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SUPPLEMENTARY INFORMATION

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Structural basis for polyglutamate chain initiation and elongation by TTLL family enzymes

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Supplementary Table 1: Chemical shift assignments for the $^{13}C/^{15}N$ TTLL6 glutamylated α1B^{445}E1 peptide.

Atom	Intermediate δ(ppm)	Terminus δ(ppm)	BMRB Glu $\delta \pm$ S.D. (ppm)
H_α	4.331	4.123	4.238 ± 0.401
$H_{\beta 2}$	2.078	2.045	2.021 ± 0.207
$H_{\beta 3}$	1.943	1.912	1.997 ± 0.213
$H_{\gamma 2}$	2.305	2.237	2.268 ± 0.209
$H_{\gamma 3}$	2.305	2.237	2.249 ± 0.213
C_α	56.239	57.966	57.330 ± 2.065
H_N	8.522	8.129	8.330 ± 0.580
C_β	30.700	31.222	29.973 ± 1.698
C_γ	36.218	36.654	36.103 ± 1.205

Atom assignments were made by comparison to protein glutamate chemical shifts derived from the Biological Magnetic Resonance Data Bank (filtered to remove proteins bound to paramagnetic or aromatic ligands, dated 22nd May 2018). Assignment to intermediate or terminal glutamate was determined using the CBCA(CO)(N)H experiment.

Supplementary Note 1

Protein expression and purification

Caenorhabditis elegans TTLL4 (106-601) was expressed in *E. coli* Arctic Express DE3 cells (Agilent) with a N-terminal GST tag. Cultures were grown at 37˚C to an OD_{600} of 2. Protein expression was induced with 0.5 mM IPTG at 13˚C for 24 hours. Cells were lysed and the clarified cell lysate was purified using GST affinity chromatography. The tag was removed using 3C Prescission protease at 1:10 molar ratio. TTLL4 was further purified on a heparin column and size exclusion chromatography using a Superdex75 column (GE). The protein eluted as a single peak.

Recombinant human α1A/βIII and α1A-Y/βIII tubulin was purified as described previously[16]. Briefly, α1A tubulin with a His-tag inserted in the acetylation loop and a C-terminally Flag-tagged βIII tubulin was expressed in insect cells using the Bac-to-Bac system (Life Technologies). Recombinant virus infected HighFive cells were lysed and tubulin was purified using Flag resin followed by Ni-NTA affinity chromatography. The eluate was buffer exchanged using PD100 columns (GE Healthcare) equilibrated with BRB80 and 20 μM GTP. Aliquots were flash frozen in liquid nitrogen and stored at -80˚C.

Peptide Synthesis

Amino acids were purchased from P3Bio or Novabiochem, except H-Glu(tBu)-OtBu, Fmoc-Glu(2-Phenylisopropyl ester)-OH which were purchased from Bachem. Novasyn TG Sieber resin was purchased from Novabiochem. The backbone peptide sequence was synthesized with orthogonally protected Fmoc-Glu(2-Phenylisopropyl ester)-OH at the branch point. Fully protected Glu polypeptides were synthesized on super acid sensitive Novasyn TG Sieber resin and

cleaved using 1% TFA in DCM. The combined 1% TFA washes were combined, neutralized with DIEA, and rotovaped under reduced pressure. The orthogonally protected Glu was deprotected with 1% TFA in DCM. The protected polypeptide Glu chains were coupled to the resin-bound peptide using PyBOP or PyOxim coupling reagents overnight. Final cleavage of the branched peptides was achieved using 95% TFA containing scavengers 1,2-ethandithiol and thioanisole (1:2). The cleavage reaction was performed at room temperature for 1.5-3h depending on the peptide length. The cleaved peptide was precipitated in diethyl ether, centrifuged and washed several times with fresh ether. Purification of the crude peptides was performed on a C18 Grace-Vydac semi-preparative reversed-phase HPLC column (250x10mm) using a Waters 600 series HPLC system. Fractions were analyzed on a Vydac C18 analytical column (150x4.6mm). Separations were achieved using linear gradients of 0-100% buffer B for 120 mins (semipreparative) or 30 mins (analytical), at a flow rate of 3ml/min or 1ml/min, respectively. Buffer A was water containing 0.045% TFA and buffer B was acetonitrile with 0.036% TFA. The identity of the peptides was confirmed using MALDI-TOF on a Waters LR mass spectrometer.

Kinetic analyses of microtubule glutamylation

K_M and k_{cat} values for TTLL6 wild-type, switch mutant (C179A/Q180R/H362I) and TTLL4 with brain and unmodified microtubules were determined by varying tubulin concentration from 1 to 20 μM for TTLL6 and 1 to 30 μM for TTLL4 while keeping enzyme concentration constant at 1 μM. The reaction condition consisted of 50 mM Hepes pH 7.0, 10 mM MgCl2, 50 mM KCl, 1 mM DTT, 1 mM ATP, 950 μM L-Glutamic acid, and 50 μM H^3-L-Glutamic acid. Reactions were initiated by enzyme addition and terminated by the addition of saturating EDTA and placing on ice for 30 mins. The reaction mix was transferred to the positively charged nylon transfer

membrane (GE Lifesciences) and washed extensively with 50 mM sodium phosphate, pH 7.8 supplemented with 25 mM KCl. Membranes were then transferred to a scintillation vial and residual radioactivity was measured using a Beckmann scintillation counter. Kinetic constants were obtained by using the one-site binding parameter equation in PRISM 6.1. Reactions were performed in duplicate on two separate days.