Methylated $\alpha$-tubulin antibodies recognize a new microtubule modification on mitotic microtubules

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ABSTRACT

Posttranslational modifications (PTMs) on microtubules differentiate these cytoskeletal elements for a variety of cellular functions. We recently identified SETD2 as a dual-function histone and microtubule methyltransferase, and methylation as a new microtubule PTM that occurs on lysine 40 of $\alpha$-tubulin, which is trimethylated ($\alpha$-TubK40me3) by SETD2. In the course of these studies, we generated polyclonal ($\alpha$-TubK40me3 pAb) and monoclonal ($\alpha$-TubK40me3 mAb) antibodies to a methylated $\alpha$-tubulin peptide (GQMPSD-Kme3-TGGGDC). Here, we characterize these antibodies, and the specific mono-, di- or trimethylated lysine residues they recognize. While both the pAb and mAb antibodies recognized lysines methylated by SETD2 on microtubules and histones, the clone 18 mAb was more specific for methylated microtubules, with little cross-reactivity for methylated histones. The clone 18 mAb recognized specific subsets of microtubules during mitosis and cytokinesis, and lacked the chromatin staining seen by immunocytochemistry with the pAb. Western blot analysis using these antibodies revealed that methylated $\alpha$-tubulin migrated faster than unmethylated $\alpha$-tubulin, suggesting methylation may be a signal for additional processing of $\alpha$-tubulin and/or microtubules. As the first reagents that specifically recognize methylated $\alpha$-tubulin, these antibodies are a valuable tool for studying this new modification of the cytoskeleton, and the function of methylated microtubules.

Abbreviations: PTM, posttranslational modification; SETD2, SET-domain containing 2; ICC, immunocytochemistry; IP, immunoprecipitation; IB, immunoblot; MAP, microtubule-interacting protein; mAb, monoclonal antibody; pAb, polyclonal antibody

Introduction

Microtubules are a major, and evolutionarily conserved, element of the cytoskeleton assembled from heterodimers of $\alpha$- and $\beta$-tubulin. Microtubules have a variety of cellular functions, including the maintenance of cell shape, polarity and motility, cell division, intracellular transport, cell differentiation in neurons, and are the main structural element of cilia, flagella, and centrosomes. The structural and functional diversity of microtubules is specified by posttranslational modifications (PTMs), which have been suggested to comprise a “tubulin code.” These post-translational modifications include tyrosination and detyrosination of the C-terminal tyrosine residue of $\alpha$-tubulin, $\Delta 2$ modification (the penultimate glutamate removal of $\alpha$-tubulin after detyrosination), acetylation of lysine 40 (K40) of $\alpha$-tubulin, phosphorylation, glutamylation of the C-terminal tails of both $\alpha$-tubulin and $\beta$-tubulin, and glycylation of the side chains of glutamate residues on the $\alpha$- and $\beta$-tubulin C-terminal tails. Detyrosination, $\Delta 2$ modification, acetylation and polyglutamylation are highly enriched on long-lived stable microtubule structures, whereas tyrosination occurs in newly polymerized microtubules. These PTMs recruit “reader” proteins, such as microtubule motor proteins and non-motor microtubule-associated proteins (MAPs), which contribute to the specialized function of these cytoskeletal elements.

Specific antibodies to the PTMs of microtubules have been developed and used to elucidate the roles of PTMs on specialized microtubules. The first antibody generated against a microtubule PTM was the rat monoclonal antibody YL1/2, which is specific for tyrosinated $\alpha$-tubulin. The mouse monoclonal antibody 6-B11-1, which is specific for acetylated K40 of $\alpha$-tubulin, has been widely used to identify acetylated microtubules, a marker of stable, long-lived microtubules. Rabbit polyclonal antibodies specific to detyr-tubulin and $\Delta 2$-tubulin were generated using detyrosinated $\alpha$-tubulin peptide (GEEEGEE) and $\Delta 2$-tubulin peptide (EGEEGE), respectively. For polyglutamylated $\alpha$-tubulin, 2 antibodies were developed, mouse monoclonal antibody GT335, which detects the branching point of the glutamate side chains staining both short and long side chains of tubulin polyglutamylation, and rabbit polyclonal antibody pollyE, which recognizes long polyglutamate stretches of more than 3 C-terminal glutamates. Using these PTM-specific antibodies, which are now commercially available, tremendous...
progress has been made in understanding the function of the microtubules on which they occur, the enzymes responsible for adding ("writers") or removing ("erasers") these modifications, and microtubule-interacting proteins (MAPs) ("readers") that specify their function.17,18

We recently reported that lysine 40 of α-tubulin is trimethylated (α-TubK40me3) by SET-domain containing 2 (SETD2), a histone methyltransferase.19 We performed genetic, biochemical, and cell-based experiments that demonstrated microtubule methylation occurs during mitosis and cytokinesis, and is required for genomic stability. During the conduct of these studies, we generated a polyclonal antibody to methylated lysine 40 of α-tubulin (α-TubK40me3 pAb). Here, we describe the characteristics of this polyclonal antibody, as well as monoclonal antibodies (α-TubK40me3 mAb) we subsequently generated to this novel methylated epitope on α-tubulin.

**Results**

We recently reported trimethylation at K40 of α-tubulin (TUBA1B-myc) purified from human HEK293T cells by mass spectrometry analysis.18 Trimethylation of the α-tubulin TUBA4A isoform, one of α-tubulin isotypes of rat brain tissue, was also reported to be methylated at K40 in a mass spectrometry screen in PhosphoSitePlus (http://www.phosphosite.org) (Fig. 1A).20 Using commercially available antibodies against SETD2-methylated lysine 36 of histones (H3K36me3) and a pan-trimethyl lysine antibody, we detected a methylated epitope on mitotic spindles and midbodies using conventional immunocytochemistry (ICC), but not using antibodies against pan-dimethyl lysine or other methylated histone epitopes.19

To generate a methylated α-tubulin antibody, a keyhole limpet hemocyanin (KLH)-linked trimethylated K40 peptide (GQMPSD-Kme3-TIGGGDC-KLH) was synthesized for immunizing rabbits. This peptide antigen could potentially generate a mixture of antibodies recognizing pan-α-tubulin, K40 unmethylated α-tubulin and trimethylated K40me3 α-tubulin (Fig. 1B). To purify a polyclonal antibody directed toward the α-TubK40me3 epitope, the immunized rabbit serum was passed through an affinity column containing unmodified K40 peptide to remove antibodies recognizing unmethylated α-TubK40 and pan-α-tubulin (Fig. 1C). The elute was passed through a second affinity column containing trimethylated K40 peptide to capture the antibody recognizing α-TubK40me3 (Fig. 1C), and the final elute was used as the polyclonal α-TubK40me3 pAb antibody for our experiments.

The rabbit monoclonal antibodies specific for trimethylated α-tubulin at K40 (α-TubK40me3 mAb) were hybridoma cell clones made from the lymphocytes of the above immunized rabbits, and media concentrated from the hybridoma clones used as the source of α-TubK40me3 mAbs in these studies. To screen the mAb clones, nitrocellulose membranes were loaded with unmodified, monomethylated, dimethylated, trimethylated and acetylated K40 peptides (Biotin-Ahx-IQPDGQMPSDKTIGGGDSDT) and blotted with hybridoma lysates. The mAb lysates from 3 hybridoma clones (clones 18, 22, and 24) showed better specificity for trimethylated K40 peptide than the α-TubK40me3 pAb, which exhibited more cross-reactivity with dimethylated K40 peptide (Fig. 2A and information below).

We next determined the specificity of the clone 18 mAb for trimethylated α-tubulin and compared it to the α-TubK40me3 pAb using a peptide competition assay (Fig. 2B). We previously established18 that fractionating cells to eliminate the majority of interphase nuclei (which contain abundant methylated histones) optimized our ability to detect methylated α-tubulin on microtubules by western blot analysis using the α-TubK40me3 pAb. We therefore performed western analyses on the cytoplasmic fraction (enriched for mitotic cells lacking a nuclear membrane) from HeLa S3 cells using the pAb and mAbs preincubated with unmodified, monomethylated (me1), dimethylated (me2), trimethylated (me3) or acetylated (ac) K40 peptide (Fig. 2B). The ability of the α-TubK40me3 pAb to recognize α-tubulin was completely blocked by trimethylated K40

![Figure 1. Strategy for generating polyclonal antibodies specific to trimethylated lysine 40 (K40) of α-tubulin polyclonal (α-TubK40me3 pAb).](image-url)
peptide, and strongly, but not completely, reduced by the dimethylated K40 peptide. The ability of the clone 18 mAb to recognize \(\alpha\)-tubulin was blocked by both tri- and di-methylated K40 peptides (Fig. 2B), indicating it recognized an epitope that was shared by di- and tri-methylated lysine of \(\alpha\)-tubulin. Initially, this recognition profile with native tubulin/microtubules as the target appeared to differ from our observations with the dot blot analysis (Fig. 2A), where clone 18 mAb failed to recognize the di-methylated tubulin peptide. However, increasing the amount of clone 18 mAb also revealed cross-reactivity with the di-methyl peptide (Fig. 2C).

Next, we determined the immunolocalization pattern of the methyl mark recognized by the 3 mAbs (clone 18, 22, 24). We previously reported that the \(\alpha\)-TubK40me3 pAb antibody stained both midbody microtubules and methylated histones in chromatin in mouse embryonic fibroblast (MEFs) cells fixed at room temperature (RT) (Fig. 3, column 1). Importantly, we observed that the microtubule staining was enhanced relative to chromatin staining when cells were fixed at 37 °C (Fig. 3, column 2), likely due to the cold-sensitive nature of microtubules. While all 3 clones recognized mitotic microtubules similar to our observations with the pAb, clone 18 mAb was highly specific for microtubules, showing little cross-reactivity with methylated histones of chromatin (Fig. 3, column 3). In contrast to clone 18 mAb, which stained methylated midbody microtubules without staining chromatin, clone 22 mAb stained both methylated midbody microtubules and chromatin (histones) (Fig. 3, column 4), and clone 24 mAb recognized chromatin (histones) but failed to recognize methylated midbody microtubules (Fig. 3, column 5).

To further characterize the species specificity of the clone 18 mAb, we performed ICC in human 786–0 and in Setd2 knockout MEFs using the clone 18 mAb. As seen with the wild-type MEFs (Fig. 3), methylation of midbody microtubules occurred in human 786–0 cells (Fig. 4A, upper panel). This midbody methylation was lost in 786–0 cells in which we knocked down SETD2 using lentiviral SETD2 shRNA (Fig. 4A, lower panel). Similarly, we failed to observe midbody methylation in Setd2-null MEFs3 d after 4-hydroxytamoxifen treatment (to induce loss of Setd2) (Fig. 4B, lower panel) compared to Setd2 expressing MEFs (Fig. 4B, upper panel). Importantly, clone 18 mAb showed minimal reactivity to methylated chromatin in human cells similar to our observations with the MEFs (Fig. 3).

We next immunoprecipitated methylated \(\alpha\)-tubulin from the cytoplasmic fraction of parental 786–0 cells, in which SETD2 had been inactivated using TALEN nucleases (SETD2-null), or SETD2-null cells rescued by expressing a truncated (but functional) SETD2 construct (tSETD2). Immunoprecipitation of methylated \(\alpha\)-tubulin with the \(\alpha\)-TubK40me3 pAb and immunoblotting with \(\alpha\)-tubulin antibody (DM1A) showed that the \(\alpha\)-TubK40me3 pAb immunoprecipitated methylated \(\alpha\)-tubulin, seen as a faster Figure 2. Specificity of the polyclonal (\(\alpha\)-TubK40me3 pAb) and monoclonal (\(\alpha\)-TubK40me3 mAb) antibodies against trimethylated K40 of \(\alpha\)-tubulin. (A) Peptide dot blot analysis using unmodified (un), monomethylated (me1), dimethylated (me2), trimethylated (me3) and acetylated (ac) K40 peptides of \(\alpha\)-tubulin (Biotin-Ahx-IQPDGQMPSDKIGSDDTFT) blotted on 0.2 μm nitrocellulose membranes (0.2 μg upper row across; 2 μg lower row across) and immunoblotted using the concentrated supernatants of 3 mAb clones (clone 18, 22, 24, 1:50), \(\alpha\)-TubK40me3 pAb (1:1,000), or a monoclonal antibody against acetylated K40 of \(\alpha\)-tubulin (\(\alpha\)-TubK40ac mAb, 1:1,000) as indicated. (B) Peptide competition assay using mitotic HeLa S3 cell lysates (30 μg) immunoblotted using clone 18 mAb (1:50) or \(\alpha\)-TubK40me3 pAb (1:1,000, 1 μg/ml) pre-incubated with/without unmodified, K40me1, K40me2, K40me3 or K40ac modified \(\alpha\)-tubulin peptide (10 μg/ml). Each lane of the membrane (from the same gel transfer) was cut prior to the peptide competition and subsequently re-aligned prior to visualization. (C) Peptide dot blot analysis with higher concentration of antibodies. One microgram of each peptide was blotted on the membrane. The membrane was immunoblotted using the same antibody concentrations as Fig. 2A (left) and immunoblotted further by adding the same amounts of antibodies (right).
migrating band just below 50 kD; immunoreactivity seen at 50 kD may be due to nonspecific cross-reactivity with IgG heavy chain as they appear in both SETD2 proficient and deficient cells. (Fig. 5A). Similar data were also obtained using clone 18 mAb (data not shown). Western blot analysis with α-tubulin (see input lanes in Fig. 5A) also showed both an ~50 kD band, and a higher mobility band seen only in cells expressing SETD2 that corresponded to the lower molecular weight band seen by co-immunoprecipitation (arrow). The difference in mobility between unmethylated and methylated α-tubulin (seen as a faster migrating band running lower than 50 kD on a 15% reduced SDS-PAGE gel using 2X sample buffer with 10% β-mercaptoethanol in Fig. 5A) suggests the methylated form of α-tubulin has a lower apparent molecular weight than unmethylated α-tubulin. This observation was confirmed by western blot analysis using mitotic HeLa S3 cells, which enriches for mitotic microtubules on which this PTM occurs (Fig. 5B). Western analysis showed an increase in α-tubulin recognized by the α-TubK40me3 pAb that significantly increased in mitotic cells, with the methylated α-tubulin exhibiting a faster mobility compared to α-tubulin (Fig. 5B). Taken together, these data demonstrate that methylated α-tubulin can be detected by immunoprecipitation using α-TubK40me3 pAb and mAb, and differentiated from unmethylated α-tubulin by its faster mobility in polyacrylamide gels.

Discussion

The results of this study have yielded several insights into detection of methylated α-tubulin using methyl-specific antibodies. First, because methylation is a modification of mitotic microtubules (spindle and midbody), only a small sub-population of cellular microtubules are methylated, and would thus be recognized by either polyclonal or monoclonal antibodies to this methyl mark. Even though α-TubK40me3 pAb and mAb exhibit specificity for methylated α-tubulin, due to the high levels of α-tubulin in cells, the vast majority of which is unmodified, western blot analysis using these antibodies will yield bands corresponding to both modified and unmodified α-tubulin. Importantly, when using western blot analysis, this means the band corresponding to methylated α-tubulin is barely detectable in unsynchronized cells. To circumvent this limitation, immunoprecipitation with α-TubK40me3 pAb and mAb followed by blotting with an α-tubulin antibody, or enriching for mitotic cells, will optimize visualization of methylated α-tubulin.

Second, methylated α-tubulin exhibits a lower apparent molecular weight than unmodified α-tubulin, which runs at
There are several possibilities that may account for this difference. First, many PTMs alter electrophoretic mobility, and methylation may increase the mobility of α-tubulin in polyacrylamide gels. Alternatively, methylation may be a signal for other proteins that modify α-tubulin and change its molecular weight. For example, the carboxypeptidase granzymeB has

\[ \text{Figure 4. Reactivity of human and mouse cells with clone 18 α-TubK40me3 mAb.} (A) Representative images of human 786–0 cells undergoing cytokinesis stained 4 d following infection with shGFP or shSETD2 #4 (to inactivate SETD2) lentivirus using clone 18 mAb (green) and α-TubK40ac mAb (red). Nuclei were counterstained with Hoechst 33342 (blue). (B) Representative images of ER-Cre transfected Setd2\textsuperscript{floX/floX} MEFs (Setd2\textsuperscript{+/+}) and Setd2\textsuperscript{floX/floX} MEFs treated with 4-hydroxytamoxifen treatment (Setd2\textsuperscript{−/−}, right lane) using clone 18 mAb (green) and α-TubK40ac mAb (red). Nuclei were counterstained with Hoechst 33342 (blue). Scale bar = 10 μm. (C) SETD2 knockdown using lentiviral shRNAs. Human 786–0 cells were infected with shGFP or 4 different shSETD2 lentiviruses and immunoblotted using SETD2 or α-tubulin antibodies at 4 d after infection. The most efficient shSETD2 #4 was chosen to perform the IC in Fig. 4A. (D) Setd2 knockout in MEFs. ER-Cre transfected Setd2\textsuperscript{floX/floX} MEFs were treated with 4-hydroxytamoxifen treatment (Setd2\textsuperscript{−/−}, right lane) for 4 d and immunoblotted using SETD2 or α-tubulin antibodies. Non-treated Setd2\textsuperscript{floX/floX} MEFs (Setd2\textsuperscript{+/+}, left lane) were included as a wild type control.\]
been shown to proteolytically process α-tubulin to a smaller form.\(^{21}\) When cleaved by granzymeB, α-tubulin was reported to exhibit a similar mobility shift due to removal of 13 a.a. at the C-terminal serine cleavage site.\(^{21}\) Interestingly, these authors proposed that granzymeB-mediated amino acid removal "would cause depolymerization of the microtubule network," consistent with the hypothesis that methylation is a destabilizing microtubule mark.\(^{19}\)

Using α-TubK40me3 pAb and mAb, additional functional studies to identify methylated α-tubulin readers and further explore the role of methylated α-tubulin in mitosis, and possibly other processes, can be done. In this regard, several functions for other microtubule PTMs have recently been discovered.\(^{1,2,17,18}\) For example, detyrosinated microtubules guide centromere protein E (CENP-E)–dependent chromosome congression during mitosis, whereas tyrosinated astral microtubules lead dynein-dependent peripheral chromosome movements to spindle poles.\(^{22,23}\) Polyglutamylation induces spastin-dependent microtubule disassembly biaxially to glutamate number on tubulin.\(^{24,25}\) The discovery of α-tubulin methylation as a new PTM of microtubules and the availability of our unique antibodies, which are the first generated against this methyl mark, open new avenues for identifying the readers of this mark and exploring further the role of microtubule methylation during mitosis, as well as the effect of loss of SETD2 in tumor cells.

**Materials and methods**

**Cell culture**

Immortalized Setd2\(^{\text{flx/flx}}\) MEFs were transfected with an ER-Cre vector expressing Cre recombinase fused with a mutated ligand-binding domain for the human estrogen receptor (ER-Cre). Cells expressing this ER-Cre were cultured in phenol-red free media (DMEM, high glucose, HEPES, no phenol red (Thermo Fisher Scientific, 21063029)) supplemented with sodium pyruvate (Thermo Fisher Scientific, 11360070) and GlutaMAX (Thermo Fisher Scientific, 35050061), to prevent any spontaneous activation of the Cre recombinase by endogenous compounds found in phenol red containing media. Stable cell lines expressing ER-Cre were generated by selection using 5 μg/ml blasticidin S (Thermo Fisher Scientific, A1113903). Parental Setd2\(^{\text{flx/flx}}\) MEFs treated with 4-hydroxytamoxifen (the active metabolite of tamoxifen, Sigma-Aldrich, H7904) and Setd2\(^{\text{flx/flx}}\) MEFs transfected with ER-Cre, treated with vehicle (0.01% ethanol) were used as controls. Setd2\(^{\text{flx/flx}}\) MEFs expressing ER-Cre were treated with 3μM 4-hydroxytamoxifen for 3 to 5 d for efficient Setd2 knockout. SETD2-null 786–0 (human renal cell carcinoma (RCC)) cells were generated using TAL effector nucleases targeted to exon 3 of SETD2.\(^{19}\) The truncated SETD2 (tSETD2) construct was subcloned into the pNDUCER20 vector.\(^{26}\) Stable cell lines expressing tSETD2 were generated as described.\(^ {19}\) MISSION\(^{\text{TM}}\) lentiviral shRNAs to GFP and SETD2 were purchased from Sigma and validated as described previously.\(^{27}\) Knockdown efficiency was examined by western blot analyses using SETD2 (1:1,000, Sigma, HPA042451) or α-tubulin (1:2,000, Santa Cruz Biotechnology, sc-32293) antibodies at 4 d after infection into 786–0 cells. The shRNA sequences were:

| shGFP | CCGGTACAACAGCCACAAGCTATCTCAGAGATAGCTGTTGCTGTTGTA TTTTTT |
|-------|--------------------------------------------------------|
| shSETD2 | CCGGCTTGAGAATGATGATAATTCTCGGAGTTCTACCATCTCTTCCAG #1 |
| shSETD2 | CCGGGGCTATGACTCTTCTGTTTACCTCGAGATTGAAACGAGTATGAGG #2 |
| shSETD2 | CCGCGAGGAAAGCAGCGGTAATAACTCGAGTTTTAGCGCTGTCTCCTT #3 |
| shSETD2 | CCGCCGAAGTAACGGCCCTCTCACTCGAGTTGAGGGAGGCTTACTTG CTTTTT |

**Generation of anti-α-TubK40me3 antibodies**

Twenty mg of the α-TubK40me3 pAb was produced in rabbits using trimethylated K40 peptide (Ac-GQMPSD-Kme3-TIGGGDC-amide) conjugated to KLH as the immunogen (Covance). α-TubK40me3 specific antibody was purified using serial columns coupled with unmethylated and trimethylated K40 peptide (Fig. 1B and 1C) using industry standard procedures (Covance). Hybirdoma cell lines from immunized rabbits were generated by Abcam, and grown in growth media (RPMI + 10% FBS + 2 vials of Abcam Rabbit Hybridoma Supplement A 40 ml each in 1000 ml media, 55 μM 2-mercaptoethanol). For the production of monoclonal antibodies, the hybridoma cell lines were expanded to 70–80% confluency in a T150 flask and cultured in low-serum (2.5% FBS) medium containing antibiotic/antimycotic (40 ml per 1L glutamax) for 3 d. After 3 days, the media was switched to serum-free media (SFM, Irvine Scientific) and the cells cultured for 10 additional days. After 10 days, the supernatant media was collected and concentrated using Amicon Ultra-15 centrifugal filter. This procedure yielded 200 μl antibody solution with protein concentration of ~2 μg/μl from 15 ml of media.

**Peptide dot blot analysis**

Unmodified, monomethyl (K40me1), dimethyl (K40me2), trimethyl (K40me3) and acetyl (K40ac) lysine K40 peptides (biotinylated at C-termini) of α-tubulin (0.2 μg and 2 μg or 1 μg) were dot-blotted on nitrocellulose membranes and immunoblotted using the α-TubK40me3 mAb (1:50), pAb (1:1,000), and acetylated K40 α-tubulin antibodies (1:1,000, Santa Cruz Biotechnology, sc-23950). After binding for overnight at 4 °C, the membranes were bound using goat anti-rabbit IgG-HRP antibody (1:5,000, Santa Cruz Biotechnology, sc-2004) for 2 hours at RT.

**Peptide competition assay**

Mitotic HeLa S3 cells were collected by gentle pipetting and lysed in phosphate-buffered saline (PBS) with 1% Triton X-100 to collect an enriched cytoplasmic fraction (Fig. 2B). Nucleic fraction was removed by centrifugation at 10,000 g for 5 min. Cell extracts (30 μg) were run on 6 lanes of a 15% SDS-PAGE gel and transferred on polyvinylidene difluoride membrane. Each lane was cut and immunoblotted using polyclonal α-TubK40me3 antibody (1μg/ml, 1:1,000), concentrated
monoclonal antibody solutions (clone 18, 1:50), or α-tubulin antibody (1:2,000) pre-incubated with/without unmodifed, K40me1, K40me2, K40me3 or K40ac peptide of α-tubulin at a ratio of 10:1 (10 μg/ml of peptide was added to 1 μg/ml of antibody solution) for 30 min at RT. After binding overnight at 4 °C, the membranes were immunoblotted using goat anti-rabbit IgG-HRP antibody (1:5,000) (rabbit clone 18 mAb and α-TubK40me3 pAb) or goat anti-mouse IgG-HRP antibody (1:5,000, Santa Cruz Biotechnology, sc-2005) mouse α-tubulin antibody). The membranes were realigned prior to exposure to X-ray film.

**Immunocytochemistry**

Cells were cultured on coverslips and immediately fixed using pre-warmed 4% paraformaldehyde solution in PEM/PEG buffer (80 mM PIPES (pH7.0), 1 mM EGTA, 1 mM MgCl2, 4% w/v PEG 8000) at 37 °C for 30 mins (we failed to observe a robust signal for methylation of microtubules using a shorter, more conventional 15 min fixation), followed by permeabilization using a 0.5% Triton X-100 solution in PEM/PEG buffer. Cells were washed using PEM/PEG buffer and blocked in blocking buffer (5% skim milk in PEM/PEG buffer) for 1 hour at RT. Cells were incubated in blocking buffer using primary antibodies overnight at 4 °C. Primary antibodies were diluted as follows; mouse anti-K40 acetylated α-tubulin (1:4,000, Santa Cruz Biotechnology, sc-23950), α-TubK40me3 pAb (1 μg/ul, 1:4,000) and concentrated rabbit monoclonal antibody solutions (4 μg/ml). After five rounds of washing for 10 mins each using PEM/PEG buffer, cells were incubated for 1 hour in blocking buffer with corresponding secondary antibodies (Alexa fluor 546 goat anti-mouse IgG (H+L) (Thermo Fisher Scientific, A11030) and Alexa fluor 488 goat anti-rabbit (Thermo Fisher Scientific, A11034)) at a dilution of 1:5,000. Following more than 3 washes, (10 mins each) using PEM/PEG buffer, cells were then post-fixed to stabilize the signal for 10 mins using 4% paraformaldehyde in PEM/PEG buffer. Cells were counterstained using Hoechst 33342 (Thermo Fisher Scientific) for subsequent visualization of nuclei. Coverslips were mounted in SlowFade Gold Antifade Mountant (Thermo Fisher Scientific) and imaged using deconvolution and confocal microscopy (DeltaVision Elite (GE), Ti Eclipse (Nikon) or InCell6000 (GE)).

**Cell fractionation and immunoprecipitation**

Cell were lysed by gentle pipetting several times and incubated on ice for 10 mins in a cell fractionation buffer (320 mM sucrose, 10 mM Tris-HCl (pH8.0), 2 mM magnesium acetate, 0.1 mM EDTA, 0.5% NP-40, fresh 1mM dithiothreitol, protease inhibitor cocktail). Cytosplasmic fractions were collected after centrifugation at 1,000 g for 5 min (Fig. 5A and 5B). Methylated α-tubulin was immunoprecipitated using 700 μg of cytoplasmic protein and α-TubK40me3 pAb (10 μL, i.e., ~10 μg) or clone 18 (100 μl) with protein G magnetic beads (Thermo Scientific) using 1X cell lysis buffer (20 mM Tris-HCl (pH7.5), 150 mM NaCl, 1mM EDTA, 1 mM EGTA, 1% Triton X-100, and protease inhibitor cocktail (Sigma Aldrich)). The immunoprecipitated complex was washed using the cell lysis buffer. Following immunoprecipitation, the samples were subjected to 15% SDS-PAGE gel electrophoresis and subsequently immunoblotted using antibodies against α-tubulin (1:2000, overnight at 4°C). Goat anti-mouse IgG-HRP was used as the secondary antibody. Mitotic HeLa S3 cells were obtained by gentle pipetting and washed twice using growth medium. Non-mitotic cells were washed using PBS and scraped after removal of mitotic cells. Mitotic cells were cultured for an additional hour in fresh growth medium to induce cells to enter cytokinesis. These cells were subsequently harvested and the cytoplasmic fraction generated as above. The total HeLa S3 cell extracts were prepared by combining detached cells in the medium following gentle pipetting and the adherent cells. Cytoplasmic fractions were immunoblotted using α-TubK40me3 pAb and α-tubulin antibody.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed by the authors. Texas A&M University has entered into an agreement with Abcam to make the clone18 mAb commercially available.

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**References**

1. Janke C, Bulinski JC. Post-translational regulation of the microtubule cytoskeleton: mechanisms and functions. Nat Rev Mol Cell Biol 2011; 12:773-86; PMID:22086369; http://dx.doi.org/10.1038/nrm3227
2. Verhey KJ, Gaertig J. The tubulin code. Cell Cycle 2007; 6:2152-60; PMID:17786050; http://dx.doi.org/10.4161/cc.6.17.4633
3. Barra HS, Rodríguez JA, Arce CA, Caputto R. A soluble preparation from rat brain that incorporates into its own proteins ( 14 C)arginine by a ribonuclease-sensitive system and ( 14 C)tyrosine by a ribonuclease-insensitive system. J Neurochem 1973; 20:97-108; PMID:4687210; http://dx.doi.org/10.1111/j.1471-4159.1973.tb12108.x
4. Hallak ME, Rodríguez JA, Barra HS, Caputto R. Release of tyrosine from tyrosinated tubulin. Some common factors that affect this process and the assembly of tubulin. FEBS Lett 1977; 73:147-50; PMID:838053; http://dx.doi.org/10.1016/0014-5793(77)80968-X
5. Gundersen GG, Kalnins MH, Bulinski JC. Distinct populations of microtubules: tyrosinated and non-tyrosinated α-tubulin are distributed differently in vivo. Cell 1984; 38;779-89; PMID:6836617; http://dx.doi.org/10.1016/0092-6674(84)90273-3
6. L’Hernault SW, Rosenbaum JL. Chlamydomonas α-tubulin is post-translationally modified by acetylation on the epsilon-amino group of a lysine. Biochemistry 1985; 24:473-8; PMID:3919761; http://dx.doi.org/10.1021/bi00323a034
7. Epper BA. Rat brain microtubule protein: purification and determination of covalently bound phosphate and carbohydrate. Proc Natl Acad Sci U S A 1972; 69:2283-7; PMID:4506098; http://dx.doi.org/10.1073/pnas.69.8.2283
8. Edde B, Rossier J, Le Caer JP, Desbruères E, Gros F, Denoulet P. Posttranslational glutamylation of α-tubulin. Science 1990; 247:83-5; PMID:1967194; http://dx.doi.org/10.1126/science.1967194
9. Redeker V, Levilliers N, Schmitter JM, Le Caer JP, Rossier J, Adoutte A, Bre MH. Polyglycylation of tubulin: a posttranslational
modification in axonemal microtubules. Science 1994; 266:1688-91; PMID:7992051; http://dx.doi.org/10.1126/science.7992051

10. Kilmartin JV, Wright B, Milstein C. Rat monoclonal antitubulin antibodies derived by using a new nonsecreting rat cell line. J Cell Biol 1982; 93:576-82; PMID:6811596; http://dx.doi.org/10.1083/jcb.93.3.576

11. Cumming R, Burgoyne RD, Lytton NA. Immunocytochemical demonstration of α-tubulin modification during axonal maturation in the cerebellar cortex. J Cell Biol 1984; 98:347-51; PMID:6707095; http://dx.doi.org/10.1083/jcb.98.1.347

12. Piperno G, Fuller MT. Monoclonal antibodies specific for an acetylated form of α-tubulin recognize the antigen in cilia and flagella from a variety of organisms. J Cell Biol 1985; 101:2085-94; PMID:2415535; http://dx.doi.org/10.1083/jcb.101.6.2085

13. Paturle-Lafanechere L, Manier M, Trigault N, Pirolet F, Mazarguil H, Job D. Accumulation of delta 2-tubulin, a major tubulin variant that cannot be tyrosinated, in neuronal tissues and in stable microtubule assemblies. J Cell Sci 1994; 107(Pt 6):1529-43; PMID:7962195; ISSN: 0021-9533

14. Wolff A, de Nechaud B, Chillet D, Mazarguil H, Desbruyeres E, Audebert S, Edde B, Gros F, Demoulet P. Distribution of glutamylated α and β-tubulin in mouse tissues using a specific monoclonal antibody, GT335. Eur J Cell Biol 2003; 21:532-8; PMID:12692561; http://dx.doi.org/10.1038/nbt819

15. Shang Y, Li B, Gorovsky MA. Tetrahymena thermophila contains a conventional γ-tubulin that is differentially required for the maintenance of different microtubule-organizing centers. J Cell Biol 2002; 158:1195-206; PMID:12356684; http://dx.doi.org/10.1083/jcb.200205101

16. Rogowski K, van Dijk J, Magiera MM, Bosc C, Deloume JC, Bosson A, Peris L, Gold ND, Lacroix B, Bosch Grau M, et al. A family of protein-deglutamylating enzymes associated with neurodegeneration. Cell 2010; 143:564-78; PMID:21074048; http://dx.doi.org/10.1016/j.cell.2010.10.014

17. Yu I, Garnham CP, Roll-Mecak A. Writing and reading the tubulin code. J Biol Chem 2015; 290:17163-72; PMID:25957412; http://dx.doi.org/10.1074/jbc.R115.637447

18. Janke C. The tubulin code: molecular components, readout mechanisms, and functions. J Cell Biol 2014; 206:461-72; PMID:25135932; http://dx.doi.org/10.1083/jcb.201406055

19. Park YJ, Powell RT, Tripathi DN, Dere R, Ho TH, Blasius TL, Chiang YC, Davis IJ, Fahey CC, Hacker KE, et al. Dual chromatin and cytoskeletal remodeling by SETD2. Cell 2016; 166:950-62; PMID:27518565; http://dx.doi.org/10.1016/j.cell.2016.07.005

20. Wu CC, MacCoss MJ, Howell KE, Yates JR, 3rd. A method for the comprehensive proteomic analysis of membrane proteins. Nat Biotechnol 2003; 21:532-8; PMID:12692561; http://dx.doi.org/10.1038/nbt819

21. Goping IS, Sawchuk T, Underhill DA, Bleackley RC. Identification of γ-tubulin as a granzyme B substrate during CTL-mediated apoptosis. J Cell Sci 2006; 119:858-65; PMID:16495481; http://dx.doi.org/10.1242/jcs.02791

22. Barisic M, Silva e Sousa R, Tripathy SK, Magiera MM, Zaytsev AV, Pereira AL, Janke C, Grishchuk EL, Maiato H. Mitosis. Microtubule detyrosination guides chromosomes during mitosis. Science 2015; 348:799-803; PMID:25908662; http://dx.doi.org/10.1126.science.aaa5175

23. Barisic M, Maiato H. The Tubulin Code: A navigation system for chromosomes during Mitosis. Trends Cell Biol 2016; PMID:27344407; http://dx.doi.org/10.1016/j.tcb.2016.06.001

24. Lacroix B, van Dijk J, Gold ND, Guizetti J, Aldrian-Herrada G, Rogowski K, Gerlich DW, Janke C. Tubulin polyglutamylation stimulates spastin-mediated microtubule severing. J Cell Biol 2010; 189:945-54; PMID:20530212; http://dx.doi.org/10.1083/jcb.201001024

25. Valenstein ML, Roll-Mecak A. Graded control of microtubule severing by tubulin glutamylation. Cell 2016; 164:911-21; PMID:26875866; http://dx.doi.org/10.1016/j.cell.2016.01.019

26. Meerbrey KL, Hu G, Kessler JD, Roarty K, Li MZ, Fang JE, Herschkowitz JI, Burrows AE, Ciccio A, Sun T, et al. The pINDUCER lentiviral toolkit for inducible RNA interference in vitro and in vivo. Proc Natl Acad Sci USA 2011; 108:3665-70; PMID:21307310; http://dx.doi.org/10.1073/pnas.1019736108

27. Ho TH, Park YJ, Zhao H, Tong P, Champion MD, Yan H, Monzon FA, Hoang A, Tamboli P, Parker AS, et al. High-resolution profiling of histone h3 lysine 36 trimethylation in metastatic renal cell carcinoma. Oncogene 2016; 35:1565-74; PMID:26073078; http://dx.doi.org/10.1038/onc.2015.221