Coordinate errorb  |  |  | 0.31 | 0.34 |
| Ramachandran  |  |  |  |  |
| favored (%) |  |  | 96 | 95 |
| disallowed (%) |  |  | 0 | 0 |

a Values in parentheses are for the highest-resolution shell.

b Maximum likelihood

**Table S2 (related to Figure 2)**

**Summary of Cryo-EM Data**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
|  | Totalimages | Images processed | segments | Segments after 2D classification | 15 pfsegments | 15pf asymmetricunits |
| TTLL7: unmodified microtubule complex(Tecnai F20 data) | 817 | 614 | 40,758 | 25,783 | 19,418 | 291,270 |
| TTLL7:brain microtubule complex | 939 | 825 | 119,534 | 35,991 | 10,047 | 150,705 |

The number of images collected, the number of images selected for further processing, the number of segments before and after 2D classification and the number of asymmetric units contributing to the final reconstructions are listed.

**SUPPLEMENTAL FIGURES**

**Figure S1 (related to Figure 1)**

**Alignment of Selected TTLL7 Sequences**

Residues are numbered according to the human TTLL7 sequence (GenBank accession number NP\_078962). Secondary structure elements are indicated above the corresponding sequence. α-helices, cylinders; β-strands, arrows; random coil, lines. Segments of the protein not built due to poorly resolved electron density are denoted by dashed lines. Sequence identity is color-coded using a gradient from white (below 50%) to red (100%). Residues important for microtubule binding are denoted by , those important for ATP binding are denoted by **+**, while those mutated in this study important for glutamylation activity are denoted by a red ★.

**Figure S2 (related to Figure 2)**

**Cryo-EM Imaging and Reconstruction of the TTLL7:Microtubule Complex**

(A,B) Undecorated unmodified human microtubules exhibit well-defined, clean edges (A), while microtubules decorated with TTLL7 show a fuzzy layer of protein surrounding them (B). The diffraction pattern of a selected area (between black lines) shows an 8nm layer line due to the ordering of the TTLL7 protein on the microtubule lattice at each tubulin dimer.

(C) Representative class averages. 2D analysis was performed on extracted segments of the microtubules, revealing the TTLL7 organization on the microtubules lattice. Only particles contributing to class averages with well-resolved TTLL7 densities were included in 3D refinement. Each class shown contains ~ 175 particles.

(D) Forward projection of the reconstructed 3D density. The refined cryoEM structure closely resembles the reference-free 2D class averages shown in Figure S2C. The tubulin density is noticeably stronger than the density of TTLL7 bound at the exterior of the tubulin polymer, suggesting that the TTLL7 interaction with tubulin allows for a degree of flexibility.

(E) Comparison of Fourier shell correlation (FSC) curves of 3D refinements. Reconstruction of the complex with brain microtubules is shown in blue, unmodified microtubules in red. The resolutions of the reconstructions according to the 0.5 FSC criterion are 10.7 and 7.95 Å, respectively.

(F) Features that identify the register of the -tubulin dimer in the EM density. Despite their overall similarity, structural features that are unique to each tubulin monomer enabled unambiguous discernment of the - register in the reconstructed density. Density for the bound taxol ligand (green ball-and-stick) is observed in the -subunit (yellow), while in the  subunit (purple) the 358-372 loop (orange) occupies the taxol binding site. Furthermore, density corresponding to the disordered K40 loop is observed in the -tubulin subunit (lower right circle), while the corresponding location in the -tubulin subunit contains the well-resolved S40 helix. The atomic model (PDBID: 3J6G) docked into the density in this correct register yields a UCSF Chimera correlation coefficient of 0.85, but docking into the incorrect register yields a value of 0.74.

(G) Axial view down three protofilaments of the TTL7-bound microtubule shows the interactions between two helices of the c-MTBD (orange) and the α-tubulin body (purple). TTLL7 secondary structure elements colored as in Figure 1A.

(H) Local resolution calculation. The tubulin dimer and associated ligase is colored according to its local resolution, calculated using the bsoft “blocres” function. Blue density corresponds to 7Å resolution, with a continuum of colors indicating increasingly lower resolution, ending with red at 11Å resolution. The local resolution calculation reveals a wide variability in resolution within the reconstruction, with the ligase density substantially less resolved than the tubulin density.

(I) Molecular surface of the TTLL7:microtubule complex rendered transparent and colored according to resolution as in Figure S2H. The c-MTBD is highlighted by a dashed ellipse. Select secondary structure elements in tubulin and TTLL7 are labeled.

(J) Consensus secondary structure prediction of TTLL7 c-MTBD. The secondary structure of TTLL7 was predicted using different algorithms (Psipred, cfssp, jpssm, Jnet, and Phyre2). Predicted α-helices and β-sheets are colored gray, while secondary structure elements revealed by X-ray crystallography or cryo-EM is aligned above and colored as in Figure 2. c-MTBD helices are colored orange.

**Figure S3 (related to Figure 3)**

**TTLL7 preference for the -tubulin tail and contribution of tubulin tails to TTLL7 microtubule binding**

(A, B) Reverse-phase LC/MS analysisof TTLL7-modified synthetic tubulin peptides and taxol-stabilized naïve microtubules. (A) Deconvoluted mass spectra of a synthetic C-terminal α1B-tubulin peptide (left panel) and βIVb-tubulin peptide (right panel) glutamylated by TTLL7 at 1:10 TTLL7:tubulin peptide molar ratio. Earlier time points show the same non-discriminatory modification of the  and -tail peptides. Unmodified peptides at t = 0h are shown in grey; peaks corresponding to glutamylated products (each separated by 129 Da) are shown in blue and purple for α1B and βIVb, respectively. The number of glutamates added to each species is indicated. (B) Deconvoluted α- and β-tubulin mass spectra of taxol-stabilized unmodified microtubules after 0h, 4h and 8h incubation with TTLL7 at a 1:20 TTLL7:tubulin molar ratio. The number of glutamates added is indicated and colored according to isoform.

(C) Normalized activity of GFP-tagged TTLL7 compared to wild-type TTLL7 (residues 1-518). Initial rates were determined via the incorporation of 3H-glutamate into taxol-stabilized brain microtubules.

(D) Left to right: distribution of durations for TTLL7-GFP interactions with unmodified microtubules (MT) and unmodified microtubules missing their -tubulin tails (MT -tail).  is the mean interaction lifetime obtained by fitting an exponential curve to the histogram and correcting for photobleaching (N = 807; R2 = 0.9528 for MT; N = 790; R2= 0.9917 for MT -tail).

(E) Sedimentation assays of TTLL7 binding to taxol-stabilized brain microtubules (black) and unmodified microtubules (grey).

(F) Affinity of TTLL7-GFP for taxol-stabilized brain microtubules (black) and unmodified microtubules (grey) as determined using our TIRF microscopy assay (Figure 3 and Extended Experimental Procedures).

(G) Kinetic parameters of the TTLL7-GFP microtubule interaction with the microtubule in the presence of various nucleotides (0.6 mM) and/or free glutamate at physiological levels (1 mM; [Blosser and Wells, 1972](#_ENREF_4)). These measured kinetic parameters were averaged from 253, 1555, 1662, 955 and 1189 binding events for no nucleotide, ADP, ATP, ATP + Glu and AMPPNP condition, respectively and were from at least two separate flow cells imaged on different days (Extended Experimental Procedures).

(H) Sedimentation assay of TTLL7 with microtubules (MTs) and microtubules missing both - and -tubulin tails (MTs , -tails). Complete digestion of the C-terminal tails was verified by mass spectrometry.

**Figure S4 (related to Figure 4)**

**Interaction of TTLL7 with Unmodified and Glutamylated Microtubules**

(A) Kinetic parameters of the TTLL7-GFP microtubule interaction.

(B) Left to right: distribution of durations for TTLL7-GFP interactions with unmodified microtubules (MT) and microtubules glutamylated to different extents.  is the mean interaction lifetime obtained by fitting an exponential curve to the histogram and correcting for photobleaching (N = 1009; R2 = 0.9879 for MTs; N = 1137; R2= 0.9927 for MTs ,  +4.2 Glu; N = 483; R2 = 0.9926 for MTs  +0.5 Glu,  +8.3 Glu). Number of glutamates on  and -tubulin were calculated from the weighted average of the mass spectra peak values for the different glutamylated species (Extended Experimental Procedures).

(C) Left to right: distribution of microtubule interaction times for all TTLL-GFP molecules, static TTLL7-GFP molecules alone and scanning TTLL7-GFP molecules with hyperglutamylated microtubules (MTs  +6.8 Glu,  +28.2 Glu). τ was photobleach corrected and obtained by fitting an exponential curve to the histogram (N = 802; R2 = 0.9835 for all TTLL7-GFP events, N = 510; R2 = 0.9947 for static TTLL7-GFP single molecues, N = 292; R2 = 0.9094 1D-diffusing TTLL7-GFP single molecules).

(D) Kymograph depicting TTLL7-GFP motion on hyperglutamylated microtubules. Scale bars, horizontal, 5 s; vertical, 2 m.

(E) Scan distance distribution for TTLL7-GFP molecules on hyperglutamylated microtubules. The histogram was fit to a single exponential decay (R2 = 0.9789).

(F) Kinetic parameters of catalytically dead TTLL7-GFP (E349Q) mutant interaction with glutamylated microtubules in various salt conditions. (N = 2223 TTLL7-GFP events in 50 mM NaCl, N = 1832 events in 105 mM NaCl, and N = 117 events in 200 mM NaCl). Dwell times distribution in the highest ionic strength condition was not determined because of the low number of binding events observed under this condition.

(G) Left to right: kymograph depicting catalytically dead TTLL7-GFP (E349Q) mutant static interaction with unmodified MTs in the presence of ATP and glutamate. Scale bars, horizontal, 5s; vertical, 2 m; Distribution of unmodified MT interaction times with catalytically dead TTLL7-GFP molecules. τ was photobleach-corrected and obtained by fitting an exponential curve to the histogram (N = 1655; R2 = 0.9702).

(H) Left to right: kymograph depicting catalytically dead TTLL7-GFP (E349Q) mutant interactions with glutamylated MTs in the presence of ATP and glutamate. Scale bars, horizontal, 5s; vertical, 2 m. Distribution of glutamylated MT interaction times with catalytically dead TTLL7-GFP molecules. τ was photobleach-corrected and obtained by fitting an exponential curve to a histogram (N = 1832; R2 = 0.9854).

**Figure S5 (related to Figure 5)**

**Molecular Determinants of TTLL7 Microtubule Recognition**

(A) Deconvoluted mass spectra of a synthetic C-terminal βIVb-tubulin peptide glutamylated by TTLL7 at 1:10 enzyme:tubulin peptide molar ratio at 0 and 20 h time points. The number of glutamates added is indicated.

 (B, F) Deconvoluted mass spectra of a synthetic C-terminal βIVb-tubulin peptide glutamylated by TTLL7 E349Q, R106E, R205E, K271E, and R352E mutant proteins at 1:10 enzyme:tubulin peptide molar ratio at 20 h. The number of glutamates added is indicated.

(G) Deconvoluted mass spectra of a synthetic C-terminal βIVb-tubulin peptide glutamylated by the TTLL7 384-446 mutant at 1:10 enzyme:tubulin peptide molar ratio. The number of glutamates added is indicated.

(H) Size exclusion chromatography traces on a S200 HiLoad 16/60 column (GE Healthcare) for structure guided TTLL7 mutants used in *in vitro* activity assays. Vo = void volume.

(I) Size exclusion chromatography traces on a S200 GL10/300 column (GE Healthcare) for structure guided TTLL7 mutants used in *in vitro* activity assays. Vo = void volume.

**Figure S6 (related to Figure 6)**

**TTLL7 Microtubule Binding Domain**

Size exclusion chromatography of wild-type and Δ384-446 TTLL7. Proteins were separated at a flow rate of 0.05 ml/min on a Superdex200 PC 3.2/30 column (GE Healthcare); Vo = void volume, Vt = total volume, Ve = elution volume.

**Figure S7 (related to Figure 7)**

**A Conserved Cationic Region Critical for Activity in TTLL Family Glutamylases**

Domain organization of TTLL family glutamylases that have been documented to have autonomous glutamylation activity. N domain, blue; central domain, hot pink; C domain, green; proposed microtubule binding insertion in the central domain, gold; C-terminal helix homologous to 9 in TTLL7, cyan. The boundaries for helix 9 are unclear in the case of TTLL6 and 11; likewise the boundaries of the c-MTBD region downstream of the predicted 9 helix for TTLL4, 5, 6, 11, and 13 are ambiguous. Residue numbers for *H. sapiens* enzymes are shown.

**Movie S1 (related to Figures 1 and 2)**

This movie shows the organization of the TTLL7 ligase (gold) on the surface of the microtubule (silver). A single tubulin dimer and its associated ligase are selected from the cryo-EM reconstruction of the microtubule and the electron density is made transparent to reveal the docked atomic models for TTLL7 (colored as in Figures 1 and 2) and the tubulin dimer (-tubulin, yellow; -tubulin magenta). The interactions between TTLL7 and the microtubule are shown from three orthogonal views. At each view the threshold of the electron density is increased by 1.5 , to show the resolved density for the modeled -helices.

**SUPPLEMENTAL INFORMATION**

**EXTENDED SUPPLEMENTAL PROCEDURES**

**Protein Expression and Purification**

*Homo sapiens* TTLL7 (1-518) was expressed in *E. coli* Rosetta2 DE3 pLysS as an N-terminal GST fusion protein. Protein expression was induced with 0.5 mM IPTG for 16 hours at 16 °C. Cells were lysed using a microfluidizer and cellular debris was removed by centrifugation at 30,500 x *g* for 1 hour. The fusion protein was purified through a GST affinity chromatography step followed by a heparin column. TTLL7 was digested with TEV protease. TTLL7 was further purified by anion exchange and size-exclusion chromatography. All *in vitro* functional studies and cryo-EM experiments were performed with this TTLL7 construct. The TTLL7 36-518 construct used for crystallization displays similar glutamylation activity to this construct (Figure 5B). The structure guided TTLL7 mutants as well as the GFP-labeled proteins were purified following the same protocol as for the wild-type protein. Mutagenesis was performed using Quikchange (Stratagene). All proteins used were monodisperse and eluted as a single peak from a Superdex 200 size exclusion chromatography column (GE) (Figures S5H, S5I and S6). Purity and integrity of wild-type TTLL7 as well as all mutants were verified by mass spectrometry and measured masses agreed with predicted masses within 0.1-2.0 Da.

**Crystallization, X-ray Structure Determination and Refinement**

Mass spectrometric analysis revealed that when overexpressed in *E. coli* TTLL7 self-modifies producing a heterogeneous mixture of polyglutamylated species that failed to crystallize. To solve this heterogeneity problem, we conducted all crystallographic and EM structural studies with a catalytic inactive mutant (E349Q). This mutant binds microtubules with the same affinity as the wild-type protein (Kd ~ 3 M). Moreover, the automodification does not alter the glutamylation activity of our TTLL7 construct as a TTLL7 construct that has all the modified glutamates mutated to Asp has the same activity as the wild-type protein (data not shown). For crystallization studies TTLL7 (36-518) yielded crystals that were suitable for structure determination. Crystals of apo, ADP, ATP or AMPPNP bound TTLL7 (8 mg/ml) grew at room temperature by hanging drop vapor diffusion from protein solution mixed at 1:1 ratio with 0.1 M MES 6.0, 10% PEG 20K, 5 mM MgCl2 and 0.5 mM nucleotide. Crystals grew with symmetry of space group C2221 with one copy of the protein in the asymmetric unit. Crystals typically appeared after 4 days and grew to maximum dimensions (~800 x 50 x 40 μm) in two weeks. SeMet crystals grew in the same condition. Crystals were flash frozen in liquid nitrogen following quick soaks in 10% PEG 20K, 0.1 M MES 6.0, 5 mM MgCl2, 1 mM nucleotide and 30% glycerol. Most crystals, regardless of bound nucleotide diffracted poorly and were not suitable for structural determination.

Data from SeMet and ethyl mercuric phosphate (EMP) soaked TTLL7-AMPNP crystals were collected at the Advanced Photon Source beamline 24IDE. Two SAD datasets from two SeMet crystals were collected at the peak of the Se K-absorption edge. Three SAD datasets were collected at the inflection point of the Hg L-III absorption edge from three crystals soaked overnight in 10% PEG 20K, 100 mM MES 5.8, 1 mM AMPPNP, 1 mM L-glutamate, 5 mM MgCl2, and 0.5 mM EMP. All three datasets of the EMP-soaked crystals were scaled together using the NECAT RAPD data processing pipeline that employs XDS ([Kabsch, 2010](#_ENREF_12)) and SCALA ([Evans, 2006](#_ENREF_10)), as were the two SeMet data sets (Table S1). The diffraction limit of the combined EMP dataset was 2.51Å and that of the combined SeMet dataset 3.24Å. (Table S1). Phenix Autosol ([Adams et al., 2010](#_ENREF_1)) identified five out of six Hg sites. Nine of the twelve possible SeMet residues were identified through anomalous difference Fourier synthesis using the Hg phases. The remaining unidentified sites are located in disordered regions of the protein. The experimental electron density maps at this stage were not interpretable, but density modification using RESOLVE ([Terwilliger, 2000](#_ENREF_25)) yielded maps in which ~43% of the protein could be built by Phenix Autobuild ([Adams et al., 2010](#_ENREF_1)). Refinement of the partial model against amplitudes from the best individual EMP dataset using Phenix and calculation of electron density maps with (2|Fobs|-|Fcalc|)model difference Fourier syntheses allowed building of 299 residues out of 487 using COOT ([Emsley and Cowtan, 2004](#_ENREF_9)).

Native data sets for AMPPNP (a=79.126, b=122.324, c=129.445, diffraction limit 2.45Å) and TTLL7-ADP (a=78.548, b=121.762, c=129.858, diffraction limit 2.47Å) were collected at Advanced Light Source beamlines 5.0.2 and 5.0.1, respectively. Diffraction data were reduced using HKL2000 ([Otwinowski and Minor, 1997](#_ENREF_17)). Several rounds of iterative model building and refinement were performed using COOT ([Emsley and Cowtan, 2004](#_ENREF_9)) and Phenix ([Adams et al., 2010](#_ENREF_1)). The current refinement model for TTLL7-AMPPNP consists of 334 residues and 20 waters. Six regions of the polypeptide chain (residues 36-39, 162-169, 176-180, 236-269, 383-450, and 485-518) are not well resolved in the electron density map and are presumed disordered. Mass spectrometry of TTLL7 crystals confirmed that the protein was intact. The current TTLL7-AMPPNP crystallographic model at a resolution of 2.60 Å has *R*work and *R*free of 21.0 and 24.6%, respectively (Table S1) MolProbity ([Chen et al., 2010](#_ENREF_4)) revealed no unfavorable () combinations, and main-chain and side-chain structural parameters consistently better than average. ATP and AMPPNP TTLL7 structures are similar and refined to similar *R*free and *R*work factors. Only the statistics for the AMPPNP bound TTLL7 structure are shown in Table S1. The structure was solved by molecular replacement using PHASER ([McCoy et al., 2007](#_ENREF_15)) by using the TTLL7-AMPPNP structure as the search model (residues around the active site were excluded from the model). Several rounds of iterative model building and refinement were performed using COOT and Phenix. The current refinement model for TTLL7-ADP consists of 335 residues and 15 waters. Six regions of the polypeptide chain (residues 36-39, 162-169, 176-180, 236-268, 383-450, and 485-518) are not well resolved in the electron density map and are presumed disordered. The current TTLL7-ADP crystallographic model at a resolution of 2.55 Å has *R*work and *R*free of 22.2 and 26.9%, respectively (Table S1). MolProbity revealed no unfavorable () combinations, and main chain and side-chain structural parameters consistently better than average. Structural figures were prepared with the programs PyMOL ([DeLano, 2002](#_ENREF_6)) and CHIMERA ([Pettersen et al., 2004](#_ENREF_18)). Maps of electrostatic surfaces were calculated with DELPHI ([Rocchia et al., 2002](#_ENREF_19)).

**Cryo-EM sample preparation**

For grid preparation, TTLL7 (1-518, E349Q) at 11 g/l was rapidly thawed and diluted at a 1:25 molar ratio in 20 mM Hepes 7.0, 0.5 mM ATP, 1 mM TCEP and 50 mM NaCl.Unmodified tubulin was purified *via* the TOG affinity method; ([Widlund et al., 2012](#_ENREF_27)). Unmodified microtubules were prepared as follows. Tubulin was thawed in a 37 °C water bath and aggregates were removed *via* ultracentrifugation at 436,000 x g for 10 minutes at 4 °C. Tubulin was supplemented with 10% (v/v) DMSO, 0.5 mM GTP and 10 mM MgCl2 and incubated at 37 °C for 1 hour. The polymerization reaction was supplemented with taxol to a final concentration of 10 µM and incubated at 37 °C for an additional 3-12 hours. Microtubules were pelleted through a 60% glycerol cushion in BRB80 (80 mM PIPES 6.8, 1 mM MgCl2, and 1 mM EGTA) supplemented with 20 µM paclitaxel (Calbiochem) at 109,000 xg for 10 minutes at 30 °C. The pellet was re-suspended in BRB80 buffer supplemented with 1 mM DTT and 20 µM paclitaxel. The brain microtubules were prepared by polymerizing 5 mg/ml bovine brain tubulin (Cytoskeleton Inc.) in polymerization buffer (80 mM PIPES, pH 6.8, 1 mM EGTA, 4 mM MgCl2, 2 mM GTP, 12% DMSO) for 30 minutes at 37ºC. 250 µM paclitaxel was added before a further incubation of 30 minutes at 37ºC. The polymerized microtubules were then incubated at room temperature for several hours before use.

All microtubule samples were prepared on 400-mesh C-flat grids (Protochips, Inc) containing 2.0 μm holes separated by 2.0 μm spacing. Grids were glow-discharged prior to sample application. The cryo samples were prepared using a manual plunger, which was placed in a homemade humidity chamber, which varied between 80 - 90% RH. 4 μl of the unmodified microtubules at ~ 200 μg/ml in 80 mM PIPES 6.8, 4 mM MgCl2, 1 mM EGTA supplemented with 20 μM taxol, was allowed to absorb, and then 4 μl of 440 μg/ml of TTLL7 in EM buffer (20 mM HEPES 7.0, 50 mM NaCl, 0.5 mM ATP, 1 mM TCEP) was added to the grid. After a short incubation, the sample was blotted (from the back side of the grid) and plunged into liquid ethane.

**Electron Microscopy and Image Processing**

Cryo-grids were loaded into a 626 single-tilt cryo-transfer system (Gatan) and imaged in a Tecnai F20 FEG electron microscope (FEI) operated at 200 keV using a dose of ~ 20 e−/Åand a nominal range of 1.0 - 3.0 μm under focus. All images were recorded on a 4k × 4k Tietz F416 CMOS detector at a nominal magnification of 62,000x (pixel size 0.136 nm) using the automated Leginon software ([Suloway et al., 2005](#_ENREF_22)). Diffraction of individual cryo-EM images of TTLL7 bound to microtubules showed an 80 Å layer line, indicating that one TTLL7 molecule binds an -tubulin heterodimer (Figure S2B). Image processing was performed within the Appion processing environment ([Lander et al., 2009](#_ENREF_13)). The contrast transfer function (CTF) was estimated by using CTFFIND3 ([Mindell and Grigorieff, 2003](#_ENREF_16)) and the best-quality micrographs were selected for further processing. Microtubules were manually selected and overlapping segments were extracted using a box size of 384 pixels every 80 Å along the filament. The particles were binned by a factor of two for 2D analysis and 3D refinement. The particle stacks were subjected to iterative multivariate statistical analysis (MSA) and multireference alignment (MRA). Particles in classes that did not clearly show TTLL7 density were excluded from further processing.

**Cryo-EM 3D Reconstruction**

Undecorated 14 and 15 protofilament microtubule densities ([Sui and Downing, 2010](#_ENREF_21)) were used as initial models for all preliminary reconstructions. The unmodified tubulin produced mainly 14 and 15 protofilament microtubules. The IHRSR ([Egelman, 2007](#_ENREF_8)) procedure was used for multi-model projection matching of microtubule specimens with varying numbers of protofilaments ([Alushin et al., 2014](#_ENREF_2)), using libraries from the EMAN2 image processing package ([Tang et al., 2007](#_ENREF_24)). ~66% of the data were classified as 15 protofilament microtubules, which do not have a seam and have the advantage of being helical. After each round of projection matching, an asymmetric back projection is generated of aligned segments, and the 3-start helical parameters (rise and twist) describing the monomeric tubulin lattice are calculated. These helical parameters are used to generate and average 15 symmetry-related copies of the asymmetric reconstruction, and the resulting models are used for projection matching during the next round of refinement. A final refinement of the microtubule segment alignment parameters was performed in FREALIGN ([Grigorieff, 2007](#_ENREF_11)) without further refinement of helical parameters. Fourier Shell Correlation (FSC) curves for microtubule reconstructions were used to estimate resolutions of each reconstruction using a cutoff of 0.5(Figure S2E). In order to more accurately estimate the resolution of each region of the reconstructed density, a local resolution calculation was performed using the “blocres” and “blocfilt” functions in the Bsoft processing package (Heymann et al 2007). This analysis revealed that the majority of the tubulin density is in the range of 7-8Å resolution, while the ligase portion ranges from 9-12Å resolution. Microtubule models were obtained through rigid-body docking of the electron crystallographic structure of tubulin (PDB ID [1JFF](http://www.rcsb.org/pdb/explore.do?structureId=1SA0); ([Lowe et al., 2001](#_ENREF_14))) and the TTLL7 crystal structure into the cryo-EM density maps using UCSF Chimera ([Pettersen et al., 2004](#_ENREF_18))

**Modeling of the c-MTBD helices**

Secondary structure prediction of the C-MTDB was performed using the Jpred, Psipred and Phyre servers (Figure S2J; ([Buchan et al., 2013](#_ENREF_3); [Cole et al., 2008](#_ENREF_5)), and polyA helices of the predicted length were generated using the “Build Structure” tool in UCSF Chimera ([Pettersen et al., 2004](#_ENREF_18)). Helices were positioned into place based on the locations of the missing regions of the TTL7 crystal structure and the observed EM density. Only helices 383-400 and 416-448 could be positioned with considerable confidence. The 379-382 loop of the docked TTLL7 crystal structure extends towards an EM density that is consistent with an -helix, providing a starting point for the 383-400 helix. The length of the EM density for this helix matched closely the length of the modeled helix. Next, a much longer helix of 33 residues was docked into the only EM density that could accommodate a helix of this length within the c-MTBD. The C-terminus of this modeled helix, residue 448, was positioned in close proximity to residue 451 of the TTLL7 crystal structure, supporting the docking of this helix. Lastly, the C-terminal helix 463-484 in the TTLL7 crystal structure was predicted to connect to an additional helix of ~16 residues, also visible as a sausage-like shape in our EM reconstruction.

**Mass Spectrometric Analysis of Glutamylation Reactions**

TTLL7 was incubated at room temperature with 5 μM taxol stabilized unmodified microtubules at a 1:20 molar ratio in 20 mM HEPES 7.0, 50 mM NaCl, 5 mM MgCl2, 0.5 mM TCEP, 1 mM ATP, 10 μM taxol and 1 mM L-glutamate. Unmodified tubulin was purified from tsa201 cells using a TOG affinity column ([Widlund et al., 2012](#_ENREF_27)). Aliquots were removed at the indicated time points and the reaction quenched by the addition of an equal volume of 0.1% trifluoroacetic acid, 20 mM EDTA, and 10% acetonitrile. Samples were analyzed by electrospray mass spectrometry as described in ([Vemu et al., 2014](#_ENREF_26)).

**Peptide Glutamylation Assays**

A synthetic βIVb-tubulin peptide acetylated at its N-terminus (Ac-DATAEEEGEFEEEAEEEVA, courtesy of Dr. Martin Tanner, University of British Columbia) was dissolved in water to a concentration of 10 mM and the pH of the solution was adjusted to 7 with 5 M NaOH. A synthetic detyrosinated α1B-tubulin peptide (YEEVGVDSVEGEGEEEGEE, Biosynthesis) was dissolved in 20 mM HEPES 7.0 to a concentration of 10 mM and the pH was adjusted to 7 using 5 M KOH. Each peptide was incubated separately with wild type TTLL7 (10 μM) for 18 hours at room temperature at a 10:1 ratio of peptide to enzyme in 20 mM HEPES 7.0, 50 mM NaCl, 5 mM MgCl2, 0.5 mM TCEP, 1 mM ATP, and 1 mM L-glutamate. Reactions were terminated by the addition of an equal volume of 0.1% TFA, 20 mM EDTA, and 10% acetonitrile. A 7-μl aliquot of each reaction was separated on Zorbax 300SB-C18 column (Agilent) using a 30-60% acetonitrile gradient in 0.05% TFA at a flow rate of 0.2 ml/min. The column was in-line with a 6224 ESI TOF LC-MS (Agilent). Data were analyzed using the MassHunter Workstation platform (Agilent). To compare the rate at which substrate is converted to product between wild type and Δ384-446 TTLL7 constructs, the βIVb peptide was incubated with each construct in the conditions described above. The decrease in m/z signal of the unmodified peptide was monitored as a function of time in the presence of each enzyme. BSA (Sigma) at a concentration of 0.2 mg/ml was used as an internal standard as it elutes separately from the tubulin peptide and TTLL7.

**Glutamylation Assays**

Initial rates of wild-type and structure guided TTLL7 mutants were determined by the incorporation of radio-labeled [3H]-glutamate into 4 μM taxol stabilized microtubules in buffer consisting of 20 mM HEPES 7.0, 50 mM NaCl, 5 mM MgCl2, 0.5 mM TCEP, 1 mM ATP, 10 μM taxol, 98 μM 2H-glutamate, and 2 μM 3H-glutamate (51.1 Ci/mmol). All reactions were performed at room temperature and initiated by the addition of enzyme to a final concentration of 200 nM. Aliquots (7.5 μl) were removed at 2, 4, 6, 8, 10, 20, 30 minutes. Reactions were quenched by addition of 20 mM EDTA and snap frozen on dry ice. Aliquots were spotted on DEAE-cellulose filters (Whatman). Tubulin binds to the filter and unincorporated radioactivity was removed by washing. Filter-bound radioactivity was measured by scintillation counting and the amount of 3[H]-Glu transferred to tubulin was calculated after measuring total activity of 3[H]-Glu in the reaction. Rates were obtained by fitting the linear range of the reaction curves using the PRISM 6 software package.

Glutamylation activity of TTLL4, 5, 6, 7, 11 and 13 and their mutants was determined in human tsa201 cells by immunoblotting cell extracts 24 hours post transfection using antibodies to glutamylation (mAB GT335; 1:1000, Adipogen). Total tubulin levels were detected using monoclonal rabbit anti-α tubulin antibody EP1332Y (1:1000, AbCam) and IRDye 800W goat anti-rabbit secondary antibody (1:18000 dilution, Li-Cor). GFP fusion protein expression levels were determined using monoclonal mouse anti-GFP antibody (Catalog 11 814 460 001; 1:1000, Roche) and IRDye 680LT goat anti-mouse secondary antibody (1:18000, Li-Cor). Western blots were quantified using Image Studio Lite (Li-Cor). The glutamylation signal was normalized for enzyme expression levels and total tubulin levels.

**Generation of Glutamylated Microtubule Substrates**

Taxol stabilized microtubules containing 98% unmodified tubulin, 1% tetramethyl rhodamine (TMR) and 1% biotin brain tubulin were incubated with TTLL7 at a 1:10 (TTLL7:tubulin) molar ratio for 1.5, 4 and 10 hours to obtain different levels of glutamate incorporation (, + 4.2 Glu,  +0.5 Glu,  + 8.3 Glu and  + 6.8 Glu,  + 28.2 Glu, respectively). TTLL7 was removed by addition of 0.3 M KCl and sedimentation through a 60% glycerol cushion in BRB80. The number of glutamates added to - and -tubulin was determined by electrospray mass spectrometry ([Vemu et al., 2014](#_ENREF_26)). The spectra display the characteristic distribution of masses with peaks separated by +129 Da corresponding to one glutamate (Figure 4B).

**Generation of microtubules missing -tails or both  and -tails**

Taxol-stabilized unmodified microtubules labeled with 1% TMR and 1% biotin were prepared as described above. Partial Δβ-tail microtubules were obtained by digesting microtubules with subtilisin at a 1:300 subtilisin:tubulin mass ratio for 90 minutes. Δβ-tail microtubules were obtained by digesting microtubules with subtilisin at a 1:200 subtilisin:tubulin mass ratio for 30 minutes and Δα,β-tail microtubules were obtained by digesting microtubules with a 1:50 subtilisin:tubulin mass ratio for 2 hours. Reactions were performed at room temperature and quenched with 5 mM phenylmethylsulfonyl fluoride (PMSF). Microtubules were recovered through a glycerol cushion. Digests were subjected to mass spectrometry as described in ([Vemu et al., 2014](#_ENREF_26)).

**TIRF Imaging and Image Analysis**

For single molecule assays, taxol-stabilized microtubules consisting of 98% tubulin (porcine brain tubulin or unmodified tubulin purified *via* the TOG affinity method; ([Widlund et al., 2012](#_ENREF_27))), 1% TMR tubulin and 1% biotin tubulin were immobilized on a glass surface using neutravidin as previously described ([Szyk et al., 2014](#_ENREF_23)). Single molecule kinetic measurements were performed with TTLL7-GFP wild-type or mutants (0.4 nM) in 14 mM HEPES 7.0, 105 mM NaCl, 7.2 mM MgCl2, 0.6 mM ATP, 16mM PIPES 6.8, 0.2 mM EGTA, 1 mM glutamate, 4 µM taxol, 1% pluronic F-127 supplemented with oxygen scavengers prepared as described in ([Ziolkowska and Roll-Mecak, 2013](#_ENREF_28)). An inverted TIRF microscope (Nikon Ti-E with TIRF attachment) was used for image acquisition. The excitation light was provided by a 532 nm Coherent Sapphire CUBE for TMR and a 488 nm Coherent CUBE for GFP both operating at 20 mW. A 100X 1.49 NA objective (Nikon CFI Apo TIRF 100X) delivered the evanescent wave excitation light to the sample and the emitted light was collected by the same objective. The emitted light was split using an Andor TuCAM equipped with a dichroic mirror (Semrock Di02-R594). Two EMCCD cameras (Andor iXON3-897) equipped with the appropriate filters (Semrock FF01-514/30 for GFP and FF01-593/LP for TMR) were used to image the emitted fluorescence. The TuCAM introduces an additional 2x magnification factor resulting in an 89 nm pixel size. First, a single frame image of the TMR-labeled microtubules using the 532 nm excitation channel was acquired at an exposure of 200 ms. Then, the 488 nm channel was used to image TTLL7-GFP at an exposure of 200 ms per frame for 1000 frames. TTLL7-GFP particles were tracked using the FIJI TrackMate plugin ([Schindelin et al., 2012](#_ENREF_20)). For the single-molecule kinetic measurements presented in Figure 3 multiple microtubules (807 TTLL7 binding events) and -tails microtubules (790 binding events) were analyzed from four separate flow cells on different days. In Figure 4, ten unmodified microtubules (1,009 TTLL7 binding events) from six separate flow cells on three different days and eight modified microtubules (483 TLLL7 binding events) from four separate flow cells on different days were used to obtain the kinetic parameters.

For the fluorescence intensity line scan analyses, TTLL7-GFP or structure guided mutants were perfused at a concentration of 7 nM in 14 mM HEPES 7.0, 50 mM NaCl, 7.2 mM MgCl2, 0.6 mM ATP, 16 mM PIPES 6.8, 0.2 mM EGTA, 1 mM glutamate, 4 µM taxol, 1% pluronic F-127 and oxygen scavengers. The chamber was left to equilibrate for 5 minutes prior to imaging. Images of the 488 nm and the 532 nm excitation channels were acquired at an exposure of 100 ms. A neutral density (ND) filter 8 was used. Multiple fields of view were imaged. Background corrected line scan intensities were measured using FIJI ([Schindelin et al., 2012](#_ENREF_20)) and intensities were normalized to microtubule length.

**Immunofluorescence**

U2OS cells were grown on poly-L-lysine coated cover slips and were co-transfected with human GFP-tagged WT or Δc-MTBD clones of TTLL7. Cells were fixed 18 hours post transfection in cytoskeletal buffer (CB) (10 mM MES 6.1, 138 mM KCl, 3 mM MgCl2, 2 mM EGTA) containing 4% paraformaldehyde and 10 μM taxol, and permeabilized in CB containing 0.5% Triton X-100. Prior to fixation, growth media was supplemented with 5 μM taxol for 30 minutes to aid in cytoskeletal preservation. Tubulin was detected using polyclonal rabbit antibody ab18251 (1:100 dilution, Abcam) and Cy3-labeled goat anti-rabbit secondary antibody (1:200 dilution, Jackson ImmunoResearch). Glutamylated microtubules were detected using monoclonal mouse antibody GT335 (1:500 dilution, Adipogen) and Cy5-labeled goat anti-mouse secondary antibody (1:200 dilution, Jackson ImmunoResearch). Cells were mounted in Prolong gold antifade with DAPI (Life technologies). Images were acquired on a Nikon Eclipse Ti microscope equipped with a CSU-X1 spinning disc confocal scanner unit (Yokogawa), Nikon Plan Apo VC 60x (NA 1.40) oil objective, and an ORCA-Flash4.0 V2 camera (Hamamatsu). Microscope automation was controlled using μManager software ([Edelstein et al., 2010](#_ENREF_7)). Excitation light for DAPI (405 nm), GFP (488 nm), Cy3 (561 nm), and Cy5 (640 nm) was provided by an Agilent MLC400B monolithic laser combiner. Appropriate filters were used to image emitted light: Chroma ET460/50m for DAPI, Chroma ET525/50m for GFP, Chroma ET605/52m for Cy3, and Semrock FF01 731/137 for Cy5. All images were acquired using 100 ms exposure times for all channels except for Cy5 (50 ms). The brightness and contrast of all images was adjusted post acquisition using ImageJ software (NIH).

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