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Site-Specific Chemistry on the Microtubule Polymer

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Supporting Information

ABSTRACT: Microtubules are hollow tube-like biological polymers required for transport in diverse cellular contexts and are important drug targets. Microtubule function depends on interactions with associated proteins and post-translational modifications at specific sites located on its interior and exterior surfaces. However, we lack strategies to selectively perturb or probe these basic biochemical mechanisms. In this work, by combining amber suppression-mediated non-natural amino acid incorporation and tubulin overexpression in budding yeast, we demonstrate, for the first time, a general strategy for site-specific chemistry on microtubules. Probes and labels targeted to precise sites on the interior and exterior surfaces of microtubules will allow analysis and modulation of interactions with proteins and drugs, and elucidation of the functions of post-translational modifications.

Site-specific modification of proteins with biochemical and biophysical probes allows unraveling dynamic properties that are crucial for function.[1] This approach provides complementary insights to those gained from static structural studies. In particular, microtubules, which are dynamic polymers composed of the highly conserved proteins α-and β-tubulin, provide tracks for intracellular transport and organization, rendering them essential for life in all eukaryotes.[2,3] Microtubules are also important targets for chemotherapeutic agents as disrupting their function blocks cell proliferation.[4] We now have good structural models for this polymer's organization and function;[5] however, advances in our understanding of the dynamic interactions of proteins and drugs with microtubules, and the role of post-translational modifications, have been limited as we have lacked good approaches for efficient and selective chemical-modification of tubulin and microtubules.

While the best-characterized microtubule-associated proteins (MAPs) interact with binding sites on the exterior surface of this polymer,[6] studies also suggest that the microtubule inner pore, measuring 15 nm in diameter, contains important sites for the binding of drugs[7] and proteins,[8,9] as well as sites of post-translational modification.[10] In order to comprehensively profile these interactions using fluorescent probes and cross-linkers, modulate interaction "hot spots"[11] with caged amino acids, and recapitulate post-translational modifications, it is critical to modify microtubules in a site- and topology-specific manner. To address this, we have devised a strategy that allows, for the first time, the incorporation of chemically modifiable residues at selected sites proposed to be on the interior or exterior of this complex biological polymer (Figure 1). Further, we demonstrate that probes can be introduced at these sites in the soluble and polymer forms of tubulin.

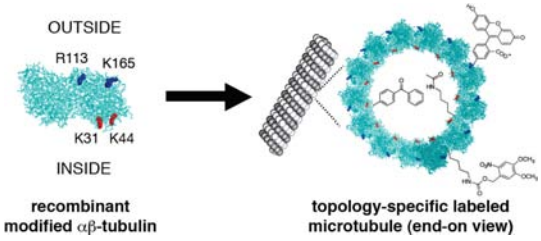

Figure 1. Site-specifically modifiable tubulin can be polymerized into microtubules. Biophysical and biochemical probes can be selectively linked to the interior or exterior surface of the tube-like polymer.

Standard methods for modifying $\alpha\beta$-tubulin with fluorescent probes and affinity handles have relied on a low-yielding protocol that involves nonspecific amide bond chemistry with polymer.[12] Cysteine modifications are even more restrictive and can block polymerization.[13] Tubulin tyrosine ligase can append modifiable derivatives of tyrosine to α-tubulin,[14] but is limited to modification of the carboxy terminus. Since these methods lack the generality and specificity to incorporate single modifications at any desired site, we focused on applying amber suppression[15]—an approach that has been successful in a variety of organisms including *Saccharomyces cerevisiae*.[16] This was critical for our study since *S. cerevisiae* permits the transient (but not constitutive) overexpression of $\alpha\beta$-tubulin,[17] whereas expression of tubulin in bacterial systems has failed to yield functional protein, likely due to bacteria lacking the chaperones required for proper tubulin folding.[18] Therefore, we chose to focus on expressing chemically modifiable forms of tubulin in budding yeast.

To generate site-specifically modifiable $\alpha\beta$-tubulin in yeast, we adapted the pyrrolysine amber suppression system developed by Chin and co-workers.[19] Prior studies using this system in *S. cerevisiae* have been limited to a proof-of-principle demonstration using the model protein hSOD, which can be expressed at high levels in yeast.[19] To probe the capabilities of amber suppression for the incorporation of non-natural amino acids into tubulin, which cannot be highly overexpressed,[17,20] we designed constructs encoding *S. cerevisiae* α-tubulin containing a TAG *amber* stop codon at different sites within the gene (Figure 2a). In addition, a hexa-histidine tag was included at the C-terminus to facilitate analysis of full-length protein expression levels and successful amber suppression. The

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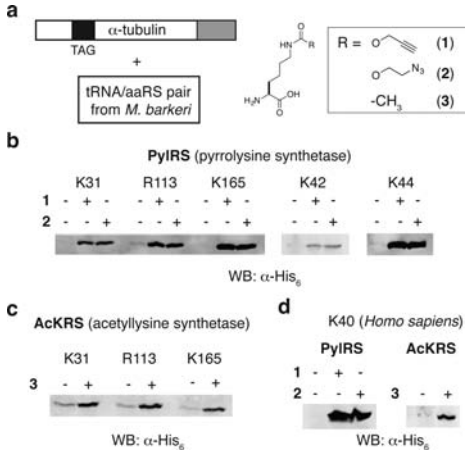

Figure 2. Site-specific incorporation of non-natural amino acids into yeast and human α-tubulin. (a) Amber suppression constructs and non-natural amino acids used in this work. His6-tag (gray box); TAG amber codon (black box). (b) Western blot of total cell lysate from *S. cerevisiae* (containing PylRS and α-tubulin amber suppression constructs) grown in the presence or absence of **1** or **2**. (c) Western blot as in (b) except yeast expressed the acetyllysine synthetase (AcKRS) and were grown in the presence or absence of **3**. (d) *S. cerevisiae* cells were treated as in (b) and (c) except that the human α-tubulin gene with an amber codon at position 40 was used.

most advanced structural models of tubulin in its polymeric form[7,21] predict that residues R113 and K165 lie on the outside of the microtubule while K31, K42, and K44 lie on the inside (Figure 1), therefore we focused on these amino acids.

S. cerevisiae were transformed with the tubulin constructs described above and amber suppression constructs, and grown in medium containing or lacking the non-natural amino acids alkynyl-lysine (**1**) or azido-lysine (**2**) (Figure 2a). We used Western blot analysis of total cell lysate (see Figure S1 for loading controls) to analyze the expression of full-length α-tubulin. In yeast expressing the wild-type pyrrolysine synthetase (PylRS), we observed efficient non-natural amino acid dependent translation of full-length tubulin when the TAG *amber* codon was placed at position 31, 44, 113, or 165 (Figure 2b). Amber suppression appeared to be less efficient at position 42 (Figure 2b). Similar results were observed at position 31, 113, and 165 when yeast expressing the engineered acetyllysine synthetase (AcKRS) were grown in the presence of acetyllysine (**3**), although translation in the absence of non-natural amino acid appeared to occur at a higher level than was observed with PylRS (Figure 2c). Using analogous methods, we were able to observe successful amber suppression at two sites (residue 32, located near the taxol binding site, and residue 412, proximal to the kinesin binding site) found within yeast β-tubulin (Figure S2), demonstrating the generality of this approach for incorporating non-natural amino acids into either subunit of the $\alpha\beta$-tubulin heterodimer.

We also probed the ability of the pyrrolysine system to incoporate non-natural amino acids into human α-tubulin expressed in *S. cerevisiae*. Gratifyingly, we were able to observe amber suppression with all three modified lysine analogues: alkynyl-lysine, azido-lysine, and acetyllysine at position K40, the known tubulin acetylation site,[22] in human α-tubulin (Figure 2d).

We next focused on generating recombinant site-specifically modifiable tubulin on a scale that would allow *in vitro* biochemical and biophysical studies. As affinity tagging is known to perturb protein function,[23] we needed to apply amber suppression to native, untagged tubulin (Figure 3a). For

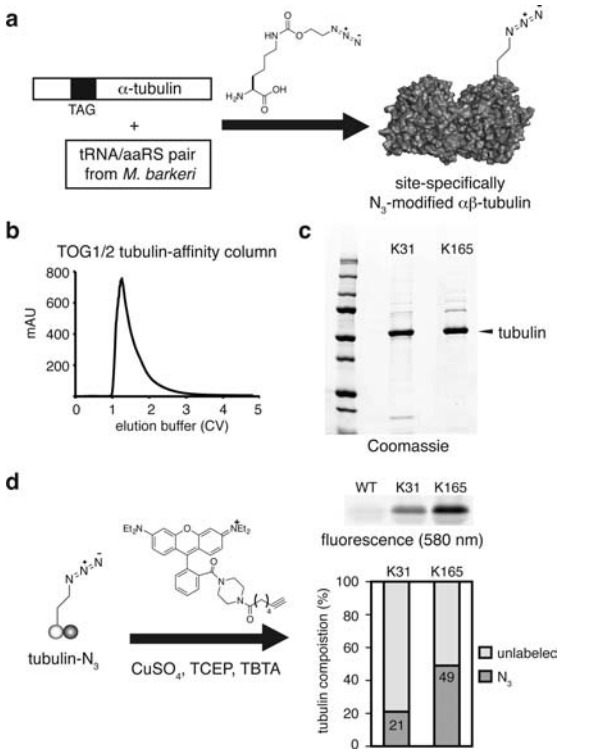

Figure 3. Generation of recombinant, site-specifically azide-modified tubulin. (a) Yeast transformed with amber suppression constructs were grown in the presence of azido-lysine. (b) Elution chromatagram of tubulin purified from yeast using the TOG1/2 tubulin-affinity column.[24] (c) SDS-PAGE of tubulin purified from yeast expressing the amber suppression constructs described in (a) with *amber* codons at position K31 or K165. (d) Purified tubulins were reacted with Rhodamine B-alkyne and analyzed by in-gel fluorescence. Tubulin labeling was compared against a fluorescent standard (Figure S3) to quantify azido-lysine incorporation.

these studies, we chose two sites in α-tubulin that could be efficiently suppressed with azido-lysine: K31, predicted to lie on the inside of a polymerized microtubule, and K165, predicted to lie on the outside (Figure 1).

To purify tubulin we used an affinity column comprised of beads to which two tandem repeats of the conserved tubulin-binding TOG domain (TOG1/2) had been covalently linked.[24] This strategy, which has been used to purify tubulins from diverse sources,[24] yielded ~250 μg of $\alpha\beta$-tubulin with >90% purity from 4 L cultures, as judged by Coomassie staining (Figure 3b,c). To verify that the purified tubulins contained azido-lysine, we performed click chemistry with an excess of Rhodamine B-alkyne (Figure 3d). Both K31(N_3)- and K165(N_3)-modified tubulins, but not wild-type tubulin, reacted with Rhodamine B-alkyne (Figure 3d). Comparison against a reference (Figures 3d and S3) showed that 21% of the purified K31(N_3)-$\alpha\beta$-tubulin and 49% of the purified K165(N_3)-$\alpha\beta$-tubulin contained azido-lysine. These results demonstrate that we can isolate biochemical quantities of site-specifically modified soluble recombinant $\alpha\beta$-tubulin and perform site-specific chemistry to install a biophysical probe.

We next turned to chemically modifying the microtubule polymer. As mutations of tubulin frequently disrupt polymer-

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ization, we first examined if our azide-modified tubulins were polymerization competent. Upon subjection of K31(N_3)- and K165(N_3)-tubulin to polymerization conditions, the majority of tubulin in both reactions could be pelleted by ultracentrifugation (Figure S4), consistent with the assembly of higher-order structures. Click chemistry with Rhodamine B-alkyne on the supernatant (unassembled) and pellet (polymer) fractions confirmed that azide-modified tubulin pelleted with the wild-type tubulin present in the polymerization reaction (Figure S4).

To analyze the structures of assembled azide-modified tubulins, we used negative-stain electron microscopy and fluorescence microscopy. Negative-stain electron microscopy on microtubules polymerized from K31(N_3)- or K165(N_3)-tubulin showed filamentous structures with an average diameter of 25 nm (Figure 4a and Table S1), similar to what has been

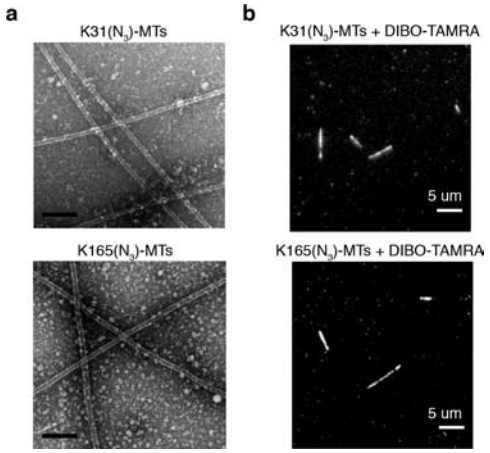

Figure 4. Recombinant azide-modified tubulin is polymerization competent and enables the selective labeling of residues on the inside and outside of microtubules. (a) Negative-stain EM of microtubules polymerized from K31(N_3)- or K165(N_3)-modified tubulin. Scale bar is 200 nm. (b) Fluorescent microscopy of DIBO-TAMRA labeled microtubules polymerized with K31(N_3)- or K165(N_3)-modified tubulin.

observed for wild-type microtubules.[5] At higher magnification, structures consistent with microtubule protofilaments were observed (Figure S5). In order to observe fluorescent microtubules and demonstrate co-assembly of modified and unmodified tubulin, we co-polymerized K31(N_3)-tubulin and K165(N_3)-tubulin with wild-type tubulin and subjected the resulting polymers to Cu-free click chemistry with DIBO-TAMRA(Figure S6); standard Cu(I)-catalyzed click chemistry disrupts microtubule structure. For microtubules polymerized with K31(N_3)-tubulin or K165(N_3)-tubulin, fluorescence imaging after DIBO-TAMRA labeling clearly showed the presence of TAMRA-modified microtubules (Figure 4b), indicating co-polymerization of azide-modified and wild-type tubulin, as well as successful reaction at both azide-modified sites. Importantly, microtubules composed entirely of wild-type tubulin were not labeled by the DIBO-TAMRA dye (Figure S7).

Taken together, our results demonstrate successful translation of *amber* codon-containing yeast α- and β-tubulin, as well as human α-tubulin, and suggest that non-natural amino acids including substrates for bioorthogonal ligation chemistry, alkynyl- and azido-lysine, and regulatory lysine acetylation can be incorporated at a number of distinct sites within tubulin. Additionally, we have demonstrated the selective modification of amino acids predicted to lie on the interior or exterior surface of the microtubule polymer and thereby shed light on tubulin biochemistry. We have shown that incorporation of modified amino acids or bulky substituents, such as fluorescent probes, at specific sites in tubulin does not disrupt microtubule polymerization or overall structure. In particular, the strictly conserved K165 (mammalian numbering: K164) residue in α-tubulin[25] lies near the lateral interface between microtubule protofilaments. Our studies show that replacement of K165 with an azide-modifed amino acid does not prevent microtubule formation even when present in 50% of the polymerizable tubulin. This suggests that the strict conservation of this residue is not for polymerization, but rather for its role in another aspect of microtubule biology. Recent proteomics studies indicate that K165 is likely to be acetylated.[26] Our data shows that amber suppression will enable the generation of K165-acetylated tubulin in amounts needed for biochemical and biophysical analysis.

Our approach will allow at least three different types of experiments, as we have addressed the challenges of producing recombinant, modifiable tubulin. First, the targeted incorporation of cross-linkers and fluorescent probes can be used to identify proteins that selectively bind the interior or exterior microtubule surface. Recent discoveries have suggested the presence of proteins in the lumen of microtubules in the primary cilium,[9] a hub of cellular signaling. However, the identity and function of these proteins remain unknown. Comprehensive profiling of microtubule lumen binding proteins is required in order to begin to investigate their function and understand how these MAPs contribute to microtubule specialization in organelles such as the primary cilium. Second, conditional control of drug binding or protein−protein interactions can be accomplished by positioning caged amino acids at critical residues. In particular, modification of β-tubulin residues implicated in taxol binding will enable control of polymerization dynamics within single cells. Finally, chemical control of microtubule composition provides the opportunity to recapitulate important microtubule post-translational modifications such as lysine acetylation, polyglutamylation, and polyglycination, enabling analysis of the role of post-translational modification on microtubule function in normal and disease cell physiology.

■ ASSOCIATED CONTENT

Supporting Information

Tubulin polymer pelleting assay, in-gel fluorescence, and β-tubulin amber suppression data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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