Charles University

Faculty of Science

Study programme: Biology

Branch of study: Cell and Developmental Biology

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Regulation of the microtubule severing activity of Katanin

Regulace štěpení mikrotubulů Kataninem

Diploma thesis

Supervisor: **RNDr. Zdeněk Lánský, Ph.D.**

Prague, 2021

Prohlášení:

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V Praze, 10. 8. 2021

Roman Podhájecký

Děkuji především svému školiteli RNDr. Zdeňkovi Lánskému, Ph.D. za neutuchající podporu, vedení a kritické zhodnocení této práce. Také děkuji všem členům Laboratoře strukturních proteinů za pomoc při řešení experimentálních potíží. Poděkování patří i mé rodině za laskavost a trpělivost.

Abstrakt

Cytoskelet je dynamický systém, který se podílí na mnoha buněčných procesech, jako například buněčné dělení nebo buněčná motilita. Katanin je protein asociovaný s mikrotubuly, jehož klíčová vlastnost je štěpení mikrotubulů. Štěpení mikrotubulů je důležité pro amplifikaci a organizaci mikrotubulových sítí. Nicméně přesná kontrola Kataninové aktivity je nutná, protože dysregulace Kataninové aktivity vede těžkým vývojovým vadám. V této práci jsme prozkoumali lokalizaci Kataninu do mikrotubulových překřížení a důsledky post-translačních modifikací tubulinu na aktivitu Kataninu. Pozorovali jsme, že lokalizace Kataninu do mikrotubulových překřížení je jeho přirozená vlastnost a podjednotka p80 se na lokalizaci Kataninu do překřížení nepodílí. Z našich výsledků vyplývá, že podjednotka p80 se do překřížení lokalizuje skrze neznámého interakčního partnera. Také jsme charakterizovali vázání Kataninu na mikrotubuly a schopnost Kataninu štěpit je v kontextu různých post-translačních modifikací a tubulinových isotypů. Nehledě na vyšší affinitu Kataninu ke značně modifikovaným mikrotubulům, schopnost Kataninu štěpit je byla nižší v porovnání s méně modifikovanými mikrotubuly. Naše výsledky poukazují na důležitost post-translačních modifikací a jejich možné role v organizaci buněčných procesů.

Klíčová slova:
cytoskelet, remodelace mikrotubulových sítí, mikrotubuly, enzymy štěpící mikrotubuly, Katanin, post-translační modifikace, tubulinové isotypy, rozpoznávání překřížení, p60, p80

Abstract

Cytoskeleton is a dynamic system which contributes to a variety of cellular processes, for example cell division or cell motility. Katanin is a microtubule associated protein and its key feature is the microtubule severing. The microtubule severing is important for microtubule array amplification and its organization. However, precise control of Katanin activity is needed, otherwise dysregulated microtubule severing results in severe developmental disorders. Here, we investigated Katanin localization to microtubule crossovers and consequences of tubulin post-translational modifications on Katanin severing activity. We found that Katanin localization to crossovers is its inherent property and the contribution of the subunit p80 to Katanin localization to crossovers is insignificant. Our results suggest that the subunit p80 localizes to crossovers via unknown interacting partner. We also conducted characterization of Katanin binding and severing in the context of different post-translational modifications and tubulin isotypes. We found that despite higher binding of Katanin to heavily-modified microtubules, its severing activity is lower. Our results emphasize the importance of post-translational modifications and its potential role in organization of cellular processes.

Key words:
cytoskeleton, microtubules, microtubule severing enzymes, Katanin, post-translational modification, tubulin isotype, crossover recognition, p60, p80

Abbreviations

αTAT1	Alpha-tubulin N-acetyltransferase
γTuRC	γ-Tubulin Ring Complex
a.u.	arbitrary unit
AAA	ATPase Associated with diverse cellular Activities
ASPM	Abnormal Spindle-like, Microcephaly-associated
ATP	Adenosine triphosphate
BRB80	Britton–Robinson buffer
DDS	Dichlorodimethylsilane
GDP	Guanosine Diphosphate
GFP	Green Fluorescent Protein
GMPCPP	Guanosin-5'-[(α,β)-methyleno]phosphate
GTP	Guanosine triphosphate
HBD	Helix Bundle Domain
HDAC6	Histone Deacetylase 6
IRM	Interference Reflection Microscopy
MAP	Microtubule Associated Protein
MCAK	Mitotic centromere-associated kinesin
MEC-17	αTAT1 found in *C. elegans*
MEI-1/MEI-2	Katanin-like protein
MIT	Microtubule Interacting and Trafficking
mmKatanin	Mammalian Katanin
MT	Microtubule
NBD	Nucleotide Binding Domain
PBS	Phosphate-buffered saline
PIPES	Piperazine-N,N-bis(2-ethanesulfonic acid)
RFC	Relative Centrifugal Force
TIRF	Total Internal Reflection Fluorescence
TTL	Tubulin tyrosine ligase
TTLL	Tubulin tyrosine ligase-like
VASH	Vasohibin 1

Contents

1 Introduction

Cytoskeleton maintains variety of cellular functions as cell motility, structural rigidity, force transfer, cellular transport and many others. Cytoskeleton consists of three significant structures, microtubules, actin filaments and intermediate filaments. Proper regulation of cytoskeletal processes is needed to achieve specific cellular roles. Katanin is microtubule associated protein responsible for microtubules severing. Both microtubules and Katanin play important role in brain development and proper plant cell wall formation. Here, we studied different regulation mechanisms of Katanin mediated microtubule severing.

2 Overview

2.1 Microtubules

2.1.1 Microtubules and dynamics

Microtubules, as a part of cytoskeleton repertoire of the euaryotic cells, are polymeric structures which maintain several key functions of the cell. Microtubules contribute sister-chromatid separation during cell division [1], vesicular transport [2], cell migration [3] or cell wall formation in plants [4]. Microtubules are hollow tubular structures composed of α- and β-tubulin. The α/β-tubulin dimer is arranged into protofilaments, which form lattice of the microtubule. Tubulin subunits alter in arrangement and thus we recognize microtubules as polarized structures, where an end capped with β-tubulin is called (+)-end and an end capped with α-tubulin is called (-)-end. Typically, microtubules are formed by 13 protofilaments. However, formations between 11 and 15 protofilaments have been observed *in vivo* [5]. Both α- a β-subunits bind a molecule of GTP. After subunit dimerization, α-tubulin bounded GTP molecule is buried within the binding interface between both subunits and cannot be hydrolysed. Second β-tubulin bond GTP molecule can be hydrolysed and thus participates in dynamic instability of microtubules [6].

New microtubule formation

Formation of new microtubules is vital for cell survival. Currently, there are two known mechanisms how new microtubules are formed, templated formation and non-templated formation [7]. Templated microtubule formation needs existing tubular structure from which the microtubule can polymerize. Such mechanism is favorable in cell environment because it bypasses the energetic barrier which has to be overcome prior to formation of microtubules *de novo*. The γ-tubulin ring complex is a typical example of a template for a new microtubule [8]. Another way how new templated microtubules are formed is by severing of existing ones. Severing enzymes, such as Katanin, Spastin or Fidgetin, are AAA-ATPases able to dislocate tubulin subunits from the microtubule wall and thus produce local damage to the lattice. Consecutive removal of the tubulin subunits in the same spot can result in destabilisation of microtubule lattice. This leads to generation of new (+)-end and new (-)-end, which can potentially serve as template for microtubule polymerization. Recently, *in vitro* work largely relies on reconstituted microtubules [9]. Non-templated formation of these microtubules is dependent on high tubulin concentration to overcome high energetic barrier prior nucleation [10].

Microtubule dynamics

Microtubules are recognized as dynamic structures, because of their ability to polymerize and rapidly disassemble. The transition event from polymerization to disassembly is called catastrophe and the opposite event is called rescue [11]. α/β-tubulin dimer has two GTP binding sites, E-site and N-site. N-site is buried within the dimer interface of α- and β-tubulin. N-site bound GTP does not hydrolyse. E-site GTP is prone to hydrolysis and thus affects conformation of the α/β-tubulin dimer. Cryo-electron microscopy imaging revealed that after GTP hydrolysis, tubulin conformation changes lead to microtubule lattice compaction which resolves in built up strain within microtubule lattice [12]. Revealing of the GDP tip of the microtubule, either by hydrolysis of the GTP cap [13] or by severing [14], leads to rapid microtubule depolymerization. Presence of GTP islands, remnants of the GTP tubulin in otherwise GDP lattice, is currently highly discussed. Present studies bring convincing evidence that GTP islands play a key role in facilitating a rescue of depolymerising microtubules. For example, the higher percentage of GMPCPP,

unhydrolisable analog of GTP, the higher chance that rapid depolymerization ceases for a short while, which promotes a chance for a rescue [15]. GTP islands were also detected *in vivo* and their presence correlates with higher rescue rate [16].

Possible mechanism behind GTP islands formation could be incorporation of free GTP tubulin form solution. *In vitro* study shows, that mechanically stressed microtubules accumulate lattice damage which can be repaired by incorporation of free GTP tubulin [17]. Study on severing enzymes opened interesting possibility for new function of these enzymes to operate as initiators of GTP islands formation. It seems that severing enzymes are able to generate microtubule lattice nanodamage which can be repaired by free GTP tubulin [18].

Generation of GTP islands is not only mechanism behind microtubule dynamics regulation. Proteins as members of microtubule associated proteins (MAPs) family including tau protein, MAP2 and MAP4 or specific kinds of kinesins can also modulate catastrophe and rescue rates as well as shrinkage or growing rates. MCAK, a member of kinesin 13 family, is playing crucial role in chromatid alignment, proper bi-oriented spindle connection and consequentially chromatid separation [19]. Chromatid separation relies on rapid microtubule depolymerization, which can be mediated by MCAK [20]. Moreover, it has been shown that MCAK is able to depolymerize stable, GTP mimicking, GMPCPP microtubules [21].

2.2 Tubulin code

Complexity of the cytoskeletal system requires precise tuning to properly execute all its cellular roles. Main players, in this fine-tuned process, are post-translational modifications of tubulin, tubulin isotypes and microtubule associated proteins. Post-translational modifications and tubulin isotypes offer large pool of variability and are collectively termed 'the tubulin code' [22] [23].

2.2.1 Isotypes

There are nine genes for α-tubulin and nine genes for β-tubulin in the human genome [23]. The term isotype is used to distinguish between different tubulin gene products but the term isoform is also sometimes used in the same context [24]. In general, the term isoform is used for the description of different protein

products of the same gene, which can sometimes lead to confusion. The importance of different tubulin isotypes is illustrated by developmental disorders correlated with loss or polymorphisms in tissue-specific tubulin isotypes [25] [26] [27] or enrichment of certain isotypes in taxol-resilient cancer cells [28] [29]. The C-termini tail of both tubulins plays crucial role in the interaction with variety of associated proteins [30] [31]. Most of the variability between tubulin isotypes is located in the C-termini tail [32], which in context of tissue specific isotypes underpins their role in regulation of cellular processes.

Neuronal isotypes

Tubulin isotype composition dramatically changes during neuronal differentiation. Tubb3 is neuron specific isotype of β-tubulin [33]. *In vitro* cultured neurons expressed Tubb3 in early differentiation stages but expression levels significantly dropped after maturation. Similar results were observed for Tubb5. For α-tubulin isotypes, the Tuba4a showed increased expression levels in matured neurons and hippocampal lysates from adult mice [32]. In brain tissue lysates it has been shown that *TUBB2A* is largely expressed together with *TUBB4* (gene for Tuba4a) [34]. Moreover, it should be noted that brain lysates contain beside neurons also non-neuronal cells.

Isotypes and microtubule dynamics

In naive cells taxol induces mitotic arrest and eventually cell death [35]. Interestingly, some cancer cell lines appear to be resistant to taxol-mediated arrest. Several mechanisms of resistance have been proposed. One of them is tied to different expression profiles of tubulin isotypes [29]. Microtubules in these cells appear to be more dynamic and thus able to compensate for the reduced dynamicity caused by taxol [28]. Taxol is a microtubule stabilising agent and important anti-cancer drug. First isolation of taxol was made from *Taxus brevifolia*, but interestingly its biosynthesis is highly entangled with an endophytic fungi [36]. Mechanism by which taxol modulates microtubule dynamics lies within its ability to bind β-tubulin subunit, strengthening lateral interaction between protofilaments [37]. Interestingly compared with other isotypes, Tubb3 appears to modulate microtubule dynamics by elevating catastrophe frequency, but does not affect growing rate of micro-

tubules [38] [39]. However, the resistance of certain cancer cell lines probably comes from other properties of Tubb3, like different affinity for taxol binding [40].

2.2.2 Post-translational modifications

Post-translational modifications are changes to chemical structure of translated proteins. These changes are often accomplished via specific enzymes and are under tight control. There are numerous described modifications of both tubulin subunits. For example acetylation, poly-glutamulation, poly-glycylation, tyrosination and detyrosination, phosphorylation and many more [23], shown in the Figure 1.

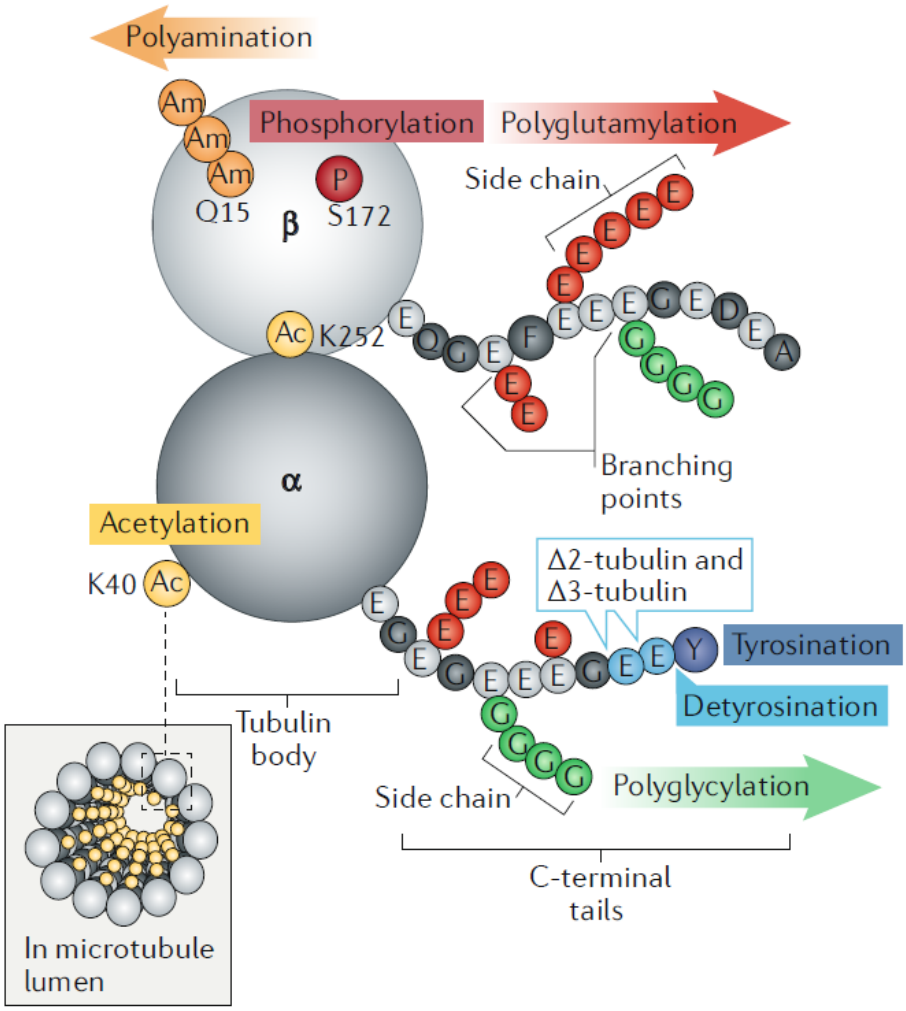

Figure 1: Illustration of different tubulin post-translational modifications. Adapted from [23]

Acetylation

Lysine side chain contains primary ϵ-amino group which can be subject of acetylation [41]. The α-tubulin contains conserved lysine at position 40 and it can be acetylated by enzyme αTAT1 (previously described as MEC-17) [42]. The enzymatic mechanism is shown in the Figure 2. The lysine 40 is located in unstructured loop facing the lumen of the microtubule [43]. In combination with observation that tubulin is acetylated preferentially in its polymeric state [44], it rises the following question: How does αTAT1 access the lumen of microtubules? The possibility that αTAT1 just diffuses in from ends was doubted because it would require days to effectively achieve center of microtubules [45], but it is highly dependent on the binding affinity of the diffusing substance. Interestingly, recent studies confirm the hypothesis that αTAT1 enters the microtubule lumen through microtubule ends, which came along with slow diffusion and acetylation accumulation to the entry point [46] [47] [48]. Additionally, these studies also bring evidence that ends are not the only entry points and lattice defects and breaks could be another way to the microtubule lumen. Disruption in microtubule acetylation in mice is connected to defective neuronal migration [49], brain development [50], sperm motility [51] and touch sensation [52]. Interestingly, altered touch sensation was also observed in other model organisms such as *Drosophila* [53] or *C. elegans* [42]. Microtubules in these scenarios are mechanically stressed, which could imply one physiological function of acetylation. Additionally, recent study suggests that acetylation weakens tubulin lateral interaction and thus makes microtubules more flexible and resilient to mechanical stress [54].

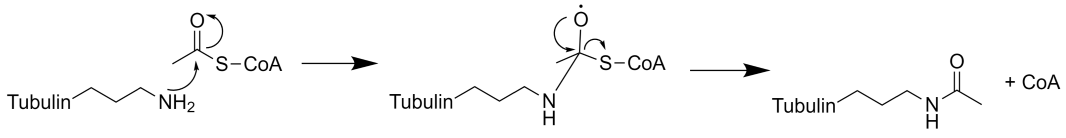

Figure 2: Mechanism of acetylation. Adapted from [41].

Polyglutamylation and Polyglycination

The C-termini tails of both tubulin subunits are rich in glutamate residues. Some of these cytosolic exposed glutamates can undergo mono- (or poly-) glutamylation

and glycination. The enzymes catalyzing these modifications are called tubulin tyrosine ligase-like (TTLL) and contain over 13 members in mammals [55]. Because TTLL are evolutionary close, the mechanism of modification also shares a lot of similarities. The process of poly-glutamylation (poly-glycination) could be divided to initiation phase and elongation phase. Initiation takes place at γ-carboxyl group of the C-tail glutamate residue and starts with phosphorylation. Phosphorylated γ-carboxyl group is subsequently attacked by amino group of ligated residue and thus form peptide bond. Similar mechanism is utilized in elongation phase, but both α- and γ-carboxyl group could form new peptide bond [56]. The Figure 3 shows reaction mechanism of the polyglutamylation. Lengths of the side chains vary tissue from tissue. For example axonemal microtubules in *Tritrichomonas mobilensis* tend to have poly-glutamylated both α- and β- tubulin tails with up to 20 glutamic residues [57]. Mammalian neurons seem to acquire 3 to 6 residues long poly-glutamylation side chain [58] [59] and the length of poly-glutamylation has to be tightly regulated because for example disruption in deglutamylases activity leads to neurodegenerative disorders [60]. Interestingly, it has been suggested that TTLL could be spatially restricted and thus generate sub-populations of differently glutamylated microtubules within one cell [61] and thus impact specific cellular processes. For example longer poly-glutamylation chains increase the processivity of kinesin-1 probably because higher negative charge enhances the interaction with the positively charged motor [62]. In a similar way poly-glutamylation affects severing enzymes, but interestingly the relationship between the length of the side chain and the enzymatic activity is not linear. Rather it's biphasic, which means that shorter side chains stimulate the enzymatic activity and on the other hand longer side chains inhibit it [63].

On the other hand poly-glycination is much more enigmatic. It has been shown that poly-glycination is enriched in cilia [64] [65]. These studies suggest that mono-glycylation and poly-glycylation participate in cilia stability and their length regulation, but precise molecular mechanism is unknown.

Detyrosination

Most of human encoded α-tubulin have the C-terminal amino acid tyrosine. This terminal tyrosine can undergo enzymatic cleavage catalyzed by vasohibin pro-

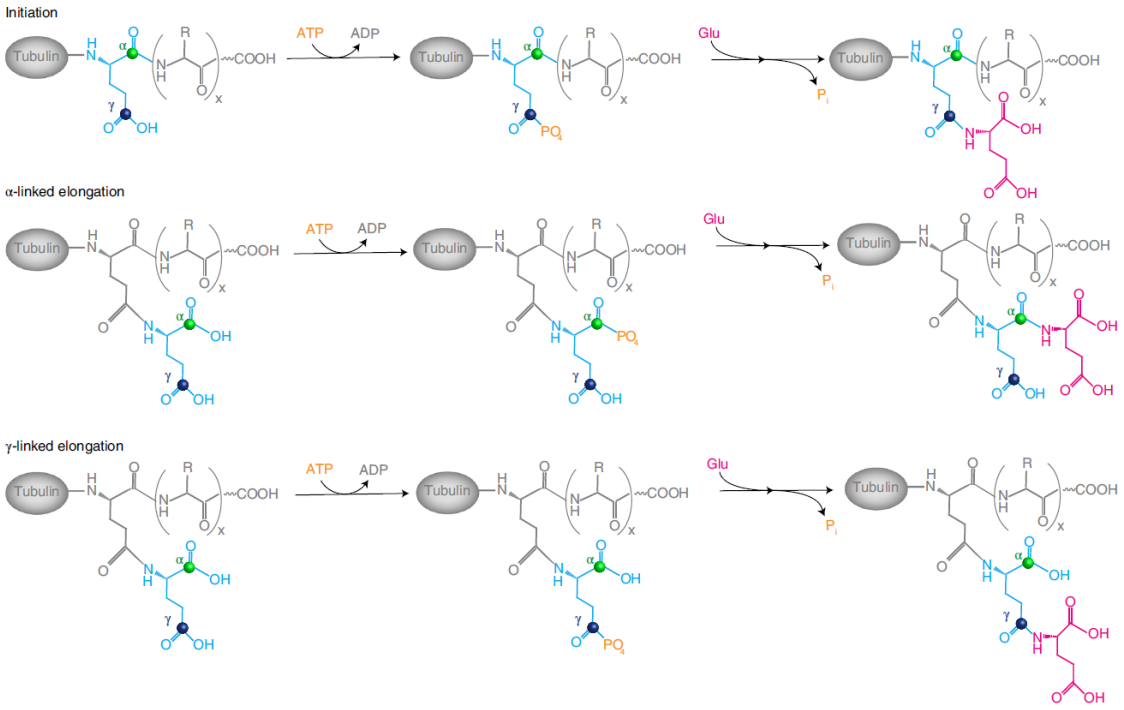

Figure 3: Mechanism of poly-glutamylation. Adapted from [56].

teins (VASH) and thus produces detyrosinated tubulin [66]. This modification is reversible by tubulin tyrosine ligase (TTL) [67]. From a physiological perspective it appears that detyrosination can affect ways how certain microtubule associated proteins interact with microtubules. For example, kinesin-1 shows preference for detyrosinated tubulin *in vivo* [68]. Since detyrosination is enriched in axons, it probably participates in mechanism by which kinesin-1 is distributed in neuron cells [69]. Although, the relationship between kinesin-1 and detyrosination is not direct as shown *in vitro* [70]. Physiological role of the detyrosination can be found in brain development. When enzymatic function of VASH or TTL is disrupted, it can result in severe developmental disorders, like microcephaly [71]. Another role for tyrosination seems to be spindle orientation in asymmetric oocyte meiosis. Interestingly, tubulin tyrosination accumulates on the meiotic spindle towards cortical side of the oocyte [72]. When the tyrosination levels are manipulated it results in disrupted meiotic plane organisation.

2.3 Katanin

Microtubules form a platform for a number of microtubule associated proteins, for example Katanin. Katanin is a microtubule severing enzyme and it was discovered in early 90' [73]. Together with its paralogues, Spastin and Fidgetin, it belongs to a larger group of the AAA ATPase family [74]. Members of the AAA family have several common features, as conserved AAA cassettes, which contain NTP binding motif Walker A and B, and second region of homology. Proteins of the AAA family often oligomerize and participate in plenty of cellular functions for example membrane fusion and protein degradation [75]. In the cell Katanin is formed by two subunits, p60 and p80, which refers to their molecular weight [73]. Katanin heterodimer can further hexamerize in presence of microtubules and thus achieve enzymatically active state [76] [77]. Katanin orhologues can be found in most eukaryotic species. Firstly described example was in sea urchin [73], afterwards it was described in *C. elegans* [78], *Drosophila* [79], *Arabidopsis thaliana* [80] and human [81]. It has been aslo associated with many different cellular processes like mitotic spindle formation [82], synapses and dendrite formation [83], cell migration [79] and plant cell wall formation [84].

2.3.1 Structure of p60

The subunit p60 is enzymatically active and is responsible for microtubule disassembly [14]. In human genome we can find one canonical subunit p60 called KATNA1 and two p60-like homologues, KATNAL1 and KATNAL2 [85]. Structurally p60 can be divided into the N-terminial microtubule interacting domain and the C-terminal AAA cassette, shown in the Figure 3. The AAA cassette can be further divided to nucleotide binding domain and helix bundle domain. Beside motifs associated with ATP hydrolysis, like Walker A and B or arginine fingers, two unstructured pore loops can be found in the nucleotide binding domain. Both pore loops interact with residues of the tubulin C-tail. Interaction between tubulin C-tail and pore loops is probably responsible for transfer of forces induced by hexamer conformation changes after ATP hydrolysis [86]. The MIT domain and AAA cassette are connected via positively charged disordered linker. In the linker we can also find serine 135, which can be phosphorylated and thus negatively regulate Katanin's

activity [87] [31]. The MIT domain is formed by three conserved α-helices rich in positively charged residues, which facilitate interaction with negatively charged surface of the microtubule [88]. Moreover, the MIT domain also provide interaction with Katanin's subunit p80. Dissociation constant of p60:p80 complex is in nano- or even pico-molar range. Such a strong interaction is probably provided by numerous hydrophobic residues [89].

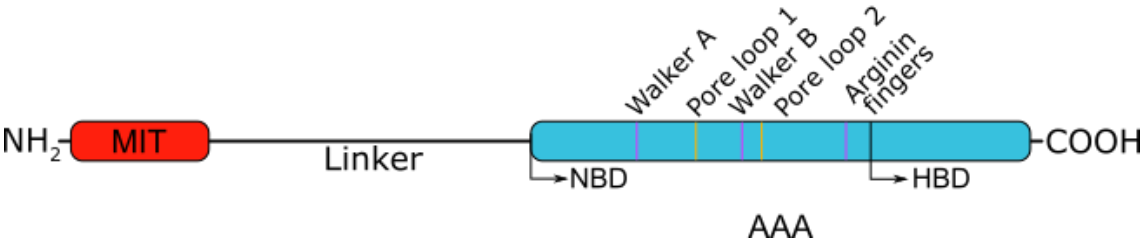

Figure 4: Sequence of the subunit p60 divided into domains and significant structures.

2.3.2 Structure of p80

Two significant structures can be found in the subunit p80, the N-terminal WD40 domain and the C-terminal con80 domain, shown in the Figure 3. The WD40 domain consists of six repeated motifs bordered by tryptophan-aspartate [14]. Although structure of full p80 is not solved yet, the WD40 proteins often share similar architecture as they form β-propeller structure. Over 300 different proteins containing WD40 repeats have been identified in human. It is also common that WD40 proteins serve as scaffolds and mediate protein-protein interactions [90]. But interestingly, the con80 domain is also able to provide binding interface to other proteins. For example, ASPM protein target whole Katanin complex to centrosome [82]. The C-terminal con80 domain is responsible for binding to the p60's MIT domain. Hetero-dimer formation relies on hydrophobic interactions and hydrogen bonds. In comparison to other members of the AAA family, the p60/p80 hetero-dimer shows one of the strongest interaction [89].

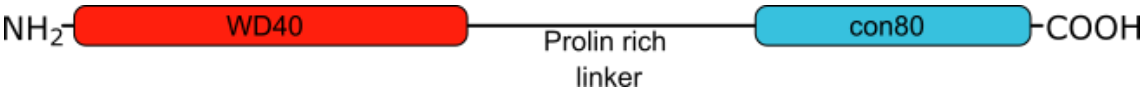

Figure 5: Sequence of the subunit p80 divided into domains.

2.3.3 Ring formation and mechanism of action

Formation of the Katanin hexamer is critical for triggering ATPase activity and thus microtubule severing [76]. The structure representation is shown in the Figure 6. Moreover, Katanin in solution can oligomerize up to dimers (p60/p80 heterotetramers) and the presence of microtubules is needed to achieve higher oligomeric state [76] [91]. Katanin affinity to microtubules plays crucial regulatory role in the process. For example, regulatory subunit p80 enhances the affinity of the subunit p60 to microtubules [81] [92] and thus increases the probability of hexamer formation. In a similar manner the tubulin C-tail polyglutamylation enhances interaction between Spastin's MIT and microtubules [63]. Since Katanin and Spastin are closely related, similar mechanism of regulation is expected. Last but not least example is the positively charged linker between MIT domain and AAA cassette. Mutation in these positively charged residues disrupts Katanin's binding to microtubules. Additionally, introduction of the negative charge, in form of serine phosphorylation, could have a similar result [93] [31]. It has been proposed that Katanin hexamer can acquire several different conformations depending on the nucleotide state. First described mechanism suggest, that Katanin in the ATP nucleotide state forms right-handed spiral, which after hydrolysis changes to closed ring conformation [86]. This transition is supposed to apply force on the tubulin C-tail and thus result in tubulin displacement form the lattice [31]. Proposed mechanism is shown in the Figure 7.

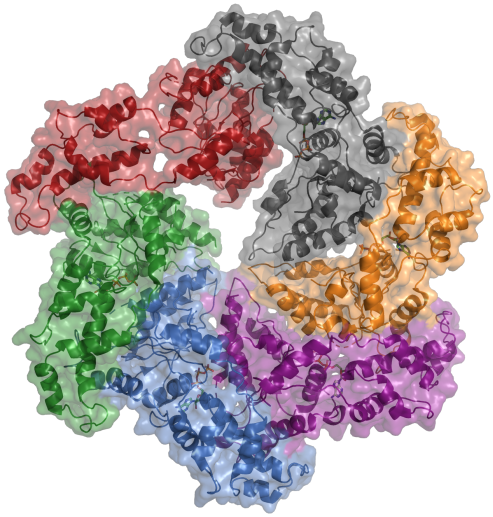

Figure 6: Structure of hexamerized AAA cassette of the subunit p60. Structure published in [86].

2.3.4 Physiological role of severing

Katanin participates in many different cellular processes and disruption of Katanin function leads to developmental disruptions in humans as well as in plants. In human, mutation in KATNB1, a gene for subunit p80, results in brain developmental disorder microlissencephaly [94]. Interestingly, loss of the subunit p80 also resulted in centriole overduplication and disruption of Shh signaling [94]. Loss of function of the subunit p80 in mice also results in spermatogenesis disruption and sperm motility diminution. Also the subunit p80 localizes to sperm head tip and its loss results in sperm morphology abnormalities [95]. Katanin also participates in cell motility. The subunit p60 localizes to the leading edge of *Drosophila* migrating cells and the loss of function results in the leading edge morphology alteration and sight decrease in directionally persistence migration [79]. Loss of Katanin function also manifests in a disruption of axon outgrowth in neuronal rat cell culture [96].

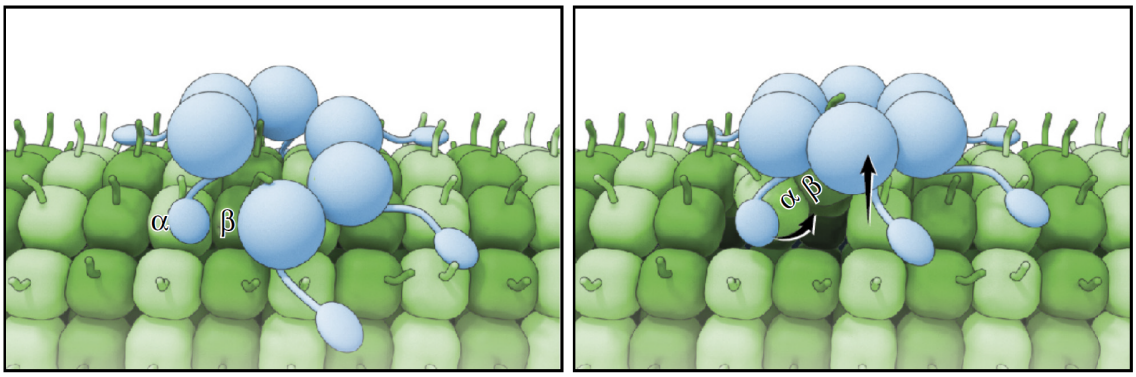

Figure 7: Proposed mechanism of Katanin-mediated severing. By this mechanism, conformation changes in Katanin hexamer result in a tubulin displacement from the microtubule lattice [86].

Another important physiological process, which Katanin participates in, is regulation of meiotic spindle length [87]. Katanin regulatory subunit p80 can target Katanin complex to centrosome and thus facilitates microtubule severing in specific cellular location [14] [87] [82]. It was shown that Katanin depleted *C. elegans* embryos have longer meiotic spindle [97]. It was also hypothesized that Katanin role is not just microtubule dissasembly but also microtubule release and array amplification via increasing polymer number by severing [98]. Similar behaviour was shown in plants. If Katanin is mutated in plants, it results in cortical microtubule array

disorganization. Cortical microtubules are responsible for cellulose deposition to the cell wall and thus responsible for cellular shape [80] [4]. Interestingly, Katanin localizes to microtubule nucleation points and microtubule crossovers and severs microtubules in that location. Moreover, such specific catalytic action is needed for massive microtubule array reorganization [84]. Indeed, in this physiological function the regulatory subunit p80 is needed. The subunit p80 promotes Katanin localization to crossovers and provides crosslinking with other proteins [99] [100].

3 Aims

Katanin localization to microtubule crossovers in plants is essential for proper development. Although this mechanism was precisely studied, molecular mechanism is still unknown. Here, we hypothesized that Katanin regulatory subunit p80 facilitates Katanin localization to crossovers. To examine this hypothesis we performed *in vitro* comparison of microtubule localization of the catalytic subunit p60 and both subunits p60&p80. Rising evidence of 'Tubulin code' importance in regulation of cellular processes leads steps in cytoskeletal research. For example, microtubules in brain tissue are heavily modified and are composed of different tubulin isotypes than microtubules in other tissue. There is little to no evidence on how post-translational modifications and tubulin isotypes affect Katanin-mediated severing, even though Katanin is needed for plethora cellular processes. Several studies examining Katanin-related protein Spastin showed effect of polyglutamylation on affinity and catalytic activity, therefore we hypothesized that Katanin-mediated severing is also regulated by post-translational modifications. Here, we used brain-derived microtubules as a model for heavily modified microtubules and HeLa cell microtubules as a model for almost non-modified microtubules. To examine effect of different substrates on Katanin activity we compared Katanin binding and severing activity in different Katanin concentrations.

4 Methods and materials

4.1 Protein purification

4.1.1 Tubulin purification

Tubulin was purified from porcine brains. Brains were kept ice-cold in PBS buffer (see below) during transport. Homogenization was performed with depolymerization buffer (see below) in 1:1 ratio via blender at 4 °C. Homogenate was centrifuged for 1 hour at 17 700 RCF, 4 °C. For the first round of polymerization, supernatant was diluted in equal volume of High-molarity PIPES (see below) heated up to 37 °C. ATP and GTP was added to final concentration of 1.5 mM ATP and 0.5 mM GTP after addition of glycerol. Warm glycerol (37 °C) was added to the mixture, added volume is equal to added PIPES. Mixture was kept at 37 °C for 1 hour to achieve microtubule polymerization. Next step was depolymerization. Solution was centrifuged at 151 000 RFC for 30 minutes at 37 °C. Supernatant was discarded. Pellets were resuspended in 100 ml of cold depolymerization buffer via douncer and incubated on ice for 30 minutes to accomplish microtubule depolymerization. Afterward, solution was centrifuged at 70 000 RFC for 30 minutes at 4 °C. Supernatant was used for next step of polymerization which followed same steps as first round except the incubation time, which was reduced to 30 minutes. Pellets were resuspended in 15 ml of ice-cold BRB80 and homogenized via douncer. Mixture was incubated for 10 minutes on ice, then it was centrifuged at 100 000 RCF for 30 minutes at 4 °C. At the end supernatant was aliquoted and sanp-freezed in liquid nitrogen. Tubulin was stored at -80 °C. HeLa tubulin was kindly provided by Carsten Janke (Inst. Curie, Paris).

4.1.2 Plant subunit p60 and p80 purification

Subunits p60 and p80 were expressed separately in an AcsI-NotI-digested pOCC destination vector. Both subunits were tagged with 3C PreScission Protease cleavage site and 6xHis-tag. Both subunits were expressed in SF9 insect cells using an opensource FlexiBAC baculovirus vector system. Insect cells were harvested after 4 days, centrifugation at 300 RFC for 10 minutes at 4 °C. The pellet was resuspended in 5 ml ice-cold PBS. Insect cells were lysed in 30 ml ice-cold His-Trap buffer. Cells

PBS

20 mM	NaH_2PO_4	Sigma-Aldrich
150 mM	NaCl	Sigma-Aldrich
pH 7.2		

Depolymerization buffer

50 mM	MES	Sigma-Aldrich
1 mM	$CaCl_2$	Sigma-Aldrich
pH 6.6		

High-molarity PIPES

1 M	PIPES	Sigma-Aldrich
10 mM	$MgCl_2$	Sigma-Aldrich
20 mM	EGTA	Sigma-Aldrich
pH 6.9	(KOH)	

BRB80

80 mM	PIPES	Sigma-Aldrich
1 mM	$MgCl_2$	Sigma-Aldrich
1 mM	EGTA	Sigma-Aldrich
pH 6.8	(KOH)	

Nucleotides

100 mM stock	ATP	Jena Bioscience
200 mM stock	GTP	Jena Bioscience

Others

	Glycerol	Penta

were centrifugated at 45000 RFC for 60 minutes at 4 °C. The lysate was incubated in a lysis buffer-equilibrated Ni-NTA column for 2 hours at 4 °C. The Ni-NTA column was washed with the His-Trap buffer supplemented with 300 mM Imidazole. Fraction containing protein was diluted 1:10 in the His-Trap buffer. The purification tag was cleaved overnight with 3C PreScisson protease. The protein was snap frozen in liquid nitrogen and stored at -80 °C.

4.1.3 mmKatanin purification

Mammalian Katanin (mmKatanin) was expressed and purified as described previously [82]. Briefly, full length mammalian subunit p60 was co-expressed with the C-terminal domain of mammalian subunit p80. Expression was performed in the pET28a vector. Transfected bacteria cells were lysed by ultrasonication in lysis buffer. Mammalian Katanin was subsequently purified by Immobilized Metal Affinity Chromatography at 4 °C. Size-exclusion chromatography was used for subsequent purification. Purified mmKatanin was snap frozen in liquid nitrogen and stored at -80 °C.

4.2 Tubulin labeling

The tubulin was labeled as described previously [101].

4.2.1 TIRF and IRM settings

Microscope Nikon Ti-E Eclipse was used for total internal reflection fluorecence (TIRF) microscopy. Microscope was equipped with 100x oil immersion objective Nikon Plan Apo and an electron multiplying CCD camera Andor Technology iXon Ultra 888. Unlabeled microtubules were visualized via internal reflection microscopy (IRM), previously described in [102]. The GFP acquisition rates were approximately 1.7 frames per second. The IRM acquisition rates were approximately one frame per 60 seconds.

His-Trap buffer

50 mM	NaH_2PO_4	Sigma-Aldrich
300 mM	KCl	Sigma-Aldrich
1 mM	$MgCl_2$	Sigma-Aldrich
10 mM	β-Mercaptoethanol	Sigma-Aldrich
0.1%	Tween20	Sigma-Aldrich
0.1 mM	ATP	Jena Bioscience
pH 5%	Glycerol	Penta
pH 7.5		

Lysis buffer

50 mM	HEPES	Sigma-Aldrich
500 mM	NaCl	Sigma-Aldrich
5 mM	β-Mercaptoethanol	Sigma-Aldrich
10 mM	Imidazole.	Sigma-Aldrich
pH 7.4		

4.3 Taxol-stabilized microtubules

To prepare taxol-stabilized microtubules 5 μl of tubulin was mixed with 1.25 μl of polymerization mix to the final concentration 4 mg.ml^{-1} of tubulin. Mixture was incubated 5 minutes on ice and afterwards incubated at least 30 minutes at 37 °C. Subsequently, 100 μl of BRB80T was added. Mixture was then centrifuged at 14 000 RFC for 30 minutes. Finally, supernatant was discarded, and the pellet was gently washed with BRB80T. The pellet was gently resuspended in 80 μl of BRB80T via trimmed pipette tip to avoid breakage of polymerized microtubules.

BRB80

80 mM	PIPES	Sigma-Aldrich
1 mM	$MgCl_2$	Sigma-Aldrich
1 mM	EGTA	Sigma-Aldrich
pH 6.8	(KOH)	

BRB80T

	BRB80	
10 μM	Taxol	Sigma-Aldrich

Polymarizetion mix

24%	DMSO	Sigma-Aldrich
20 mM	$MgCl_2$	Sigma-Aldrich
5 mM	GTP	Jena Bioscience
	BRB80	

4.4 Microscopy

4.4.1 Flow chambers preparation

Flow chambers were assembled out of two DDS (dichloredimethylsilane) coated coverslips connected by several thin parafilm rails to create channels (see the Figure 8). Channels were subsequently coated by antibody solution. After 10 minutes of incubation 4x 10 μl F127 solution was flushed into channels to passivate the coverslip surface. Flow chambers were kept in wet chambers to prevent evaporation of samples. At least 1 hour F127 passivation preceded any assay.

4.4.2 Microscopy assay

Channels were flushed 4x with 10 μl BRB80T just before the experiment. Subsequently, previously made microtubules were flushed into the channel and incubated from 10 seconds to 1 minute. Channels were afterwards flushed with 2x 10 μl of Oxygen scavenger mix. Finally, proteins of interest were diluted in Oxygen scav-

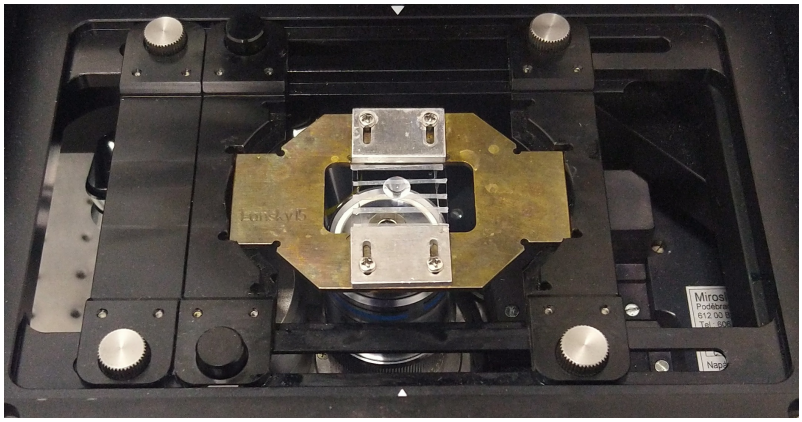

Figure 8: Photo of the flow chamber. Two coverslips separated by thin parafilm rails in brass holder.

F127 solution

100 mg	F127	Sigma-Aldrich
10 ml	BRB80	
Filtred 0.22 μm		VWR

Antibody solution

245 μl	PBS	Sigma-Aldrich
5 μl	Anti-biotin antibody (1 mg.ml^{-1})	Sigma-Aldrich

Motility buffer

	BRB80	
10 μM	Taxol	Sigma-Aldrich
10 mM	DTT	Sigma-Aldrich
20 mM	D-glucose	Sigma-Aldrich
0.1 % v/v	Tween20	Sigma-Aldrich
0.5 mg.ml^{-1}	Casein	Sigma-Aldrich
1 mM	ATP	Jena Bioscience

Oxygen scavenger mix

	Motility buffer	
0.22 mg.ml^{-1}	Glucose oxidase	Sigma-Aldrich
0.02 mg.ml^{-1}	Catalase	Sigma-Aldrich

enger mix and flushed into channels. Microtubules were visualized with laser wave length 640 nm or via internal reflection microscopy (IRM). The GFP was visualized with laser wave length 488 nm.

4.5 Image analysis and data processing

4.5.1 Crossover assembly

All movies were analyzed via ImageJ. The GFP signal was manually tracked and measured form 7x7 pixel rectangle around the signal. Signal was measured form 7 frames before assembly event and 7 frames after assembly event. Background was subtracted from measured signal.

4.5.2 Relative cross/non-cross intensity

Gathered GFP signal was processed via ImageJ sum slices function. Relative cross/non-cross intensity was used to compare differences between experiments. Relative cross/non-cross intensities were calculated by dividing average intensity measured at crossover by the average intensity measured at randomly selected point on the microtubule. The background was subtracted for each measurement. Same procedure was performed for p60 as well as for complex p60&p80.

4.5.3 Kymograph

Kymographs were made using ImageJ function Multi kymograph using line width 7 pixels.

4.5.4 Manual analysis of Katanin dwell time

To get Katanin dwell time spent at the microtubule crossover we tagged all measured crosses with rectangle 7x7 pixels. Whenever GFP signal entered this rectangle and spent there at least 2 frames it was counted as interaction event. As an end of an event was counted when there were at least 2 consecutive frames without GFP signal in the rectangle. Event times were sorted and used to calculate survival probability for every movie using custom Python script.

4.5.5 Peak Katanin Density

To measure Peak Katanin Density we tagged all microtubules with line using width 7 pixels and measured GFP signal. For each microtubule, background was subtracted using the same line shifted out of the line of microtubules. GFP signal from each microtubule was divided by its microtubule length to get Katanin density [$\mu\mathrm{m}^{-1}$]. For each movie, frame and microtubule type Katanin density was averaged and plotted against time. The peak of the measured Katanin density was manually tracked and final Peak Katanin Density is average of 7 frames around the peak.

4.5.6 Microtubule disassembly assay

Microtubule signal was gathered from IRM channel. Microtubules were tagged with line, with width 5 pixels. Background was subtracted. Fraction of microtubule length was calculated as microtubule signal divided by microtubule signal from the first frame. Data was plotted against time and fitted with an exponential.

5 Results

5.1 Katanin interaction and assembly at crossovers

In plants Katanin localization and severing in microtubule crossovers is essential for the cortical microtubule array reorientation [84]. Similarly, there is evidence that MEI-1/MEI-2 complex, *C. elegans* analog of Katanin, has higher severing activity at crossovers *in vitro* [103]. How does Katanin recognize microtubule crossovers is, however, unclear. It is unknown whether the subunit p60 is capable of crossover recognition by itself or requires the subunit p80 for its function. There are two possible explanations for higher severing rate at crossovers. Firstly, structure of Katanin complex suggests that crossovers offer more interaction points and therefore reduce the chance of Katanin dissociation. Therefore, crossovers could serve as docking point for Katanin and thus facilitate Katanin oligomerization. Secondly, the subunit p80 enhanced affinity of the subunit p80 to microtubules could also promote crossover recognition.

To answer these questions, we visualized the interaction of Katanin with microtubules with single molecule resolution (see methods). Prior to imaging, the subunit p60 was diluted to final concentration ideal for visualizing single molecules. It appeared that for our conditions ideal concentration for single molecule microscopy was 13,8 nM of the subunit p60 only. The same assay was performed for complex p60&p80 with the ideal final concentration 6,9 nM of the subunit p60. The mixing ratio of the subunit p60 and p80 was 1:1.

We found that the subunit p60 moves diffusively along the microtubule, as shown in the Figure 9. When the subunit p60 encountered a microtubule crossover, we occasionally observed that it can interact with and switch to the second microtubule in the cross (see Figure 10). Moreover, we occasionally observed the Katanin assembly of two independent oligomers at crossovers. The Figure 11 shows example assembly of two oligomers. First oligomer diffuses along the microtubule and the second one is still at the crossover. When the diffusing oligomer encountered crossover, it fused with the still oligomer and after a while the fused oligomer diffused away. To quantitatively asses the fusion of oligomers, we measured GFP signal intensities before fusion and after. Measured signal of the fused oligomer was significantly higher

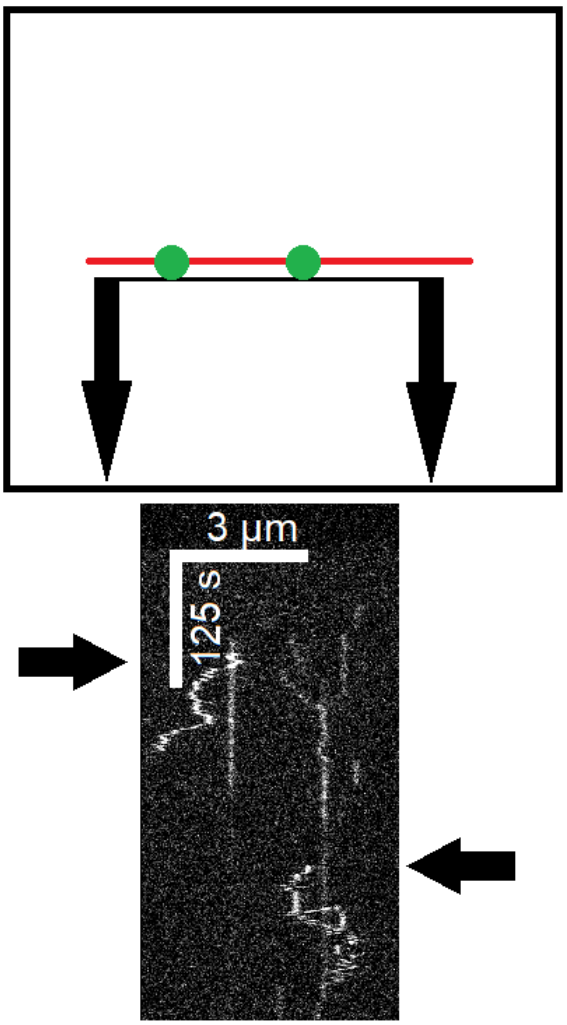

Figure 9: Kymograph shows several events of the subunit p60 diffusion along the microtubule. Schematic cartoon on top shows principle how kymograph was measured. Horizontal arrows indicate diffusion events.

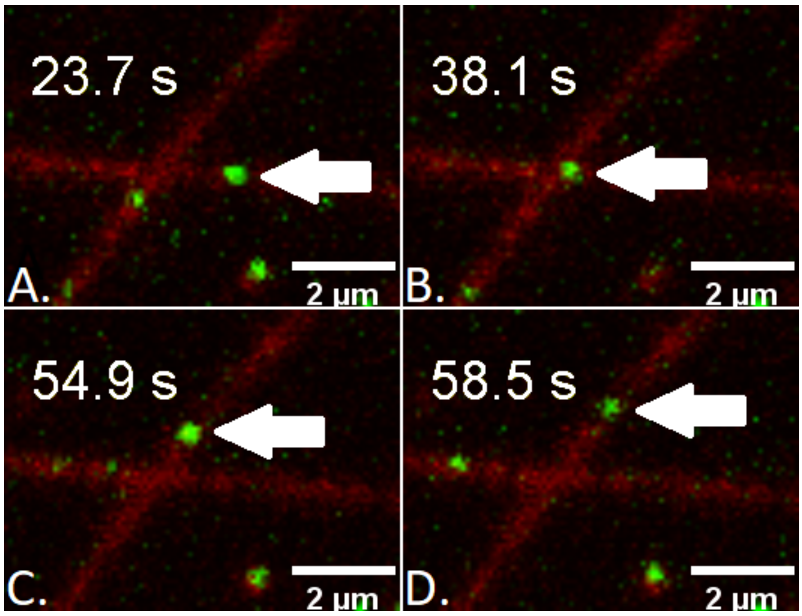

Figure 10: TIRF microscopy time-lapse images show oligomer of the subunit p60 (green) diffusing along the microtubule (red). When the subunit p60 diffused to the crossover it switched to the second microtubule.

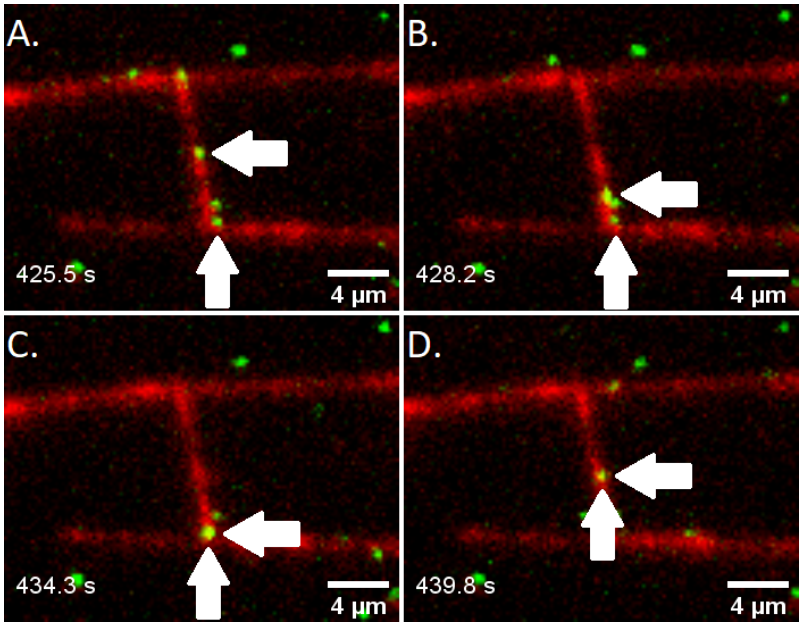

Figure 11: TIRF microscopy time-lapse images show two independent p60 oligomers (green) on the microtubule (red). The oligomer marked by horizontal arrow diffused along the microtubule to the crossover. The oligomer marked by vertical arrow was still in the cross until diffusive oligomer encountered crossover. After a while fused oligomer diffused away from the crossover.

and approximately equals to the sum of pre-fusion oligomer signals as shown in the Figure 12.

These observations suggest, that crossovers serve as Katanin oligomerization points. Katanin diffusion further promotes oligomerization as it provides stochastic 'scanning' of the microtubule.

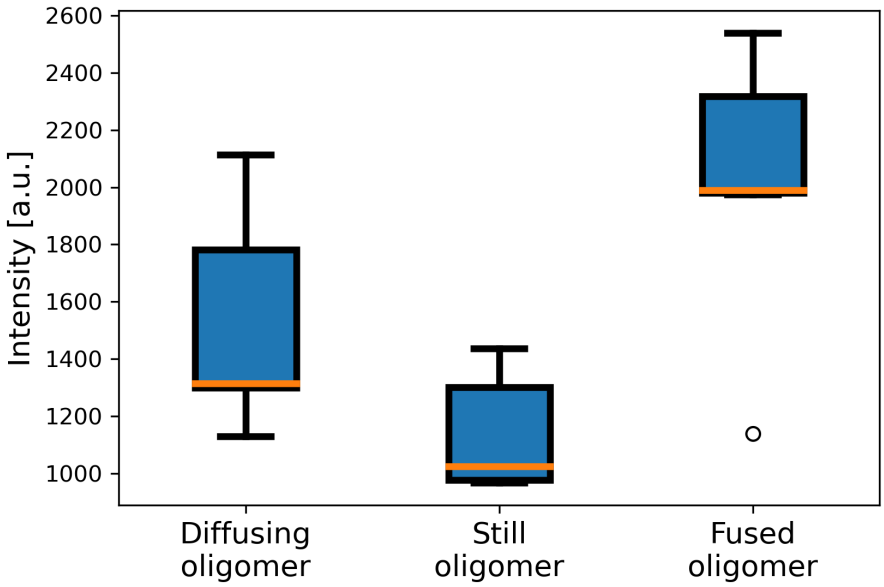

Figure 12: Measurement of intensities of diffusing oligomer, still oligomer and fused oligomer. Measurement shows that when diffusive oligomer encountered still oligomer, signal summed. Measurement was conducted on 7 frames before and after fusion.

5.2 Katanin's interaction dynamics at crossovers

Previous studies showed that the subunit p60 hexamerization is preferred in presence of microtubules [76]. Solution-microtubule equilibrium of the subunit p60 can be further shifted towards microtubules when the subunit p80 is present, as the subunit p80 increases the affinity of the subunit p60 to microtubules [92]. Recently, it was presented that the subunit p80 promotes formation of heterotetrames (dimer of p60/p80 heterodimers) in solution [77][91]. Heterotetramer formation is possible explanation for higher affinity of the complex p60&p80 towards microtubules. Based on these observations we hypothesized that the presence of the subunit p80 could alter the interaction and the recognition of microtubule crossovers. We hypothesised that higher affinity to microtubules leads to accumulation at crossovers by prolonging the dwell time which the Katanin complex resides at the microtubule crossover.

To determine whether the subunit p80 contributes to accumulation at crossovers, we analyzed obtained movies from microscopy assay performed at single molecule level. The subunit p80 contribution to crossover accumulation was characterized via relative cross/non-cross intensities. Relative cross/non-cross intnsities is defined as GFP signal in the crossover divided by GFP signal measured in random location on the microtubule (see methods).

Relative cross/non-cross intensity of the subunit p60 and subunits p60&p80 are shown in the Figure 13. To test the null hypothesis, if two sets of data have the same average, two-sided t-test was used.

We found no statistical difference and thus we cannot reject the null hypothesis. Surprisingly thus, our results indicate that p80 does not significantly participate in microtubule crossover recognition.

To further investigate the role of the subunit p80 in the crossover recognition, we performed manual analysis of crossover interaction time of just the subunit p60 and mixture of both subunits p60&p80 (see methods).

The kymograph in the Figure 14 shows typical Katanin crossover event. Gathered interaction times were summarized in the survival probability plot shown in the Figure 15. To examine whether dwell times of the subunit p60 alone and both subunits p60&p80 in the crossover come form the same distribution, we used two-sample Kolmogorv-Smirnov test. P-value equals 0.5 for obtained set of measurements shows

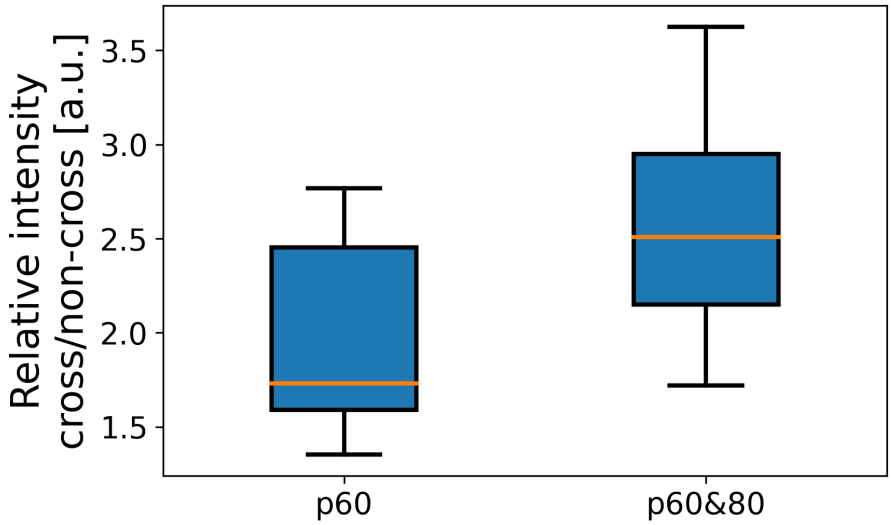

Figure 13: Comparison of the subunit p60 (measured from 7 independent experiments) and subunits p60&p80 (measured from 4 independent experiments) relative cross/non-cross intensities. Two-sided t-test were used to determine if obtained means are significantly different from each other. (p-value=.2)

that there is no significant difference. This result is consistent with our previous observation that regulatory subunit p80 does not increase accumulation of the subunit p60 at crossovers. Combined, these results show that the subunit p80 alone does not affect crossover recognition of the subunit p60.

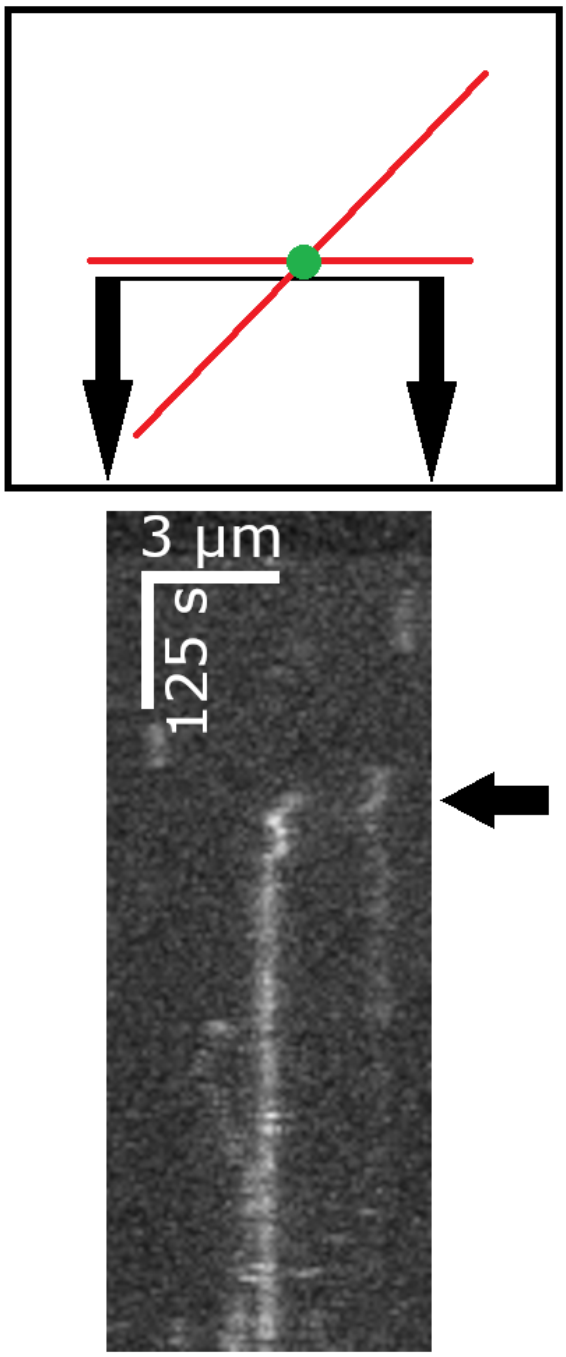

Figure 14: Kymograph of the subunit p60 interaction with the crossover. Schematic cartoon shows how kymograph was measured. Horizontal arrow indicates crossover recognition event.

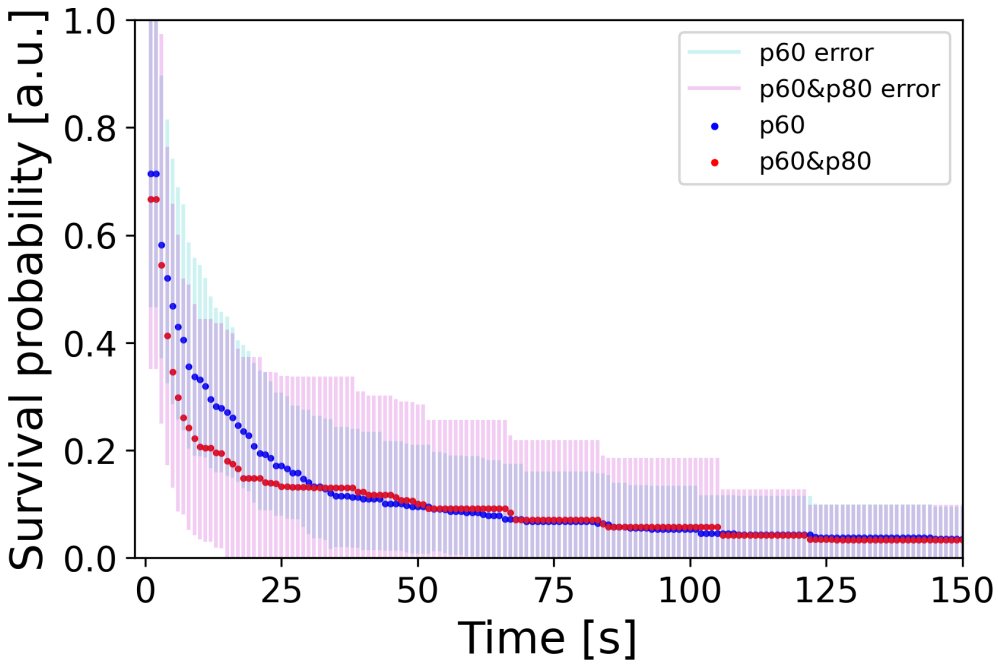

Figure 15: Survival plot of the subunit p60 and both subunits p60&p80 showing the distribution of dwell times in crossovers. To test whether dwell times come from the same distribution, we used two-sample Kolmogorv-Smirnov test. P-value equals 0.5. Median dwell time for the subunit p60 was 4.4 $\pm$ 0.19 seconds and for both subunits p60&p80 the median dwell time was 3.3 $\pm$ 0.28 seconds.

5.3 The role of tubulin post-translational modifications and isoforms on Katanin binding

Tubulin post-translational modifications and tubulin isoforms are highly diverse both among and between tissues and even at the cellular level. Most of post-translational modifications take place at the C-terminus tail of both α and β-tubulin. It is known that the C-terminus tail is a substrate for Katanin-mediated severing [31].Therefore, we decided to examine the effect of post-translational modifications naturally occurring on microtubules in the brain on Katanin function.

We performed microscopy assays with porcine brain-derived microtubules, which are heavily modified [104], and compared them to microtubules derived from HeLa cell line, which microtubules are low in post-translational modifications [105].To achieve same conditions we flushed both microtubule types in the same channel, as shown in the Figure 16. To address how post-translational modifications of microtubules affect the affinity of mmKatanin, composed of mammalian catalytic subunit p60 and C-terminal domain of p80. We performed assays at several different mmKatanin concentrations, 43 nM, 86 nM and 430 nM. To compare affinity for different microtubule type, we measured Peak Katanin Density. We chose this method because in most experiments microtubules were not stable enough to reach mmKatanin equilibrium. The example of GFP intensity recording is shown in the Figure 17. Only exception when mmKatanin reach equilibrium without disassembly of microtubules was in presence of 43 nM concentration of mmKatanin. In this concentration HeLa cell microtubules were stable while brain-derived microtubules were disassembled.

In all performed experiments we recorded higher GFP signal on brain-derived microtubules. Peak Katanin Density was almost 9.6-times higher in 43 nM concentration of mmKatanin and almost 1.4-times higher in 430 nM concentration of mmKatanin, see the Figure 18. These results suggests that mmKatanin has higher affinity to brain-derived microtubules.

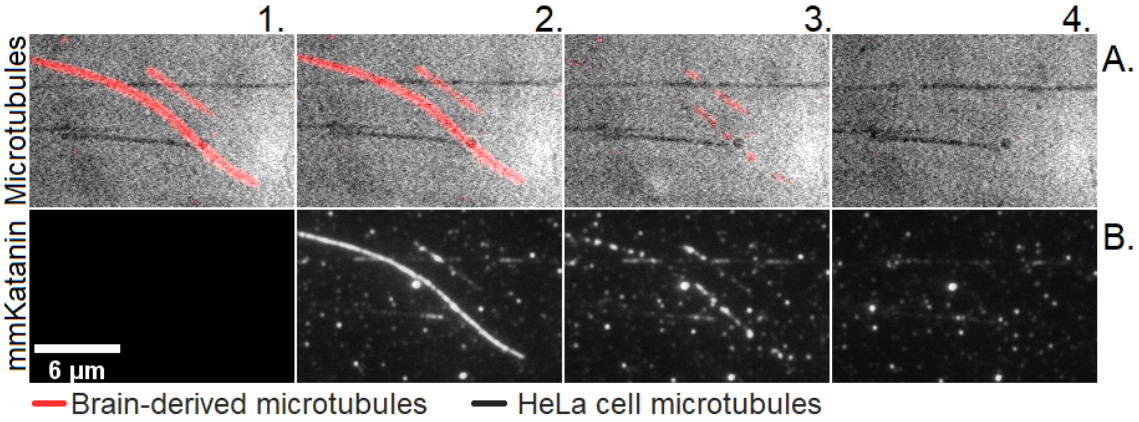

Figure 16: Representative time-lapse of microscopy assay with brain-derived microtubules and HeLa cell microtubules in Katanin concentration 43 nM. Brain-derived microtubules are shown in red and HeLa cell microtubules are unlabled shown in dark gray. In row 2. shows Katanin GFP signal. In the picture 2.B. there is noticeable difference in Katanin GFP signal on brain-derived microtubules and HeLa cell microtubules. Also, row A. shows how are brain-derived microtubules disassembled, while HeLa cell microtubules are intact.

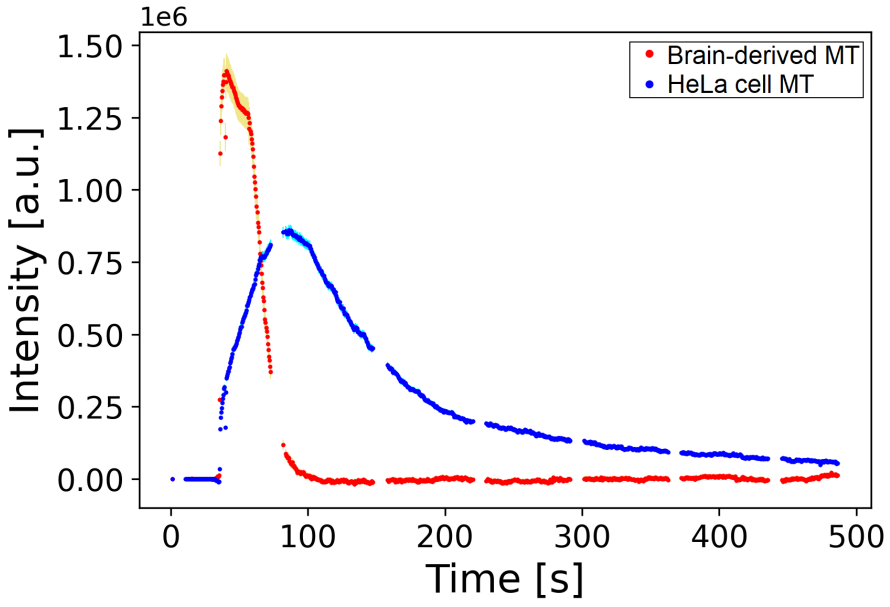

Figure 17: Example measurement of Katanin GFP signal in experiment with Katanin concentration 86 nM. Red represents signal from brain-derived microtubules and blue represents signal from HeLa cell microtubules.

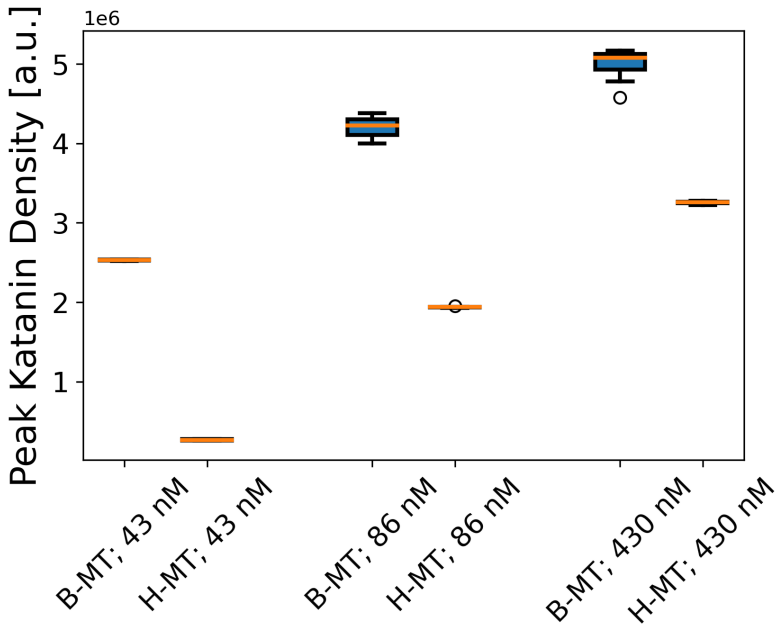

Figure 18: Peak Katanin Density measurements. Peak Katanin Density was almost 9.6-times higher in 43 nM concentration of mmKatanin, almost 2-times higher in 86 nM concentration of mmKatanin and almost 1.4-times higher in 430 nM concentration of mmKatanin. B-MT = brain-derived microtubules, H-MT = HeLa cell microtubules.

5.4 Brain-derived microtubules deferentially affect Katanin catalytic activity

We measured microtubule disassembly rate by direct observation of microtubules in DIC channel. In all experiments brain-derived microtubules were preferably disassembled, see the Figure 19. In experiment with mmKatanin concentration 43 nM, HeLa cell microtubules were stable to the end of recording. To quantify Katanin activity we measured the decay of microtubule signal over the time. The microtubule signal was fitted with an exponential. Obtained disassembly rates are summarized in the Table 1. Increasing concentration of added mmKatanin resulted in higher disassembly rate in both microtubule types, as shown in the Figure 20. To address the effect of higher Katanin affinity for brain-derived microtubules, we plotted microtubule disassembly rate against Peak Katanin Density, shown in the Figure 21. At the Katanin concentrations for which the Peak Katanin Density is similar on brain-derived and HeLa-derived microtubules, the microtubule disassembly rate seems to be higher for the HeLa microtubules (Figure 21). Surprisingly, this experiment thus suggests that HeLa microtubules are a preferential substrate of Katanin, as shown in the Figure 21. In conclusion, our data suggest that mmKatanin has higher affinity for brain-derived microtubules but severing activity on these microtubules is lower.

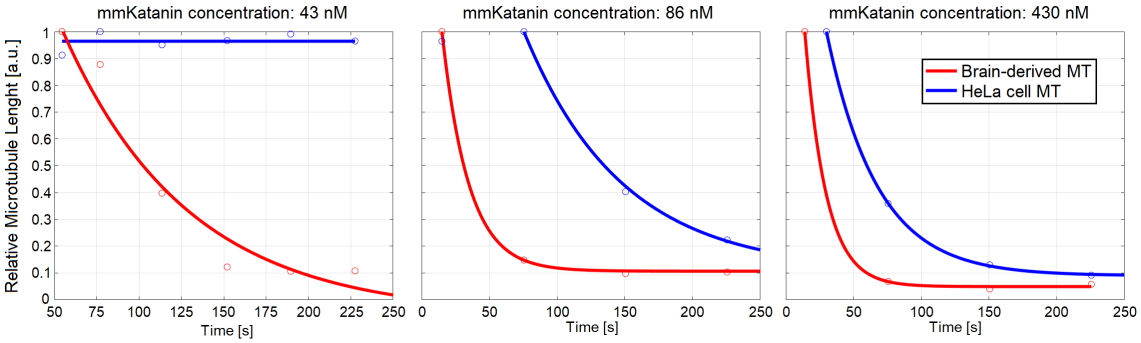

Figure 19: Measured microtubule disassembly from three different Katanin concentration fitted with an exponential. Red = Brain-derived microtubules, Blue = HeLa cell microtubules.

	43 nM	86 nM	430 nM
Brain-derived MT	0.003 ± 0.0025 s^{-1}	0.025 ± 0.004 s^{-1}	0.032 ± 0.014 s^{-1}
HeLa MT	—	0.0069 ± 0.0006 s^{-1}	0.0133 ± 0.0018 s^{-1}

Table 1: Table of disassembly rates measured from microtubule disassembly.

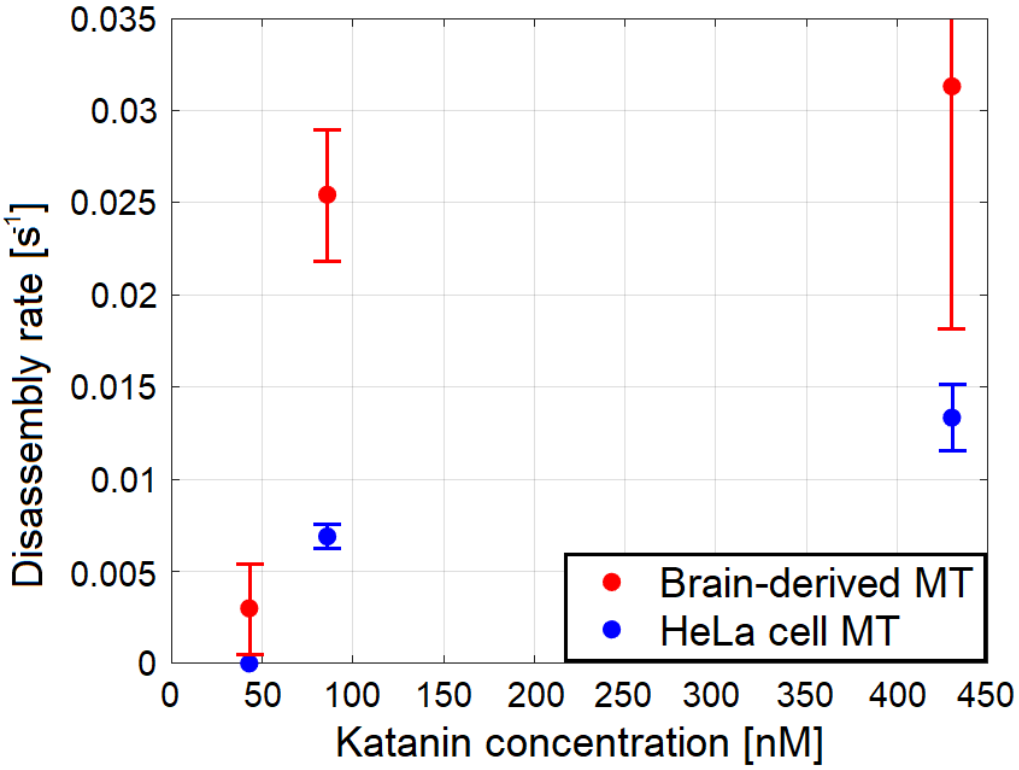

Figure 20: Microtubule disassembly rate plotted against Katanin concentration. The higher Katanin concentration the higher disassembly rate means. Red = Brain-derived microtubules, Blue = HeLa cell microtubules.

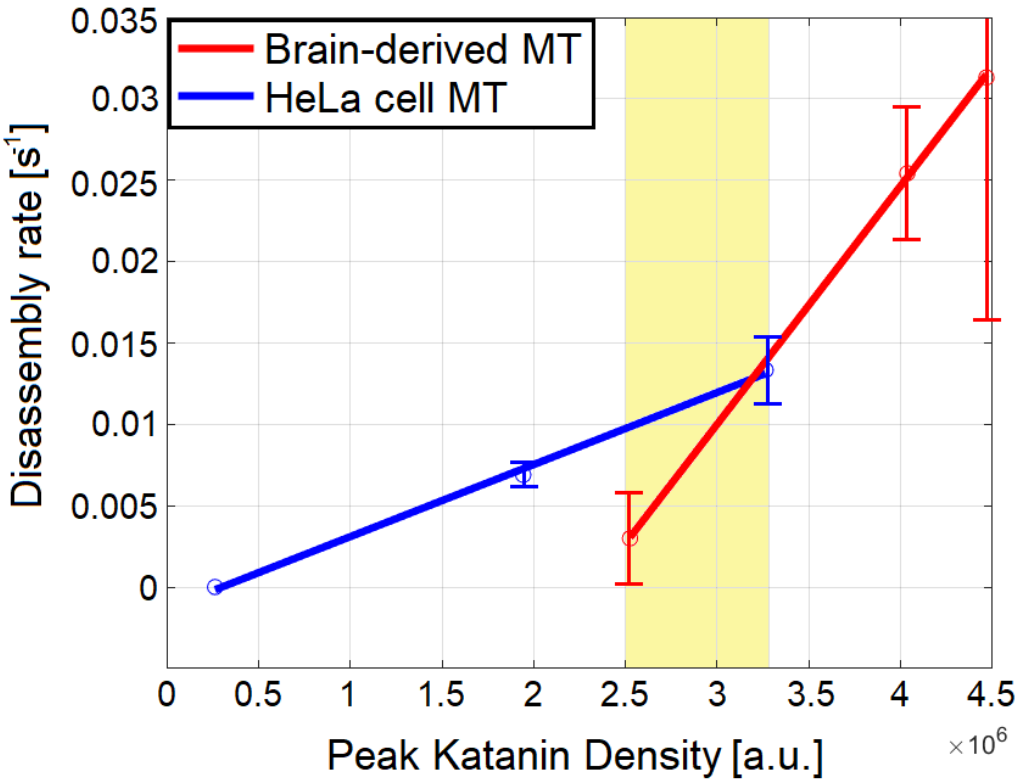

Figure 21: Microtubule disassembly rate plotted against Peak Katanin Density. Peak Katanin densities which are comparable between tested microtubule types is marked yellow. Red = Brain-derived microtubules, Blue = HeLa cell microtubules.

6 Discussion

Katanin severing activity is essential for phototropic induced reorientation of cortical microtubules in plants [80]. Precise Katanin targeting to microtubule crossovers and microtubule side branching points promotes massive microtubule array reorientation [84]. In the first part of the thesis, we characterized behaviour of plant Katanin at the single molecule level. We observed that the plant Katanin subunit p60 is able to diffuse along microtubules. We also identified crossovers as potential oligomerization points, which can facilitate formation of Katanin hexamers. Hexamer formation is required for Katanin ATPase activity [76]. Consistently with our result MEI1/MEI2, Katanin *C. elegans* orhologue, shows increased severing activity at crossovers *in vitro* and *in vivo* [103] [84]. Taken together, our results support the hypothesis that Katanin is able to recognize and interact with crossovers as its inherent property, but additional factors might further enhance this ability of Katanin.

To assess the role of the subunit p80 in the crossover recognition process, we compared enrichment at microtubule crossovers of only subunit p60 and both subunits p60&p80. Interestingly, we didn't observe any significant difference. No difference was also observed in dwell times of only subunit p60 and both subunits p60&p80 in the crossover. Together, these results are in contrast with previously observed accumulation of the subunit p80 at microtubule crossovers *in vivo*, independently of the subunit p60 [99]. Independence of the subunit p80 suggests that involvement of another crosslinking protein is needed. As an example of crosslinking protein was previously described Katanin specific targeting to centrosome via ASPM [82]. Moreover, study investigating Katanin targeting to branching points uncovered a novel Katanin interacting partner Msd1-Wdr8, which colocalizes with γTuRC. Interestingly, they reported that Msd1-Wdr8 is not involved in the crossover recognition [100], thus underlying mechanism behind microtubule crossovers recognition remains to be uncovered.

Another level of regulation of cortical microtubule reorientation might be mediated by substrate specificity. During microtubule reorientation newer microtubules are preferentially severed at crossovers [84]. Since Katanin dominant function is array amplification [106], it probably helps with establishment of new microtubule

array order. Potential mechanism responsible for preferential severing of new microtubules could involve differential post-translational modifications or selective shielding of the older microtubule. Attractive target of interest could be polyglutamylation [107] as it is responsible for attenuation of severing activity [63]. Another potential post-translational modification could be tyrosine nitration. Root cells of *A. thaliana* in presence of elevated concentrations of 3-nitrotyrosine show a similar morphology as plants with disrupted Katanin function [108]. Our method did not allow us to quantify Katanin severing activity at crossovers because of the low Katanin concentrations used. Further experiments could involve higher concentrations of Katanin at the loss of single molecule resolution or using a mixture of labeled and unlabeled Katanin at the loss of oligomer size information. Also it should be noted that experiments were performed with mammalian brain-derived microtubules because of difficult access to sufficient amounts of plant tubulin. We assume, based on high tubulin similarity between species [109], that fundamental and qualitative features are unchanged. However, future experiments with plant tubulin will be needed.

In the second part of the thesis, we characterized mammalian Katanin binding to two types of microtubules, HeLa cell microtubules and brain-derived microtubules. HeLa cell microtubules serve as a model with low amount of post-translational modifications [105]. On the other hand brain-derived microtubules serve as model of heavily post-translationaly modified microtubules [104]. Commonly found post-translational modification in mammalian brain are for example poly-glutamylation [58] [59] and acetylation [110]. We observed that mammalian Katanin has higher affinity to brain-derived microtubules. Our observation is consistent with previously reported increased affinity of Katanin paralogue Spastin to polyglutamylated microtubules [63]. Possible explanation for such behaviour could be higher density of negative charge caused by γ-carboxyl group of glutamate residues. Similarly reasoned is increased kinesin-1 processivity on polyglutamylated microtubules [62]. Role of tubulin C-tail in Katanin binding to microtubules further supports low affinity to subtilisin treated microtubules, microtubules which C-tails were artificially removed by a protease [111]. Interestingly sedimentation experiments revealed that also Katanin nucleotide state plays role in binding to microtubules. Katanin in ATP-like

state was bound stronger than Katanin in ADP state [111]. Since Katanin severing activity is strongly dependent on binding to microtubules and hexamerization [76], affinity and its manipulation is important indirect regulation of the microtubule severing activity.

We also measured mammalian Katanin severing activity and compared two different substrates as with the binding experiment. At first, brain-derived microtubules showed higher microtubule disassembly rates in comparison to HeLa cell microtubules in all examined concentrations of mmKatanin. In light of previously discussed increased affinity by post-translational modifications we asked, how do post-translational modifications alter katanin catalytic activity? To asses this question we compared Peak Katanin Density and microtubule disassembly rate in several different mmKatanin concentrations. Surprisingly, we discovered, despite the fact that mmKatanin has higher affinity to brain-derived microtubules, HeLa cell microtubules are disassembled faster at a given Peak Katanin Density in comparison to brain-derived microtubules. This result indicates that mmKatanin catalytic activity is slightly inhibited by brain-derived microtubules. Together these results are consistent with observation that length of polyglutamylation side-chain negatively regulates Spastin catalytic activity [63]. Although it is tempting to assume that polyglutamylation is responsible for such difference in our experiments, further aspects should be considered. For example, poorly characterized effect of other post-translational modifications on Katanin catalytic activity such as acetylation or detyrosination. Interestingly, in *Drosophila* neuron cultures Katanin overexpression phenotype can be reverted by overexpression of HDAC6, enzyme responsible for tubulin deacetylation [83]. But direct effect is doubtful because *in vitro* experiments did not reveal any significant effect of acetylation on Spastin-mediated severing [63]. Similarly, previously observed *in vivo* increase in kinesin-1 binding to microtubules [112] was subsequently adjusted as indirect effect, because kinesin-1 did not show any significant increase in binding to acetylated microtubules *in vitro* [70]. Also an effect of other microtubule associated protein should not be overlooked, as en example tau protein binds to microtubules and forms protective layers, which restricts Katanin binding and severing [113].

Next difference between HeLa cell microtubules and brain-derived microtubules,

which has to be considered, is isotype composition. HeLa cell microtubules are mostly composed of βI+βIVb tubulin [38]. On the other hand, brain-derived microtubules are composed mainly of approximately 25% of βIII tubulin and 58% mixture of βIIa and βIIb [114]. Interestingly, isotype composition affects microtubule dynamics as brain-derived microtubules show higher catastrophe frequency and slower growth and depolymerization rate, than microtubules composed of βI and βIVb [38]. Interestingly, slightly higher depolymerization rate observed on microtubules composed of βI and βIVb is consistent with our observations. On the other hand, documented difference in microtubule depolymerization rate was conducted on dynamic microtubules, while we used taxol-stabilized microtubules. If we consider possibility that βIII tubulin has lower affinity to taxol binding [40] we assume that the effect of lower taxol binding would be opposing to our results. However, how does tubulin isotype composition affect microtubule dynamics and how does it affect microtubule interaction with microtubule associated proteins is still unclear and more research is needed. Our method is limited to rely on taxol stabilization of microtubules, which slightly alters microtubule properties. To discriminate taxol-stabilizing effect, experiment with dynamic microtubules is needed. Although, it is impossible to measure Katanin severing activity on dynamic microtubules of both types, brain-derived and HeLa cell derived, in the same channel as we did. To further generalize result that HeLa cell microtubules are better substrate, presented in the Figure 21, a measurement in larger concentration range is required.

Presented results provide evidence that post-translational modifications and tubulin isotype composition may play important role in Katanin-mediated severing. To further elucidate role of specific post-translational modifications, tubulin with controlled post-translational modifications is needed. Tubulin expressed and purified from transgenic mouse models lacking specific post-translational modifying enzymes promises precise control over post-translational modification and also tubulin composition [105].

7 Conclusion

Here we examined properties of plant and mammalian Katanin, a microtubule severing protein. In the first part of the thesis, we reconstructed *in vitro* system similar to microtubule cortical arrays found in plants. In plants, Katanin localizes to microtubule crossovers and catalyzes microtubule severing. We examined Katanin localization to crossovers *in vitro* and assessed contribution of the Katanin regulatory subunit p80 to crossover recognition of the Katanin catalytic subunit p60. Surprisingly, we found that the Katanin regulatory subunit p80 does not participate in crossover recognition and thus we suggest that another interacting partner is needed. In second part of the thesis, we compared mammalian Katanin activity in context of two different microtubule types, brain-derived microtubules and HeLa cell derived microtubules. Brain microtubules are rich in post-translation modifications and certain tubulin isotype. On the other hand, HeLa cell microtubules, we used, are low on post-translational modifications. Our results suggest that Katanin has higher affinity for brain-derived microtubules. Surprisingly, our results indicate that even though Katanin has higher affinity to brain-derived microtubules, it shows higher catalytic activity on HeLa cell derived microtubules.

8 Bibliography

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