



TTLL10 can perform tubulin glycylation when co-expressed with TTLL8

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ABSTRACT

Tubulin can undergo unusual post-translational modifications, glycylation and glutamylation. We previously failed to find glycylase (glycine ligase) for tubulin while identifying TTLL10 as a polyglycylase for nucleosome assembly protein 1. We here examine whether TTLL10 performs tubulin glycylation. We used a polyclonal antibody (R-polygly) raised against a poly(glycine) chain, which does not recognize monoglycylated protein. R-polygly strongly reacted with mouse tracheal cilia and axonemal tubulins. R-polygly detected many proteins in cell lysates co-expressing TTLL10 with TTLL8. Two-dimensional electrophoresis revealed that the R-polygly-reactive proteins included α - and β -tubulin. R-polygly labeling signals overlapped with microtubules. These results indicate that TTLL10 can strongly glycyrate tubulin in a TTLL8-dependent manner. Furthermore, these two TTLL proteins can glycyrate unidentified 170-, 110-, 75-, 40-, 35-, and 30-kDa acidic proteins.

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1. Introduction

Microtubules undergo a variety of post-translational modifications (PTMs), including highly unusual modifications—detyrosination/tyrosination [1,2], glutamylation [3], and glycylation [4]—as well as common modifications such as acetylation [5] and phosphorylation [6]. Recently, the physiological roles of the modifications are beginning to be understood. Glutamylase of α -tubulin is required for trafficking of the KIF1A kinesin motor [7], while that of β -tubulin is essential for neurite growth [8]. The level of tubulin glutamylation is likely to affect ciliary motility [9,10]. Tyrosination of α -tubulin is essential for CLIP170 to enter growing neurites and plays a pivotal role in normal brain development [11]. Tyrosination of α -tubulin has also been demonstrated to regulate the recruitment of CAP-Gly proteins to microtubule plus ends [12]. More recently, we have demonstrated that α -tubulin tyrosination navigates the KIF5 kinesin motor to axons [13].

Tubulin acetylation enhances the interaction of KIF5 with microtubules, promoting KIF5-dependent cargo transport [14]. A more recent report demonstrates that tubulin acetylation plays an important role in the maturation of cortical projection neurons [15].

This understanding of PTM's physiological roles can be attributed to identification of the modifying enzymes. Tyrosination is mediated by tubulin tyrosine ligase (TTL) [16,17]. A number of researchers, including those of our research group, have recently identified enzymes that perform tubulin glutamylation. These enzymes belong to a family of proteins characterized by a domain homologous to TTL [9]. A TTL-like (TTLL) protein, TTLL7 is a highly specific enzyme that performs β -tubulin polyglutamylase [8,18]. Tubulin glutamylase activities have been detected in other TTLL proteins, TTLL4, 5, 6, 11, and 13, which have close phylogenetic relations with TTLL7 [19]. A large enzyme complex containing TTLL1 and a subunit, PGs1, is also essential for α -tubulin glutamylation in the brain [7]. An acetyltransferase for α -tubulin has been identified more recently [14]. Despite this remarkable progress, the enzyme(s) mediating tubulin glycylation have not yet been identified, although we have succeeded in identifying TTLL10 as a glycylase for nucleosome assembly protein 1 (NAP1) [20]. Here, we investigate whether TTLL10 also has tubulin glycylation activity.

Abbreviations: NAP1, nucleosome assembly protein 1; PTM, post-translational modification; TTL, tubulin tyrosine ligase; TTLL, TTL-like; R-polygly, anti-polyglycine polyclonal antibody; mAb, monoclonal antibody; cDNA, complementary DNA; PCR, polymerase chain reaction; PBS, phosphate-buffered saline

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2. Materials and methods

2.1. Antibodies

Both the anti-polyglycine polyclonal antibody (R-polygly), which was generated by using Cys-(Gly)₉ as an immunogen [21], and the monoclonal antibody (mAb) 4A8 for NAP1 [22] were obtained gratis from external sources. In addition, the following antibodies were obtained commercially: anti- α -tubulin (mAb DM1A), anti- β -tubulin (mAb Tub2.1), anti-acetylated-tubulin (mAb 6-11B-1), anti-FLAG[®] tag (mAb M2) from Sigma-Aldrich, St. Louis, MO; anti-GFP polyclonal antibody (Medical & Biological Laboratories, Nagoya, Japan); Alexa Fluor[®]-conjugated secondary antibodies for immunofluorescent analyses (Invitrogen, Carlsbad, CA); and horseradish peroxidase-conjugated secondary antibodies for immunoblotting (Jackson ImmunoResearch Laboratories, West Grove, PA).

2.2. Plasmids

Complementary DNA (cDNA) containing whole coding sequences of murine TTLL8 and TTLL10 was cloned by polymerase chain reaction (PCR) as described in a previous study [20]. Primers were designed using reference sequences published in the NCBI database. Instead of *Taq*, *Pfx* (Invitrogen), which possesses a proof-reading activity, was used in PCR. Amplified coding regions were inserted in pCMV-Tag 5 vector (Stratagene, La Jolla, CA) or pEGFP-C vector (Clontech Laboratories, Mountain View, CA). Sequences were verified with the sequencing equipment Prism[®] 3100 (Applied Biosystems, Foster City, CA) using the BigDye[®] Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems).

2.3. Cell culture

COS7 cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum (Hyclone, Logan, UT). Cells were seeded on pieces of thin cover glass (Matsunami, Osaka, Japan) at half confluency. Plasmids were transfected using Lipofectamine[™] 2000 (Invitrogen) according to the manufacturer's instructions. Cells were harvested for immunoblotting or fixed for immunocytochemistry at 24 h.

2.4. Immunohistochemistry

The trachea of an anesthetized adult mouse was dissected and fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) at 37 °C, followed by overnight fixation at 4 °C. After fixation, the trachea was immersed in 20% sucrose solution overnight. It was then embedded in Tissue Tech O.C.T. compound (Sakura Finetek Japan, Tokyo, Japan) with dry ice. Coronal thin sections were cut at 10 μ m with cryosectioning equipment (Leica CM 1950; Leica Microsystems, Wetzlar, Germany). The sections were rinsed in PBS and then blocked with 5% goat serum in PBS containing 0.1% Triton X-100. The sections were incubated with the primary antibodies at the following dilution rates: R-polygly, 1:5000; 6-11B-1, 1:5000. The primary antibodies were labeled with the secondary antibodies at 4 μ g/ml. Fluorescence images were obtained with a laser scanning confocal microscope (Fluoview FV1000; Olympus, Tokyo, Japan) equipped with a 100 \times , 1.45 N.A. objective lens.

2.5. Two-dimensional electrophoresis

Harvested cells were directly dissolved in lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 40 mM DTT, 2% IPG buffer, pH range 3.5–5.0), containing Complete[™] EDTA-free protease inhibitor cocktail

(Roche, Indianapolis, IN). First and second-dimension electrophoresis was performed according to the manufacturer's instructions (GE Healthcare). Resolved protein spots were electrically transferred onto polyvinylidene fluoride membrane (Millipore, Beverly, MA).

2.6. Immunoblotting

Cells were lysed in a lysis buffer, 20 mM Tris-HCl, pH 7.4, containing 1% Triton X-100, and centrifuged supernatants were used. Axonemes of tracheal cilia were isolated, as described previously [23], with some modifications to scale down the procedures. In brief, dissected mouse tracheas were minced to small pieces and vortexed for 5 min in the cilia-removing buffer, 20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 10 mM CaCl₂, 1 mM EDTA, and 0.1% Triton X-100. The vortexed mixture was centrifuged at 1500 \times g for 2 min to remove tissue debris. The centrifuged supernatant was subjected to further centrifugation at 20 000 \times g for 5 min to precipitate ciliary axonemes. The precipitated axonemes were directly dissolved in the sample buffer.

In tubulin-separating electrophoresis, 95% purity SDS (Sigma) was used as described in an earlier research [8]. Separated proteins were transferred onto polyvinylidene fluoride membranes. The membranes were then blocked with 10% goat serum in Tris-buffered saline and probed with the primary antibodies at the following dilutions: R-polygly, 1:5000; DM1A, 1:10 000; Tub2.1, 1:2000; 4A8, 1:50; M2, 1:1000; and anti-GFP, 1:2000. The primary antibodies were labeled with the secondary antibodies, followed by detection with an enhanced chemiluminescence system (GE Healthcare, Waukesha, WI).

2.7. Immunocytochemistry

Cells were fixed with –25 °C cold methanol to leak cytoplasmic glycylation nucleosome assembly protein 1. The fixed cells were rinsed once with PBS, and then blocked with 5% goat serum in PBS containing 0.1% Triton X-100. The cells were incubated with the primary antibodies at the following dilution rates: R-polygly, 1:5000; DM1A, 1:10 000; and M2, 1:2000. The primary antibodies were labeled with the secondary antibodies at 2 μ g/ml. Zenon[®] antibody labeling kits (Invitrogen) were used when it was necessary to perform tricolor labeling with primary antibodies derived from the same hosts, e.g. co-labeling with DM1A and M2. Fluorescence micrographs were taken with the FV1000.

3. Results

3.1. R-polygly recognizes both α - and β -tubulins of mammalian ciliary axonemes

In previous work, we used a polyclonal antibody against poly(glycine) chains (R-polygly) [21] to identify TTLL10 as a glycylation for nucleosome assembly protein 1 (NAP1) [20]. To this day, it remains unclear whether R-polygly can recognize glycylation mammalian tubulin. Thus, we first examined whether R-polygly reacted with murine tracheal cilia, which contain polyglycylated tubulins [24] that were detected with a mAb, AXO49, specific for polyglycylated tubulin [25].

We first carried out an immunohistochemical analysis. R-polygly strongly labeled the mouse tracheal cilia (Fig. 1A; magenta). Double staining for acetylated tubulin revealed that R-polygly stained the middle region of cilia (Fig. 1A; magenta versus yellow). We next examined whether R-polygly detected axonemal tubulin by immunoblotting. R-polygly detected two bands (Fig. 1B). A clear band with stronger intensity migrated to almost the same position

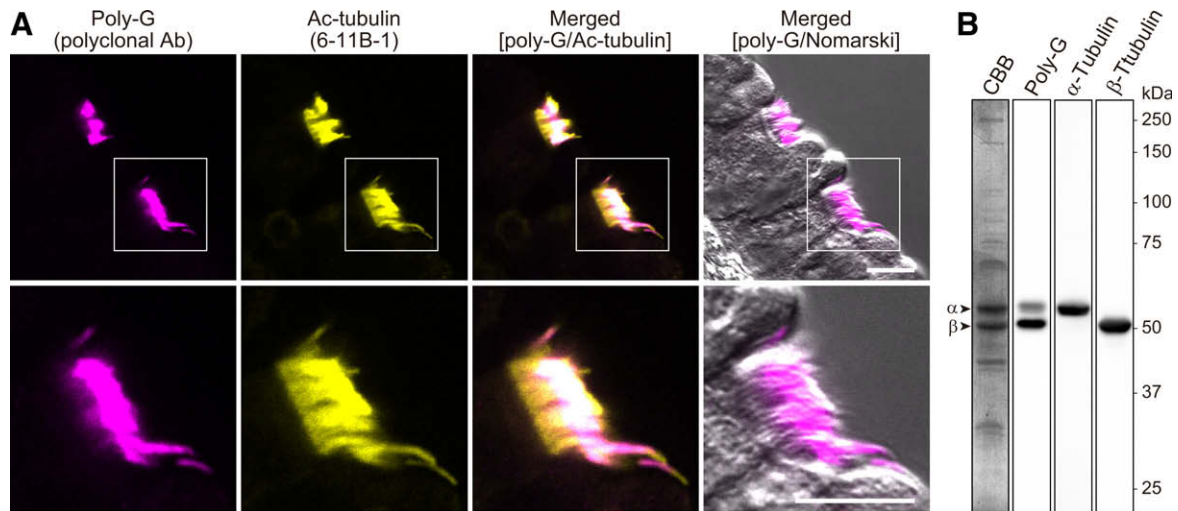


Fig. 1. R-polygly can bind to murine tracheal cilia and recognize axonemal tubulin. (A) Immunostaining of a mouse trachea section. Substances immunoreactive with R-polygly (magenta) were distributed throughout the cilia, which were labeled with anti-acetylated tubulin (Ac-tubulin; yellow). The boxed area was further magnified. Note that the R-polygly-labeled signal was detected particularly in the middle region of cilia. Scale bar, 5 μ m. (B) Immunoblotting of isolated ciliary axonemes. A membrane was repeatedly probed with R-polygly, anti- α -tubulin, and β -tubulin antibodies, after removing antibodies in each step. R-polygly raised a strong band with almost the same migration position as that of the β -tubulin band and an additional weaker band migrating at almost the same position as that of the α -tubulin band. Note that both R-polygly-detected bands migrated slightly slower than those of α - and β -tubulins.

as β -tubulin (Fig. 1B). The other, blurred band showing weaker intensity migrated to almost the same position as α -tubulin (Fig. 1B). The two R-polygly-detected bands were shifted slightly upward in comparison with the bands of α - and β -tubulins. This is reasonably consistent with a report that showed slightly slower migration of AXO49-detected α - and β -tubulins extracted from spermatozoa of many mammalian species including ram, boar, and mouse [26]. A blurred and weaker signal of polyglycylated α -tubulin has also been reported [26]. Taken together, these results indicate that R-polygly can recognize polyglycylated forms of both mammalian α - and β -tubulin; thus, the antibody is useful for the further investigation of whether TTLL10 can perform tubulin polyglycylation.

3.2. TTLL10 produces R-polygly-recognized α - and β -tubulins when co-expressed with TTLL8

Phylogenetic analysis revealed that TTLL3 and 8 were phylogenetically nearest to TTLL10 (Fig. 2A). Despite the close relationship between TTLL3/8 and TTLL10, we found that neither TTLL3 nor 8 produced signals detected with R-polygly [20]. Glutamylases are clearly divided into “mono”-glutamylase and “poly”-glutamylase [21]. Thus, it is reasonable to expect that tubulin glycylation is also separated into mono-glycylation and poly-glycylation. We hypothesized that TTLL3, 8, and 10 were able to produce a subpopulation of tubulin that could be detected with R-polygly under combinational expressions in cells.

We overexpressed TTLL10 tagged with a small artificial peptide, FLAG[®], along with EGFP-fused TTLL8 in COS7 cells to prove our hypothesis. Co-expression of TTLL10 and TTLL8 produced several protein bands that were detected by R-polygly; the major bands included 170-, 110-, 75-, 60-, 52-, 50-, 40-, 35-, and 30-kDa proteins (Fig. 2B). The protein bands, except for the 60- and 50-kDa proteins, were specifically detected in the cell lysates co-expressing TTLL10 and TTLL8 (Fig. 2B). The 52-kDa protein band migrated at almost the same position as the α -tubulin band (Fig. 2B; arrowhead). TTLL8 produced no extra band, as we have previously reported [20].

We carried out two-dimensional electrophoreses with a narrow pH range, pH 3.0–5.6, to clarify whether tubulins are included in

the proteins that are detected specifically by R-polygly in cell lysates co-expressing TTLL10 and TTLL8. Almost all proteins detected by R-polygly migrated to highly acidic positions (Fig. 2C). Both α - and β -tubulin spots were strongly labeled by R-polygly in the cell lysates expressing both TTLL10 and TTLL8 (Fig. 2D; arrowheads). In comparison with the case in untransfected control cells, R-polygly detected faint spots corresponding to α - and β -tubulins even in the cell lysates expressing TTLL10 alone (Fig. 2D; asterisks). In contrast, R-polygly reacted to the 60- and 50-kDa protein spots that were more acidic than tubulins in TTLL10-expressing cell lysates independently of the presence of TTLL8 (Fig. 2D; arrows). Those spots were labeled with the mAb 4A8, which was generated against NAP1 [22]. These results indicate that TTLL10 can effectively produce polyglycylated α - and β -tubulins in cells where TTLL8 is also present, while it can polyglycylate NAP1 without TTLL8 expression as we have previously reported [20].

3.3. TTLL10 produces R-polygly-recognized microtubules when co-expressed with TTLL8

We next examined whether the co-expression of TTLL10 with TTLL8 raised strong signals in immunocytochemical analysis as well. Co-expression of TTLL10 and TTLL8 produced microtubule-like strong signals that were labeled with R-polygly (Fig. 3A; magenta). In addition to the microtubule-like staining, R-polygly detected nuclear and strong nucleolar components in the cells expressing TTLL10 and TTLL8 (Fig. 3A; arrowheads). Single expression of either TTLL8 or TTLL10 raised neither microtubule-like nor nuclear immunoreactivity (Fig. 3A).

Finally, we examined whether the microtubule-like structure detected with R-polygly actually overlapped with microtubules. The cytoplasmic cytoskeletal signals completely overlapped with microtubules visualized with DM1A (Fig. 3B; compare magenta with yellow). Taken together, these findings indicate that TTLL10 can produce polyglycylated tubulin in a TTLL8-dependent manner.

4. Discussion

In this study, we found that the R-polygly reacts to mouse tracheal cilia and axonemal tubulins (Fig. 1), which have been re-

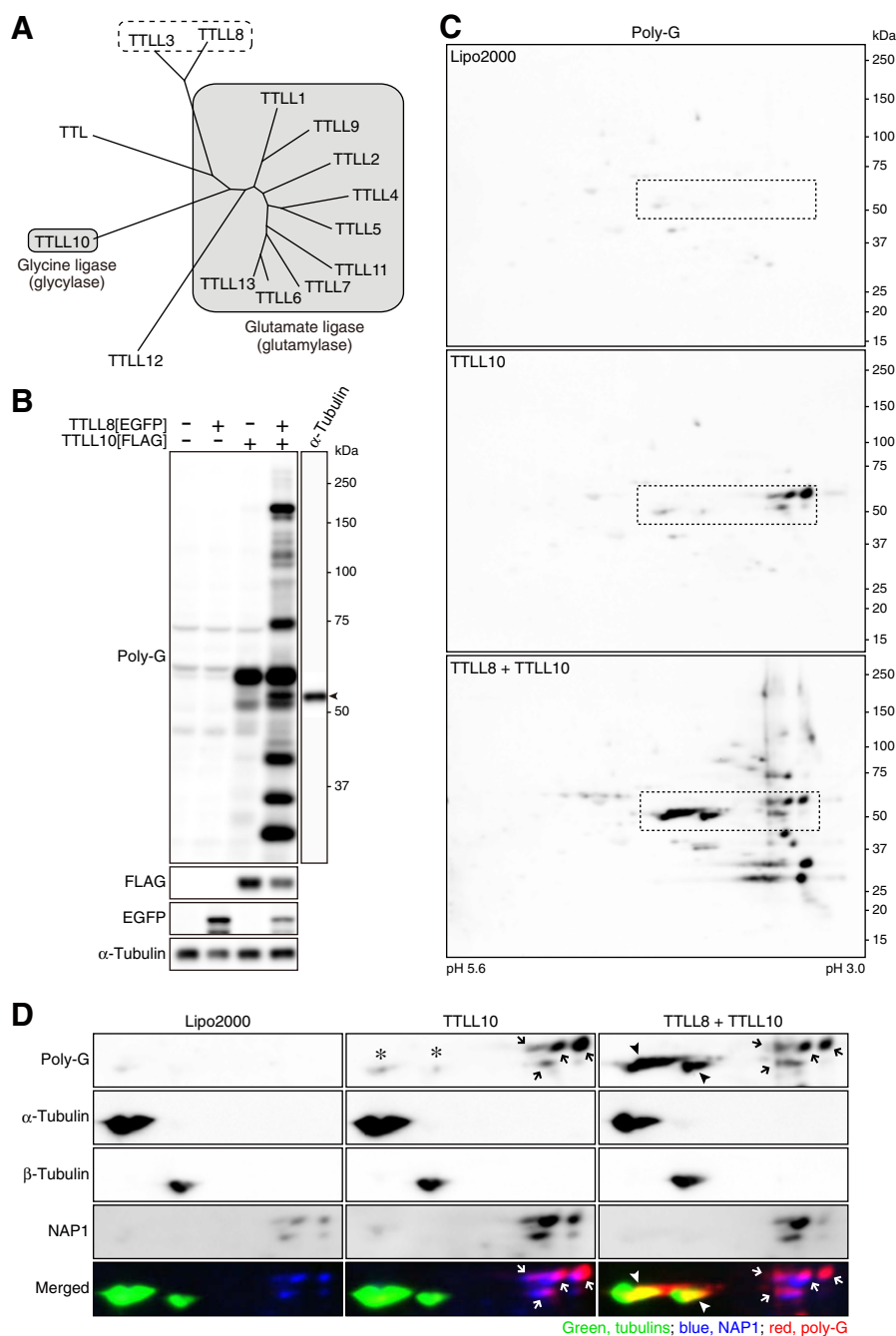


Fig. 2. Co-expression of TTLL10 with TTLL8 produces strong polyglycylation signals. (A) Phylogenetic tree of mouse TTLL proteins. Identified glutamylases and a glycylase are highlighted in a shaded box. TTLL3 and 8 are boxed with a dashed line. (B) Immunoblotting of COS7 lysates. The R-polygly antibody detected several protein bands with strong signal intensities in lysates of cells expressing both TTLL10 and TTLL8. One of the detected bands migrated at the same position as α -tubulin (arrowheads). The strong 60-kDa and weak, blurred 50-kDa bands were also detected even in the sample possessing only TTLL10. (C, D) Two-dimensional electrophoresis. Two-dimensionally resolved proteins were detected with R-polygly. (C) In the cell lysates expressing both TTLL10 and TTLL8, many acidic proteins were detected. The area boxed with a dashed line is enlarged in panel D. (D) The blots were repeatedly reprobated with anti- α -tubulin, β -tubulin, and NAP1 antibodies, after removing antibodies in each step. The bottom panels are merged images of pseudocolor photographs of each blot. Co-expression of TTLL10 and TTLL8 generated tubulin spots that were strongly detected by R-polygly (arrowheads). NAP1 spots were detected by R-polygly in both cell lysates expressing TTLL10 alone and co-expressing TTLL10 with TTLL8 (arrows). Note that the intensities of spots corresponding to tubulin increased slightly even in TTLL10-only cell lysates (asterisks).

ported to have polyglycylation [24]. By two-dimensional electrophoresis, we demonstrated that both α - and β -tubulins are strongly recognized by R-polygly when TTLL10 and TTLL8 co-exist (Fig. 2). We have also found that R-polygly reacts to cytoskeletal structures that completely overlap with microtubules when TTLL10 is co-expressed with TTLL8 (Fig. 3). Given that the R-polygly was generated against a chain composed of nine glycines [21], our findings indicate that TTLL10 can produce poly-

glycylation forms of both α - and β -tubulin in cells in which TTLL8 also exists.

Our interpretation postulates that tubulin glycylation can be separated into two steps: initiation and elongation. This concept of two-step modification is plausible based on the demonstration that tubulin glutamylation is clearly divided into two steps [19]. In this scenario, TTLL8 may function as a putative initiase that initiates the modification by attaching the first glycine to tubulin. It is

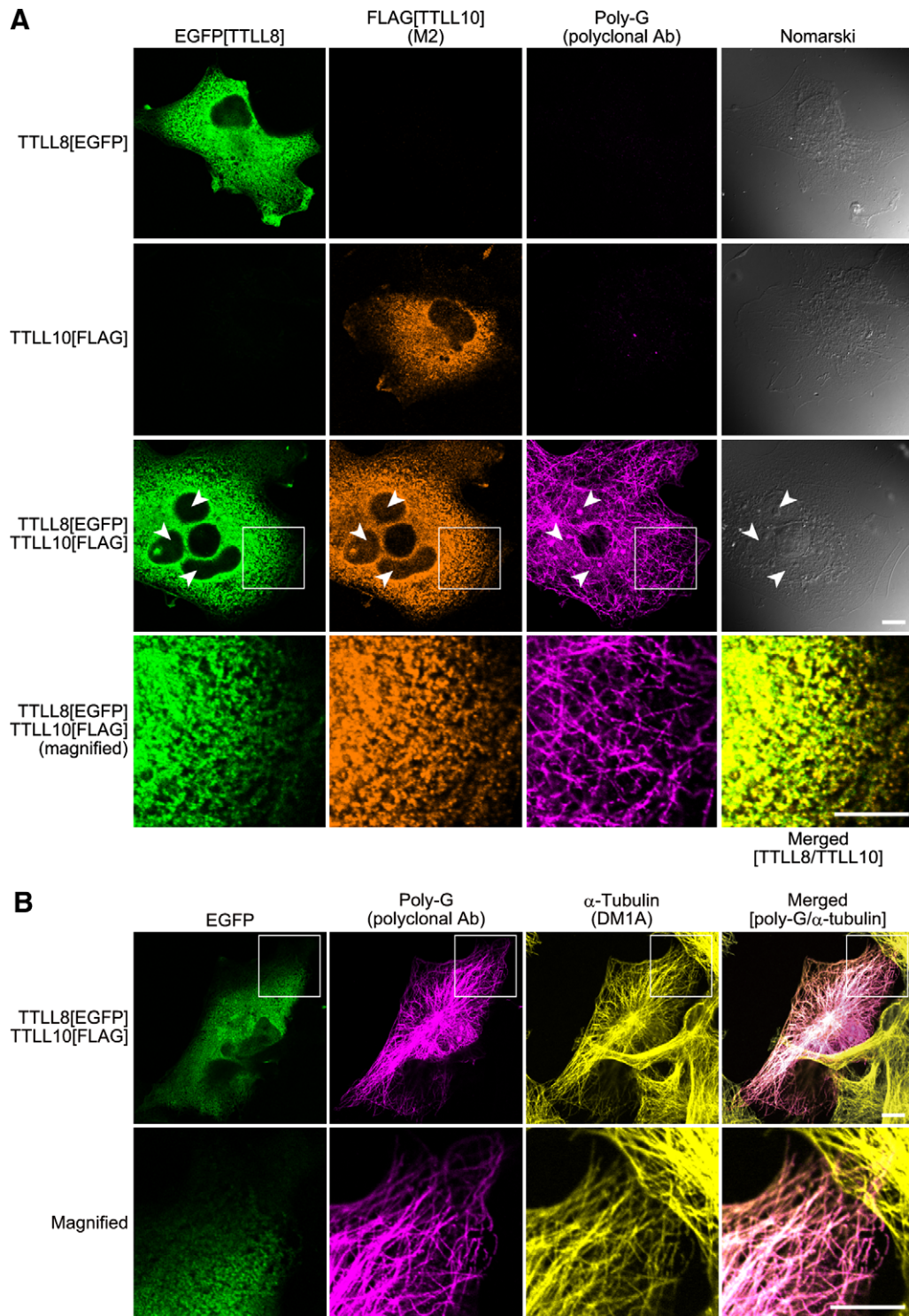


Fig. 3. Polyglycylation signal overlaps microtubule in cells co-expressing TTLL8 and TTLL10 (magenta of the third row). (A) R-polygly detected cytoskeletal structures in cells expressing TTLL8 and TTLL10 (magenta of the third row). The arrowheads indicate nuclear and nucleolar components detected by R-polygly. The bottom row is a magnified photomicrograph of the boxed region in the third row. Note that the signals of EGFP (green) overlapped well with FLAG signals (orange), making a merged yellow signal. Scale bar, 10 μ m. (B) The cytoskeletal structure detected with R-polygly overlapped with microtubules (magenta versus yellow). The bottom row is a magnified image of the boxed area of the top row. Due to the difficulty of quadruple staining in our detection system, the staining of the FLAG signal was not available in this sample. Scale bar, 10 μ m.

well understood that a TTLL protein close to TTLL10 performs glycylation, given that all identified glutamylases, TTLL1, 4, 5, 6, 7, 9, 11, and 13 are phylogenetically close (Fig. 2A) [19]. The expression pattern of TTLL8 also supports this idea. TTLL8 is expressed intensively in the testis [19], which contains a huge number of sperm flagella that are rich in glycylation tubulins [26]. TTLL10 may function as

an elongase that elongates the glycine chain by adding multiple glycines to the first added glycine residue. The functional interaction between TTLL8 and TTLL10 could be explained by the strong colocalization of TTLL8 and TTLL10 (Fig. 3A). Nonetheless, we still cannot exclude the possibility that TTLL8 functions as an enhancer of TTLL10 or as only a co-factor for TTLL10.

Tubulin glycylation is reported to have a crucial involvement in flagellar and ciliary function. Sea urchin sperm motility is inhibited by the injection of monoclonal antibodies to mono- or polyglycylated tubulin [26]. Injection of anti-monoglycylated tubulin antibody also impairs ciliary motility in cultured human nasal epithelia [27]. Recent genetic studies have provided stronger evidence in support of the importance of β -tubulin glycylation in ciliary motility and cell division [28]. *Tetrahymena*, with a mutation in the glycylation sites of β -tubulin, shows a deleterious structural abnormality in its ciliary axonemes: the absence of central double microtubules [29]. A microtubule-severing enzyme, katanin, is a molecular candidate to account for the phenotype [30]. Cross-talk between C-terminal polymeric modifications of α - and β -tubulin could underlie the katanin-regulated microtubule dynamics of axonemes [31]. It is anticipated that knockout organisms deficient in glycylation and glutamylases will yield further evidence at the molecular level to support these deductions.

In Fig. 1A, we found that R-polygly labeled the middle region of cilia. The staining pattern was somewhat different from that of polyglycylated tubulin detected with another antibody, AXO49. AXO49 stained only the proximal region of gerbil tracheal cilia [24]. This difference might be attributed to the different species tested in the study and our study. The state of axonemal tubulin polyglycylation differs between species [26,27]. In addition, the developmental stages of tracheal cilia could produce such variations [24]. The fine-tuned regulation of glycylation and glutamylation during ciliary development requires further study.

In Fig. 1B, axonemal β -tubulin was more strongly detected by R-polygly than α -tubulin. A similar finding was reported in a work where the polyglycylated tubulin-specific mAb AXO49 is used [26]. This result is reasonably consistent with the observation of more intensive glycylation of β -tubulin in bull sperm axonemes [32], and could be attributed to the presence of more glycylation sites in β -tubulin than in α -tubulin [28]. In addition, tubulin bands detected by R-polygly were shifted slightly upward in comparison with the corresponding bands of α - and β -tubulins. The upward shift probably reflects extensive modifications of axonemal tubulins by glycylation and glutamylation [26,27]. The lack of detection of such highly modified tubulins could arise from a limited amount of the modified tubulins or a decrease in antibody accessibility to modified tubulins [33]. This concept can explain our finding that R-polygly does not react with all tubulin spots in two-dimensional electrophoresis (Fig. 2D; merged image).

In Fig. 2B, we found that without TTL8 expression, TTL10 produced a blurred 50-kDa protein band that was recognized by R-polygly. Whether the 50-kDa protein is tubulin or not is an important question. Our two-dimensional electrophoresis results have revealed that the majority of the 50-kDa proteins are NAP1 or NAP-related species (Fig. 2D). This is substantially consistent with our previous work, where a 50-kDa protein immunopurified with R-polygly was recognized by the anti-NAP1 antibody [20]. However, TTL10 alone probably produces polyglycylated tubulins, albeit to a small extent, because tubulin spots were faintly labeled by R-polygly even in cells expressing TTL10 alone (Fig. 2D). Given that we consider TTL10 to be an elongase of tubulin polyglycylation, the most plausible explanation is that COS7 cells contain a limited amount of monoglycylated tubulins, which can be subjected to polyglycylation. Indeed, monoglycylated tubulins have been detected in mammalian cytoplasmic microtubules [24]. A similar situation is reported in polyglutamylation, where a small amount of slightly glutamylated tubulins are polyglutamylated by an elongase, TTL6, without co-expression of the initiase, TTL5 [19]. Nevertheless, there still remains the interesting question of why TTL10 shows different glycylation activity on tubulins and NAP1, i.e., it requires TTL8 to polyglycylate tubulin while it can polyglycylate NAP1 independently of TTL8. TTL10 could both ini-

tiate and elongate polyglycylation of NAP1. Alternatively, NAP1 might be endogenously monoglycylated, which could allow TTL10 to polyglycylate NAP1 effectively. This question will be addressed in future work.

Co-expression of TTL8 and TTL10 produced several protein bands strongly detected by R-polygly (Fig. 2B). Almost all these proteins had highly acidic isoelectric points (Fig. 2C). This finding suggests that TTL10, in combination with TTL8, can polyglycylate many proteins other than tubulin and NAP1. This broad substrate specificity has already been reported for glutamylation by TTL4, which can glutamylate multiple proteins [34]. The majority of proteins glutamylated by TTL4 are nuclear proteins with acidic isoelectric points [34]. Similar or identical proteins undergo glycylation by co-expression of TTL8 and TTL10. In our experiment, R-polygly detected nuclear and nucleolar proteins in cells co-expressing TTL8 and TTL10 (Fig. 3A; arrowheads). Further studies should focus on identifying the additional glycylatable proteins.

In summary, we have demonstrated that TTL10 can modify tubulin in addition to nucleosome assembly protein 1 when TTL8 is also present. The enzymatic properties of TTL8 and TTL10 require further study to ascertain whether TTL8 or TTL10 can function as an initiase (monoglycylase) or elongase (polyglycylase) and act on other glycylatable proteins such as 14-3-3 [35] and heat shock proteins [36].

Acknowledgments

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References

- [1] Argaraña, C.E., Barra, H.S. and Caputto, R. (1978) Release of [14 C]tyrosine from tubulin-[14 C]tyrosine by brain extract. Separation of a carboxypeptidase from tubulin-tyrosine ligase. *Mol. Cell Biochem.* 19, 17–21.
- [2] Gundersen, G.G., Kalnoski, M.H. and Bulinski, J.C. (1984) Distinct populations of microtubules: tyrosinated and nontyrosinated α tubulin are distributed differently in vivo. *Cell* 38, 779–789.
- [3] Eddé, B., Rossier, J., Le Caer, J.P., Desbrières, E., Gros, F. and Denoulet, P. (1990) Posttranslational glutamylation of α -tubulin. *Science* 247, 83–85.
- [4] Redeker, V., Levilliers, N., Schmitter, J.M., Le Caer, J.P., Rossier, J., Adoutte, A. and Bré, M.H. (1994) Polyglycylation of tubulin: a posttranslational modification in axonemal microtubules. *Science* 266, 1688–1691.
- [5] L'Hernault, S.W. and Rosenbaum, J.L. (1985) *Chlamydomonas* α -tubulin is posttranslationally modified by acetylation on the ϵ -amino group of a lysine. *Biochemistry* 24, 473–478.
- [6] Eipper, B. (1974) Properties of rat brain tubulin. *J. Biol. Chem.* 249, 1407–1416.
- [7] Ikegami, K., Heier, R.L., Taruishi, M., Takagi, H., Mukai, M., Shimma, S., Taira, S., Hatanaka, K., Morone, N., Yao, I., Campbell, P.K., Yuasa, S., Janke, C., MacGregor, G.R. and Setou, M. (2007) Loss of α -tubulin polyglutamylation in ROSA22 mice is associated with abnormal targeting of KIF1A and modulated synaptic function. *Proc. Natl. Acad. Sci. USA* 104, 3213–3218.
- [8] Ikegami, K., Mukai, M., Tsuchida, J.L., Heier, R.L., MacGregor, G.R. and Setou, M. (2006) TTL7 is a mammalian β -tubulin polyglutamylase required for growth of MAP2-positive neurites. *J. Biol. Chem.* 281, 30707–30716.
- [9] Janke, C., Rogowski, K., Wloga, D., Regnard, C., Kajava, A.V., Strub, J.M., Temurak, N., van Dijk, J., Boucher, D., van Dorsselaer, A., Suryavanshi, S., Gaertig, J. and Eddé, B. (2005) Tubulin polyglutamylase enzymes are members of the TTL domain protein family. *Science* 308, 1758–1762.
- [10] Wloga, D., Rogowski, K., Sharma, N., van Dijk, J., Janke, C., Eddé, B., Bré, M.H., Levilliers, N., Redeker, V., Duan, J., Gorovsky, M.A., Jerka-Dziadosz, M. and Gaertig, J. (2008) Glutamylation on α -tubulin is not essential but affects the assembly and functions of a subset of microtubules in *Tetrahymena thermophila*. *Eukaryot. Cell* 7, 1362–1372.
- [11] Erck, C., Peris, L., Andrieux, A., Meissirel, C., Gruber, A.D., Vernet, M., Schweitzer, A., Saoudi, Y., Pointu, H., Bosc, C., Salin, P.A., Job, D. and Wehland, J. (2005) A vital role of tubulin-tyrosine-ligase for neuronal organization. *Proc. Natl. Acad. Sci. USA* 102, 7853–7858.

- [12] Peris, L., Thery, M., Fauré, J., Saoudi, Y., Lafanechère, L., Chilton, J.K., Gordon-Weeks, P., Galjart, N., Bornens, M., Wordeman, L., Wehland, J., Andrieux, A. and Job, D. (2006) Tubulin tyrosination is a major factor affecting the recruitment of CAP-Gly proteins at microtubule plus ends. *J. Cell Biol.* 174, 839–849.
- [13] Konishi, Y. and Setou, M. (in press) Tubulin tyrosination navigates the kinesin-1 motor domain to axons. *Nat. Neurosci.*, doi:10.1038/nn.2314.
- [14] Reed, N.A., Cai, D., Blasius, T.L., Jih, G.T., Meyhofer, E., Gaertig, J. and Verhey, K.J. (2006) Microtubule acetylation promotes kinesin-1 binding and transport. *Curr. Biol.* 16, 2166–2172.
- [15] Creppe, C., Malinowskaya, L., Volvert, M.L., Gillard, M., Close, P., Malaise, O., Laguesse, S., Cornez, I., Rahmouni, S., Ormenese, S., Belachew, S., Malgrange, B., Chapelle, J.P., Siebenlist, U., Moonen, G., Chariot, A. and Nguyen, L. (2009) Elongator controls the migration and differentiation of cortical neurons through acetylation of α -tubulin. *Cell* 136, 551–564.
- [16] Barra, H.S., Arce, C.A. and Argarana, C.E. (1988) Posttranslational tyrosination/detyrosination of tubulin. *Mol. Neurobiol.* 2, 133–153.
- [17] Ersfeld, K., Wehland, J., Plessmann, U., Dodemont, H., Gerke, V. and Weber, K. (1993) Characterization of the tubulin-tyrosine ligase. *J. Cell Biol.* 120, 725–732.
- [18] Mukai, M., Ikegami, K., Sugiura, Y., Takeshita, K., Nakagawa, A. and Setou, M. (2009) Recombinant mammalian tubulin polyglutamylase TTL7 performs both initiation and elongation of polyglutamylation on β -tubulin through a random sequential pathway. *Biochemistry* 48, 1084–1093.
- [19] van Dijk, J., Rogowski, K., Miro, J., Lacroix, B., Eddé, B. and Janke, C. (2007) A targeted multienzyme mechanism for selective microtubule polyglutamylation. *Mol. Cell* 26, 437–448.
- [20] Ikegami, K., Horigome, D., Mukai, M., Livnat, I., MacGregor, G.R. and Setou, M. (2008) TTL10 is a protein polyglucylase that can modify nucleosome assembly protein 1. *FEBS Lett.* 582, 1129–1134.
- [21] Duan, J. and Gorovsky, M.A. (2002) Both carboxy-terminal tails of α - and β -tubulin are essential, but either one will suffice. *Curr. Biol.* 12, 313–316.
- [22] Fujii-Nakata, T., Ishimi, Y., Okuda, A. and Kikuchi, A. (1992) Functional analysis of nucleosome assembly protein, NAP-1. The negatively charged COOH-terminal region is not necessary for the intrinsic assembly activity. *J. Biol. Chem.* 267, 20980–20986.
- [23] Hastie, A.T., Dicker, D.T., Hingley, S.T., Kueppers, F., Higgins, M.L. and Weinbaum, G. (1986) Isolation of cilia from porcine tracheal epithelium and extraction of dynein arms. *Cell Motil. Cytoskeleton* 6, 25–34.
- [24] Dossou, S.J., Bré, M.H. and Hallworth, R. (2007) Mammalian cilia function is independent of the polymeric state of tubulin glycylation. *Cell Motil. Cytoskeleton* 64, 847–855.
- [25] Bré, M.H., Redeker, V., Vinh, J., Rossier, J. and Levilliers, N. (1998) Tubulin polyglycylation: differential posttranslational modification of dynamic cytoplasmic and stable axonemal microtubules in paramecium. *Mol. Biol. Cell* 9, 2655–2665.
- [26] Bré, M.H., Redeker, V., Quibell, M., Darmanaden-Delorme, J., Bressac, C., Cosson, J., Huitorel, P., Schmitter, J.M., Rossler, J., Johnson, T., Adoutte, A. and Levilliers, N. (1996) Axonemal tubulin polyglycylation probed with two monoclonal antibodies: widespread evolutionary distribution, appearance during spermatozoan maturation and possible function in motility. *J. Cell Sci.* 109, 727–738.
- [27] Million, K., Larcher, J., Laoukili, J., Bourguignon, D., Marano, F. and Tournier, F. (1999) Polyglutamylation and polyglycylation of α - and β -tubulins during in vitro ciliated cell differentiation of human respiratory epithelial cells. *J. Cell Sci.* 112, 4357–4366.
- [28] Xia, L., Hai, B., Gao, Y., Burnette, D., Thazhath, R., Duan, J., Bré, M.H., Levilliers, N., Gorovsky, M.A. and Gaertig, J. (2000) Polyglycylation of tubulin is essential and affects cell motility and division in *Tetrahymena thermophila*. *J. Cell Biol.* 149, 1097–1106.
- [29] Thazhath, R., Liu, C. and Gaertig, J. (2002) Polyglycylation domain of β -tubulin maintains axonemal architecture and affects cytokinesis in *Tetrahymena*. *Nat. Cell Biol.* 4, 256–259.
- [30] Sharma, N., Bryant, J., Wloga, D., Donaldson, R., Davis, R.C., Jerka-Dziedzic, M. and Gaertig, J. (2007) Katanin regulates dynamics of microtubules and biogenesis of motile cilia. *J. Cell Biol.* 178, 1065–1079.
- [31] Redeker, V., Levilliers, N., Vinolo, E., Rossier, J., Jaillard, D., Burnette, D., Gaertig, J. and Bré, M.H. (2005) Mutations of tubulin glycylation sites reveal cross-talk between the C termini of α - and β -tubulin and affect the ciliary matrix in *Tetrahymena*. *J. Biol. Chem.* 280, 596–606.
- [32] Rüdiger, M., Plessmann, U., Rüdiger, A.H. and Weber, K. (1995) β -Tubulin of bull sperm is polyglycylation. *FEBS Lett.* 364, 147–151.
- [33] Bré, M.H., de Néchaud, B., Wolff, A. and Fleury, A. (1994) Glutamylated tubulin probed in ciliates with the monoclonal antibody GT335. *Cell Motil. Cytoskeleton* 27, 337–349.
- [34] van Dijk, J., Miro, J., Strub, J.M., Lacroix, B., van Dorsselaer, A., Eddé, B. and Janke, C. (2008) Polyglutamylation is a post-translational modification with a broad range of substrates. *J. Biol. Chem.* 283, 3915–3922.
- [35] Lalle, M., Salzano, A.M., Crescenzi, M. and Pozio, E. (2006) The *Giardia duodenalis* 14–3–3 protein is post-translationally modified by phosphorylation and polyglycylation of the C-terminal tail. *J. Biol. Chem.* 281, 5137–5148.
- [36] Xie, R., Clark, K.M. and Gorovsky, M.A. (2007) Endoplasmic reticulum retention signal-dependent glycylation of the Hsp70/Grp170-related Pgp1p in *Tetrahymena*. *Eukaryot. Cell* 6, 388–397.