Supplemental Methods

All chemicals used were ACS quality or better, and purchased from Sigma, Invitrogen, CalBiochem, or Polysciences. Animals used include: Sprague/Dawley rats (200-225 grams, Harlan), male C57BL/6 mice (Jackson Laboratories) and male TG2 KO mice from the colony of G.V.W.Johnson, founders obtained from R.M. Graham (Nanda et al., 2001)

Proteins carried by axonal transport in rat optic nerve were labeled by intraocular injection of radioactive amino acids (^{35}S-methionine) or polyamines (^{3}H or ^{14}C-PUT) into the eye as described previously (Brady and Lasek, 1982). An injection-sacrifice interval (ISI) of 21 days was used to position the SCa wave containing both stable and labile MTs fully in the optic nerve. Radioactive proteins were separated by SDS-PAGE and fluorography (Kirkpatrick et al., 2001). Our standard protocol for cold/Ca^{2+} fractionation of neuronal tubulins was used (Brady et al., 1984) (see suppl. Fig 1 and suppl. methods).

Following cold/Ca^{2+} fractionation, samples were separated on gradient gels as described then transferred to Immobilon-P transfer membrane (Millipore) as described previously. The primary antibodies used throughout these studies are DM1A (1:20,000, Sigma) for alpha-tubulin, TGMO1 (1:4000) for TG2, H2 (1:50,000) (Pfister et al., 1989)) for kinesin heavy chain, Tu27 (1:10,000, provided by Dr. A. Frankfurter (Caceres et al., 1984)) for β-tubulin, A2066 (1:5000, sigma) for beta-actin. pab0022 and pab0023 (1:1200, Covalab) for SPM/SPD (SPM/SPD).

For radioactive quantitative immunoblots, the secondary antibody was rat- anti-mouse IgG (1:1000, Jackson) detected using ^{125}I-Protein A and measured by PhosphorImager (Molecular Dynamics). Scanned images were quantitated using ImageQuant. For fluorescent quantitative immunoblots**,** the secondary antibodies were Cy5-anti-rat (1:400, Jackson) for TG MO1, Cy5-anti-rabbit (1:4K, Invitrogen) for pab022 and pab023, and Cy5-anti-mouse (1:400, Jackson) for the rest. The blots were imaged by Typhoon 9410 (GE healthcare).

Difluoromethylornithine (DFMO, Merrell Dow Pharmaceuticals) was administered as a 2% solution in the drinking water. DFMO was given to rats 16-18 hours prior to labeling of axonal transport with ^{35}S-methionine, and continued for 48hours, or the first 7 days of a 21-day ISI, or for all 21 days of a 21-day ISI. Control rats were injected and sacrificed as with experimental rats, but did not receive DFMO. At the time of sacrifice, the entire labeled optic nerve-optic tract was removed and processed through cold/Ca^{2+} fractionation.

To lower endogenous polyamine levels and facilitate protein labeling with labeled PUT (^{14}C or ^{3}H), DFMO was administered 16-18 hours prior to labeling and removed at the time of injection as above. Following a 21-60 day ISI, the rats were sacrificed and optic nerve-optic tract removed for cold/Ca^{2+} fractionation. The resulting P2 fraction was resuspended in 8M urea and then loaded on a 60 ml Toyopearl HW-55F (Supelco) column equilibrated in 6M guanidine-HCl in 100 mM MES, pH 6.8. A 200-µL aliquot of each fraction was used to detect elution of radioactivity. Fractions around the eluted peak of radioactivity were methanol-precipitated and analyzed by SDS-PAGE and immunoblot as above. Total α-tubulin was detected with DM1A antibody.

Tubulin was purified from mouse brains through two cycles of polymerization-depolymerization in high-molarity PIPES buffer as described(Castoldi and Popov, 2003). For polymerized MTs, tubulin (50 µM) were incubated in Brinkley BR buffer 1980 (BRB80) supplemented with 2 mM GTP at 37 °C for 30min described. For Taxol-stabilized MTs, taxol was added stepwise to equimolar of tubulins in warm BRB80 buffer supplemented with 1mM DTT and 1mM GTP and incubated at 37 °C for 30min in total. Polymerized MTs were pelleted over a glycerol/BRB80 cushion as described (http://mitchison.med.harvard.edu/protocols.html).

In vitro polyamination assay used 1) N,N'-dimethylcasein (a known transglutaminase substrate) or tubulin/MT 2) 4mM monodansylcadaverine (MDC) or PA mix (SPM/SPD) 3) 1µM guinea pig TG (similar to TG2 in brain) 4) 5mM DTT in a Tris-HCl based reaction buffer (PH 7.5) with or without 10mM Ca^{2+} at 37 °C for 60min. The reaction was stopped by 20mM cystamine and

centrifuged for SDS-PAGE gel as above. Fluorescence due to MDC incorporation into tubulins was detected with a Gel Doc 2000 (Bio-Rad).

For endogenous transglutaminase activity assays, 150µg of transglutaminase extract was incubated with 0.2mg/ml N,N-dimethylcasein, 4mM MDC, 5mM DTT in a Tris-HCl based reaction buffer (PH 7.5) with 10mM Calcium at 37 °C for 60min. Transglutaminase extracts were prepared from mouse by homogenization in a cold 50mM Tris-HCl, 1mM EDTA, 0.25M sucrose, 0.4mM DTT and protease inhibitor cocktail. Homogenates were centrifuged at 16,000g at 4°C for 20min. Pellets were discarded and supernatant centrifuged at 100,000g at 4°C for 1hour to produce the transglutaminase extract.

Brain transglutaminase extract was prepared as above, and contained endogenous transglutaminase, soluble tubulins and PAs. A tubulin pellet was obtained by centrifugation after *in vitro* polyamination was stopped by cystamine and subjected to cold/Ca^{2+} fractionation. Fractions were analyzed by SDS-PAGE and immunoblots with DM1A and Tu27 antibodies.

Polyaminated and native tubulins were analyzed in 2D PAGE after solubilization in 8 M urea, 4% CHAPS, 65 mM DTE, 0.5% ampholytes, and bromophenol blue. Protein solution was spun at 100,000 RPM for 30 minutes at 4°C and absorbed onto a 7 cm immobilized pH gradient pH 3-10 IPG strip (IPG strip, GE Bioscience) overnight. IEF was run for 60 kVhr (PROTEAN IEF cell, Bio-Rad) at room temperature. Before SDS-PAGE, IPG strips were equilibrated with 50 mM Tris-HCl (pH 8.8), 6 M urea, 30% glycerol, 2% SDS, a trace of bromophenol blue, and DTE (1% w/v) for 20 min, followed by a second equilibration in equilibrium solution containing iodoacetamide (2.5% w/v) instead of DTE. Finally, IPG strips were transferred to the top of 4-12% Nu-Page gels and held in position with molten 0.5% agarose in 25 mM Tris, 0.192 M glycine, 0.1% SDS. Gels were run at constant voltage of 200 V for 45 minutes followed by immunoblotting procedure as above.

Freshly purified mouse brain tubulins were modified by various polyamines in the presence of transglutaminase as described above then pelleted. Pellets were dissolved in 8M urea, 2M thiourea

containing dithiothreitol and separated by preparative isoelectric focusing using an Agilent 3100 OFFGEL fractionator using 13 cm pI 3-10 IPG strips (GE Healthcare) according to manufacturer's instructions. Aliquots were run on 10% SDS gels, stained with Coomassie and the tubulin band excised, reduced with dithiothreitol, alkylated with iodoacetamide and subjected to in gel digestion using overnight digestion with trypsin (Promega) at 37°C. Peptides were extracted with ammonium bicarbonate and 50% acetonitrile, concentrated by speedvac and subjected to LC/MS/MS as above. Data was searched as above using Mascot followed by manual validation of putative polyamine modifications.

Freshly prepared S1 (cold and calcium labile tubulin fraction) and P2 (cold and calcium stable tubulin fraction) were subjected to isoelectric fractionation and electrophoresis as above but without any exogenous polyamine addition to detect endogenous transglutaminase modifications by both immunoblots and mass spectrometry (figure S5).

In vitro polyaminated tubulins were prepared fresh with MDC, PUT, SPM and SPD separately as above and subjected to overnight trypsin digestion, tubulin without modifications serving as a control. Samples were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS-MS, Thermo LTQ-FT Ultra using Agilent Zorbax SB300-C18 0.075 mm ID x 150 mm Capillary Analytical Column with 0.3 mm ID x 5 mm Zorbax SB300 C18 trapping 250 nL/min gradient, 5% ACN to 65% ACN. Nano ESI Positive Ion mode (Resolution 50,000 @m/z 400, Scan 400-1800 m/z) was performed. Top 3 +2 and greater charged ions for MS/MS from each MS scan were selected. Putative modified peptides were picked based on major mass shift in MS1, and specific modification sites were further evaluated in MS2 spectra and confirmed through target runs. Searches were conducted using MASCOT (version 2.2.04, Matrix Sciences, Inc) and MassMatrix search engines against the SwissProtKB using mouse as taxonomy, carbamidomethylation and methionine oxidation as variable modifications in addition to each PUT, SPM, SPD as variable modifications of Q.

Searches were performed using 10 ppm peptide tolerance and 0.6 Da fragment tolerance using decoy database searches to ensure false discovery rates were below 5%.

For the semi quantitative analysis of the brain fractions, an Agilent chip cube (Agilent Technologies Inc, Santa Clara, CA) was modified as described earlier (Crot et al., 2010) and mounted on the LTQ-FT Ultra instrument using a custom source mount. Chromatography was performed using an Agilent 1260 series nano/capillary pumping system equipped with an Agilent 1200 series nano WPS autosampler. Chromatography was performed using a Zorbax SB300 C18 Protein ID Chip (G4240-62001) with a 75 µm x 43 mm internal column length and a 40 nL internal enrichment column. The samples were chromatographed using a binary solvent system consisting of A: 0.1% formic acid and 5% acetonitrile and B: 0.1% formic acid and 95% acetonitrile at a flow rate of 200 nL/min. A gradient is run from 3% B to 65%B over 15 minutes with injection volumes of 2-4 µL. Enrichment column conditions used an isocratic flow of 0.1% formic acid in water at 4µL/min. The mass spectrometer conditions employed a custom method for each of the putatively modified EIVH tubulin peptides. A 3 scan event program was used for each sample in which an initial full scan MS was recorded from 400-1800 Da in the isolation cell of the FT portion of the LTQ-FT Ultra instrument. Then targeted MS/MS scan events for the 2+ and 3+ precursor ions for each type of modified peptide were run in the low resolution LTQ part of the instrument allowing for the acquisition of two spectra per MS/MS scan produced from each precursor ion. In this way a continuous chromatogram was acquired for each possible modified form of the EIVH peptide in MS/MS mode as opposed to the acquisition of just a few MS/MS scans of a given precursor ion at any arbitrary retention time that characterizes the typical protein ID experiment. Separate runs were performed for each brain fraction for each of the putative modified forms of the EIVH peptide. Data analysis examined summed chromatograms for each of the A, B or Y series ions predicted in the fragmentation of each precursor ion. Given the low *in vivo* levels of the modified tubulin expected, no precursor ion signals could be observed in the MS scan part of each experiment but clearly observed extracted ion chromatograms

for the fragment ions of each peptide were observed and the areas of each peak were integrated and listed in table S4. A modified peptide was considered to be detected only if co-eluting peaks were observed in all the summed A, B and Y series fragment ion extracted ion chromatograms for each respective modified peptide.

Synthesis and purification of TG2 Inhibitors

An irreversible inhibitor of transglutaminase 2, IR072, was synthesized. All Fmoc protected amino acids, resins, and coupling reagents were purchased from GL Biochem. Wang resin was purchased from NovaBiochem. All other reagents were obtained from Sigma-Aldrich. Reactions requiring anhydrous conditions were carried out under a dry nitrogen atmosphere employing conventional benchtop techniques. ^{1}H- and ^{13}C-NMR spectra were recorded on AMXR400 and AMX300 spectrometers and were referenced to the residual proton or ^{13}C signal of the solvent. Mass spectra were determined by FAB+ ionization on an AutoSpec Q spectrometer at the Regional Mass Spectrometry Centre at the Université de Montréal.

Reactor tubes for solid-phase peptide synthesis were obtained from Supelco. All resins were swelled in DMF and washing steps were performed using DCM and DMF (EMD Chemicals). Purification of all peptides was performed using a preparative HPLC method. Mass spectral data (MS, LCMS) were all obtained using two different columns: Column A: Gemini C18, 150 × 4.6 mm, 5 µm (Phenomenex, Torrance, CA); column B : Synergi Polar-RP, 150x4.6 mm, 4 µm (Phenomenex, Torrance, CA). The crude peptide was purified using a preparative Synergi Polar-RP, 100x21.20 mm (Phenomenex, Torrance, CA) on a Varian (Prep Star) HPLC system. at the Centre Régional de Spectrométrie de Masse de l'Université de Montréal.

All peptides were synthesized using standard solid phase Fmoc chemistry. Briefly, the first Fmoc protected amino acid (5.5 mmol) was coupled to Wang resin (1.1 mmol) using DIC (5.5 mmol) and DMAP (0.11 mmol). The level of loading of the amino acid on the resin after the first coupling step was used as the resin loading capacity for all subsequent steps, as determined by spectroscopic

measurement of the UV absorbance of the piperidine dibenzofulvalene adduct. The remaining free hydroxyl groups of the resin were then capped by treating the resin with a mixture of acetic anhydride/pyridine (2:3) and shaking for 2 h. After washing with DMF (3 × 10 resin volumes), DCM (3 × 10 resin volumes) and ether (3 × 10 resin volumes), the Fmoc group was removed by incubating three times with piperidine in DMF (20% v/v; 10 resin volumes) for 5 min, followed by washing with DMF (3 × 10 resin volumes), DCM (3 × 10 resin volumes) and ether (3 × 10 resin volumes) in preparation for the next amide coupling. Deprotection was verified by positive Kaiser test on a sample of a few beads. Then each Fmoc protected amino acid (1.7 mmol) was coupled to Wang resin preloaded with the necessary carboxyl-terminal amino acid (0.68 mmol) in DMF (5 resin volumes) using HOBT (1.7 mmol) and DIC (1.7 mmol). This operation was effected twice for 30 min. Coupling was verified by negative Kaiser test on a sample of a few beads. In the case of rhodamine (1.7 mmol) and acrylic acid (1.7 mmol), the coupling reagent was EEDQ (1.7 mmol) and the shaking time was 1 h. The peptide was cleaved from the resin (1 g) by incubating with TFA:DCM (1:1) for 2 h. The peptide was precipitated from the cleavage solution using diethyl ether and hexane. Peptide sequence purity was performed using two different columns: Column A : Gemini C18, 150 × 4.6 mm, 5 µm (Phenomenex, Torrance, CA); column B : Synergi Polar-RP, 150 × 4.6 mm, 4 µm (Phenomenex, Torrance, CA). The crude peptide was purified using a preparative Synergi Polar-RP, 100 × 21.20 mm column (Phenomenex, Torrance, CA) on a Varian (Prep Star) HPLC system.

Boc deprotection protocol

To a reactor containing 1 g of resin (0.68 mmol, according to the loading), was added 30 mL of deprotection mixture, freshly prepared from 470 µL TEA (2 eq.), 1.09 mL of TMSOTf (0.2 M) and 28.44 mL of anhydrous DCE. The resin was shaken for 10 min then filtered and washed with 5 × 5 mL of DCM, 2 × 5 mL of DIEA 10% in DCM, and 3 × 5 mL of DCE. This procedure was then repeated with fresh deprotection mixture for another 10 min. The resin was filtered then washed with 5 × 5 mL of DCM, 2 × 5 mL of DIEA 10% in DCM, 2 × 5 mL of DCM, 2 × 5 mL of DMF and 2 × 5 mL

of Et_2O.

Acrylation protocol

To the Wang resin supported Fmoc-(Me)GABA-lysinyl-prolinyl-leucinyl-phenylalaninate (1 g, 0.68 mmol) swollen in anhydrous DCM (5 resin volumes) was added acrylic acid (1.7 mmol) and EEDQ (1.7 mmol). The reaction was shaken for 1 h, followed by washing with DMF (3 × 10 resin volumes), DCM (3 × 10 resin volumes) and ether (3 × 10 resin volumes). This coupling operation was effected twice.

HPLC analysis and purification

For HPLC analysis, the peptide was cleaved from the resin as described above. The crude material was purified by preparative Synergi Polar-RP (100 × 21.20 mm) column, on a Varian (Prep Star) HPLC system using MeOH in water as eluant, at a flow rate of 8 mL/min and the detector set at 254 nm or 214 nm. The collected fractions were freeze-dried to yield the desired compounds. The areas under the peaks were determined using LC/MSD Chem Station (Agilent Technologies).

Fmoc-(Me)GABA-lysinyl(acryloyl)-leucinyl-prolinyl-phenylalaninate (IR072)

After the peptide was cleaved from the resin as described in the General Procedure above, the crude material was purified by preparative Synergi Polar-RP (100 × 21.20 mm) column, on a Varian (Prep Star) HPLC system using 80-95 % MeOH in water as eluant, at a flow rate of 8 mL/min with detection at 254 nm. The collected fractions were freeze-dried to give the desired irreversible peptidic inhibitor **IR072** as a white powder (Fig. S7). Yield : 11%; 99% purity by column A, 98% by column B. HRMS m/z ($M+H^+$): calcd 879.4651; found 879.46567.

Immunochemistry and measurement of neurite extension

SH-SY5Y cells were grown on coverslips under 3 different conditions, undifferentiated, differentiated, and differentiated with 10μM IR072, an irreversible TG2 inhibitor (synthesized by JWK and NC) (figure S7). The cells were fixed with 4% paraformaldehyde, permeablized and washed with

0.1% triton in PBS. 4% donkey serum was used to block the non-specific binding at room temperature for 1 hour followed by incubation in primary antibody diluted in PBST at room temperature for 2 hours, 3 times of washing, incubation in secondary antibody diluted in PBST at room temperature for 1 hour and 3 times of washes. Primary staining included tubulin (chicken βIII antibody 1:1000, Abcam and DM1A 1:600, as described above), transglutaminase 2 (Ab3 1:200, Thermo Scientific). Secondary antibodies included Alexa 488-anti-mouse (Invitrogen, 1:400) and Cy5-anti-chicken (1:400, Jackson ImmunoResearch). Images acquired by Zeiss LSM 510 meta confocal (Z-stack) were processed with Volocity 5.2.1(Improvision, Boston, MA). Neurite length was measured in Openlab 5.5.1 (Improvision, Boston, MA), differences in mean neurite lengths with different treatments were evaluated for significance by Student's t test in Microsoft Excel ($p \geq 0.05$) and plotted in Deltagraph.

Statistics: Paired sample t-test function was performed using Datadesk.

Supplemental Tables

Table S1: Related to Figure 1. Pretreatment with DFMO decreased cold/Ca^{2+} stable tubulin in rat optic nerve labeled by slow axonal transport without affecting stability of neurofilament proteins.

TUBULIN	**S1**	**S2**	**P2 % ± SEM**
Control (n=5)	40.8 ± 5.4	6.9 ± 2.5	52.4 ± 5.7
18h DFMO (n=9)	37.9 ± 4.6	8.6 ± 4.1	53.5 ± 7.0
7d DFMO (n-5)	57.1 ± 6.9	9.9 ± 2.4	33.1 ± 8.9
21d DFMO (n-5)	55.7 ± 4.5	5.7 ± 7.6	38.7 ± 3.7
NFM			
Control (n=3)	26.5 ± 3.4	9.0 ± 3.8	64.6 ± 7.2
7d DFMO (n-5)	25.4 ± 4.2	8.8 ± 2.9	65.8 ± 5.5

Table S2: Table of ions identified by the tandem mass spectrum in figure 4B. Note that after y7 all further y ions were double charged, consistent with a positive charge in Q due to the modification. Provided as an Excel files.

Table S3: Table of ions used to create extracted ion chromatograms (EIC) for PUT and SPM modified EIVH tubulin peptide as shown in figure 4C/D and Supplemental figure S1 respectively.

Table of ions used to create extracted ion chromatograms (EIC) for PUT and SPM MS/MS data

Modification		Putrescine			Spermine		
Sequence on beta tubulin	Amino Acid	A ions	B ions	Y ions	A ions	B ions	Y ions
3	E	-	-	-	-	-	-
4	I	-	-	-	-	-	-
5	V	-	-	-	-	-	-
6	H	-	-	-	-	-	-
7	I	-	-	-	-	-	1529.858056
8	Q	-	-	-	-	-	1416.773992
9	A	763.446100	791.441014	1174.599716	-	-	1288.715415
10	G	820.467563	848.462478	1103.562603	820.467563	848.462478	1217.678301
11	Q	948.526141	976.521056	1046.541139	948.526141	976.521056	1160.656837
12	**C**	1108.556789	1136.551704	918.482562	1108.556789	1136.551704	1032.598260
13	G	1165.578253	1193.573167	758.451913	1165.578253	1193.573167	872.567612
14	N	1279.621180	1307.616095	701.430450	1279.621180	1307.616095	815.546148
15	**Q**	1478.753257	1506.748171	587.387522	1592.868955	1620.863870	701.503221
16	I	1591.837321	1619.832235	388.255446	1705.953019	1733.947934	-
17	G	-	-	-	1762.974483	1790.969397	-
18	A	-	-	-	-	-	-
19	K	-	-	-	-	-	-

Table S4: Table of summarized peak areas for polyaminated EIVH-derived ions in Brain MT fractions. A, B and Y ions derived from modified EIVH peptide as one of the representative digests of polyaminated tubulin (by putrescine and spermine) were shown in relative abundance, as indicated by peak areas, in mouse brain fractions. The modification was found significantly higher in the P fraction composed largely of cold/Ca^{2+} stable tubulin, compared with that in the S fraction consisting mainly cold/Ca^{2+} labile tubulin, consistent with the distributions of the total ions shown in figure 4E.

Table S4

SUMMARY OF PEAK AREAS FOR POLYAMINATED EIVH-DERIVED IONS IN BRAIN MT FRACTIONS

PUT (m/z: 632)

	A ions			B Ions			Y ions	
Fraction	RT	Area		RT	Area		RT	Area
P7	NF	NF		NF	NF		NF	NF
P8	2.5	2087		2.51	2623		2.52	5649
P9	NF	NF		NF	NF		NF	NF
S7	2.46	264		2.46	47		2.46	457
S8	2.47	122		2.48	47		2.47	279
S9	NF	NF		NF	NF		NF	NF

SPM (m/z: 670.04)

	A ions			B Ions			Y ions	
Fraction	RT	Area		RT	Area		RT	Area
P7	2.55	873		2.5	314		2.52	4594
P8	2.53	253		2.53	574		2.53	1088
P9	NF	NF		NF	NF		NF	NF
S7	2.44	180		2.48	134		2.46	51
S8	2.45	210		2.4	67		2.42	256
S9	2.42	85		2.42	78		2.42	48

PUT: Putrescine
SPM: Spermine
RT: Retention Time
NF: Not Found

Supplemental Figures and Legends

Figure S1 Related to Figure 1. A diagram of synthetic pathways for polyamines with their structures and molar masses indicated. The rate-limiting enzyme in polyamine synthesis is ornithine decarboxylase (ODC). Difluoromethylornithine (DFMO) is an irreversible inhibitor of ODC and consequently inhibits polyamine synthesis.

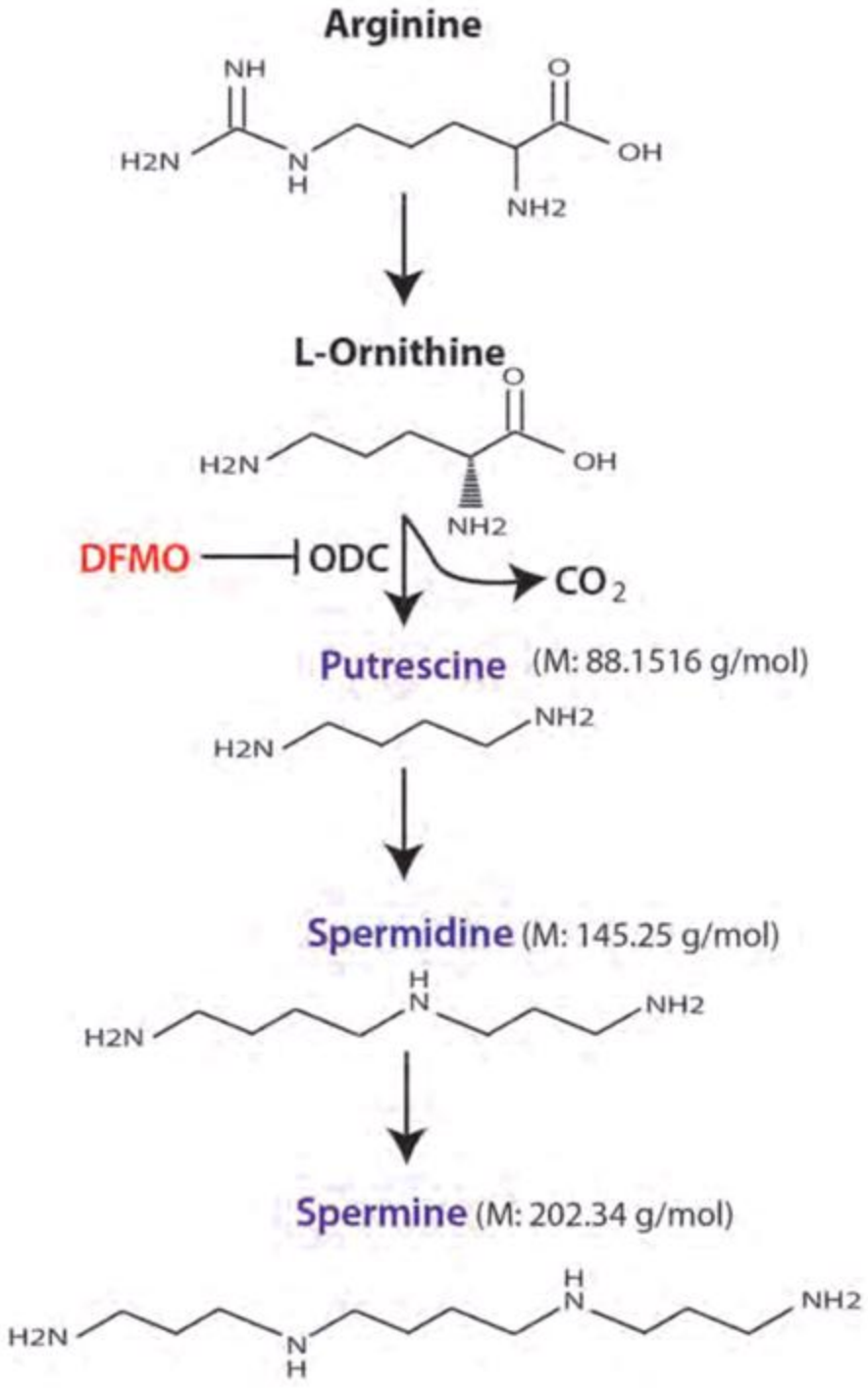
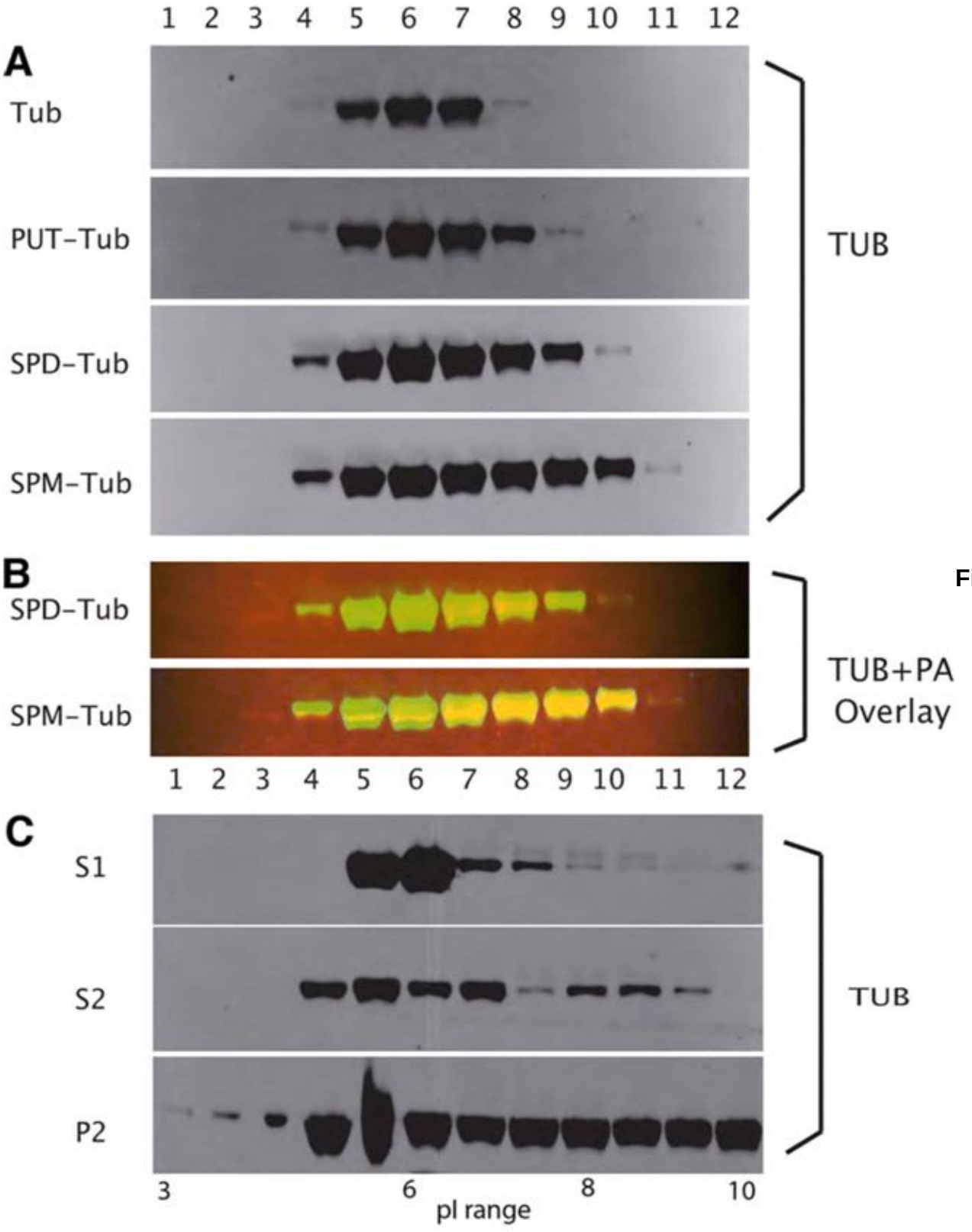


Figure S2 Related to Figure 3. Immunoblots from IEF gels of 12 off-gel fractionations on modified tubulins both *in vitro* and *in vivo*. (A) Blotted with pan-tubulin antibody, it was shown that purified mouse brain tubulins (Tub in the 1st lane) have PIs of 4.75~7 (in fractions 4-8), with a significant PI shift toward the basic end while polyaminated *in vitro* by putrescine (PUT-Tub in the 2nd lane), spermidine (SPD-Tub in the 3rd lane) and spermine (SPM-Tub in the 4th lane). The positive charges increase from putrescine to spermine (as would be predicted from the structures shown in figure S6), resulted in continuous basic PI shifts. (B) Double blotted with polyamine antibodies (red), which recognize spermine and spermidine conjugated proteins, and pan-tubulin antibody (green), polyaminated tubulins were shown mainly in fractions with basic PIs (P7 and P8 in SPD-Tub and P7-P10 in SPM). (C) Blotted with pan-tubulin antibody, the *in vivo* mouse brain microtubule samples after cold and Ca^{2+} fractionation showed notable differences in PI distributions among the cold/Ca^{2+} labile tubulins (S1), cold stable and Ca^{2+} labile tubulins (S2) an cold/ Ca^{2+} stable tubulins (P2).

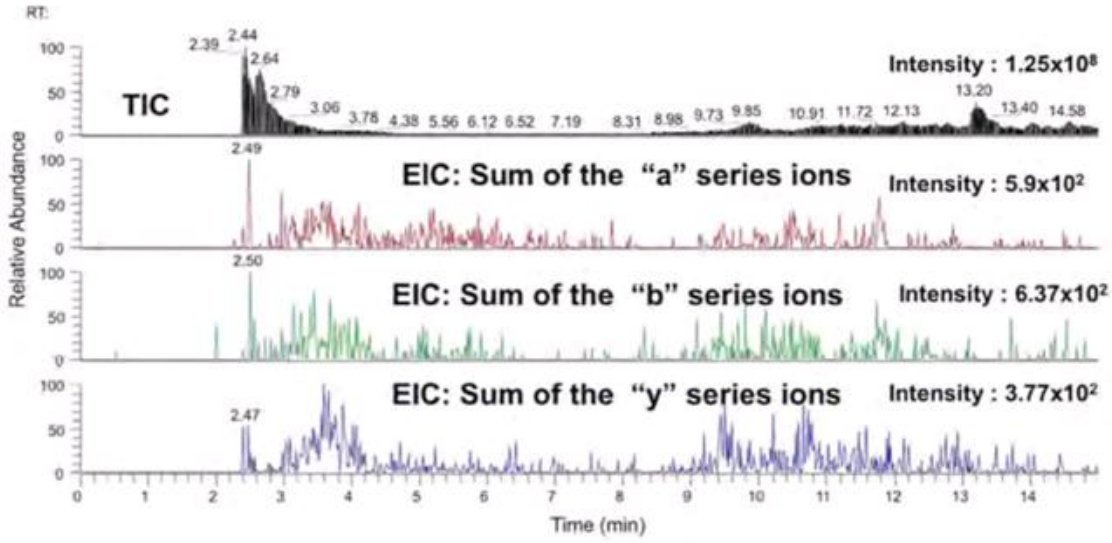


Figure

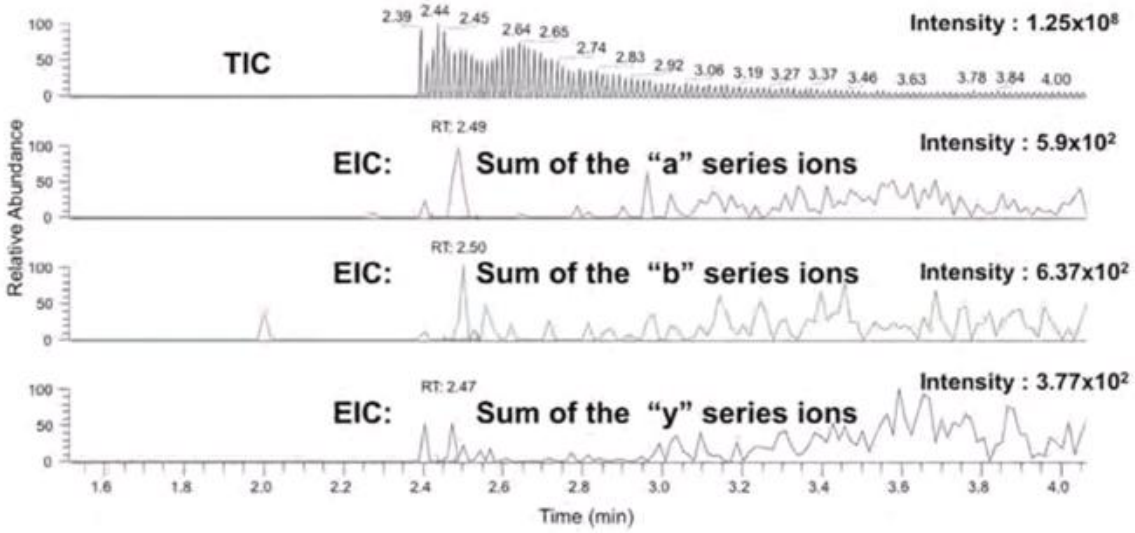
Figure S3 Related to Figure 4 and mass spectrometry text. **A-B)** Chromatograms of brain stable

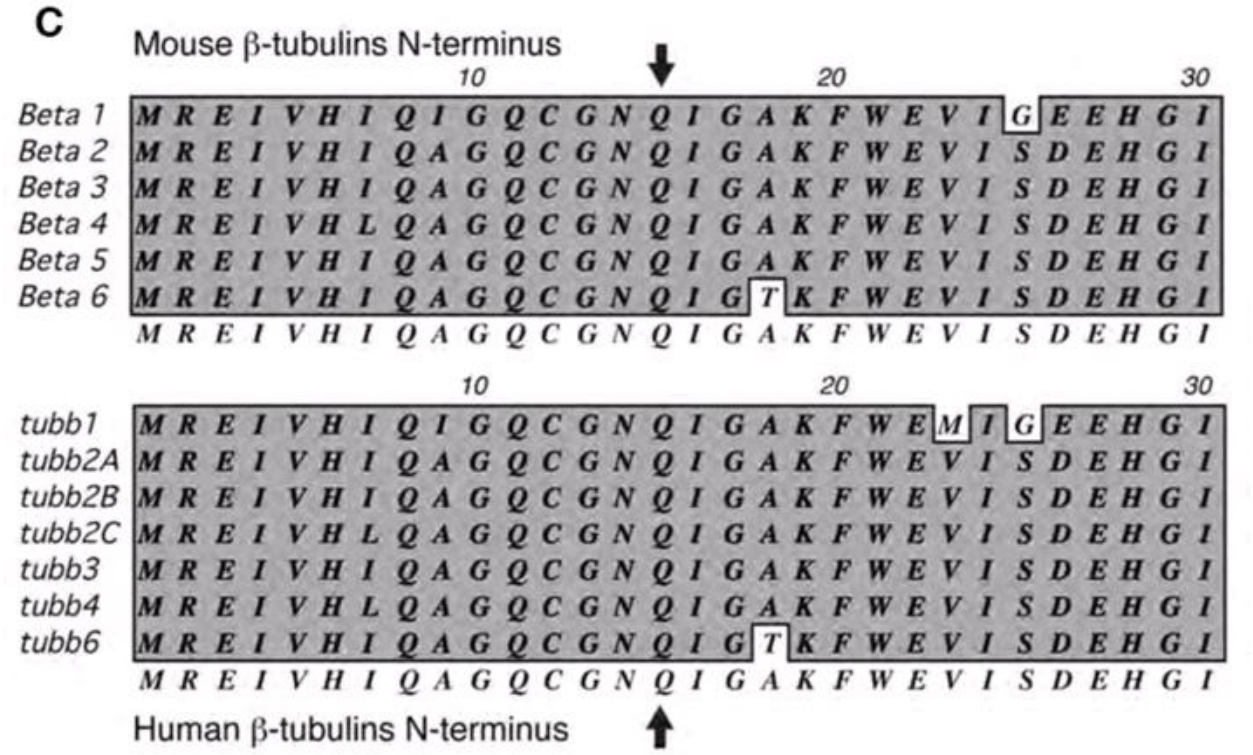
microtubule fraction confirmed the presence of modified EIVH peptide by spermine (SPM- P_{EIVH}) as well as putrescine, suggesting that the same modification occurs *in vivo* and that the modification is sequence specific. C**)** Alignments of N-termini of major beta tubulin isoforms in both the mouse and the human indicate a high degree of conservation. The N-terminus, including the putative Q15 polyamination site, is highly conserved in both species. This suggests that modification on this specific residue may play a fundamental role in microtubule structure and function. **D)** Enlarged image of polyaminated Q15 site on the β–tubulin structure highlights the potential for polyamine interaction with GTP. The presence of the charged polyamine at the GTP binding site would have the potential to alter hydrolysis of GTP by β–tubulin, which is closely linked to dynamic instability of microtubules.

A


RT:
TIC
Intensity : 1.25x10⁸
EIC: Sum of the "a" series ions
Intensity : 5.9x10²
EIC: Sum of the "b" series ions
Intensity : 6.37x10²
EIC: Sum of the "y" series ions
Intensity : 3.77x10²
Relative Abundance
Time (min)

B


TIC
Intensity : 1.25x10⁸
EIC: Sum of the "a" series ions
RT: 2.49
Intensity : 5.9x10²
EIC: Sum of the "b" series ions
RT: 2.50
Intensity : 6.37x10²
EIC: Sum of the "y" series ions
RT: 2.47
Intensity : 3.77x10²
Relative Abundance
Time (min)


C
Mouse β-tubulins N-terminus
10
20
30
Beta 1 M R E I V H I Q I G Q C G N Q I G A K F W E V I G E E H G I
Beta 2 M R E I V H I Q A G Q C G N Q I G A K F W E V I S D E H G I
Beta 3 M R E I V H I Q A G Q C G N Q I G A K F W E V I S D E H G I
Beta 4 M R E I V H L Q A G Q C G N Q I G A K F W E V I S D E H G I
Beta 5 M R E I V H I Q A G Q C G N Q I G A K F W E V I S D E H G I
Beta 6 M R E I V H I Q A G Q C G N Q I G T K F W E V I S D E H G I
M R E I V H I Q A G Q C G N Q I G A K F W E V I S D E H G I
10
20
30
tubb1 M R E I V H I Q I G Q C G N Q I G A K F W E M I G E E H G I
tubb2A M R E I V H I Q A G Q C G N Q I G A K F W E V I S D E H G I
tubb2B M R E I V H I Q A G Q C G N Q I G A K F W E V I S D E H G I
tubb2C M R E I V H L Q A G Q C G N Q I G A K F W E V I S D E H G I
tubb3 M R E I V H I Q A G Q C G N Q I G A K F W E V I S D E H G I
tubb4 M R E I V H L Q A G Q C G N Q I G A K F W E V I S D E H G I
tubb6 M R E I V H I Q A G Q C G N Q I G T K F W E V I S D E H G I
M R E I V H I Q A G Q C G N Q I G A K F W E V I S D E H G I
Human β-tubulins N-terminus

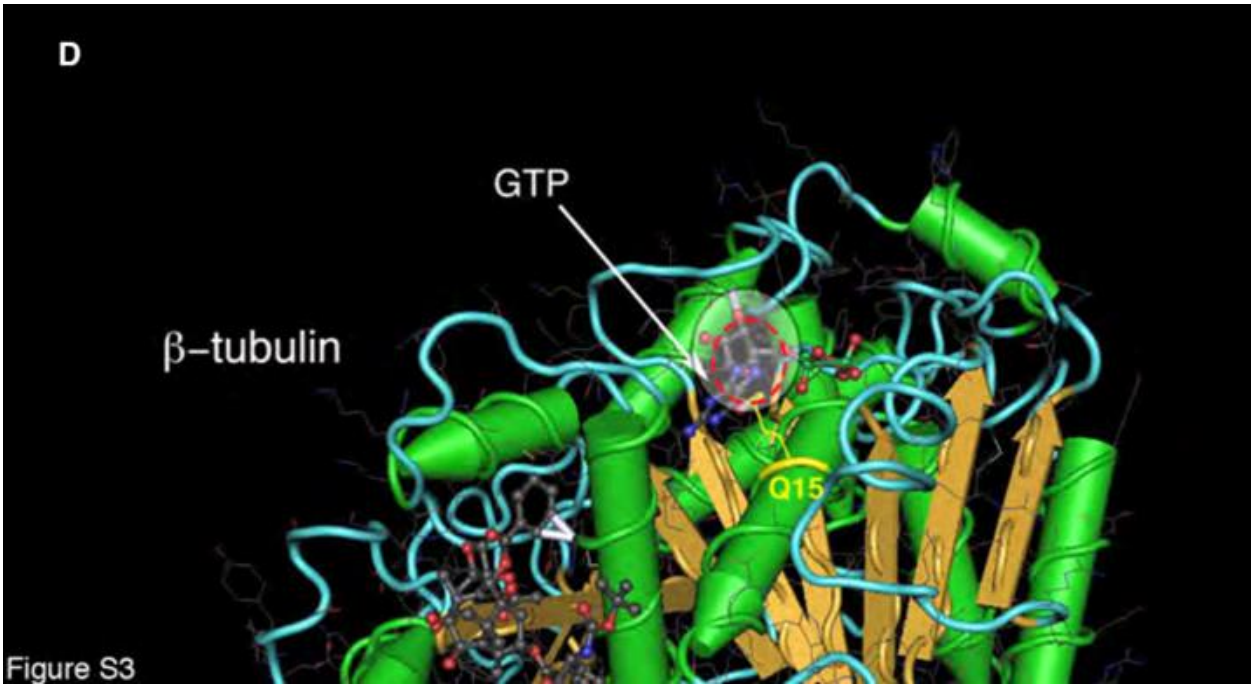

D
GTP
β–tubulin
Q15

Figure S3

S4 Related to figure 5 and methods: Flow chart illustrating the procedures used to analyze the biochemical characteristics of *in vitro* polyaminated brain tubulins by endogenous brain transglutaminase and polyamines. These procedures were used to produce the results shown in figure 5.

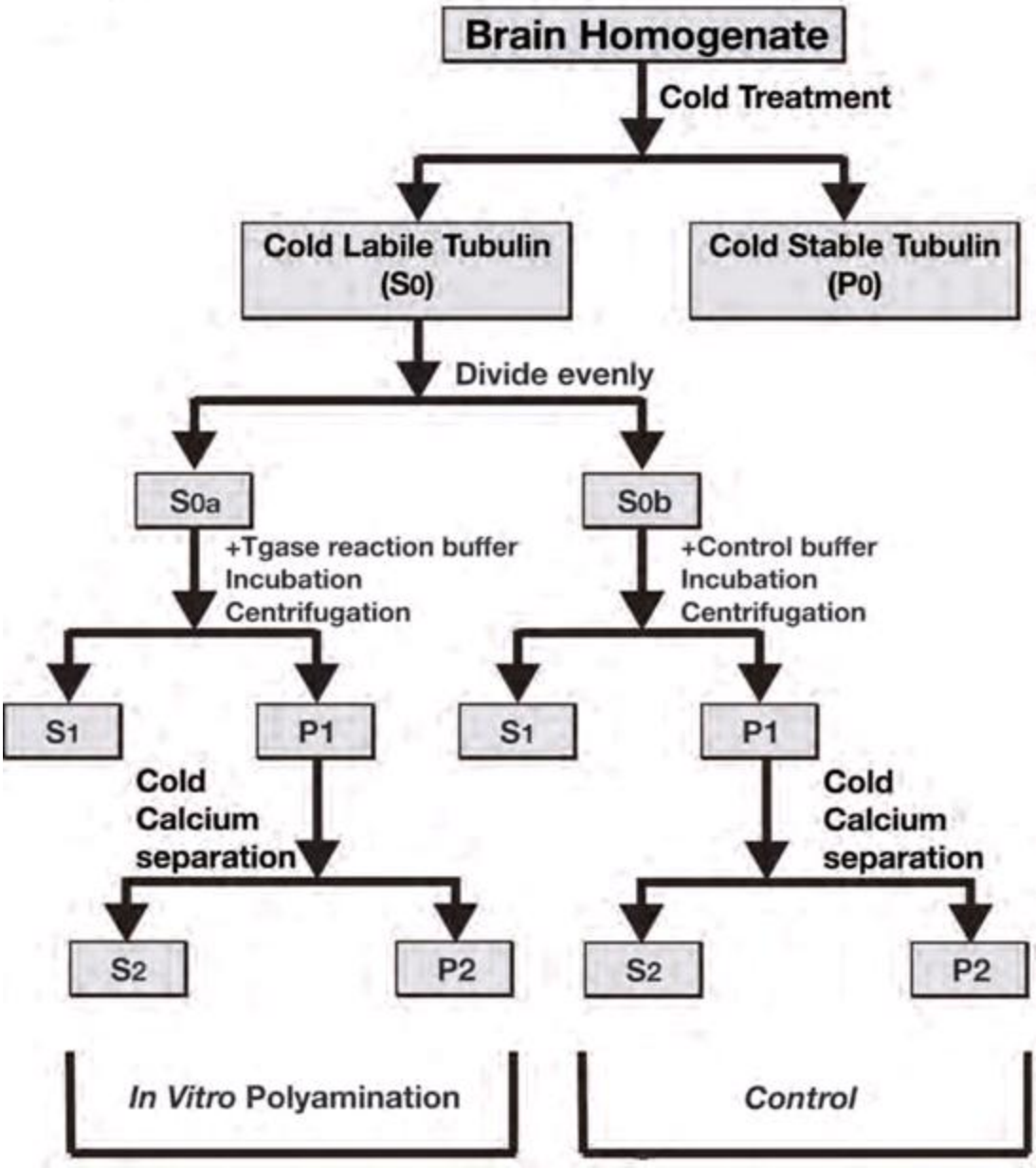


Figure S5 Related to Figure 7: Structure for IR072, an irreversible inhibitor of TG2 used in this study to inhibit TGase during neurite outgrowth and differentiation.

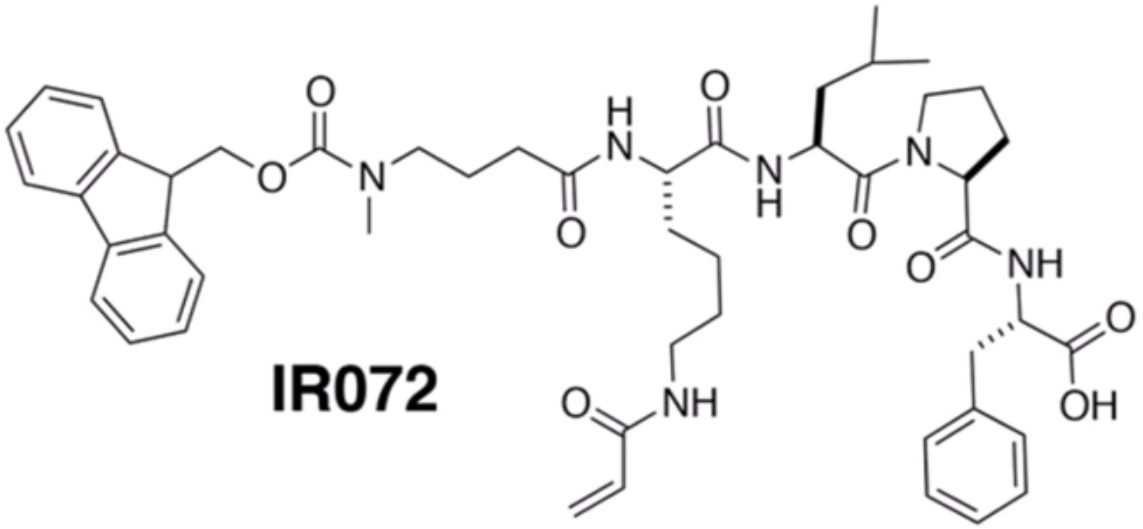
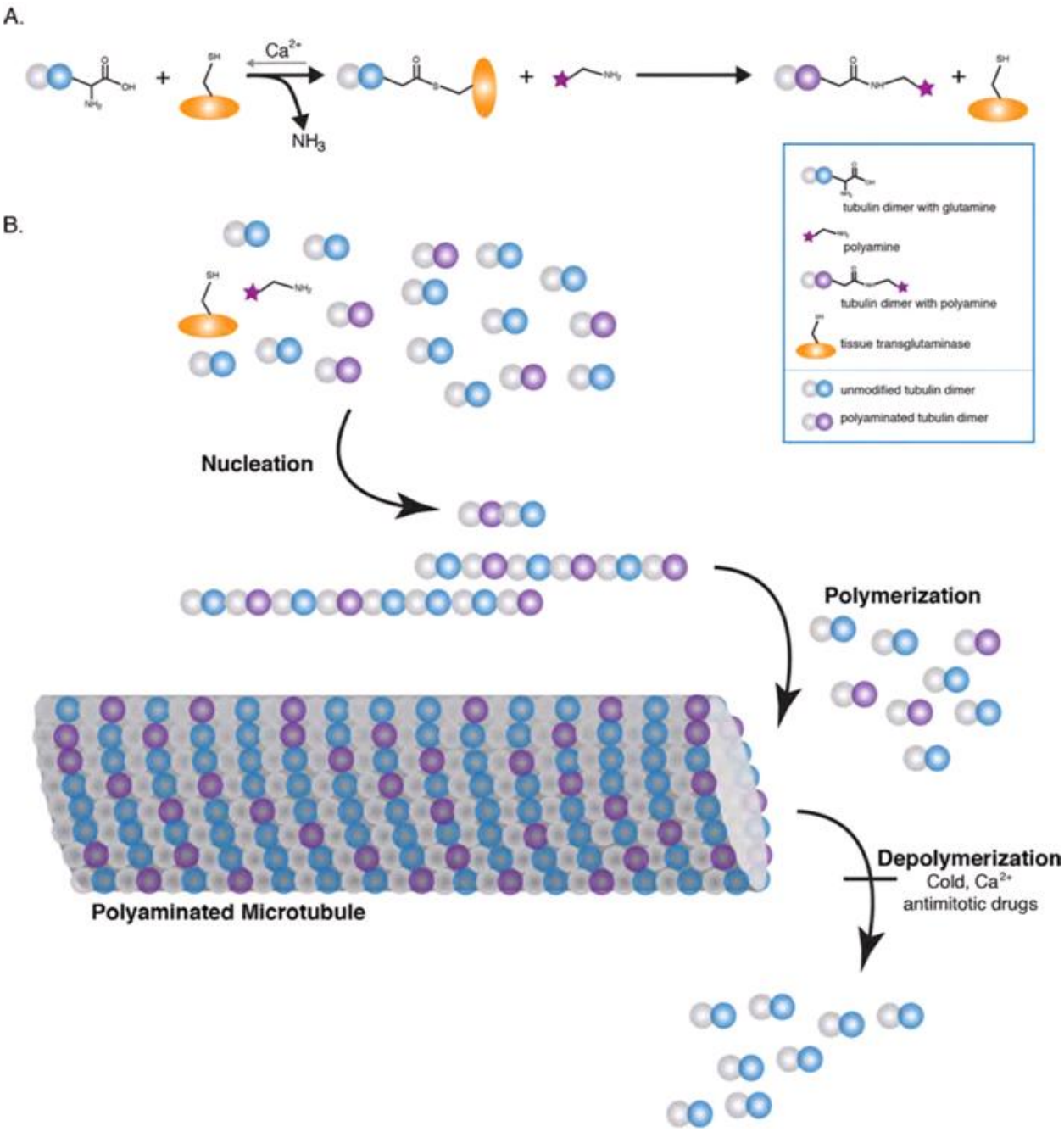


Figure S6: Related to Discussion. Proposed model for stabilization of microtubules by polyamines and transglutaminase (TG). (A) TG-catalyzed polyamination of neuronal tubulin shows that polyamines are covalently added to selective glutamine sites on tubulin in the presence of active TG and Ca^{2+}. (B) Potential functional roles of polyaminated tubulins in regulating neuronal microtubule dynamics include facilitating microtubule nucleation and polymerization, as well as stabilizing polymerized microtubules against depolymerizing factors, such as cold, Ca^{2+} and some anti-mitotic drugs.



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Supplemental Table S2

#	Immon.	a	a++	a+++	b	b++
3	102.06					
4	86.1	215.14			243.13	
5	72.08	314.21			342.2	
6	110.07	451.27			479.26	
7	86.1	564.35			592.35	
8	101.07	692.41			720.4	
9	44.05	763.45			791.44	
10	30.03	820.47	410.74		848.46	424.74
11	101.07	948.53	474.77	316.85	976.52	488.76
12	76.02	1108.56	554.78	370.19	1136.55	568.78
13	30.03	1165.58	583.29	389.2	1193.57	597.29
14	87.06	1279.62	640.31	427.21	1307.62	654.31
15	101.07	1478.75	739.88	493.59	1506.75	753.88
16	86.1	1591.84	796.42	531.28	1619.83	810.42
17	30.03	1648.86	824.93	550.29	1676.85	838.93
18	44.05	1719.9	860.45	573.97	1747.89	874.45
19	101.11					

EIVH peptide-derived ions for figure 4 panel B

b+++	b+++-H2O	b+++-NH3	b++-H2O	b++-NH3	b-H2O	b-NH3
					225.12	
					324.19	
					461.25	
					574.34	
					702.39	703.38
					773.43	774.41
			415.73	416.22	830.45	831.44
326.18	320.18	320.5	479.76	480.25	958.51	959.5
379.52	373.52	373.85	559.77	560.27	1118.54	1119.53
398.53	392.53	392.85	588.29	588.78	1175.56	1176.55
436.54	430.54	430.87	645.31	645.8	1289.61	1290.59
502.92	496.92	497.25	744.87	745.36	1488.74	1489.72
540.62	534.61	534.94	801.41	801.91	1601.82	1602.81
559.62	553.62	553.95	829.93	830.42	1658.84	1659.83
583.3	577.3	577.63	865.44	865.94	1729.88	1730.86

Seq.	y	y++	y+++	y+++-H2O	y+++-NH3	y++-H2O
E	1894	947.5	632	626	626.33	938.5
I	1764.95	882.98	588.99		583.31	
V	1651.87	826.44	551.29		545.62	
H	1552.8	776.9	518.27		512.6	
I	1415.74	708.37	472.59		466.91	
Q	1302.66	651.83	434.89		429.22	
A	1174.6	587.8	392.2		386.53	
G	1103.56	552.28	368.53		362.85	
Q	1046.54	523.77	349.52		343.84	
C	918.48	459.74	306.83		301.16	
G	758.45					
N	701.43					
Q	587.39					
I	388.25					
G	275.17					
A	218.15					
K	147.11					

y++-NH3	y-H2O	y-NH3	#
938.99	1875.99	1876.97	19
874.47		1747.93	18
817.92		1634.84	17
768.39		1535.77	16
699.86		1398.72	15
643.32		1285.63	14
579.29		1157.57	13
543.77		1086.54	12
515.26		1029.51	11
451.23		901.46	10
		741.42	9
		684.4	8
		570.36	7
		371.23	6
		258.14	5
		201.12	4
		130.09	3