Causes and Consequences of Microtubule Acetylation

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SUMMARY

Among the different types of cytoskeletal components, microtubules arguably accumulate the greatest diversity of post-translational modifications (PTMs). Acetylation of lysine 40 (K40) of α-tubulin has received a particular attention because it is the only tubulin PTM to be found in the lumen of microtubules - most other tubulin PTMs are found at their outer surface. As a consequence, the enzyme catalyzing K40 acetylation needs to penetrate the narrow microtubules lumen to find its substrate. Acetylated microtubules have been considered as stable, long-lived microtubules, however until recently there was little information about whether the longevity of these microtubules is the cause or the consequence of acetylation. Current advances suggest that this PTM helps the microtubule lattice to cope with mechanical stress, thus facilitating microtubule self-repair. These observations now shed a new light on the structural integrity of microtubules, as well as on mechanisms and biological functions of tubulin acetylation. Here we discuss the recent understanding on how acetylation is generated in the lumen of microtubules, and how this 'hidden' PTM can control microtubule properties and functions.

MAIN TEXT

Introduction

Microtubules are hollow, polarized tubes, which dynamically assemble from heterodimers of α- and β-tubulin. Their dynamics allow microtubules to constantly switch between growth and shrinkage, thus exploring and probing the intracellular space, and to rapidly re-organize into highly specialized structures, such as the mitotic spindle during cell division. Consequently, the strict regulation of dynamics and stability is of key importance for microtubule functions, and is thus controlled by several complementary mechanisms.

One mechanism expected to control microtubule properties and functions is the posttranslational modification (PTM) of their tubulin subunits. Tubulin PTMs have recently attracted a growing interest, as the discovery of many modifying enzymes has allowed to determine biological functions of these previously barely studied modifications (reviewed in Janke 2014 [1]). While most known tubulin PTMs occur on the outer surface of polymerized microtubules, acetylation has been identified on lysine 40 (K40) of α-tubulin [2, 3], a residue exposed at the inner microtubule surface, i.e. in the microtubule lumen (Figure 1A).

The luminal localization of the acetylation site has puzzled investigators for many years, particularly because acetylation of α-tubulin K40 is only observed in subunits that are incorporated in the microtubule lattice, and not in cytosolic tubulin heterodimers. This implied that enzymes catalyzing the addition of the acetyl moiety to K40 must somehow enter the narrow, 15-nm-wide lumen of microtubules. In addition, it remained unclear to which extent such a luminal PTM could affect microtubule properties, or the interaction between microtubules and other factors modulating microtubule functions, such as microtubule-associated proteins (MAPs). While an excellent antibody directed against K40-acetylated α-tubulin [4] has early allowed to study the distribution of this PTM, the tubulin acetyltransferase aTAT1 has been discovered much later [5, 6], thus genetic, cell biological and biophysical studies have only recently emerged. Here we discuss current studies that

have strongly advanced the understanding of the mechanisms and functions of microtubule acetylation.

Molecular mechanisms controlling microtubule acetylation

For many years, strong biochemical evidence had suggested that α-tubulin acetylation only takes place on polymerized microtubules [7-10]. Accordingly, upon the discovery of aTAT1, it was shown that this enzyme has an over 100-times higher catalytic activity with tubulin in its polymeric compared to its unpolymerized state. The question of how the enzymes that control K40 acetylation can reach their modification site inside the microtubules is thus of key importance for understanding how the dynamics of this PTM is regulated.

The acetylation enzyme and the challenge of luminal access

Although aTAT1 has not been formally visualized inside the microtubule lumen, it is likely to be there. The presence of so-far unidentified intraluminal material has been demonstrated by electron microscopy [11], and strikingly this material was missing inside the microtubules of a C. elegans strain with mutations in Mec-17 (aTAT1 [12]). At present, several hypotheses attempt to explain how the α-tubulin acetyl-transferase could access its luminal K40 target. One possibility is that aTAT1 enters the microtubule lumen via irregularities in the microtubule structure [13] (Figure 1B), which is supported by the existence of switches in the number of protofilaments and loss of tubulin dimers in microtubule lattices in vivo [14, 15]. ATAT1 would use these microtubule defects to locally enter and acetylate microtubules (Figure 1C). The drawback of this model is that microtubules would require a high frequency of defects in order to spread acetylation over their entire length. Lattice defects and protofilament switches have been visualized on microtubules purified under physiological conditions using atomic-force microscopy [16, 17], however it is possible that these defects occur only transiently and get repaired by the recently discovered microtubule self-repair mechanism [15] (Figure 1C). This could provide aTAT1 with transient, but frequent luminal access sites, and result in

efficient spreading of K40-acetylation along microtubules. Indeed, aTAT1 binds to the external wall of microtubules [18, 19] ideally positioning it to enter the microtubule lumen upon the appearance of transient microtubule defects. The externally bound aTAT1 might, similar to other MAPs, scan the microtubule surface to channel the enzyme toward its luminal entry sites (Figure 1C).

Another, non-exclusive possibility is that aTAT1 enters the microtubule lumen from the open microtubule ends (Figure 1B). Along this line, Coombes et al. recently showed that aTAT1 has a higher affinity for microtubule ends, most likely because of the high density of exposed luminal sites at the tapered extremities, which could be used as 'traps' for the enzyme [13]. In addition, acetylation could be controlled by the contacts between microtubules and aTAT1-enriched structures, such as focal adhesions or clathrin-coated pits [20, 21]. To date, several reports provide evidence for a preferential entry of aTAT1 at the open microtubule ends [13, 19, 22], however a quantitative evaluation of how much of the enzyme enters through open ends versus defects in the microtubule lattice is lacking. Moreover, the ends of microtubules may be partially obstructed inside living cells, as they bind a plethora of specific tip-binding proteins that could hamper luminal access [23].

The mechanism by which aTAT1 enters the microtubule lumen may have important consequences on the dynamics of acetylation by controlling which microtubules are acetylated in cells. The difficulty of elucidating this mechanism in vitro could in particular be related to the fact that microtubule features, and thus the frequency of microtubule defects, vary dramatically depending on how microtubules are polymerized. For instance, microtubules assembled in the test tube from purified tubulin seem to be more permissive to lateral entry of aTAT1 as compared to microtubules extracted from cells, or purified axonemes [5, 19]. Lateral entry into the lumen of cellular microtubules could be obstructed by different factors. One possibility is that the multitude of MAPs binding microtubules in cells limit the access of aTAT1 to the lattice. Alternatively, the presence of microtubule polymerases and nucleation factors in cells may contribute to the generation of microtubules with much fewer defects, and thus a lower frequency of lateral aTAT1 entry. Doublecortin, for instance, is

assuring that all microtubules in a cell are homogenously built from 13 protofilaments, thus reducing chances to accumulate switches in protofilament numbers [24]. On the other hand, microtubules are exposed to mechanical stress in living cell clearly, which favors their buckling and bending. Microtubule bending had recently been shown to result in the lateral incorporation of tubulin dimers into the microtubule lattice, suggesting that the bent microtubules acquire defects that are subsequently repaired [15]. Thus, the relative contribution of open ends and lattice defects to aTAT1 luminal access is likely to vary depending on the way microtubules are assembled, and which mechanical challenges they experience.

The luminal diffusion conundrum

Szyk et al. were the first to suggest that aTAT1 diffuses in the lumen of in-vitro-assembled microtubules [22]. They proposed that once aTAT1 enters an open microtubule extremity, it diffuses rapidly throughout the entire microtubule lumen thus stochastically acetylating more and more tubulin units. Although the diffusion hypothesis has the merit of simplicity, it was initially ruled out by a mathematical model, which had suggested that diffusion in the lumen of microtubules would require years to reach equilibrium [25]. However, as this model was dominated by the affinity of the protein of interest for the luminal microtubule surface, it did not exclude that proteins with low affinity, such as aTAT1, are able to make their way through the lumen. In the model proposed by Szyk et al., the fact that K40 acetylation becomes detectable only after aTAT1 diffusion has reached an equilibrium results from the very low catalytic activity of this enzyme. Consequently, acetylation was proposed to occur randomly all along the length of microtubules [22]. Nevertheless, the conclusion that aTAT1 diffusion is fast inside microtubules was derived from fluorescent microscopy experiments, which make it difficult to determine whether aTAT1 scans the luminal or the external surface of microtubules. In addition, the presence of discontinuous acetylation patterns, as observed by the authors [22], are difficult to explain with a stochastic acetylation model, but would better fit with the presence of aTAT1 entry sites at microtubule defects as discussed above. Indeed, using a

similar in vitro approach, Coombes et al. proposed that aTAT1 luminal diffusion is actually slow due to frequent rebinding of the enzyme to nearby non-acetylated K40 residue which impedes luminal mobility [13] (Figure 1C). Accordingly, the authors found that acetylation marks accumulate at the entry sites of the enzyme (open ends or lateral openings), and not randomly along microtubule.

These discordant observations on aTAT1 entry into the microtubule lumen may be a consequence of the way microtubules were prepared and stabilized in different studies, as we discussed above. Using microtubules extracted from HeLa cells, Ly et al. observed that K40 acetylation is biased towards open extremities, and spreads longitudinally into the microtubule lumen [19], perhaps because these microtubules have less irregularities. Whatever the precise experimental determinants of the different observations, the results of Coombes et al. and Ly et al. suggest that aTAT1 diffusion is slower than initially proposed [13, 19]. Their observations might be closer to the in-vivo situation, as it was shown that acetylated K40 marks are first detected at the extremities of most microtubules after releasing cells from nocodazole-induced microtubule depolymerization [19, 21]. In this assay, the acetylated microtubule segments progressively extended over time, suggesting a mechanism in which aTAT1 slowly diffuses inside the microtubule lumen thereby spreading acetylated K40 marks [10, 21].

The mechanism of deacetylases

The tubulin deacetylases HDAC6 [26] and sirtuin type 2 (SIRT2) [27] were identified before aTAT1, but their precise mode of action is so far less understood. Initial observations in vivo indicated that tubulin deacetylation correlates with microtubule depolymerization [9], suggesting that deacetylases work on free, cytosolic tubulin dimers. This was contradicted by two studies, which used immuno-purified HDAC6 to suggest that the enzyme efficiently deacetylates polymerized microtubules in vitro [26, 28]. However more thorough in vitro experiments later demonstrated that HDAC6 preferentially works on the free tubulin dimer [29, 30].

Notwithstanding these controversies, the initial studies have shown that deacetylation of polymerized microtubules is possible, implying that HDAC6 should, similar to aTAT1, be able to enter the microtubule lumen. Along these lines, HDAC6 was shown to interact with the plus-end tracking protein EB1, suggesting that HDAC6 may be funneled to the lumen at this entry site [31]. A recent report further showed that incubation of acetylated microtubules with recombinant HDAC6 results in random deacetylation all-along the length of microtubules [30]. This feature is reminiscent of the stochastic aTAT1-dependent acetylation pattern, and could be mediated by similar mechanisms (i.e. holes in the in-vitro-assembled microtubule lattices) as discussed for aTAT1.

Impact and biological functions of microtubule acetylation

For many years following the discovery of acetylated microtubules, the biological functions of tubulin acetylation have remained an intriguing, unsolved problem. After decades of intensive work, the impact of K40 acetylation on microtubule properties, and the biological roles of this modification only begin to emerge.

Microtubule stability and K40 acetylation: the chicken and the egg

Soon after the discovery of K40 acetylation, investigators found that this PTM is associated with long-lived subsets of microtubules in cells [8, 9, 32]. Since then, a central question has been if acetylated microtubules are stable because they are acetylated, or if they are acetylated because they are stable. For more than two decades of research, the prevailing model was that acetylation is a consequence of microtubule stabilization. This was based on observations that chemical stabilization of microtubules leads to their acetylation, whereas artificially increasing tubulin acetylation level did not stabilize microtubules [33].

Another mechanism by which tubulin acetylation at K40 might affect microtubule dynamics is to alter the structural rearrangement of the lattice, which would most likely modulate its physical properties. While structural studies did not find an acetylation-induced changes in tubulin conformation [18], it

was demonstrated that K40 acetylation weakens lateral interactions between protofilaments, which softens the microtubules [34]. As microtubules in living cells are frequently exposed to mechanical forces, which can damage the lattice and subsequently result in microtubule depolymerization (Figure 2A), an acetylation-induced increase in their flexibility would allow microtubules to better resist mechanical stress [35], and consequently making acetylated microtubules longer-lived. The observation that acetylation marks are often found at curved region of microtubules [35], which are the very same regions that accumulate lattice openings, or cracks [17], strongly suggests that those stress-induced cracks in microtubules lattice are the luminal entry points for aTAT1. The enzyme would then locally generate acetylation, which in turn could render the partially damaged microtubule regions resistant to mechanical stress, thus allowing their subsequent repair [15] (Figure 2B). At the same time, longer-lived microtubules are still more likely to experience mechanical stress, thus further accumulating acetylation marks, which may reflect to the notion that acetylation is a marker of microtubule age.

Functional consequences of K40 acetylation at the cellular and tissue level

Functional insight into the role of K40 acetylation has mostly been obtained by manipulation of the acetyltransferase aTAT1, or of the deacetylases SIRT2 and HDAC6. K40 acetylation has so far been linked to cell migration [20, 21], autophagy [36], neuronal-dependent touch sensation in C. elegans [6] and mouse [37], intracellular trafficking [38, 39], and cell adhesion [40]. Despite these remarkable advances, the precise mechanisms through which acetylated K40 marks could regulate those functions had remained completely unknown until recently [34, 35]. For instance, the observations that molecular motors preferentially run along acetylated microtubules [38, 39] were correlative and not confirmed by in-vitro experiments with purified components. Once differentially acetylated tubulin was experimentally available, direct measurements of kinesin-1 on in-vitro polymerized microtubules showed that this motor is not affected by the tubulin acetylation of the microtubule track [41, 42]. While acetylation of K40 is less probable to affect motor-based transport on

microtubules, its recently discovered role in protecting microtubules from mechanical breakage [34, 35] may actually be the key to most observations regarding functional consequences of microtubules acetylation. In the light of these new findings, it will now be possible to re-evaluate a number of former studies.

An intriguing feature of tubulin acetylation is that aTAT1-knockout mice show no gross developmental and physiological abnormalities, despite the fact that in these mice, no K40-acetylated microtubules could be detected. Yet, aTAT1-KO mice exhibit a deformation of the dentate gyrus [43], as well as defects in touch sensation [37] and sperm motility [44]. Cells from these mice show reduced capacity of contact-inhibition during cell proliferation, and have a lower number of focal adhesions [40].

Additional insights into the impact of tubulin acetylation on microtubule structure and function came from the model organism C. elegans, which contains a unique set of 15-protofilament microtubules in touch-sensation neurons [45, 46]. The assembly of these unusual microtubules depends on aTAT1 (Mec-17), as the depletion of this enzyme completely abolishes their assembly and results in aberrant touch sensation in this organism [47]. It thus appears that the biological processes regulated by tubulin acetylation are not essential for organism development and survival in controlled experimental conditions, but might provide robustness in competitive situations, such as survival in challenging environments, diseases, or during evolution.

Other sites of acetylation at tubulin

When talking about tubulin acetylation, we almost invariably refer to acetylation of K40 at α-tubulin. However, there are several studies demonstrating the existence of additional acetylation sites on both, α- and β-tubulin, which have mostly been identified by mass spectrometry. For instance, a study that aimed for a proteome-wide discovery of novel acetylation sites in human cell lines identified 8 new acetylation sites on α-, and one on β-tubulin [48]. In this study, tubulin acetylation was discovered amongst many other acetylation events, and thus, the novel acetylation sites of

tubulin have not been verified with complementary approaches. Another study focused on tubulin, and found that almost all lysine residues of α- and β–tubulin can be acetylated [49], a result that might need some future verification as some of these potential modification sites are hardly accessible in the folded tubulin structure, and therefore the mechanism of their acetylation is difficult to envision. Finally, one functional study showed the importance of acetylation of K252 of β-tubulin for the control of microtubule polymerization [50]. Overall, our current knowledge on other acetylation sites on tubulin is rather weak, and a more thorough investigation of these sites will be needed to determine their presence in different organisms and cell types, as well as their functional implications. Most importantly, the in-vivo acetylation state of these residues has so far not been measured, and should be the focus of future explorations of their potential functional and mechanistic roles.

Perspectives

Acetylation of tubulin has been discovered over 30 years ago, and an excellent antibody detecting the acetylation of K40 at α-tubulin has paved the way for frequent observation of acetylated microtubule species in different cell types. Most of the past studies have used acetylated-tubulin staining synonymous for detecting stable microtubules, which, despite the fact that this was a simplification, has become a common practice over the years. Only very recently it was found that acetylation of K40 does indeed protect microtubules from destabilization, but strikingly by making them softer and thus more resistant to damages induced by mechanical bending. The discovery of this mechanism, as well as the studies that have investigated the entry of the acetyl transferase aTAT1 into the lumen of microtubules have also revived a discussion about the structure of microtubules in cells; it seems that there are many more imperfections in the structure of these microtubules than our textbook pictures suggest. Even more strikingly, these imperfections can be repaired without disassembling and reassembling the microtubules – instead, tubulin units can be re-added into damaged lattices [15]. All these discoveries confirm a current trend in cell biology that

points towards the necessity of rethinking well-established models, and consider the possibility that temporal fluctuations in biological systems, together with regulatory events that only gradually alter the properties of biological molecules, play important roles in allowing cells to adapt to varying environmental conditions or changing functions. Acetylation as a part of the tubulin code might play important roles in these processes, and could thus be involved in a number of human pathologies linked to dysfunction of the microtubule cytoskeleton.

ACKNOWLEDGMENTS

The authors wish to thank S. Bodakuntla, D. Chrétien, M.V. Nachury and M.M. Magiera for critical and insightful discussions on the manuscript, as well as K. Natarajan (Institut Curie) for help with figure panels. This work was supported by the Gustave Roussy Institute and the INSERM (GM); the Institut Curie and the CNRS (CJ), and by grants from the *ATIP/Avenir Program* (GM), the *Agence Nationale de la Recherche* (ANR-15-CE15-0005-03 to GM, ANR-12-BSV2-0007 to CJ), *la Ligue Nationale Contre le Cancer* (GM), and the Institut National du Cancer (INCA) (2013-1-PL BIO-02-ICR-1 and 2014-PL BIO-11-ICR-1 to CJ).

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FIGURE LEGENDS

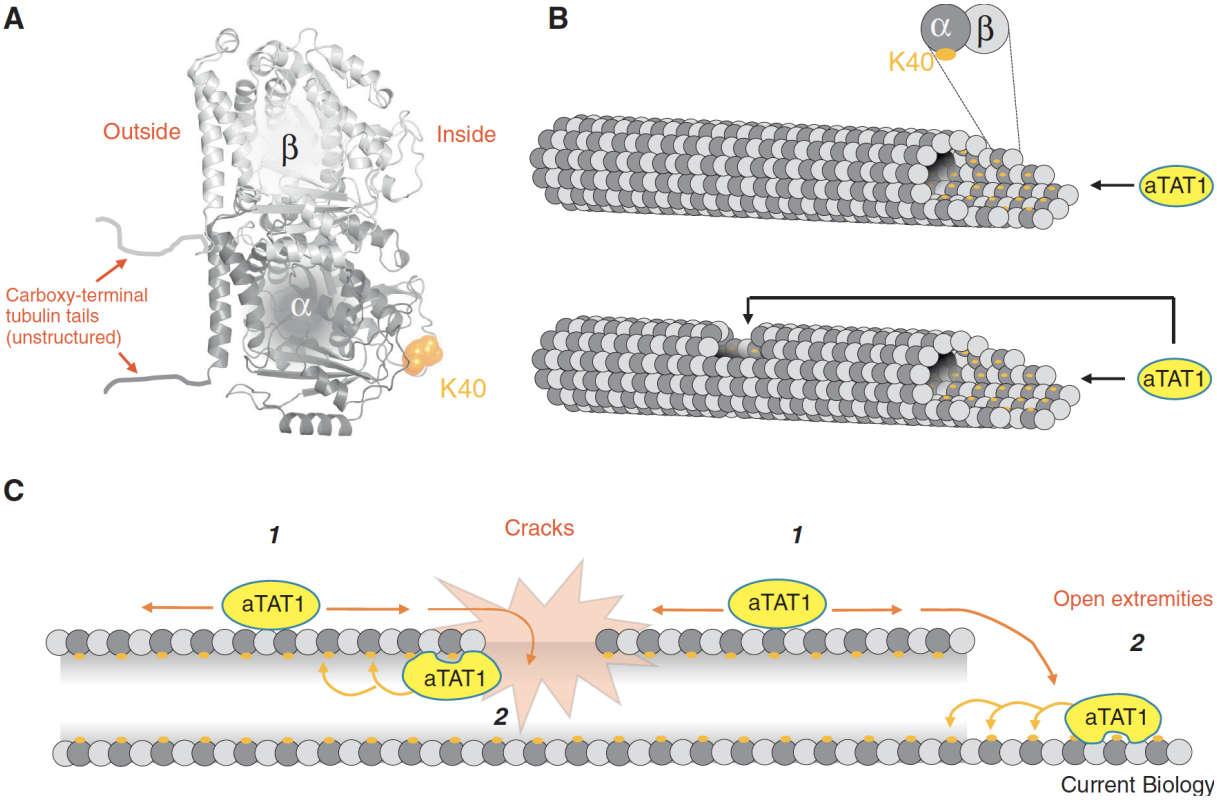

Figure 1: Modes of K40 acetylation by aTAT1.

A) Modelled structure of the α/β-tubulin dimer. The position of K40 is highlighted in yellow. The dimer will insert into microtubules in a way that locates K40 at the luminal surface.

B) Schematic representation of different entry sites of aTAT1 into the lumen of microtubules. The availability of these sites depends on the integrity of microtubules.

C) A proposed mechanistic model for aTAT1 access to the lumen involving a first step whereby aTAT1 scans the outer surface of microtubules (1) in order to find accessible K40 modification sites at microtubule ends, or at cracks in the microtubule lattice (2). Once in the lumen, aTAT1 modifies available K40 sites. Its diffusion is slowed down by the fast rebinding of the enzyme to nearby K40 residues. Acetylation confers extra flexibility to the microtubule lattice thus allowing for better resistance to mechanical stress and potential repair cracks by local addition of new α/β-tubulin subunits (3).

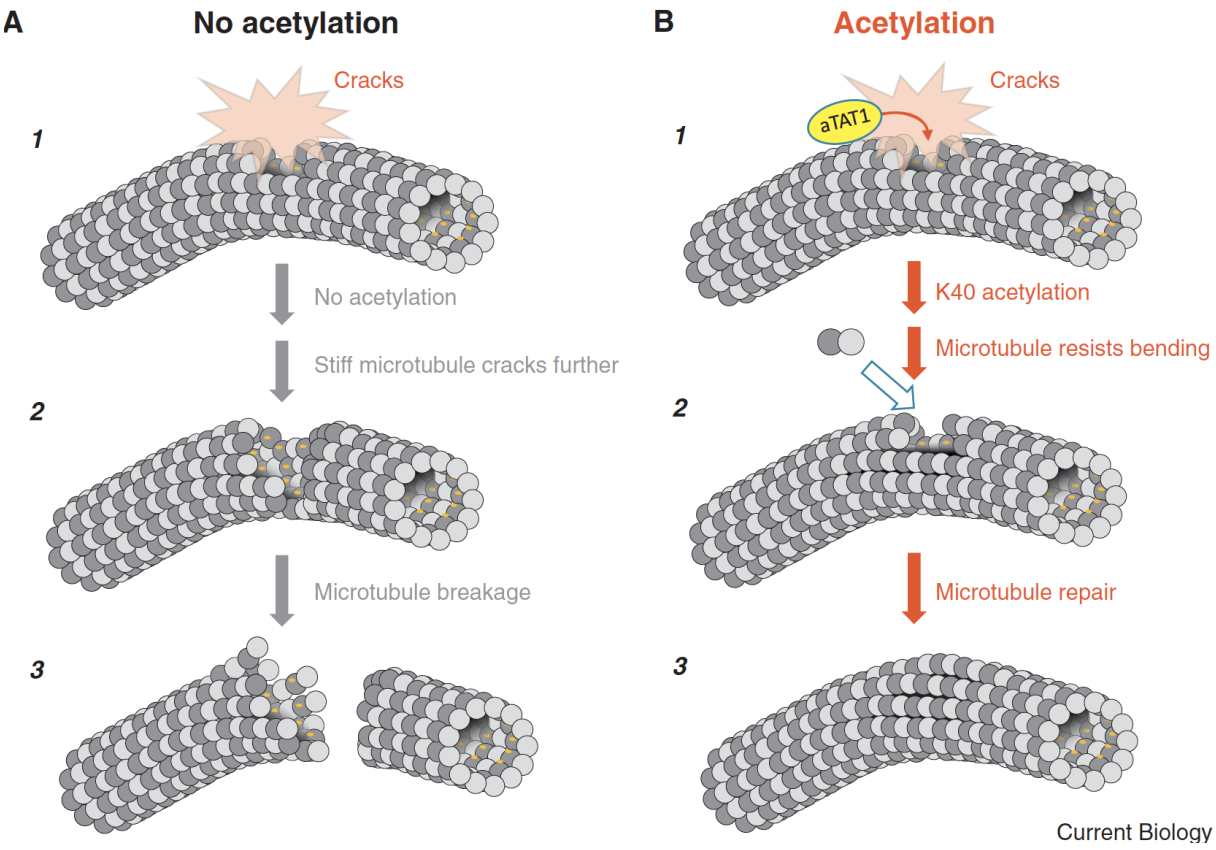

Figure 2: K40 acetylation protects microtubules from mechanical stress

Bent microtubules are subjected to mechanical stress that can damage the microtubule lattice and open cracks. The presence of aTAT1 can avoid breakage of the damaged microtubules by rendering them more flexible.

A) In absence of aTAT1 and K40 acetylation, microtubule cracks (1) further lead to the loss of tubulin dimers if the microtubule remains in the bent state (2) leading to microtubule breakage (3) which can induce the complete depolymerization of the microtubule.

B) ATAT1 can enter the microtubule lumen via cracks (1) to acetylate K40 of the damaged microtubule. This acetylation increases the flexibility of the microtubule, which makes microtubules more resistant to mechanical stress, thus avoiding further breakage of the bent microtubule (2). This in turn will give the damaged microtubule more time for self-repair, and further accumulation of acetylation will keep the bent microtubule intact (3).