

University of Colorado Boulder

**Investigations of Interactions between the Microtubule Binding Domain of
Centrosomal P4.1 Associated Protein and Polyglutamylated Microtubules**

Undergraduate Thesis

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Table of Contents

ABSTRACT	3
KEY WORDS	4
INTRODUCTION.....	5
Figure 1. Cartoon of post-translational modifications occurring on tubulin's C-terminal tails.....	5
Figure 2. Model of theoretical in vivo fluorescent sensors for specific post-translation modifications occurring on tubulin's C-terminal tail.	6
Figure 3. Map of CPAP domains.....	7
Figure 4. Diagram of steps completed for co-sedimentation assay.....	8
Figure 5. Cartoon of the tubulin's post-translational modifications produced in each <i>T. thermophila</i> strain.	11
Figure 6. Plasmid map of pGEX-2T_A5N.....	12
METHODS	13
2.1 – TOG GROWTH AND INDUCTION IN BL21 (DE3) E. COLI CELLS.....	13
2.2 – TOG PURIFICATION	13
2.3 – LOADING TOG ONTO HiTrap-NHS HP GE COLUMN.....	14
2.4 – TTLL3KO <i>T. THERMOPHILA</i> CULTURES	14
2.5 – TTLL3KO TUBULIN PURIFICATION	15
2.6 – FORMATION OF MTs	15
2.7 – AMPLIFICATION AND PURIFICATION OF pGEX-2T_A5N PLASMID FROM DH5 α E. COLI CELLS	16
2.8 – PREPARATION OF GLYCEROL CELL STOCKS OF TRANSFORMED ROSETTA (DE3) E. COLI CONTAINING pGEX-2T_A5N.....	16
2.9 – EXPRESSION OF GST-A5N PROTEIN IN ROSETTA (DE3) E. COLI CELLS.....	17
Figure 7. Representation of transformed Rosetta (DE3) E. coli growth sequence.....	17
2.10 – PROTEIN PURIFICATION OF GST TAGGED A5N (GST-A5N)	17
2.11 – CLEAVAGE OF GST TAG AND FINAL A5N PURIFICATION.....	18
2.12 – CO-SEDIMENTATION ASSAY	18
RESULTS.....	20
3.1 – TOG PURIFICATION, 12% SDS-PAGE GEL	20
Figure 8. TOG purification results, 12% SDS-PAGE.....	20
3.2 – TUBULIN PURIFICATION, 12% SDS-PAGE GEL	21
Figure 9. TTLL3KO tubulin purification results, 12% SDS-PAGE.....	21
3.3 – pGEX-2T_A5N AMPLIFICATION AND PURIFICATION, 1% AGAROSE GEL	22
Figure 10. Restriction digest of pGEX-2T_A5N, 1% agarose gel electrophoresis.	22
3.4 – CHROMATOGRAM OF GST-A5N PURIFICATION.....	23
Figure 11. Chromatogram of GST-A5N purification.....	23
3.6 – GST-A5N PURIFICATION, 12% SDS-PAGE GEL	24
Figure 12. GST-A5N purification results, 12% SDS-PAGE.....	24
3.7 – A5N PURIFICATION, 12% SDS-PAGE GEL	25
Figure 13. Thrombin cleavage reaction results: purified A5N, 12% SDS-PAGE.....	25
3.8 – CO-SEDIMENTATION ASSAY, 12% SDS-PAGE GEL.....	26
Figure 14. Co-Sedimentation assay of A5N with variously post-translationally modified MTs (1), 12% SDS-PAGE.....	27
Figure 15. Co-Sedimentation assay of A5N with variously post-translationally modified MTs (2), 12% SDS-PAGE.....	28
DISCUSSION.....	29
CONCLUSIONS.....	29
LIMITATIONS	30
FUTURE INVESTIGATIONS	30
REFERENCES	33

Abstract

Microtubules maintain particularly diverse and important functions within the cell despite being polymers made up of two highly conserved proteins: α - and β -tubulin. Some of these diverse roles are explained by the presence of post-translational modifications occurring on the C-terminal tail of tubulin proteins, primarily detyrosination, polyglutamylation and polyglycylation. Misregulation of C-terminal tail post-translational modifications are observed in disease phenotypes and may increase the risk of some forms of cancers; however, the role of these post-translational modifications is not well understood. Working towards a goal of designing *in vivo* methods to study specific post-translational modifications, this paper examines the specificity of the interaction between the microtubule binding domain of centrosomal P4.1 associated protein and polyglutamylated microtubules using co-sedimentation assays.

Key Words

- 1) Microtubule (MT)
- 2) α/β – tubulin's C-terminal tails
- 3) Post-translational modifications
- 4) Polyglutamylation
- 5) Polyglycylation
- 6) Centrosomal P4.1 associated protein (CPAP)

Introduction

Microtubules are macromolecular structures in the cell comprised of α/β -tubulin dimers and are responsible for important cellular functions, such as division, maintaining cellular shape, motility, assisting in localization of proteins, and other cellular products. The functional diversity of microtubules may partly be attributed to the intrinsically disordered C-terminal tails of α - and β -tubulin, the site of substantial post-translational modifications including detyrosination, polyglutamylation, and polyglycylation (figure 1) (Janke and Chloë Bulinski 2011). Microtubule

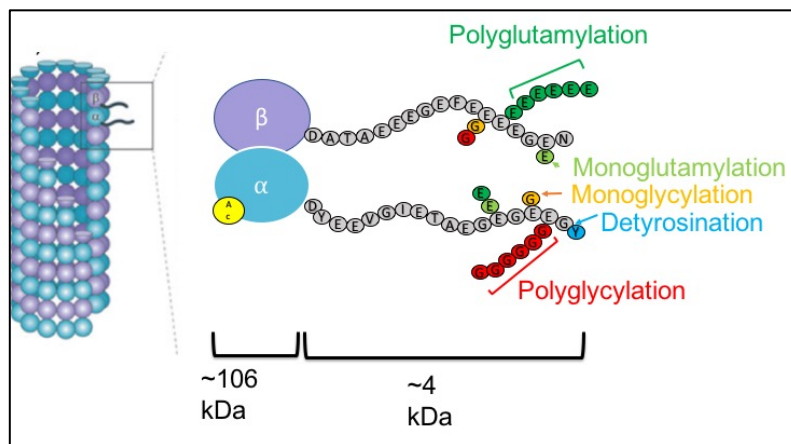


Figure 1. Cartoon of post-translational modifications occurring on tubulin's C-terminal tails. Figure adapted from Janke & Bulinski, Nature Reviews Molecular Cell Biology volume 13, page 276 (2012)

(MT) dynamics, flexibility, polymerization, and MT associated protein affinities are affected by the amount and category of post-translational modification on the C-terminal tails of α - and β -tubulins (Song and Brady 2015; Wall et al.

2016). Tubulin tyrosine ligase like (TTLL) enzymes are responsible for monoglycylation, polyglycylation, monoglutamylation, and polyglutamylation of glutamate residues within the C-terminal tails of α - or β -tubulin (Song and Brady 2015). The misregulation of post-translational modifications (polyglutamylation or polyglycylation) on the C-terminal tails is often caused by a faulty TTLL and can lead to malfunctioning MTs that may ultimately cause disease phenotypes like Retinitis Pigmentosa, neurodegenerative diseases and increase risk of some cancers (Baird and Bennett 2013; Das et al. 2014; Sun et al. 2016; Dias et al. 2017). There is limited knowledge about the function of post-translational modifications on the C-terminal tails, its association with

various MT associated proteins and disease phenotypes; hence, there is much to be gained by studying these post-translation modifications and their effect on the cell.

Studying C-terminal tails' post-translational modifications *in vivo* would be ideal to understanding the function of these post-translational modifications but, currently there is no direct way to incorporate a specific label for C-terminal tails' post-translational modifications *in vivo*. Antibodies have been developed but they are not very specific, and they can only be used in dead, preserved cells. *In situ* studies using antibodies only provide limited data because there is no way to efficiently and accurately perform time point experiments. *In vivo* time point experiments would offer the most direct data to understanding the function of the C-terminal tails' post-translational modifications and the consequences of misregulated modifications.

One of the Hough lab's long-term goals is to design a fluorescently labeled protein sensor that will bind specifically to one type of post-translationally modified MT *in vivo* without affecting its function; in doing this, we will be better able to study and monitor specific post-translational modifications *in vivo* and gain insights into post-translational modifications

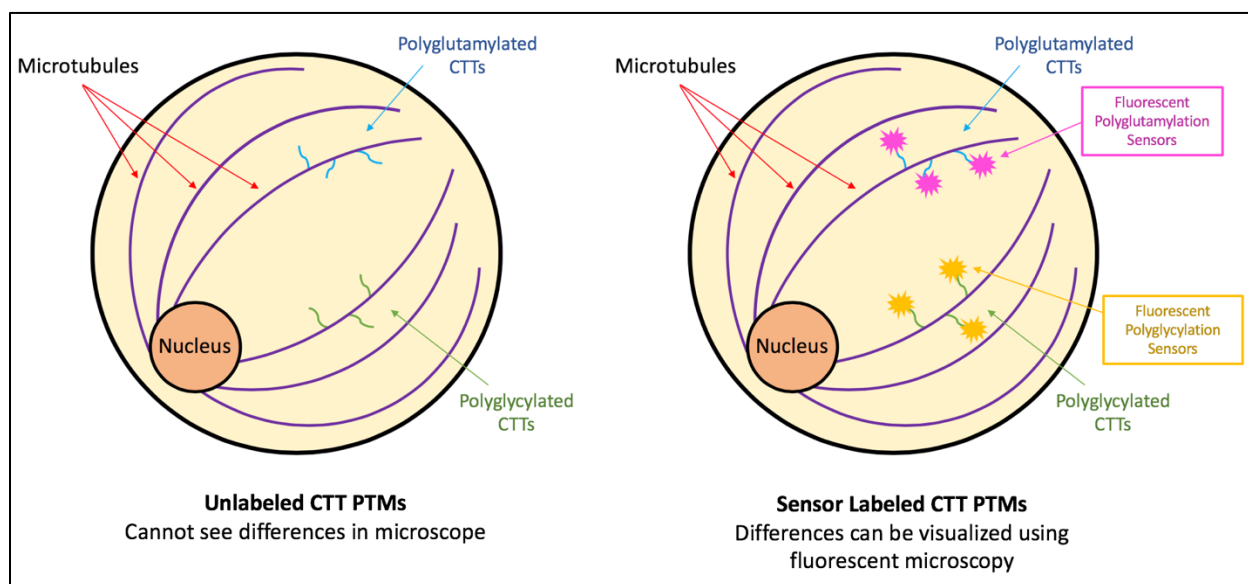


Figure 2. Model of theoretical *in vivo* fluorescent sensors for specific post-translation modifications occurring on tubulin's C-terminal tail. (Left) Where scientists currently are in research methods (cannot see *in vivo* localization of modifications), (Right) Fluorescent visualization of the localization of specific post-translational modifications on tubulin's C-terminal tail by the sensor protein.

dynamics as well as associated effects on MT dynamics, affinity for MT associated proteins, flexibility, and polymerization (figure 2).

Working towards this goal, various MT associated proteins were analyzed to access its potential for specificity to one post-translational modification. We focused on MT binding proteins whose MT binding region was a distinct domain, which we hope will mean that the specific MT binding domain can be used ideally separated from any enzymatic or regulatory functions. This paper will examine the MT binding domain of *H. sapien* centrosomal P4.1 associated protein (CPAP), abbreviated as A5N, in the prospects of gaining insights into the interaction between CPAP and various types of MT post-translational modifications as well as discovering if A5N could potentially be useful in the goal of designing *in vivo* sensors for post-translationally modified MTs.

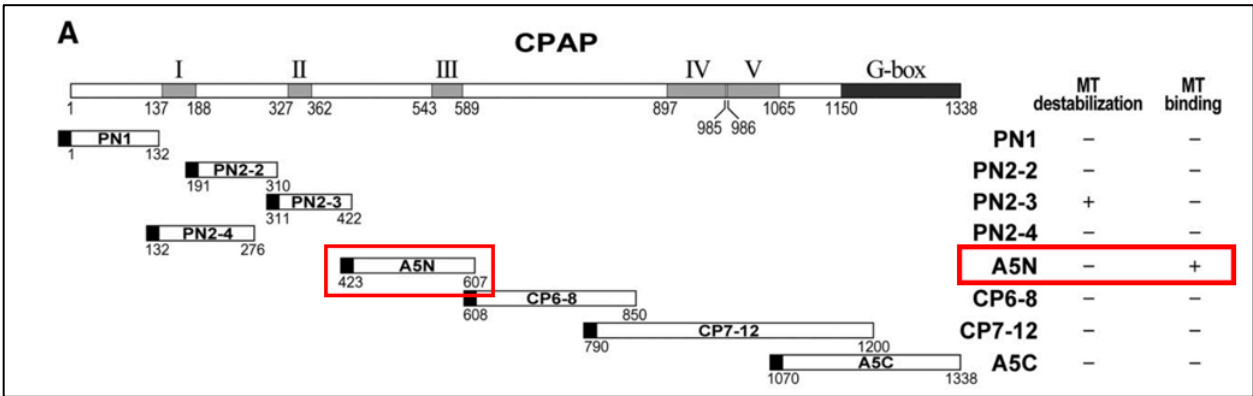


Figure 3. Map of CPAP domains. MT binding region (A5N) boxed in red. Adapted from Hsu et al. Experimental Cell Research. 2008.

CPAP, also known as CENPJ, is associated with the γ -tubulin ring complex and contains a MT-destabilizing domain and a MT binding domain of 184 amino acids long called A5N (figure 3) (Hsu et al. 2008). CPAP is one of six genes involved in autosomal recessive microcephaly (MCPH) (Hsu et al. 2008). MCPH is a neurodevelopmental disorder that leads to a reduction in brain size (Hsu et al. 2008). CPAP is also required for centriole biogenesis in human

cells (Gudi et al. 2014). Parental centrioles have been shown to be polyglutamylated (Kleylein-Sohn et al. 2007). Thus, this may indicate that CPAP could have higher affinity for polyglutamylated MTs compared to other types of MTs. In addition, CPAP has also been shown to interact with other polyglycylylated proteins like 14.3.3 (Chen et al. 2006). In light of this limited and contradicting evidence, the A5N domain was tested with polyglycylylated MTs, polyglutamylated MTs, and reduced polyglycylylated MTs using a co-sedimentation assay. To perform the co-sedimentation assay, purified A5N and purified MTs of various post-translational modifications had to be obtained; an overview of procedures is depicted in figure 4.

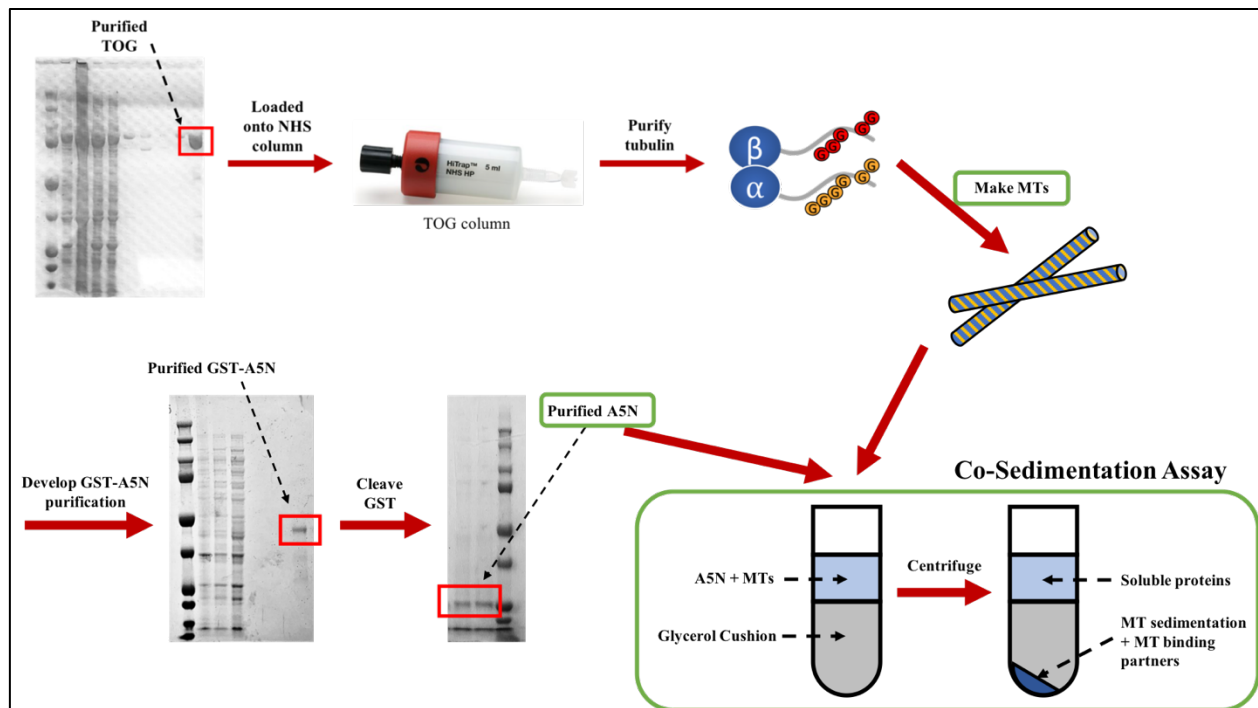


Figure 4. Diagram of steps completed for co-sedimentation assay.

The theory behind the co-sedimentation assay is based on the fact that MTs are large polymers of tubulin dimers and during centrifugation, MTs will sediment into a pellet. Given that A5N is a soluble protein that will not sediment on its own, if mixtures of A5N and MTs are incubated and centrifuged and A5N is found in the pellet, then it can be inferred that it did, in

fact, bind to MTs before they sedimented out. We found that A5N co-sedimented with polyglutamylated MTs but not with polyglycylated MTs.

Reagents for the co-sedimentation assay were acquired as follows: A5N was purified using GST affinity fast-flow liquid chromatography (FPLC). Rosetta (DE3) *E. coli* cells were transformed with a pGEX-2T plasmid of 5,507 base-pairs (bps) containing the A5N domain of CPAP with an N-terminus GST tag separated by a thrombin cleavage site, regulated by the bacterial LAC operon and containing an ampicillin resistance gene for positive selection, notated as pGEX-2T_A5N (figure 6). This plasmid was generously donated by Professor Tang from Academia Sinica, Institute of Biomedical Sciences in Taipei, Taiwan. The GST-A5N protein was induced in a 1 L culture containing 1 mM IPTG, an analog of lactose, which activates the Lac promoter without getting degraded to create an abundance of the GST-A5N mRNA and subsequently the GST-A5N protein. After expressing GST-A5N in Rosetta (DE3) *E. coli* and purifying using two tandem 5 mL HiTrap-GST HP GE columns on FPLC, the resulting GST-A5N protein was cleaved using thrombin protease and purified a second time to get A5N in isolation. Thrombin is a site-specific protease that cleaves the amino acid sequence L-V-P-R-G-S between R and G, leaving G-S- attached to our A5N protein, now 186 amino acids long and 21 kilo-Daltons (kDa).

Next, *T. thermophila* cultures were used to create tubulin proteins, *T. thermophila* is a ciliate and the cells' total protein content contains a substantial portion of tubulin, making this organism a good choice for tubulin purification. Tubulin proteins were purified using a TOG affinity column (Widlund et al. 2012). TOG is a protein isolated from the MT binding domain of *S. cerevisiae* Stu2 protein and selectively binds many isoforms and isotypes of tubulin (Widlund et al. 2012). TOG is grown in BL21 (DE3) *E. coli* cells, induced with 1 mM IPTG, positively

selected for by ampicillin and linked to a GST tag for purification. Once TOG is purified, it is covalently attached to a 5 mL N-hydroxysuccinimide ester-activated Sepharose column (HiTrap-NHS HP GE), which is then used to purify tubulin proteins. During purification a wash including ATP helps to wash away proteins that remained bound to tubulin, and tubulin is later eluted with a high salt concentration.

A TTLL3 knock-out (TTLL3KO) strain of *T. thermophila* was used to purify for polyglutamylated tubulin proteins. TTLLs attach the initial glutamate or glycine to glutamate residues to a glutamate residue in the C-terminal tail by a γ -carboxyl (Ikegami and Setou 2010). Elongation of glycine or glutamate chains occurs on the initial addition by traditional peptide bonds (Ikegami and Setou 2010). Typically, initiation is catalyzed by separate TTLLs from elongation TTLLs, and separate types of TTLLs are responsible for either glycylation or glutamylation. Because glycylation and glutamylation occur on the same sites of the C-terminal tails, if one of these post-translational modifications is present in a tubulin protein, the other will not be. Likewise, if one of the post-translational modifications is reduced, the other will increase (Bulinski 2009). TTLL3 is the only enzyme in *T. thermophila* responsible for glycylation initiation; in the TTLL3KO strain of *T. thermophila* (Wloga et al. 2009), glycylation will not occur because glycylation elongation TTLLs cannot elongate without the initial glycylation, and when no glycylation is present, there is no site competition for polyglutamylation and polyglutamylation will occur in excess (figure 5). It should be noted that the tubulin from wild-type (WT) *T. thermophila* is naturally polyglycylated, so producing polyglycylated tubulin will come from culturing a WT strain of *T. thermophila* (figure 5) (Wall et al. 2016). The last strain of *T. thermophila* used is called ATU1-6D, and this strain produces tubulin where all six of the glutamate residues in the α -tubulin C-terminal tail are replaced with aspartates. As glutamates in

the C-terminal tail are the sites of glutamylation and glycylation, once glutamates are mutated to aspartates, the TTLs cannot function on these tubulin tails, and there is no polyglutamylation or polyglycylation present on the α -tubulin proteins (figure 5).

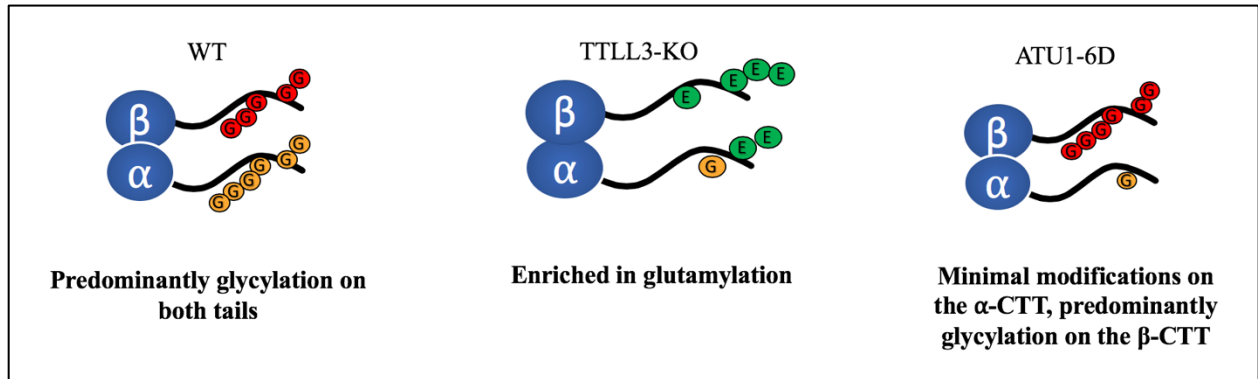


Figure 5. Cartoon of the tubulin's post-translational modifications produced in each *T. thermophila* strain.

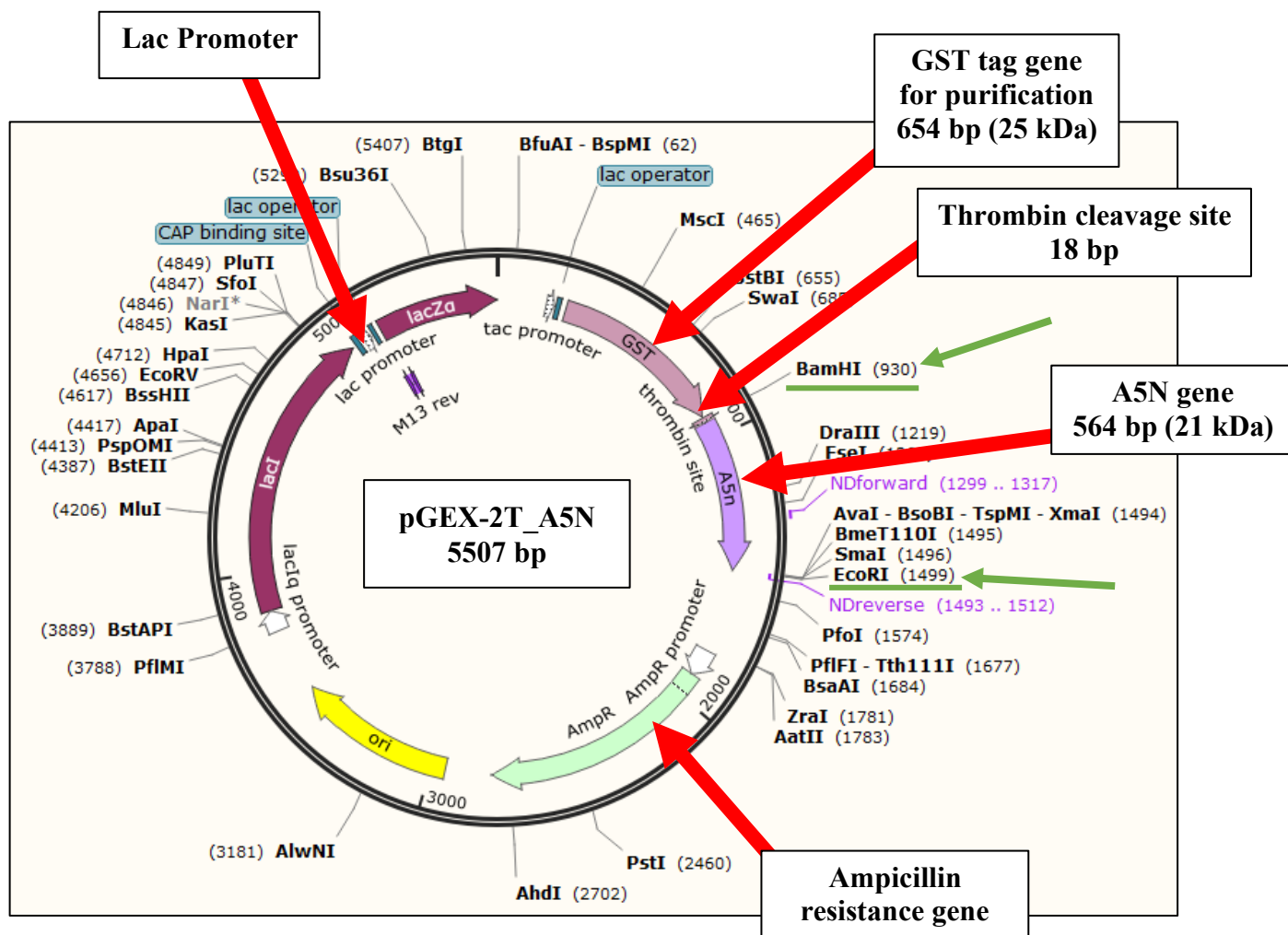


Figure 6. Plasmid map of pGEX-2T_A5N. Showing ampicillin resistance gene, lac promoter, GST tag gene, thrombin cleavage site, A5N gene, and restriction sites BamHI and EcoRI. Diagram was created using SnapGene.

Methods

2.1 – TOG Growth and Induction in BL21 (DE3) *E. coli* Cells

Courtesy of Allison Holt, BL21 (DE3) cells transformed with an expression vector containing a GST-TOG domain under control of the bacterial lac promoter with a positive selection ampicillin resistance gene were cultured and induced using the following methods: a 50 mL preculture containing a small amount transformed BL21 (DE3) cells, lysogeny broth (LB) media, 100 µg/mL ampicillin, pH 7.5 was incubated at 37°C, 125 rpm overnight. The following morning, the 50 mL preculture was added to 1 L of media containing the same mixture and incubated at 37°C, 125 rpm until OD₆₀₀ reached 0.5. At this point the culture was shifted to incubation at 18°C, 125 rpm for 1 hour after which 1 mM IPTG was added and the culture was allowed to incubate in these conditions over-night. The following morning the culture was centrifuged at 3,000 × *g* and stored at -80°C.

2.2 – TOG Purification

BL21 (DE3) *E. coli* cell pellets overexpressing TOG protein were provided by Allison Holt. The cell pellet was resuspended in 30 mL containing 2× phosphate buffered saline (PBS) pH 7.4, 50 µL of 1× protease inhibitor cocktail (PIC) and 2 µL of 1× benzonase and sonicated 30 seconds on, 59 seconds off for a total ‘on’ time of 2.5 minutes on ice. The resulting lysed cells were centrifuged at 80,000 × *g* for 40 minutes at 4°C and supernatant was loaded onto a HiTrap-GST HP GE column (equilibrated with 2× PBS pH 7.5 containing 1 mM dithiothreitol (DTT)) at 0.5 mL/min. After loading the column was washed with 200 mL 2× PBS, 1mM DTT and 0.1% Tween20 (wash 1) then 40 mL of 2× PBS, 5 mM ATP and 10 mM MgCl₂ (wash 2) then 100mL of 6× PBS (wash 3) then finally, 100 mL of 2× PBS and 1 mM DTT (wash 4) all at 3 mL/min.

TOG was eluted with 2× PBS, 1 mM DTT and 5 mM reduced glutathione then dialyzed in 3 L of 100 mM NaHCO₃, 100 mM NaCl, pH 8.2. Results were analyzed on 12% SDS-PAGE (figure 8).

2.3 – Loading TOG onto HiTrap-NHS HP GE Column

After TOG elution has been concentrated to around 12 mL, 2.4 mL of 0.5 M MgCl₂ (final of 80mM) and 50 µL of 1× PIC was added. Meanwhile a 5 mL HiTrap-GST HP GE column was washed with 25 mL of ice cold 1 mM HCl. TOG was loaded onto the column at 1.5 mL/min and recirculated for 30 minutes. Once there is little to no protein in the flow-through, the column was washed with 30 mL of 0.5 M ethanolamine, 0.5 M NaCl, pH 8.3 and incubated for 30 minutes. After incubation, the column was washed with 50 mL of 6× PBS then 25 mL of 1× PBS, 50% glycerol and stored at -20°C.

2.4 – TTLL3KO *T. thermophila* Cultures

A 50 mL pre-culture containing 1× SPP media, 1× Pen/Strep and 50 µL of TTLL3KO *T. thermophila* culture stock incubated in a 200 mL flask at 30°C for 24 hours at 100 rpm. Prepare the 1 L culture with the same reagents and a final initial cell concentration of 1x10³ cells/mL from the pre-culture and incubate at 30°C at 100 rpm for 36-48 hours. Cultures were centrifuged at 3,000 × g for 25 minutes at 4°C. Decanted pellets were resuspended in 15 mL 10 mM Tris-HCl, pH 7.4 and stored at -80°C. The same procedure was used for culturing WT *T. thermophila* and ATU1-6D *T. thermophila*.

2.5 – TTLL3KO Tubulin Purification

250 mL of tubulin purification (TP) buffer was made containing 1× BRB80 (80 mM PIPES, 1 mM MgCl₂ and 1 mM EGTA) pH 6.7, 0.021 g PMSF and 0.03 g benzamidine. TTLL3KO *T. thermophila* pellets were resuspended in 8 mL of TP buffer, 7 µL of 25 KU benzonase and 1 mM DTT. The solubilized pellet was sonicated for 30 seconds on and 2 minutes off for 25 minutes on ice and spun at 100,000 × g for 30 minutes. The supernatant was immediately loaded onto an equilibrated TOG column at 0.5 mL/min (equilibrated with 50 mL TP buffer and 0.1 mM guanosine triphosphate (GTP)). The column was washed at 1.5 mL/min with 40 mL of TP buffer with 0.1 mM GTP (wash 1) then with 30 mL of TP buffer with 0.1 mM GTP, 4.4 mM ATP and 10 mM MgCl₂ (wash 2) then with 50 mL of TP buffer with 0.1 mM GTP (wash 3) and finally with 20 mL of TP buffer with 10% glycerol and 0.1% Tween20 (wash 4). Tubulin was eluted with 40 mL of 0.5 M ammonium sulfate in MES buffer pH 6.7 and immediately desalted by loading 2.5 mL of elution onto an equilibrated PD-10 desalting column GE, flow-through was discarded. 3.5 mL of MES buffer pH 6.7 was added and flow-through collected and stored at 20°C. Results were tested with 12% SDS-PAGE (figure 9). The same procedure was used to prepare WT tubulin and ATU1-6D tubulin.

2.6 – Formation of MTs

A mixture containing 40 µL of 20 µM tubulin in 1×BRB80 buffer pH 6.7, 3 µL neat DMSO, 1 µL of 100 mM GTP, 1 µL of 500 mM MgCl₂, 9 µL of 3 M glutamate and 1.2 µL of 1 mM fluorescent taxol was incubated at 37°C for one hour, centrifuged at 350,000 × g and stored in the dark at 25°C. Verification was done using a confocal microscope (results not shown).

2.7 – Amplification and Purification of pGEX-2T_A5N Plasmid from DH5α *E. coli* Cells

5 µL (10 pg-100 ng) of pGEX-2T_A5N DNA was added to 50 µL of competent DH5α *E. coli* cells and incubated on ice for 30 minutes followed by 60 seconds in a 42°C water bath and moved back onto ice for another 2 minutes. 1 mL of LB pH 7.5 was added to the mixture and incubated on a rocker at 37°C for 1 hour. Finally, 100 µL of transformed culture was plated on a 10 cm plate containing LB agar, 100 µg/mL ampicillin, incubated over-night at 37°C and stored at 20°C. Five isolated colonies were chosen and each was grown separately in 10 mL culture tubes containing 5 mL LB and 100 µg/mL ampicillin, incubated over-night at 37°C and 125 rpm. The following morning the cultures were each lysed and purified for the pGEX-2T_A5N plasmid using a mini-prep Omega Bio-tek kit. A 50 µL restriction digest of 1 µg of purified plasmid was performed for verification using 18.75 µL of 55.8 ng/µL DNA, 5 µL of 10× cutsmart buffer, 1 µL of 20,000 units/mL BamH1 HF, 1 µL of 20,000 units/mL EcoR1 HF and 23.25 µL of nuclease free water. The restriction digest was incubated at 37°C, samples were taken after 2 hours and after 18 hours and tested with 1% agarose gel electrophoresis (figure 10). pGEX-2T_A5N was also verified by sequencing (results not shown).

2.8 – Preparation of Glycerol Cell Stocks of Transformed Rosetta (DE3) *E. coli* Containing pGEX-2T_A5N

Rosetta (DE3) cells were transformed with the pGEX-2T_A5N plasmid using the same transformation procedure as was used for DH5α cells. One 5 mL culture was grown, also using the same procedure as was used for the 5 mL DH5α cell cultures. Ten 1 mL glycerol stocks were made by combining 500 µL of cell stock with 500 µL 100% glycerol and storing at -80°C.

2.9 – Expression of GST-A5N Protein in Rosetta (DE3) *E. coli* Cells

Two pre-cultures containing 50 mL LB pH 7.5, a small amount of glycerol cell stock and 100 µg/mL ampicillin were grown over-night at 37°C, 125 rpm in 200 mL flasks. The following day each pre-culture was transferred into a 4 L flask containing 1 L LB and 100 µg/mL ampicillin. Cultures were grown until the OD₆₀₀ reached 1.2, at this point the cultures were spun at 3,000 × g, and the resulting cell pellets were combined into the same 4 L flask containing 1 L fresh LB and 100 µg/mL ampicillin and incubated at 37°C, 125 rpm. After 1 hour, 1 mM IPTG was added and incubated until the OD₆₀₀ reached 3.5-4. To stop culture growth the culture was spun at 3,000 × g and pellets were stored at -80°C.

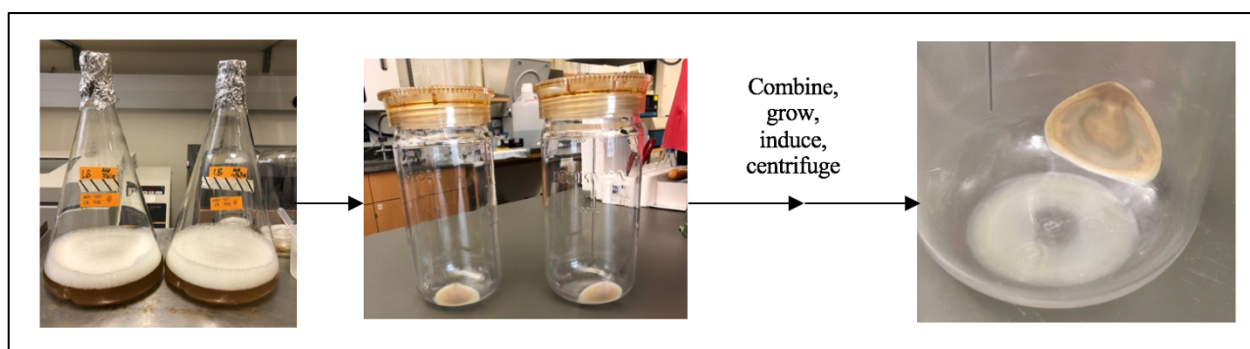


Figure 7. Representation of transformed Rosetta (DE3) *E. coli* growth sequence.

2.10 – Protein Purification of GST Tagged A5N (GST-A5N)

After the cell pellet was thawed on ice, it was resuspended in 30-40 mL lysis buffer containing 2× phosphate buffered saline (PBS) pH 7.4, 1 µg/mL pepstatin, 3 µL of 25 KU benzonase, 0.02 g lysozyme, 1 µg/mL leupeptin, 5 µL 1× protease inhibitor cocktail (PIC), 0.15 mM PMSF, 1 mM DTT, 5 mM EDTA, 0.8% Tween20 and stirred at RT for 40 minutes. Next the solubilized pellet was sonicated for 1 second on, 3 seconds off for 30 minutes total on ice. The sonicated mixture was centrifuged at 80,000 × g for 40 minutes at 4°C and the supernatant was

loaded directly onto two tandem 5 mL GStrap-HP GE columns (pre-equilibrated with 2× PBS and 1 mM DTT) at 1 mL/min. Once all of the supernatant was loaded, equilibration buffer containing 2× PBS and 1 mM DTT was run through the column at 3 mL/min until UV absorbance levels stabilized (wash 1). The column was then washed at 3 mL/min with 100 mL of 2× PBS, 1 mM DTT and 0.1% Tween20 (wash 2) followed by another 100 mL of 6× PBS (wash 3). A5N-GST was eluted with 2× PBS, 1 mM DTT and 10 mM reduced glutathione and stored at 20°C. Results were tested on 12% SDS-PAGE (figure 12).

2.11 – Cleavage of GST Tag and Final A5N Purification

Eluted GST-A5N protein was dialyzed in 1× PBS pH 7.4 then mixed with 300 µL of 100 NIH units/100 µL thrombin and nutated for 18 hours at 4°C. Cleavage reaction was terminated by adding 20 µL of 100 mM PMSF and mixing by pipetting up and down for 10-15 minutes. The terminated reaction mixture was added to an equilibrated 7 mL of Glutathione Sepharose 4 Fast Flow GE in a gravity column (equilibrated in 2× PBS pH 7.4, 1 mM DTT) and allowed to incubate at 25°C for 2 hours. The flow-through containing purified A5N was collected. Results were tested on 12% SDS-PAGE (figure 13).

2.12 – Co-Sedimentation Assay

A5N was dialyzed in 1× BRB80 pH 6.8 and concentrated to 5 µM. 20 µM of WT, ATU1-6D and TTLL3KO MTs were prepared in 1× BRB80 + 20 µM taxol. 60 µL the A5N solution was mixed with 60 µL of each type of MT solution for a total of 3 experimental samples plus one control containing 60 µL 10 µM A5N and 60 µL 1× BRB80 buffer and three more controls containing 60 µL 1× BRB80 and 60 µL 20 mM of each type of MT (WT, TTLL3KO and ATU1-

6D) in 1× BRB80 and 20 μM taxol. All samples of 120 μL were allowed to incubate at 25°C in the dark for one hour. After incubation, 100 μL of each sample was delicately layered onto 150 μL of glycerol cushion containing 1× BRB80, 40% glycerol and 20 μM taxol at a pH of 6.8 and centrifuged at 350,000 × g, 25°C for 30 minutes. The resulting supernatant and pellet were tested for presence of proteins using 12% SDS-PAGE (figures 14 and 15).

Results

3.1 – TOG Purification, 12% SDS-PAGE gel

Allison Holt provided us with cell pellets over-expressing the TOG protein. We successfully reproduced Winlund's results and purified TOG (figure 8, lane 10) for conjugation onto an NHS column to create a TOG column, from which tubulin was successfully purified (Widlund et al. 2012).

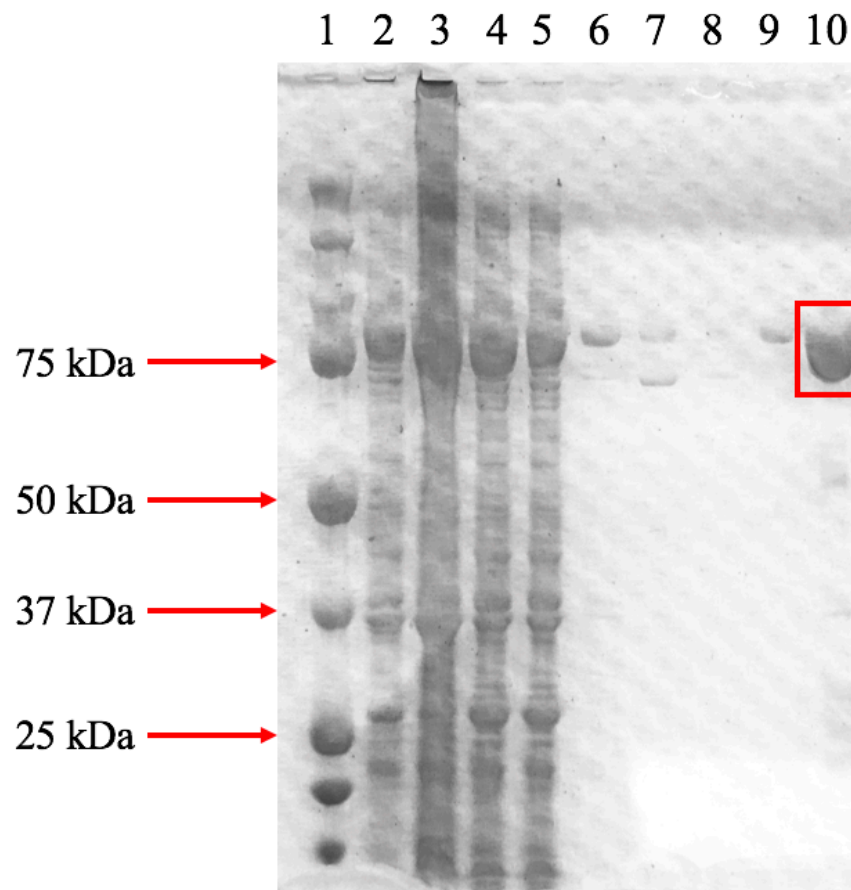


Figure 8. TOG purification results, 12% SDS-PAGE. BL21 (DE3) E. coli cells were lysed by sonication and the resulting whole cell lysate was centrifuged to remove cell debris. Lysate supernatant was loaded onto a GST column, washed and eluted with 10 mM reduced glutathione. Lanes: 1 – Ladder, 2 – Whole cell lysate, 3 – Lysate supernatant, 4 – Lysate pellet, 5 – Flow-through from loading, 6 – Wash 1, 7 – Wash 2, 8 – Wash 3, 9 – Wash 4, 10 – Elution of purified GST-TOG protein.

3.2 – Tubulin Purification, 12% SDS-PAGE gel

TTLL3KO tubulin was successfully purified using a TOG column (figure 9, lane 7). WT and ATU1-6D strains of tubulin were also successfully purified, each with their own specified TOG column to prevent contamination (results not shown).

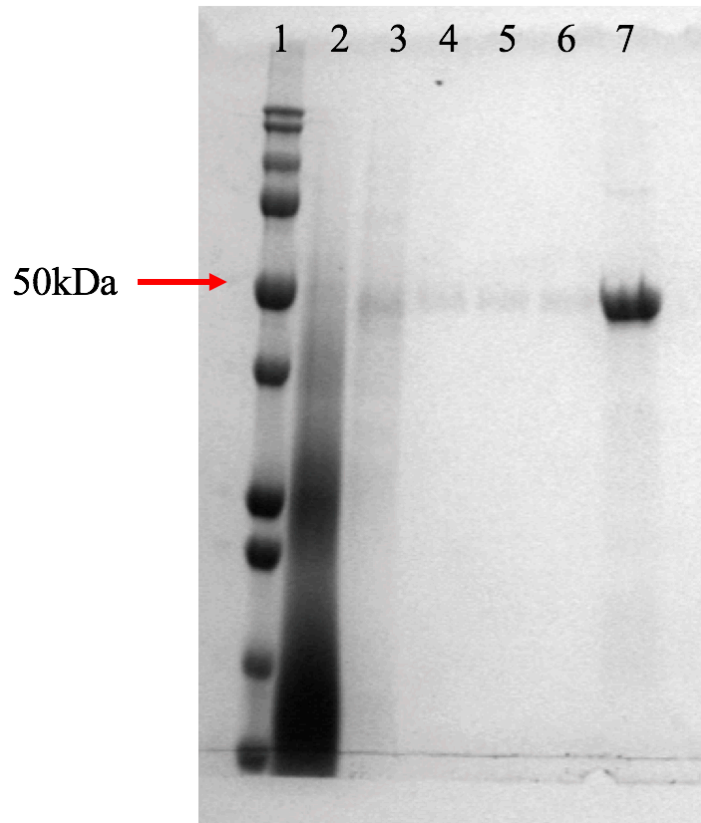


Figure 9. TTLL3KO tubulin purification results, 12% SDS-PAGE.

TTLL3KO *T. thermophila* cells were lysed by sonication and the resulting whole cell lysate was centrifuged to remove cell debris. Lysate supernatant was loaded onto a TOG column, washed and eluted with 0.5 M ammonium sulfate.

Lanes: **1** – Ladder, **2** – Flow-through, **3** – Wash 1, **4** – Wash 2, **5** – Wash 3, **6** – Wash 4, **7** – Elution of TTLL3KO tubulin proteins.

3.3 – pGEX-2T_A5N Amplification and Purification, 1% Agarose gel

Concerning figures 6 and 11, BamHI successfully cleaved the plasmid at the 930th bp and EcoRI successfully cleaved the plasmid at the 1499th bp, leaving two fragments of 4,938 bp and 569 bp. The first bands in lanes 2 and 3 (from the wells in figure 10) near the 5 kbp marker are the 4,938 bp fragments, which contain most of the pGEX-2T_A5N plasmid, excluding the A5N gene. The second band from the wells in lanes 2 and 3 is mostly the A5N gene at 569 bp, a few extra bps that are not a part of the gene remained attached (see figure 6). Note that the 5.5kbp band in lane 4 has migrated slightly farther than the 5kbp fragment in lanes 2 and 3, this is because this fragment is circular rather than linear which, tends to migrate slightly faster than linear DNA of the same bps. Due to the fact that there is no visible difference in concentrations between lane 2 and 3, it can be inferred that the restriction digest was complete after 2 hours.

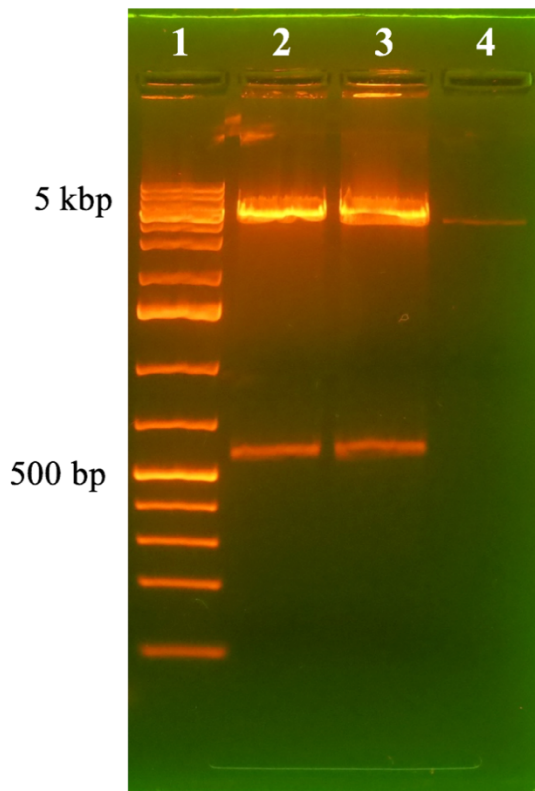


Figure 10. Restriction digest of pGEX-2T_A5N, 1% agarose gel electrophoresis. The pGEX-2T_A5N plasmid was digested with BamHI and EcoRI at 37°C for 2 hours (lane 2) and 18 hours (lane 3).

Lanes: **1** – Ladder, **2** – restriction digest after 2 hour incubation, **3** – The same RD reaction after 18 hour incubation, **4** – pGEX-2T_A5N before cleavage.

3.4 – Chromatogram of GST-A5N Purification

After 120 mL of preparing and equilibrating the column, the lysate supernatant was loaded onto the column from 120 mL – 150 mL, resulting in a rise of UV absorbance to ~2,000 mAU due to a high concentration of unbound cellular proteins flowing through the column (figure 12). All of the buffers used were PBS based and maintained similar UV absorbance levels of around ~100 mAU. The column was washed with equilibration buffer until the absorbance was at levels seen before loading (~100 mAU) and constant. The column then underwent washes 2 and 3 where only minimal UV absorbance spikes were observed, presumably additional proteins that were not able to bind tightly to the column and/or minimal amounts of GST-A5N; these steps help with getting a purer elution of GST-A5N. From 262 mL – 290 mL, the protein was eluted with a buffer containing reduced glutathione resulting in a UV absorbance peak of ~800 mAU due to the GST-A5N protein coming off of the column.

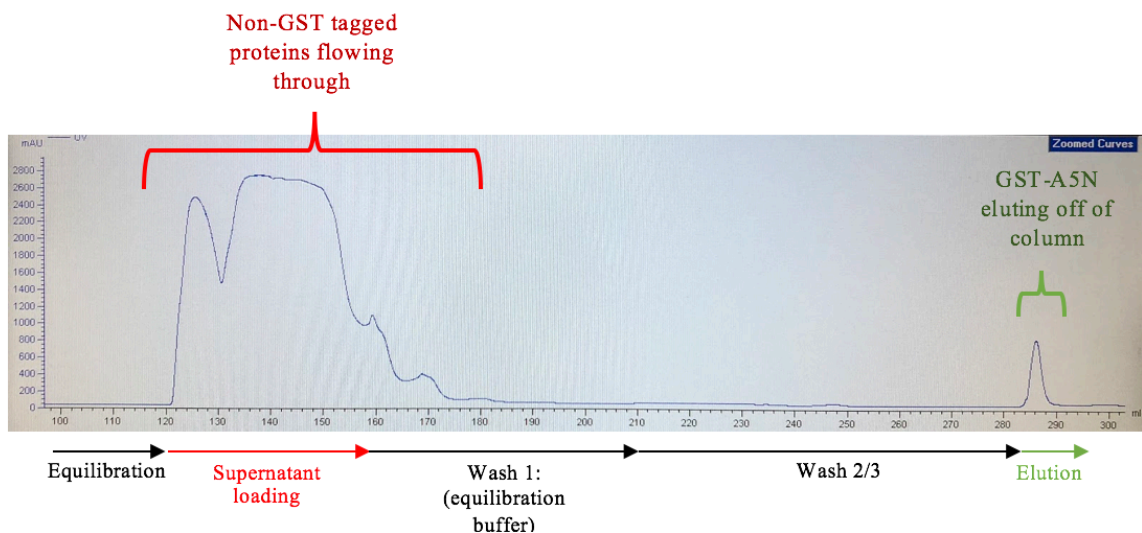


Figure 11. Chromatogram of GST-A5N purification. UV absorbance in milli-Absorbance Units (mAU) versus total volume (mL) passed through the column. After Rosetta (DE3) E. coli cells expressing GST-A5N were lysed by sonication, the resulting whole cell lysate was centrifuged to remove cell debris and lysate supernatant was loaded onto a GST purification column. The UV spectrophotometer measured the UV absorbance ($\lambda=280$ nm) of the liquid directly after passing through the column.

3.6 – GST-A5N Purification, 12% SDS-PAGE gel

Figure 12 complements the chromatograph in figure 11, figure 11 shows when proteins left the column and figure 12 shows what proteins (based on kDa) came off in these stages leading to the conclusion that GST-A5N was successfully eluted in presumably near isolation (figure 12, lane 7).

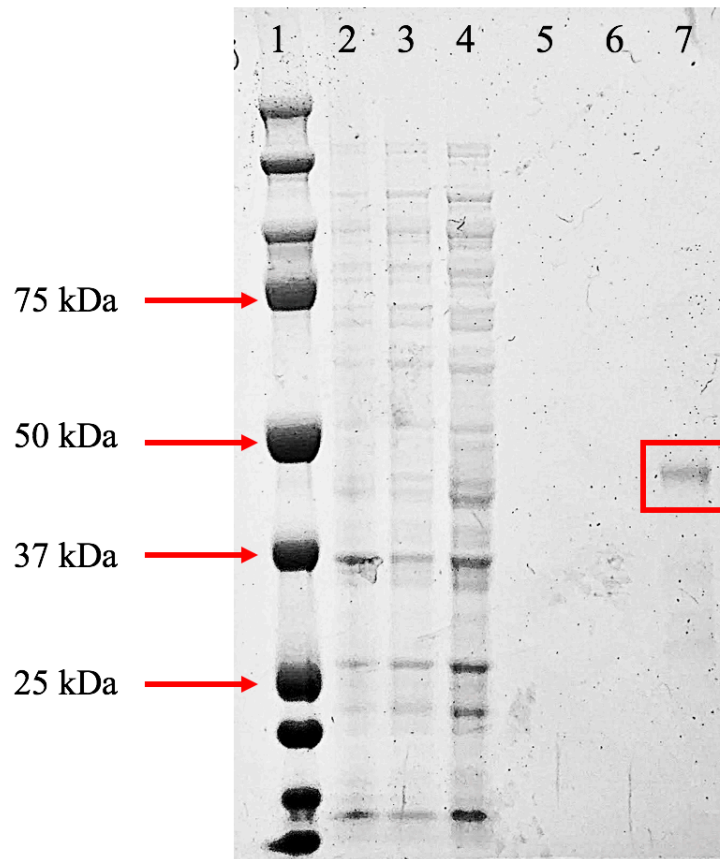


Figure 12. GST-A5N purification results, 12% SDS-PAGE. After Rosetta (DE3) *E. coli* cells expressing GST-A5N were lysed by sonication, the resulting whole cell lysate was centrifuged to remove cell debris and lysate supernatant was loaded onto a GST purification column, washed and eluted with 10 mM reduced glutathione. Lanes: 1 – Ladder, 2 – Whole cell lysate, 3 – Lysate supernatant, 4 – Flow-through, 5 – Wash 1, 6 – Wash 2, 7 – Elution of GST-A5N protein.

3.7 – A5N Purification, 12% SDS-PAGE gel

Elutions from the GST-A5N purification (figures 11 and 12) were concentrated and subjected to a thrombin cleavage reaction (discussed in methods). After termination, the cleavage reaction was then loaded onto a GST column to purify A5N from its cleaved GST tag. The results of this final purification are depicted in figure 13. The boxed region in lanes 1 and 2 highlights the A5N protein in isolation.

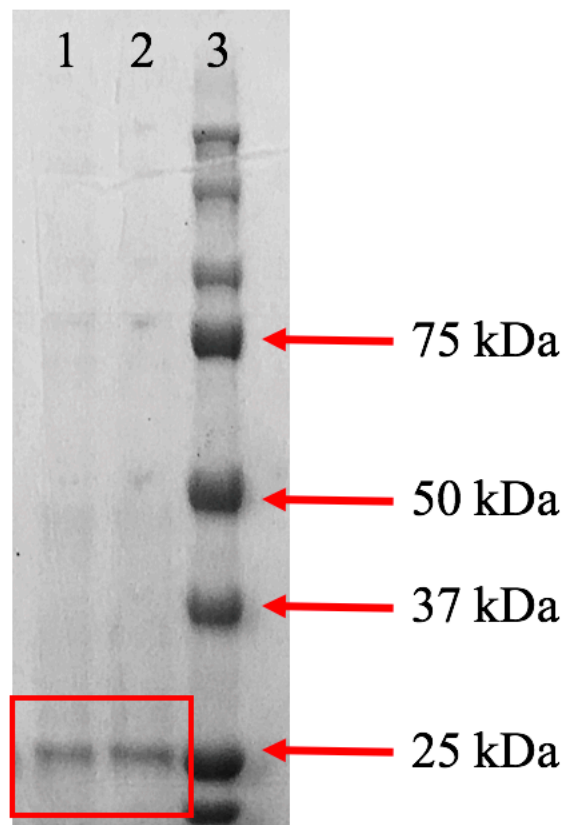


Figure 13. Thrombin cleavage reaction results: purified A5N, 12% SDS-PAGE. After GST-A5N was purified it was subjected to a thrombin cleavage reaction at 4°C for 18 hours and purified again on a GST column to remove the GST tag, leaving the A5N protein in isolation.
Lanes: **1** – Purification flow-through of thrombin cleavage reaction, **2** – repeat of lane 1, **3** – ladder.

3.8 – Co-Sedimentation Assay, 12% SDS-PAGE gel

The co-sedimentation assay (figures 14 and 15) was done in triplicate (third result not shown due to a poor quality SDS-PAGE gel). A5N proteins are 21 kDa and the α - and β -tubulin proteins are 49.5 kDa and 49.8 kDa respectively. All of the samples for the SDS-PAGE were taken after centrifugation. Lanes 2, 3, 4, 5, 8, 9, 12 and 13 represent controls. The A5N control in figure 15 did show some sedimentation, which was not expected; however, the other trials' A5N control (no MTs) showed the expected result of no pellet hence, no A5N sedimentation (see figure 15). In addition, other titration trials of this co-sedimentation assay (not shown or discussed) showed anticipated A5N control lane results. This might be explained because there were some aggregation issues with the A5N protein during dialysis. Usually, fractions that had aggregated were thrown away but if there was some aggregation that was not visible by eye and was used for this control then that may explain why A5N had pelleted (figure 14, lane 3). For the experimental samples, A5N remained in the supernatant and did not pellet with WT MTs or ATU1-6D MTs (figures 14 and 15, lanes 7 and 15) in this trial or in any other trial. Presumably, A5N was not able to bind to polyglycylated MTs (WT and ATU1-6D) with great affinity. Lanes 10 and 11 show that more A5N sedimented with the TTLL3KO MTs (polyglutamylated) than remained in the supernatant (highlighted box in figures 14 and 15).

Co-Sedimentation, result #1

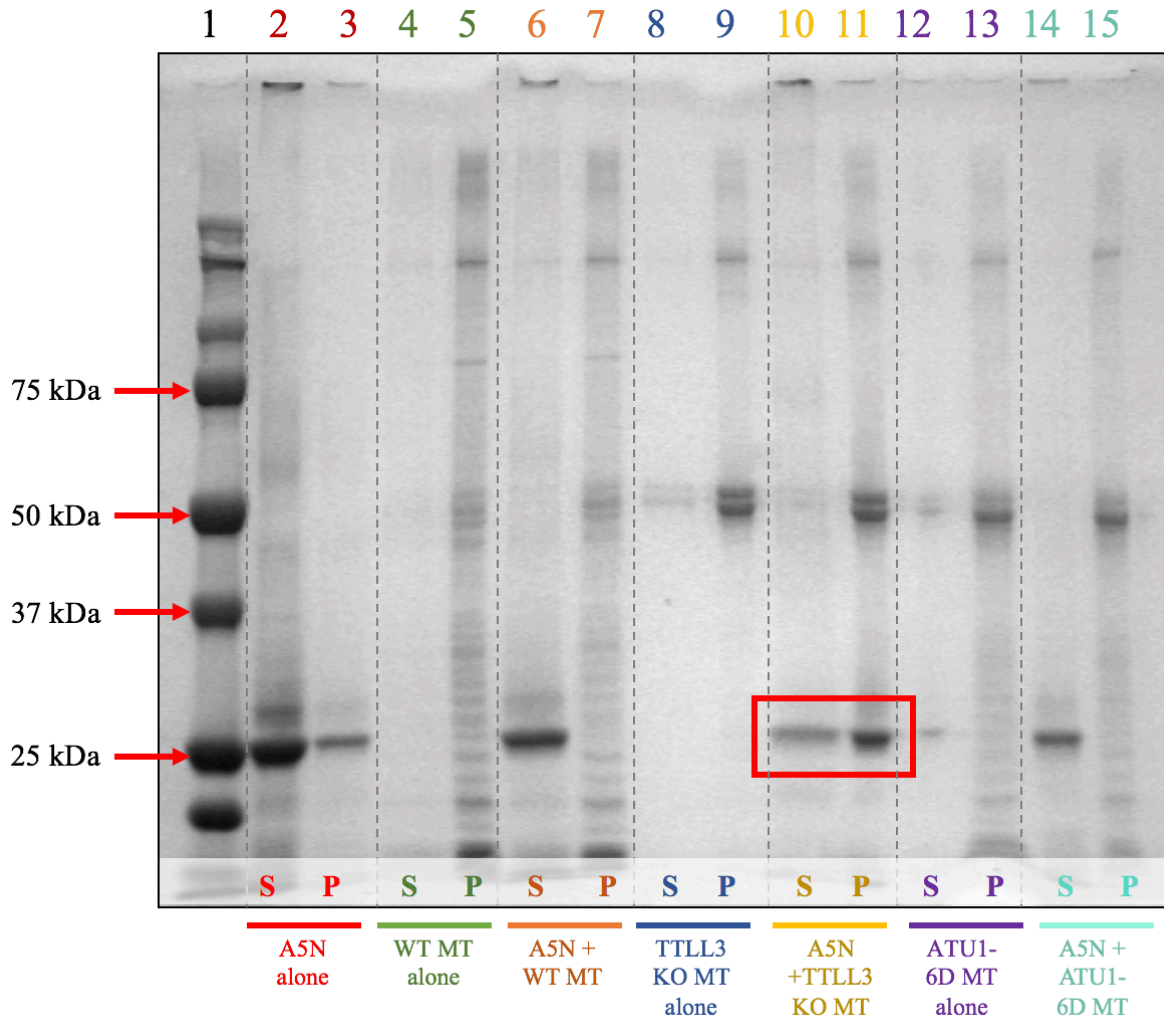


Figure 14. Co-Sedimentation assay of A5N with variously post-translationally modified MTs (1), 12% SDS-PAGE.

Reaction mixtures incubated at 25°C for 1 hour in the dark before being layering 100 μ L of each reaction mixture onto 150 μ L of a 40% glycerol cushion and centrifuging at 90,000 $\times$ g. 12% SDS-PAGE gel depicts samples of the resulting supernatants (S) and pellets (P) after centrifugation. WT MTs are polyglycylated, ATU1-6D MTs have reduced polyglycylation and TTLL3KO MTs are polyglutamylated (figure 5).

Lanes: **1** – Ladder, **2** – supernatant of reaction containing 60 μ L of 5 μ M A5N + 60 μ L 1 $\times$ BRB80 buffer, **3** – pellet of reaction containing 60 μ L of 5 μ M A5N + 60 μ L 1 $\times$ BRB80 buffer, **4** – supernatant of reaction containing 60 μ L of 20 μ M WT MTs + 60 μ L 1 $\times$ BRB80 buffer, **5** – pellet of reaction containing 60 μ L of 20 μ M WT MTs + 60 μ L 1 $\times$ BRB80 buffer, **6** – supernatant of reaction containing 60 μ L of 5 μ M A5N + 60 μ L of 20 μ M WT MTs, **7** – pellet of reaction containing 60 μ L of 5 μ M A5N + 60 μ L of 20 μ M WT MTs, **8** – supernatant of reaction containing 60 μ L of 20 μ M TTLL3KO MTs + 60 μ L 1 $\times$ BRB80 buffer, **9** – pellet of reaction containing 60 μ L of 20 μ M TTLL3KO MTs + 60 μ L 1 $\times$ BRB80 buffer, **10** – supernatant of reaction containing 60 μ L of 5 μ M A5N + 60 μ L of 20 μ M TTLL3KO MTs, **11** – pellet of reaction containing 60 μ L of 5 μ M A5N + 60 μ L of 20 μ M TTLL3KO MTs, **12** – supernatant of reaction containing 60 μ L of 20 μ M ATU1-6D MTs + 60 μ L 1 $\times$ BRB80 buffer, **13** – pellet of reaction containing 60 μ L of 20 μ M ATU1-6D MTs + 60 μ L 1 $\times$ BRB80 buffer, **14** – supernatant of reaction containing 60 μ L of 5 μ M A5N + 60 μ L of 20 μ M ATU1-6D MTs, **15** – pellet of reaction containing 60 μ L of 5 μ M A5N + 60 μ L of 20 μ M ATU1-6D MTs.

Co-Sedimentation, result #2

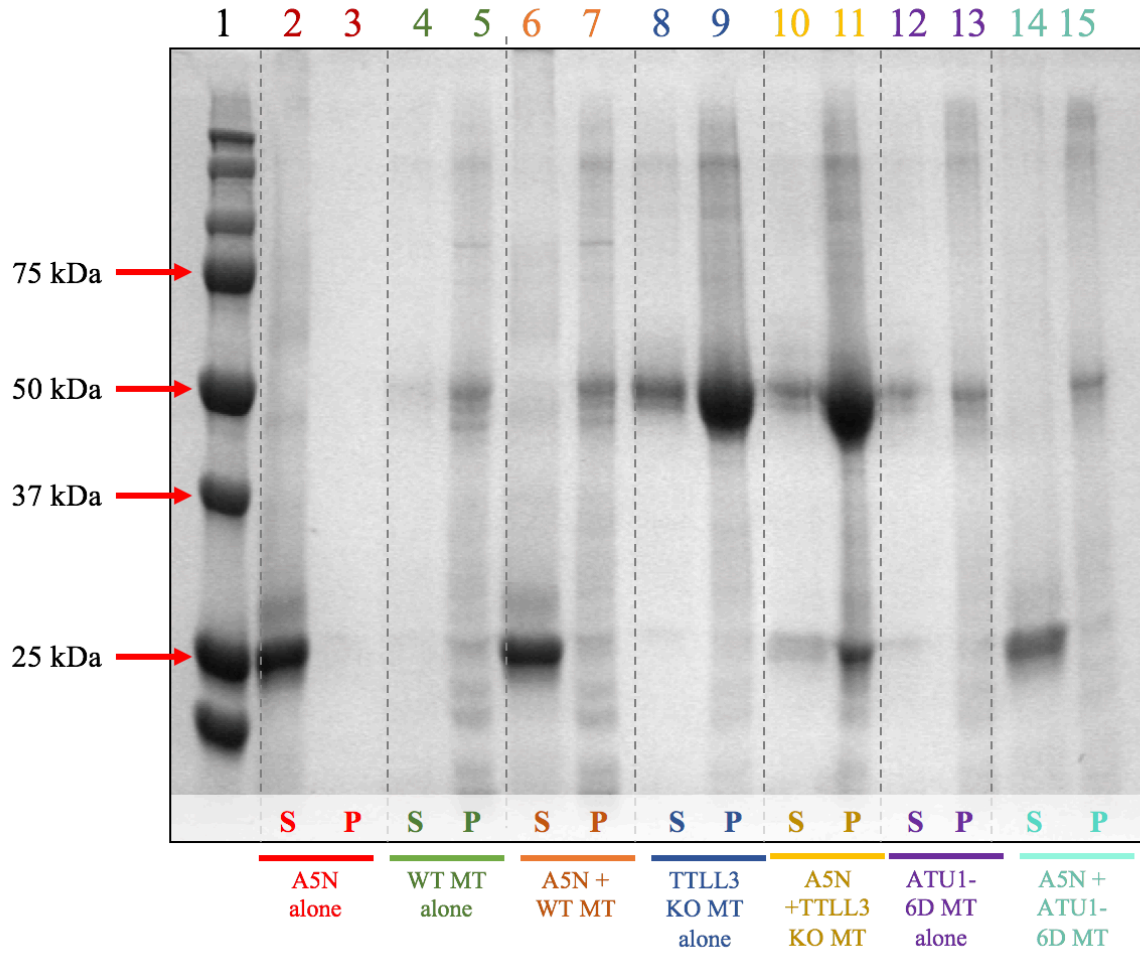


Figure 15. Co-Sedimentation assay of A5N with variously post-translationally modified MTs (2), 12% SDS-PAGE.

Reaction mixtures incubated at 25°C for 1 hour in the dark before being layering 100 μ L of each reaction mixture onto 150 μ L of a 40% glycerol cushion and centrifuging at 90,000 $\times$ g. 12% SDS-PAGE gel depicts samples of the resulting supernatants (S) and pellets (P) after centrifugation.

Lanes: 1 – Ladder, 2 – supernatant of reaction containing 60 μ L of 5 μ M A5N + 60 μ L 1 $\times$ BRB80 buffer, 3 – pellet of reaction containing 60 μ L of 5 μ M A5N + 60 μ L 1 $\times$ BRB80 buffer, 4 – supernatant of reaction containing 60 μ L of 20 μ M WT MTs + 60 μ L 1 $\times$ BRB80 buffer, 5 – pellet of reaction containing 60 μ L of 20 μ M WT MTs + 60 μ L 1 $\times$ BRB80 buffer, 6 – supernatant of reaction containing 60 μ L of 5 μ M A5N + 60 μ L of 20 μ M WT MTs, 7 – pellet of reaction containing 60 μ L of 5 μ M A5N + 60 μ L of 20 μ M WT MTs, 8 – supernatant of reaction containing 60 μ L of 20 μ M TTLL3KO MTs + 60 μ L 1 $\times$ BRB80 buffer, 9 – pellet of reaction containing 60 μ L of 20 μ M TTLL3KO MTs + 60 μ L 1 $\times$ BRB80 buffer, 10 – supernatant of reaction containing 60 μ L of 5 μ M A5N + 60 μ L of 20 μ M TTLL3KO MTs, 11 – pellet of reaction containing 60 μ L of 5 μ M A5N + 60 μ L of 20 μ M TTLL3KO MTs, 12 – supernatant of reaction containing 60 μ L of 20 μ M ATU1-6D MTs + 60 μ L 1 $\times$ BRB80 buffer, 13 – pellet of reaction containing 60 μ L of 20 μ M ATU1-6D MTs + 60 μ L 1 $\times$ BRB80 buffer, 14 – supernatant of reaction containing 60 μ L of 5 μ M A5N + 60 μ L of 20 μ M ATU1-6D MTs, 15 – pellet of reaction containing 60 μ L of 5 μ M A5N + 60 μ L of 20 μ M ATU1-6D MTs.

Discussion

Conclusions

CPAP was investigated due to its interaction with parental centriolar MTs which, happen to be polyglutamylated. However, CPAP also binds to polyglycylated proteins like 14.3.3. and nobody has specifically studied if CPAP binds to specific C-terminal tail post-translational modifications, leaving the literature with unanswered questions about the nature of CPAP's interaction with MTs. We discovered that the MT binding domain preferentially binds to polyglutamylated MTs over polyglycylated MTs. This discovery sheds light on CPAP's interaction with MTs as well as directing us in our long term goal of developing an *in vivo* sensor for polyglutamylated MTs. Moving forward with our goal we can make mutations in A5N to remove the post-translational modification specificity of this MT binding domain to better understand the mechanism of specificity of the MT binding interaction.

Purified A5N and differential post-translationally modified MTs were successfully created for the co-sedimentation assay. To get A5N in isolation, a procedure for culture growth and purification was optimized, this included adjusting timing of growth and induction, and fine-tuning lysis and purification buffers. All three strains of tubulin were productively purified using prepared TOG columns for each strain.

The results of the co-sedimentation assays were confirmed in triplicate. A5N was shown to bind to polyglutamylated MTs but not polyglycylated MTs. We have confirmed that A5N is in fact binding specifically to polyglutamylated MTs, which is the first step towards building an *in vivo* sensor for polyglutamylated tubulin.

Limitations

MS/MS would have been very useful in verifying the A5N protein. However, as the pGEX-2T_A5N plasmid was sequenced, we do have more confidence that our A5N protein is correct than we would have from just the SDS-PAGE gels showing the expected kDa. MS/MS would have also been valuable in determining the amount of polyglycylation and polyglutamylolation in the three strains of tubulin, especially because there is virtually no difference in size (kDa) between polyglutamylated, polyglycylation and reduced polyglycylation tubulin. Fortunately, these protocols have been established and performed many times by members of the lab with MS/MS data, so it is known that these protocols for making polyglutamylated or polyglycylation tubulin do provide the desired post-translational modifications on MTs. Still, the MS/MS data from these experiments is insensitive so we don't have reliable mapping of these modifications. This limitation also plays into the limitations with the co-sedimentation assay as the degree of post-translational modifications the MTs has not been verified. However, western blots have confirmed the presence of polyglutamylolation in our experiments. In addition, co-sedimentation assays do not provide information on the mechanism of interaction, the K_d of the interaction or the specificity of A5N for polyglutamylated tubulin. Nevertheless, it can still be concluded from our experiments that A5N is interacting with polyglutamylated MTs and not with WT MTs or polyglycylation MTs.

Future Investigations

Using $^{15}\text{N}^{13}\text{C}$ isotopically labeled A5N, we will be able to get a Nuclear Magnetic Resonance (NMR) spectra of A5N with and without tubulin. The differences in spectra can be used to help determine the mechanism of interaction and identify the binding sites. Making

isotopically labeled A5N can be done by growing the transformed *E. coli* in M9 salt media (void of carbon and nitrogen) with ^{13}C labeled glucose and ^{15}N labeled ammonium instead of LB media.

In situ imaging of GFP-A5N localization with polyglutamylated MT localization in mammalian cell lines will provide information on if interactions within the cell are similar to *in vitro* binding assays, which have limitations considering buffer composition, allosteric restrictions in the cell and other cellular conditions that are not predictable in *in vitro* assays. This will be particularly helpful in determining if A5N can be used as a polyglutamylation sensor *in vivo*. This will be done by transfecting mammalian cells lines with a plasmid for a GFP labeled A5N protein. After imaging for GFP, the cells will be fixed and stained with fluorescently labeled antibodies for polyglutamylated MTs. Images of individual cells can be superimposed to assess if co-localization is present.

In addition, a pull-down assay of A5N will indicate if A5N also binds to polyglutamylated or other types of modified α/β -tubulin dimers, this can give us further insights into the nature of the interaction between A5N and MTs by answering the question about if A5N needs the polymerized MT structure to bind or if it is simply interacting with exposed regions of the α/β -tubulin dimers. This can be done by re-loading purified tubulin onto a TOG column, before elution the A5N protein will be slowly run through the column and the flow-through and elution fractions can be tested for the presence of the A5N. Presumably, if A5N is found in the flow-through, it did not bind to tubulin and if it is found in the elution with tubulin then it did show interaction with tubulin dimers.

Finally, additional experiments will include Microscale Thermophoresis (MST) and Isothermal Titration Calorimetry (ITC) experiments to determine the K_d of the interaction between A5N and polyglutamylated MTs.

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