THE CLASP FAMILY REGULATES MICROTUBULE DYNAMICS BY USING AN ARRAY OF TOG-LIKE DOMAINS

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ABSTRACT

Jonathan B. Leano: The CLASP family regulates microtubule dynamics by using an array of TOG-like domains (Under the direction of Dr. Kevin C. Slep)

CLASP is a key regulator of microtubule (MT) dynamics and bipolar mitotic spindle formation, with mutants displaying chromosome aggregation, aberrant monopolar spindle morphologies, and aneuploidy. How CLASP binds the microtubule lattice to regulate MT dynamics and facilitate proper spindle assembly remains unknown; however, it has been postulated that cryptic TOG domains underlie CLASP's ability to regulate MT dynamics. In this work, we report the crystal structure of the first cryptic TOG domain (TOG2) from human CLASP1, confirming the presence of a TOG array in CLASP. CLASP1 TOG2 displays a bent architecture at the tubulin-binding surface that contrasts with the flat tubulinbinding surface from XMAP215 family TOG domains. Mutating key tubulin-binding determinants along the tubulin-binding surface of TOG2 abrogated the ability of CLASP to 1) rescue mitotic bipolar spindle formation in Drosophila S2 cells 2) associate CLASP with the MT lattice, and 3) promote in vitro MT polymerization. These findings highlight the mechanistic use of a cryptic TOG domain in CLASP to facilitate bipolar spindle formation and MT polymerization. Determining the crystal structure of TOG1 and the second cryptic TOG-like domain (TOG3) is ongoing. Structural characterization of CLASP's array of TOG domains we shows that differential TOG domain architecture confers distinct functions for each TOG domain including MT lattice association, MT polymerization, and MT

stabilization. In addition, CLASP's C-terminal domain (CTD) associates with the coiled-coil regions of various associating factors to recruit CLASP at specific cellular locations and is also a necessary component for CLASP dimerization. To determine the role of CLASP CTD in promoting dimerization and interacting with known CLASP-associating factors, we are structurally and biochemically characterizing the interaction between CLASP CTD and the coiled-coil (CC) domain of CLIP-170, a known CLASP-associating factor. CLASP CTD and CLIP-170 CC form a complex in SEC-MALS and ITC experiments. In addition, CLASP CTD alone exists as a monomer, suggesting that CLASP CTD is necessary, but not sufficient, for dimerization. Further analysis to structurally characterize the interaction between CLASP CTD and CLIP-170 CC is an ongoing goal for this thesis work.

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PREFACE

The work in Chapter 2 is a manuscript that was published in the journal, Structure, in 2013, under the direction of Dr. Kevin Slep and with the support of Dr. Stephen Rogers' reagents, equipment, and advice. This work focused on characterizing CLASP1 TOG2 using structural, biochemical, and cellular studies. Kevin Slep crystallized TOG2 and collected diffraction data, while I built the model and refined the structure. After the work was published, I solved another crystal structure of TOG2 at resolution of 1.8Å. The citation to the manuscript is: Leano, J.B., Rogers, S., Slep, K.C. (2013). A Cryptic TOG Domain with a Distinct Architecture Underlies CLASP-Dependent Bipolar Spindle Formation. *Structure*. 21(6):939-50.

The work in Chapter 3 is an ongoing project attempting to crystallize TOG1 (CLASP1 1-257) and TOG3 (843-1092). TOG1 crystals diffract to 2.0Å, but failed attempts in SAD phasing and molecular replacement has prevented us from obtaining a crystal structure. TOG3 crystals have been obtained and will be screened for higher resolution diffraction in future studies. Trent Wei, an undergraduate student I mentored for two years, designed constructs for TOG3 and performed initial expression and purification of these constructs.

The work in Chapter 4 is also an ongoing project characterizing the interaction between CLASP C-terminal and CLIP-170 coiled coil domain using structural and biochemical studies. Trent Wei designed several constructs of CLASP C-terminal and CLIP-

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170 coiled coil domain. After screening these constructs for binding, CLASP 1253-1522 and CLIP-170 331-456 were used for biochemical studies and crystallization. CLASP 1253-1522 and CLIP-170 331-456 formed a complex under SEC-MALS and ITC experiments. Crystals of these proteins alone and in complex with each other were obtained, but did not diffract or diffracted to low-resolution.

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LIST OF ABBREVIATIONS and symbols

Å	Angstrom
β-ΜΕ	beta-mercaptoethanol
Br	bromide
CaCl ₂	calcium chloride
CD	circular dichroism
CENP-E	Centromere-associated protein E
ch-TOG	colonic and hepatic tumor-overexpressed gene
CLASP	CLIP170 associated protein
CLIP170	cytoplasmic linker protein 170
Cu(II)Cl ₂	copper(II) chloride
CuSO ₄	copper sulfate
Da	Dalton
DARPin	designed ankryin repeat protein
DMSO	dimethyl sulfoxide
dsRNA	double-stranded RNA
DTACC	Drosophila transforming acidic coiled-coil
DTT	dithiothreitol
EB1	end binding protein 1
eGFP	enhanced green fluorescent protein
FBS	fetal bovine serum
GCC185	Grip and Coiled-coil domain containing protein 185kDa
GDP	guanosine diphosphate

GFP	green fluorescent protein
GMPCPP	guanosine-5'-[(α , β)-methyleno]triphosphate
GTP	guanosine triphosphate
HC1	hydrochloric acid
HEAT	Huntingtin, elongation factor 3, protein phosphatase 2A, target of rapamycin 1
Hg	mercury
HR	HEAT repeat
IPTG	isopropyl β-D-1-thiogalactopyranoside
KCl	potassium chloride
kDa	kilo Dalton
kMTs	kinetochore microtubules
КОН	potassium hydroxide
L	liter v. leucine
LL5β	Pleckstrin homology-like domain family member 2
М	molar
MAP	microtubule associated protein
mg	milligram
MgCl ₂	magnesium chloride
min	minute
ml	milliliter
ML	maximum-likelihood
MLHL	phased maximum-likelihood
mM	millimolar

MPa	megapascal
Msps	Minispindles
МТ	microtubule
MWCO	molecular weigh cut off
NaCl	sodium chloride
NaN ₃	sodium azide
Ni ²⁺ -NTA	nickel nitrilotriacetic acid
nm	nanometer
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffer saline
PEG	polyethylene glycol
PIP ₃	phosphatidylinositol (3,4,5)-trisphosphate
rmsd	root-mean-square deviation
res	residues
RNAi	RNA interference
rpm	rotations per minute
SAD	single-wavelength anomalous diffraction
SASA	solvent accessible surface area
SDS	sodium dodecyl sulfate
SEC-MALS	size exclusion chromatography coupled with multi-angle light scattering
SeMet	selenomethionine
TACC	transforming acidic coiled-coil
TOG	tumor-overexpressed gene

μg	microgram
μl	microliter
μΜ	micromolar
μm	micron
UTR	untranslated region
UV	ultraviolet
WT	wild type
Xkid	Xenopus kinesin-like protein kif22a
XMAP215	Xenopus microtubule associated protein 215
+TIP	microtubule plus-end tracking protein

CHAPTER 1: INTRODUCTION

Microtubules are dynamic and physically robust polymers that are essential for a host of cellular processes

Microtubules (MTs) are dynamic components of the cellular cytoskeleton that facilitate cell-shape change, cell migration, intracellular transport, and mitotic spindle formation. (Desai and Mitchison, 1997). Microtubules are tubular polymers composed of heterodimers of α - and β -tubulin (55 kDa each) (Figure 1-1A). These $\alpha\beta$ -tubulin heterodimers polymerize in a head-to-tail fashion to form the protofilament (Figure 1-1B), and approximately thirteen protofilaments associate laterally to form the microtubule (Figure 1-1C) (Mandelkow and Mandelkow, 1989) (Akhmanova and Steinmetz, 2008). The αβtubulin heterodimer is made up of two different 55-kDa proteins that assemble into a specific, asymmetric orientation. As a consequence of this asymmetry, $\alpha\beta$ -tubulin is intrinsically polar. This polarity is propagated along the microtubule lattice to form microtubules ends with two different polymerization rates. The microtubule end that consists of the exposed β -tubulin is the fast-growing "plus" end while α -tubulin is oriented towards the slow-growing "minus" end (Figure 1-1C) (Allen and Borisy, 1974) (Desai and Mitchinson, 1997). In eukaryotic cells, the minus end is tethered to microtubule-organizing centers (MTOC) such as the centrosome, while the plus end explores cytoplasmic space and can extend towards the cell cortex (Wiese and Zhang, 2000).



Figure 1-1. Microtubules are polymers of \alpha\beta-tubulin. (A) Cartoon representation of the crystal structure of $\alpha\beta$ -tubulin (1JFF). (B) Tubulin heterodimers interact in a head-to-tail fashion to form the protofilament. (C) Thirteen protofilaments interact laterally to form the hollow, tubular structure of the microtubule. The asymmetric orientation of $\alpha\beta$ -tubulin gives microtubules its inherent polarity. The end that comprises of exposed α -tubulin is referred to as the "minus-end" while the end that comprises of exposed β -tubulin is referred to as the "plus-end." Figure adapted from (Akhmanova and Steinmetz, 2008).

The most essential and fundamental property of microtubules is dynamic instability, the ability of microtubules to stochastically switch between states of growth [polymerization], pause, and shrinkage [depolymerization] (Mitchison and Kirschner, 1984) (Figure 1-2). Cryo-EM images that capture growing microtubules display a sheet of MT protofilaments forming at MT ends (Figure 1-2A), while shrinking microtubules display frayed ends (Figure 1-2B) (Simon and Salmon, 1990) (Mandelkow et al., 1991). Events that characterize abrupt, stochastic transitions between states of growth and shrinkage are called rescue and catastrophe (Figure 1-2). The transition from growth to rapid shrinkage is referred to as "catastrophe," while the transition from shrinkage to a state of growth is called "rescue" (Walker et al., 1988). To transition between states of growth and shrinkage, the microtubule goes through a kinetically metastable intermediate state called the "pause" state, in which the microtubule is neither growing nor shrinking (Tran et al., 1997). Mechanical and UVmicrobeam severing of self-assembled microtubules and axoneme-nucleated microtubules produces stable MT ends that do not undergo states of polymerization and depolymerization (Walker et al., 1989) (Tran et al., 1997). Cryo-electron microscopy (Cryo-EM) images that captured different populations of dynamic microtubules *in vitro* showed a large population of blunt MT ends along with MT sheets and frayed ends. These blunt MT ends suggest they are occupying a metastable intermediate "pause" state between growing and shrinking microtubules (Figure 1-2C) (Simon and Salmon, 1990) (Mandelkow et al., 1991) In addition, the blunt MT ends can switch to phases of growth or shortening, suggesting that dynamic MTs must occupy the pause state to undergo rescue or catastrophe (Arnal et al., 2000).

The ability of $\alpha\beta$ -tubulin to bind, hydrolyze, and exchange guanine nucleotide states underlies MT dynamic instability. The $\alpha\beta$ -tubulin heterodimer has two guanine nucleotide binding sites: 1) non-exchangable site (N-site), located at the interface between α - tubulin and β -tubulin, always has GTP present, and 2) nucleotide exchange site (E-site), located at the exposed surface of β -tubulin, binds GTP, hydrolyzes GTP, and exchanges GDP to GTP (Figure 1-3) (Nogales et al., 1998) (Heald and Nogales, 2002). Tubulin bound to GTP at the E-site (GTP-tubulin) adds to pre-existing protofilament sheets on growing MT plus ends



Figure 1-2. Overview of microtubule dynamics. (A) GTP-tubulin (red and green spheres) adds to pre-existing sheets of protofilaments on growing microtubules. The transition from shrinking to growing microtubules is referred to as rescue. (B) GDP-tubulin (purple and green spheres) adopts a "curved" conformation, resulting in frayed ends that promote shrinking microtubules. The transition from growing to shrinking microtubules is referred to as catastrophe. (C) The blunt-ended MT is displaying the pause state, in which the MT is neither growing nor shrinking. Eventually, the paused MT will undergo growing or shrinking phases. Figure adapted from (Akhmanova and Steinmetz, 2008).



Figure 1-3. $\alpha\beta$ -tubulin binds guanine nucleotides at two sites. Cartoon representation of the crystal structure of $\alpha\beta$ -tubulin (1JFF). Sphere representation of GDP molecule that occupies the nucleotide exchange site (E-site), which is located on β -tubulin. Sphere representation of GTP molecule that occupies the non-exchangeable site (N-site), which is located on α -tubulin.

(Figure 1-2A) (Simon and Salmon, 1990; Akhmanova and Steinmetz, 2008). Tubulin hydrolyzes GTP to GDP at the E-site during or shortly after polymerization (Hyman et al., 1992). Consequently, GDP-bound tubulin (GDP-tubulin) comprises most of the microtubule lattice (Figure 1-1C and 1-2). However, GTP hydrolysis promotes tubulin to adopt a curved conformation that disrupts lateral contacts between protofilaments (Müller-Reichert et al., 1998). This causes the protofilaments to "peel" away from the MT lattice, creating frayed ends (Figure 1-2B). These frayed ends will disassemble to into protofilament rings or nonpolymerized tubulin subunits (Mandelkow et al., 1991). Lateral and longitudinal contacts between GDP-tubulin within the MT lattice force GDP-tubulin to remain in a "straight" conformation. This constraint builds tension, causing protofilaments to peel away and promote MT depolymerziation. To prevent microtubule depolymerization, studies have proposed that a thin layer of GTP-bound tubulin (GTP cap) is sufficient to stabilize the microtubule plus end and facilitate polymerization (Figure 1-2A) (Mitchison and Kirschner, 1984) (Voter et al., 1990) (Walker et al., 1991). Similar studies involving the addition of tubulin bound to GMCPMP, a non-hydrolyzable analog of GTP, forms a GMCPMP cap that is one or two tubulin heterodimers deep (longitudinally) and is sufficient to prevent depolymerization (Drechsel et al., 1994) (Caplow et al., 1996).

As mentioned previously, microtubule assembly involves converting tubulin between curved and straight conformations. Structural studies observing both non-polymerized tubulin and polymerized microtubules reveal insights into straight-bent conformational changes. Electron crystallography structures of zinc-induced sheets of tubulin bound to taxol reveals tubulin protofilaments in a straight conformation, which mimics the state of tubulin found in microtubules (Figure 1-4A) (Nogales, et al., 1998) (Löew et al., 2001) (Nettles et al., 2004). The structures of incorporated MTs contain only GDP-tubulin. In contrast, X-ray crystallography structures of non-polymerized tubulin bound to depolymerizing factors such as the stathmin homologue RB3 (Ravelli, et al., 2004) and a designed ankryin repeat protein (DARPin) (Figure 1-4 B and C) (Pecqueur et al., 2012), show the tubulin heterodimers in a curved conformation. In these structures, either GDP (Figure 1-4B) or GTP (Figure 1-4C) was bound to β-tubulin indicating that non-polymerized tubulin adopts a curved conformation despite binding to different guanine nucleotides. This suggests that the curved conformation is the energetically favorable state for non-polymerized microtubules. Tubulin



Figure 1-4. Structures of $\alpha\beta$ -tubulin are observed in different conformations. (A) Crystal structure of $\alpha\beta$ -tubulin (1JFF) in a "straight" conformation that mimics tubulin incorporated into the microtubule lattice. Sphere representation of a GDP molecule occupies the E-site located on β -tubulin. (B) Crystal structure of $\alpha\beta$ -tubulin (4FFB) captured in a "curved" conformation by a DARPin molecule (not shown). GDP molecule occupies the E-site in a curved tubulin, which emulates the depolymerizing state of tubulin. (C) Crystal structure of $\alpha\beta$ -tubulin (4DRX) also captured in a "curved" conformation by a DARPin molecule (not shown), but containing GTP molecule at the E-site, which emulates a state of tubulin that is primed for polymerization.

only changes from a curved to straight conformation during MT assembly when lateral

interactions between tubulin heterodimers force tubulin to adopt the straight conformation

(Peng et al., 2014). These structures reveal that tubulin adopts different conformations

depending on the state of MT assembly/disassembly.

Microtubule associated proteins (MAPs) and plus-end tracking proteins (+TIPs) regulate MT dynamics

Most of the research on microtubule structure and dynamics explained above were characterized through *in vitro* experiments that reconstituted microtubule dynamics using purified or isolated tubulin, guanine nucleotides, and microtubule-binding proteins. While microtubules are inherently dynamic *in vitro*, microtubule-associated proteins (MAPs) are a diverse family of proteins that regulate intracellular microtubule dynamics to ensure proper assembly and disassembly of microtubule at specific locations within the cell and precise times during the cell cycle (Amos and Schliper, 2005). Their ability to regulate microtubule dynamics in accordance with temporal and spatial cues controls fundamental processes such as mitosis, cell shape and differentiation, and intracellular trafficking. Defects in MAPs have been implicated in pathological conditions including cancer, cardiovascular, and neurodegenerative diseases (Akhmanova and Steinmetz, 2008). MAPs associate with nonpolyermized tubulin, the microtubule minus end, the microtubule lattice, and the microtubule plus end (Halpain and Dehmelt) (Akhmanova, Steinmetz, 2008) to alter rates of polymerization, depolymerization, and pause.

Although MAPs can associate with the minus end and plus end of microtubules, classical MAPs bind along the microtubule lattice and non-polyermized tubulin to stabilize or destabilize microtubules (Halpain and Dehmelt, 2006). MAPs that stabilize microtubules are referred to as structural MAPs and include doublecortin and the Tau/MAP2 family of proteins. Structural MAPs usually have repeating domains that enables one MAP molecule to bind multiple tubulin subunits. Doublecortin is a structural MAP that is required for neuronal migration into the cerebral cortex (Glesson et al., 1999). Doublecortin contains two tandem

microtubule-binding repeats (DCX1/R1 and DCX2/R2) that bind and stabilize microtubules (Figure 1-5A) (Taylor, K.R., et al., 2000). Mutations of doublecortin result in misguided neuronal migration, leading to brain developmental disorders, including the formation of a "double cortex." (Amos and Schlieper 2005). One of the most well characterized structural MAPs is the Tau/MAP2 family of microtubule stabilizers. Tau is an important stabilizer of axonal microtubules and is primarily expressed in the nervous system. Tau binds microtubules through short microtubule-binding motifs that repeat two or four times depending on the isoform (Figure 1-5B) (reviewed by Butner and Kirschner, 1991). Hyperphosphorylation of Tau detaches Tau from microtubules, resulting in a loss of microtubule stability and eventually leads to the aggregation of Tau into neurofibrillary tangles (NFTs). These tau-containing NFTs are one of the hallmarks for Alzheimer's disease (Mandelkow and Mandelkow, 1998). The multiple microtubule-binding repeats that comprises these structural MAPs enables it to decorate the microtubule lattice and crosslink tubulin subunits along the microtubule, providing overall microtubule stability and promoting microtubule growth.

In opposition to structural MAPs, microtubule destabilizers reduce the rate of microtubule assembly, sever microtubules, and promote microtubule disassembly. This class of destabilizing MAPs includes katanin and Stahmin/Op18. Katanin is a member of the AAA (ATPase associated with different cellular activities) superfamily. It directly binds to the microtubule lattice and uses ATPase activity to break contacts between tubulin subunits, resulting in the severing of microtubules into smaller pieces or the rapid disassembly of microtubules (Figure 1-5C) (Quarmby, 2000) (Vale, 2000). Stathmin/Oncoprotein18 belongs to a family of phosphoproteins that promotes microtubule depolymerization when activated



Figure 1-5. MAPs bind along the microtubules lattice to alter microtubule dynamics. Various MAPs (microtubule-associated proteins) bind to microtubule using a microtubule-binding domains or repeats (purple boxes). (A) Doublecortin uses two tandem microtubule-binding repeats (DCX1 and DCX2) and a serine-proline rich region (S/P rich) (red box) to bind and stabilize microtubules. (B) Tau uses 2-4 short microtubule-binding repeats (R1-4) and a basic proline-rich region (red box) to bind microtubules. Tau contains an N-terminal projection domain that extends from the outer surface of the microtubule when Tau is bound to microtubules. (C) The Katanin p60 subunit is a 60kDa ATPase subunit that binds microtubule along its microtubule-binding domain, in which it severs microtubules using its AAA ATPase domain (green box). Katanin's C-terminal domain (brown box) is used to oligomerize with the regulatory Katanin p80 subunit. (D) RB3/Stathmin 4 sequesters tubulin subunits using its stathmin-like domain (SLD) which contains a coiled-coil region.

through phosphorylation (Marklund et al., 1996). Stathmin overexpression is found in many malignant cancers including breast, lung, and ovarian cancer (Bièche, et al., 1998; Chen et al., 2003; Price et al., 2000). Stathmin sequesters free tubulin through a stathmin-like domain (SLD) domain, preventing microtubule incorporation and thereby inhibiting microtubule polymerization (Figure 1-5D). Biochemical studies reveal that one molecule of stathmin tightly binds two tubulin heterodimers to form a T₂S complex (Jourdain et al. 1997). Crystal structures of the T₂S complex showed a long alpha-helical domain of stathmin interacting with a pair of tubulin heterodimers and capping the α -tubulin end, preventing longitudinal contacts with another tubulin heterodimer (Gigant et al., 2000) (Ravelli et al. 2004). Microtubule destabilizers such as katanin, and stathmin facilitate the rearrangement of the microtubule cytoskeleton in accordance with temporal and spatial cues. In summary, classical MAPs including microtubule stabilizers (structural MAPs) and destabilizers coordinate with each other to maintain the critical balance between microtubule polymerization/depolymerization and to alter the microtubule cytoskeleton in accordance with temporal and spatial cues.

Plus-end tracking proteins (+TIPs) are a diverse group of specialized MAPs that accumulate at plus ends to modulate MT dynamics (Schuyler and Pellman, 2001). +TIPs were initially discovered by time-lapse video microscopy of green fluorescent protein (GFP) fusion construct of CLIP-170 displaying "comet-like" trajectories that coincided with the growing tips of microtubules (Perez, et al. 1999). Additional +TIPs have since been discovered, all varying in their ability to modulate MT dynamics. Cytoplasmic linker proteins (CLIP) promote stability and stimulate rescue. CLIP-associated proteins (CLASP) promote the pause state, stabilize microtubules, and prevent MT depolymerization (Sousa et al.,

2007). The colonic and hepatic tumor overexpressed gene (ch-TOG) protein belongs to a family of proteins that include *Xenopus* member XMAP215 and *Drosophila* member minispindles, which functions as microtubule polymerases to enhance the rate of microtubule growth (Brouhard et al., 2008). End-binding proteins (EB) promote microtubule growth and suppresses catastrophe. +TIPs, including the ones mentioned previously, function in a vast network to accumulate at the MT plus end and alter its dynamics.

EB1 and its family members act as the central hub and master regulator for the +TIP network. EB1 autonomously tracks growing microtubules ends through its recognition of a specific tubulin structural state at the growing plus end (Bieling et al., 2008). EB1 uses conserved surface residues on the N-terminal Calponin Homology (CH) domain of EB1 to form the hydrophobic and electrostatic interactions needed bind to microtubules (Slep et al., 2007) (Hayashi et al., 2003). The C-terminal domain contains a coiled-coil EB-homology (EBH) domain that forms a four-helix bundle followed by a short disordered tail and contains a hydrophobic cavity that interacts with other various +TIPs (Figure 1-6A) (Honnappa et al., 2005; Slep et al., 2005; Buey et al., 2011). +TIPs that contain a SxIP-motif or a cytoskeletonassociated protein-glycine-rich (CAP-Gly) domain can bind the EBH domain to interact with EB1 (Figure 1-6B and C). The SxIP (x denotes any amino acid) motif is a short polypeptide that acts as broad a microtubule localization signal (MtLS) across diverse +TIPs, including CLASP (Honnappa et al., 2009; Buey et al., 2012). SxIP motifs (x denotes any amino acid) are usually located on intrinsically disordered regions enriched with serine, proline, and basic regions, including CLASP (Figure 1-6B). The SxIP motif forms extensive contacts with the hydrophobic cavity and the disordered C-terminal tail (Honnappa et al., 2009). To date, over fifty SxIP motif-containing proteins have been identified; one of these proteins, CLASP, is



Figure 1-6. Plus-end tracking proteins (+TIPs) interact in a network to modulate MT plus-end dynamics. Different types of +TIPs interact with the MT plus-end. (A) Endbinding protein 1 (EB1) can automatously track growing microtubule plus-ends (red arrow). EB1 contains an N-terminal calponin homology domain (orange circle) to interact with microtubules, a coiled-coil region (light blue), an EB homology domain (yellow box), and an EEY/F-like motif at its C-terminus. (B) CLASP's SxIP motif (vertical black line), which is located within a basic serine-rich linker region, directly binds with the EB homology domain on EB1 (green arrow). (C) CLIP-170's directly binds EB1 using CLIP-170's CAP-gly domains (purple boxes) to interact with EEY/F-like motifs (red boxes) on EB1 (blue arrow). CLIP-170 contains a coiled-coil domain (light blue) and a EEY/F-like motifs at its C-terminus, similar to that of EB1. (Figure adapted from van der Vaart et al., 2011)

a focus of this thesis. Another interacting mode of EB1 is the CAP-Gly domain, which is characterized by highly conserved glycine and hydrophobic residues. +TIPs, including CLIP-170, may contain single or multiple copies of CAP-Gly domains (Akhmanova and Steinmetz, 2008). CAP-Gly domains contain a conserved GKNDG motif that interacts with the EEYmotif located at the C-terminal tail of the EBH domain (Figure 1-6C) (Weisbrich et al., 2007). Although the ch-TOG family does not contain either a SxIP motif or a CAP-Gly domain, the SxIP-motif-containing protein SLAIN2 binds both ch-TOG and EB1, thereby conferring ch-TOG plus end localization. SLAIN2 is required for ch-TOG-dependent persistent MT growth (van der Vaart et al., 2011) (Kumar and Wittman, 2012). Because EB1 is a critical microtubule regulator and the central hub of the +TIP network, loss-of-function mutations or depletion in EB1 results in a decrease of microtubule dynamics.

MAPs and +TIPs play important roles in the formation and maintenance of the mitotic bipolar spindle

During the onset of mitosis, destabilizing MAPs and +TIPs dismantle the inter microtubule array by increasing catastrophe events and suppressing microtubule growth and rescue (Belmont et al., 1990; Rusan et al., 2001; Gadde and Heald, 2004). A combination of MAPs, microtubule-based motor proteins, and +TIPs rearrange the microtubule network to form the bipolar spindle – a molecular machine composed of microtubules that segregate chromosomes into daughter cells (Figure 1-7) (Walczak, 2005; Tamura and Draviam, 2012). The bipolar spindle forms when a pair of centrosomes that were duplicated during S phase spatially separates to form two opposing spindles poles. Microtubules nucleate from these spindle poles to form three different spindle microtubules: 1) kinetochore microtubules, 2)



Figure 1-7. The mitotic bipolar spindle is a microtubule-based machine that segregates chromosomes. A simplified model of the bipolar spindle during metaphase. Microtubules nucleate from two spindle poles (red circle) located on opposite sides. Astral microtubules (light green lines) extend towards the cell cortex. Interpolar microtubules (medium green lines) grow towards the central region of the spindle and connect with other interpolar MTs nucleated from the opposite spindle pole. Dozens of kinetochore microtubules (dark green lines) attach to the kinetochore (small orange circles) located on chromosomes (blue) and bundle with each other to form the kinetochore fiber.

astral microtubules, and 3) interpolar microtubules. Kinetochore microtubules (kMTs) attach

to specialized protein structures on the chromosomes, called kinetochores. Astral

microtubules grow with their plus ends extending towards the cell cortex. Interpolar

microtubules grow towards the central region of the spindle and interconnect with

microtubules from the opposite spindle pole (Figure 1-7) (Glotzer, 2009).

Dozens of kMTs bundle to form the physically robust, kinetochore fiber. The forces

required for chromosome segregation are generated from the kinetochore fibers (Kitamura et

al., 2009). Poleward microtubule flux is one of the major forces that powers chromosomal motion during mitosis. This force occurs when microtubules polymerize at the kinetochore, the kMT lattice continuously moves towards the spindle pole, and tubulin subunits are removed at the spindle pole (Figure 1-8) (Margolis et al., 1978). Consequently, controlled regulation in balancing polymerization/depolymerization rates of the fluxing kMTs at the kinetochore and spindle pole is required to coordinate the position and movement on chromosomes through mitosis (Buster et al., 2007). During metaphase, the bipolar spindle reaches a steady-state of poleward microtubule flux, in which the rate of polymeration at the kMT plus end is matched by deplymerization at the kMT minus end. This balance is essential in maintaining the proper positioning of the chromosomes at the spindle center or metaphase plate (Mitchison et al., 1989; Rogers et al., 2005; Jaqaman et al., 2010). During anaphase, kMT polymerization stops and poleward MT flux slows to approximately half the metaphase rate (Rogers et al., 2005). This decreases the length of the kinetochore fiber and promotes the proper separation of sister chromatids into daughter cells.

+TIPs are required to attach microtubules to the cellular cortex to control spindle positioning and to attach microtubules to the kinetochore to coordinate chromosome segregation (Maiato et al., 2004; Tamura and Draviam, 2012). EB1 also acts as a major regulator of MT plus end dynamics during mitosis, interacting with the dynamic network of +TIPs to differentially regulate mitotic MT plus ends (Steinmetz and Akhmanova, 2008). Depletion of EB1 using double-stranded RNA interference (RNAi) in *Drosophila* S2 cells produces several defects including astral microtubule reduction, defocused spindle poles, and misalignment of the bipolar spindle, away of the cell center (Rogers et al., 2002). The *Xenopus* member of the ch-TOG family, XMAP215, serves as a microtubule polymerase that



Figure 1-8. Microtubule poleward flux. During microtubule poleward flux kinetochore microtubules continuously grow at the kinetochore (orange ellipse). CLASP (green box) is an essential component of the outer kinetochore that promotes MT polymerize at the kinetochore. At the spindle pole (red circle), kinetochore microtubules continuously depolymerize. The MT growth at the kinetochore coordinated with the MT depolymerize at the spindle pole generates MT poleward flux that is directs towards the spindle pole. Controlling the growth/shrinkage rates of fluxing kinetochore microtubules determines the rate of poleward flux.

increases the rate of microtubule polymerization (Brouhard et al., 2008). XMAP215 and ch-TOG proteins promote MT growth and play an essential role in stabilizing spindle microtubules (Matthews et al. 1998; Tournebize et al. 2000; Popov et al. 2001). The *Drosophila* member of the CLASP family, MAST/Orbit, stabilizes spindle microtubules and incorporates tubulin subunits into the fluxing kinetochore fibers (Maiato et al., 2005). CLASP interacts with EB1 and LL5β to attach and stabilize MT plus end to the cell cortex (Mimori-Kiyosue et al., 2005; Lansbergen et al, 2006). In *C. elegans* embryos, the three *C. elegans* CLASP homologs (CLS-1, CLS-2, CLS-3) function redundantly to regulate astral microtubule attachment to the cell cortex. In a one-cell *C. elegans* embryo, depleting CLS-2 in combination with either CLS-1 and CLS-2 results in defects in nuclear rotation, spindle displacement, and spindle length maintenance (Espiritu et al., 2013). CLIP-170 plays an essential role during early mitosis, facilitating kinetochore-microtubule attachment and chromosome alignment (Tanenbaum et al., 2006; Amin et al., 2014). A diverse network of +TIPs, including EB1, ch-TOG, CLASP, and CLIP-170, interact with one another to form and maintain the mitotic bipolar spindle. Their precise regulation of MT plus end dynamics is required to form microtubule-kinetochore attachments, generate poleward microtubule flux, and maintain correct spindle length and positioning, all required to coordinate the faithful segregation of chromosomes into daughter cells.

ch-TOG promotes MT polymerization through a pentameric array of TOG domains

The ch-TOG (colonic and hepatic tumor overexpressed gene) family is a conserved family of MT polymerases that accelerates MT growth rates (Kinoshita et al., 2002; Brouhard et al., 2008). The ch-TOG gene was identified through the characterization of a cDNA clone amplified from human brain tumor tissue and was found to be overexpressed in colon and hepatic tumor cells, leading to name the gene ch-TOG [colonic and hepatic tumor-overexpressed gene] (Charrasse et al., 1995). The *Xenopus* member, XMAP215 was originally isolated from *X. laevis* egg extract to identify the specialized MAP that could stimulate microtubule polymerization. XMAP215 was identified as a 215kDa protein that accelerates the MT plus end assembly rate eight-fold in extracts compared to MT assembly rates in extracts depeleted of XMAP215 (Gard and Kirschner, 1987). Since its initial discovery, additional members of the XMAP215 family were identified, revealing that this protein family is conserved from yeast to mammals and plants (Kinoshita et al., 2002).
Members of this conserved family of +TIPs and mitotic MT regulators include *S. pombe* Dis1 (fission yeast), *S. cerevisiae* Stu2 (budding yeast), *Drosophila* minispindles , and human ch-TOG (Ohkura et al., 1988; Wang et al., 1997; Cullen et al., 1999).

In addition to regulating microtubule plus end, ch-TOG family members were found to play essential roles in forming and maintaining the mitotic bipolar spindle. Dis1 was discovered as a cold-temperature sensitive mutant that blocks mitotic chromosome separation (Ohkura et al., 1988). Stu2 localizes to spindle pole bodies (SPB), along spindle microtubules and kinetochores (Wang et al., 1997; He et al., 2001). Ch-TOG/XMAP215 promotes MT growth and plays an essential role in stabilizing spindle microtubules (Matthews et al. 1998; Tournebize et al. 2000; Popov et al. 2001). Minispindles (msps) mutants disrupt spindle structural integrity, resulting in small or abnormally organized spindles and short astral MTs (Cullen et al., 1999). Confirming support was produced from a full-genome RNAi screen in Drosophila S2 cells in which msps RNAi resulted in a shortened metaphase spindle phenotype (Goshima et al., 2007). Msps localizes to spindle microtubules and is also highly concentrated at the centrosome (Cullen et al., 1999). Msps interacts with the centrosomal protein D-TACC to stabilize microtubule minus ends growing out of the centrosome/spindle pole (Lee et al., 2001). TACC is a centrosome-associated protein family, conserved across metazoans that uniformly recruit ch-TOG members to the centrosome during mitosis (Lee et al., 2001). The ch-TOG family plays an essential role in promoting microtubule growth and stabilizing spindle microtubules. Depletion or mutation of ch-TOG family members results in blocked chromosome segregation and leads to aberrant phenotypes including shortened metaphase spindle and additional spindles.

The ch-TOG family contains an array of TOG domains. Sequence and secondary



Figure 1-9. TOG domains are tubulin-binding domains. Cartoon representation of Stu2 TOG1 (PDB: 4FFB). TOG (tumor overexpressed gene) domains are comprised of six HEAT (huntingtin, elongation factor 3, the PR65/A subunit of protein phosphatase 2A and the lipid kinase Tor) repeats (HR A-F). The face that is composed of intra-HEAT loops (face containing red-highlighted residues) forms the tubulin-binding surface. Conserved tubulin-determinant determinants (highlighted red) located within the intra-HEAT loops of HR A-E are essential in promoting TOG-tubulin interactions.

structure analysis of ch-TOG revealed an N-terminal repeating unit referred to as TOG domains. These TOG domains are composed of ~200 residues that repeat in an array of five units (Ohkura et al., 2001). Higher eukaryotic members of the XMAP215 family contain an array of five TOG domains, while lower eukaryotic yeast members have two tandem TOG domains and a homodimerization coiled-coil domain (Wang et al., 1997; Spittle et al., 2000; Nakaseko et al., 2001; Slep, 2010).

TOG domains bind tubulin heterodimers and recruit them to MTs (Al-Bassam et al.

2011). TOG domains are composed of 12 helices that consecutively pair into a series of



Figure 1-10. TOG domains interact with \alpha\beta-tubulin. (A) Cartoon representation of Stu2 TOG1 (PDB: 4FFB) (grey-white) in complex with $\alpha\beta$ -tubulin (α -tubulin: light green) (β -tubulin: light blue). (B) Sphere representation of same structure shown on (A). The face composed of intra-HEAT loop regions on Stu2 TOG1 form extensive contacts with $\alpha\beta$ -tubulin. HR A-D forms contacts with β -tubulin, while HR E-F interacts with α -tubulin.

six *H*untingtin, *e*longation factor 3, protein phosphatase 2*A*, *t*arget of rapamycin 1 (HEAT) repeats (HR) (Al-Bassam et al., 2007; Slep and Vale, 2007). HEAT repeats consists of a helix-loop-helix motif and are found in a number of proteins including Huntingtin and protein phosphate 2A to mediate protein-protein interactions (Neuwald and Hirano, 2000). In the case of TOG domains, six HEAT repeats linearly align to form an oblong, paddle-like structure that binds tubulin (Figure 1-9) (Slep et al., 2009). Intra-HEAT loops define one face of the domain and contain conserved tubulin-binding determinants that interact with the $\alpha\beta$ -tubulin heterodimer (Figure 1-10) (Ayaz et al., 2012). These tubulin-binding determinants are a combination of conserved hydrophobic and positively charged residues (Figure 1-9) (Slep et al., 2007). Mutating a conserved solvent-exposed tryptophan on the first intra-HEAT loop ablated the ability of a Msps TOG1-2 construct to interact with $\alpha\beta$ -tubulin over gel filtration

(Slep et al., 2007).

The pentameric TOG domain array underlies the ability of ch-TOG members to promote MT plus end polymerization. One of the proposed mechanisms for MT polymerization is that the TOG domain array serves as a template for multiple tubulin heterodimers to form on the MT plus end (Gard and Kirschner, 1987; Slep et al., 2010). The crystal structure showing Stu2 TOG1 interacting along the side of the tubulin heterodimer that is exposed on the outside of the MT, arranged with the TOG domain's long axis aligned with the MT longitudinal axis, suggest that the array may template tubulin to assemble in a linear fashion, forming a short protofilament (Ayaz et al., 2012).

CLASP is a conserved family of +TIPs that promotes MT stability

Human CLASP was first identified as a CLIP-family-associated protein. It was identified as having two family members, CLASP1 and CLASP2. CLASP1 ubiquitously expresses across human tissue, while CLASP2 primarily expresses in neurons and testes tissue (Akhmanova et al., 2001). CLASP shares homology with two previously identified proteins: *Saccharomyces cerevisiae* Stu1 (a suppressor of a β-tubulin mutation) and *Drosophila* multiple asters (MAST)/orbit/chromosome bows, named after its mutant monopolar spindle phenotypes (Pasqualone and Huffaker, 1994; Lemos et al., 2000; Inoue et al., 2000; Fedorova et al., 1997). Homologous members have since been identified across eukaryotic species, forming the CLASP family of conserved microtubule regulators (Hannak and Heald, 2006; Bratman and Chang, 2007).

In cells, CLASP localizes to the polymerizing MT plus end, classifying CLASP as a plus-end tracking protein. Like other +TIPs, CLASP MT plus end association is EB1-

dependent, mediated through a central EB1-binding SxIP motif (Mimori-Kiyosue et al., 2005). At the MT plus end, CLASP modulates MT dynamics, promoting MT pause, stabilization, and rescue during interphase (Akhmanova et al., 2001; Sousa et al., 2007; Drabek et al., 2006). RNAi depletion of the *Drosophila* homologue MAST/Orbit in *Drosophila* S2 cells resulted in a lower frequency of MT pause events, suggesting that MAST/Orbit promotes the pause state. During mitosis, CLASP localizes to kinetochores and promotes tubulin incorporation into fluxing kinetochore fibers (Maiato et al., 2003, 2005; Cheeseman et al., 2005; Pereira et al., 2006). Inhibiting CLASP function in mitosis, either through RNAi or deletion of CLASP's C-terminal domain, induces spindles to collapse into a monopolar spindle and cause abnormal chromosome congression (Maiato et al., 2002) (Maiato et al., 2003). Overall, CLASP is a key spatial regulator of MT dynamics as well as mitotic spindle structure and dynamics.



Figure 1-11. CLASP and ch-TOG/XMAP215 family uses an array of TOG domain to regulate microtubule dynamics. Ch-TOG/XMAP215 family is a microtubule polymerase that consists of five arrayed TOG domains (purple boxes) and a conserved C-terminal domain (brown box). CLASP is a microtubule stabilizer that consists of an N-terminal TOG domain (green box; TOG1) and C-terminal domain (orange box) that associates with other binding partners. Two central conserved regions are predicted to be cryptic TOG-like domains (green box; crTOG2 and crTOG3), suggesting that CLASP contains an array of TOG domains similar to ch-TOG/XMAP215.

CLASP has a distinct domain architecture compared to other microtubule regulators and plus-end tracking proteins. CLASP was originally annotated as having a conserved Cterminal domain used to bind CLIP-170 as well as an N-terminal TOG domain (Figure 1-11) (Akhmanova et al., 2001). This TOG array works as a multivalent-tubulin binding platform, collectively modulating MT dynamics (Widlund et al., 2011; Ayaz et al., 2012). It was thus surprising that CLASP would only have a single TOG domain, because ch-TOG/XMAP215 activity requires an array. When the structure of ch-TOG/XMAP215 family TOG domains were determined from Drosophila and yeast, the authors noted discontinuous determinants in two conserved central regions of CLASP that bore sequence similarity to TOG domain intra-HEAT loops (Slep and Vale, 2007). Secondary structure predictions of the two central conserved regions in CLASP showed 12 alpha helices that corresponded to the six arrayed HEAT repeats found in previously solved crystal structures of ch-TOG/XMAP215 TOG domains. The authors hypothesized that these two central conserved regions were cryptic TOG-like domains, whose discontinuous tubulin-binding determinants were retained, but the intervening regions had diverged, specifically the composition of the inter-HEAT loops and the lengths and surface-exposed residues of the HEAT-repeat helices. Other HEAT-repeat containing proteins are made up of degenerate HEAT-like sequences, suggesting that they form a structural scaffold that facilitates protein-protein interactions despite diverging in amino acid sequences (Takano and Gusella, 2002). The discontinuous sequence similarity with ch-TOG/XMAP215 TOG domains prevented standard BLAST searches from identifying CLASP's cryptic TOG domains as TOG domains and required structure-based insight. This cryptic TOG domain hypothesis suggested that CLASP, like ch-TOG/XMAP215, might use a TOG array to regulate MT dynamics. In support of this, early

work characterizing the yeast CLASP member Stu1 mapped β-tubulin binding activity to the region encompassing the first predicted cryptic TOG domain (Yin et al., 2002). Subsequent work with *Drosophila*, *Schizosaccharomyces pombe*, and *Xenopus* CLASP members has implicated this region as a key mechanistic determinant in CLASP function (Slep and Vale, 2007; Al-Bassam et al., 2010; Patel et al., 2012).



Figure 1-12. CLASP's C-terminal domain (CTD) interacts with various factors to promote specific functions of CLASP at different cellular locations. CLASP's CTD (red arrow) interacts with coiled-coil regions (blue lines) of known CLASP-associating factors. (A) CLASP interacts with CLIP-170 to regulate MT plus-end dynamics. (B) CLASP interacts with LL5 β to stabilize and attach microtubules to the cellular cortex during interphase. (C) CLASP interacts with GCC185 to nucleates microtubules at the trans golgi network (TGN) independent of centrosomes.

CLASP's C-terminal domain (CTD) mediates CLASP dimerization and interacts with

other factors to recruit CLASP to different locations in the cell. Secondary structure analysis

of the CLASP CTD predicts a short coiled-coil domain, suggesting that this domain may

mediate CLASP dimerization. Biochemical analysis of purified full-length CLASP indicates identified a monomer and a homodimer population (Patel et al., 2012). The CLASP CTD binds directly to the coiled-coiled domains of CLASP-interacting proteins (Figure 1-12). The coiled-coiled domain of CLIP-170 directly binds the CLASP CTD (Figure 1-12A) (Akhmanova et al., 2001) and forms a complex with EB1 to regulate MT plus-end dynamics (Mimori-Kiyosue, et al., 2005). CLASP also forms a complex with ELKS and the phosphatidylinositol-3,4,5-triphosphate (PIP3) binding protein, $LL5\beta$, to attach and stabilize MT plus ends to the cell cortex (Figure 1-12B) (Lansbergen et al., 2006). The trans-Golginetwork (TGN) protein GCC185 recruits CLASP to the TGN in order to nucleate noncentrosomal MTs at the TGN (Figure 1-12C) (Efimov et al., 2007). In mitosis, CLASP interacts with kinetochore-associated protein CENP-E to tether MT plus ends to kinetochores and mechanistically promote bipolar spindle assembly and chromosome segregation (Hannak et al., 2006). The common feature that these CLASP-interacting proteins share is that a section of their coiled-coil domain is required to interact with CLASP's CTD. Predicted secondary structure elements similar to cryptic TOG2 and TOG3 were found in CLASP CTD, such as the six HEAT repeats of a TOG domain. However, the tubulin-binding determinants that mediate TOG-tubulin interactions are not present in the intra-HEAT loops, suggesting that the CLASP CTD may retain the structural identity of a TOG domain but does not have TOG-like tubulin-binding activity. Interestingly, two short coiled-coil regions were predicted to exist in CLASP CTD, which may underlie CLASP homodimerization and its interaction with the coiled-coil of its binding partners. Whether the CLASP CTD requires the structural elements of a divergent TOG-like domain or its two coiled-coil regions for dimerization and interaction with other binding partners remains to be determined.

My thesis work involves establishing the presence of a TOG array in CLASP and investigating the role of these TOG domains in differentially promoting microtubule pause and growth. I am also investigating the role of CLASP's C-terminal domain in CLASP dimerization and its interaction with known CLASP-associating factors. My work employs a combination of X-ray crystallography, biochemical, and cellular assays on human CLASP and its Drosophila homologue, MAST/Orbit. We present the crystal structure of the first cryptic TOG-like domain from human CLASP1 that we designate as TOG2. CLASP1 TOG2 has a bent architecture not observed in TOG domain structures determined to date. The bent TOG architecture has concomitant implications for the conformation of $\alpha\beta$ -tubulin in complex with CLASP and potentially underlies CLASP-dependent MT pause and rescue events. We support our structural investigation with analysis of TOG2 structural determinants and their key role in bipolar mitotic spindle formation, *in vitro* MT polymerization, and *in vivo* MT lattice association. We also have purified TOG1 and the second cryptic TOG-like domain (TOG3) of human CLASP1. We are currently optimizing crystallization conditions that will produce high-resolution crystal structures of each individual TOG domain. Additionally, we are performing in vitro binding studies to further map the interaction of CLASP CTD to a \sim 150 residue region of the CLIP-170 coiled coil domain. We are also optimizing crystallization conditions to determine a crystal structure of the CLASP CTD in complex with a segment of the CLIP-170 coiled-coil domain. The overall goal of this work will provide mechanistic insight into how CLASP's arrayed TOG domains stabilize MTs and promotes the pause state, as well as how CLASP's CTD domain interacts with itself and known CLASP-associating factors.

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CHAPTER 2: A Cryptic TOG Domain with a Distinct Architecture Underlies CLASP-Dependent Bipolar Spindle Formation¹

Summary

CLASP is a key regulator of microtubule (MT) dynamics and bipolar mitotic spindle structure with CLASP mutants displaying a distinctive monopolar spindle phenotype. It has been postulated that cryptic TOG domains underlie CLASP's ability to regulate MT dynamics. Here, we report the crystal structure of a cryptic TOG domain (TOG2) from human CLASP1, demonstrating the presence of a TOG array in CLASP. Strikingly, CLASP1 TOG2 exhibits a convex architecture across the tubulin-binding surface that contrasts with the flat tubulin-binding surface of XMAP215 family TOG domains. Mutations in key conserved TOG2 determinants abrogate the ability of CLASP mutants to rescue bipolar spindle formation in Drosophila cells depleted of endogenous CLASP. These findings highlight the common mechanistic use of TOG domains in XMAP215 and CLASP families to regulate MT dynamics and suggest that differential TOG domain architecture may confer distinct functions to these critical cytoskeletal regulators.

¹ Leano, J.B., Rogers, S., Slep, K.C. (2013). A Cryptic TOG Domain with a Distinct Architecture Underlies CLASP-Dependent Bipolar Spindle Formation. *Structure*. 21(6): 939-50.

Introduction

Microtubules (MTs) are polymers of $\alpha\beta$ -tubulin that facilitate cell-shape change, cell migration, intracellular transport, and mitotic spindle formation. The dynamic nature of the MT polymer underlies these activities. MT dynamics are tightly regulated by MT-associated proteins, including the key regulator CLASP. Human CLASP was first identified as a CLIPfamily-associated protein (Akhmanova et al., 2001). It forms a family with two previously identified proteins: *Saccharomyces cerevisiae* Stu1(a suppressor of a β-tubulin mutation) and Drosophila multiple asters (MAST)/orbit/chromosome bows, named after its mutant spindle phenotypes (Pasqualone and Huffaker, 1994; Lemos et al., 2000; Inoue et al., 2000; Fedorova et al., 1997). Homologous members have since been identified across eukaryotic species, positioning the CLASP family as conserved regulators of MT dynamics (Hannak and Heald, 2006; Bratman and Chang, 2007). In cells, CLASP localizes to the polymerizing MT plus end. CLASP MT plus end association is EB1 dependent, mediated through a central EB1-binding SxIP motif (Mimori-Kiyosue et al., 2005). At the MT plus end, CLASP modulates MT dynamics, promoting MT pause, stabilization, and rescue during interphase (Akhmanova et al., 2001; Sousa et al., 2007; Drabek et al., 2006). During mitosis, CLASP localizes to kinetochores and promotes tubulin incorporation into fluxing kinetochore fibers (Maiato et al., 2003, 2005; Cheeseman et al., 2005; Pereira et al., 2006). How CLASP differentially promotes MT pause and growth remains to be determined. Overall, CLASP is a key spatial regulator of interphase MT dynamics as well as spindle structure and dynamics, but its mechanism remains to be determined.

CLASP has a distinct domain architecture. CLASP was originally annotated as having a conserved C-terminal domain used to bind CLIP-170 as well as an N-terminal TOG

domain (Akhmanova et al., 2001). TOG domains are approximately 220–250 residues in length and form a pentameric array in the XMAP215 MT polymerase family (Cullen et al., 1999; Brittle and Ohkura, 2005). This TOG array works as a multivalent-tubulin binding platform, collectively modulating MT dynamics (Widlund et al., 2011; Ayaz et al., 2012). It was thus surprising that CLASP would only have a single TOG domain, because XMAP215 activity requires an array. TOG domains are composed of 12 helices, consecutively paired in six Huntingtin, elongation factor 3, protein phosphatase 2A, target of rapamycin 1 (HEAT) repeats (HR) (Al-Bassam et al., 2007; Slep and Vale, 2007). Intra-HEAT loops define one face of the domain, which interacts with the $\alpha\beta$ -tubulin heterodimer (Ayaz et al., 2012). When the structure of ch-TOG/XMAP215 family TOG domains were determined from *Drosophila* and yeast, the authors noted discontinuous determinants in two conserved central regions of CLASP that bore sequence similarity to TOG domain intra-HEAT loops (Slep and Vale, 2007). The authors hypothesized that these two conserved regions were cryptic TOG-like domains, whose discontinuous tubulin-binding determinants were retained, but the intervening regions had diverged, specifically the composition of the inter-HEAT loops and the lengths and surface-exposed residues of the HEAT-repeat helices. The discontinuous sequence similarity with XMAP215 member TOG domains prevented standard BLAST searches from identifying CLASP's cryptic TOG domains as TOG domains and required structure-based insight. This cryptic TOG domain hypothesis suggested that CLASP, like XMAP215, might use a TOG array to regulate MT dynamics. In support of this, early work characterizing the yeast CLASP member Stu1 mapped β-tubulin binding activity to the region encompassing the first predicted cryptic TOG domain (Yin et al., 2002). Subsequent work with Drosophila, Schizosaccharomyces pombe, and Xenopus CLASP

members has implicated this region as a key mechanistic determinant in CLASP function (Slep and Vale, 2007; Al-Bassam et al., 2010; Patel et al., 2012).

Here, we present the crystal structure of the first cryptic TOG-like domain from human CLASP1. This reveals that the first cryptic TOG-like domain is a bona fide TOG domain. Accordingly, we designate it TOG2. Unexpectedly, CLASP1 TOG2 has a bent architecture not observed in TOG domain structures determined to date. The bent TOG architecture has concomitant implications for the conformation of $\alpha\beta$ -tubulin complexed with CLASP and potentially underlies CLASP-dependent MT pause and rescue events. This structure of TOG2 from CLASP, establishes the presence of a TOG array in CLASP, and suggests that a potential TOG array mechanistic paradigm underlies the activities of the CLASP and XMAP215 families. We support our structural investigation with analysis of TOG2 structural determinants and their key role in bipolar mitotic spindle formation and MT polymerization.

Experimental Procedures

Cloning and Expression

Human CLASP1 TOG2 (residues 284-552) was subcloned into pET28 (Novagen), generating a thrombin-cleavable N-terminal H6 tag. CLASP1 TOG2 was transformed into BL21 DE3 (pLysS) E. coli, and grown in 61 Luria Broth (50 μ g/l kanamycin) at 37° C to an optical density at 600 nm of 0.8. The temperature was lowered to 20° C and protein expression induced with 100 μ M IPTG (final concentration) for 16 hours. Cells were centrifuged at 2100 x g for 10 min and pellets resuspended in 250 ml buffer A (25 mM Tris pH 8.0, 300 mM NaCl, 10 mM Imidazole, 0.1% β-ME) and stored at -20° C. To produce

selenomethionine-substituted CLASP1 TOG2, expression was conducted in the B834 methionine auxotrophic E. coli strain, grown in L-selenomethionine-containing minimal media as described (Leahy et al., 1994). Drosophila MAST TOG1 (residues 6- 230) and TOG2 (residues 278-544) were cloned into pET28 and expressed similar to CLASP1 TOG2. *Protein Purification*

Native and selenomethionine-substituted CLASP1 TOG2 cell pellets were thawed, 0.5 mM Phenylmethylsulfonyl fluoride added and the cells lysed by sonication. After lysis, additional Phenylmethylsulfonyl fluoride was added to 1mM final concentration and the lysate was centrifuged at 23,000 x g for 45 min. Supernatant was loaded onto a 10 ml Ni2+-NTA column (Qiagen), washed with 500 ml buffer A and TOG2 eluted over a 250 ml linear gradient between buffer A and B (buffer B = buffer A supplemented with 290mM Imidazole). TOG2-containing fractions were pooled and CaCl2 added to 1 mM. 0.1 mg bovine α-thrombin was added to cleave off the H6-tag, leaving a four-residue N- terminal GSHM cloning artifact. Thrombin digest proceeded for 16 hours at 4° C. Digested protein was filtered over 0.5 ml Benzamadine sepharose (GE Healthcare), concentrated in a Millipore Ultrafree 10,000 MWCO concentrator, and exchanged into buffer C (25 mM HEPES pH 7.0, 0.1 % β -ME). Protein was loaded onto a 10 ml SP- sepharose fast flow column (GE Heathcare), washed with 200 ml buffer C and eluted over a 250 ml linear gradient between buffer C and D (buffer D = buffer C supplemented with 1 M NaCl). Protein factions were pooled, concentrated and exchanged into 25 mM HEPES pH 7.0, in a Millipore Ultrafree 10,000 MWCO concentrator to 50 mg/ml, frozen in liquid nitrogen and stored at -80° C. Purification of selenomethionine-substituted protein proceeded according to the above protocol, except buffers were supplemented with 5 mM L-methionine. Drosophila MAST

TOG1 was purified using a protocol similar to CLASP1 TOG2 except that ion exchange chromatography was performed using Q- sepharose fast flow resin (GE Healthcare). Drosophila MAST TOG2 and MAST TOG2 mutant constructs were subcloned into pET28 using the respective pMT/V5-His A-full length wild-type or mutant MAST construct as a PCR template. MAST TOG2 constructs were expressed and purified using Ni2+-NTA chromotography (Qiagen) as described for CLASP1 TOG2, pure fractions pooled, concentrated and exchanged into 10 mM sodium phosphate buffer (pH 7.5), 50 mM NaF. *Crystallization*

CLASP1 TOG2 was crystallized via hanging drop: 2 µl of 10 mg/ml protein plus 2 µl of a 1 ml well solution containing 22% PEG 3350 and 200 mM sodium citrate (pH 8.25), 20°C. Another crystallization condition yielded crystals: 200mM ammonium phosphate, 16% PEG 3350. Crystals were frozen in paratone-N or 200mM ammonium phosphate, 16% PEG 3350, 20% glycerol. Native crystals were used to seed selenomethionine-substituted crystals. *Data Collection, Structure Determination, and Refinement*

A selenium SAD peak data set was collected on a single crystal at the Advanced Photon Source 22-ID beamline at 100 K. Data were processed using HKL2000 (Otwinowski and Minor, 1997). Phases were determined using PHENIX (Adams et al., 2010) AutoSol. The CLASP1 TOG2 construct contains two endogenous methionines as well as a third Nterminal methionine that was introduced as a cloning artifact. Four selenium sites were found in the heavy atom search, correlating with two molecules in the asymmetric unit (47.5% solvent content), with a figure of merit equal to 0.43. An initial model was built using AutoBuild (PHENIX) that placed 476 residues and yielded an Rfree of 0.27. Reiterative buildings in Coot (Emsley et al., 2010) followed by refinement runs using phenix.refine

(PHENIX) were performed using real space, simulated annealing refinement protocols (temperatures: 5,000 K start, 300 K final; 50 steps), and individual B-factor refinement, using experimental phase restraints against a maximum-likelihood Hendrickson-Lattman (MLHL) target. Once the Rfree reached 0.24, individual B-factor and translation libration screwmotion (TLS) refinement was performed using experimental phase restrains against a MLHL target. The final refinement run produced a figure of merit equal to 0.85 and an Rfree value of 0.21. The final model includes residues 296–538 for protomers A and B and 385 water molecules. Figures were generated using PyMol (Schrödinger) and APBS (Baker et al., 2001).

Drosophila S2 Cell Expression Plasmids

Full-length MAST/Orbit complementary DNA (residues 1–1491) was subcloned into a modified pMT/V5-His A vector (Invitrogen, Carlsbad, CA, USA), engineering a Cterminal myc tag. The vector drove eGFP expression off a separate promoter to mark transfected cells. MAST TOG1-2-linker (residues 1–830) was subcloned using the Gateway TopoD pEntr system (Invitrogen) into a destination vector with an N-terminal GFP tag under a pMT-inducible promoter (Invitrogen). MAST/Orbit mutant constructs were generated using the QuikChange protocol (Stratagene, LaJolla, CA, USA) and confirmed by DNA sequencing.

Cell Culture and Transfection

Drosophila S2 cells were cultured and treated with dsRNA as previously described (Rogers and Rogers, 2008). MAST constructs were transfected into Drosophila S2 cells using the Amaxa Nucleofector Kit II (Lonza, Basel, Switzerland) as described by the manufacturer's protocol. Each transfection used 2 µg of plasmid DNA. Construct expression

off the pMT promoter was induced using 500 µM copper sulfate, at which point, RNAimediated depletion of endogenous MAST was initiated. MAST TOG1-2-linker overexpression was assayed in the absence of RNAi treatment, and cells were induced for 16 hr before imaging.

RNAi

RNAi was performed as previously described (Rogers and Rogers, 2008). Templates for in vitro transcription were generated by PCR amplification from FlyBase cDNA clone LD31673. The T7 promoter sequence was added to the flanking regions of primers specific to the MAST cDNA 5'UTR (5'-TTTTTGCAACGCAACGGGCTGG-3', 5'-CGTTGCAATGTCTATTCGTCCTTC-3'). The pBluescript SK plasmid was used as a template to generate control dsRNA with minimal homology with the Drosophila genome. RNA was synthesized using a RiboMAX Large Scale RNA Production System- T7 (Promega). RNAi was performed in 12-well plates in which cells (50-90% confluent) were treated with 10 μg/mL dsRNA (final concentration). Cells were replenished with fresh media, including dsRNA and 500 μM copper sulfate each day for 3 days.

Western Blots

Trans expression of MAST-Myc constructs was monitored by western blot analysis, using rabbit anti-c-Myc primary antibody (Sigma) (Figure 2-S2). Samples were normalized against actin protein levels, detected using mouse anti-actin antibody (Millipore). Purified guinea pig anti-MAST polyclonal antibodies generated against MAST TOG1 residues 6- 230 was used (1:500) to monitor RNAi-mediated depletion of endogenous MAST. Primary antibodies were detected using HRP-conjugated goat secondary antibodies (Sigma). Primary and secondary antibodies were used at a 1:1000 dilution unless otherwise noted.

Immunofluorescence Microscopy and Mitotic Spindle Classification

S2 cell fixation protocols were adapted from previously described methods (Rogers and Rogers, 2008). Cells transfected with full-length MAST constructs were plated on concanavalin-A-coated coverslips for 1.5-3.0 hr and fixed with 10% paraformaldehyde in BRB80 (80 mM PIPES [pH 6.8], 1 mM MgCl2, and 1 mM EGTA) for 10 min. PBS-Triton 0.1% (PBST) was used to permeabilize the cells, and antibodies were diluted in PBST supplemented with 5% normal goat serum (Sigma-Aldrich, St. Louis). Antibodies used for immunofluorescence included mouse anti-tubulin (DM1a;1:1,000) (Sigma-Aldrich), rabbit anti-DPLP (1:3,000) (Rogers et al., 2009), and DAPI (300 nM) (Invitrogen). Primary antibodies were detected using Cy3- α -mouse (1:200) and Cy5- α -rabbit (1:1,000) (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) conjugated secondary antibodies. Cells were mounted in a 10% PBS, 90% glycerol, and 4% N-propyl gallate solution. Images were acquired using an Eclipse Ti microscope with a 100× oil NA-1.45 objective, driven by NIS Elements software (Nikon, Tokyo, Japan). Images were processed using Photoshop CS5 (Adobe Systems, San Jose, CA, USA) and ImageJ (National Institutes of Health, Bethesda, MD, USA). Spindles were classified as bipolar biastral, bipolar monoastral, or monopolar monoastral. Each MAST construct was scored in three independent experiments with a minimum of 50 spindles scored in each experiment. The p values were calculated using twoway ANOVAs. Cells transfected with MAST TOG1-2-linker constructs were treated as above, except that after plating, cells were washed briefly in BRB80 and fixed with -20° C methanol for 10 min. MAST localization was scored for MT lattice binding and MT plus end binding.

Circular Dichroism

Circular dichroism (CD) spectra of Drosophila TOG2 proteins (wild-type, W334E, R460E, and R502E) were collected at room temperature (23 °C) using a Chirascan-plus CD spectrometer (Applied Photophysics, Leatherhead, UK). Each protein sample was diluted to a final concentration of 0.1 mg/ml in 10 mM sodium phosphate buffer (pH 7.5), 50 mM NaF. The spectra were recorded from 260 to 185 nm with a step size of 0.5 nm using a 1-mm path-length cuvette. The time per point was kept at 1.25 s. A base-line CD spectrum of the buffer was taken that was subtracted from each spectrum. All spectra were smoothed in the Chirascan-plus software.

Light Scattering Tubulin Polymerization Assay

Tubulin polymerization was monitored using a SPEX Fluorolog-3 spectrofluorometer (Horiba Jobin Yvon) in T-format, high voltage mode with the excitation and detection wavelength set at 350 nm and the excitation and emission slits set at 0.5 and 0.75 nm respectively. The cuvette holder was maintained at 37 °C. Clarified tubulin samples (15 µM final concentration) were prepared in the presence or absence of MAST TOG2 constructs (WT, W334E, R460E, and R502E; each at 1.4 µM final concentration) in polymerization buffer (50 mM MES pH 6.6, 3.4 M glycerol, 5 mM DTT, 1 mM EGTA, 5 mM MgSO4, 1 mM GTP) and maintained at 4 °C prior to the polymerization assay. 300 µL samples were injected into a 4 mm path length quartz cuvette at room temperature, then immediately placed into the 37 °C cuvette holder and scattering recorded at 350 nm in 1 second intervals over 1500 seconds. Spikes in scattering within the first 100 seconds were the result of samples equilibrating to 37 °C.

Microscopic Analysis of Tubulin Polymerization in Vitro

Tubulin was polymerized in the presence or absence of MAST TOG2 as described in the light scattering assay. At 200 and 500 second time points, samples were diluted 10-fold in polymerization buffer supplemented with 4% glutaraldehyde for 3 minutes at 37 °C, and fixation quenched by the addition of Tris pH 6.8, 100 mM final concentration. Samples were then flowed into base-washed, poly-lysine coated flow chambers, allowed to incubate for 30 minutes, then fixed with PBS supplemented with 4% para-formaldehyde. Chambers were then washed with PBS supplemented with 100 mM Tris pH 6.8, then incubated with a PBS-BSA(2%) blocking solution. Microtubules were detected using mouse anti-tubulin (DM1 α ;1:1000) (Sigma) and a Cy3- α -mouse (1:200) (Jackson ImmunoResearch Laboratories) conjugated secondary antibody. Dako fluorescent mounting media (Dako) was added and images were acquired using an Eclipse Ti microscope with a 40x oil NA-1.0 objective, driven by NIS Elements software (Nikon). Images were processed using Photoshop CS5 (Adobe Systems, Inc.) and ImageJ (NIH).

Results

The First Cryptic TOG-like Domain is a True TOG Domain

Secondary structure prediction of the human CLASP1 sequence predicted a dodecahelical domain spanning residues 284–552 that bore high conservation across species (Figures 2-1A and 2-1B; Figure 2-S1). This region was subcloned, expressed, crystallized, and used to seed selenomethionine-substituted crystals. A single-wavelength anomalous dispersion (SAD) data set was collected to 2.0 Å resolution. The structure was phased and refined to Rwork and Rfree values of 18.3 and 21.0, respectively. Crystallographic statistics are presented in Table 2-1. The asymmetric unit contains two CLASP1 molecules that are nearly identical, with a root-mean-square deviation (rmsd) of 0.5 Å across 243 Cα atoms (Figure 2-S2A). The architecture of the domain is an elongated, helical solenoid, composed of six HRs, largely conforming to the arrangement observed in TOG domains (Figures 2-1B and 2-1C) (Al-Bassam et al., 2007; Slep and Vale, 2007). The six HRs, designated HR A-F, are arranged into triads. HR A–C have a right-handed twist and abut the HR D–F triad, which is shifted laterally relative to the axes of the first triad's helices. The HR D–F triad is righthanded between HR D-E and left-handed between HR E-F. A feature of the CLASP1 TOG2 domain is the N-terminal helix $\alpha 2N$, which runs orthogonal to HR B–C helices $\alpha 2B'$ and $\alpha 2C'$. We mapped conserved residues onto the CLASP1 TOG2 structure, contouring conservation at 80% identity (green) and 80% similarity (yellow) based on a multispecies alignment (Figures 2-1B and 2-S1). This revealed a high conservation of solvent-exposed residues clustered on the TOG domain surface formed by the intra-HEAT loops; these match residues in the XMAP215 family that mediate tubulin-binding (Figure 2-1D) (Ayaz et al., 2012). This surface also has a net positive charge (Figure 2-1E) that would complement the negatively charged MT surface. Additional residues, on other faces of the domain, also show a high degree of conservation. These residues either form the domain core or are dispersed across the domain surface and are involved in inter-HR salt bridges or $\alpha 2N$ binding. We hypothesize that these additional conserved residues confer structural support and stability. The architecture of this conserved domain confirms our prediction that CLASP is composed of arrayed TOG domains, similar to the arrayed TOG domains of the XMAP215 family.



Figure 2-1. CLASP Contains a Conserved, Cryptic TOG Array. (A) Human CLASP1 domain architecture showing the four conserved domains: the annotated TOG1 domain, two cryptic TOG-like domains (TOG2 and TOG3), and the CLIP interaction domain (CLIP-ID).(B) Sequence alignment of human (*Hs*) CLASP1, CLASP2, and *Drosophila* (*Dm*) MAST/Orbit TOG2. Identity (green) and similarity (yellow) are contoured at 80% based on the alignment in Figure 2-S1. Numbering corresponds to human CLASP1. *Drosophila* Msps TOG2 intra-HEAT loop sequences are aligned. MAST/Orbit residues mutated for cellular studies are boxed in red. See also Figure 2-S1.



Figure 2-1 (continued). CLASP TOG2 is a bona fide TOG Domain. (C) Human CLASP1 TOG2 structure, composed of HR A–F. The N-terminal helix, $\alpha 2N$, runs orthogonal to the HRs. See also Figure 2-S2. (D) CLASP1 TOG2 oriented as in (C) and shown in surface representation with conservation mapped as in (B). See also Figure 2-S2. (E) Electrostatic surface potential mapped on CLASP1 TOG2 and oriented as in (D). (F) Intra-HEAT loops from HR 2A and 2B. Hydrogen bonds (<3.5 Å) shown in red. The conserved TOG domain tubulin-binding determinant, W338, is solvent exposed. (G) The HR 2C intra-HEAT loop contains the conserved, solvent-exposed residue K423, implicated in tubulin binding (Al-Bassam et al., 2010). (H) The HR 2D intra-HEAT loop contains the conserved, solvent-exposed residue K426, which correspond to TOG-tubulin binding determinants in Stu2 (Ayaz et al., 2012). Noted residues are shown with red arrows.

The Intra-HEAT Loops Carry Canonical TOG Domain Tubulin-Binding Determinants

The conserved surface-exposed intra-HEAT loop residues share similarity to characterized tubulin-binding determinants from XMAP215 family TOG domains, identified through mutagenesis and structural analysis (Al-Bassam et al., 2007; Slep and Vale, 2007). Particular insight comes from the recent low-resolution structure of the S. cerevisiae XMAP215 member Stu2 TOG1 domain in complex with αβ-tubulin (Ayaz et al., 2012). This structure confirmed the TOG intra-HEAT loops as prime tubulin-binding determinants. Stu2 TOG1 HR A–D engage β -tubulin, while HR E–F engage α -tubulin. Many of the tubulinbinding determinants identified in these XMAP215 family studies are conserved in CLASP1 TOG2: a conserved, solvent-exposed tryptophan in the HR A loop (W338, Figure 2-1F), a leucine residue in the HR B loop (L380, Figure 2-1F), a lysine residue in the HR C loop (K423, Figure 2-1G), and basic residues in the HR D loop (K456 and R462, Figure 2-1H). The role of these residues in tubulin-binding is supported by mutagenesis conducted in the S. pombe CLASP family member, Cls1p (Al-Bassam et al., 2010). In this study, a construct comprising Cls1p TOG1 and TOG2 was capable of binding and shifting $\alpha\beta$ -tubulin over gel filtration, indicative of Cls1p- $\alpha\beta$ -tubulin complex formation. When mutations in the nowidentified TOG2-conserved HR A and C loops were introduced, the ability of this Cls1p construct to shift $\alpha\beta$ -tubulin was ablated. Likewise, these same mutations prevented Cls1p from stabilizing MTs in vivo (Al-Bassam et al., 2010). The structure and mutational studies support the hypothesis that CLASP TOG2 interacts directly with tubulin in a mode generally similar to XMAP215 family TOG domains.

The CLASP1 TOG2 Structure Adopts a Bent Conformation that Contrasts with XMAP215 Family TOG Domains

To compare CLASP1 TOG2 to previously determined TOG domain structures, we structurally aligned CLASP1 TOG2 to Stu2 TOG1 using the Dali server (Hasegawa and Holm, 2009). The TOG domains aligned poorly overall, with an rmsd equal to 3.4 Å over 193 Ca residues (13% identity, Figure 2A). Relative to the straight tubulin-binding surface of Stu2 TOG1, this alignment revealed a surprisingly dramatic 30° shift between the predicted tubulin-binding surface of CLASP1 TOG2's first and second HR triads (Figures 2-2B and 2-2C, red arrow). To examine this architectural difference in more detail, we structurally aligned the first HR triad from CLASP1 TOG2 and Stu2 TOG1 and examined how this positioned the respective second HR triad of each domain. The N-terminal HR triads (HR A-C) again aligned poorly with an rmsd equal to 3.0 Å across 102 C α atoms (15% identity) (Figure 2D). Whereas the CLASP1 TOG2 intra-HEAT A and B loops align well to the Stu2 TOG1 tubulin-binding determinants, HR C is shifted toward the tubulin-binding surface as well as toward the second HR triad. In contrast to the Stu2 TOG1 HR C antiparallel helices, the CLASP1 TOG2 HR C helices are angled relative to one another due to the absence of bulky, buried hydrophobic side chains at the $\alpha 2C'$ N-terminal region. This effectively compresses the $\alpha 2C'$ N-terminal region against $\alpha 2C$, shifting the intra-HEAT loop toward the tubulin-binding surface and rotating the $\alpha 2C'$ C-terminal region outward (Figure 2-2E, red arrows).

The CLASP1 TOG2 α2N N-terminal helix is likely to promote this change in HR C positioning, as it inserts two conserved phenylalanine residues (F302 and F306) between HR B and HR C (Figure 2-2F). The differential conformation in HR C causes the second HR
triad to undergo an en bloc rotation away from the tubulin-binding plane established by the first HR triad, effectively displacing HR F 14 Å relative to the position of HR F in Stu2 TOG1 (Figure 2-2D, red arrow). When we structurally aligned the C-terminal HR triads (HR D-F) of Stu2 TOG1 and CLASP TOG2, we were surprised to find that these triads aligned quite well, with an rmsd equal to 1.9 Å across 89 C α atoms (10% identity) (Figure 2-2G). The highest degree of structural similarity in the second HR triad occurs at the intra-HEAT loops used by Stu2 TOG1 to engage tubulin. Thus, determinants in CLASP1 TOG2 HR C and the α 2N helix impart a bent architecture to the TOG domain. On either side of this bend point, the CLASP TOG2 intra-HEAT loops bear structural similarity to the tubulin-binding determinants of Stu2 TOG1. Both CLASP1 TOG2 protomers in the asymmetric unit have the same bent structure with relatively low B-factors at the domain's bend site (Figures 2-S2A and 2-S2B). Crystal packing interactions are limited to the domain's flanking HRs, suggesting that the bent architecture is not due to crystal packing (Figures 2-S2C and 2-S2D). CLASP TOG2's bent architecture likely affects its interaction with αβ-tubulin and is probably a mechanistic determinant of CLASP-mediated MT regulation.

The CLASP1 TOG2-Tubulin Interaction Is Predicted to Be Dramatically Different from XMAP215 Family TOG-Tubulin Interactions

To gain insight into how CLASP1 TOG2 might interact with tubulin, we compared the structure of CLASP1 TOG2 to Stu2 TOG1 in complex with $\alpha\beta$ -tubulin (Ayaz et al., 2012). In the Stu2 TOG1- $\alpha\beta$ -tubulin structure, Stu2 forms extensive contacts with both β tubulin and α -tubulin (Figure 2-3A). Stu2 TOG1 binds a curved tubulin conformation, predicted to mimic the structure of $\alpha\beta$ -tubulin in solution. This contrasts with the straight conformation of tubulin determined from zinc-induced tubulin sheets, predicted to represent the structure of tubulin in the MT lattice (Nogales et al., 1998; Löwe et al., 2001). When βtubulin from the straight tubulin structure is aligned to β -tubulin in the Stu2 TOG1- $\alpha\beta$ tubulin structure, α-tubulin does not engage Stu2 TOG1 HR E–F (Figure 2-3B). We docked the CLASP1 TOG2 structure onto the Stu2 TOG1- $\alpha\beta$ -tubulin complex, aligning the first HR triad of each TOG domain (as was done in Figure 2-2D) because this region contains the HR A tryptophan, a well-characterized TOG domain tubulin-binding determinant. Whereas this enabled the first HR triad to engage β -tubulin effectively, the second HR triad was dramatically angled away from the curved αβ-tubulin structure (Figure 3C, red arrow). This gap was exacerbated if CLASP1 TOG2 was structurally aligned to the straight $\alpha\beta$ -tubulin structure (Figure 2-3D). This was surprising because the CLASP1 TOG2 and Stu2 TOG1 second HR triads structurally align and contain similar predicted α -tubulinbinding determinants (Figure 2-2G). If the CLASP1 TOG2 second HR triad is to engage αtubulin, a dramatic rearrangement in the TOG domain and/or $\alpha\beta$ -tubulin will be required. It is of note that (1) the stabilizing $\alpha 2N$ helix does not extend past the first HR triad to the second triad, suggesting that the two triads may undergo relative en bloc movement and (2) the bend between the first and second HR triad is positioned at the $\alpha\beta$ -tubulin hinge region that changes between the straight and curved tubulin conformations. Perhaps CLASP binds an even more highly curved αβ-tubulin conformation, allowing it to stabilize the curved protofilaments observed during MT depolymerization, preventing further depolymerization. Stabilizing a curved MT plus end conformation would also inhibit polymerization because lateral tubulin contacts would be disrupted. This may underlie CLASP's ability to promote



Figure 2-2. CLASP1 TOG2 Has a Bent Structure. (A) CLASP1 TOG2 (colored as in Figure 2-1C) and S. cerevisiae XMAP215 member Stu2 TOG1 (4FFB, HR triads: HR A–C and HR D–F shown in two shades of gray) aligned over HR A–F. The alignment highlights the bent architecture of CLASP1 TOG2 (red arrows). (B and C) Surface representation of Stu2 TOG1 (B) and CLASP1 TOG2 (C) orientated as in (D), showing the flat Stu2 TOG1 tubulin-binding surface versus the bent CLASP1 TOG2 surface. The intra-HEAT A–C and D–F loops form two surfaces and are angled 30° relative to each other (red arrow). (D) CLASP1 TOG2 and Stu2 TOG1 as in (A) are aligned over HR A–C. (E) CLASP1 TOG2 (green) and Stu2 TOG1 (gray) HR C are aligned as in (D), highlighting the structural divergence in the α C and α C' helices (red arrows), which offsets the relative position of HR D–F. (F) Structure of CLASP1 TOG2 α 2N residues F302 and F306 (MAST F298 and F302, respectively) that pack between HR B and C (2Fo-Fc electron density map in gray, 1.5 σ). (G) CLASP1 TOG2 and Stu2 TOG1 as in (A) are aligned over HR D–F.



Figure 2-3. CLASP1 TOG2 and/or αβ-Tubulin Is Likely to Undergo Conformational Change upon Binding. (A) Structure of the Stu2 TOG1-αβ-tubulin complex (4FFB). Stu2 is colored as in Figure 2A. Stu2 TOG1 binds curved αβ-tubulin. (B) Model of Stu2 TOG1 docked onto straight (MT-like) αβ-tubulin (1JFF) by aligning β-tubulin from IJFF and 4FFB. The straight αβ-tubulin conformation creates a gap between α-tubulin and Stu2 TOG1 HR D–F. (C) CLASP1 TOG2 superimposed on the 4FFB structure after alignment across HR A– C as in Figure 2D (Stu2 not shown). Whereas CLASP1 TOG2 HR A–C can engage β-tubulin using similar binding determinants, HR D–F is splayed 30° from α-tubulin (red arrow). (D) Model of CLASP1 TOG2 docked onto straight tubulin yields a larger gap between CLASP1 TOG2 and α-tubulin.



Figure 2-4. MAST Depletion Causes Bipolar Spindle Defects that Can Be Partially Rescued in Trans. (A) Mitotic spindle classification from Drosophila S2 cells treated with control or mast 5-UTR-directed dsRNA and transfected with the indicated MAST construct. Spindles were classified as bipolar biastral, bipolar monoastral, or monopolar monoastral. Error bars represent SD. See also Table 2-S1. (B) Representative images of spindles used to tally the distribution showed in (A). Cells were stained with anti-PLP, DAPI, and DM1 α to label centrosomes, DNA, and MTs, respectively. Scale bar: 10 µm. (C) Western blot showing effective MAST depletion.

MT pause. In contrast, an inter-TOG2 structural transition to a straighter conformation may underlie the CLASP-dependent MT polymerization activity observed in mitosis.

TOG2 is Required for CLASP-Mediated Bipolar Spindle Formation

To probe the role of TOG2 and its unique structural determinants in CLASP function, we performed rescue assays in Drosophila S2 cells. The single Drosophila CLASP member, MAST/Orbit, regulates interphase MT dynamics as well as mitotic spindle structure and dynamics (Lemos et al., 2000; Maiato et al., 2002; Maiato et al., 2005; Sousa et al., 2007). Here, we concentrated our analysis on mitotic spindle structure. We treated S2 cells with control dsRNA and scored mitotic spindle structure (Figure 2-4A-B, Table 2-S1). 76% of cells formed normal bipolar biastral spindles, while 10% formed bipolar monoastral spindles and 14% formed monopolar monoastral spindles. In contrast, RNAi-mediated MAST depletion produced the established monopolar monoastral spindle structure with "orbiting" "chromosome bows" (Figure 2-4A-B; we confirmed MAST depletion via western blot analysis; Figure 2-4C). 72% of cells had monopolar monoastral spindles, 14% had bipolar monoastral spindles, and only 14% had normal bipolar biastral spindles. eGFP control transfections did not alter either the wild-type or MAST RNAi distributions. This monopolar phenotype could be substantially but not completely rescued by FL MAST-Myc, decreasing the monopolar monoastral spindle index to 46% and increasing the bipolar biastral index to 42%. This failure to fully rescue spindle structure is likely due to a dominant effect of overexpression, as cells treated with control dsRNA and transfected with FL MAST-Myc had a monopolar monoastral index increased to 44%. Similar effects have been noted with the Xenopus member Xorbit (Patel et al., 2012). The MT stabilizing agent taxol also increases

the monopolar monoastral spindle index, suggesting that MAST overexpression may hyperstabilize MTs, causing monopolar spindle formation (Maiato et al., 2002).

This provided us with an assay to assess the ability of different MAST mutants to maintain spindle architecture when endogenous MAST was depleted. We examined the role of TOG2's conserved determinants in MAST function. We first examined a mutant completely lacking TOG2. The Δ TOG2 construct failed to rescue spindle structure relative to the eGFP control, suggesting that MAST activity requires TOG2 (Figures 2-5A and 2-5B). We next examined whether a residue known to make a critical tubulin contact in other TOG domains was important for MAST function. To do so, we mutated the conserved, surfaceexposed HR A loop tryptophan to glutamate, a mutation known to disrupt tubulin binding activity in XMAP215 family TOG domains (Figure 2-5C) (Slep and Vale, 2007). MAST W334E failed to rescue bipolar biastral spindle structure and yielded spindle distributions on par with the $\Delta TOG2$ construct, indicating that this key residue used in XMAP215 TOG domains to bind tubulin and promote polymerization is similarly required in MAST TOG2 to promote bipolar spindle formation (Figures 2-5A and 2-5B). We next individually mutated conserved basic residues in the second HR triad to glutamate: R460E in HR D, and R502E in HR E. Both R460E and R502E failed to rescue bipolar biastral spindle structure. Collectively, our data indicate that TOG2 tubulin-binding determinants in both the first and second HR triad are utilized in CLASP-dependent bipolar spindle formation. Bacterially expressed TOG2 constructs containing these mutations were purified and analyzed using circular dichroism (CD). All constructs behaved like wild-type TOG2 and produced similar CD spectra, indicative of a folded, α -helical domain (Figures 2-5D–G).



Figure 2-5. Point Mutations across the TOG2 Tubulin-Binding Surface Fail to Rescue MAST-Mediated Bipolar Spindle Formation. (A) Mitotic spindle classification from Drosophila S2 cells treated with control or mast 5'-UTR-directed dsRNA and transfected with the indicated MAST construct. Spindles were classified as in Figure 4A. Error bars represent SD. See also Table 2-S1. (B) Representative images of spindles used to tally the distribution showed in (A). Cells were stained as in Figure 4B. Scale bar: 10 µm. See also Figure S3. (C) Domain architecture of human CLASP1 TOG2 showing the location of identical, counterpart residues in Drosophila MAST TOG2 that were individually mutated to glutamate in α 2N (Drosophila melanogaster F298), HRA (D. melanogaster W338), HRD (D. melanogaster. R461), and HRE (D. melanogaster R503). (D–G) Circular dichroism spectra of wild-type MAST TOG2 (D), W334E (E), R460E (F), and R502E (G), each showing similar spectra characteristics of an α -helical protein, indicating that all mutants retained a similar folded state.

We next explored the role of the α 2N helix, which is a feature of TOG2 that is not present in other TOG domain structures determined to date. The α 2N helix engages HR B and C, using two conserved phenylalanine residues, F302 and F306 (F298 and F302 in MAST, respectively) (Figure 2-2F), and may serve to stabilize and/or promote the bent TOG2 architecture. To assess whether this helix is important for MAST function, we systematically mutated each conserved phenylalanine, individually or in pairs, to either alanine or glutamate to potentially disengage it from the TOG domain. The individual phenylalanine to alanine mutations did not alter the mitotic spindle index from levels observed with FL MAST; however, the double F298A/F302A mutant as well as the individual or paired phenylalanine to glutamate mutations failed to rescue spindle structure (Figures 2-5A and 2-5B). Bacterially expressed MAST TOG2 constructs containing the F298E or F302E mutation proved insoluble, strongly suggesting that the α 2N helix plays a key role in TOG2 domain structure and stability.

TOG2 Promotes Microtubule Lattice Association In Vivo

While CLASP localizes to growing MT plus ends, overexpression has been shown to result in MT lattice binding (Sousa et al., 2007). We analyzed MAST truncation constructs and found a minimal construct, TOG1-2-linker, that binds the MT lattice when overexpressed (Figures 2-6A and 2-6B). The TOG1-2-linker construct contains the EB1-binding SxIP motif and also localizes to MT plus ends. We systematically introduced the same TOG2 point mutations that we tested in our bipolar spindle rescue assay and found that all three



Figure 2-6. MAST TOG2 Determinants Mediate MT-Lattice Binding. (A) Domain organization of the MAST TOG1-2-linker construct (MAST 1-830). The relative location of TOG2 domain mutations are indicated as is the linker region's EB1-binding SxIP motif. (B) Drosophila S2 cells transfected with the indicated GFP MAST TOG1-2-linker construct. Cells were strained with DM1 α to label MTs. Merge shows GFP in green and tubulin in red, and the box inset is shown in zoom view in the three panels to the right. Scale bar: 10 µm.

mutations, W334E, R460E, and R502E, individually reduced the relative amount of MAST1-2-linker on the microtubule lattice but retained MT plus end association, likely mediated through EB1 binding to the SxIP motif. This indicates that TOG2 tubulin-binding determinants are used for MT lattice binding and further supports a direct TOG2-tubulin interaction.

TOG2 Increases Microtubule Polymerization Rates In Vitro

Given that CLASP promotes MT polymerization in mitosis, we next assayed if MAST TOG2 could promote microtubule polymerization in vitro. We performed a 90° light scattering assay to measure bulk microtubule polymerization over time in the presence of wild-type or mutant MAST TOG2 constructs (Figure 2-7A). Tubulin alone began to polymerize after an ~400 s lag time. In contrast, the addition of TOG2 dramatically decreased the lag time to ~50 s and promoted rapid MT polymerization. No scattering was evident with the MAST TOG2 control. The ability of MAST TOG2 to enhance MT polymerization kinetics was diminished by point mutations in HR D (R460E) and HR E (R502E) and nearly abrogated with the W334E HR A mutation. Microscopy-based analysis of the MT polymerization reaction at fixed times showed little MT polymerization for tubulin alone at 200 and 500 s, whereas the reaction conducted in the presence of MAST TOG2 showed the clear presence of MTs (Figure 2-7B). Larger CLASP constructs containing TOG2 have been shown to promote MT polymerization in vitro (Slep and Vale, 2007; Patel et al., 2012), but our findings distinctly ascribe MT polymerization activity to TOG2 and pinpoint key residues that underlie both this activity and bipolar mitotic spindle formation.



Figure 2-7. MAST TOG2 **Promotes MT Polymerization** In Vitro. (A) Light scattering curves (90°, 350 nm) of tubulin (15 µM) polymerized at 37°C in the presence or absence of MAST TOG2 constructs $(1.4 \,\mu\text{M})$ (see legend). Initial spikes in scattering within the first 100 s were the result of samples equilibrating to 37°C. The TOG2 alone control showed no scattering contribution over time (gray trace). (B) Images of tubulin alone and tubulin plus MAST TOG2 polymerization samples fixed and diluted at 200 and 500 s time points. MTs were stained with DM1a. Scale bar: 50 µm.

D



Discussion

Our results confirm the prediction that CLASP carries a TOG array (Slep and Vale, 2007; Slep, 2009). However, in this TOG array, TOG2 has a distinct domain architecture not observed in other TOG domains: it is bent and has an N-terminal helix that runs along its side—structural features that suggest distinctive interactions with tubulin. Whereas the bent TOG architecture may explain CLASP-induced microtubule pause, we note that our model represents only a single energy state. Whether CLASP TOG2 can sample different conformational states that underlie its differential activities—promoting microtubule pause during interphase versus microtubule polymerization during mitosis—is an interesting question awaiting further studies. Our findings highlight a common TOG-domain-mediated mechanism for the XMAP215 and CLASP families. It will be exciting to probe how CLASP's TOG2 domain interacts with tubulin and whether the flanking TOG domains, TOG1 and TOG3, also adopt bent conformations and how these domains work collectively to regulate MT dynamics in interphase and mitosis.

Since the manuscript on TOG2 was published in the journal *Structure*, we solved and refined another crystal structure of CLASP1 TOG2 (Table 2-S2, Figure 2-S3). Crystals containing CLASP1 TOG2 were obtained using a different condition. The crystals diffracted to a resolution of 1.8Å. The second structure of CLASP1 TOG2 is nearly identical to the original crystal structure, displaying a bent architecture across the tubulin-binding surface. The structure aligned well with an rmsd of 0.45Å (Figure 2-S3). Despite obtaining crystals of CLASP1 TOG2 in another crystallization, the second crystal structure displayed the same conformation as the original CLASP1 TOG2 structure. This finding further supports that

notion that the bent architecture in CLASP1 TOG2 is functionally relevant and was not created through a crystallization artifact.

A long-term goal we wish to accomplish is obtaining a structure of CLASP1 TOG2 bound to $\alpha\beta$ -tubulin. This structure would provide insight into how TOG2 interacts with $\alpha\beta$ tubulin, and will reveal the conformations TOG2 and $\alpha\beta$ -tubulin adopts through their interaction with each other. We predict that TOG2 binds to a conformation of tubulin that corresponds to MT pause or a state of tubulin that mimics tubulin in a stabilized, curved MT protofilament, which may differ from tubulin conformations found in previously determined structures. Solving the structure of TOG2 bound to $\alpha\beta$ -tubulin may reveal a mechanism into how CLASP associate with tubulin through different modes of interactions which contributes to CLASP's ability to regulate MT dynamics.

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Tables

Table 2-1. Data Collection and Refinement Statistics for human CLASP1 TOG2

Data Collection		
Wavelength (Å)	0.980377	
Space group	$P2_{1}2_{1}2_{1}$	
Cell dimensions: a,b,c (Å)	62.3, 66.5, 138.9	
Resolution (Å)	20.0-2.00 (2.07-2.00)	
# Reflections: Measured / Unique	68,739 (4,858) / 10,919 (1,278)	
Completeness (%)	92.3 (64.9)	
Mean redundancy	6.3 (3.8)	
<i σi=""></i>	16.7 (3.9)	
R _{sym} ^a	0.09 (0.278)	
Refinement		
Resolution (Å)	20.0-2.00 (2.05-2.00)	
$\mathbf{R}^{\mathbf{b}} / \mathbf{R}_{\text{free}} (\%)^{\mathbf{c}}$	18.3 (22.3) / 21.0 (28.7)	
# Reflections, R/R _{free}	34474 (1525) / 1960 (79)	
Total atoms: Protein / Water	3,912 / 377	
Stereochemical ideality (rmsd): Bonds /	0.014 / 1.15	
Angles (Å/°)		
Mean B-factors (Å ²): Overall / Protein /	34.8 / 34.1 / 41.3	
Water		
Ramachandran Analysis: Favored /	99.2 / 0.8	
Allowed (%)		

^a $R_{sym} = \sum_h \sum_i |I_i(h) - \langle l(h) \rangle | / \sum_h \sum_i I_i(h)$ where $I_i(h)$ is the integrated intensity of the *i*th

reflection with the Miller Index *h* and $\langle I(h) \rangle$ is the average over Friedel and symmetry

equivalents.

^{**b**} R value = $\Sigma(|F_{obs}| - k|F_{calc}|) / \Sigma|F_{obs}|$.

^c R_{free} is calculated using a 5% subset of the data that are removed randomly from the original data and excluded from refinement.

Table 2-S1. Mitotic spindle morphology in *Drosophila* S2 cells transfected with MAST constructs

Construct	dsRNA	% Bipolar Biastral (Std. Dev; Std. Error)	% Bipolar Monoastral (Std. Dev; Std. Error)	% Monopolar Monoastral (Std. Dev; Std. Error)
Not transfected	control	76 (4; 3)	10 (4; 2)	14 (5; 3)
eGFP	control	72 (4; 3)	11 (3; 2)	17 (2; 1)
FL MAST	control	47 (5; 3)	9 (4; 2)	44 (8; 5)
Not transfected	MAST 5'UTR	14 (3; 2)	14 (5; 3)	72 (3; 2)
eGFP	MAST 5'UTR	17 (8; 4)	12 (4; 2)	71 (6; 4)
FL MAST	MAST 5'UTR	42 (3; 2)	12 (4; 2)	46 (4; 2)
ΔTOG2	MAST 5'UTR	12 (2; 1)	9 (6; 4)	79 (4; 2)
W334E	MAST 5'UTR	17 (4; 2)	8 (5; 3)	68 (5; 3)
F298A	MAST 5'UTR	35 (3; 2)	11 (6; 3)	54 (9; 5)
F298E	MAST 5'UTR	24 (4; 2)	10 (2; 1)	66 (6; 3)
F302A	MAST 5'UTR	43 (2; 1)	8 (3; 2)	49 (5; 3)
F302E	MAST 5'UTR	17 (3; 2)	10(1;1)	73 (4; 2)
F298A/F302A	MAST 5'UTR	21 (3; 2)	8 (4; 2)	71 (5; 3)
F298E/F302E	MAST 5'UTR	24 (2; 1)	8 (5; 3)	68 (5; 3)

Table 2-S2. Data Collection and Refinement Statistics for human CLASP1 TOG2 solved to 1.8Å resolution.

Data Collection	
Wavelength (Å)	1.0000
Space group	P2 ₁
Cell dimensions: a,b,c (Å)	51.6, 67.4, 81.0
Resolution (Å)	50.0-1.78 (1.84-1.78)
# Reflections: Measured / Unique	319,655 (19,398) / 51,928 (4,492)
Completeness (%)	98.1 (85.4)
Mean redundancy	6.8 (4.3)
<i σi=""></i>	15.1 (2.8)
$\mathbf{R}_{sym}^{\mathbf{a}}$	0.95 (0.278)
Refinement	
Resolution (Å)	38.1-1.78 (1.83-1.78)
$\mathbf{R}^{\mathbf{b}} / \mathbf{R}_{\text{free}} (\%)^{\mathbf{c}}$	17.6 (29.8) / 21.7 (38.3)
# Reflections, R/R _{free}	48318 (2654) / 1874 (114)
Total atoms: Protein / Water	3,920 / 678
Stereochemical ideality (rmsd): Bonds /	0.0073 / 0.96
Angles (Å/°)	
Mean B-factors (Å ²): Overall / Protein /	29.1 / 27.1 / 40.2
Water	
Ramachandran Analysis: Favored /	99.2 / 0.8
Allowed (%)	

^a $R_{sym} = \Sigma_h \Sigma_i |I_i(h) - \langle l(h) \rangle | / \Sigma_h \Sigma_i I_i(h)$ where $I_i(h)$ is the integrated intensity of the *i*th reflection with the Miller Index *h* and $\langle I(h) \rangle$ is the average over Friedel and symmetry equivalents.

^b R value = $\Sigma(|F_{obs}| - k|F_{calc}|) / \Sigma|F_{obs}|$.

^e R_{free} is calculated using a 4% subset of the data that are removed randomly from the original data and excluded from refinement.

Supplemental Figures



Figure 2-S1, related to Figure 2-1. CLASP Family Members are Composed of an Array of Conserved Cryptic TOG Domains. Sequence alignment of TOG2 from diverse CLASP family members. Identity and similarity are highlighted in green and yellow respectively, each contoured at 80%. Amino acid numbering corresponds to human CLASP1. Secondary structure elements are shown above the alignment. Solvent accessible surface area (SASA) for each residue is plotted below the alignment. The sequences of the Drosophila Msps TOG2 intra-HEAT loops are aligned to the corresponding intra-HEAT loops of CLASP TOG2. Species abbreviated: Hs: Homo sapiens, XI: Xenopus laevis, Dm: Drosophila melanogaster, Sc:Saccharomyces cerevisiae, Sp: Schizosaccharomyces pombe.



Figure 2-S2, related to Figure 2-1. CLASP TOG2 Protomers Align Well and Form Minimal Contacts With Symmetry Mates. (A) CLASP TOG2 protomers A and B aligned using the Dali Pairwise server with an overall C α rmsd equal to 0.5 Å across 243 residues. Protomers A and B are shown as $C\alpha$ traces, with protomer A colored in a spectrum as in Figure 2-1C and protomer B colored light green. The image at top is rotated 90° about the xaxis to generate the image below. (B) CLASP TOG2 protomers A and B as shown in (A) except that each protomer is colored according to its Ca B-factor (see color key). The domain's flanking HEAT repeats (HR A and HR F) show higher B-factors than the central HEAT repeats (HR B – HR E) where the domain's bend occurs. (C-D) Crystallographic interactions between CLASP TOG2 protomers A and B with symmetry mates. Protomers A and B have a 731 Å2 interface while interfaces formed between either protomer A or B with crystallographic symmetry mates form smaller interfaces ranging from 494 Å2 to 15 Å2. Packing interactions occur primarily at the two ends of the TOG domain and not at the location of the domain's bend. The image in (D) is generated by rotating the image in (C) 40° about the x-axis. Protomers A and B are colored in a spectrum as in Figure 2-1C and symmetry mates are shown in light grey.



Figure 2-S3. Second crystal structure of CLASP1 TOG2 at 1.8Å resolution. (A) A higher-resolution structure of CLASP1 TOG2, also composed of HR A-F, was solved to 1.8Å resolution. (B) The higher-resolution structure is nearly identical to the original CLASP1 TOG2 structure (PDB id: 4K92) with an r.m.s.d. of 0.5 Å.

CHAPTER 3: Expression, Purification, and Crystallization of TOG1 and cryptic-TOG3 Domains from Human CLASP1

Summary

The CLASP family of proteins is a conserved class of plus-end tracking proteins that promote MT stabilization. Mutations in CLASP proteins cause highly dynamic microtubules and aberrant monopolar spindle morphologies. CLASP consists of an N-terminal TOG domain and two cryptic TOG domains that have been predicted to underlie CLASP's ability to regulate MT dynamics. In this chapter, we report on the expression, purification, and preliminary crystallographic analysis of hCLASP1 TOG1 and cryptic-TOG3 (crTOG3). With the reported crystal structures of MAST/Orbit TOG1 and hCLASP1 TOG2, the crTOG3 is the only position in the TOG array where a structure has not been determined. The crTOG3 protein was cloned, expressed and purified and initial conditions that yielded crTOG3 crystals were identified. In addition, hCLASP1 TOG1 was cloned, expressed, crystallized, and diffracted to 2.1-3.5 Å resolution. Attempts at obtaining phasing information of TOG1 using single-anomalous dispersion and molecular replacement were unsuccessful. Our work is still ongoing, and we plan on determining the crystal structures of hCLASP1 TOG1 and crTOG3 to elucidate structural and mechanistic insights into the role of arrayed TOG domains in CLASP's ability to regulate microtubule dynamics.

Experimental Procedures

Cloning and Expression

Human CLASP1 TOG1 (residues 1-257) and TOG3 (843-1092) were subcloned into pET28 (Novagen), generating a thrombin-cleavable N-terminal hexa-histidine tag. CLASP1 TOG1 was transformed into BL21 DE3 (pLysS) *E. coli*, and grown in 6 liters of Luria Broth (50 μ g/l kanamycin) at 37° C to a 0.8 optical density at 600 nm. The temperature was lowered to 18° C and protein expression induced with 100 μ M IPTG (final concentration) for 16 hours. CLASP1 TOG3 was transformed into Rosetta 2 DE3 (pLysS) *E. coli*, and grown in 6 liters of Luria Broth (50 μ g/l kanamycin and 34 μ g/l chloramphenicol) at 37° C to a 0.8 optical density at 600 nm. The temperature was 10 hours. CLASP1 TOG3 was transformed into Rosetta 2 DE3 (pLysS) *E. coli*, and grown in 6 liters of Luria Broth (50 μ g/l kanamycin and 34 μ g/l chloramphenicol) at 37° C to a 0.8 optical density at 600 nm. Cells were harvested at 2100 x g for 10 minnutes, resuspended in 250 ml buffer A (25 mM Tris pH 8.0, 300 mM NaCl, 10 mM Imidazole, 0.1% β -ME) and stored at -20° C. To produce selenomethionine-substituted CLASP1 TOG1, expression was conducted in the B834 methionine auxotrophic *E. coli* strain, grown in L-selenomethionine-containing minimal media as previously described (Leahy et al., 1994).

Protein Purification

Native and selenomethionine-substituted CLASP1 TOG1 cell pellets were lysed by sonication with the addition of 0.5 mM Phenylmethylsulfonyl fluoride. After lysis, additional Phenylmethylsulfonyl fluoride was added to 1mM final concentration and the lysate was clarified at 23,000 x g for 45 min. Supernatant was loaded onto a 5 mL Ni²⁺-NTA column (Qiagen), washed with 500 ml buffer A and protein eluted over a 250 ml linear gradient between buffer A and B (buffer B = buffer A supplemented with 290mM Imidazole). TOG1-containing fractions were pooled and CaCl₂ added to 1 mM. The hexahistidine-tag was cleaved using 0.1 mg bovine α -thrombin overnight at 4° C. Digested protein was filtered over

0.5 ml Benzamadine sepharose (GE Healthcare), concentrated in a Millipore Ultrafree 10,000 MWCO concentrator, and exchanged into buffer C (25 mM Tris pH 8.0, 0.1 % β-ME). Protein was loaded onto a 10 ml Q-sepharose fast flow column (GE Heathcare), washed with 200 ml buffer C and eluted over a 250 ml linear gradient between buffer C and D (buffer D =buffer C supplemented with 1 M NaCl). Protein fractions were pooled, concentrated to 50 mg/ml, exchanged into storage buffer (10 mM Tris pH 7.0, 150mM NaCl, 0.1 % β -ME) using a Millipore Ultrafree 10,000 MWCO concentrator, frozen in liquid nitrogen and stored at -80° C. Purification of selenomethionine-substituted protein proceeded according to the above protocol, except buffers were supplemented with 5 mM L-methionine. Human CLASP TOG3 was purified using a protocol similar to CLASP1 TOG1 except that pooled CLASP TOG3 fractions were not digested with bovine α -thrombin, leaving the N-terminal hexahistidine tag intact. Protein was exchanged into buffer C (25 mM Tris pH 7.0, 0.1 % β-ME). Protein was loaded onto a 10 ml SP-sepharose fast flow column (GE Heathcare), washed with 200 ml buffer C and eluted over a 250 ml linear gradient between buffer C and D (buffer D = buffer C supplemented with 1 M NaCl). Protein was exchanged into storage buffer in a Millipore Ultrafree 10,000 MWCO concentrator to 80 mg/ml, frozen in liquid nitrogen, and stored at -80° C.

Crystallization

Initial screening of crystallization conditions was carried out by hanging-drop vapordiffusion method using HR-126 sparse matrix and HR-110 PEG/Ion commercial screens (Hampton), as well as custom PEG/ion, ammonium sulfate, and sodium malonate screens. Crystallization droplets contained 2µL of 15mg/ml protein solution mixed with 2µL reservoir solution and were equilibrated against 1ml reservoir solution in a 24-well plate at 18° C.

CLASP1 TOG1 crystals appeared within 1-2 days under from the following separate well conditions: 1) 2.0M Ammonium sulfate, and 2) 1.5M Sodium malonate pH 6.25. The Sodium malonate condition yielded crystals sufficient for diffraction. Additional screening of crystallization conditions for CLASP1 TOG1 was carried out by the sitting-drop vapordiffusion method using an automated Phoenix crystallization robot (Art Robbins Instruments) and JCSG suite (Qiagen). The crystallization droplets contained 200 nl protein solution mixed with 100 nl reservoir solution and were equilibrated against 100 μ l reservoir solution in a 96-well Intelliplate (Art Robbins Instruments) at 20°C. Crystals appeared after three days from a well condition containing 0.1M MES pH 6, 30% PEG 600, 5%(w/v) PEG 1000, and 10% glycerol. The volume of crystallization droplets was scaled up to contain $2\mu L$ protein solution mixed with 2µL reservoir solution and were equilibrated against 1ml reservoir solution in a 24-well Linbro plate (Hampton). Refinement of this condition consisting 0.1M MES pH 6.0, 30% PEG 600, and 10% glycerol produced crystals large enough for diffraction. Crystals were flash-frozen in liquid nitrogen after soaking in a cryoprotectant solution consisting of Fomblin-Y for the first Sodium malonate condition and 0.1M MES pH 6.0, 30% PEG 600, and 20% glycerol for the second crystallization condition.

Initial crystallization conditions for CLASP1 TOG3 was carried out similar to CLASP1 TOG1 using the commercial Hampton screen previously mentioned. Several conditions containing potassium and PEG 3350 produced phase separation. Lowering the concentration of PEG 3350 yielded an optimized condition for crystallization (0.2M Potassium sodium tartrate tetrahydrate and 5-10% PEG 3350). Crystals appeared using drops containing 2 μ l of 5 mg/ml protein and 2 μ l of reservoir solution equilibrated against 1 ml reservoir solution at 20°C.

Heavy-atom and halide soaks

Mercury soaks of CLASP1 TOG1 crystals were prepared using the quick-soak method (Sun et al., 2002). Crystals were transferred into stabilization buffer containing 1.5M-1.75M Sodium malonate pH 6.25 with 10mM Mercury(II) acetate and soaked for 10-20 minutes. Bromide-derivatives of CLASP1 TOG1 crystals were prepared using an established halide soak protocol (Dauter et al., 2000) (Dauter and Dauter, 2007). Crystals were transferred into stabilization buffer containing 0.1M MES pH 6, 0.2M Sodium acetate, 30% PEG 600, 20% glycerol and 0.5M Sodium bromide and soaked for a range of 30 seconds to 1 minute. Both mercury- and bromide-soaked CLASP TOG1 crystals were flash frozen in liquid nitrogen.

Data Collection and Structure Determination Attempts

Native, selenium SAD peak, Hg peak, and Br peak data sets were collected on native, selenomethionine-substituted, mercury-soaked, and bromide-soaked CLASP1 TOG1 crystals respectively at the Advanced Photon Source 22-ID beamline at 100 K. Multiple data sets were processed using HKL2000 (Otwinowski and Minor, 1997). Attempts at obtaining SAD phasing angles were performed using PHENIX AutoSol (Adams et al., 2010). The CLASP1 TOG1 construct contains six endogenous methionines as well as a seventh N-terminal methionine that was introduced as a cloning artifact. Attempts at obtaining phasing angles through molecular replacement from a *Drosophila* MAST TOG1 crystal structure (PDB entry 4G3A; De la Mora-Rey et al., 2013) were performed using Phaser through AutoMR in the PHENIX software package (Adams et al., 2010; McCoy et al., 2007; Storoni et al., 2004; McCoy et al., 2005).

Results

Expression, Purification, and Crystallization of CLASP1 TOG1

Cloned constructs of CLASP1 TOG1 (residues 1-257) were expressed using a pET28 expression vector in *E. coli*. TOG1 protein was highly expressed in soluble form and purified to homogeneity after tandem purification of Ni²⁺-NTA and ion exchange column chromatography (Figure 3-1A,B and C). The yield of TOG1 protein was 11 mg per liter of culture. The high yield and homogeneity of TOG1 allowed us to obtain crystals using the hanging drop method. Native and selenomethionine-substituted CLASP1 TOG1 were crystallized in two conditions: 1) 1.5M Sodium malonate pH 6.25 (Figure 3-1D) and 2) 0.1M 2-(N-morpholino)-ethanesulfonic acid (MES) pH 6, 30% PEG 600, 10% glycerol. Crystals that grew under condition 1 were soaked in Mercury(II) acetate to produce Hg-derivatives of TOG1, while crystals that grew under condition 2 were soaked in Sodium bromide to produce Br-derivatives of TOG1.

Data collection and attempts at obtaining phasing information for CLASP1 TOG1

All of the crystals were sent to 22-ID beamline at the Advanced Photon Source (APS) synchrotron. One native TOG1 crystal grown from a condition containing 1.5M Sodium malonate pH 6.25 diffracted to a resolution of 2.0Å (Figure 3-1D, Table 3-1). However, assigning the correct space group proved to be difficult due to the primitive hexagonal space groups. Xtriage and CCP4 Pointless programs estimated the most likely space group as P622 (Zwart et al., 2005a; Zwart et al., 2005b; Evans, 2011). However, the assignment of this space group was not possible since the composition of a 25-kDa protein is too large for the higher symmetry of primitive hexagonal lattice. Consequently, lower symmetry space groups belonging to the primitive trigonal system, including P3, P3₁, and P3₂, were suspected as





possible space group assignments. Single-wavelength anomalous dispersion (SAD) on selenomethionine-substituted crystals was attempted to obtain the required phasing information for structure determination. A SeMet TOG1 crystal diffracted to a resolution of 2.5Å at the Se anomalous peak wavelength (Table 3-1). However, the anomalous signal was too weak for successful Se-SAD phasing. To collect a complete data set, I am currently reproducing the SeMet TOG1 crystals.

We then attempted to obtain SAD phasing from the mercury- and bromide-soaked TOG1 crystals. Mercury-soaked crystals were produced by soaking the crystal 1.5M Sodium malonate, supplemented with 10mM Merucry(II) acetate. Similarly, Br-soaked crystals were obtained by quickly soaking the crystal in 0.1M MES pH 6, 0.2M Sodium acetate, 30% PEG 600, 20% glycerol and 0.5M Sodium bromide. Data sets were collected at the corresponding absorption peaks for mercury and bromide. The mercury-derivative data set diffracted to 2.5Å was assigned to P3₁ or P3₂ space groups (Table 3-1). The Br-derivative data sets diffracted to 2.5Å and were assigned to the P2₁ space group (Table 3-1). Despite producing heavy atom and halide derivatives of CLASP TOG1 crystal, SAD phasing was unsuccessful to date.

During our attempts at SAD phasing, the structure of TOG1 from the *Drosophila* homolog MAST/Orbit was published (PDB: 4G3A; De la Morey et al., 2012). We attempted to obtain phasing information through molecular replacement by using MAST/Orbit TOG1 as the search model. However, molecular replacement yielded low-scoring solutions in P3, P3₁, P3₂, and in lower symmetry space groups including P2₁. Model building and refinement of these low-scoring solutions yielded high R factors, indicating the model built from the molecular replacement solutions was incorrect. Originally, we had suspected that a large

conformational rearrangement impeded a molecular replacement solution. As a result, the MAST/Orbit TOG1 structure was divided into smaller units to produce search models appropriate for the suspected conformational rearrangement. However, modifying the search model into smaller units did not produce a successful molecular replacement solution.

Since the search model for molecular replacement was the Drosophila homolog of CLASP1 TOG1 with 40% sequence identity, a successful molecular replacement solution should have been produced. However, obtaining a molecular replacement solution was unsuccessful. We suspect that crystal twinning added difficulty to assigning a correct space group and obtaining a molecular replacement solution. One clue for twinning was that Xtriage and Pointless identified P622 as the most likely space group, despite the large protein composition of TOG1 that would physically prevent formation into a primitive hexagonal lattice. One explanation for this incorrect space group assignment is psuedomerohedral twinning, which can arise if point-group symmetry is higher than the point-group symmetry of the crystal unit cell (Rudolph et al., 2004; Zwart et al., 2008; Kim et al., 2009). In the cases of Rosendal et al. and Barends et al., a particular case of pseudomerohedral twinning called tetrahedral twinning added 222 symmetry to the P3 space-group, resulting in apparent 622 point-group symmetry of the crystal (Rosendal et al., 2004; Barends et al., 2005). Considering that an apparent space group of P622 was assigned for our dataset, the CLASP1 TOG1 may have pseudomeroheral twin operators that appears as a P622 space group. Xtriage twinning analysis of CLASP TOG1 detected twinning through intensity statistics that were significantly different from expected values of untwined data.

Secondary structure prediction, purification and crystallization of cryptic TOG3

Secondary structure predictions of cryptic TOG3 (TOG3) spanning residues 843-



Figure 3-2. Predicted secondary structure of CLASP1 TOG3. Sequence alignment of TOG3 from diverse CLASP family members. Identity (green) and similarity (yellow) are contoured at 80% based on the alignment. Secondary structure elements are shown above the alignment.Amino acid numbering corresponds to human CLASP1. *Drosophila* Msps TOG2 intra-HEAT loop sequences are aligned. Species abbreviated: Hs: Homo sapiens, XI: Xenopus laevis, Dm: Drosophila melanogaster. Figure adapted from (Slep et al., 2008).

1087 identified a dodeca-helical domain that showed sequence conversation across several species (Figure 3-2). In addition, alternating loops within TOG3 exhibited high homology to intra-HEAT loops from XMAP215 TOG domains (Slep et al., 2007) (Figure 3-2). The characteristics of a dodeca-helical domain with conserved intra-HEAT loops were also identified in CLASP1 TOG2, which was shown to be a true TOG domain (Leano et al., 2013). In addition, tubulin-binding determinants previously identified in structural and biochemical studies of XMAP215 and CLASP TOG domains were present and conserved in the predicted intra-HEAT loops of TOG3 (Slep et al., 2007; Al-Bassam et al., 2010; Ayaz et

al., 2012; Leano et al., 2012). These tubulin-binding determinants located on TOG3 include: W869 on intra-HEAT loop A, V913 on intra-HEAT loop B, K958 on intra-HEAT loop C, K993 on intra-HEAT loop D, and R1040 on intra-HEAT loop E. The secondary structure prediction of a dodeca-helical domain and the appearance of conserved tubulin-binding determinants located on the intra-HEAT loops suggest that cryptic TOG3 is a TOG domain that binds to tubulin in a mechanism similar to CLASP TOG2 and XMAP215 TOG domains.

To determine if TOG3 is a true TOG domain and to characterize its binding mechanism with tubulin, we cloned, expressed, and purified a TOG3 construct in preparation for crystallization and subsequent X-ray crystallography studies (Figure 3-3). We amplified a construct of TOG3 (CLASP1 843-1092) from CLASP1 cDNA and cloned it into a pET28 *E. coli* expression vector. Similar to TOG1, the TOG3 protein was highly expressed in soluble form and purified to homogeneity after tandem purification of Ni²⁺-NTA and ion exchange column chromatography (Figure 3-3 A and B). The yield of TOG3 was 20 mg per liter of culture. During ion exchange purification, a fraction of TOG3 precipitated when the protein was exchanged into low salt buffer. TOG3 solubility increased when the protein was eluted from SP-sepharose column using high salt buffer. The TOG3 protein remained stable in buffers that contained a minimum of 150 mM NaCl.

When screening crystallization conditions for TOG3, several conditions that contained the potassium ion (i.e. Potassium chloride, Potassium acetate, Sodium Potassium Tartrate) and 20% PEG 3350 produced phase separation. Optimizing these potassium-based conditions by lowering the concentration of PEG 3350 yielded a crystallization condition that produced crystals. Optimizing the condition to 0.2M Sodium potassium tartrate and 5-10% PEG 3350 produced medium-sized crystals that grew within a week (Figure 3-3D).



Figure 3-3. Expression, purification, and crystallization of CLASP1 TOG1 (residues 843-1092). CLASP TOG3 eluted as a single peak during (A) Ni-NTA chromatography and (B) ion -exchange chromatography. (C) SDS-PAGE gel of purified CLASP TOG3. Molecular weight markers labeling sizes from 25-40kDa. Lane 1: Pre-induction cell lysate; Lane 2: Post-induction cell lysate; Lane 3: Post-induction supernatant; Lane 4: Post-induction cell pellet. Lanes 5-12 are Ni-NTA purified fractions containing purified TOG3. (D) Multiple crystals of CLASP TOG3 grown under saturating conditions of 200mM Sodium potassium tartrate and 5% PEG 3350.
Discussion

In an effort to elucidate the mechanism underlying CLASP's ability to regulate MT dynamics using its array of TOG domains, the work in this chapter provides preliminary steps toward characterizing CLASP1 TOG1 and TOG3 for future structural and biochemical studies. We have expressed and purified CLASP1 TOG1 and TOG3. We identified and optimized crystallization conditions that yield crystals for X-ray crystallography. The crystallography work is still ongoing since a structure of human CLASP1 TOG1 or TOG3 has not been determined. TOG1 crystals were readily produced under multiple crystallization conditions, but obtaining phasing information using SAD and molecular replacement has not produced a viable solution to date. One explanation for the difficulty in determining the TOG1 structure is that the crystal lattice exhibits psuedomerohedral twinning, adding difficulty in producing a molecular replacement solution. Additional screening and optimization of crystal condition that will not produce twinning is ongoing. In addition, crystals of TOG3 will be sent to 22-ID at APS for data collection. Determining the crystal structures of TOG1 and TOG3 will provide structural insights into how these individual TOG domains bind to tubulin. Comparing these structures to CLASP1 TOG2 will provide mechanistic insights into how each of CLASP's TOG domain collectively work in an array to modulate MT dynamics.

CLASP TOG1 is a peculiar TOG domain in comparison to other TOG domains found in CLASP and XMAP215. *In vitro* studies of TOG1 in *Drosophila* MAST/Orbit and yeast Stu1p reveal that TOG1 alone does not bind to $\alpha\beta$ -tubulin and microtubules through MT cosedimentation and co-immunoprecipitation (De la Mora-Rey et al., 2013; Funk et al., 2014). Instead, TOG1 domain has been shown to interact with actin filaments. Tsvetkov et al.

demonstrated that CLASP1a and CLASP2a isoforms co-immunoprecipited with actin, CLASP2a co-localized with actin stress fibers, and exhibited retrograde flow with actin at the leading edges of motile cells (Tsvetkov et al., 2007). The crystal structure of MAST/Orbit TOG1 resembles a canonical TOG domain, displaying a straight tubulin-binding architecture that is found in other XMAP215 TOG domains. However, sequence analysis of predicted intra-HEAT loops located in TOG1 indicates that conserved tubulin-binding determinants found in other CLASP and XMAP215 TOG domains are not present (Funk et al., 2014). In particular, the invariant tryptophan in HEAT loop A has been replaced with smaller hydrophobic residues (Slep et al., 2007; Slep et al., 2009). In addition, the shortest isoform of CLASP2 (CLASP2) lacks TOG1 domain, but efficiently localizes with growing MT plus ends and stabilizes MTs in neurons (Akhmanova et al., 2001). These findings suggest that the TOG1 domain alone may not contribute to the regulation of MT dynamics but may act to facilitate actin-microtubule cross-linker. Although a crystal structure of MAST/Orbit TOG1 has been published, a crystal structure of CLASP TOG1 alone and in complex with actin can provide insights into TOG-actin interactions. Whether actin interacts with the face consisting of intra-HEAT loops or if it interacts with another face of the TOG domain would be an interesting question to answer. Although TOG1 alone does not associate with microtubules, constructs consisting of TOG1 and TOG2 bind soluble $\alpha\beta$ -tubulin, associate with the MT lattice, and recruit tubulin to the MT lattice to promote rescue (Al-Bassam et al., 2010). These findings suggest that TOG1 interacts with microtubules in the presence of TOG2. However, this conflicts with the studies suggesting that TOG1 can interact with actin. One hypothesis that can reconcile these two sets of findings is that TOG1 can interact with both microtubules and actin. Whether TOG1 binds tubulin and/or actin, and whether binding these

cytosekeletal components is mutual or exclusive remains to be determined. The interchangeable actin- and microtubule-binding activity may be determined by particular spatial and temporal situations. Understanding why TOG1 can bind to actin and microtubules would be a new, interesting aspect of CLASP to discover. Future studies include determining the crystal structure of TOG1 and correlating structural findings with biochemical and cellular assays to help uncover the peculiar role of CLASP TOG1 in cytoskeletal network association and regulation of dynamics.

Although TOG3 has been implicated as a conserved microtubule-binding domain, whether TOG3 is structurally a true TOG domain remains to be determined. Secondary structure prediction suggests a dodeca-helical domain with conserved tubulin-binding determinants found in other TOG domains. Determining the crystal structure of CLASP1 TOG3 will reveal if the domain adopts the straight architecture found in canonical TOG domains or if it adopts a curved architecture similar to CLASP1 TOG2. Comparing the predicted secondary structure of TOG3 with the known secondary structure of TOG2 offers a clue in determining which architecture TOG3 will adopt. In contrast to the HR C anti-parallel helices found in canonical TOG domains, the CLASP1 TOG2 HR C helices are angled relative to one another due the absence of bulky, buried hydrophobic side chains at the $\alpha 2C'$ N-terminal region. The differential conformation in HRC causes the second HR triad to rotate down, causing TOG2 to adopt the curved architecture (Leano et al., 2013). Secondary structure prediction at the $\alpha 2C'$ N-terminal region of TOG3 reveal the presence of buried hydrophobic side chains, suggesting that HRC consists of anti-parallel helices found in XMAP215 TOG domains (Figure 3-S1). This analysis predicts that TOG3 will adopts the canonical, straight architecture. Previous studies have shown that constructs containing

TOG3 and the linker region between TOG3 and CTD display MT lattice binding activity in growth cone and lamella microtubules (Wittmann and Waterman-Storer, 2005; Kumar et al., 2009; Hur et al., 2011). These TOG3-containing protein constructs decorate and stabilize the MT lattice in the leading edge of migrating cells. These findings suggest that TOG3 serves as a MT-lattice binding domain and is important for promoting MT stability during cell migration and motility.

Although the crystal structure of MAST/Orbit TOG1 and human CLASP1 TOG2 have been determined, crystal structure of TOG3 is still unknown. Therefore, structural insights into the TOG array as a whole has yet to be determined. As mentioned previously, secondary and tertiary structure prediction suggests TOG3 adopts a straight tubulin-binding architecture similar to TOG1. In addition, the MT-lattice binding activity of TOG3 plays an important role in adding stability in MT promoting cell protrusion in the leading edge of motile cells. This information allows us to speculate on a possible model for the role of CLASP's arrayed TOG domain in the regulation of MT dynamics (Chapter 5; Figure 5-1). We hypothesize that TOG3 binds to the MT lattice towards the plus end of a depolymerizing microtubule. TOG2 binds to the curved protofilament of a depolymerizing microtubule and stabilizes it, possibly induced MT pause state. TOG1 binds to non-polymerized tubulin to incorporate it into the MT lattice, inducing rescue and promoting MT growth. In the case of motile cells, CLASP TOG3 decorates and stabilizes the MT lattice in leading edge. This allows TOG1 to serve as an actin-microtubule cross-linker and coordinate movements between actin and microtubule network to promote cell motility and migration. Determining why CLASP is needed to crosslink actin and microtubules in leading edge of a motile cell would be an interesting question to answer for future studies.

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Tables

Table 3-1. Data colle	ection on native and r	nulti-derivative cryst	tals of CLASP1 TOG1.
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Crystal Space Group Unit Cell	Native P31 ^ª		SeMet P2 ₁		Mercury (Hg) P3 ₁ ª	Bromide (Br) P2 ₁	
a, b, c (Å)	38.0, 139.7	38.0,	47.0, 82.6	113.9,	37.5, 37.5,	40.1, 47.9	114.1,
Wavelength (Å) Resolution (Å) No. observations: measured / unique	1.000 50.0-2.50 45388/15	355	0.979 50.0-2.9 94592/34	0 4843	1.008 50.0-2.14 67025/12020	0.919 50.0- 2.3 39217/1	39 5172
Mean redundancy Completeness	3.0 99.6		5.3 91.3		5.6 99.9	2.6 88.3	
(/ο) I/σ R _{sym} ^b	21.0 0.075		9.07 0.209		14.8 0.127	7.08 0.162	

^aBest guessed space group. One of the possible space groups in primitive hexagonal lattice.

 ${}^{b}R_{sym} = \Sigma_h \Sigma_i |I_i(h) - \langle I(h) \rangle| / \Sigma_h \Sigma_i I_i(h)$ where $I_i(h)$ is the integrated intensity of the *i*th reflection with the Miller Index *h* and $\langle I(h) \rangle$ is the average over Friedel and symmetry equivalents.



Figure 3-S1. CLASP1 TOG3 HR C is predicted to consist of anti-parallel helices. MAST/Orbit TOG1 (grey) contains buried, hydrophobic side chains that force HR C to adopt anti-parallel helices. CLASP1 TOG2 (green) HR C does not contain hydrophobic side chains along the α C' helix. As a result, α C and α C' helices are angled relative to each other. This local arrangement facilitates the bent architecture of CLASP 1 TOG2. The predicted CLASP1 TOG3 HR C contains several buried, hydrophobic residues and also contains a large polar residue (Q116). These residues facilitate HR C helices to arrange in anti-parallel fashion. This suggests that TOG3 adopts a straight architecture across the tubulin-binding surface, similar to the arrangement of HR C found in XMAP215 TOG domains.

CHAPTER 4: CLASP C-terminal Domain interacts with CLIP-170 Coiled-coil Domain

Summary

The C-terminal domain of CLASP mediates CLASP homodimerization and associates with other factors to recruit CLASP to different locations in the cell. Structural and mechanistic insights into how CLASP C-terminal promotes homodimerization and interacts with other factors are poorly understood. In this chapter, we report preliminary work characterizing CLASP dimerization and the interaction between CLASP C-terminal domain (CTD) and CLIP-170 coiled-coil (CC) domain using in vitro binding assays and X-ray crystallography. We have further mapped the interaction of CLASP CTD to a \sim 125 residue region of the CLIP-170 coiled coil domain. ITC and SEC-MALS assays reveal CLASP CTD and CLIP-170 CC to form a weakly interacting complex. In addition, SEC-MALS analysis showed CLASP CTD to be a monomer, suggesting that CLASP CTD is necessary but not sufficient for CLASP dimerization. Crystals consisting of CLASP CTD and CLIP-170 CC alone and in complex were obtained, but neither diffracted. Our preliminary findings provide initial insight into understanding the role of CLASP C-terminal in promoting CLASP dimerization and interacting with other factors, but additional work is required to understand a domain of CLASP that is not well characterized through structural and biochemical studies.

Experimental Procedures

Cloning and Expression

Various constructs of human CLASP1 C-terminal domain (residues 1171-1538; residues 1253-1522; residues 1270-1538; residues 1302-1522) were subcloned into a customized pET28 (Novagen), generating a Prescission protease-cleavable N-terminal H6 tag. CLASP1 C-terminal domain constructs were transformed into BL21 DE3 (pLysS) E. coli, and grown in 6 liters of Luria Broth (50 µg/l kanamycin) at 37° C to an optical density at 600 nm of 0.8. The temperature was lowered to 18° C and protein expression induced with 100 µM IPTG (final concentration) for 16 hours. Cells were centrifuged at 2100 x g for 10 min and pellets resuspended in 250 ml buffer A (25 mM Tris pH 8.0, 300 mM NaCl, 10 mM Imidazole, 0.1% β-ME) and stored at -20° C. One of the CLASP1 C-terminal domain constructs (1253-1522) was selected to produce selenomethionine-substituted CLASP1 Cterminal domain protein (CTD). Selenomethionine-substituted CLASP1 CTD protein expression was conducted in the B834 methionine auxotrophic E. coli strain, grown in Lselenomethionine-containing minimal media as described (Leahy et al., 1994). Four constructs of human CLIP-170 (residues 277-588; residues 331-588; residues 331-418; and residue 331-456) were cloned into pET28 and expressed similar to CLASP1 CTD, except they were transformed into Rosetta2 DE3 (pLysS) cells (Novagen), and grown in 6 liters of Luria Broth (50 µg/l kanamycin and 34 µg/l chloramphenicol).

Protein Purification

Native and selenomethionine-substituted CLASP1 CTD cell pellets were thawed, 0.5 mM Phenylmethylsulfonyl fluoride added and the cells lysed by sonication. After lysis, additional Phenylmethylsulfonyl fluoride was added to 1mM final concentration and the

lysate was centrifuged at 23,000 x g for 45 min. Supernatant was loaded onto a 10 ml Ni2+-NTA column (Qiagen), washed with 500 ml buffer A and TOG2 eluted over a 250 ml linear gradient between buffer A and B (buffer B = buffer A supplemented with 290mM Imidazole). CTD-containing fractions were pooled and 500µg of Prescission protease (GE Healthcare) was added to cleave off the H6-tag, leaving a three-residue N- terminal GPM cloning artifact. Prescission protease digest proceeded for 16 hours at 4° C. Digested protein was filtered over 0.5 ml glutatione S-transferase (GST) column (GE Healthcare), concentrated in a Millipore Ultrafree 10,000 MWCO concentrator, and exchanged into buffer C (25 mM Tris pH 8.0, 0.1 % β-ME). Protein was loaded onto a 10 ml Q-sepharose fast flow column (GE Heathcare), washed with 200 ml buffer C and eluted over a 250 ml linear gradient between buffer C and D (buffer D = buffer C supplemented with 1 M NaCl). Protein factions were pooled, concentrated and exchanged into 10 mM Tris pH 8.0, 150mg/ml NaCl, 0.1% β-ME in a Millipore Ultrafree 10,000 MWCO concentrator to 50 mg/ml, frozen in liquid nitrogen and stored at -80° C. Purification of selenomethionine-substituted protein (CLASP1 1253-1522) proceeded according to the above protocol. Human CLIP-170 coiledcoil domains were purified using a protocol similar to CLASP1 C-terminal domain except that CLIP-170 coiled-coil domain constructs were exchanged into anion exchange chromatography buffer C (25mM Tris pH 7.3, 0.1 % β-ME) and eluted with buffer D (25mM Tris pH 7.3, 0.1 % β-ME, 1M NaCl).

Crystallization and Data Collection

Initial screening of crystallization conditions was carried out by hanging-drop vapordiffusion method using HR-126 sparse matrix and HR-110 PEG/Ion commercial screens (Hampton). Crystallization droplets contained 2µL of protein solution at various

concentrations mixed with 2µL reservoir solution and were equilibrated against 1ml reservoir solution in a 24-well plate. Human CLIP-170 coiled-coil domain (residues 331-456) crystals were grew under 0.1M Sodium cacodylate pH 6.35, 1.4M Sodium acetate, 20°C at a protein concentration of 5mg/ml. Native crystals of human CLASP C-terminal domain (residues 1253-1522) were produced via hanging drop method using 0.2M Sodium acetate, 0.2M Sodium potassium tartrate, 12% PEG 3350, 20°C at a protein concentration of 2mg/ml. Crystals consisting of selenomethionine-substituted human CLASP C-terminal domain (1253-1522) and human CLIP-170 coiled-coil domain (331-456) were produced via hanging drop method: 2 µl of 2 mg/ml protein plus 2 µl of a 1 ml well solution containing 0.1M MES pH 6, 3% PEG 3350, 1mM Copper(II) chloride, 20°C. CLIP-170 crystals, native CLASP1 Cterminal domain crystals, and selenomethionine-substituted CLASP1 C-terminal domain (SeMET CLASP CTD) in complex with CLIP-170 coiled-coil domain (CLIP-170 CC) crystals were sent to the Advanced Photon Source 22-ID beamline. Crystals were screened for diffraction using an X-ray beam wavelength of 1.0Å at a temperature of 100 K. Isothermal Titration Microcalorimetry

Isothermal titration calorimetry experiments were performed in 25 mm HEPES, pH 6.8, 50 mm NaCl, and 0.1% β -mercaptoethanol on a MicroCal AutoITC200 (GE Healthcare). 20 × 2- μ l injections of 1.25 mM or 1mM human CLASP1 CTD (residues 1253-1522) were automatically injected into 200 μ l of 50 or 100 μ m CLIP-170 CC (residues 331-456). Binding isotherms were analyzed using the Origin 7.0 software package (OriginLab) and were fit to a one-site binding model.

Size Exclusion Chromatography and Multi-angle Light Scattering

Three separate proteins solutions were prepared in 25 mm HEPES pH 6.8, 150mM NaCl, 0.1% β -mercaptoethanol, and 0.2 g/liter sodium azide: 1) 161 μ M of CLASP CTD (residues 1253-1522), 2) 327 μ M of CLIP-170 CC (residues 331-456), and 3) mixture of 161 μ M of CLASP CTD and 327 μ M of CLIP-170 CC. 100 μ l of each prepared protein solution was injected onto a Wyatt WTC030S5 silicone size exclusion column (for elution of 5,000– 1,250,000-Da proteins) in 25 mm HEPES pH 6.8, 150mM NaCl, 0.1% β -mercaptoethanol, and 0.2 g/liter sodium azide and passed in tandem through a Wyatt DAWN HELEOS II light scattering instrument and a Wyatt Optilab rEX refractometer. The light scattering and refractive index data were used to calculate the weight-averaged molar mass and the mass fraction in each peak using the Wyatt Astra V software program (Wyatt Technology Corp.) (Wyatt, 1993).

Results

Cloning, Expression, and Purification of CLASP1 C-terminal domain and CLIP-170 coiledcoil domain

Various constructs of CLASP1 C-terminal domain (CTD) and CLIP-170 coiled-coil (CC) domains, ranging in different amino acid lengths, were cloned, expressed, and purified for binding assays that mapped the interaction between CLASP1 and CLIP-170 (Figure 4-1). For CLASP1 CTD domains, four constructs were designed: 1) residues 1171-1538, 2) residues 1253-1522, 3) residues 1270-1538, 4) residues 1302-1522. With CLIP-170 CC domains four constructs were designed: 1) residues 331-588 3) residues 331-456, 4) residue 331-418. Out of the CLASP1 CTD domains, CLASP1 residues 1171-



Figure 4-1. Various constructs of CLASP C-terminal domain and CLIP-170 coiled-coil domain. CLIP-170 is a well-characterized binding partner to CLASP. CLIP-170 consists of two CAP-gly domains that interact with EB1 to localize to the MT plus end. CLIP-170 also contains a coiled-coil domain that interacts with CLASP CTD. Several constructs of both CLASP CTD and CLIP-170 CC were made to screen for compatible binding partners. CLASP 1253-1522 and CLIP-170 331-456 were selected for further binding studies.

1538 degraded during protein purification. Constructs consisting of CLASP1 residues 1253-1522, residues 1270-1538, and residues 1302-1522 were highly expressed in soluble form and purified to homogeneity after tandem purification of Ni²⁺-NTA and ion exchange column chromatography (Figure 4-2A and B; Figure 4-S1). The yields for CLASP1 CTD constructs were: 1) residues 1253-1522, 16.5mg per liter of cell culture, 2) residues 1270-1538, 7mg per liter of cell culture, and 3) residues 1302-1522, 0.5mg per liter of cell culture. For CLIP-170 coiled-coil domains, CLIP-170 residues 331-418 and 331-456 highly expressed in soluble form and purified to homogeneity after tandem purification of Ni²⁺-NTA and ion exchange column chromatography (Figure (4-3A,B and C). The yields for CLIP-170 CC constructs were: 1) residues 277-588, 0.5mg per liter of cell culture, 2) residues 331-418, 0.8mg per liter of cell culture, and 3) residues 331-456, 4.2mg per liter of cell culture.



Figure 4-2. Expression, purification, and crystallization of CLASP1 CTD (residues 1253-1522). CLASP CTD eluted as a single peak during (A) Ni-NTA chromatography and (B) anion-exchange chromatography. (C) SDS-PAGE gel of purified CLASP CTD after Ni-NTA²⁺ affinity chromatography. Molecular weight markers labeling sizes between 25 and 40kDa. Lanes 1-12 are fractions corresponding to the purification peak in (A). Lanes 13 and 14 are post-induction cell lysate and supernatant, respectively. (D) SDS-PAGE gel of purified CLASP CTD after ion exchange chromatography.Molecular weight markers labeling sizes between 25 and 35kDa. Lane 1: pre-treatment of Prescission protease; Lane 2: post-treatment of Prescission protease. Lane 3-14 are purified fractions of CLASP CTD corresponding to the chromatogram in (B). (D) Crystals of CLASP CTD and CLIP-170 CC grown under saturating conditions of 200mM MES pH 6, 200mM sodium acetate, 1mM Copper chloride(II), and 3% PEG 3350. (F) Low-resolution (~6Å) diffraction of CLASP CTD.



Figure 4-3. Expression, purification, and crystallization of CLIP-170 coiled coil domain constructs. Ion-exchange chromatograms of (A) CLIP-170 residues 331-456 and (B) CLIP-170 residues 331-418. (C) SDS-PAGE gel of purified CLIP-170 residues 331-418: lanes 1-7 and CLIP-170 residues 331-456: lanes 8-14. (D) Needle-like crystals of CLIP-170 residues 331-456 that were too small for diffraction.

Binding analysis of CLASP1 C-terminal domain and CLIP-170 coiled-coil domain

Native gel electrophoresis was used as an initial assay to characterize CLASP CTD-

CLIP-170 CC interactions. Various constructs of CLASP1 CTD were mixed and incubated at

different ratios with CLIP-170 331-456 before electrophoresis. Out of the constructs,

CLASP1 residues 1253-1522 and CLIP-170 residues 331-456 at a minimum ratio of 1:2

shifted in electrophoretic mobility (Figure 4-S1). This shift suggested that CLASP1 residues

1253-1522 and CLIP-170 residues 331-456 were potential candidates for further binding

studies.

To determine binding affinities between the selected candidates of CLASP1 CTD and CLIP-170 CC, isothermal titration calorimetry was performed. CLASP 1253-1522 was titrated into a calorimetric cell containing CLIP-170 331-456. Two separate binding experiments were performed, both showing an exothermic binding isotherms which was fitted into a one-site binding model. The binding affinity (K_D) calculated from the first was 23µM (Figure 4-4A). However, the binding curve calculated from the binding isotherm did not reach saturation, suggesting the binding affinity may be higher than the apparent K_D . When protein concentrations of CLASP CTD and CLIP-170 CC were adjusted and optimized in the second experiment, the binding isotherm reached saturation and the binding affinity was calculated to 7.1µM (Figure 4-4B).

To further characterize the interaction between CLASP1 CTD and CLIP-170 CC and to determine their oligomeric states, we utilized size-exclusion chromatography coupled with multi-angle light scattering (SEC-MALS). We analyzed the elution profiles of CLASP residues 1253-1522 and CLIP-170 residues 331-456 alone and mixed with each other. The CLASP residues 1253-1522 elution profile contained a single peak calculated at 31kDa, suggesting CLASP CTD eluted as a monomer, instead of a dimer as was expected. Surprisingly, CLIP-170 residues 331-456 eluted as a monomer (16kDa) earlier than CLASP residues 1253-1522, indicating a larger hydrodynamic radius despite a smaller molecular weight. This suggests that the CLIP-170 residues 331-456 folded into a coiled-coil conformation but did not dimerize. The CLASP CTD and CLIP-170 CC mixture elution profile contained one peak followed by a long shoulder edge that overlapped into the peak eluted from CLIP-170 CC alone. Analyzing the first half of the peak (exclusing the shoulder peak) calculated an avarage moelcular weight of 73kDa, corresponding to a complex that



Figure 4-4. CLASP C-terminal domain forms weak interactions with CLIP-170 coiledcoil domain. (A) 20 x 2µl of 1.25mM CLASP 1253-1522 was injected into 200 µl of 50µM CLIP-170 331-456. (B) 20 x 2µl of 1mM CLASP 1253-1522 was injected into 200 µl of 100µM CLIP-170 331-456. Both thermograms (upper panel) show exothermic reactions and displays µcal/sec over the injection period (min). K_D values were calculated as $K_D=7.1\mu$ M for experiment (A) and $K_D=23\mu$ M for experiment (B).

consists of two molecules of CLASP CTD and one molecule of CLIP-170 CC (76kDa)

(Figure 4-5). We noticed that the elution profile of each protein solution showed a descending trend in molecular weight as a function of time. One possible explanation for this trend is that the protein may be forming oligomers that dissociates as it traverses the SEC column. Additionally, we performed SEC-MALS analysis on a larger construt of CLIP-170 coiled-coil domain (residues 331-588). The longer construct eluted as a single peak with an average molecular weight calculated at 67kDa, which corresponds to a dimer (Figure 4-S2).



Figure 4-5. Size exclusion chromatography and multiangle light scattering (SEC-MALS) analysis of CLASP CTD and CLIP-170 CC. Differential refractive index elution profiles of CLASP CTD (1253-1522), CLIP-170 CC (331-456), and CLASP CTD with CLIP-170CC. CLASP CTD (blue line) elutes as a monomer with a calculated molecular weight at 31kDa. CLIP-170 CC (red line) elutes earlier than CLASP CTD despite having a calculated molecular weight at 16kDa. CLIP-170 CC is expected to fold into a long coil, which increases its hydrodynamic radius. However, CLIP-170 CC elutes as a monomer. CLASP CTD and CLIP170 CC (purple line) elutes as a dissociating complex with a calculated molecular weight of 73kDa.

Crystallization of CLASP1 C-terminal domain and CLIP-170 coiled-coil domain

Based on characterizing the interaction between CLASP 1253-1522 and CLIP-170 331-456 through ITC and SEC-MALS, crystallization conditions for CLASP 1253-1522 and CLIP-170 331-456 alone and in complex with each other were screened using commercial crystallization screens. Small needle-like crystals of CLIP-170 (residues 331-456) were produced through an initial crystallization condition consisting of 0.1M MES pH 6.35, 1.4M Sodium acetate (Figure 4-3D). However, these crystals did not diffract using the APS 22-ID X-ray beam line. Screening conditions for native and selenomethionine-substituted CLASP 1253-1522 initially produced droplets displaying phase separation. Optimizing one of these conditions to 0.2M Sodium acetate, 0.2M Sodium potassium tartrate, and 12% PEG 3350 produced a selenomethionine-substituted crystal that initially diffracted to 6.0Å (Figure 42F). However, optimizing and reproducing these crystals resulted in the formation of salt crystals. A droplet containing 2mg/ml of SeMET CLASP 1253-1522 and CLIP-170 331-456 produced several spear-shaped crystals using a crystallization condition consisting of 0.1M MES pH 6, 0.2M Sodium Acetate, 1mM Copper (II) chloride, and 3% PEG 3350 (Figure 4-2E). However, these crystals did not produce diffraction using the APS 22-ID X-ray beam line. In summary, crystals containing CLASP 1253-1522 and CLIP-170 331-456 either did not diffract or diffracted to low-resolution.

Discussion

To begin uncovering the role of the CLASP's C-terminal domain in CLASP dimerization and its interaction with known CLASP-associating factors, we characterized the interaction of CLASP's C-terminal domain with the coiled-coil domain from CLIP-170. CLASP was originally identified as a CLIP-associating factor using a yeast two-hybrid system (Akhmanova et al., 2001). The regions that mediated the interaction between these two proteins were mapped to CLASP's C-terminal domain (residues 1253-1538) and the N-terminal region of CLIP-170's coiled-coil domain (residues 277-588). Using a series of binding assays including native gel electrophoresis, ITC, and SEC-MALS, we narrowed down the interacting regions of CLASP's C-terminal domain to residues 1253-1522 and CLIP-170's coiled-coil domain to 331-456. Native gel electrophoresis was initially used as a robust screen in identifying interactions between various constructs of CLASP's CTD and CLIP-170's coiled-domain (Figure 4-S1). CLASP (1253-1522) and CLIP-170 (331-456) were identified as potential candidates for additional binding studies. ITC calculated the dissociation constant between these two proteins in the micromolar range, suggesting a

relatively weak interaction. SEC-MALS analysis revealed a complex, possibly consisting of 2 molecules of CLASP CTD and 2 molecules of CLIP-170 CC, undergoing dissassciation as it traversed the size-exclusion column.

Based on the finding that deleting CLASP's C-terminal domain ablated CLASP dimerization (Patel et al., 2012), we expected CLASP 1253-1522 to elute as a dimer under SEC-MALS. However, CLASP 1253-1522 eluted as a monomer. These findings suggest that CLASP1 1253-1522 is necessary but not sufficient for dimerization, but requires another component such as CLIP-170 to facilitate CLASP dimerization. In yeast member Stulp, FLAG-tag construct of TOG3-linker-CTD (residues 716-1513) copurified with WT Stu1p using affinity purification (Funk et al., 2014), suggesting that TOG3-linker may be a necessary component for dimerization. In addition, a construct of FLAG-Stu1p CTD alone (residues 1181-1533) copurified with WT Stu1p. The region of Stu1p CTD that corresponds to CLASP1 CTD consists of residues 1229-1538, encompassing 24 additional residues compared to CLASP1 1253-1522, suggesting that the constructed we used in SEC-MALS was too short and incomplete for dimerization. To confirm whether CLASP 1253-1522 construct as too short for dimerization or if the TOG-linker-CTD is required for dimerization, new constructs of CLASP1 CTD (residues 1229-1538) and CLASP TOG-linker-CTD (residues 843-1538) will be subjected to SEC-MALS to determine if they elutes as a dimer or as a monomer.

CLASP CTD domain exhibits a dual role in promoting CLASP function. The domain interacts with the coiled-coil domain of CLASP-associating factors and is a necessary component for CLASP dimerization (Akhmanova et al., 2001; Lansbergen et al., 2006. (Efimov et al., 2007; Patel et al., 2012; Funk et al., 2014; Hannak et al., 2006). In addition,

several known CLASP-associating factors exist as dimers through their coiled-coil domains (Akhmanova et al., 2001; Hannak et al., 2006; Lansbergen et al., 2006; Efimov et al., 2007). One interesting aspect to explore is determining if CLASP exists as a dimer or a monomer when interacting with CLASP-associating factors. Interestingly, our SEC-MALS analysis reveals a peak whose average molecular weight was calculated 73kDa. We speculate that a complex consisting of a dimer of CLASP CTD and dimer of CLIP-170 CC (96kDa) initially formed, but dissociated during size-exclusion chromatography. For future SEC-MALS studies, we plan on adding CLASP (1253-1522) to CLIP-170 (331-588), which elutes as a dimer in SEC-MALS (Figure S1). We expect to see a single peak that unambiguously corresponds to dimer-dimer interaction between CLASP CTD and CLIP-170 CC.

We plan on extrapolating our investigation into the mechanism underlying the interaction of CLASP to CLIP-170 into other CLASP-associating factors. Several known CLASP-associating factors, including LL5β, CENP-E, Xkid, and GCC185, interact with CLASP CTD through a specific region of their coiled-coil domains (Hannak et al., 2006; Lansbergen et al., 2006; Efimov et al., 2007; Patel et al., 2012). The determinants in CLIP-170 coiled domain that mediate CLASP interaction may be conserved in the coiled-coil domain of these other known CLASP-associating factors. Identifying and characterizing these common determinants can provide broad insight into how CLASP interacts with various associating factors to localize to different areas of the cell and perform different functions at specific locations in the cells, such as promoting MT polymerization at the kinetochore, linking MT plus ends to the cell cortex, and nucleation of MTs at the transgolgi-network. The interaction of CLASP's C-terminal domain with other associating factors is an area of research that is poorly understood and warrants further investigation.

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Supplemental Figures



Figure 4-S1. CLASP CTD and CLIP-170 CC display interaction through native gel electrophoresis. Native gel showing the electrophoretic mobility of CLASP CTD and CLIP-170 CC alone and in complex at various molar ratios. 5µl of CLASP CTD (1mg/ml) were mixed with 2.5µl of CLIP-170 CC (1mg/ml) to form a mixture at 1:1 molar ratio. Increasing volumes of CLASP CTD were adding to produce increasingly higher molar ratio solutions. Electrophoretic mobility of CLASP CTD and CLIP-170CC occurs at molar ratios of 2:1 and above.



Figure 4-S2. Larger construct of CLIP-170 (residues 331-588) forms a dimer under SEC-MALS. Elution profile of CLIP-170 CC (residues 331-588) alone (green line), CLASP CTD (residues 1302-1522) (brown line), and CLASP CTD and CLIP-170 CC mixture (pink line). CLIP-170 (residues 331-588) eluted as a dimer with a calculated peak of 67kDa, which is consistent with the expected molecular weight of a dimer of CLIP-170 CC. However, the CLIP-170 CC construct did not form a complex with CLASP CTD (1302-1522).

CHAPTER 5: DISCUSSION AND FUTURE WORK

CLASP uses an array of structurally distinct TOG domains to regulate microtubules

CLASPs are a conserved class of plus-end tracking proteins that are essential in regulating microtubules, which they do by promoting MT pause during interphase and MT polymerization during mitosis (Akhmanova et al., 2001; Maiato et al., 2003; Cheeseman et al., 2005; Maiato et al., 2005; Drabek et al., 2006; Pereira et al., 2006; Sousa et al., 2007). How CLASP differentially promotes MT pause and growth remains to be determined. CLASP was originally annotated as having a conserved C-terminal domain used to bind CLIP-170, an N-terminal TOG domain, and two central conserved regions (Akhmanova et al., 2001). These two conserved regions were hypothesized to be cryptic TOG-like domains (Slep and Vale, 2007). Under this hypothesis, CLASP uses a TOG array to regulate MT dynamics similar to that of ch-TOG/XMAP215 family (Slep and Vale, 2007; Slep, 2010). To address this hypothesis, the work in this thesis characterizes these TOG domains individually and in an array using structural, biochemical, and cellular analysis. Our work on the first cryptic TOG-like domain (TOG2) confirms our prediction that CLASP is composed of arrayed TOG domains. Additional work characterizing TOG1 and the second cryptic TOGlike domain (TOG3) to further support our central hypothesis is ongoing. Our long-term goal is to characterize these TOG domains individually and as an array using structural, biochemical, and cellular analyses to discover mechanistic insights into how CLASP uses arrayed TOG domains to deferentially promote MT pause and growth.

Despite difficulties in solving the structure of CLASP1 TOG1, the work solving the crystal structure is ongoing. Although CLASP1 TOG1 crystals diffracted to resolution of 2.0Å, obtaining phasing information using SAD and molecular replacement was unsuccessful, possibly due to crystal twinning. While we were attempting to solve the crystal structure of CLASP1 TOG1, the crystal structure of MAST/Orbit TOG1 was determined and revealed a straight architecture across the tubulin-binding surface (PDB ID: 4G3A; De la Mora-Rey et al., 2013). Further analysis into TOG1 showed that the determinants that promote tubulin-binding activity in TOG domains are not well conserved in TOG1. In addition, TOG1 in Drosophila MAST/Orbit and yeast Stu1p does not bind microtubules in MT co-sedimentation and co-immunoprecipitation assays (De la Mora-Rey et al., 2013; Funk et al., 2014). Although TOG1 alone does not associate with microtubules, constructs consisting of TOG1 and TOG2 bind soluble $\alpha\beta$ -tubulin, associate with the MT lattice, and recruit tubulin to the MT lattice to promote rescue (Al-Bassam et al., 2010). These findings suggest that TOG1 interacts with tubulin and promotes MT polymerization in the presence of TOG2. In addition, TOG1 may also interact with actin filaments, increasing confusion about the role of TOG1. Tsvetkov et al. demonstrated that CLASP1 α and CLASP2 α isoforms coimmunoprecipite with actin, and that CLASP2 α co-localizes with actin stress fibers and exhibites retrograde flow with actin at the leading edges of motile cells (Tsvetkov et al., 2007). Further investigation is needed to reconcile these conflicting findings on about TOG1. To confirm which cytoskeleton network TOG1 interacts with, constructs containing TOG1 alone or TOG1-2 can be used in separate microtubule and actin polymerization assays. Another question to answer is why TOG1 does not contain tubulin-binding determinants found in other TOG domains. One explanation is that residues with the intra-HEAT loops

have been modified to specifically interact with actin, while another is that TOG1's tubulinbinding determinants are still present, but have slightly diverged compared to other TOG domains. In Drosophila MAST/Orbit TOG1, a methionine residue, which is still capable of forming hydrophobic interactions with tubulin, replaced the conserved tryptophan on the HR A intra-HEAT loop. Solving the crystal structure of TOG1 in complex with either tubulin or actin will determine which cytoskeleton network TOG1 can interact with and will also reveal the determinants that mediate these interactions. Probing the role of these structural determinants in biochemical and cell-based assays will provide mechanistic insight into how TOG1 contributes to CLASP function and confirm if TOG1 binds actin, microtubules, or both cytoskeletal elements.

Most of our completed work on CLASP focused on TOG2. We solved the crystal structure of the first cryptic TOG domain (TOG2) from human CLASP1, which confirmed the existence of a *bona fide* TOG array in the CLASP. This TOG2 structure exhibits a curved architecture across the tubulin-binding surface that contrasts with the straight, flat tubulin-binding surface from ch-TOG/XMAP215 TOG domains. We employed a series of biochemical and cell-based assays to probe the role of TOG2 and its unique structural determinants in CLASP function. Our cell-based assays show that TOG2 promotes MT lattice association and CLASP-dependent bipolar spindle formation and increases the rate of MT polymerization in vitro. These findings are consistent with other studies that show that larger constructs of CLASP containing TOG2 also promote microtubule polymerization in vitro (Slep and Value, 2007; Patel et al., 2012). We plan to expand these biochemical and cell-based assays within the context of the entire TOG array and are designing a library of constructs of full-length MAST/Orbit, including wild type and a series of single, double, and

triple TOG mutants that ablate TOG-tubulin interactions. These constructs will be used in the Drosophila S2 cell system to analyze their ability to perform CLASP-dependent bipolar spindle formation. Additionally, we plan to measure microtubule dynamics using live cell imaging, including measuring rates of polymerization and quantifying the frequency of pause states of MT plus end during interphase and mitosis. These experiments will provide insights into how CLASP's arrayed TOG domains can promote MT polymerization during mitosis and MT stabilization and pause during interphase.

The second cryptic TOG domain (TOG3) is the only TOG domain whose crystal structure has not been determined. TOG3 crystals have been obtained and will be optimized for high-resolution diffraction. Although the crystal structure of CLASP1 TOG3 has not been solved, we predict that TOG3 will adopt a straight architecture across the tubulin-binding surface, similar to that of CLASP1 TOG1 and ch-TOG/XMAP215 TOG domains. Attempts at crystallizing and solving the structures of CLASP1 TOG1 and TOG3 are on going and will provide insights into how each structurally distinct TOG domain contributes to CLASP function. Previous studies showed that constructs containing TOG3 and the linker region between TOG3 and CTD exhibit MT lattice binding activity in growth cone and lamella microtubules (Wittmann and Waterman-Storer, 2005; Hur et al., 2011; Kumar et al., 2009). These TOG3-containing protein constructs decorate and stabilize the MT lattice in the leading edge of migrating cells. These findings suggest that TOG3 serves as a MT-lattice binding domain and is important for promoting stability in microtubules that drives cell migration and motility at the leading edge.

No structural information exists detailing how CLASP TOG domains interact with $\alpha\beta$ -tubulin. To determine if TOG2's bent architecture contributes to CLASP's ability to

stabilize and pause microtubules, we attempted to co-crystallize CLASP1 TOG2 with a Designed Ankryin Repeat Protein (DARPin) that has been used to crystallize tubulin in complex with other proteins (Pecquer et al., 2012; Gigant et al., 2013). Although we produced a complex of DARPin-tubulin in vitro, CLASP1 TOG2 did not associate with the DARPin-tubulin complex during size-exclusion chromatography or crystallization. One reason that explains why a TOG2-DARPin-tubulin complex failed to form is that TOG2 interacts with a specific, non-polymerized conformation of tubulin. Colchine, a potent inhibitor of MT polymerization, could be used to fix tubulin into a curved conformation (Ravelli et al., 2004). In addition, cryo-EM studies show that dolastatin-induced tubulin rings mimic the properties of MT ends (Moores et al., 2008; Mulder et al., 2009). Studies of these dolastatin-induced tubulin rings in the presence of CLASP TOG domains, alone or in an array, can provide insights how TOG domains interact with MT ends. Structural studies of CLASP TOG domains in complex with tubulin will provide insight into how these TOG domains associate with tubulin through different modes of interaction, which collectively contribute to CLASP's overall function to regulate microtubule dynamics.

Comparing the crystal structures of MAST/Orbit TOG1 and CLASP1 TOG2 with the predicted secondary structure of TOG3 reveals that each CLASP TOG domains is structurally distinct and contains unique features in relation with each other. For example, TOG1 displays a straight tubulin-binding surface, but tubulin-binding determinants are not well conserved, while TOG2 displays a curved tubulin-binding surface that may promote MT stabilization. TOG3 is predicted to exhibit a straight tubulin-binding surface, similar to that of TOG1, but still retain conserved tubulin-binding determinants. Information about these three TOG domains allows us to speculate on a possible model that illustrates the role of

CLASP's arrayed TOG domain regulating MT dynamics (Figure 5-1). CLASP localizes to the MT-plus end to promote stabilization and the pause state, but each TOG domain interacts with microtubules differently. First, TOG3 binds an area of the MT lattice that directly precedes the MT plus end, which allows TOG2 to interact with the curved protofilament of a depolymerizing microtubule, inducing it into a pause state and promoting MT stabilization (Wilbur and Heald, 2013). Depending on whether CLASP is needed for MT polymerization, MT stabilization, or actin interaction, TOG1 serves multi-functional roles in MT dynamics or actin-microtubule crosslinking. If CLASP is needed to promote MT polymerization, such as fluxing kinetochore microtubules during mitosis, TOG1 binds soluble $\alpha\beta$ -tubulin subunits and incorporates them into the plus end of kinetochore microtubules. For microtubule polymerization to occur, TOG2 disengages from the MT-plus end to permit TOG1 to promote MT polymerization. Conversely, when CLASP is needed to stabilize interphase microtubules, TOG2 persistently engages the MT-plus end to induce the pause state. The CLASP2 γ isoform, which lacks the TOG1 domain, stabilize microtubules and is highly expressed in neuron cells (Akhmanova et al., 2001), which suggests that TOG1 may not play a role in promoting microtubule stabilization. In the case of motile cells, CLASP TOG3 decorates and stabilizes the MT lattice in leading edge, which allows TOG1 to serve as an actin-microtubule cross-linker that coordinates movements between the actin and microtubule networks to promote cell motility and migration.

This multi-functional model of CLASP may reconcile conflicting findings about whether CLASP promotes MT polymerization, or MT pause, or if CLASP directly interacts with actin filaments. However, supporting this multi-functional model requires extensive characterization of each TOG domains. A series of structural, biochemical, and cellular

assays will be needed to determine if 1) TOG1 can play multiple roles that include incorporating $\alpha\beta$ -tubulin subunits into a growing microtubules and interacting with actin filaments, 2) TOG2 stabilizes microtubules and promotes the pause state, and 3) TOG3 specifically binds along the MT lattice (Figure 5-1).

In conclusion, our work and supporting literature highlights key features of the CLASP TOG array that further our mechanistic understanding of this critical MT regulator. The crystal structure of TOG2 is distinct from the crystal structures of MAST/Orbit TOG1 and other XMAP215 TOG domain, revealing that CLASP consists of at least one unique TOG domain to promote CLASP function. CLASP TOG1 and TOG3 may also contain distinct features that promote different roles in CLASP's ability to convert between promoting MT pause and growth. These TOG domains also contribute to CLASP's ability to promote bipolar spindle formation, associate with the MT lattice, and promote MT polymerization. Collectively, these findings suggest that each TOG domain is structurally distinct and performs specific functions that collectively promote CLASP's ability to regulate MT dynamics.

The C-terminal domain of CLASP promotes dimerization and interaction with CLASPassociating factors

The C-terminal domain of CLASP mediates CLASP dimerization and associates with other factors to recruit CLASP to different locations in the cell (Hannak et al., 2006; Lansbergen et al., 2006; Efimov et al., 2007; Patel et al., 2012; Funk et al., 2014). However, structural and mechanistic insights into how CLASP C-terminal promotes homodimerization and interacts with other factors are poorly understood. Our study aims to expand understanding of CLASP C-terminal, a domain of CLASP that has not been well

characterized when compared to CLASP's TOG domains. A construct of CLASP's Cterminal domain (residues 1253-1538) was found to interact with a region coiled-coil domain of CLIP-170 (residues 277-588) (Akhmanova et al., 2001). Our study aims to further characterize the interaction between CLASP CTD and CLIP-170 CC. We narrowed the interacting regions that have been identified to produce smaller CLASP CTD and CLIP-170 CC constructs that would be amenable for crystallization. We mapped the interaction of a shorter CLASP CTD construct (residues 1253-1522) to a ~ 150 residue region of coiled-coil domain from CLIP-170 (residues 331-456). An initial native gel electrophoresis assay showed that these two constructs interact; however, ITC and SEC-MALS analysis showed that they interact weakly. For future binding studies, we will use a construct of CLIP-170 coiled-coil domain (residues 331-588) that elutes as a dimer in SEC-MALS. We predict that having these larger constructs will improve its binding affinity to CLASP CTD.

We obtained crystals of CLASP CTD and CLIP-170 CC and its complex, but these crystals did not diffract or diffracted to a low resolution. Crystallography work is ongoing, in which we will obtain crystals that will diffract and produce a crystal structure identifying the structural determinants that mediate the interaction between CLASP CTD and CLIP-170 CC. We will probe the role of structural determinants in biochemical assays including ITC and SEC-MALS.

CLASP C-terminal domain has been found to be a necessary component for CLASP dimerization (Patel et al., 2012); however, SEC-MALS analysis of CLASP 1253-1522 showed that the constructs eluted as monomers. This conflicts with a study in CLASP yeast member Stu1p, which showed that Stu1p CTD alone co-purified with WT Stu1p using affinity purification (Funk et al., 2014). For future work, we plan to design larger CLASP

CTD constructs that include TOG3-linker-CTD and linker-CTD to determine if these constructs are dimers in SEC-MALS. One aspect of CLASP CTD to explore is to determine if CLASP dimerization is required to interact with other factors. SEC-MALS analysis on a mixture of CLASP CTD and CLIP-170 CC showed a peak whose average molecular weight was calculated at 80kDa, which is near the expected molecular weight (96kDa) of a dimer of CLASP CTD interacting with a dimer of CLIP-170 CC. Although CLASP CTD alone eluted as a monomer, CLASP CTD in the presence of CLIP-170 CC induced CLASP dimerization. SEC-MALS analysis suggests that CLIP-170 induces CLASP dimerization, forming a dimer-dimer complex. CLASP interacts as a dimer when interacting with the coiled-coil domains of other CLASP-associating factors.

Another aspect of CLASP C-terminal domain to explore to determine if CLASP dimerization enhances the ability of arrayed TOG domains to contribute to MT binding (Patel et al., 2012; Funk et al., 2014). CLASP protein constructs containing the CTD bound more tightly to MTs through MT pelleting assay (Patel et al., 2012), which supports the finding that CLASP dimerization enhances MT binding activity. Because of this finding, we propose using full-length CLASP in biochemical and cellular studies. Additionally, since CLASP interacts with various factors to localize to different parts of the cell, it would be interesting to determine if enhanced MT binding via CLASP dimerization is needed to differentially regulate MT dynamics at specific locations in the cell. This work would unify two aspects of our thesis on CLASP: 1) arrayed TOG domains underlie CLASP is ability to regulate MT dynamics, and 2) C-terminal domain is necessary for CLASP dimerization and to interact with associating factors. Future work characterizing the full-length CLASP may

further establish CLASP as an essential MT regulator that differentially promotes MT-based functions throughout the entire cell.

Concluding remarks

Microtubules play essential roles in fundamental cellular processes including cell differentiation, intracellular tracking and cell division (Akhmanova and Steinmetz, 2008). CLASP family members are important regulators of MT dynamics and are crucial in maintaining these cellular processes. CLASP plays an important role in neural development, by mediating axon growth and guidance (Lee et al., 2004; Hur et al., 2011) and is an essential component of mitosis, in which inhibition or depletion of CLASP causes the bipolar spindle to collapse into monopolar spindles (Maiato et al., 2002; Maiato et al., 2003). The persistence of monopolar spindles leads to chromosome mis-segregation, aneuploidy, and cell death. An euploidy is a condition in which the cell has gained or lost one or more chromosomes during cell division and is a hallmark of birth defects, tumors, and many types of cancers (Cimini and Degrassi, 2005). Homozygous CLASP mutants cause early embryonic lethality in Drosophila (Lemos et al., 2000; Maiato et al., 2002). Analysis of these embryos revealed cells that displayed severe aneuploidy, findings that suggest that CLASP mutations are implicated in birth defects, tumor, and cancer. A structural, biochemical, and cellular understanding of CLASP function will be key in determining how CLASP and other MAPs regulate microtubules to maintain fundamental cellular processes. This comprehensive understanding of CLASP may contribute to understanding aneuploidy, embryonic development, and neurological disorders.


Figure 5-1. CLASP promotes MT pause and MT growth. Model proposing a mechanism underlying CLASP's ability to promote MT pause and growth using an array of TOG domains. (A) CLASP stabilizes a depolymerizing MT. CLASP CTD (white box) and TOG3 (light grey box) interact with the MT lattice. TOG2 (dark grey box) binds to curved GDPtubulin (purple and green spheres) in curving protofilaments. TOG1 (black box) does does not interact with tubulin or the MT lattice. Once CLASP stabilizes depolymerizing microtubules, it may promote MT growth or pause. (B) CLASP promoting MT growth. TOG1 binds GTP-tubulin (red and greens spheres) and incorporates it into a TOG2-stabilized protofilament. (C) CLASP promoting MT pause state. TOG2 stabilizes curving protofilaments and promotes lateral interactions among protofilaments, causing the MT lattice to form blunt-ended MTs, which characterizes the MT pause state. TOG1 does not play a role in promoting MT pause. The CLASP2 γ isoform stabilizes microtubule despite lacking TOG1. Interplay between TOG1 or TOG2 determines whether CLASP promotes MT pause or growth. If MT pause is needed, MT stabilization through TOG2 dominates, while TOG1 may be interacting with actin filaments (Tsvetkov et al., 2007). If MT growth is needed, then tubulin incorporation by TOG1 dominates. CLASP homodimerization via CLASP CTD (white box) may enhance is ability to associate with microtubules and increase CLASP's ability to promote microtubule stabilization or growth.

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