Invited Review
Microtubule Severing Enzymes Couple ATPase Activity with Tubulin GTPase Spring Loading

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ABSTRACT:

Microtubules are amazing filaments made of GTPase enzymes that store energy used for their own self-destruction to cause a stochastically driven dynamics called dynamic instability. Dynamic instability can be reproduced in vitro with purified tubulin, but the dynamics do not mimic that observed in cells. This is because stabilizers and destabilizers act to alter microtubule dynamics. One interesting and understudied class of destabilizers consists of the microtubule-severing enzymes from the ATPases Associated with various cellular Activities (AAA+) family of ATP-enzymes. Here we review current knowledge about GTP-driven microtubule dynamics and how that couples to ATP-driven destabilization by severing enzymes. We present a list of challenges regarding the mechanism of severing, which require development of experimental and modeling approaches to shed light as to how severing enzymes can act to regulate microtubule dynamics in cells. © 2016 Wiley Periodicals, Inc. Biopolymers 105: 547–556, 2016.

Keywords: *tubulin; microtubule; microtubule-severing enzyme; katanin; spastin*

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GTPASE FILAMENTS: MICROTUBULES AND TUBULIN STRUCTURE AND FUNCTION

Microtubules are stiff, noncovalent biopolymers responsible for creating a rigid network inside the cell. Microtubules can be thought of the "bones" of the cell that make up the "cytoskeleton" which literally means cell skeleton. Like bones in your body, the microtubule network is mechanical and rigid to give the cell shape and structure. Unlike the bones of your body, the network of microtubules is labile, versatile, and deformable. The network is easily organized and reorganized based on the state of the cell in development and the cell cycle. The microtubule network of a plant cell, a neuron, a gut cell, or a dividing cell in a culture dish each has unique organizations to perform the cell's functions (Figure 1), yet the basic building block and structural subunit, the microtubule, is the same for each of these cell types. For example, there is 63% sequence identity between the proteins that make microtubules in yeast and in mouse.[1]

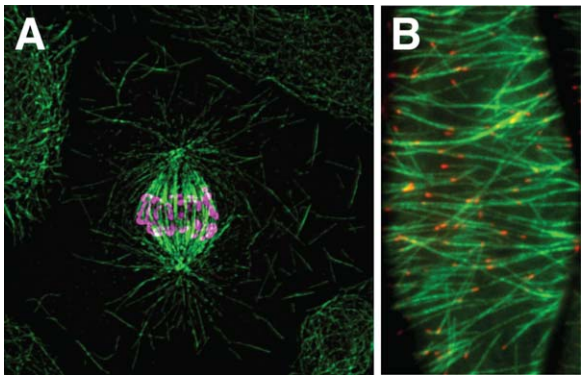

FIGURE 1 Microtubules in live cells form a variety of structures to perform specific tasks. A: Microtubules (green) in a dividing S2, fly cell attach to chromosomes (magenta). Image courtesy of Tom Maresca. B: Microtubules (green) in a plant cell are dynamic and tipped with an end-binding protein, EB1 (red). Image courtesy of Ram Dixit.

Further, many functions of microtubules are essential and similar between different cell types.

The microtubule network has the amazing ability to rapidly reorganize because the individual filaments are inherently dynamic (Figure 2).[2] The filament is a non-covalent polymer of GTPase enzymes. One benefit of biological systems over synthetic systems is that biological systems are inherently nonequilibrium. Their ability to consume energy gives biological systems the ability to transform and create fast reactions unobtainable in many synthetic systems. Making a polymer out of an enzyme is a fascinating concept that allows the polymer to store energy and quickly trigger changes in shape. In the case of microtubules, the polymer is rapidly self-destructive.

The GTP enzyme that polymerizes into the microtubules is called tubulin, which comes as a heterodimer of alpha and beta forms. While there is only 45% amino-acid sequence similarity between alpha and beta tubulin isoforms,[1] the three dimensional structures of the monomers are very similar, each consisting of three domains of similar length and secondary structure composition: the N-terminal, the middle, and the C-terminal domain. Both alpha and beta bind GTP. GTP binding to the alpha occurs in the dimerization region between the alpha and beta monomers and remains unhydrolyzed. This is called the "non-exchangeable site" or N-site. GTP binding to beta tubulin sits at the interface between two dimers within the "protofilament," the longitudinal lattice of tubulin dimers running parallel to the microtubule filament long axis (Figure 2). GTP binding to the beta tubulin is hydrolyzable and this site is called the "exchangeable site" or E-site. Hydrolysis of GTP at the E-site is required for microtubule dynamic instability.

To determine the mechanistic detail of how tubulin controls microtubule dynamics, high resolution structural studies have been performed. Nogales and coworkers have used cryo-electron microscopy to reconstruct microtubules at 3.5–5 Å resolution.[3,4] These studies provided almost atomistic details of the effect of GTP hydrolysis on the lattice. They showed that GTP hydrolysis leads to the compaction of the lattice around the interdimer longitudinal interface sandwiching the E-site nucleotide. This compaction in turn results in a conformational rearrangement in all alpha-tubulin monomers corresponding to a small rotation of the intermediate domain and C-terminal H11-H12 helices with respect to the N-terminal domain in alpha-tubulin. Additionally, helix H8 from alpha-tubulin is also distorted in the GDP-state. Interestingly, in all the solved microtubule structures, Nogales and coworkers did not detect any significant changes in the identity of lateral contacts between protofilaments.[3,4] Thus, upon GTP hydrolysis, the dimers store the potential energy in their straight conformation within the wall of the filament.

Experimental and theoretical evidence points to newly added dimers catalyzing the hydrolysis of the tubulin dimer to which they bind.[5] This is not to say that the hydrolysis is immediate, but the probability of hydrolysis within a certain time is increased when the next dimer binds. Such catalysis leads to a situation where dimers at the end of the filament typically have GTP, and are in a non-compacted, straight conformation. This is called the "GTP cap."[6,7] Dimers within the body of the filament are typically in the GDP-state and prefer to be in the compacted, bent conformation. Due to binding to neighbors, the GDP dimers cannot compact and are held straight. Thus, GDP dimers in the body are in a high potential energy state, spring-loaded to compact whenever constraints are relaxed. The purpose of the GTPase is to force dimers within the body into this spring-loaded state. When the dimers at the top are lost or hydrolyzed stochastically, the end cap loses coherence and the entire microtubule bends back to relax the dimers to their lowest energy state. The longitudinal binding is less affected by the hydrolysis and protofilaments peel back into rings unraveling the microtubule (Figure 2).

The above standard scenario of microtubule depolymerization is rooted in the idea that the lowest free energy state of GTP dimers corresponds to a straight conformation, while for GDP dimers the respective state corresponds to a bent conformation. However, all high-resolution X-ray crystal structures of tubulin to date have captured the heterodimer in a bent conformation, regardless of the nucleotide state.[8] Further, recent microtubule cryo-EM reconstructions from the Nogales lab described above strongly suggest that changes along protofilaments due to GTP hydrolysis result in the compaction of

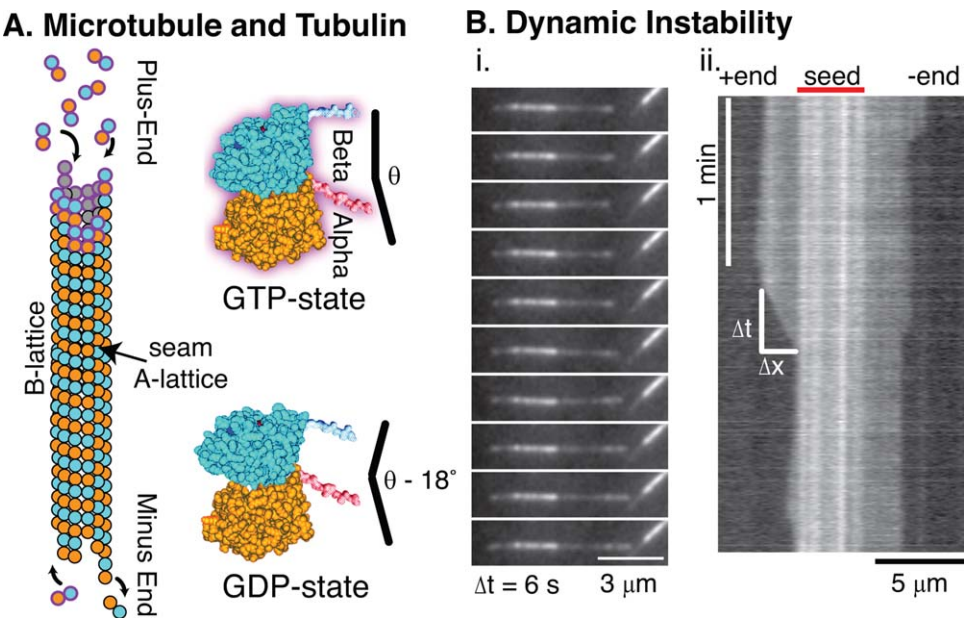

FIGURE 2 Microtubules of polymers of tubulin GTPases that exhibit a spontaneous dynamics due to the conformational changes within the dimer that occur upon hydrolysis of the GTP. A: Microtubule is polymerized into a lattice of tubulin dimers. The structure is a B-lattice with alpha-alpha and beta-beta lateral contacts except at the seam, where there is an A-lattice with alpha-beta lateral contacts. Tubulin dimers polymerize in the "straight" GTP-state and become hydrolyzed in the body of the microtubule causing a bending back of the dimer by 18°. The GDP-dimers within the body of the filament are spring-loaded to cause depolymerization once the geometrical constraints of nearby binding partners are released. B: Microtubule dynamic instability can be visualized using total internal reflection fluorescence microscopy. (i) Time series of microtubule growing and shrinking over time. The time between frames is 6 s; the scale bar is 3 μm. (ii) Kymographs, or space-time plots, can be made from movies of dynamic microtubules. The plus-end (+end) is identified as the faster growing end. The minus-end (-end) is the slower growing end. The growth velocity is measured by the change in length (Δx) over the change in time (Δt). The bright central region is the GMPCPP-stabilized seed. The vertical scale bar is 1 min. The horizontal scale bar is 5 μm.

GDP dimers in the lattice.[3,4] Based on these findings, an alternative depolymerization scenario can be envisioned: depolymerization starts with breaking lateral contacts, which are under stress in the lattice after the compaction due to hydrolysis. This is followed by outward bending of protofilaments likely due to steric constraints—as bending inward would cause crashing on the walls of the still intact microtubule. This scenario also correlates with the results of the MD atomistic simulations from the Voth group, which showed that an isolated (broken from its lateral neighbors) protofilament, regardless of the nucleotide state, does not have any intrinsic bending preference.[9]

GTP Analogs

A number of GTP-analogs have been tested to tease out the conformations adopted by the tubulin dimers during hydrolysis. The slowly hydrolyzable analog, GMPCPP, was found to be the best at nucleating and stabilizing the filament. Many studies of GMPCPP microtubules show that they nucleate microtubules and polymerize filaments faster, are stable to cold, preferentially have 14 protofilaments, and are mechanically stiffer than GDP microtubules stabilized with paclitaxel.[7,10–19] Because a non-hydrolyzable analog can nucleate and stabilize microtubules without the use of GTP-energy, it is clear that the polymerization is spontaneous and entropically driven at 37°C.

Interestingly, another non-hydrolyzable analog of GTP, GTPγS, is a poor nucleator of microtubules, and was understudied for decades.[20,21] Tubulin with GTPγS creates double rings and can be polymerized into microtubules if grown off a nucleation site or seed or in the presence of paclitaxel.[4,19,22–26] Recent work with GTPγS microtubules shows that they are not stiff like GMPCPP microtubules.[19,27] Further, end-binding proteins (EB) that tip-track growing microtubules have been shown to have enhanced binding for GTPγS microtubules.[24,28] Taken together, these results point to an altered conformational state for GTPγS compared to GTP, GMPCPP, or GDP. Specifically, GTPγS-tubulin may more closely resemble the tip of

growing microtubules, which is in a transition state as the dimers of the tip "mature" to the spring-loaded state found in the microtubule body. In agreement with these conclusions, Nogales found that GTPγS is an intermediate state between GTP and GDP states, which they attributed to a GDP-Pi state.[4]

Small Molecule Stabilizers and Destabilizers

Microtubules can be stabilized or destabilized through small molecules. One particularly important small molecule stabilizer is paclitaxel. Paclitaxel, (Taxol™) is a chemotherapeutic drug that binds to the inside surface of the microtubule near the E-site on the beta tubulin subunit. Interestingly, paclitaxel-stabilized microtubules are more flexible than regular or GMPCPP-stabilized microtubules.[19,27,29,30] This enhanced flexibility is likely the reason behind their stability. Nogales and coworkers showed that paclitaxel relieves the effects of GTP hydrolysis on the microtubule lattice by removing the compaction around the E-site effectively restoring the GDP lattice to a GTP-like state similar to the state observed with the slowly hydrolyzable analog GMPCPP.[3,4] This in turn allows individual dimers to take on more conformations and relax the spring-loaded state. In essence, paclitaxel lowers the potential energy stored in the filament and implies that stabilizers generally could remove the elastic spring-loading effect.

Small molecule destabilizers include calcium, which apparently competes for the magnesium binding site at the E-site to cause rapid hydrolysis of GTP to GDP.[31–33] Other small molecule inhibitors, such as colchicine and vinblastine, work by binding to dimers and rendering them unable to incorporate into filaments.[34,35] These molecules do not "destabilize" actively, but rather inhibit repolymerization after regular depolymerization occurs.

Open Questions and New Avenues

Despite over 30 years of work on microtubules, there are still many open questions about the mechanisms and regulation of microtubule dynamics. For instance, what are the roles and the differences between the various isoforms of tubulin? We know some are enriched in particular tissues, but we do not know the effect these subtle sequence variations have on microtubule structure or dynamics.[36] Further, tubulin can be post-translationally modified in a number of places, mostly on the carboxy-terminal tails.[37,38] Yet, we do not understand the role or effect of these modifications on filament structure or dynamics. Further, there are over 300 known microtubule-associated proteins (MAPs) and enzymes that act to regulate microtubules. The effects of individual species of MAPs and the combination of MAPs are only beginning to be tested for a handful of types. Finally, all these activities are happening together in a coordinated manner in the cell. Bottom-up experimental and theoretical studies must increase in complexity to illuminate top-down cellular studies that can manipulate the same regulators in cells. Below, we discuss some modern experimental and theoretical avenues that are being used to begin to unpack this rich and exciting problem.

There is currently a renaissance of microtubule studies thanks to new techniques to create and purify recombinant tubulin. Most prior work has been performed on tubulin from pig or cow brains, which are a mix of isoforms and post-translational states.[39] The assumption that isoforms or post-translational states can be averaged or overlooked appears to be untrue. Recent work has shown that sequence and modification state can change the dynamics, and the binding of associated proteins and enzymes.[40–46] Recombinant tubulin from yeast can achieve high purity and has been systematically studied with various mutants.[47,48] Other groups have used Sf9 cells to express and purify human tubulin.[49,50] These new techniques are being exploited to learn more than ever before about tubulin structure-function mechanisms.

Another recent breakthrough in theory has advanced our fundamental understanding of microtubule dynamics and mechanics. Previously, microtubule polymerization dynamics was understood using a simplified one-dimensional model from Oosawa.[51] In this model, the on and off-rates for tubulin during polymerization were deduced from the growth velocity as a function of tubulin concentration, and estimated to be on the order of 4 dimers per second. It is clear that many aspects of microtubule polymerization dynamics cannot be described using this model. The fundamental issue is that the microtubule is not a one-dimensional filament. By adding the geometry back to the system, a "two-dimensional" model has been able to recapitulate many more features of microtubule polymerization dynamics.[52] Another crucial concept of this new model was that the tubulin dimer off-rates are not concentration-independent, as previously assumed. Interestingly, the same concentration-dependent velocity data reveals tubulin dimer association/dissociation rates in the kHz range—three orders of magnitude faster than estimated by the one-dimensional model and more in line with diffusion-limited polymerization rates. This model explains how MAPs that stabilize microtubules can also cause faster polymerization through the reduction of the dissociation rate of dimers at the ends of the filaments. It also coarse-grains the dimer-level activity of conformational changes to differential affinities for the neighboring dimers.

Other theoretical models of microtubule instability support the roles of missing dimers and changes in number of protofilaments in depolymerization dynamics.[53,54] Starting from the hypothesis that the GDP-tubulin dimer is a conformationally bistable molecule, able to switch rapidly between a curved and

a straight state, Kulic and collaborators developed a model for polymorphic dynamics of the microtubule lattice.[54] This theoretical model explains the unusual dynamic fluctuations seen in microtubules and formation of helical loops by microtubules.[55] One limitation of this model is that it does not take into account the findings of atomistic simulations in solution that in short protofilaments interdimer and intradimer contacts in both GDP and GTP-bound tubulin dimers and protofilaments bend.[9,56] In these simulations, there were no observable differences between the mesoscopic properties of the contacts in GTP and GDP-bound dimers, in accord with the recent structural information on microtubule states.[3,4] Grishchuk and collaborators constructed a molecular-mechanical model of microtubule dynamics, with explicit Brownian dynamics simulation of tubulin-tubulin interactions, which allowed the inclusion of thermal fluctuations of protofilaments shape.[57] This model revealed detailed molecular events that precede and accompany microtubule catastrophe. Namely, the authors concluded that catastrophe events can be quantitatively described by random fluctuations in the number and extent of protofilament bending, rather than simply due to changes in the size of the GTP-cap at the tip of the microtubule.

ATPases: Microtubule Severing Enzymes

One particularly interesting aspect of microtubules is that they are polymers of GTPases whose dynamics and structure is controlled by interactions with a variety of ATPases. Some ATPases, such as kinesins, are transport motors that shuttle cargos, including microtubules themselves, to rearrange the network in cells and they are the focus of other contributions to this special issue.

Another class of ATPases acts on the filament structure itself. These ATPases are unique because they couple the energy of ATP hydrolysis with the already spring-loaded GTPase conformational changes of the tubulin to control microtubule length, location, and dynamics in cells. There are two families of ATPases that control microtubules: depolymerizing kinesins and severing enzymes. Depolymerizing kinesins use ATP to uncap microtubules at their ends. Severing enzymes remove dimers from the middle of the filament to create new ends. Both perform work to remove constraints on the GTPase dimers. They act to unleash the stored energy within the microtubule filament and cause rapid depolymerization. There are three types of microtubule severing enzymes: katanin, spastin, and fidgetin. All severing enzymes are members of the ATPases Associated with various cellular Activities (AAA+) family of enzymes.

Severing Enzymes

Katanin activity was first detected when Xenopus egg extract was added to paclitaxel-stabilized microtubules, and they were destroyed rapidly.[58] Purification of the ATP-utilizing enzyme that was responsible for destroying the microtubules revealed a dimer with a 60 kD and 80 kD component in sea urchin egg extracts.[58,59] It was named katanin after the Japanese Samurai sword, the "katana," because it can cut microtubules in the middle. Sequence analysis showed that the catalytic 60 kD subunit was an enzyme from the AAA+ family. The 80 kD subunit contains WD-repeat motifs and targets the enzymatic subunit to specific cellular regions.[60] The p60 domain can hexamerize separately and together with the p80 domain.[60] The enzymatic p60 can sever microtubules in vitro independent of p80.

Spastin was discovered as the protein that is most frequently mutated in the adult-onset neuromuscular disease of hereditary spastic paraplegia (HSP).[61,62] Spastin is also an AAA+ enzyme with several identified domains including the AAA+ domain and a microtubule-binding domain, required for microtubule binding and severing activity,[63] a microtubule-interacting and trafficking (MIT) domain required for interacting with other proteins, and a transmembrane domain required for interacting with organelles.[63,64] A truncated version of spastin, missing the first 87 amino acids in mammalian systems that contains the membrane-binding domain. When expressed in cells or reconstituted in vitro, this protein is capable of severing paclitaxel-stabilized microtubules.[58–61] Interestingly, recent work has demonstrated that the expression of both the full length and naturally truncated versions are regulated in neuronal cells, but that the truncated version expresses higher in most neuronal tissues.[65]

Fidgetin is the protein missing from a spontaneous mouse mutant called the "fidget mouse." The mice display head shaking and weaving phenotype. They were "discovered" in the 1940's by irradiating mouse eggs to cause random mutations.[66] Many decades were spent characterizing the anatomy of the fidget mice, which have reduced inner ear canals causing the head shaking, extra fingers and toes, malformed pelvis and skulls, and a variety of bone and cartilage deformities.[67–69] It was not until 2000 that the genetic sequence of the fidgetin mouse was analyzed. The analysis showed that the missing protein was a AAA+ enzyme.[70] The protein was named fidgetin, and the sequence looked similar to a microtubule severing enzyme. Indeed, cellular studies showed that it colocalized with spastin during mitosis.[71] Upon expression and purification, we have shown that fidgetin does, in fact, sever paclitaxel-stabilized microtubules.[72]

Severing proteins have been shown to play a number of roles in cells (Figure 3A).[73] They have roles in mitosis, meiosis, and cytokinesis.[71,74–76] These enzymes also regulate cell motility directionality and speed and have strong effects in wound healing.[74,77] All three severing enzyme families have been implicated

in neuronal development and maintenance.[78–81] Moreover, severing proteins affect microtubule organization in plant cells thus controlling the mechanical fragility of the cell wall and of the plant itself.[82,83]

Enzyme Characteristics

Microtubule severing enzymes and dynein motor proteins are both members of the AAA+ enzyme group, but dynein is highly modified to perform its transport functions. Severing enzymes look much closer to their AAA+ relatives present in all species from bacteria, yeast, plants, and animals. Like other AAA+ enzymes, severing enzymes appear to hexamerize to function.[60,84] Members of the AAA+ family typically assemble into ring-shaped oligomers, and only in this state can they bind their substrate with high affinity.[85,86] However, unlike most AAA+ ATPases, katanin, and spastin are monomeric when bound to ADP, and form hexamers only in the presence of ATP.[60,87] Moreover, studies strongly suggest that these enzymes bind microtubules in their monomeric form and only subsequently assemble into higher order oligomers on the microtubules.[88–90] ATPase activity and severing are reduced in the presence of high concentrations of microtubule substrate.[60,82] This result is also consistent with a scenario in which monomers bind to substrate. When there is a high concentration of substrate, the concentration of monomers is relatively dilute and inhibits hexamerization.

Upon hexamerization, there is a small, 2 nm, pore in the center, which constitutes the active site of the enzyme (Figure 2B).[87] For severing proteins, particularly for spastin, it has been shown that the pore specifically binds to the carboxy-terminal tail (CTT) of tubulin to perform severing (Figure 2B).[63,87] This was demonstrated with "pull-down assays" using the CTT as bait to retrieve spastin hexamers.[63] Interestingly, the authors found that spastin monomers cannot retrieve free tubulin, while the complete pore of a hexamer exhibits this interaction.[63] Treatment of tubulin with subtilisin protease causes the specific cleavage of the CTT. Katanin and spastin are both unable to sever subtilisin-treated microtubules.[59,63,64] Interestingly, katanin enzymes can still bind to subtilisin-treated microtubules, but spastin cannot.[59,89] Structural studies showed that the AAA+ motor module is connected via a linker, with low sequence conservation, to an N-terminal domain, which contains a microtubule interacting and trafficking (MIT) domain consisting of a three-helix bundle.[87,91] Modeling and NMR studies have shown that the N-terminus of katanin also has a structurally similar domain, which has been named an MIT domain, as well.[92,93] There is some confusion in the literature about the MIT domain and a different microtubule-binding domain (MTBD). In human spastin, the MIT domain is located from amino acids 116 – 194[63,91,93] and the MTBD is located from amino acids 270 – 328.[63] Unfortunately, several prominent papers in the literature assign the name MIT domain to the MTBD, which is closer to the AAA+ domain.[64,87] If one uses the original definition and location of the MIT domain, it contains a transmembrane domain, which can be cleaved without affecting spastin activity both in cells and in vitro.[63,64] It is clear that the MTBD is required for spastin binding and severing to microtubules, whereas the MIT domain is likely required to mediate membrane-protein and protein-protein interactions.

Both katanin and spastin are microtubule-stimulated ATPases, that require ATP hydrolysis to sever stable microtubules. The ATP hydrolysis takes place in their highly conserved C-terminal AAA+ ATPase domains. Slowly hydrolyzable nucleotide analogs, such as AMPPNP and ATPγS, enhance binding of katanin to the microtubule and inhibit severing.[59,94,95] In spite of the high affinity of katanin for microtubules in the presence of ATPγS, we have recently shown that tubulin dimers can induce dissociation of katanin from the microtubule.[90] This is surprising considering the high affinity measured for katanin binding to microtubules in the presence of ATPγS.

Severing Regulation

As described above, tubulin post-translational modifications are likely to alter microtubule structure and dynamics. Post-translational modifications have also been shown to have strong effects on the affinity of stabilizing and destabilizing MAPs for microtubules. Several studies have demonstrated that severing enzymes are specifically regulated by post-translational modifications. Cellular studies with neurons have demonstrated that katanin is attracted to acetylated microtubules, which have an acetylated lysine on alpha tubulin positioned in the inner luminal surface of the microtubule.[96] Other post-translational modifications of tubulin occur on the CTTs and can inhibit or enhance binding and severing activity. Specifically, spastin has been shown to be up-regulated by polyglutamylation and down-regulated by detyrosination.[42,63,87] We have recently shown that katanin is inhibited by beta and alpha CTTs, but detyrosinated alpha CTTs are far less effective at inhibiting severing, implying that detyrosination is a poor target for katanin.[90] New advances in the ability to purify homogenous modified tubulin from yeast and insect expression systems will enable more information about how the post-translational state of tubulin can alter katanin's severing activity. Indeed, during the review of this manuscript, a new paper was published using recombinant tubulin to show that glutamylation has a graded effect on spastin severing. Specifically,

because there are a variety of glutamylation sites and the number of glutamates residues can vary, the amount of glutamylation specifically dials the activity of spastin.[50]

Open Questions

Despite all the knowledge we have gained from the few groups studying severing, many open questions remain about severing enzymes.

There are a number of questions about how ATP-hydrolysis is communicated around the AAA+ ring. Do neighboring subunits need to be in the same or different states of hydrolysis? Does one monomer catalyze the hydrolysis on the neighboring monomer? Is the activity of hydrolysis "processive" to allow many ATP hydrolysis cycles without substrate release? Recent work has examined how poisoning the ATPase by adding increasing levels of "dead" monomer can affect severing activity.[88] Interestingly, the best enzymatic model to fit the data was a "neighbor model" where the neighboring subunit state must be intact. Because the addition of dead mutants within the hexamer is random, some severing enzymes were fully functional causing an overall reduction of severing. Future experiments with engineered severing enzymes that are concatenated monomers of dimers or trimers are needed to address these questions exactly. Such constructs can constrain an enzymatically dead monomer to bind adjacent to an active monomer to test if neighbors affect each other and to test processivity. Similar constructs have addressed a number of questions in the ClpX family of AAA+ enzymes used for protein unfolding.[103]

It is completely unknown how severing enzymes bind and dock onto the microtubule lattice. Assuming the severing enzyme is a hexamer it resembles a disk (Figures 3B and 3C). The disk can either bind flat along the microtubule, like a plate sits on a table, or the disk can sit up, like a wheel. In either possible orientation, the severing enzyme has very few monomers in contact with the microtubule lattice due to a mismatch between the structures (Figure 3D). Namely, the microtubule filament, being a rhombic lattice, is incompatible with strong binding to all six severing enzyme proteins if the monomers are hexamerized.

Several studies have imaged severing enzyme hexamers.[60,84,87,89] While one study has shown hexamers docked onto microtubules,[89] no one has performed high resolution imaging with docked severing enzyme at high enough resolution to observe how the lattices fit together. Knowledge of how severing enzymes actually bind and dock onto microtubules is crucial for building mechanical models for how such enzymes can create force to remove dimers from the lattice. Other unknowns are how many enzyme sites apply force to the filament to rip it apart or where those forces are applied. We also do not know in what directions force is being applied. To begin to address these questions, we have created mechanical models of microtubules and applied both pulling forces[97] and pushing forces[98] on the lattice to determine where the filament breaks and how that would potentially affect severing. We found that, irrespective of the point of force application, be it on a protofilament or between protofilaments, indentation of a typical GDP-like microtubule lattice, for which the free energy of dissociation of the longitudinal tubulin-tubulin bonds is double that of lateral bonds,[98] leads to the initial fracture of lateral contacts between protofilaments. Breakage of longitudinal contacts inside protofilaments is delayed. When it finally occurs, it leads to the loss of dimers from the microtubule, in accord with the findings of AFM microtubule indentation experiments.[17,99–101] Because breakage of longitudinal rather than lateral contacts is a requirement for severing, our simulations suggest that, if pushing forces are involved in severing then microtubule states corresponding to lattices where the stability of longitudinal contacts decreases compared to that of lateral bonds will be easier to sever. Simulations carried out to probe the response of microtubule fragments to pulling forces[97] revealed that lower forces lead to the extraction of dimers from a microtubule compared with the pushing (indentation) setup. If the magnitude of the applied force is a criterion for severing, our simulations suggest that pulling would be preferable to pushing. However, because in the pulling simulations we only used a fragment rather than the full microtubule from the indentation simulations and, more importantly, due to the considerable uncertainty in the number (and identity) of the sites that could be pulled by severing enzymes, as discussed before, a direct comparison between these two sets of simulations is not possible.

Severing Mechanism

Finally, the most obvious and most difficult question to tackle is the mechanism of severing itself. How does the ATPase harness the power of hydrolysis to result in the disintegration of stable microtubules? A number of experimental studies, including our own, have shown conclusively that severing proteins cannot perform their severing action on microtubules polymerized from dimers with the acidic residue-rich CTT unstructured segment of either alpha- or beta-tubulin removed.[59,95,102] Thus, as originally proposed by Roll-Mecak,[87] the first step in the severing mechanism is to grip the CTT likely using the three highly conserved loops that are essential for microtubule severing that line the 2 nm diameter pore of the hexameric structure. This action leads to stretching of the CTT tail region of tubulin to the point of making it taut.

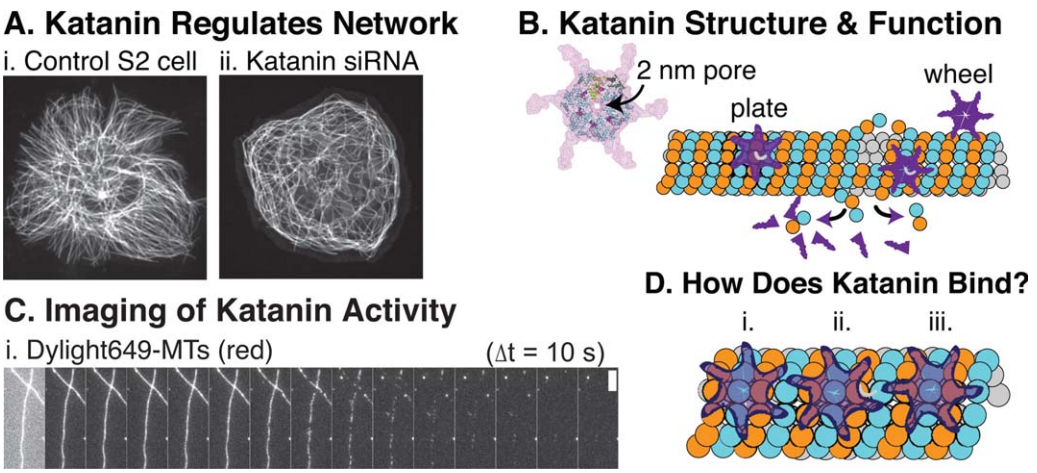

FIGURE 3 Katanin, a microtubule severing enzyme, regulates microtubules length, density, and distribution in live cell through cutting microtubules and regulating their dynamics. A: Image showing how katanin regulates microtubule networks in S2 fly cells comparing (i) a normal, control, cell to (ii) a cell with katanin knocked down using siRNA. Figure reproduced from Ref. 74. B: Spastin structure and possible severing function. Katanin is a AAA+ enzyme that forms a hexamer. The 2 nm pore in the center is the likely site for severing activity through interactions with the CTT of tubulin. The mechanism of katanin severing is currently unknown. Spastin structure reproduced here with permission from Nature publishing group.[87] Severing enzymes could bind to the microtubule surface, like a plate or like a wheel. C: Direct visualization of katanin severing of microtubules. Image reproduced with permission from Cell Press from Ref. 90. D: Models of complexes between a severing enzyme and the microtubule filament showing different modes of binding to the lattice. The different configurations, (i–iii), demonstrate three possible orientations between the lattice and the severing enzyme hexamer. Each possible binding orientation has a mismatch between the hexamer and the rhombic lattice of the microtubule. We display three possible states of a continuum of possible orientations.

Beyond this initial action, two possible second steps have been proposed for severing that are not mutually exclusive. We term them the "Unfoldase" model and the "Wedge" model.

Unfoldase Model

Roll-Mecak and Vale proposed that severing enzymes tear out tubulin dimers from the wall of microtubules by latching onto the CTT of tubulin using the 2 nm pore, threading through the pore, likely driven by nucleotide-driven conformational changes of the pore loops (Figure 3B). This mechanism is based on the known action of other AAA+ enyzmes, such as ClpX, that unfold peptides through threading the folded peptide through the pore through successive cycles of ATP hydrolysis.[103] The activity of severing enzymes could be to unfold the tubulin dimer to pluck it out of the body of the microtubule or, as Ogura and coworkers proposed, katanin pulling the CTT from both alpha- and beta-tubulin in a subunit at the same time inside the pore of the hexamer might create tension in the dimer leading to its destabilization and eventual break from the microtubule lattice.[102] The unfolding of most of the tubulin dimer is not at all likely given that dimers removed from severed microtubules are indistinguishable from free tubulins being competent for repolymerization[59] and because tubulin requires the presence of chaperones to fold. These two facts indicate that the removed dimers are likely not unfolded considerably.

Wedge Model

An alternative mechanism for tubulin dimer removal still assumes that the CTT threads through the 2 nm pore and is held taut inside the pore. The main severing action occurs through the AAA+ motor exerting forces on the tubulin-tubulin interfaces—not on the CTT peptide—and would work to wedge out the dimer. Such forces could be generated, for example, by the interaction between the MIT domains of katanin and the surface of the microtubule lattice. This scenario has been envisioned by Roll-Mecak and Vale who acknowledged that "spastin may not need to completely translocate the tubulin polypeptide substrate, but instead just grip the C-terminal tubulin tail and exert mechanical tugs that might partially unfold tubulin or locally destabilize dimer-dimer interactions, leading to catastrophic breakdown of the microtubule

lattice."[87] If this is the mechanism of action of severing proteins on microtubules then any tubulin removed will still be in its folded state. Indeed, this agrees with prior work that states most removed dimers are capable of repolymerizing into microtubules in the absence of chaperones.[59]

SUMMARY

In this review article, we have described how microtubules are polymers of GTPase enzymes. We described recent advances in theory and experiment that are opening new windows onto the mechanisms of microtubule dynamics, especially with respect to their polymerization-depolymerization behavior. Considerably less is known about the mechanisms of microtubule severing enzymes. Major stumbling blocks are the lack of knowledge of the binding sites of severing enzymes on microtubules, and the directionality and magnitude of the forces exerted by enzymes during severing. Recent experimental advances still do not completely addressing the two possible mechanisms for microtubule severing. Thus, new experiments and models are needed to determine how microtubule-severing enzymes can actually exert forces on the microtubule lattice to remove dimers.

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