Dissecting the Regulatory Mechanism of the Mammalian Microtubule-Severing Protein Katanin in Mitosis

A Dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Biochemistry and Molecular Biology

by

Chingnam Cheung

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ABSTRACT OF THE DISSERTATION

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By

Chingnam Cheung

Doctor of Philosophy in Biochemistry and Molecular Biology

University of California, Los Angeles

Professor Jorge Torres, Chair

The Katanin family of microtubule-severing enzymes is critical for remodeling microtubule-based structures that influence cell division, motility, morphogenesis and signaling. Katanin is composed of a catalytic p60 subunit (A subunit, KATNA1) and a regulatory p80 subunit (B subunit, KATNB1). The mammalian genome also encodes two additional A-like subunits (KATNAL1 and KATNAL2) and one additional B-like subunit (KATNBL1) that have remained poorly characterized.

To better understand the human Katanins and more broadly the mechanisms controlling mammalian microtubule-severing, we first analyzed the human Katanin interactome (Katan-ome) through biochemical tandem affinity purifications and mass proteomic analyses. This revealed that all Katanin subunits could reciprocally co-purify with each other, with the exception of KATNAL2 that only purified with KATNA1. These interactions were verified by in vitro and in cell reciprocal co-immunoprecipitations, with
the exception of the KATNAL2-KATNA1 interaction. Analysis of the cell cycle subcellular localization of all Katanin subunits showed that KATNA1, KATNAL1 and KATNB1 localized to the cytoplasm during interphase and to the spindle poles during mitosis. Surprisingly, KATNBL1 localized to the nucleus during interphase (dependent on an N-terminal nuclear localization signal) and to the spindle poles during mitosis. In cell microtubule-severing assays with each A subunit revealed that unlike KATNA1 and KATNAL1, which showed microtubule-severing activity, KATNAL2 lacked detectable microtubule-severing activity. Interestingly, in vitro microtubule-severing assays showed that full-length KATNBL1 regulated KATNAL1 microtubule-severing activity in a concentration dependent manner. Furthermore, KATNB1 was able to compete the KATNBL1-KATNA1 and KATNBL1-KATNAL1 interactions.

These results indicate that KATNBL1 is a regulator of microtubule-severing activity and that it cooperates/competes with KATNB1 for binding to KATNA1 and KATNAL1 and that KATNB1 and KATNBL1 together regulate Katanin A subunit microtubule-severing activity.
The dissertation of Chingnam Cheung is approved.

Margot E. Quinlan

Kent L. Hill

Jorge Torres, Committee Chair

University of California, Los Angeles

2016
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<th>Description</th>
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<tbody>
<tr>
<td>+TIPs</td>
<td>Plus-end tracking proteins</td>
</tr>
<tr>
<td>2D-LC</td>
<td>Two-dimensional liquid chromatography</td>
</tr>
<tr>
<td>AAA</td>
<td>ATPase associated with various cellular activities</td>
</tr>
<tr>
<td>AD-HSP</td>
<td>Autosomal dominant hereditary spastic paraplegia</td>
</tr>
<tr>
<td>CC</td>
<td>Coiled-coil</td>
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<tr>
<td>CDK1</td>
<td>Cyclin-dependent kinase 1</td>
</tr>
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<td>CDK2</td>
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<td>CIN</td>
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<td>Control</td>
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<tr>
<td>EGFP</td>
<td>Enhanced green fluorescent protein</td>
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<tr>
<td>ESCRT-III</td>
<td>Endosomal sorting complex required for transport-III</td>
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<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
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<td>Guanosine triphosphate</td>
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<td>Localization and affinity purification</td>
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<td>Leucine (L), alanine (A), proline (P), serine (S), glutamate (E) and arginine (R)-rich protein</td>
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<tr>
<td>LZTS2</td>
<td>Leucine zipper putative tumor suppressor 2</td>
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<tr>
<td>MAP</td>
<td>Microtubule-associated protein</td>
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<td>Meiotic spindle formation protein-1</td>
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<td>PLK2</td>
<td>Polo-like kinase 2</td>
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Curriculum Vitae

Chingnam Cheung

Education

University of California, San Diego  
**M.S. in Biochemistry, June 2010**

University of California, San Diego  
**B.S. in Biochemistry, June 2008**

Research Experience

Dr. Susan Taylor’s Lab, UCSD  
**August 2009 – June 2010**

*Undergraduate Researcher*

**Project: Role of phosphorylation on Protein Kinase A (PKA)**
- Characterized mutants of the PKA regulatory subunit
- Purified recombinant PKA subunits using a combination of chromatography techniques
- Determined activities of mutant regulatory subunits by coupled activation assays and inhibition assays
- Performed initial crystallization condition screening of PKA holoenzyme using an automated system
- Identified potential agonists and antagonists of the PKA regulatory subunit utilizing a screening assay

Dr. Elizabeth Komives’ Lab, UCSD  
**March 2007 – July 2009**

*Undergraduate Researcher*

**Project: Mechanism of IκBα-mediated Nuclear factor-kappa B (NF-kB) regulation**
- Purified regulatory protein nuclear factor-kappa B (NF-kB) and its inhibitor IκBα
- Analyzed protein binding efficiency using Electrophoretic Mobility Shift Assay (EMSA)
- Measured relative Protein-DNA interaction using a fluorescent quenching approach
- Performed and analyzed trypsin-digested IκBα using mass spectrometry

Nanogen Inc., San Diego, CA  
**November 2006 – December 2007**

*Research Associate*

**Project: Optimization of user protocol for genomic-based electronic microarray diagnosis system**
- Evaluated several automated sample purification methods
- Identified and detected target DNA from clinical samples using electronic microarray
Teaching experiences

Undergraduate Mentor
UCLA Biochemistry & Molecular Biology (Los Angeles, CA) March 2011 – Present

Teaching Assistant
University of California, Los Angeles, Department of Biochemistry & Molecular Biology
• CHEM 153L – Introduction to Protein Science Laboratory
University of California, San Diego, Department of Biochemistry & Chemistry
• CHEM 143B – Organic Chemistry Laboratory

Fellowships and Conferences

• University Fellowship, UCLA 2010
• Philip Whitcome Pre-doctoral Fellowship, UCLA July 2011– July 2014
• 2016 ASBMB conference poster presentation (San Diego, CA)
• 2014 EMBO conference: Microtubules: structure, regulation and functions poster presentation (Heidelberg, Germany)
• 2014 UCLA Molecular Biology Institute annual retreat poster presentation
• 2013 UCLA Molecular Biology Institute annual retreat poster presentation
• 2012 UCLA Molecular Biology Institute annual retreat poster presentation
• 2008 UCSD Undergraduate Student Research Conference poster presentation

Publications


Under Review


Chapter 1 - Background

1.1 Microtubules are essential in many cellular processes, especially in cell division

Microtubules are dynamic, biopolymers that serve as an essential component of the cytoskeleton network. The cytoskeleton provides mechanical support for maintaining internal organization and cell shape in eukaryotic cells. The inherent dynamics of microtubules allow them to rapidly rearrange into functional structures that adapt to various cellular processes, such as intracellular transport, motility, neurogenesis, ciliogenesis and cell proliferation (1-3). During cell division, microtubules reorganize into an anti-parallel bipolar structure called the mitotic spindle. The integrity of the mitotic spindle is critical for ensuring faithful segregation of daughter chromosomes. Defects in mitotic spindles often lead to chromosome missegregation and result in aneuploidy. Aneuploidy could subsequently lead to increased rates of chromosome missegregation, termed chromosomal instability (CIN), a hallmark of cancers (4,5).

1.2 Molecular structures and properties of microtubules

Microtubules are 25-nm-diameter, non-covalent biopolymers consisting of the 55-kDa GTPases called tubulin (6,7) (Figure 1.1). Two tubulin isoforms, α-tubulins and β-tubulins, spontaneously interact to form heterodimers, which are the basic building blocks of microtubules. Tubulin heterodimers join head-to-tail to form protofilaments, which organize into the spiral and hollow cylindrical-structures of microtubules (6). The number of protofilaments in individual microtubules varies across organisms. In mammalian cells, microtubules are composed of 13 protofilaments and may range
between 10-15 protofilaments in other organisms (8). The opposite ends of microtubules have different properties. The β-tubulin-exposed plus-end grows more rapidly, while the α-tubulin-exposed minus-end grows at a much slower rate (9). All protofilaments within an individual microtubule point to the same end, giving it a distinct direction and polarity. The microtubule ends have high affinity toward GTP-bound tubulin heterodimers and this property drives polymerization. GTP hydrolysis occurs shortly after tubulins are added to microtubules and the unstable GDP-bound tubulin heterodimers spontaneously dissociate from microtubule ends (Figure 1.1) (10). Thus, microtubules are constantly undergoing polymerization and depolymerization, known as dynamic instability (1). Whether a microtubule grows or shrinks depends on the rate of GTP hydrolysis and the rate of tubulin addition. When the rate of tubulin addition is faster than GTP hydrolysis, the microtubule ends maintain GTP-caps and continue to grow, termed “rescue” (11). When the rate of GTP hydrolysis is faster than tubulin addition, GDP-bound tubulin heterodimers start to dissociate from microtubule ends and the microtubule undergoes rapid depolymerization, termed “catastrophe” (11).
Figure 1.1 - Molecular structures and properties of microtubules.

Microtubules consist of α- (blue sphere) and β- (purple sphere) tubulin heterodimers. Mammalian microtubules consist of 13 protofilaments (between 10-15 protofilaments in other organisms) that arrange spirally into a cylindrical structure that is 25-nm in diameter. GTP-bound tubulin (dark purple sphere) heterodimers add to the fast-growing plus-end (points to the top) of microtubule. The newly added GTP-bound tubulin heterodimer is then hydrolyzed to GDP-bound tubulin (light purple sphere) heterodimer shortly after polymerization. At the microtubule-organizing center (MTOC), the slow-growing minus-end of the microtubule is stabilized by γ-tubulin (green spheres) and capping proteins. Microtubules undergo constant polymerization and depolymerization, termed “dynamic instability”. When new tubulin heterodimers are added to the microtubules faster than GTP hydrolysis, the microtubule maintains a stable “GTP-cap” and grows rapidly, a process termed “rescue”. When GTP hydrolysis occurs faster than the addition of new tubulin heterodimers, the microtubule loses the GTP-cap and spontaneously undergoes rapid depolymerization, a process termed “catastrophe”. (Figure modified from Cecilia Conde and Alfredo Cáceres, “Microtubule Assembly, Organization and Dynamics in Axons and Dendrites,” Nature Reviews. Neuroscience 10, no. 5 (May 2009): 319–332, doi:10.1038/nrn2631.)
1.3 Microtubule structures in cell division

In eukaryotes, microtubules merge into a tubulin structure called the microtubule-organizing center (MTOC), or the centrosome. The centrosome nucleates microtubules and regulates microtubule arrays. In interphase, the centrosome controls the formation of microtubule-based cellular structures such as cilia and flagella, whereas in cell division, the centrosome mediates the formation of mitotic spindle. The centrosome consists of a pair of centrioles surrounded by a matrix of proteins called the pericentriolar material (PCM) (12). The centrioles are two cylindrical structures that are positioned perpendicularly to each other. Each centriole contains nine short microtubule-structures that are composed of three microtubules bundled to each other. The pair of centrioles serves as an anchor for the mass of proteins that form the PCM including the γ-tubulin ring complex. The γ-tubulin ring complex consists of γ-tubulins and five other proteins, which are arranged into a 25-nm-wide open-ring structure (13). The γ-tubulin ring complex serves as a microtubule minus-end cap and is responsible for microtubule nucleation.

During mitosis, each pair of the duplicated centrioles moves toward the opposite sides of the dividing cell and microtubules quickly reorganize to form the bipolar spindle. Microtubules nucleate toward the opposite poles of the cell and form an antiparallel-array that defines the spindle. Microtubules that extend towards the cell cortex from the centrosomes are called astral microtubules and are responsible for spindle poles positioning. Spindle assembly and other cellular processes often require complete remodeling of microtubule structures. Rapid growth and shrinkage of microtubules
require precise spatial and temporal control of microtubule dynamics modulated by a broad class of proteins called microtubule-associated proteins (MAPs).

1.4 Microtubules dynamic are regulated by the MAP proteins

MAPs are classified as a broad class of proteins that co-purify with microtubules, which include proteins that promote microtubule polymerization, depolymerization or stabilization. For example, Tau is a classic MAP that promotes axonal outgrowth and maintains neuronal processes (14). The C-terminal repeats of Tau bind to the hydrophobic pockets in between tubulin heterodimers to stabilize microtubules, suppress catastrophe and promote rescue (15-17). EB1 and CLIP-170 are plus-end tracking proteins (+TIPs) that regulate microtubule dynamic instability by promoting plus-end dynamics (18-20). EB1 tracks the microtubule plus-end and activates the auto-inhibited CLIP-170. The EB1/CLIP-170 complex then co-polymerizes with heterodimer tubulins to promote microtubule polymerization (18,19). In contrast, the mitotic centromere-associated kinesin (MCAK) is a microtubule destabilizer that depolymerizes microtubules on both the plus- and minus-ends. MCAK binds loosely to the microtubule lattice and diffuses along the microtubule protofilament until it reaches the end, where the protofilament spontaneously adopts a curved conformation (21) (Figure 1.1). MCAK then binds tightly to stabilize the curved conformation of the protofilament and promotes microtubule catastrophe (22). Stathmin is a microtubule destabilizer that sequesters tubulin heterodimers to prevent microtubule assembly. By reducing the rate of microtubule polymerization, Stathmin destabilizes microtubules by increasing the frequency of microtubule catastrophe (23,24).
Microtubule-severing proteins are unique MAP destabilizers that disassemble microtubules by generating an internal break within the microtubule lattice. Through ATP hydrolysis, these proteins undergo a conformational change to release tubulin heterodimers from the microtubule lattice and break microtubules into shorter fragments. By generating additional microtubule-ends, microtubule-severing proteins can complement end-regulating MAP destabilizers such as MCAK to enhance microtubule depolymerization. Alternatively, microtubule fragments generated by microtubule-severing can polymerize into individual microtubules. Thus, microtubule-severing proteins can also promote formation of cellular structures that require microtubule enrichment, such as the mitotic spindle.

1.5 Structures of microtubule-severing proteins

Microtubule-severing proteins belong to the AAA (ATPase Associated with various cellular Activities) superfamily, which is characterized by one or more of the highly conserved, ~250-amino-acid AAA ATPase domain. AAA proteins are conserved across all organisms and are involved in a wide range of cellular processes such as molecular transport, protein degradation, membrane fusion, transcriptional activation and spindle formation. The AAA subfamily that includes the microtubule-severing proteins contains an N-terminal microtubule-interacting and trafficking (MIT) domain and a single C-terminal AAA domain (25). There are currently three known microtubule-severing proteins: Katanin was the first microtubule-severing protein that was discovered in mitotic Xenopus egg extract and has been studied extensively; Spastin was identified from a mutated gene that causes autosomal dominant hereditary spastic
paraplegia (AD-HSP) in humans; and Fidgetin was identified in mutant mice that had reduced or absent semicircular canals, microphthalmia, various skeletal abnormalities and displayed “fidgeting” behavior (26-28). VPS4 is the only non-microtubule-severing protein in this AAA subfamily (29). Instead, VPS4 disassembles the endosomal-sorting-complex-required-for-transport (ESCRT)-III polymers in the budding membrane neck to drive the final abscission step of cytokinesis in endosomal membrane trafficking (30).

### AAA domain structure

All AAA proteins share the common features of forming oligomeric rings and harvesting chemical energy from ATP hydrolysis to remodel nucleic acid or protein substrates. The microtubule-severing proteins Katanin, Spastin and Fidgetin form hexameric rings upon binding to ATP, while VPS4 has been reported to form a dodecameric double-ringed structure (31-33). Both the structures of human and Drosophila Spastin AAA domains have been solved by X-ray crystallography, and the core structures of the two orthologs are highly conserved (33,34). The AAA domain consists of a central α/β nucleotide-binding domain (NBD) and a smaller four-helix bundle domain (HBD) that form the nucleotide pocket. The NBD contains the conserved Walker A and Walker B motifs that are involved in nucleotide binding and hydrolysis. Inside the Walker A motif is a highly conserved phosphate-binding loop (P-loop) that is critical for nucleotide hydrolysis; a single mutation within the P-loop can abolish the ATPase activity of the AAA domain (33). Atomic docking coupled with small-angle X-ray scattering (SAXS) reconstruction of hexameric Spastin shows three highly conserved solvent-exposed pore loops that are projected into the hexameric ring (33). These loops
are critical for substrate remodeling activity and mutation of residues in any of the three loops can completely inhibit or severely cripple the enzyme (33,35). In the current model, the pore loops interact with the C-terminal tail of tubulin and translocate it through the central pore of the AAA ATPase ring. This process partially unfolds the tubulin subunit, destabilizes the microtubule lattice, and eventually generates an internal break within the microtubule. The partially unfolded tubulin subunit is then released by the microtubule-severing protein and becomes available to repolymerize into microtubules. This model is supported by recent studies showing that: 1) mutations within the C-terminal tails of tubulin perturb the microtubule-severing activity of Katanin, and 2) addition of tubulin C-terminal fragments inhibits Katanin activity in severing assays (35-37).
MIT domain structure

In addition to the AAA domain, the MIT domain is also conserved in the microtubule-severing proteins and the VPS4 AAA subfamily. NMR analysis of mouse Katanin p60 MIT domain reveals the structure of a three-stranded helix bundle arranged in an anti-parallel manner, where the second and third helices form a positively charged surface that binds to microtubules. In contrast, the VPS4 MIT domain recognizes the ESCRT-III MIT interacting motifs (MIM) and recruit VPS4 to the ESCRT-III polymer lattice. SAXS reconstruction of the Spastin hexamer predicts the N-terminal MIT domains extend outward from the hexameric ring, possibly reaching out to "latch" onto the microtubule lattice and position the AAA ATPase ring for microtubule disassembly (25,33,38). Although the MIT domain increases microtubule affinity of microtubule-severing proteins, it is not required for microtubule-severing.

Figure 1.2 - Structures and mechanisms of microtubule-severing proteins.

A) Structural model combining crystal structure of Drosophila Spastin AAA domain (light blue ribbons) and small-angle X-ray scattering (SAXS) reconstruction of full-length (MIT + AAA domain) Spastin hexamer (pink envelope). The ribbon representation shows the N-terminal helix/loop (magenta) and the C-terminal helix (dark blue) of an individual AAA subunit in the hexameric ring. The nucleotide-binding domain (NBD, dark green) and the four-helix bundle domain (HBD, light green) are colored to denote one subunit. The hexameric ring is about 220-Å in diameter. B) Current model of microtubule-severing by Spastin. The Spastin hexameric ring (cyan) is shown via equatorial cross-section view with the AAA domain pore loops (blue ribbons, labeled 1, 2 and 3). The functions of the MIT domains (gold ovals) are not included in this model. A microtubule is shown via cross-section and each tubulin heterodimer (green ribbons) represents a subunit within an individual protofilament. The schematic shows the pore loops "grabbing" the tubulin C-terminal tail (red) and translocate it through the core of the ring to partially unfold the tubulin subunit and destabilize it. (Figure modified from Antonina Roll-Mecak and Ronald D Vale, "Structural Basis of Microtubule Severing by the Hereditary Spastic Paraplegia Protein Spastin," Nature 451, no. 7176 (January 17, 2008): 363–7, doi:10.1038/nature06482.)
1.6 Katanin is a heterodimer consisting of a catalytic subunit and a regulatory subunit

Katanin was originally discovered as a heterodimer consisting of one 60-kDa and one 80-kDa subunit, named p60 and p80, respectively (39). The catalytic p60 (KATNA1) subunit contains the conserved MIT domain and AAA domain, a functionally unknown coiled-coil domain, and a C-terminal VPS4 oligomerization domain that is required for forming hexameric rings. The regulatory p80 (KATNB1) subunit interacts with the catalytic subunit and modulates its microtubule-severing activity (40). KATNB1 contains a WD40 domain, a functionally unknown proline-rich region, and a conserved p80 domain (con80). The WD40 domain, which consists of six WD40 repeats, does not directly interact with KATNA1, but is required for spindle pole targeting of KATNA1 during cell division (41). Co-transfection of KATNA1 and WD40-less KATNB1 results in a dominant negative effect and abolishes localization of KATNA1 to the spindle pole. In addition, the WD40 domain inhibits KATNA1 microtubule-severing activity when co-transfected with KATNA1 in vivo (40). Interestingly, the con80 domain interacts with KATNA1 and increases both microtubule-binding affinity and microtubule-severing activity of KATNA1 as shown by in vitro experiments (40). It remains unclear why N-terminal and C-terminal of KATNB1 have opposite effects on Katanin microtubule-severing regulation. In addition, in vitro microtubule-severing assays showed that Xenopus egg extract co-transfected with both KATNA1 and full-length KATNB1 had higher microtubule-severing activities than extract transfected with KATNA1 alone (41). However, this observation could be due to the presence of other proteins in the extract.
and has yet to be verified using purified KATNA1 and full-length KATNB1 (31,40,41). Although KATNA1 and KATNB1 were isolated as heterodimers, KATNA1 can self-assembles into hexameric ring and severs microtubules with or without KATNB1. Similar to Spastin, Katanin assembles into hexameric rings upon binding to ATP and disassembles back into monomers upon ATP hydrolysis (31).

1.7 The functions of Katanin in cell division are species-specific

Katanin plays an essential role in regulating spindle morphology. In C. elegans, the homologs of KATNA1 and KATNB1, MEI-1 and MEI-2, contribute to spindle shortening at the spindle poles to maintain proper spindle size in embryos during meiosis (42,43). In addition, MEI-1 microtubule-severing and γ-tubulin-dependent nucleation increase the number of microtubule polymers during spindle assembly to enrich spindle density. (42,43). Partial loss-of-function mutations in the MEI-1/MEI-2 complex leads to elongated spindle and reduced spindle density. However, MEI-1 must be degraded prior to entering mitosis, and persistence of MEI-1 in mitosis leads to defect in chromosome segregation (44,45). In contrast, mammalian Katanin is required in both meiosis and mitosis. In addition to spindle assembly regulation, KATNA1 and KATNB1 are also required for proper cytokinesis progression (46,47). Depletion of KATNA1 or KATNB1 leads to incomplete cleavage-furrow ingression, resulting in multinucleate cells (47). Finally, Drosophila Katanin Dm-Kat60 primarily functions at the chromosomes instead of the spindle poles to facilitate plus-end depolymerization during anaphase, suggesting a major difference in its role and regulation in mitosis (48).
Together, these results show distinctive species-specific functions of Katanin in cell division.

1.8 Katanin is subject to multiple models of regulations in cell division

To ensure Katanin severs microtubules at the right place and the right time, it is important to precisely control the localization and activity of Katanin. For example, overexpression of KATNA1 leads to complete disassembly of cytoskeleton microtubules in HeLa cells, indicating that KATNA1 microtubule-severing activity is controlled by precise mechanisms (40). Unsurprisingly, multiple models of regulation, including phosphorylation, ubiquitination and protein-protein interaction, have been shown to regulate Katanin in cell division.

Phosphorylation

A number of studies from various species have shown that the microtubule-severing function of Katanin is inhibited by phosphorylation. In C. elegans, the microtubule-severing activity of MEI-1 is enhanced by protein phosphatase 4 complex (PP4)-mediated dephosphorylation during meiosis (49). PP4 recognizes MEI-1 through a PP4 regulatory subunit PPFR-1, and depletion of PPFR-1 results in an enlarged polar body and elongated spindle, similar to the MEI-1 loss-of-function phenotype. These observations are consistent with in vitro studies showing that a Xenopus laevis phosphomimetic mutant p60 has reduced microtubule-severing compared to wild type (50). Furthermore, phosphorylation-dependent inhibition can be overcome by increasing
the concentration of phosphomimetic mutant p60, suggesting a switch-like mechanism in Katanin phosphorylation regulation (50).

**Ubiquitination**

In *C. elegans*, MEI-1 is required to form meiotic spindles in meiosis but must be inactivated before entering mitosis (51,52). At the end of meiosis, Mel-26, a substrate-specific adaptor of the Cul-3 E3 ubiquitin ligase, recruits MEI-1 and PPFR-1 for Cul-3-mediated polyubiquitination and subsequent proteasomal degradation (49). Persistence of MEI-1 beyond meiosis leads to failure in mitosis and is lethal to *C. elegans* embryos.

In higher eukaryotes, Katanin is regulated by at least two ubiquitination pathways in cell division. In the first pathway, the Cul-3 binding protein Ctb9/KLHDC5 targets KATNA1 for Cul-3-mediated polyubiquitination and subsequent proteasomal degradation in mitosis (53). In the second pathway, DYRK2, a kinase that also serves as a scaffold for the E3 ligase complex containing EDD, DDB1 and VPRBP proteins (EDVP) complex, phosphorylates KATNA1 and promotes its polyubiquitination and subsequent proteasomal degradation by the EDVP complex (54). Further studies are needed to understand why multiple ubiquitination pathways regulate Katanin and the mechanism by which these pathways regulate Katanin in different stages of mitosis.

**Protein-protein interaction**

In addition to KATNB1, a number of proteins have been shown to regulate Katanin microtubule-severing activity. XMAP230 is a *Xenopus* homolog of MAP4 that regulates microtubule dynamics by promoting microtubule assembly (55). McNally *et al.*
showed that XMAP230 isolated from *Xenopus* interphase extract inhibits KATNA1 microtubule-severing activity *in vitro*; this inhibitory effect is reversed by pre-incubating interphase XMAP230 with CyclinB/Cdk1 prior to the *in vitro* assay (56). These results indicate that XMAP230 specifically inhibits KATNA1 during interphase to prevent disassembly of the cytoskeleton. KATNB1 has been shown to interact with a pluripotent tumor suppressor, LAPER1/LZTS2, to inhibit KATNA1 microtubule-severing activity by weakening the microtubule binding affinity of the heterodimer (57). Overexpression of LAPER1 leads to cytokinesis defects and results in multinucleate cells, a phenotype similar to KATNB1 depleted cells. NDEL1 is primarily a dynein regulator involved in neurogenesis but also localizes to centrosomes in cell division and may play a role in astral microtubule regulation (58). KATNA1 localizes to the nucleus in NDEL1-null cells, suggesting that NDEL1 regulates localization of KATNA1 during interphase (59). Together, these results demonstrate multiple models of Katanin regulation throughout the cell cycle, but the temporal control of Katanin in cell division is still not well understood.

**Chapter 2 – Introduction and Rationale**

The remodeling of microtubule structures is important for many aspects of cell physiology including cell division, motility, morphogenesis and signaling (60). A major group of proteins that remodel microtubules through microtubule-severing activities are the AAA ATPase containing proteins that include Spastin, Fidgetin and the Katanins (26,48,60,61). Katanins are composed of a catalytic p60 subunit (A subunit, which contains the AAA ATPase domain) and a regulatory p80 subunit (B subunit) (26).
However, *in vitro* biochemical studies have shown that the A subunit can form an unstable 14-16 nm hexameric ring structure, which can sever microtubules in the presence or absence of the B subunit (26,31,62), indicating that the A subunit does not require the B subunit for microtubule-severing activity. However, the B subunit has been shown to regulate the rate of microtubule-severing by the A subunit (40,41), hence its designation as a regulatory subunit. The Katanins have been implicated in the regulation of multiple processes that are important for cellular homeostasis, proliferation, and invasion including the severing of spindle microtubules to generate microtubule density during meiosis and mitosis, the severing of the cilia axoneme microtubules during ciliary resorption, the severing of intercellular bridge microtubules during cytokinesis and the severing of cytoplasmic microtubules to promote cell morphogenesis and migration (47,52,62-65). For example, in *Caenorhabditis elegans*, the microtubule-severing activity of the Katanin A subunit MEI-1 is required for regulating spindle length and density during meiotic spindle formation and for stabilizing the association of the meiosis I spindle to the oocyte cortex (42,43,66). Additionally, inactivation of a temperature sensitive MEI-1 mutant during metaphase disrupts bipolar spindle assembly and the alignment of chromosomes to the metaphase plate (67). In *Drosophila melanogaster*, the microtubule-severing activity of the Katanin p60-like 1 (Kat-60L1) A subunit is critical for neuronal development (68). Kat-60L1 mutants exhibit a reduced number and length of dendrites in neurons and a diminished neuronal responsiveness to chemical and thermal stimuli (69). Additionally, during mitosis the Katanin p60 (DmKat-60) A subunit localizes to chromosomes and facilitates microtubule depolymerization during chromosome segregation (48). In other organisms such as
Katanin A subunits are important for flagellar biogenesis and cell division (64,70-72). Finally, the microtubule-severing activity of Katanin A subunits in Arabidopsis thaliana are critical for regulating cell specification, cell growth and cell wall biosynthesis (73-79).

Although the majority of Katanin studies have focused on understanding the function of the canonical p60 and p80 subunits in lower organisms, many organisms encode additional p60-like and p80-like proteins known as Katanin-like proteins. For example, the human genome encodes two alternatively spliced isoforms of the canonical p60 A subunit (KATNA1 chromosomal locus 6q25.1, protein IDs NP_001191005.1 and NP_008975.1) and two additional p60-like proteins (KATNAL1 chromosomal locus 13q12.3, protein ID NP_115492.1; KATNAL2 chromosomal locus 18q21.1, protein ID NP_112593.2). Similarly, in addition to the canonical p80 B subunit (KATNB1 chromosomal locus 16q21, protein ID NP_005877.2), the human genome encodes an additional p80-like protein (KATNBL1 chromosomal locus 15q14, protein ID NP_078989.1) (25). Interestingly, all human Katanin subunits are ubiquitously expressed across most tissue types including brain, lung, kidney, liver, pancreas and skin and in established cell lines like HeLa cells (80). Human Katanins have important roles in regulating microtubule-dependent processes like mitotic spindle length and structure during cell division and mutation of Katanin subunits has been linked to human disorders like cerebral cortical malformation and male infertility (42,46,81-83). Domain analyses of the human Katanin subunits indicate that KATNA1 and KATNAL1 share a similar domain architecture with an N-terminal microtubule interacting and trafficking domain (MIT) followed by a coiled coil domain (CC), a AAA ATPase domain (AAA) and
a C-terminal VPS4 domain (VPS4_C) (84) (Fig. 1A). In contrast, KATNAL2 only contains the AAA domain, lacks the MIT, CC and VPS_4 domains, and has an N-terminal LisH (LIS1 homology) domain (Fig. 1A). Because KATNAL2 harbors a AAA domain that is required for Katanin microtubule-severing activity, it is predicted to have a role in microtubule-severing, however this has yet to be tested. Additionally, KATNAL2 lacks the MIT domain that is important for microtubule binding in other Katanin A subunits and whether the MIT domain is critical for microtubule-severing activity remains to be determined (38). Although KATNB1 and KATNBL1 both harbor a conserved C-terminal region (con80), only KATNB1 contains additional N-terminal proline-rich (Pro-rich) and WD40 domains that are absent in KATNBL1 (25,40) (Fig. 1A). Interestingly, full length KATNB1 and the KATNB1 con80 domain alone have been shown to stimulate KATNA1 microtubule-severing activity, whereas the KATNB1 WD40 domain alone inhibits microtubule-severing (40,41). However, the underlying mechanism of how the B subunits regulate the activity of the A subunits is still unclear. Additionally, outside of KATNA1 and KATNB1, the remaining human Katanin subunits remain poorly characterized.

Our previous proteomic analyses of human mitotic microtubule co-purifying proteins identified a then hypothetical p60-like protein KATNAL1 (85). Tandem affinity purification and mass proteomic analysis of KATNAL1 identified KATNA1, KATNAL1, KATNB1 and a then hypothetical p80-like protein C15orf29 (KATNBL1) as interactors, indicating that in humans multiple Katanins were likely involved in microtubule-severing (86). Consistent with this idea, depletion of human KATNA1 or KATNAL1 alone leads to mild changes in spindle size and mitotic defects (83,87), indicating that multiple
Katanins may be involved in regulating spindle size in mammals and/or that other microtubule-severing activities are able to compensate in the absence of either Katanin. Additionally, whether KATNAL2 has microtubule-severing activity that can compensate for the absence of other Katanin A subunits remains to be determined. Finally, the effect, if any, that KATNBL1 has on Katanin A subunit microtubule-severing activity also remains to be determined. To better understand the human Katanins and more broadly the mechanisms controlling mammalian microtubule-severing, we analyzed the human Katanin interactome (Katan-ome) through biochemical tandem affinity purifications and mass proteomic analyses. We further focused on the characterization of the poorly understood KATNBL1 subunit and its role in microtubule-severing through binding assays, microtubule-severing assays, competition binding assays, and subcellular localization studies. Our results showed that KATNBL1 is uniquely sequestered to the nucleus during interphase and associates with spindle poles in mitosis, is a regulator of KATNAL1 microtubule-severing activity, and competes with KATNB1 for binding to KATNA1 and KATNAL1. These results indicate that in humans microtubule-severing is complex and likely regulated by the concerted action of KATNB1 and KATNBL1.

Chapter 3 - Results

3.1 Defining the mammalian Katanin interactome (Katan-ome)

Our recent proteomic screen to identify novel mitotic microtubule co-purifying proteins led to the identification of KATNAL1 (NP_115492.1), a hypothetical KATNA1-like protein (85,86). Interestingly, KATNA1, KATNB1 and a KATNB1-like protein 1 (C15orf29, KATNBL1) co-purified with KATNAL1, indicating that microtubule-severing
was complex in higher eukaryotes and that potentially multiple Katanin heterodimers were involved in microtubule-severing (86). To better understand the human Katanins and more broadly the mechanisms controlling mammalian microtubule-severing, we sought to define the mammalian Katanin interactome. To do this, we generated doxycycline-inducible localization and affinity purification (LAP= EGFP-TEV-S-Peptide)-tagged-KATNA1, KATNAL1, KATNAL2, KATNB1 and KATNBL1 HeLa stable cell lines (86) (Fig. 1A). These cell lines were used to express and tandem affinity purify LAP-Katanins from cells arrested in mitosis with the treatment of Taxol. The purification eluates were resolved by SDS-PAGE and six gel slices containing the entire eluates were excised, trypsinized, and the interacting proteins were identified by 2D-LC MS/MS (supplemental Tables S1 and S2). The mass spectrometry data from each purification was used to generate an interaction module for each subunit and the Katanin interactome (Katan-ome) was assembled from the five individual interaction modules using Cytoscape (88) (Fig. 1B). Several insights were gained from the Katan-ome. First, while KATNA1, KATNAL1, KATNB1 and KATNBL1 were able to co-purify with each other in reciprocal purifications, KATNAL2 only co-purified with KATNA1 (Fig. 1B and supplemental Tables S1 and S2). Second, each Katanin interacted with a unique subset of proteins composed primarily of spindle-associated proteins. Third, all Katanins shared interactions with microtubule-associated proteins and finally, all Katanins interacted with various tubulin isoforms (Fig. 1B and supplemental Tables S1 and S2).
3.2 Validation of Katanin subunit interactions

To further validate the interactions between the Katanin subunits and to determine if these interactions were direct, we performed pairwise binding assays from cell extracts and an *in vitro* system in the presence of the microtubule depolymerizing agent nocodazole. First, LAP-Katanin B subunit expressing cell lines were transfected with HA-tagged Katanin A subunits and the A subunits were immunoprecipitated and the immunoprecipitates were immunoblotted for LAP-Katanin B subunits (Fig. 2A and B). Similar reciprocal co-IPs were carried out with LAP-Katanin A subunit expressing cell lines that had been transfected with HA-tagged Katanin B subunits (supplemental Fig. S1A-B). Finally, reciprocal co-IPs were carried out with LAP-Katanin B subunit expressing cell lines that had been transfected with HA-tagged Katanin B subunits (supplemental Fig. S1C-D). This subunit binding analysis revealed that KATNB1 and KATNBL1 were each able to associate with KATNA1 and KATNAL1 but not with...
KATNAL2. Additionally, no interaction was detected between KATNB1 and KATNBL1. Next we sought to determine whether the observed interactions between KATNB1 and KATNBL1 with KATNA1 and KATNAL1 were direct. To do this, we performed pairwise binding *in vitro* co-IPs with *in vitro* transcribed and translated $^{35}$S labeled A and B subunits. Consistent with our cell extract binding assays both KATNB1 and KATNBL1 bound to KATNA1 and KATNAL1 directly (Fig. 2C and D and supplemental Fig. S1E). Together these data indicated that KATNB1 and KATNBL1 could each bind to either KATNA1 or KATNAL1 directly and that KATNAL2 failed to interact with any of the Katanin subunits (Fig. 2E).
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**Autoradiography**

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**Autoradiography**

### E

**Summary of Katanin subunit interactions**

- **A1**
- **A1**
- **AL2**
- **B1**
- **BL1**
3.3 KATNBL1 has dynamic cell cycle dependent subcellular localization

Because KATNBL1 bound directly to KATNA1 and KATNAL1, we sought to determine if it also localized to the cytoplasm in interphase and the spindle poles during mitosis, as previously shown for KATNB1 (40). Immunofluorescence microscopy of HeLa cells expressing LAP-Katanin A or B subunits showed that KATNA1, KATNAL1, KANTAL2 and KATNB1 localized within the cytoplasm, partially overlapping with microtubules, in interphase and to the mitotic spindle and spindle poles during mitosis, consistent with previous reports (39,40,83,86,89) (Fig. 3A-C). Surprisingly, KATNBL1 failed to localize to the cytoplasm in interphase cells and instead was enriched in the nucleus (Fig. 3A and B). However, similar to KATNA1, KATNAL1, KATNAL2 and
KATNB1 is also localized to the spindle poles in mitosis (Fig. 3C). Together, these results indicated that compared to other Katanin A or B subunits, KATNBL1 was uniquely localized to the nucleus during interphase and only associated with spindle poles during mitosis.
Figure 3.3 - Katanin subunit cell cycle subcellular localization.

A-C) Immunofluorescence microscopy of paraformaldehyde fixed LAP-tagged GFP-Katanin subunit expressing HeLa cells stained with Hoechst 33342 to detect the DNA, anti-α-tubulin antibodies to detect microtubules, and anti-GFP antibodies to detect GFP-Katanin subunit localization. Images show the subcellular localization of each Katanin subunit during interphase, either whole view (A) or zoomed view of cytoplasmic microtubules (B), and metaphase of mitosis (C). Note that KATNA1, KATNAL1, KATNAL2 and KATNB1 localize to the cytoplasm in interphase and the spindle poles during mitosis, while KATNBL1 localizes to the nucleus in interphase and spindle poles during mitosis. Bar= 5µm.

3.4 Regulation of Katanin microtubule-severing activity by KATNBL1

Although KATNA1 and KATNAL1 had been shown to have microtubule-severing activities (26,40,83), there had been no characterization of the putative KATNAL2 microtubule-severing activities. Therefore, we overexpressed KATNAL2 and analyzed its ability to sever microtubules by immunofluorescence microscopy. In contrast to cells overexpressing KATNA1 or KATNAL1 that displayed a loss of α–tubulin signal indicative of microtubule-severing, overexpression of KATNAL2 had no effect on the microtubule lattice, indicating that it had no detectable microtubule-severing activity (supplemental Fig. S2). Similarly, although KATNB1 had been shown to regulate microtubule-severing activity of KATNA1 (40), the effect of KATNBL1 on A subunit microtubule-severing had not been determined. Thus, we analyzed the effect of KATNBL1 on the KATNAL1 microtubule-severing activity using in vitro microtubule-severing assays coupled to live total internal reflection fluorescence microscopy (TIRFM). Briefly, recombinant KATNAL1 and KATNBL1 subunits were added to immobilized rhodamine labeled microtubules at various ratios and microtubule-severing was monitored every 10 seconds for seven minutes using TIRFM. Consistent with
previous results, KATNAL1 showed microtubule-severing activity, and this activity was enhanced by the addition of the KATNB1 procon80 domain at a 1:1 ratio (40) (Fig. 4A and B and supplemental Fig. S3). Interestingly the addition of full-length KATNBL1 at a 1:0.125 ratio (KATNAL1:KATNBL1) inhibited the microtubule-severing activity of KATNAL1 and increasing the concentration of KATNBL1 to a 1:0.5 ratio did not further inhibit KATNAL1 activity (Fig. 4A and B and supplemental Fig. S3). However, the addition of KATNBL1 at a ratio of 1:0.0625 enhanced KATNAL1 microtubule-severing activity and decreasing the concentration of KATNBL1 further to a ratio of 1:0.03125 did not further enhance the KATNAL1 activity (Fig. 4A and B and supplemental Fig. S3). Together, these results indicated that KATNBL1 was able to regulate the KATNAL1 microtubule-severing activity in a concentration dependent manner.

![A and B - Summary of Katanin microtubule-severing activity](image)

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<td>N/A</td>
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<tr>
<td>AL1</td>
<td>N/A</td>
<td>++</td>
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<tr>
<td>AL1 + B1 procon80 (1:1)</td>
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<td>+++</td>
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<tr>
<td>AL1 + BL1 (1:0.125)</td>
<td>1:0.5</td>
<td>+</td>
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<td>AL1 + BL1 (1:0.0625)</td>
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<tr>
<td>AL1 + BL1 (1:0.03125)</td>
<td>1:0.03125</td>
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*-, No activity; +, Weak activity; ++, Medium activity; +++, Fast activity
3.5 Sequestration of KATNBL1 to the nucleus is dependent on a nuclear localization signal

The strong localization of KATNBL1 to the nucleus during interphase indicated that KATNBL1 was being actively localized to the nucleus and could potentially harbor a nuclear localization signal (NLS). Analysis of the KATNBL1 amino acid sequence with cNLS Mapper (90) indicated that KATNBL1 had at least two putative bipartite NLS's; NLS1 corresponding to amino acids 9-28 (basic cluster 1 amino acids 9-11 and basic cluster 2 amino acids 26-28 ) and NLS2 corresponding to amino acids 71-87 (basic cluster 1 amino acids 71-74 and basic cluster 2 amino acids 85-87) (Fig. 5A). Therefore, we performed an analysis of NLS1 and NLS2 mutants, by substituting amino acid residues within these motifs to alanines and assessing their subcellular localization with immunofluorescence microscopy. Mutation of the NLS1 basic cluster 1 or basic cluster 1 and 2 had no effect on the localization of KATNBL1 and it remained in the nucleus (Fig. 5A and B). Consistently, a KATNBL1 truncation mutant missing the first 55 amino acids (Δ1-55) also maintained its nuclear localization and fusion of the first 69 N-terminal amino acids of KATNBL1 to GFP did not localize it to the nucleus (Fig. 5A and B and supplemental Fig. S4). This indicated that NLS1 was not necessary for KATNBL1
nuclear localization. Next, we mutated NLS2 basic cluster 1 or basic cluster 1 and 2. Interestingly, mutation of the NLS2 basic cluster 1 led to the redistribution of KATNBL1 from the nucleus to both the nucleus and cytoplasm, while mutation of both basic cluster 1 and 2 did not further redistribute KATNBL1 to the cytoplasm (Fig. 5A and B and supplemental Fig. S4). This indicated that NLS2 basic cluster 1 was important for the localization of KATNBL1 to the nucleus. Consistently, fusion of the first 98 N-terminal amino acids of KATNBL1 to GFP localized it to the nucleus (Fig. 5A and B). Furthermore, mutation of NSL1 basic cluster 1 or 2 in combination with NLS2 basic cluster 1 did not further redistribute KATNBL1 to the cytoplasm (Fig. 5A and B). However, mutation of all three (NLS1 basic cluster 1 and 2 and NLS2 basic cluster 1) led to the complete redistribution of KATNBL1 to the cytoplasm (Fig. 5A and B). Together, these data indicated that NLS2 basic cluster 1 was important for targeting KATNBL1 to the nucleus during interphase and that NLS1 basic cluster 1 and 2 also played a role.
Figure 3.5 - KATNBL1 localization to the nucleus during interphase requires an N-terminal nuclear localization sequence.

A) cNLS Mapper analysis of KATNBL1 identified two putative bipartite nuclear localization signals (NLS) corresponding to amino acids 9-28 (NLS1) and 71-87 (NLS2). The generated KATNBL1 NLS1 and NLS2 mutants and KATNBL1 truncation mutants are indicated. B) Immunofluorescence microscopy, as in Fig. 2.3, was used to visualize the subcellular localization of KATNBL1 wildtype (WT), NLS1 mutants, NLS2 mutants, deletion mutants and N-terminal fusions as indicated. + indicates nuclear localization, +/- indicates partial nuclear localization and – indicates no nuclear localization. Bar= 5µm. Note that wildtype KATNBL1 localizes to the nucleus, whereas mutation of NLS2 basic cluster 1 (amino acids 71-74) in combination with NLS1 basic cluster 1 (amino acids 9-11) and 2 (amino acids 26-28) inhibits its transport to the nucleus and it remains in the cytoplasm. See supplemental Fig. S4 for the localization of additional mutants. For a list of primers used for generating KATNBL1 mutants see supplemental Table S3.
3.6 KATNB1 competes with KATNBL1 for binding to KATNA1 and KATNAL1

Because KATNB1 and KATNBL1 both interacted directly with KATNA1 and KATNAL1 it was possible that they were competing for binding to KATNA1 and KATNAL1. To test this we performed *in vitro* Katanin A and B subunit binding experiments as described previously, except that increasing concentrations of recombinant B subunits were added. Indeed, KATNB1 was able to compete the KATNA1-KATNBL1 and KATNAL1-KATNBL1 interactions in a dose dependent manner (percent KATNBL1 bound decreased from 100% to ~20%) (Fig. 6A and B). However, reciprocal competition studies with increasing concentrations of KATNBL1 showed that KATNBL1 was only able to weakly compete the KATNA1-KATNB1 and KATNAL1-KATNB1 interactions (percent KATNB1 bound decreased from 100% to ~84%) (Fig. 6C and D). Together, these results indicated that KATNB1 and KATNBL1 were likely in competition for binding to KATNA1 and KATNAL1 and that KATNB1 had a higher affinity for KATNA1 and KATNAL1 than KATNBL1.
Figure 3.6 - KATNB1 competes the KATNA1-KATNBL1 and KATNAL1-KATNBL1 interactions.

A-D) Competition binding assays, using increasing concentrations of recombinant KATNB1 or KATNBL1. HA or Myc-tagged in vitro 35S-radiolabeled Katanin subunits were used in pairwise binding competition reactions as indicated and the results of the competition binding assays were visualized by autoradiography. A-B, Increasing concentrations of KATNB1 competes the KATNA1-KATNBL1 and KATNAL1-KATNBL1 interactions. The intensity of the KATNBL1 bands were measured using ImageJ and the percent KATNBL1 bound is indicated. Note that the percent KATNBL1 bound decreases as the concentration of KATNB1 increases. C-D, Increasing concentrations of KATNBL1 weakly compete the KATNA1-KATNB1 and KATNAL1-KATNB1 interactions. The intensity of the KATNB1 bands were measured using ImageJ and the percent KATNB1 bound is indicated. Note that the percent KATNB1 bound decreases only slightly as the concentration of KATNBL1 increases.
Chapter 4 - Discussion and Conclusions

By coupling Katanin subunit tandem affinity purifications from mitotic cells with mass proteomic analyses, we identified the protein interaction module for each of the five human Katanin subunits. These interaction modules were combined to generate the human Katanin interactome (Katan-ome): a mitotic protein network comprised of all the Katanin subunits and their interacting partners (Fig. 1B). The identified protein-protein interactions could be used to further understand the cells microtubule-severing machinery, their mechanisms of action and their regulation. For example, the Katanin interactors may represent factors that regulate Katanin activity, localization, complex assembly or that coordinate with the Katanins to promote microtubule-severing and the generation of microtubule density that is required for proper spindle assembly and cell division. Along these lines, reports in *Xenopus laevis* have indicated that Katanin function may be regulated through posttranslational modifications like phosphorylation (50,56,91). Interestingly several kinases were identified in the Katan-ome, including Aurora A (KATNA1 interactor); PLK2 (KANTAL1 interactor); CDK1 and CDK2 (KATNAL1 and KATNAL2 interactors); and the kinase regulatory protein CDK5RAP2 (KATNAL2 interactor) (Fig. 1B and supplemental Tables S1 and S2). Aurora A, PLK2, CDK1, CDK2 and CDK5RAP2 all have critical roles in mitosis predominantly through the regulation of centriole and centrosome homeostasis and could be modulating Katanin activity at the spindle poles during mitosis through phosphorylation (92-97). Interestingly, the Katan-ome contained a good degree of connectivity between the individual Katanin subunits, indicating that they may share common modes of inhibition/activation and/or may have redundant roles. However, our analysis of
mitotically associated Katanin interacting proteins could have precluded the identification of important interactors that only associate with Katanins during interphase. Therefore, additional proteomic analyses of Katanin purifications from interphase cells could elucidate additional interacting proteins important for regulating microtubule-severing.

Our binding experiments indicated that KATNAL2 did not bind to other Katanin subunits, however it is possible that the epitope-tagged KATNAL2 was not properly folded or that different KATNAL2 isoforms were binding to themselves and potentially excluding other interactions, as has been shown for the five alternatively spliced mouse KATNAL2 isoforms (89). However, there is no evidence for alternatively spliced forms of KATNAL2 in humans. Additionally, KATNAL2 had more mitotic interactors than any other Katanin subunit in the network. KATNAL2 interactors included CDK5RAP2, and CEP295, which are important regulators of centriole amplification (92,98); CDK5RAP2, WDR62, and CEP97, which are critical for maintaining a bipolar spindle and CDK5RAP2 also plays a role in promoting cytokinesis and maintaining a normal nuclear size (92,99,100); and CEP97 and PCM1, which have roles in primary cilium formation (99,101). This is interesting in light of a recent study, which showed that mouse KATNAL2 has multiple roles in microtubule-based processes such as centriole amplification, spindle bipolarity, cytokinesis, nuclear morphology and ciliogenesis (89). Although we were unable to detect KATNAL2 microtubule-severing activity in cells (supplemental Fig. S2), it is possible that KATNAL2 can regulate these cellular processes through its protein-protein interactions. Along these lines, in Caenorhabditis elegans the spindle assembly function of the Katanin A subunit MEI-1 does not require
its microtubule-severing activity, indicating that Katanins may have microtubule-severing independent roles in spindle assembly (102).

A key unanswered question is how does KATNBL1 localize to the spindle poles during mitosis. KATNB1 has been shown to localize to the spindle poles through its WD40 domain, however KATNBL1 lacks the WD40 domain (41). Interestingly, KATNBL1 interacted with WDR62, which is known to localize to the spindle poles and is required for spindle organization (103). Therefore it is possible that WDR62 may be targeting KATNBL1 to the spindle poles during mitosis and should be explored further. Additionally, KATNBL1 contains the C-terminal con80 domain that in KATNB1 binds to KATNA1 and our binding studies indicated that KATNBL1 was able to interact with KATNA1 and KATNAL1 (40,102) (Fig. 2). Thus it is also possible that KATNBL1 localizes to the spindle poles through its interaction with KATNA1 and KATNAL1. Whereas KATNB1 was able to robustly compete the KATNA1-KATNBL1 and KATNAL1-KATNBL1 interactions in a dose dependent manner (Fig. 6A and B), KATNBL1 was only able to weakly compete the KATNA1-KATNB1 and KATNAL1-KATNB1 interactions (Fig. 6C and D), indicating that in vitro KATNBL1 had a weaker association with KATNA1 and KATNAL1 compared to KATNB1. Interestingly, these observations are consistent with a previous study showing that the N-terminus of KATNAL1 bound to either KATNB1 or KATNBL1 and that depletion of the first 29 amino acids of KATNAL1 drastically decreased its association with KATNBL1 and only had a minor effect on its association with KATNB1 (102). These data indicate that KATNB1 likely has multiple sites of interaction with KATNAL1 and therefore we would expect KATNB1 to have a stronger association with KATNAL1 compared to KATNBL1. Finally,
the inhibition of KATNAL1 microtubule-severing activity with equimolar concentrations of KATNBL1 was surprising (Fig. 4A and B), however a possible explanation could be that each KATNBL1 monomer binds to a KATNAL1 monomer and associates it with microtubules, thereby depleting the levels of free KATNAL1 monomers that are available to form homo-hexamers needed for microtubule-severing. Consistent with this, the con80 domain that is found at the C-terminus of KATNBL1 has been shown to bind to both microtubules and to the Katanin A subunit (40). Therefore, lower concentrations of KATNBL1 would facilitate KATNAL1 microtubule-binding and still allow the formation of KATNAL1 homo-hexamers, thereby stimulating microtubule-severing.

To our knowledge this is the first characterization of KATNBL1’s function in regulating microtubule-severing. KATNBL1 co-purified and co-immunoprecipitated with KATNA1 and KATNAL1 indicating that it was associating directly with these enzymes in cells (Figs. 1B and 2). In vitro, KATNBL1 was able to regulate the microtubule-severing activity of KATNAL1 in a concentration dependent manner (Fig. 4 and supplemental Fig. S3). However, the localization of KATNBL1 to the nucleus during interphase implies that KATNBL1 is only able to bind and regulate KATNA1 and KATNAL1 microtubule-severing during mitosis, when the nuclear membrane is dissolved. This also implies that KATNB1 and KATNBL1 may be in competition/equilibrium to stimulate or inhibit Katanin microtubule-severing during mitosis. How KATNB1 and KATNBL1 coordinate and are regulated to ensure the appropriate amount of microtubule-severing during mitosis remains to be explored.
Both KATNA1 and KATNAL1 have been shown to sever microtubules and regulate spindle morphology in cell division. However, it is still unclear why mammalian cells express multiple Katanin subunits that have redundant roles in cell division. Since KATNA1 and KATNAL1 co-purified in LAP-tag purification, it is possible that mammalian Katanins can form hetero-hexamers in addition to homo-hexamers. Further, it is not clear how Katanin catalytic subunits form hexameric ring in the presence of regulatory subunits. The Katanin hexamer is very unstable and structural characterization of the intact hexamer remains challenging for NMR and X-ray crystallography. High-resolution cryo-electron microscopy (cryo-EM) requires less proteins and minimal sample manipulation and may provide a valuable tool for visualizing Katanin hexameric rings with different combinations of mammalian Katanin subunits.

Although the role of KATNAL1 in mitosis has been characterized, it is still unclear how KATNAL1 is regulated or if these pathways overlap with the regulation of KATNA1 (83). KATNA1 and KATNAL1 have redundant microtubule-severing function in mitosis, which could explain why depletion of KATNA1 only leads to mild mitotic defects in dividing cells. Our proteomic analysis identified a number of proteins that can potentially regulate KATNAL1 through post-translational modification, such as CDK1, CDK2 and PLK2. Interestingly, KATNA1 and KATNAL1 are the only pair of mammalian Katanin proteins that did not share any common interactors (except tubulins). These results suggest that separate pathways may regulate microtubule-severing activities of KATNA1 and KATNAL1 during mitosis. Future work should focus on the regulation of
KATNAL1 to understand how KATNA1 and KATNAL1 complement each other to regulate microtubule dynamics during cell division.

Our results identified KATNBL1 as a novel Katanin regulator in mammalian cells, which suggest a more complex microtubule-severing regulatory mechanism than previously expected. KATNB1 has been shown to regulate Katanin either directly or through interactions with other proteins and can modulate both microtubule-severing activity and localization of KATNA1 (40,57). Future studies should also focus on understanding how KATNBL1 regulates the Katanin catalytic subunits and on the identified KATNBL1 interactors that can regulate Katanin catalytic subunits through KATNBL1-interactions.

Mammalian KATNAL2 colocalizes with all other Katanin family members to the spindle poles during mitosis. Although we did not observe microtubule-severing activity of KATNAL2, it interacts with a broad number of mitotic proteins in mitosis. Consistent with our results, KATNAL2 depleted cells show a variety of spindle defects including multipolar spindles, mis-aligned chromosomes and cytokinetic failure. Finally, it will be interesting to investigate the functional relationship between KATNAL2 and its mitotic interactors such as CDK5RAP2, CDK1 and CDK2, which regulate centriole and centrosome homeostasis in mitosis.

Chapter 6 - Materials and Methods

6.1 Cell culture and cell cycle synchronization

All chemicals were purchased from Thermo Scientific unless otherwise noted. HeLa and HeLa Flp-In T-REx LAP-tagged stable cell lines were grown in F12:DMEM 50:50
medium (GIBCO) with 10% FBS, 2 mM L-glutamine and antibiotics, in 5% CO₂ at 37°C. Cells were induced to express the indicated LAP-tagged proteins by the addition of 0.2µg/ml doxycycline (Sigma-Aldrich). For synchronization of cells in mitosis, cells were treated with 100 nM Taxol (Sigma-Aldrich) for 16 hours.

6.2 Plasmids, mutation and generation of LAP-tagged Katanin inducible stable cell lines

For full-length KATNA1, KATNAL1, KATNAL2, KATNB1, and KATNBL1, or STARD9-START and STARD9-MD expression, cDNA corresponding to the full-length open reading frame of each Katanin or the indicated STARD9 domains was fused to the C-terminus of either HA (pCS2-HA-DEST vector), Myc (pCS2-Myc-DEST vector), FLAG (pCDNA3-FLAG-DEST vector), GST (pGEX-6p-1-DEST vector) or EGFP (pGLAP1 vector) using the Gateway cloning system (Invitrogen) as described previously (85). The pGLAP1-Katanin vectors were used to generate doxycycline inducible HeLa Flp-In T-REx LAP-KATNA1, KATNAL1, KATNAL2, KATNB1, and KATNBL1 stable cell lines that express the fusion protein from a specific single locus within the genome as described previously (86). For KATNBL1 mutations, pGLAP1-KATNBL1 was mutated using the QuickChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies) with primers carrying the desired mutations, as described previously (104), whereas truncation mutants were generated by PCR amplification and cloning into pGLAP1 as described above. All primers were purchased from Fisher Scientific. For a list of primers used see supplemental Table S3.
6.3 Immunoprecipitations

For cell extract immunoprecipitations (IPs), LAP-KATNA1, LAP-KATNAL1, LAP-KATNB1, or LAP-KATNBL1 HeLa stable cells lines were transfected with the indicated HA-tagged Katanin subunit expression vectors for 24 hours and whole cell extracts were prepared in LAP150 lysis buffer (50 mM Tris pH 7.4, 150 mM KCl, 1 mM EDTA, 1 mM MgCl$_2$, 10% glycerol) plus 250 µM ATP, 0.3% NP40, 0.5 mM DTT and protease and phosphatase inhibitor cocktail (Thermo Scientific). Cell extracts were cleared by centrifugation at 15K RPM for 10 minutes. 140µg of cleared lysate was incubated with 5µl packed bead volume of anti-HA antibody conjugated magnetic beads (MBL) for 1 hour at 4°C. The beads were then washed 3 times with 50µl of LAP150 lysis buffer and bound proteins were eluted with 20µl of 1X Laemmli SDS sample buffer (Bio-Rad). 6% of the sample inputs, 6% of the unbound fractions and the entire eluates from the immunoprecipitations were resolved on a 10% Tris gel (Bio-Rad) with Tris-Glycine SDS running buffer, transferred to a Immobilon PVDF membrane (EMD Millipore), immunoblotted with the indicated antibodies, and imaged with a LI-COR Odyssey imager (LI-COR Biosciences).

6.4 In vitro binding assays

For in vitro binding assays, HA, Myc, or FLAG-tagged KATNA1, KATNAL1, KATNAL2, KATNB1, or KATNBL1 were in vitro transcribed and translated (TnT® Quick Coupled Transcription/Translation System, Promega) in 50µl reactions. Two different Katanin reactions were combined and incubated with 5µl packed bead volume of anti-HA antibody conjugated magnetic beads (MBL) for 1 hour. Beads were washed four times
with a wash buffer containing 10 mM Tris pH 7.4, 100 mM NaCl, and 0.1% NP40. The beads were then boiled in 20 µl of 1X Laemmli SDS sample buffer (Bio-Rad). 6% of the sample inputs, 6% of the unbound fractions (where indicated), and the entire eluates from the immunoprecipitations were resolved on a 10% Tris gel (Bio-Rad) with Tris-Glycine SDS running buffer, transferred to a Immobilon PVDF membrane (EMD Millipore), and binding was monitored by radiometric analysis with a PharosFX Plus molecular imaging system (Bio-Rad).

6.5 Tandem affinity purification of Katanins

The LAP-KATNA1, KATNAL1, KATNAL2, KATNB1, and KATNBL1 inducible stable cell lines were grown in roller bottles and induced with .2 µg/ml Dox for 16 hours in the presence of 100 nM Taxol prior to harvesting cells, as described in (86). Mitotic cells were then harvested in the presence of protease (Thermo Scientific), phosphatase (Thermo Scientific), and proteasome inhibitors (MG132, Enzo lifesciences). LAP-KATNA1, KATNAL1, KATNAL2, KATNB1, and KATNBL1 were purified from cleared extracts using a previously established tandem affinity purification protocol (86).

6.6 In gel protein digestions

Sample lanes from SDS-PAGE were sliced into six pieces and placed into individual micro-centrifuge tubes. Each gel slice was dehydrated with 100% acetonitrile for 30 minutes. Cysteines were reduced with 100 mM dithiothreitol in 50 mM ammonium bicarbonate for 60 minutes at 37°C, followed by the removal of buffer, and subsequently alkylated with 55 mM iodoacetamide in 50 mM ammonium bicarbonate for 45 minutes at
room temperature in the dark. Buffer was decanted and gel slices were dehydrated with 100% acetonitrile followed by rehydration with 50 mM ammonium bicarbonate, repeated twice, except swelling in 5ng/µL trypsin with the second 50 mM ammonium bicarbonate rehydration step on ice for 45 minutes. Trypsin solution was decanted and samples were incubated at 37°C overnight. Peptides were extracted from the gel slices using 100µL of 50% acetonitrile for 20 minutes using water-bath sonication, repeated twice. Extracted peptides were dried using Speed-Vac and reconstituted in 80µL of 3% acetonitrile with 0.1% formic acid. Peptides were desalted using C18 StageTips as previously described (105).

6.7 Nano-liquid chromatography with tandem mass spectrometry (LC-MS/MS) analysis

Nano-LC-MS/MS with collision-induced dissociation was performed on an Orbitrap XL (Thermo Fisher) integrated with an Eksigent 2D nano-LC instrument. A laser-pulled reverse-phase column, 75 µm x 200 mm, containing 5-µm C18 resin with 300-Å pores (AcuTech) was used for online peptide chromatography. Electrospray ionization conditions using the nanospray source (Thermo Fisher) for the Orbitrap were set as follows: capillary temperature at 200°C, tube lens at 110 V, and spray voltage at 2.3 kV. The flow rate for reverse-phase chromatography was 300nl/min for loading and analytical separation (buffer A, 0.1% formic acid and 3% acetonitrile; buffer B, 0.1% formic acid and 100% acetonitrile). Peptides were loaded onto the column for 30 minutes and resolved by a gradient of 0–40% buffer B over 60 minutes. The Orbitrap was operated in data-dependent mode with a full precursor scan at high resolution.
(60,000 at m/z 400) from 300-1,800 m/z and 10 MS/MS fragmentation scans at low resolution in the linear trap using charge-state screening excluding both unassigned and +1 charge ions. For collision-induced dissociation, the intensity threshold was set to 500 counts, and a collision energy of 40% was applied. Dynamic exclusion was set with a repeat count of 1 and exclusion duration of 30 seconds.

6.8 Experimental design and statistical rational

Database searches of the acquired spectra were analyzed with Mascot (v2.4; Matrix Science). The UniProt human database (March 19, 2014; 88,647 sequences; 35,126,742 residues) was used. The following search parameters were used: trypsin digestion allowing up to 2 missed cleavages, carbamidomethyl on cysteine as a fixed modification, oxidation of methionine as a variable modification, 10-ppm peptide mass tolerance, and 0.5-Da fragment mass tolerance. With these parameters, an overall 5% peptide false discovery rate, which accounts for total false positives and false negatives, was obtained using the reverse UniProt human database as the decoy database. Peptides that surpassed an expectation cut-off score of 20 were accepted. All raw mass spectrometry files can be accessed at the UCSD Center for Computational Mass Spectrometry MassIVE datasets ftp://MSV000079358@massive.ucsd.edu (login: Torres, password: mitosis1). Peptides meeting the above criteria were filtered further. First, peptides corresponding to common contaminants identified in the CRAPome (106) and an internal LAP purification database were excluded from further analysis. The lists of proteins identified from each of the Katanin subunit purifications as a single pass are listed in supplemental Table S1 and the spectrums of significant single peptides are in
supplemental Table S2. The final Katanin interactor lists were compiled and Cytoscape (88), an open access software used for complex network analysis and visualization, was used to generate the human Katanin interactome (Katan-ome) (Fig. 1B).

6.9 Immunofluorescence microscopy
Immunofluorescence was carried out essentially as described previously (107) with minor modifications. HeLa stable cell lines were induced to express LAP-KATNA1, KATNAL1, KATNAL2, KATNB1, and KATNBL1 for 16 hours, fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100/PBS, and co-stained with 0.5µg/ml Hoechst 33342 and the indicated antibodies. Images were captured with a Leica DMI6000 microscope (Leica DFC360 FX Camera, 63x/1.40-0.60 NA oil objective, Leica AF6000 software) at room temperature. Images were deconvolved with Leica Application Suite 3D Deconvolution software and exported as TIFF files.

6.10 Preparation of GOPTS-PEG-biotin functional coverslips
Coverslips (22 mm × 30 mm) were cleaned by incubating with 2% Hellmanex® II solution at 60°C for 60 minutes, rinsed five times with Milli-Q water and dried in an oven. In the silanization step, a drop of (3-Glycidyloxypropyl)-trimethoxysilane (GOPTS) (Sigma, 440167) was sandwiched between every two coverslips and baked at 70°C for 60 minutes. Each coverslip was then rinsed through three beakers of 30mL glass-distilled acetone (Electron Microscopy Sciences, 10015) and air-dried. To functionalize coverslips with PEG-Biotin, 144mg of methoxy PEG Amine MW 3000 (Jenkem Technology) and 16mg of biotin-PEG-NH₂ MW 3,400 (Laysan Bio) were dissolved in
700µL DMF and 35µL was applied between each pair of GOPTS silanized coverslips followed by 75°C incubation in an oven for 8 hours. The coverslip sandwiches were quickly disassembled, rinsed in Milli-Q water, air-dried and stored at 4°C.

6.11 Katanin microtubule-severing TIRF assays

Flow cells were constructed with GOPTS-PEG-biotin functional coverslips and glass slides were separated by double-sided tape, four chambers were made for each coverslip. To prepare for microtubule immobilization, each chamber was first incubated with blocking buffer (5% pluronic F-127, 100µg/mL casein, 1× PBS) for 1 minute, followed by 1 minute incubation in 80 nM streptavidin in BRB80 (80 mM PIPES pH 7.5, 2 mM MgCl₂, 2 mM EGTA). Chambers were rinsed with wash buffer (80 mM PIPES pH 7.5, 2 mM MgCl₂, 2 mM EGTA, 40 mM D-glucose, 10 mM DTT) after each step. Microtubules labeled with 10% rhodamine and 10% biotin were added and allowed to bind for 3 minutes. Chambers were then rinsed with TIRF buffer (80 mM PIPES pH 7.5, 2 mM MgCl₂, 2 mM EGTA, 40 mM D-glucose, 10 mM DTT, 5 mM ATP, 1mg/mL glucose oxidase, 1mg/mL catalase) to remove unattached microtubules. To record time-lapse assays, the flow cell was placed on a DMI6000 TIRF microscope (Leica) and microtubules were brought to focus. 20µL of Katanin subunits were quickly flowed through the chamber at the indicated concentrations in TIRF buffer and images were collected every 10 seconds for 7 minutes. To measure the rate of microtubule-severing, the average fluorescence intensities of the time-lapse images were measured using ImageJ and the background fluorescence was subtracted.
6.12 Recombinant protein expression and purification

GST-fusion Katanin protein constructs were expressed in *Escherichia coli* BL21 (DE3) cells, grown in 6 L LB media with ampicillin (100 µg/mL) to an optical density of 0.6-0.8, and induced using 0.3 mM isopropyl thio-β-D-galactoside for ~18 hours at 16°C. Fresh cultures were harvested and resuspended in 100mL lysis buffer [50 mM Tris-HCl pH 8.0, 300 mM NaCl, 2 mM MgCl₂, 10% glycerol, 0.5 mM ATP, 1 mM DTT and complete EDTA-free protease inhibitor cocktail (Roche)] followed by lysis with a microfluidizer® M110-P. Lysates were cleared by centrifugation at 15K RPM, 45 minutes, 4°C and incubated with Glutathione Agarose (Pierce) for 2 hours at 4°C. Bound proteins were washed three times with wash buffer (50 mM Tris-HCl pH 8.0, 200 mM NaCl, 2 mM MgCl₂, 10% glycerol, 0.5 mM ATP, 1 mM DTT) and eluted with 50 mM glutathione in storage buffer (20 mM HEPES pH 7.8, 50 mM KCl, 1 mM MgCl₂, 10% glycerol, 0.25 mM ATP, 1 mM DTT), followed by dialysis into storage buffer at 4°C overnight. Dialyzed elutions were flash frozen in aliquots and stored at -80°C until use to prevent multiple freeze-thaw cycles. Katanin protein concentrations were determined using the Bradford reagent (Bio-Rad) and comparing to bovine serum albumin (BSA) standards with a NanoDrop 2000 (Thermo Scientific). For *in vitro* binding experiments, ³⁵S-radiolabeled Katanins were expressed using the TnT® Quick Coupled Transcription/Translation System (Promega) (104).
6.13 Antibodies

Immunofluorescence, immunoblotting, and immunoprecipitations were carried out using the following antibodies: GFP (Abcam), Gapdh (GeneTex), α-tubulin (Serotec), HA (Cell Signaling). Secondary antibodies conjugated to FITC, Cy3 and Cy5 were from Jackson Immuno Research (Affinipure).
* Asterisks indicate HA-tagged immunoprecipitated proteins

★ Arrows indicate Myc-tagged B1 or BL1 that co-precipitate with HA-tagged A1 and AL1
Figure S1 - Katanin subunit interactions.

A-D) In cell Katanin subunit pairwise binding reactions. (A-B) LAP-tagged GFP-A1 (A) or GFP-AL1 (B) HeLa stable cell lines were transfected with HA-tagged B1 or BL1 subunits. HA-B1 or HA-BL1 were immunoprecipitated from 140\(\mu\)g of protein extracts. 6% of the input and all of the immunoprecipitates were western blotted (WB) for the indicated GFP-tagged A subunits (using anti-GFP antibodies) and the HA-tagged B subunits (using anti-HA antibodies). NT denotes non-transfected. (C-D) same as in A-B, except that LAP-tagged GFP-B1 (C) or GFP-BL1 (D) HeLa stable cell lines were transfected with HA-tagged B1 or BL1 subunits. E) In vitro \(^{35}\)S-radiolabeled Katanin subunit pairwise binding reactions. In vitro transcribed and translated \(^{35}\)S-radiolabeled HA-tagged A subunits or B subunits or Myc-tagged A subunits or B subunits were used for pairwise in vitro binding reactions as indicated. HA-tagged Katanin subunits were then immunoprecipitated and the radiolabeled Katanin subunits in the immunoprecipitates were visualized by autoradiography. The bands corresponding to the immunoprecipitated HA-tagged Katanin subunits contain an asterisk below them. PME denotes the negative control protein PME-1. Note that Myc-B1 and Myc-BL1 (indicated by arrows) co-precipitate with both HA-A1 and HA-AL1.

![Image](image.png)

Figure S2 - KATNAL2 does not display microtubule-severing activity.

Immunofluorescence microscopy of cells transfected with GFP-KATNA1, GFP-KATNAL1 or GFP-KATNAL2 for 48 hours. Cells were fixed with paraformaldehyde and stained with Hoechst 33342 to detect the DNA, anti-\(\alpha\)-tubulin antibodies to detect microtubules, and anti-GFP antibodies to detect GFP-Katanin subunit localization. Note
that in cells overexpressing GFP-KATNA1 or GFP-KATNAL1 (see arrows) microtubules are severed, whereas microtubules remain stabilized in cells overexpressing GFP-KATNAL2. Bar= 5µm.

Figure S3 - Quantification of time-lapse microtubule TIRF fluorescence signals in the presence (or absence) of various katanin subunits.

Quantification of microtubule fluorescence signals of the TIRF data presented in Fig. 4B. Each data point represents the average of three individual experiments. Error bars indicate +/- SD. Average fluorescence signals of time-lapse TIRF images were measured with ImageJ. Each time point represents the percentage of the average fluorescence signal remaining in comparison to the initial amount of fluorescence signal at t = 0s. (+++) indicates fast microtubule-severing activity, (++) indicates medium microtubule-severing activity, (+) indicates weak microtubule-severing activity, and (-) no microtubule-severing activity.
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+ = Nuclear localization, 
-/+ = Partial nuclear localization 
- = No nuclear localization
Figure S4 - KATNBL1 localization to the nucleus during interphase requires an N-terminal nuclear localization sequence.

Immunofluorescence microscopy of cells transfected with wildtype (WT) GFP-KATNBL1, GFP-KATNBL1 NLS1 and NLS2 mutants, and GFP-KATNBL1 truncation mutants as indicated for 24 hours. Cells were fixed with paraformaldehyde and stained with Hoechst 33342 to detect the DNA, anti-α-tubulin antibodies to detect microtubules, and anti-GFP antibodies to detect GFP-KATNBL1 localization. + indicates nuclear localization, +/- indicates partial nuclear localization and – indicates no nuclear localization. Bar= 5µm. See Fig. 5 for the localization of additional mutants. For a list of primers used for KATNBL1 mutagenesis see supplemental Table S3.

KATNA1

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Table S1 - Summary of the Katanin interactome (Katan-ome).

Summary of LAP-KATNA1, KATNAL1, KATNAL2, KATNB1 and KATNBL1 interacting proteins identified by biochemical tandem affinity purifications and mass spectrometry analyses from mitotic cells. List includes protein name, UniProt accession number, number of peptides identified, the percent protein coverage and a description of each of the identified proteins.
KATNA1

AURKA

MS/MS Fragmentation of ATAPVGGPK
Found in A5KEF0 in UniProt-Human, Aurora kinase A OS=Homo sapiens GN=AURKA PE=1 SV=1

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Monoisotopic mass of neutral peptide Mr(calc): 796.4443
Fixed modifications: Carbamidomethyl (C) (apply to specified residues or termini only)
Variable modifications:
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Ions Score: 22  Expect: 0.056
Matches	:	6/64	fragment	ions	using	7	most	intense	peaks			(help)

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SEC16A

MS/MS Fragmentation of FRELLLYGR
Found in F79O11 in UniProt-Human, Protein transport protein Sec16A OS=Homo sapiens GN=SEC16A PE=1 SV=1

Match to Query 7241: 1245.629124 from(623.821838,2+) index(8969)
Title: jc_012214_Torres_AL-MIT-4.314.314.2.dta
Data file C:\Program Files (x86)\Matrix Science\Mascot Daemon\mgf\125 jc-012214-Torres-AL-MIT\mascot_daemon_merge.mgf
Monoisotopic mass of neutral peptide Mr(calc): 1245.6271
Fixed modifications: Carbamidomethyl (C) (apply to specified residues or termini only)
Variable modifications:
Y7     : Phospho (Y)
Ions Score: 31  Expect: 0.051
Matches	:	8/78	fragment	ions	using	10	most	intense	peaks			(help)

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KATNAL1

PCM1

MS/MS Fragmentation of ALYALQDIVSR
Found in H0YBA1 in UniProt-Human, Pericentriolar material 1 protein (Fragment) OS=Homo sapiens GN=PCM1 PE=1 SV=1

Match to Query 9167: 1247.06624 from(624.850588,2+) index(9970)
Title: jc_112413_IGD_Torres_AL2-2.2733.2.dta
Data file C:\Program Files (x86)\Matrix Science\Mascot Daemon\mgf\77 Astrin\mascot_daemon_merge.mgf

Monoisotopic mass of neutral peptide Mr(calc): 1247.6866
Fixed modifications: Carbamidomethyl (C) (apply to specified residues or termini only)
Ions Score: 40  Expect: 0.00061
Matches : 13/100 fragment ions using 22 most intense peaks  (help)

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DIAPH3

MS/MS Fragmentation of LVTCLESLR
Found in Q9NSV4 in UniProt-Human, Protein diaphanous homolog 3 OS=Homo sapiens GN=DIAPH3 PE=1 SV=4

Match to Query 6221: 1089.587014 from(545.800783,2+) index(881)
Title: jc_112413_IGD_Torres_AL2-1.2010.2.dta
Data file C:\Program Files (x86)\Matrix Science\Mascot Daemon\mgf\77 Astrin\mascot_daemon_merge.mgf

Monoisotopic mass of neutral peptide Mr(calc): 1089.5852
Fixed modifications: Carbamidomethyl (C) (apply to specified residues or termini only)
Ions Score: 33  Expect: 0.024
Matches : 7/64 fragment ions using 9 most intense peaks  (help)

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KATNAL1

PLK2

MS/MS Fragmentation of QIVSGLK
Found in A0A087WUH9 in Uniprot-Human, Serine/threonine-protein kinase PLK OS=Homo sapiens GN=PLK2 PE=1 SV=1

Match to Query 1985: 823.422764 from(412.718658,2+) index(7357)
Title: jc_112413_IGD_Torres_AL2-3.581.581.2.dta
Data file C:\Program Files (x86)\Matrix Science\Mascot Daemon\mgf\77 Astrin\mascot_daemon_merge.mgf

Monoisotopic mass of neutral peptide Mr(calc): 823.4205
Fixed modifications: Carbamidomethyl (C) (apply to specified residues or termini only)
Variable modifications: 
S4     : Phospho (ST), with neutral losses 0.0000(shown in table), 97.9769

Ions Score: 32  Expect: 0.021
Matches	:	20/108	fragment	ions	using	41	most	intense	peaks			(help)

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Ionen Score: 32  Expect: 0.021
Matches: 20/108 Fragment ions using 41 most intense peaks (help)
SPAG5

MS/MS Fragmentation of TITLE11R
Found in K7ELC8 in UniProt-Human, Sperm-associated antigen 5 (Fragment) OS=Homo sapiens GN=SPAG5 PE=1 SV=1

Match to Query 1787: 830.486664 from(416.250608,2+) index(2516)
Title: jc_012214_Torres_AL2-2.1586.1586.2.dta
Data file C:\Program Files (x86)\Matrix Science\Mascot Daemon\mgf\124 jc-012214-Torres-AL2\mascot_daemon_merge.mgf

Monoisotopic mass of neutral peptide Mr(calc): 830.4862

Ions Score: 29  Expect: 0.0029
Fixed modifications: Carbamidomethyl (C) (apply to specified residues or termini only)

Ions Score: 26  Expect: 0.017

Monoisotopic mass of neutral peptide Mr(calc): 830.4862

Data file C:\Program Files (x86)\Matrix Science\Mascot Daemon\mgf\124 jc-012214-Torres-AL2\mascot_daemon_merge.mgf
Title: jc_012214_Torres_AL2-2.3167.3167.2.dta

Found in MS/MS Fragmentation of
Kif26B

MS/MS Fragmentation of AALPPDAALASR
Found in K7ELC8 in UniProt-Human, Kinesin-like protein KIF26B OS=Homo sapiens GN=KIF26B PE=2 SV=1

Match to Query 8064: 1251.664034 from(626.839293,2+) index(3856)
Title: jc_012214_Torres_AL2-2.3167.3167.2.dta
Data file C:\Program Files (x86)\Matrix Science\Mascot Daemon\mgf\124 jc-012214-Torres-AL2\mascot_daemon_merge.mgf

Monoisotopic mass of neutral peptide Mr(calc): 1251.6642

Fixed modifications: Carbamidomethyl (C) (apply to specified residues or termini only)

Monoisotopic mass of neutral peptide Mr(calc): 1251.6642

Data file C:\Program Files (x86)\Matrix Science\Mascot Daemon\mgf\124 jc-012214-Torres-AL2\mascot_daemon_merge.mgf
Title: jc_012214_Torres_AL2-2.1586.1586.2.dta

Found in MS/MS Fragmentation of
KATNAL2

DIAPH3

MS/MS Fragmentation of LVTCLSLELR
Found in Q96SV4 in UniProt-Human, Protein diaphanous homolog 3 OS=Homo sapiens GN=DIAPH3 PE=1 SV=4
Match to Query 5446: 1089.585794 from(545.800173,2+) index(2963)
Title: jc-012214_Torres_AL2-2.2082.2082.2.dta
Data file C:\Program Files (x86)\Matrix Science\Mascot Daemon\mgf\124 jc-012214-Torres-AL2\mascot_daemon_merge.mgf
Monoisotopic mass of neutral peptide Mr(calc): 1089.5852
Fixed modifications: Carbamidomethyl (C) (apply to specified residues or termini only)
Variable modifications: Oxidation (M)  (apply to specified residues or termini only)
Data file C:\Program Files (x86)\Matrix Science\Mascot Daemon\mgf\124 jc-012214-Torres-AL2\mascot_daemon_merge.mgf
Title: jc_012214_Torres_AL2-6.1003.1003.2.dta
Match to Query 6442: 1154.606054 from(578.310303,2+) index(11874)
Match to Query 6442: 1154.606054 from(578.310303,2+) index(11874)
Match to Query 6442: 1154.606054 from(578.310303,2+) index(11874)
Match to Query 6442: 1154.606054 from(578.310303,2+) index(11874)
Found in MS/MS Fragmentation of DIAPH3

KATNA1

MS/MS Fragmentation of DIISQNPVIR
Found in B7ZBC8 in UniProt-Human, Katnatin p60 ATPase-containing subunit A1 (Fragment) OS=Homo sapiens GN=KATNA1 PE=1 SV=1
Match to Query 6442: 1154.600054 from(579.310303,2+) index(11874)
Title: jc-012214_Torres_AL2-6.1003.1003.2.dta
Data file C:\Program Files (x86)\Matrix Science\Mascot Daemon\mgf\124 jc-012214-Torres-AL2\mascot_daemon_merge.mgf
Monoisotopic mass of neutral peptide Mr(calc): 1154.6044
Fixed modifications: Carbamidomethyl (C) (apply to specified residues or termini only)
Variable modifications: Oxidation (M)  (apply to specified residues or termini only)
Data file C:\Program Files (x86)\Matrix Science\Mascot Daemon\mgf\124 jc-012214-Torres-AL2\mascot_daemon_merge.mgf
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Found in MS/MS Fragmentation of KATNA1
KATNAL2

CDKSAP2

MS/MS Fragmentation of SITTSSTLQSR
Found in: A0A046M629 in UniProt-Human, CDKSAP2 regulatory subunit-associated protein 2 Osl-Homo sapiens GN=CDKSAP2 PH=1 SV=1
Match to Query 7263: 1207.605804 from(604.810178,2+) index(4673)
Title: jc_012214_Torres_AL2-6.2841.2841.2.dta
Data file C:\Program Files (x86)\Matrix Science\Mascot Daemon\mgf\124 jc-012214-Torres-AL2\mascot_daemon_merge.mgf
Monoisotopic mass of neutral peptide Mr(calc): 1754.9778

Found in: A0A046M629 in UniProt-Human, Mitotic spindle assembly checkpoint protein MAD2A Osl-Homo sapiens GN=MAD2L1 PE=1 SV=1
Match to Query 7263: 1207.605804 from(604.810178,2+) index(4673)
Title: jc_012214_Torres_AL2-2.772.772.2.dta
Data file C:\Program Files (x86)\Matrix Science\Mascot Daemon\mgf\124 jc-012214-Torres-AL2\mascot_daemon_merge.mgf
Monoisotopic mass of neutral peptide Mr(calc): 1207.6044

MAADL1

MS/MS Fragmentation of LJVVDNHSVGEVLER
Found in: Q12175 in UniProt-Human, Mitotic spindle assembly checkpoint protein MAD2A Osl-Homo sapiens GN=MAD2L1 PH=1 SV=1
Match to Query 12457: 1754.980044 from(878.497678,2+) index(15202)
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Data file C:\Program Files (x86)\Matrix Science\Mascot Daemon\mgf\124 jc-012214-Torres-AL2\mascot_daemon_merge.mgf
Monoisotopic mass of neutral peptide Mr(calc): 1754.9773

Found in: Q12175 in UniProt-Human, Mitotic spindle assembly checkpoint protein MAD2A Osl-Homo sapiens GN=MAD2L1 PH=1 SV=1
Match to Query 12457: 1754.980044 from(878.497678,2+) index(15202)
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Monoisotopic mass of neutral peptide Mr(calc): 1754.9773

64
### MS/MS Fragmentation of TTDFSDFLSIVGCTK

**Found in Q9UHD1 in UniProt-Human, Cysteine and histidine-rich domain-containing protein 1 OS=Homo sapiens GN=CHORDC1 PE=1 SV=2**

**Match to Query 12126: 1689.795264 from(845.904908,2+) index(4107)**

**Data file C:\Program Files (x86)\Matrix Science\Mascot Daemon\mgf\124 jc-01224-Torres-AL2\mascot_daemon_merge.mgf**

**Monoisotopic mass of neutral peptide Mr(calc): 1689.7920**

**Fixed modifications: Carbamidomethyl (C) (apply to specified residues or termini only)**

**Ions Score: 100  Expect: 2.1e-09**

**Matches : 27/138  fragment ions using 28 most intense peaks (help)**

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</table>

**Note:** The table shows a list of fragment ions that were matched to the query sequence. Each row represents a fragment ion, with columns for the fragment type (b or y), charge state (q), and mass values. The ions were identified using Mascot software with a Monoisotopic mass and fixed modifications. The data file used for this analysis is located at C:\Program Files (x86)\Matrix Science\Mascot Daemon\mgf\124 jc-01224-Torres-AL2\mascot_daemon_merge.mgf.

**Extractions:**

- MS/MS Fragmentation of TTDFSDFLSIVGCTK
- Match to Query 12126: 1689.795264 from(845.904908,2+) index(4107)
- Data file C:\Program Files (x86)\Matrix Science\Mascot Daemon\mgf\124 jc-01224-Torres-AL2\mascot_daemon_merge.mgf

**Fixed modifications:** Carbamidomethyl (C) (apply to specified residues or termini only)

**Ions Score:** 100  **Expect:** 2.1e-09

**Matches : 27/138  fragment ions using 28 most intense peaks (help)**
### KATNB1

**MS/MS Fragmentation of MLQQQEQLR**

Found in UTES in UniProt-Human. Pericentriolar material 1 protein OS=Homo sapiens GN=PCM1 PE=1 SV=1

Match to Query 9125: 1188.9904 from(593.6823,2+) index(1)

Title: jc_07111414_Torres_KATNB1-1.1001.1001.2.dta

Data file C:\Program Files (x86)\Matrix Science\Mascot Daemon\mgf\29 071114-jc-Torres-KATNB1\mascot_daemon_merge.mgf

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<th>Matches: 32/216 fragment ions using 49 most intense peaks</th>
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<td>Oxidation (M), with neutral losses 0.0000(shown in table), 63.9983</td>
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### LMNB1

**MS/MS Fragmentation of LSSEMTSTVNSAR**

Found in F20708 in UniProt-Human. Lomb-1 OS=Homo sapiens GN=LMNB1 PE=1 SV=2

Match to Query 12905: 1511.700294 from(756.857423,2+) index(10517)

Title: jc_07111414_Torres_LMNB1-1.1001.1001.2.dta

Data file C:\Program Files (x86)\Matrix Science\Mascot Daemon\mgf\29 071114-jc-Torres-LMNB1\mascot_daemon_merge.mgf

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### Fragmentation Data

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### Conclusion

The fragmentation data for KATNB1 and LMNB1 show significant matches with the theoretical masses, indicating successful identification of these proteins.
Table S2 - Spectra of one peptide IDs in the Katan-ome.

Spectra of one peptide IDs in the LAP-KATNA1, KATNAL1, KATNAL2, KATNB1 and KATNBL1 mass spectrometry analyses from mitotic cells.
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**Table S3 - List of primers used to mutate KATNB1.**

List of primers used to generate KATNB1 nuclear localization sequence mutants and truncation mutants.
References/Bibliography


microtubule instability around the midbody and facilitates cytokinesis in rat cells. *PLoS One* **8**, e80392


