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Role of CLASP2 phosphorylation in regulating kinetochore-microtubule interactions

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Role of CLASP2 phosphorylation in regulating kinetochore-microtubule interactions

by

Hayley Pemble

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REFERENCE TO PUBLISHED MATERIALS

- Figures 1.1 through 1.4 (and figure legends) are from the published article: Kumar, P., M.S. Chimenti, H. Pemble, A. Schönichen, O. Thompson, M.P. Jacobson, and T. Wittmann. 2012. Multisite phosphorylation disrupts arginine-glutamate salt bridge networks required for binding of cytoplasmic linker-associated protein 2 (CLASP2) to end-binding protein 1 (EB1). *Journal of Biological Chemistry*. 287:17050–17064. doi:10.1074/jbc.M111.316661.
- Chapter 2 is from a manuscript article currently in preparation: Pemble, H., Wittmann, T. CLASP2 phosphorylation regulates kinetochore-microtubule interactions. The coauthor listed in this publication, Torsten Wittmann, helped formulate and provided supervision for the research in this dissertation.
- Chapter 3 refers to published articles that represent co-authorships and collaborations based largely on reagents I developed during this dissertation research:
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    - Basu, S., S. Sladecek, H. Pemble, T. Wittmann, J.A. Slotman, W. van Cappellen, H.R. Brenner, and N. Galjart. 2014. Acetylcholine receptor (AChR) clustering is regulated both by glycogen synthase kinase 3 (GSK3)-dependent phosphorylation and the level of CLIP-associating protein 2 (CLASP2) mediating the capture of microtubule plus-ends. *Journal of Biological Chemistry*. doi:10.1074/jbc.M114.589457.

- Liz, M.A. F.M. Mar, T.E. Santos, H.I. Pimentel, A.M. Marques, M.M. Morgado, S. Vieira, V.F. Sousa, H. Pemble, T. Wittmann, C. Sutherland, J.R. Woodgett, and M.M. Sousa. 2014. Neuronal deletion of GSK3beta increases microtubule speed in the growth cone and enhances axon regeneration via CRMP-2 and independently of MAP1B and CLASP2. *BMC Biol*. 12:47. doi:10.1186/1741-7007-12-47.

Role of CLASP2 phosphorylation in regulating kinetochore-microtubule interactions

Hayley Pemble

ABSTRACT

Proper chromosome segregation requires dynamic regulation of kinetochore-microtubule attachments throughout mitosis. Multiple kinetochore proteins display microtubule-binding activity, yet how exactly these proteins are spatially and temporally regulated is unclear. Cytoplasmic linker-associating proteins (CLASPs) are present in the outer kinetochore, in close proximity to microtubule ends, and are required for mitosis. Here, we test whether phosphorylation of CLASP2 during mitosis serves as a mechanism to regulate kinetochore-microtubule interactions and the fidelity of chromosome segregation. We show that cyclin-dependent kinase 1 (Cdk1) and glycogen synthase kinase β (GSK3β)-dependent mitotic phosphorylation of CLASP2 within its microtubule end-binding domain inhibits its microtubule end-binding activity but does not affect CLASP2 kinetochore binding. Deregulation of CLASP2 phosphorylation does not affect initial chromosome congression, but weakens kinetochore-microtubule attachments. In the presence of endogenous CLASP2, phosphorylation-deficient CLASP2 increases average interkinetochore distance, while phosphomimetic CLASP2 decreases average interkinetochore distance measurements. Together, these results suggest that CLASP2 microtubule binding at the kinetochore is required for stable,

tension-generating kinetochore-microtubule attachments. Furthermore, cells expressing CLASP2 phosphomutants have abnormal kinetochore dynamics and in some cells, kinetochore pairs flip about the metaphase plate, suggesting absent or imbalanced microtubule attachments. Expression of either nonphosphorylatable or phosphomimetic CLASP2 fails to rescue depletion of CLASP2 and ultimately leads to an increase in lagging chromosomes. Together, these results characterize specific phosphorylation sites in CLASP2 that negatively regulate microtubule binding and present a novel mechanism of Cdk1 and GSK3β-mediated control of kinetochore-microtubule interactions.

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INTRODUCTION

I. Chromosome segregation

A. Mitosis produces two genetically identical daughter cells

a. Background

Mitosis is the process by which cells divide, thereby creating two genetically identical daughter cells. Mitotic divisions are essential for the development of an organism; remarkably, it takes approximately one trillion cell division cycles to produce a human from one fertilized cell (Bloom and Joglekar, 2010). Mitosis is also required for normal growth, repair, and maintenance of cells and tissues, such as those in the gut, skin, and hair follicles (Radtke, 2005). Cell division gives rise to all cells of an organism and despite occurring in many different cell types, it is made up of stereotypical phases which have been loosely defined based upon the behavior of chromosomes (Pines and Rieder, 2001; Walczak et al., 2010). As cells transition from G2 phase to M, they first enter prophase, in which chromosomes become highly condensed and the duplicated centrosomes migrate to opposite sides of the cell (Kline-Smith and Walczak, 2004). As the nuclear envelope breaks down, dynamic microtubules emanating from the centrosomes are able to interact with and attach to chromosomes (Tanaka, 2012). Once chromosomes become attached either laterally or end-on to microtubules, they begin aligning in the middle of the cell in prometaphase. After reaching alignment, chromosomes oscillate back and forth across the center of the metaphase plate, a behavior that is powered by dynamic microtubules. Once all chromosomes are stably attached to microtubules from opposite spindle poles, the cohesion holding sister chromatids together is degraded, allowing sister chromatids to be pulled to opposite

sides of the cell during anaphase. Telophase is the last phase of mitosis, and culminates in cytokinesis, or the separation of the cell into two identical daughter cells (Maddox and Oegema, 2003).

b. Disease relevance

In addition to cellular components, DNA is equally separated during mitosis to ensure that each resulting daughter cell receives exactly one complement of the genome (Cheeseman, 2014). Proper segregation of DNA, packaged into chromosomes during mitosis, has important implications for many human diseases, including cancer (Malumbres, 2011). Missegregation of chromosomes can have deleterious effects and result in aneuploidy or genomic instability (Kops et al., 2005). In fact, many solid tumors are aneuploid and exhibit high rates of chromosome missegregation (Thompson et al., 2010). While the causal link between aneuploidy and cancer has long been debated, recent evidence suggests that in small doses aneuploidy contributes to cancer formation and progression, while larger doses results in cell death (Weaver and Cleveland, 2006; Weaver et al., 2007). There are many underlying causes of chromosome missegregation, including merotelic kinetochore-microtubule attachments, which occur when one kinetochore is attached to both spindle poles (Holland and Cleveland, 2009); perturbed spindle checkpoint signaling (Hanks et al., 2004); multipolar spindles (Brinkley, 2001); and perturbed kinetochore microtubule dynamics (Bakhoum et al., 2009a; b). Because cells are particularly vulnerable to perturbations during mitosis, many cancer therapeutics are anti-mitotics that either arrest mitosis or cause genomic damage (Chan et al., 2012a). While chromosome segregation mechanisms underlie development and cancer formation, progression, and

therapeutics, the molecular mechanisms that control chromosome segregation are not completely understood.

B. Kinetochores attach microtubules to chromosomes

a. Kinetochore structure

The critical task of chromosome segregation is carried out by the mitotic spindle, a highly dynamic microtubule-based structure that attaches to chromosomes to facilitate their dynamic movements throughout mitosis (Kline-Smith and Walczak, 2004). Microtubules bind to chromosomes through kinetochores, large protein structures that assemble on centromeric DNA (Cheeseman, 2014). To date, there are over 100 identified proteins that localize to kinetochores, and amazingly, many of these proteins rapidly assemble and disassemble during each division (Gascoigne and Cheeseman, 2013; Cheeseman and Desai, 2008). Proteins that bind closer to the centromere, referred to as inner kinetochore proteins, provide structure for the rest of the kinetochore to assemble on. CENP-A, a histone H3 variant (Palmer et al., 1987; 1991), stably associates with centromeres (Jansen et al., 2007) and specifies where kinetochores are assembled during mitosis. CENP-A is required for the localization of all other kinetochore proteins (Regnier et al., 2005). A group of 16 proteins, known as the constitutive centromere-associated network (CCAN), bind to CENP-A and recruit additional kinetochore proteins, most of which are transiently localized to kinetochores during mitosis (Perpelescu and Fukagawa, 2011). Outer kinetochore proteins lie closer to the microtubule and facilitate attachment to microtubules or serve as signaling proteins for the spindle checkpoint. Using super resolution microscopy techniques, the precise localization of 16 kinetochore proteins was determined relative to Hec1 with

nanometer accuracy in both wildtype and drug-treated cells (Wan et al., 2009). Treatment with microtubule drugs resulted in localization changes in some of the 16 proteins, suggesting that kinetochore structure is somewhat dynamic. This dynamic rearrangement of proteins has also been seen in EM images of kinetochores in the presence or absence of microtubules (Suzuki et al., 2011). Far more than 16 kinetochore proteins have been identified to date; therefore, future experiments are necessary to get a more completely defined kinetochore protein organization.

Proteomic studies have helped researchers compile an almost complete list of kinetochore proteins, yet the structural determination of the kinetochore has proven to be more challenging. Early electron microscopy (EM) images suggested that the kinetochore was composed of a series of clearly defined plates, with an electron dense structure at the centromere and a less dense zone of proteins in close proximity to microtubules (O'Connell et al., 2012; Kern and Cheeseman, 2012; Cleveland et al., 2003). However, more recent EM imaging utilizing milder fixation conditions suggest that the kinetochore is much less structured than originally thought (Kern and Cheeseman, 2012). Using high-pressure freezing methods, the outer kinetochore appears as a meshwork of proteins surrounding the microtubule end (Dong et al., 2007). In an additional study using cryo-EM, fibrils from the outer kinetochore can be seen contacting the curved ends of microtubule protofilaments, however, the identity of the fibrils remains unclear (McIntosh et al., 2008). Remarkably, kinetochore particles from *S. cerevisiae* have been purified and visualized in the presence of microtubules using EM (Gonen et al., 2012). Budding yeast kinetochores form complexes that are 126 nm in diameter and when mixed with microtubules, form both lateral and end-on

attachments to microtubules (Kern and Cheeseman, 2012). Interestingly, multiple attachments are formed between microtubules and these purified kinetochores, although the exact identity of all of these attachments is not known.

b. Kinetochore functions

i. Microtubule binding

The main function of the kinetochore is to bind microtubules and serve as a link between chromosomes and the mitotic spindle. Budding yeast kinetochores attach to a single microtubule, while vertebrate kinetochores bind between 12 and 30 microtubules, depending on the cell type (Wendell et al., 1993; Mcewen et al., 1997; Winey, 1995). There are multiple proteins that mediate kinetochore-microtubule attachments, which will be outlined later in the Introduction (Part II.C.b.).

ii. Checkpoint signaling and improper kinetochore-microtubule attachments

In addition to binding microtubules, kinetochores serve as signaling platforms for spindle assembly checkpoint (SAC) proteins that ensure all kinetochores are stably attached to microtubules prior to anaphase onset (Sacristan and Kops, 2014). The multiple SAC proteins that localize to kinetochores include both kinases (Aurora B, Bub1, BubR1, Mps1) and the proteins Mad1, Mad2, and Bub3 (Hauf, 2013). Together, they prevent activation of the anaphase promoting complex/cyclosome (APC/C), a ubiquitin ligase that marks securin and cyclin B for degradation and thereby freeing separase to cleave cohesin between sister chromatids, allowing anaphase to continue (Peters, 2006; Lesage et al., 2011).

How exactly improper or unstable kinetochore-microtubule attachments are monitored in cells is a major question in the field. Aurora B plays a central role in recognizing improper attachments and signaling to downstream components in order to delay anaphase onset (Biggins et al., 1999; Lampson and Cheeseman, 2011). The prevailing model is that Aurora B acts a tension sensor at the kinetochore (Akiyoshi et al., 2010). When kinetochores are improperly attached and under low tension, Aurora B at the centromere is in close proximity to and phosphorylates substrates with microtubule-binding activity at the outer kinetochore (Liu et al., 2009). Phosphorylation of Aurora B substrates weakens kinetochore-microtubule attachments and allows for error correction (Cheeseman et al., 2006). Kinetochores that are stably and properly attached are under tension and Aurora B is spatially separated from its substrates, preventing their phosphorylation (Khodjakov, 2010). Recently, this model has been challenged by data in budding yeast showing that localization of Aurora B to the outer kinetochore has no effect on the stability of kinetochore-microtubule attachments (Campbell and Desai, 2013). Substrates of Aurora B include Ndc80 (Deluca et al., 2006), Dam1 in budding yeast (Cheeseman et al., 2002), KNL1, Mis12 (Welburn et al., 2010), MCAK, and the more recently described Ska1 complex (Chan et al., 2012b; Schmidt et al., 2012a).

Despite the robust response of the SAC to prevent anaphase onset until all chromosomes are properly attached, some kinetochore-microtubule attachments are formed that fly under the radar of the SAC. Merotelic attachments, in which one sister kinetochore is attached to both spindle poles, generate tension and thus do not activate the spindle checkpoint (Gregan et al., 2011). Not surprisingly, the formation of merotelic

attachments that do not get resolved before anaphase leads to chromosome missegregation and aneuploidy (Cimini, 2008; Cimini et al., 2001).

iii. Force generation

Kinetochore-attached microtubules remain dynamic throughout mitosis to facilitate proper chromosome alignment and separation. By remaining bound to dynamic microtubules, the kinetochore acts as a coupler that converts energy generated during rounds of microtubule polymerization and depolymerization to power chromosome movements (Umbreit and Davis, 2012; Asbury et al., 2011). In a famous experiment conducted by Bruce Nicklas in insect cells during meiosis, the force generated by kinetochore-microtubule attachments was measured. He used a glass microneedle to pull on chromosomes during meiosis and by comparing the amount of kinetochore stretch to *in vivo* observations, he deduced that the kinetochore can withstand 7pN of force per attached microtubule (Nicklas, 1988).

The ability of the kinetochore to generate force relies on microtubule-binding proteins as well as components at the kinetochore that regulate the dynamics of microtubules. Importantly, the microtubule-binding protein at kinetochores, Ndc80, has been shown to couple microtubule end dynamics to bead movement *in vitro* (Powers et al., 2009). In *S. cerevisiae*, the Dam1 complex, which is essential for kinetochore-microtubule attachments, can also couple microtubule tip dynamics to bead movement *in vitro* (Asbury et al., 2006). The Ska1 complex, the presumed human homologue of the Dam1 complex, localizes to outer kinetochores, binds to microtubules, and Ska-coated beads can track depolymerizing microtubule ends *in vitro* (Welburn et al., 2009; Hanisch, 2006; Raaijmakers et al., 2009). Since microtubule dynamics are the driving

force behind chromosome movements, it is critical to understand how kinetochore proteins locally influence microtubule dynamics. This topic will be covered later in the Introduction (Part III B). In addition, dynein and kinesin microtubule motors contribute to kinetochore-generated forces and chromokinesins in particular make significant contributions to metaphase chromosome oscillations (Stumpff et al., 2012; 2008; Varma et al., 2008).

II. Microtubule-binding proteins

A. Microtubules

a. Structure

The microtubule cytoskeleton is an organized array of dynamic polymers that plays a critical role during many cellular processes, such as cell migration and division (Walczak and Heald, 2008; Etienne-Manneville, 2013; Kaverina and Straube, 2011). Microtubules are hollow cylinders 25nm in diameter made up of protofilaments of α/β-tubulin dimers. In most cases, microtubules contain 13 protofilaments, but this number can vary with organism, cell type, or in microtubule preparations *in vitro* (Böhm et al., 1984; Wade, 2009). Tubulin dimers are added to the microtubule end in a head-to-tail fashion, such that the β-tubulin subunit is always remains at the end, giving the polymer an intrinsic polarity. The addition of tubulin subunits occurs at the plus end, which is characterized by faster growth kinetics *in vitro* (Wade, 2009). The minus end grows slower *in vitro* and is usually anchored in cells, often at the centrosome, or protected by minus-end capping proteins, such as those in the CAMSAP family, that anchor non-centrosomal microtubules in differentiated polarized cells (Silva and Cassimeris, 2014).

b. Dynamic Instability

Following the purification of tubulin from animal brains, Mitchison and Kirschner discovered that microtubules assembled *in vitro* were extremely dynamic. They observed microtubules undergoing continuous switching between phases of growth and shrinkage and they called this behavior 'dynamic instability' (Mitchison and Kirschner, 1984). This behavior was later confirmed using time-lapse microscopy of microtubules both *in vitro* and in cells (Walker et al., 1988; Sammak and Borisy, 1988). The energy required to power microtubule dynamic instability is derived from GTP hydrolysis of α/β-tubulin dimers within the microtubule (Desai, 1997). The β-tubulin subunit binds GTP and after incorporation of the α/β-tubulin dimer into the growing microtubule, GTP is hydrolyzed to GDP by β-tubulin's intrinisic GTPase activity. GTP-tubulin has a straight conformation, and thus favors microtubule assembly, whereas GDP-tubulin adopts a curved conformation and drives microtubule depolymerization (Müller-Reichert et al., 1998). These structural differences between polymerizing and depolymerizing microtubules have been observed using cryo-EM (Simon and Salmon, 1990; Mandelkow et al., 1991). The presence of GTP-tubulin at the plus-end, known as a 'GTP cap', is thought to protect the microtubule from depolymerization (Caplow, 1992). Immunostaining using a GTP-tubulin antibody suggests that this cap might be much smaller than previously thought and demonstrated the presence of GTP-tubulin remnants along the microtubule lattice (Dimitrov et al., 2008). However, the specificity of this antibody is still very much under debate. Once the rate of GTP hydrolysis exceeds that of tubulin dimer addition, the GTP cap is lost and the microtubule depolymerizes. Therefore, the difference in the rates of addition of GTP-bound tubulin dimers and the hydrolysis of GTP-tubulin determine whether a microtubule polymerizes or

depolymerizes. A microtubule that switches from a growing state to a shrinking state undergoes a ‘catastrophe’ and conversely, a shrinking or disassembling microtubule undergoes a ‘rescue’ event when it begins growing again (Gardner et al., 2013). If the addition and removal of tubulin subunits is equal such that the microtubule does not change in length, then it is said to be in a ‘pause’ state (Howard and Hyman, 2003).

c. Functions

The ability of microtubules to persistently grow and shrink allows them to play vital roles in dynamic cellular processes in both interphase and mitosis. Microtubules serve as tracks for motor proteins to deliver intracellular components to various locations within cells, such as the terminus of axons in neurons (Prokop, 2013). In addition, microtubules direct the movement and organization of organelles such as Golgi and ER (Miller et al., 2009; Shen et al., 2012); serve as structural components of cilia, flagella, and centrioles (Kim and Dynlacht, 2013; Gönczy, 2012); and stabilize the leading edge of cells during migration (Stehbens and Wittmann, 2012; Vasiliev et al., 1970). During mitosis, microtubules undergo a dramatic reorganization to form the mitotic spindle, which facilitates chromosome movements necessary for alignment in metaphase and separation in anaphase (Walczak et al., 2010).

B. Microtubule-binding proteins

a. Modes of microtubule binding

There are hundreds of proteins that interact with microtubules. They can bind unpolymerized tubulin subunits, along the microtubule lattice, indirectly through other microtubule-interacting proteins, or specifically to either the minus or plus end of microtubules (Akhmanova and Steinmetz, 2008). Since the bulk of the research in this

dissertation pertains to the plus-end binding protein CLASP, I will mainly focus on +TIPs in this chapter, a class of proteins that bind specifically to the microtubule plus-end and play critical roles in the regulation of microtubule binding to cellular structures (Tamura and Draviam, 2012).

b. +TIPs

The ability to create GFP fusion proteins and observe protein dynamics by live-cell microscopy led to identification of the first +TIP, cytoplasmic linker protein 170 (CLIP-170) (Perez et al., 1999; Diamantopoulos et al., 1999). Since then, dozens of +TIPs have been identified and the majority of these proteins bind to microtubule plus-ends through end-binding protein 1 (EB1) (Kumar and Wittmann, 2012). EB1 recognizes a structural conformation on the growing ends of microtubules that is consistent with a GTP hydrolysis transition state (Maurer et al., 2011).

+TIP proteins can be classified depending on their mechanism of binding to the microtubule plus-end. *End-binding (EB) proteins* contain calponin homology (CH) domains that bind to microtubules in a hydrophobic manner (Akhmanova and Steinmetz, 2008). Examples include EB1, 2, and 3. CH domains are found in actin binding proteins and interestingly, in Ndc80, a highly conserved protein that serves as the core microtubule-binding site at kinetochores (Wei et al., 2007). Proteins that bind to microtubule ends through EB1 contain one or more short, hydrophobic (S/T)x(I/L)P sequences (SxIPs) (Kumar and Wittmann, 2012). The list of SxIP-containing proteins is continually expanding and includes proteins with diverse cellular functions (van der Vaart et al., 2011). *CAP-Gly proteins* are cytoskeleton-associated proteins (CAPs) that have glycine-rich (Gly) domains, which mediate interactions with microtubules or EB

proteins (Galjart, 2005). Examples include CLIP-170, CLIP-115, and p150^{Glued}, a component of the dynactin complex that associates with dynein (Schroer, 2004). +TIP proteins that contain *basic and serine-rich regions* bind to microtubules or EBs through electrostatic interactions with negatively-charged tubulin or the C-terminus of EB1 (Honnappa et al., 2005; Kumar et al., 2012). CLASP1/2, APC, MACF/ACF7, and STIM1 all contain basic or serine-rich regions (Kumar and Wittmann, 2012). Several +TIPs contain multiple *tumor overexpressed gene (TOG) domains* that mediate binding to tubulin and microtubules. TOG domains contain arrayed helices formed by HEAT repeats and highly conserved residues between the helical regions mediate tubulin binding (Slep, 2009). Proteins with TOG domains include Lis1, the microtubule polymerase XMAP215/chTOG, and CLASPs. *Microtubule motors*, both plus-end and minus-end-directed can be considered +TIPs because of their ability to processively track growing microtubules, either directly or through binding to other +TIPs (Wu et al., 2006).

c. CLASPs

Cytoplasmic linker-associating proteins (CLASPs) were first identified as interacting proteins of the +TIPs CLIP-115/170 in yeast two-hybrid screens (Akhmanova et al., 2001a). There are two CLASPs in mammalian cells that share 77% sequence similarity, and they are largely thought to be functionally redundant, although this has not been experimentally confirmed. During interphase in mammalian cells, CLASPs track microtubule plus-ends in the cell body and bind along the microtubule lattice in the lamella (Akhmanova et al., 2001a; Wittmann, 2005). A middle serine and arginine-rich unstructured region is responsible for binding to EB1 and thus the plus-end-tracking of

CLASPs (Wittmann, 2005) (Figure 2.1). CLASP2 EB-binding is mediated by two SxIP motifs and positively charged residues in this region (Kumar et al., 2012). In addition to plus-end tracking, CLASP binds to the lattice of microtubules through a TOG-like domain in the c-terminal half of the protein (Figure 2.1) (data not shown). This asymmetric localization of CLASP to microtubules suggests that CLASPs may functions to stabilize microtubules in the cell periphery. Indeed, overexpression of GFP-CLASP causes an increase in stabilized microtubules, assayed by acetylated tubulin staining (Akhmanova et al., 2001a; Bulinski et al., 1988). Conversely, injection of CLASP-specific antibodies or depletion of CLASPs results in a decrease in microtubule density and stabilized microtubules (Mimori-Kiyosue et al., 2005). CLASPs bind directly to polymerized microtubules *in vitro* (Wittmann, 2005; Mimori-Kiyosue et al., 2005; Akhmanova et al., 2001a). In addition, the fission yeast homolog of CLASP, Cls1p, has been shown to increase microtubule rescue frequency, decrease catastrophe frequency, and slightly decrease disassembly rate *in vitro* (Al-Bassam et al., 2010). These data suggest that CLASP acts as a rescue factor by either recruiting tubulin dimers to the microtubule end to promote assembly or by protecting the microtubule end from depolymerization. However, this microtubule rescue factor model has not been tested in mammalian cells (Al-Bassam and Chang, 2011), and yeast and mammalian CLASPs are structurally and functionally quite divergent. For example, yeast CLASP does not track microtubule ends and lacks a SxIP motif. In addition to microtubules, CLASPs localize to Golgi, where they direct Golgi organization, and during cell migration, CLASPs at the cortex are required for focal adhesion disassembly (Miller et al., 2009; Liu et al., 2007; Stehbens et al., 2014).

C. Microtubule binding during mitosis

a. Microtubule behaviors during mitosis

As cells enter mitosis, microtubules become increasingly dynamic and undergo a dramatic reorganization to form the mitotic spindle (Rusan et al., 2001). There are many different types of microtubules in the spindle. Astral microtubules grow out toward the edge of the cell and mediate interactions with the cortex and cortical-associated proteins. They are important for location of cytokinetic furrow ingression and spindle orientation (Rappaport, 1990). Interpolar microtubules grow across the spindle but do not attach to kinetochores, and their crosslinking provides stability to the spindle. In fact, the Eg5 kinesin crosslinks interpolar microtubules and inhibition of Eg5 with the drug, STLC, results in spindle collapse (Skoufias et al., 2006). Microtubules that attach to kinetochores, either laterally or end-on, are collectively referred to as kinetochore microtubules and direct chromosome movements throughout mitosis. In addition, microtubules are nucleated from other areas of the spindle in addition to centrosomes (Tulu et al., 2003; Wollman et al., 2005). Kinetochores can generate microtubules, and this is thought to occur through kinetochore-localized gamma-turc components (Mishra et al., 2010; Tulu et al., 2006). In addition, a gradient of Ran activity near chromosomes promotes microtubule assembly (Clarke and Zhang, 2008).

Directly following nuclear envelope breakdown, kinetochores begin interacting with microtubules. Much of these initial interactions are lateral attachments to the sides of the microtubule lattice, and mostly mediated by motor proteins (Tanaka et al., 2005). These lateral attachments are converted to more stable end-on attachments as chromosomes biorient on the spindle. Recent evidence suggests that CENP-E mediates

lateral interactions with the microtubule and the microtubule depolymerase, MCAK, releases laterally-attached microtubules once partial end-on attachments are formed (Shrestha and Draviam, 2013). Biorientation is not required for chromosomes to congress to the metaphase plate, but ultimately required for proper segregation of sister chromatids during anaphase (Magidson et al., 2011).

b. Microtubule-binding proteins at kinetochores

Chromosomes remain dynamic throughout mitosis; therefore, it is critical that kinetochores remain stably attached to microtubules that are undergoing continuous cycles of polymerization and depolymerization. The primary microtubule attachment at kinetochores is through the Ndc80 complex, a four subunit complex that forms a long rod-shaped structure (Deluca and Musacchio, 2012). The Ndc80 complex contains calponin homology (CH) domains (Wei et al., 2007) and a positively charged amino terminal tail that bind microtubules *in vitro* and *in vivo* (Cheeseman et al., 2006; Deluca et al., 2005; Wei et al., 2007; Ciferri et al., 2008). By EM, both Ndc80 and another protein in the complex, Nuf2, make contact with the microtubule lattice (Wilson-Kubalek et al., 2008; Alushin et al., 2010). Not surprisingly, cells exhibit extensive missegregation of chromosomes following depletion of Ndc80 (Deluca, 2002; Desai et al., 2003). The Ndc80 complex is part of a larger network of proteins known as the KMN network, made up of KNL-1 and the Mis12 complex, which together, synergistically bind to microtubules. In addition to Ndc80, KNL-1 has microtubule-binding activity, and although it binds more weakly to microtubules, it increases the affinity of Ndc80 for microtubules (Cheeseman et al., 2006). In *S. cerevisiae* kinetochores, Dam1 confers additional microtubule-binding to the kinetochore by forming a ring around the

microtubule end (Westermann et al., 2005). There is no clear mammalian homolog of Dam1 and no such ring around microtubules has been observed at kinetochores.

In addition to the KMN network, there are many other kinetochore proteins that have microtubule-binding activity. CENP-E, a plus-end-directed kinesin, is required for the congression of chromosomes from spindle poles to the metaphase plate and may also play a role in regulating microtubule dynamics (Kim et al., 2008; Sardar et al., 2010). CENP-E directly binds microtubules *in vitro* (Musinipally et al., 2013). Because CENP-E is required for CLASP kinetochore localization, the role for CENP-E in regulating microtubule dynamics may be attributed to CLASP's effect on microtubules (Maffini et al., 2009a). Another component of the outer kinetochore, CENP-F, binds weakly to microtubules *in vitro* and is required for stable kinetochore-microtubule attachments and stable kinetochore fibers (Bomont et al., 2005; Musinipally et al., 2013). Interestingly, CLASPs interact with CENP-F in *C. elegans* (Cheeseman et al., 2005). The recently discovered Astrin/SKAP complex at kinetochores is required for stable kinetochore-microtubule attachments and furthermore, both Astrin and SKAP bind directly to polymerized microtubules *in vitro* (Schmidt et al., 2010; Manning et al., 2010). SKAP tracks microtubule plus-ends *in vitro* in an EB1-dependent manner and contains one SxIP motif (Wang et al., 2012). Astrin tracks microtubule plus-ends during interphase, however, it lacks a SxIP motif, strongly suggesting that it binds to microtubule ends in an EB1-independent manner (Dunsch et al., 2011).

In addition to CLASP, there are many other kinetochore-associated +TIPs that are implicated in kinetochore-microtubule attachment. Both EB1 and APC localize to kinetochores that are attached to microtubules and depletion of either protein causes

chromosome misalignment (Kaplan et al., 2001; Green et al., 2005; Draviam et al., 2006). EB1 binds preferentially to antipoleward-moving kinetochores that are attached to polymerizing microtubules in Ptk cells, however, this has not been verified in human cells (Tirnauer et al., 2002). CLIP-170 is an outer kinetochore component that is only present on unattached kinetochores (Tanenbaum et al., 2006). Recently, the formin mDia3, which binds to EB1 but is not considered a +TIP, has been shown to be required for stable kinetochore-microtubule attachments (Cheng et al., 2011). Because mDia3 localizes to kinetochores through mitosis and EB1 and APC do not, the current model is that mDia3 provides kinetochores a link to polymerizing microtubules (Cheng, 2011). While multiple +TIPs localize to kinetochores during mitosis, their functions and regulation are not well understood.

c. Mitotic functions of CLASPs

A critical mitotic function for CLASPs was originally discovered through genetic screens in *Drosophila melanogaster* for maternal effect lethal genes (Inoue et al., 2000; Lemos, 2000). Mutants of Orbit/Mast, the fly homolog of CLASP, are viable but exhibit developmental delays and abnormal mitoses in immunofluorescence experiments (Inoue et al., 2000; Lemos, 2000). In HeLa cells, CLASPs localize to kinetochores, centrosomes, and the spindle midzone and midbody (Pereira et al., 2006; Mimori-Kiyosue et al., 2006). They exhibit fast turnover rates at kinetochores (Pereira et al., 2006). Specifically, CLASPs are present in the outer kinetochore and by super resolution protein localization methods, they are one of the outermost kinetochore components and appear farther away from the kinetochore than the ends of microtubules (Mimori-Kiyosue et al., 2006; Wan et al., 2009). This raises the question of

whether CLASP lattice-binding or end-binding activities are utilized at kinetochores. Depletion of CLASPs using siRNA in human cells, immunodepleted *Xenopus* extracts, and mouse embryonic fibroblasts (MEFs) from a CLASP2 knockout mouse cause a range of mitotic phenotypes including monopolar and multipolar spindles, increased mitotic time, wider metaphase plate, and lagging chromosomes (Pereira et al., 2006; Hannak, 2006; Mimori-Kiyosue et al., 2006). Together, these data suggest that CLASP is required for proper chromosome segregation.

CLASP binds directly to microtubules *in vitro* and is present at kinetochores close to microtubules, which begs the question if CLASP mediates kinetochore-microtubule interactions (Wittmann, 2005; Wan et al., 2009; Akhmanova et al., 2001b). However, electron microscopy (EM) images of kinetochores in anti-CLASP antibody-injected cells show normal kinetochore-microtubule interactions, while CLASP-depleted cells show unperturbed kinetochore-microtubule attachments (Mimori-Kiyosue et al., 2006; Maiato et al., 2003). Whether or not these methods resulted in incomplete CLASP perturbations, or if CLASP functional redundancy masks mitotic phenotypes remain unclear. A current model for CLASP mitotic function is that it is not required for kinetochore-microtubule attachment per se, but instead it regulates the dynamics of attached microtubules (Maiato, 2003). This model has been tested in *Drosophila* S2 cells by monitoring poleward flux, the movement of tubulin subunits within a kinetochore fiber that is mediated by microtubule polymerization at kinetochores and depolymerization at spindle poles, using photobleaching of GFP-alpha-tubulin (Rogers, 2005; Maddox et al., 2002). Depletion of the *Drosophila* CLASP homologue abrogates poleward flux, and the authors showed that this was not due to a defect in

depolymerization of microtubules at spindle poles (Maiato et al., 2005). This suggests that *Drosophila* CLASP is required for microtubule polymerization at kinetochores. In human U2OS cells, CLASP depletion decreases, but does not abolish poleward flux, possibly because of incomplete depletion or redundant poleward flux mechanisms (Maffini et al., 2009a). Interestingly, EB1 is not required for poleward flux in S2 cells, possibly indicating a EB1-independent role for CLASP during mitosis (Maiato et al., 2005).

There are many different CLASP-interacting proteins identified through immunoprecipitation experiments, including CLIP-170, LL5beta, ninein, and the mitotic proteins CENPE, CENPJ, and Astrin (Maffini et al., 2009b). CENPE, a mitotic kinesin required for chromosome alignment, is essential for CLASP kinetochore localization (Kim et al., 2008; Maffini et al., 2009a). It has been proposed that CLASP exists in two mutually exclusive complexes during mitosis; during prometaphase, CLASP interacts with Kif2b and is associated with increased Aurora B activity and together with Astrin, CLASP promotes stabilization of kinetochore-microtubule attachments during metaphase (Manning et al., 2010). In addition to kinetochore-microtubule interactions, CLASP has also been shown to contribute to spindle pole integrity and spindle positioning (Logarinho et al., 2012; Samora et al., 2011; Bird et al., 2013).

III. Regulation of microtubule-binding activity

A. Microtubule-binding proteins regulated through mitotic phosphorylation

a. Function

Microtubules serve many functions during mitosis, such as chromosome movements and cytokinesis, therefore it is no surprise that they are spatially and

temporally regulated throughout mitosis. Remarkably, spindle microtubules undergo a drastic increase in dynamics as cells enter mitosis (Rusan et al., 2001). Microtubules at the cortex are stabilized, much like that during cell migration, to allow for delivery of components to the cortex and spindle positioning (Tamura and Draviam, 2012). Kinetochore microtubules, classified as microtubules that are stably attached to both a spindle pole and kinetochore, are selectively stabilized as compared to other microtubules in the spindle, as evidenced by their resistance to cold treatment (Brinkley and Cartwright, 1975). Centrosomal microtubules are highly dynamic, yet, once bound to kinetochores, they become stabilized, suggesting that the kinetochore is a localized zone of microtubule regulation. Kinetochore-microtubule attachments must be extremely stable to maintain attachment to chromosomes, yet flexible to allow for error correction mechanisms and microtubule dynamics that power chromosome movements. Microtubules within a kinetochore bundle must have coordinated dynamics in order to productively push or pull a chromosome. In addition to being able to withstand cold treatment, microtubules at kinetochores have a decreased rate of turnover as compared to spindle microtubules. Also, depolymerizing microtubule ends at kinetochores display a distinct curvature; they are much less flared than spindle microtubules (McIntosh et al., 2013). Together, these data strongly suggest the existence of kinetochore factors that locally stabilize microtubule ends.

b. Major regulators of microtubule-binding and dynamics (Aurora B, Cdk, Plk, PP1 and PP2A, GSK3)

There are multiple kinases and phosphatases at kinetochores that locally regulate kinetochore-microtubule interactions. The kinase, *Aurora B,* plays an important

role in the correction of improper kinetochore-microtubule attachments by regulating microtubule-binding activities and protein-protein interactions of many kinetochore substrates (Tanaka, 2012). Components of the core microtubule-binding complex at kinetochores, the KMN network, most notably, Ndc80, are substrates of Aurora B (Cheeseman et al., 2006). Phosphorylation decreases Ndc80's affinity for microtubules and allows for release of improper attachments (DeLuca et al., 2011). Interestingly, Ndc80 phosphorylation has also been shown to directly regulate microtubule dynamics *in vitro* (Umbreit, 2012). The recently discovered microtubule-binding Ska complex is also a substrate of Aurora B (Chan et al., 2012b). Phosphorylation of Ska components inhibits interaction with the KMN network and the formation of stable kinetochore-microtubule attachments, and represents another mechanism by which Aurora B phosphorylation inhibits the formation of stable kinetochore-microtubule attachments. MCAK, a microtubule depolymerase, is also phosphorylated by Aurora B, which controls MCAK targeting within kinetochores (Andrews et al., 2004). Phosphorylation of the kinesin CENP-E is required for chromosome congression (Kim et al., 2010). Intriguingly, CLASP2 contains a putative Aurora B phosphorylation site in close proximity to the CDK and GSK3 sites within its microtubule plus-end-tracking domain (Figure 2.1). It will be important to conduct future experiments to determine if Aurora B phosphorylates CLASP2 and if phosphorylation negatively regulates kinetochore-microtubule interactions or dynamics.

Glycogen synthase kinase 3 beta (GSK3β) is an important enzyme that participates extensively in many signaling pathways, including those in neurons where GSK3β regulates axonal growth (Jope and Johnson, 2004). The microtubule-binding

activities of several microtubule-binding proteins are regulated through GSK3β phosphorylation, including MAP1B, APC, tau (Jope and Johnson, 2004; Buttrick and Wakefield, 2008), and CLASP2 (Kumar et al., 2009; Watanabe et al., 2009). During interphase, a gradient of GSK3β restricts CLASP2 lattice binding to the lamella of cells, where it acts to stabilize microtubules (Kumar et al., 2009; Stehbens et al., 2014). GSK3β is required for proper mitotic spindle formation, however, the mitotic substrates of GSK3β and the molecular mechanisms underlying its role in chromosome segregation have remained elusive (Wakefield, 2002; Tighe et al., 2007). In Chapter 2 of this thesis, I report that dynamic regulation of GSK3β phosphorylation of CLASP2 is required for proper kinetochore-microtubule interactions during mitosis. The mitotic *cyclin-dependent kinase 1 (CDK1)*, known for its function in cell cycle progression, also plays a role in regulating microtubule binding during mitosis. CDK1 regulates MAP4 and its role in stabilizing microtubules (Ookata et al., 1997), Op18/stathmin and microtubule catastrophes (Cassimeris, 2002), the microtubule polymerase XMAP215 (Vasquez et al., 1999), and CLASP2-EB1 binding (Kumar et al., 2012). *Polo-like kinase 1 (Plk1)* also has a role in stabilizing kinetochore-microtubule attachments and is thought to oppose Aurora B phosphorylation. CLASP2 is phosphorylated by Plk1 and phosphorylation is required for normal chromosome alignment and mitotic timing (Maia et al., 2012). Phosphatases localize to kinetochores and act to oppose kinase activity. *Protein phosphatase 1 (PP1)* at kinetochores is required for stable microtubule attachments and actively inhibits Aurora B localization to kinetochores (Liu et al., 2010). In addition, the B56-PP2A phosphatase is required for the stability of kinetochore fibers (Foley et al., 2011).

B. Regulation of microtubule binding at kinetochores

During mitosis, kinetochore-microtubule interactions must be precisely regulated in space and time to ensure accurate chromosome segregation. In particular, kinetochore-microtubule attachments must be stable enough to power chromosome movements during both metaphase alignment and anaphase separation. In addition, microtubule attachments must be extremely dynamic to allow for correction of improper kinetochore-microtubule attachments detected by the spindle checkpoint and to allow for dynamics and coordination of attached kinetochore microtubule bundles. Microtubules at kinetochores display unique properties as compared to other microtubules in the spindle, strongly suggesting that they are under localized regulation at kinetochores. Kinetochore microtubules are much more stable than spindle microtubules as evidenced by their ability to withstand cold-induced depolymerization (Rieder, 1981). By EM, microtubule ends at kinetochores appear to have a more straight conformation, possibly due to multiple microtubule-binding activities at kinetochores (McIntosh et al., 2013). In addition, not all microtubules in a kinetochore bundle are coordinated in their polymerization state, suggesting a more complex regulation of microtubule dynamics (VandenBeldt et al., 2006). How exactly kinetochore-microtubule interactions are spatially and temporally regulated remains unclear.

I have previously described the concept of tension-dependent phosphoregulation of microtubule-binding proteins at kinetochores through Aurora B phosphorylation in the context of error correction mechanisms (Introduction Part I. B. b. ii.). Recently, Aurora B phosphorylation of the Ndc80 complex has been shown to directly affect microtubule

dynamics in vitro, which suggests a more complex Aurora-B-mediated phosphoregulation (Umbreit, 2012). Previous work in the Wittmann lab showed that CLASP2 phosphorylation by Cdk1 and GSK3β inhibits its ability to bind microtubule ends and because CLASP2 is present in the outer kinetochore close to the ends of microtubules, this begs the question of whether CLASP2 phosphorylation is a further mechanism by which kinetochore-microtubule attachments are regulated.

Chapter 1: CLASP2 phosphorylation inhibits microtubule binding during mitosis

I. Introduction

Previous work in the Wittmann lab demonstrated that CLASP2 asymmetrically localizes to microtubules in interphase cells, showing microtubule plus-end binding in the cell body and binding along the microtubule lattice in the cell periphery (Wittmann, 2005). Subsequently, it was shown that many microtubule +TIP proteins contain SxIP motifs that are required for EB1-binding and microtubule plus-end tracking. Interestingly, CLASP2 contains two SxIP motifs in its previously characterized microtubule end-binding, central unstructured region (Honnappa et al., 2009). However, EB1-dependent plus-end tracking had not been directly shown for CLASP2. CLASP2 microtubule binding is regulated through GSK3β phosphorylation of serine residues that also reside in the central, plus-end tracking domain of CLASP2. Phosphorylation of CLASP2 inhibits its microtubule-binding activity and a gradient of GSK3β activity was hypothesized to control CLASP microtubule binding spatially (Kumar et al., 2009). During my rotation in the Wittmann lab, I noticed that wildtype CLASP2 constructs failed to localize to microtubule plus-ends during mitosis. In addition, GSK3β activity was required for proper chromosome segregation (Tighe et al., 2007; Wakefield, 2002), which raised the possibility of GSK3β-mediated regulation of CLASP2 during mitosis.

II. Results

A. CLASP2 plus-end-tracking is EB1-dependent

To test if CLASP2 microtubule-plus-end binding is dependent on EB1, we used purified, recombinant proteins in an *in vitro* assay in which dynamic microtubules are polymerized from biotinylated GMPCPP microtubule seeds to reconstitute plus-end-tracking (Bieling et al., 2007; 2010). The central domain of CLASP2(497-794) bound weakly along microtubules, however, when EB1 was added to the assay mixture EGFP-CLASP2(497-794) bound to the growing ends of microtubules (Figure 1.1). When microtubule ends were paused or depolymerizing, EGFP-CLASP2(497-794) dissociated from the microtubule end. This behavior is consistent with that of an EB1-dependent +TIP *in vitro*, such as CLIP-170 (Bieling et al., 2008; Dixit et al., 2009).

B. Mitotic phosphorylation of CLASP2 inhibits its microtubule plus-end binding

Previous work in the Wittmann lab demonstrated that CLASP2 microtubule binding is regulated through GSK3β-mediated phosphorylation (Kumar et al., 2009). Specifically, phosphorylation of serine residues in the central domain of CLASP2 inhibits binding to EB1 and thus microtubule plus-end-tracking(Kumar et al., 2009; 2012). After joining the Wittmann lab, I found that CLASP2 is hyperphosphorylated during mitosis (Figure 1.5 A). To test if mitotic phosphorylation of CLASP2 occurred in the central domain at the previously-identified serines, we used a metabolic labeling assay in cells arrested in metaphase with the Eg5 kinesin inhibitor, S-trityl-L-cysteine (STLC) (Skoufias et al., 2006). Wildtype EGFP-CLASP2 displayed an upshift in metaphase-arrested cells and addition of a GSK3β inhibitor, SB216763, or mutation of the nine serines to nonphosphorylatable alanines (9xS/A) partially abrogated the mitotic upshift (Figure 1.2). Neither perturbation completely abrogated the mitotic upshift, suggesting that CLASP2 is phosphorylated by other kinases within the central domain (Figure 1.1

A, CLASP2(497-794)), likely mitotic CDKs. To test the effect of mitotic phosphorylation on CLASP2 microtubule end binding, we expressed EGFP-tagged versions of CLASP2 in which all serines were mutated to nonphosphorylatable alanines (9xS/A) or versions of CLASP in which just the CDK priming sites were mutated (S741/775A) in HaCaT cells. While wildtype EGFP-CLASP2 was largely absent from microtubule ends in metaphase, nonphosphorylatable EGFP-CLASP2(9xS/A) and (S741/775A) bound to plus-ends in a manner identical to EB1 (Figure 1.3). To further test if priming phosphorylation of CLASP2 by CDK1 inhibited CLASP2 plus-end binding during mitosis, we treated EGFP-CLASP2-expressing cells in metaphase with the specific CDK1 inhibitor, RO-3306. EGFP-CLASP2 re-associated with microtubule ends following addition of RO-3306 to metaphase cells (Figure 1.4). Together, these data show that CLASP2 is phosphorylated during mitosis by CDK1 and subsequently GSK3β on serines within the central plus-end-tracking domain and that phosphorylation of CLASP2 inhibits microtubule end binding during mitosis.

C. Generation of a phospho-specific CLASP2 antibody

We have shown that phosphorylation of CLASP2 by CDK1 and GSK inhibits CLASP2-microtubule association during mitosis (Kumar et al., 2009; 2012) (Figure 1.3 and 1.4). To test if CLASP2 microtubule-binding activity, and thus phosphorylation, is regulated temporally and spatially during mitosis we generated a custom phospho-specific antibody to CLASP2 through the company, Phosphosolutions (Archuleta et al., 2011). The polyclonal antibody was raised against the peptide sequence S(p)QGCS(p)REAS(p) (Figure 1.1 A). We reasoned that this sequence would serve as a successful epitope because these residues exist in both stretches of GSK3β

phosphorylation sites within plus-end-binding domain of CLASP2 (Figure 1.1 A). We have shown that serines in the second GSK3β stretch are phosphorylated during interphase and that during mitosis, there is likely phosphorylation in both stretches (Kumar et al., 2009) (data not shown). Therefore, we expected the antibody to be specific for phospho-CLASP2, although it may recognize both interphase and mitotic phosphorylated CLASP2.

First, we tested the ability of the phospho-specific antibody to recognize CLASP2 in biochemical assays of asynchronously growing HaCaT cells or cells arrested in mitosis using STLC (Skoufias et al., 2006). In immunoblotting experiments, we observed a slight upshift of phospho-CLASP2 signal in metaphase-arrested cells, suggesting that the antibody is able to recognize phosphorylated CLASP2 (Figure 1.5 A). While a larger upshift is seen with a total CLASP2 antibody, this is likely due to phosphorylation outside of this motif or modifications other than phosphorylation. To test the phosphospecificity of our antibody, we immunoblotted lysates from cells treated with a GSK3β inhibitor, SB216763, or overexpressing constitutively active mRFP-GSK3β(S9A). While a total CLASP2 antibody recognizes CLASP2 in cells in which GSK3β is both inhibited and constitutively active, the phospho-specific antibody fails to recognize CLASP2 in cells treated with GSK3β inhibitors (Figure 1.5 B). This strongly suggests that the antibody is specific for phosphorylated CLASP2, although it seems to recognize CLASP2 with a lower affinity than the total CLASP2 antibody.

To test if CLASP2 phosphorylation is regulated in a spatial manner in cells, such as at kinetochores during mitosis, we compared kinetochore staining of phospho-CLASP2 in metaphase cells with perturbed GSK3β activity. We found that the phospho-

CLASP2 antibody stains cells in a punctate pattern and that this staining was not decreased upon inhibition of GSK3β using treatment with LiCl, strongly suggesting that the cytoplasmic punctae antibody staining is nonspecific (Figure 1.6 A). Next, we tested the phospho-CLASP2 antibody in cells expressing nonphosphorylatable EGFP-CLASP2(9xS/A). We reasoned that EGFP-CLASP2(9xS/A) would compete with endogenous CLASP2 for kinetochore binding sites and thus if this antibody was specific for the indicated serines, then we would observe loss of any kinetochore signal. However, we observed an overlap of signal between nonphosphorylatable EGFP-CLASP2(9xS/A) expression and staining of phospho-CLASP2 at kinetochores, further suggesting that the phospho-CLASP2 antibody does not specifically stain phosphorylated CLASP2 in cells. In an attempt to optimize staining, we tested multiple fixation methods, including methanol, gluteraldehyde, paraformaldehyde (PFA), and a combination of gluteraldehyde/PFA. In addition, we tested pre-permeabilizing cells prior to fixation. Lastly, we tested both bovine serum albumin (BSA) and fish gelatin as blocking buffers. However, none of these experimental conditions led to a decrease in background staining and thus we concluded that this phospho-specific antibody does not specifically stain phosphorylated CLASP2 by immunostaining methods.

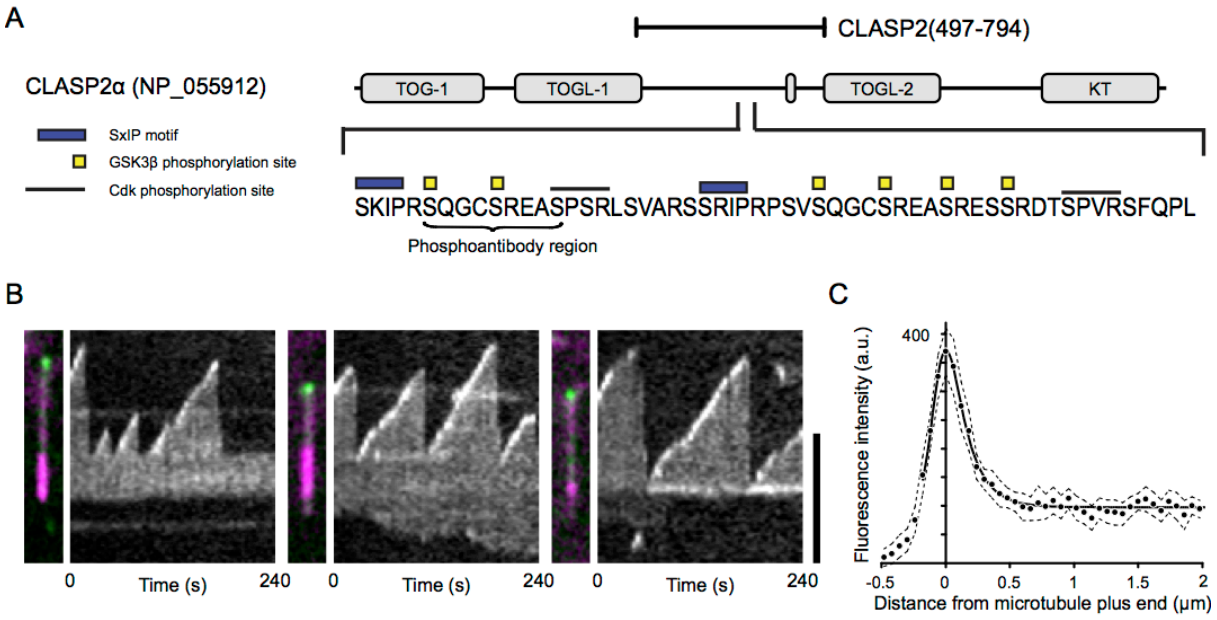

Figure 1.1 CLASP2 microtubule-plus-end association in vitro is EB1-dependent. (A) Domain structure of human CLASP2 showing SXIP motifs (blue), previously identified GSK3 phosphorylation sites (yellow), and CDK phosphorylation sites (underlined) (Kumar et al., 2009). Amino acid numbering is based on the most recent NCBI reference sequence NP_055912.2 and is different from the numbering we previously used (Kumar et al., 2009). Also shown is the sequence used for generation of a phospho-specific CLASP2 antibody. (B) Three representative examples of in vitro microtubule plus-end-tracking of the central SXIP motif-containing CLASP domain, EGFP-CLASP2(497–794). Panels on the left are overlays of the EGFP channel (green) and X-rhodamine-labeled tubulin (purple; the bright region is the GMPCPP-stabilized microtubule seed). Panels on the right are kymographs of the EGFP channel showing EGFP-CLASP2-(497–794) accumulation at growing microtubule plus ends. Scale bar, 5 μm. (C) An average fluorescence intensity profile of EGFP-CLASP2(497–794) along growing microtubules (n = 20). The dashed line indicates the 95% confidence interval, and the solid line is an exponentially modified Gaussian fit. a.u., absorbance units.

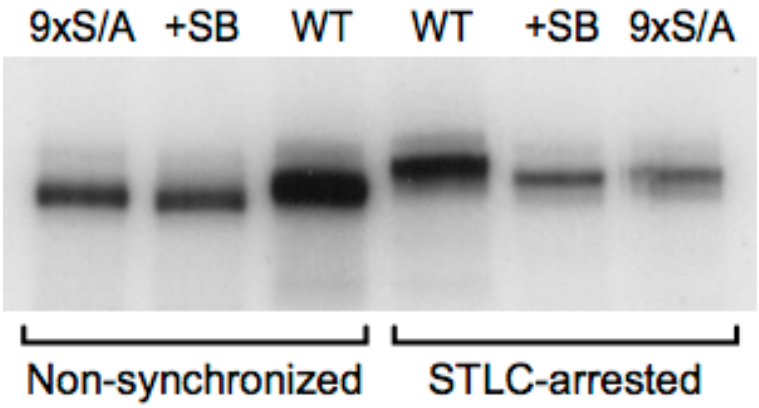

Figure 1.2 CLASP2 is phosphorylated by GSK3 during mitosis. Metabolic labeling of HeLa cells with 32P-labeled phosphate and treated with a GSK3 inhibitor, SB216763. EFP-CLASP2(497–1238) WT or 9xS/A was immunoprecipitated and analyzed by SDS-PAGE and autoradiography. Radioactivity incorporation is not directly comparable between non-synchronized and STLC-arrested cells because of differences in loading due to limited recovery of mitotic cells.

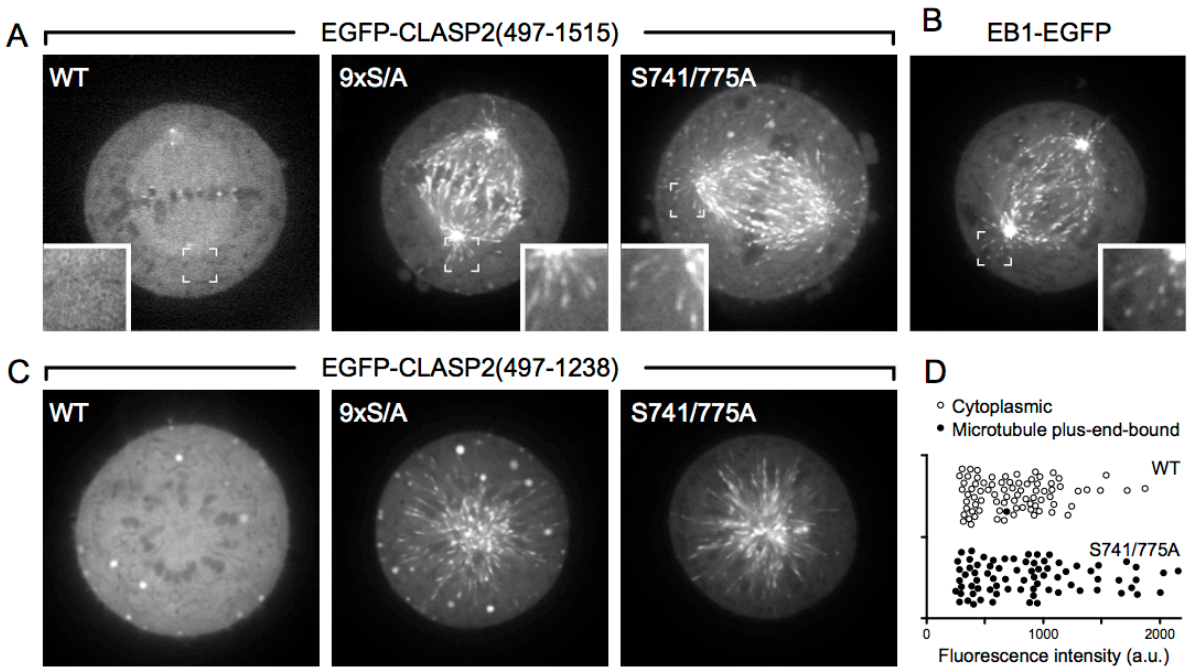

Figure 1.3 CLASP2 plus-end-tracking is inhibited during mitosis due to phosphorylation. (A) HeLa cells in metaphase expressing wildtype (WT) EGFP-CLASP2(497–1515) or constructs in which all GSK3 sites (9xS/A) or only SXIP motif-associated CDK priming sites (S741A/S775A) were mutated. Insets show the indicated regions at higher magnification. (B) A metaphase cell expressing EB1-EGFP. (C) HeLa cells expressing EGFP-CLASP2(497–1238) constructs lacking the kinetochore binding domain and treated with STLC, which results in monopolar spindles arrested in a metaphase-like state. (D) Quantification of data in C demonstrates that mutation of the priming sites alone restores CLASP2 plus-end-tracking during mitosis. Each symbol represents the average EGFP-CLASP2(497–1238) fluorescence intensity in the cytoplasm of one cell (n = 80). a.u., arbitrary units.

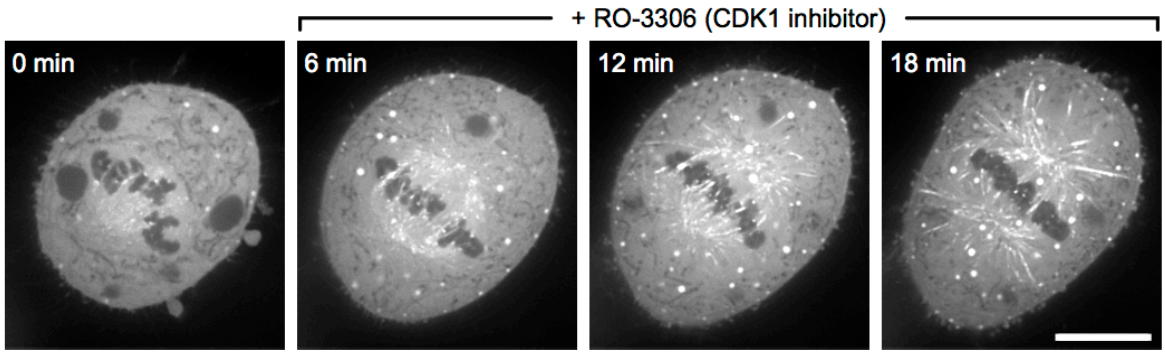

Figure 1.4 CLASP2 plus-end-tracking is inhibited during mitosis by CDK1 priming phosphorylation. EGFP-CLASP2(497–1515)-expressing HeLa cells arrested in metaphase with MG132 and subsequently treated with the CDK1 inhibitor, RO-3306. Scale bar, 10 μm.

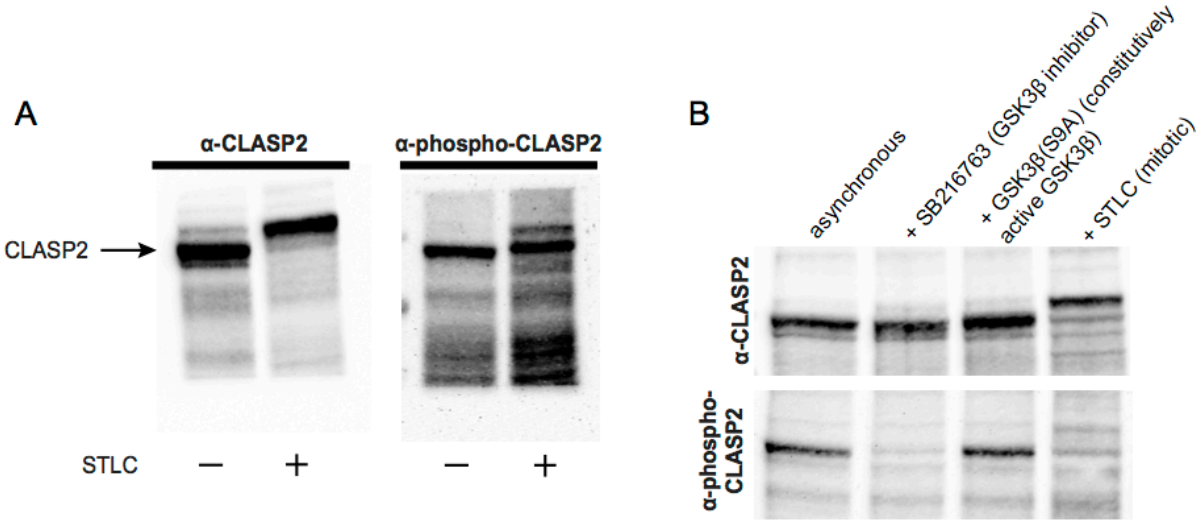

Figure 1.5 Biochemical characterization of a phospho-CLASP2 antibody. (A, B) Immunoblots of endogenous CLASP2 and phospho-CLASP2 in lysates from asynchronously-growing cells, cells arrested in mitosis with STLC, or with perturbed GSK3β activity (20 μM SB216763 - GSK3β inhibitor, GSK3β(S9A) - constitutively active GSK3β).

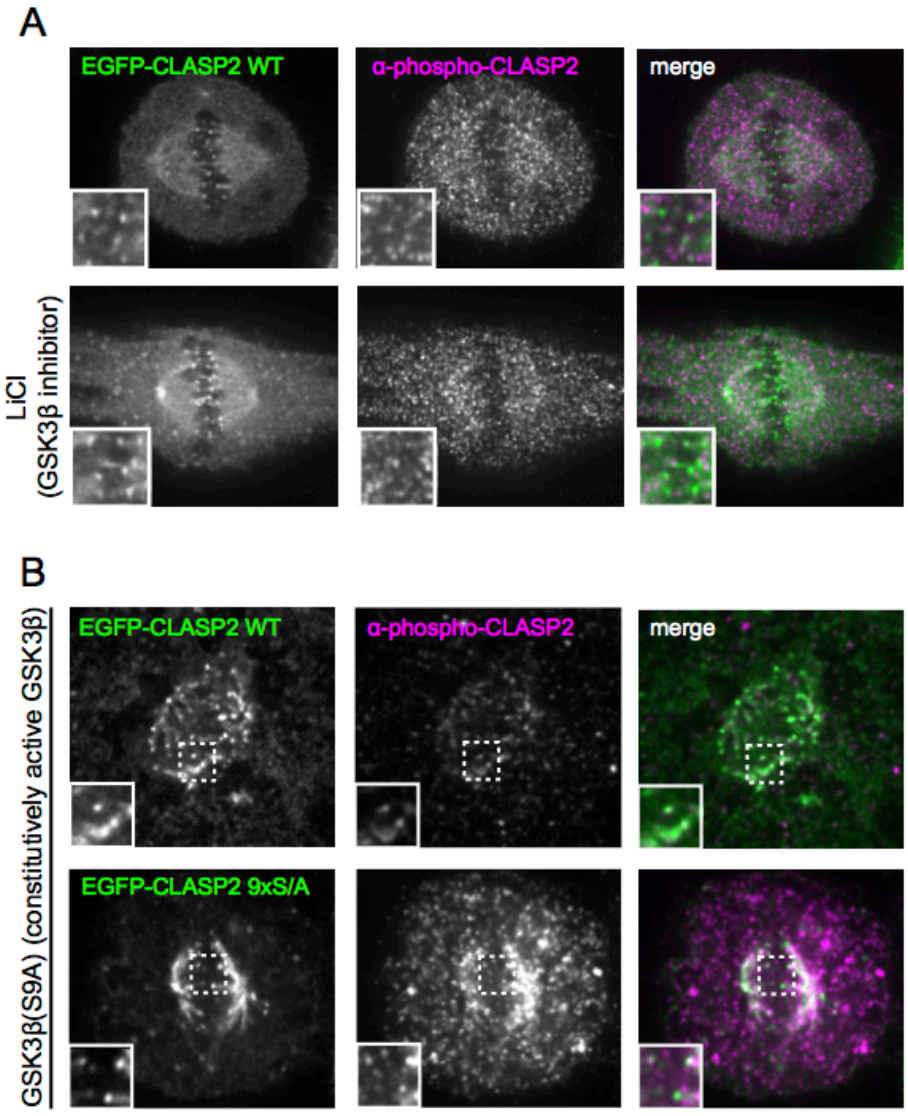

Figure 1.6 Immunofluorescence experiments to detect phosphoCLASP2 during mitosis. (A) HeLa cells at metaphase expressing wildtype (WT) EGFP-CLASP2 (green) and stained for phospho-CLASP2 (magenta). Bottom row images are from cells treated with LiCl, a potent inhibitor of GSK3. (B) HeLa cells at metaphase expressing either wildtype (WT) or nonphosphorylatable(9xS/A) EGFP-CLASP2 constructs (green), treated with constitutively active GSK3β, and stained for phospho-CLASP2 (magenta). Insets show sister kinetochore pairs.

Chapter 2: Mitotic phosphorylation of CLASP2 regulates kinetochore-microtubule interactions

I. Introduction

Proper chromosome segregation requires dynamic regulation of kinetochore-microtubule attachments throughout mitosis. There are multiple kinetochore proteins with microtubule-binding activity, yet how exactly these proteins are regulated in space and time is unclear. CLASP2 is present in the outer kinetochore, in close proximity to microtubule ends, and is required for proper chromosome segregation (Wan et al., 2009; Mimori-Kiyosue et al., 2006; Maiato et al., 2005; Maia et al., 2012). We have shown that CLASP2 is hyperphosphorylated during mitosis, which inhibits its ability to bind microtubule ends (Chapter 1). Together, these data beg the question of whether phosphorylation of CLASP2 is locally regulated at kinetochores and serves as a mechanism to regulate kinetochore-microtubule interactions during mitosis. We show that Cdk1 and GSK3β-dependent mitotic phosphorylation of CLASP2 within its microtubule plus-end-binding domain inhibits its microtubule plus-end-binding activity but does not affect CLASP2 kinetochore binding. While deregulation of CLASP2 phosphorylation has little effect on initial chromosome congression, it weakens kinetochore-microtubule attachments. Phosphorylation-deficient CLASP2 increases average interkinetochore distance, while phosphomimetic CLASP2 decreases average interkinetochore distance measurements. These results suggest that CLASP2 microtubule binding at the kinetochore is required for stable kinetochore-microtubule attachments that can generate normal levels of tension. Furthermore, CLASP2 phosphomutant-expressing cells display abnormal kinetochore dynamics and in some

cells, kinetochore pairs flip about the metaphase plate, suggesting loss or imbalance of microtubule attachments. Ultimately, expression of either nonphosphorylatable or phosphomimetic CLASP2 fails to rescue depletion of CLASP2 and their expression ultimately leads increased lagging chromosomes. Together, these results characterize specific phosphorylation sites in CLASP2 that regulate microtubule binding and present a novel mechanism of GSK3β-mediated control of kinetochore-microtubule interactions.

II. Results

A. CLASP2α is phosphorylated during mitosis

We previously reported that binding to growing microtubule ends of an N-terminally truncated CLASP2(497-1515) construct is greatly reduced during mitosis due to multisite phosphorylation by cyclin-dependent kinases and GSK3β (Kumar et al., 2012). Although this construct is similar to the shorter CLASP2γ isoform and contains EB1-binding SxIP motifs and a TOG-like domain that we previously identified as being required for direct CLASP2-microtubule binding (Wittmann, 2005), many cells express a longer isoform, CLASP2α, that contains two additional N-terminal TOG-like domains (Figure 2.1 A). Although the function of these TOG domains is unclear, evidence exists that they may contribute to microtubule binding (Patel et al., 2012). To first test how phosphorylation levels of endogenous CLASPs change during the cell cycle, we immunoblotted HeLa cell lysates synchronized by double thymidine block with CLASP1 and CLASP2 specific antibodies. We detected an upshift of CLASP2, but not CLASP1, beginning ~8 hours after release from cell cycle arrest when cyclin B1 levels peaked and cells entered mitosis, which also confirms published data (Figure 2.1 B) (Maia et al., 2012). In addition, we observed hyperphosphorylation of endogenous CLASP2 in

human HaCaT keratinocytes arrested in mitosis with the Eg5/KIF11 inhibitor, S-Trityl-L-cysteine (STLC) (Figure 2.1 C) (Skoufias et al., 2006). We previously demonstrated in HeLa cells that this mitotic upshift is largely due to GSK3β-mediated multisite phosphorylation of sites within the plus-end-binding domain of CLASP2 (Kumar et al., 2012).

B. Mitotic phosphorylation of CLASP2α inhibits its microtubule end-binding activity

To determine how mitotic phosphorylation of CLASP2 affects microtubule binding, we quantified the amount of transiently expressed EGFP-CLASP2α on microtubule ends as a function of cell cycle phase in asynchronously growing HaCaT cells stably expressing histone H2B-mCherry (Figure 2.1 D, E). As expected, EGFP-CLASP2α microtubule binding decreased after nuclear envelope breakdown and reached a minimum in metaphase. CLASP2 microtubule binding increased again during anaphase, and surprisingly, spiked above interphase levels in telophase, possibly indicating an overshoot of protein phosphatase activity during exit from mitosis. To test whether this regulation of CLASP2α microtubule binding was due to GSK3β multisite phosphorylation at the previously identified sites, we compared CLASP2α microtubule end-association in metaphase-arrested cells expressing nonphosphorylatable(9xS/A) or phosphomimetic(8xS/D) versions of full-length or truncated CLASP2(497-1515). We previously showed that EGFP-CLASP2(8xS/D) abolishes EB1 binding both *in vitro* and in cells, confirming that these mutations mimic phosphorylation (Kumar et al., 2012). Compared with wildtype, nonphosphorylatable EGFP-CLASP2α(9xS/A) was enriched several-fold on metaphase microtubule ends. The difference between wildtype and

phosphomimetic EGFP-CLASP2α(8xS/D) was small and not significant. Truncated EGFP-CLASP2(497-1515) constructs behaved similarly, except that wildtype EGFP-CLASP2(497-1515) showed weaker microtubule binding than CLASP2α, indicating that the N-terminal TOG domains contribute to a small extent to CLASP2 microtubule binding in mitotic cells. For this reason, we used full-length CLASP2α for the remainder of this study. Together, these results confirm that microtubule binding of CLASP2 is greatly reduced in metaphase and is regulated by GSK3β-mediated phosphorylation.

C. CLASP2 kinetochore binding is not regulated by phosphorylation

Next, we tested whether GSK3β-mediated multisite phosphorylation directly influences kinetochore binding by measuring the relative amount of wildtype EGFP-CLASP2α at kinetochores compared with phosphorylation variants. Only the nonphosphorylatable EGFP-CLASP2α(9xS/A) intensity at kinetochores was increased several-fold over wildtype (Figure 2.2 A,B). However, this difference disappeared in nocodazole-treated cells, indicating that this increase reflects increased binding of EGFP-CLASP2α(9xS/A) to microtubule ends at kinetochores rather than direct kinetochore binding itself. These data indicate that phosphorylation does not regulate CLASP2 kinetochore binding, and may be explained because phosphorylation occurs in a separate domain from kinetochore binding (Figure 2.1 A).

D. Nonphosphorylatable CLASP2α(9xS/A) accumulates preferentially on sister kinetochores attached to polymerizing microtubules

Since EGFP-CLASP2α(9xS/A) shows increased binding to microtubule ends at kinetochores, we tested if this construct preferentially accumulated on sister kinetochores attached to polymerizing microtubules. During sister kinetochore

oscillations, only the kinetochore moving antipoleward is expected to contain polymerizing microtubule ends, and thus accumulate increased amounts of +TIPs, which has been shown for EB1 (Tirnauer et al., 2002). To test whether CLASP2α(9xS/A) binds preferentially to antipoleward kinetochores, we acquired time-lapse sequences of mitotic cells expressing EGFP-CLASP2α constructs and mCherry-CENPA to mark kinetochores. We quantified the amount of EGFP-CLASP2α on kinetochores by fitting an exponentially modified Gaussian function to fluorescence intensity profiles and determining the area under the curve for sister kinetochores (Figure 2.2 C, D). We calculated a ratio between the two sisters and normalized to mCherry-CENPA signal to correct for kinetochore movements in and out of focus. EGFP-CLASP2α(9xS/A) signal was significantly increased at antipoleward kinetochores, clearly demonstrating kinetochore movement-coupled switching of microtubule polymerization dynamics (Figure 2.2 E). In contrast, wildtype EGFP-CLASP2α bound to sister kinetochores equally during oscillations, indicating that if local phosphoregulation of CLASP2α microtubule binding occurs, it is limited to kinetochore-bound CLASP2α and does not result in additional CLASP2α recruitment.

E. CLASP2 phosphorylation weakens kinetochore-microtubule attachments

For most of the functional experiments described in this chapter, we expressed either nonphosphorylatable or phosphomimetic EGFP-CLASP2α in the presence of endogenous CLASP2. We reasoned that dominant negative effects following expression of EGFP-CLASP2 phosphomutants might occur if these constructs competed with endogenous CLASP2 for kinetochore binding sites and prevented proper regulation of CLASP2 microtubule binding at kinetochores. We first tested if

kinetochore-microtubule attachments in CLASP2 phosphomutant-expressing cells are able to generate tension across sister kinetochores, we measured interkinetochore distance in cells expressing CENPA-mCherry and EGFP-CLASP2α constructs (Figure 2.2 D). We used Gaussian distributions fit to intensity profiles across sister kinetochores to calculate the distance between CENPA-mCherry peaks in order to precisely determine interkinetochore distance. In both control and wildtype EGFP-CLASP2α-expressing cells we observed similar interkinetochore distances (1.125μm and 1.134μm, respectively, Figure 2.2 F, G). Expression of EGFP-CLASP2α(9xS/A) in which microtubule-end-binding is constitutively active, slightly increased interkinetochore distance (1.168μm). Interestingly, EGFP-CLASP2α(8xS/D)-expressing cells displayed decreased interkinetochore distances (1.05μm). This strongly suggests that CLASP2 microtubule-end-binding activity must be activated at kinetochores to generate wildtype levels of tension across sister kinetochores. If interkinetochore distance measurements are plotted as a histogram, populations of kinetochores with abnormally low or abnormally high interkinetochore distances can be observed in EGFP-CLASP2α(8xS/D) or (9xS/A)-expressing cells, respectively (Figure 2.2 G). These outlier interkinetochore distances are very significant given the total size of a kinetochore being ~150-200nm, as measured from centromere to outermost kinetochore component (Wan et al., 2009).

Because expression of EGFP-CLASP2α(8xS/D) decreased microtubule-generated tension across sister kinetochores, we tested the possibility that CLASP2 phosphorylation decreases the stability of kinetochore fibers. To test this, we treated HaCaT cells expressing EGFP-CLASPα constructs with a short incubation on ice, which causes depolymerization of non-kinetochore microtubules while leaving kinetochore

microtubules intact (Rieder, 1981). Expression of either EGFP-CLASP2α(8xS/D) or (9xS/A) had no discernible effect on the stability of kinetochore fibers using this assay (Figure 2.3). Because phosphomimetic EGFP-CLASP2α(8xS/D)-expressing cells decreased interkinetochore distance, we tested the status of the spindle checkpoint by staining for BubR1, a mitotic kinase that monitors tension at kinetochores (Chan et al., 1999; Skoufias et al., 2001). In prometaphase control cells, we observed bright staining of BubR1 at unaligned kinetochores as compared to aligned kinetochores, confirming the ability of the BubR1 antibody to recognize kinetochores under low tension (data not shown). However, in cells expressing EGFP-CLASP2α phosphomutants we could not detect any systematic differences in BubR1 kinetochore levels (Figure 2.4, quantification not shown). This could be because BubR1 staining in this assay is either not sensitive enough to detect the small changes in interkinetochore distances we observe in phosphomimetic CLASP2-expressing cells or BubR1 could be unaffected by small changes in interkinetochore distance.

F. CLASP2 phosphorylation regulates kinetochore microtubule dynamics

Since CLASP2 phosphorylation regulates CLASP2 binding to microtubule ends and CLASP2 localizes to outer kinetochores, we tested if regulation of the CLASP2 phosphorylation state is required for proper kinetochore microtubule dynamics. We expressed EGFP-CLASP2α constructs in HaCaT cells stably expressing CENPA-mCherry to mark kinetochores and used spinning disk confocal microscopy to monitor sister kinetochores during metaphase oscillations, a behavior that is driven by the dynamics of attached kinetochore microtubules (Amaro et al., 2010). We optimized

imaging conditions to achieve fast acquisition and low illumination intensities in order to capture rapid kinetochore movements during metaphase (detailed in Chapter 2, III. B).

To visualize sister kinetochore movements over time, we created two-dimensional kymographs of cropped sister kinetochore pairs (Figure 2.4). Using this method, we observed abnormal kinetochore movements in cells expressing EGFP-CLASP2α(9xS/A) (red arrows, Figure 2.4). Interestingly, the uncoordinated behavior of sister kinetochores in these cells occurred at points of kinetochore direction reversal, which corresponds to changes in polymerization status of attached kinetochore microtubules. However, accurate two-dimensional kymographs requires that sister kinetochores remain in the same z-plane over time and that no other kinetochore pairs move into the cropped field, conditions that ultimately bias the analysis. Together, these requirements prevented us from obtaining enough kymographs to properly quantitate kinetochore dynamics across different conditions. To more clearly visualize sister kinetochore pairs, we applied pseudo-coloring to kinetochores based on their z-position in the metaphase plate (Figure 2.5 A). Doing this allows us to see abnormal kinetochore behaviors, such as the flipping of a kinetochore pair about the metaphase plate (Figure 2.5 B). In an example EGFP-CLASP2α(9xS/A)-expressing cell, a kinetochore pair moves out of the metaphase plate and the kinetochores flip around each other, as shown by their paths in Figure 2.5 C. This flipping pair also undergoes drastic changes in interkinetochore distance, between 0.6 – 1.8 μm over this time sequence. Since EGFP-CLASP2α(9xS/A) is expected to bind microtubule ends more tightly, the ability of these kinetochores to flip suggests an uneven balance of forces across sister kinetochores. We have also observed such abnormal behaviors in EGFP-

CLASP2α(8xS/D)-expressing cells, with kinetochore pairs moving far out of the plate. Indeed, we observe more abnormal events in cells expressing either nonphopshorylatable or phosphomimetic CLASP2 (38% and 32% of cells, respectively, from a total of 26 and 25 cells examined in independent experiments, respectively). This is compared to only 9% of wildtype CLASP2-expressing cells exhibiting abnormal kinetochore events (from a total of 22 cells). We attempted to utilize a Matlab-based kinetochore-tracking algorithm to quantify kinetochore dynamics in an unbiased manner, still, this approach had many limitations for our purposes (Jaqaman et al., 2010) (detailed in Chapter 2 III).

G. Regulation of CLASP2 phosphorylation is required for timely chromosome segregation

To test the effects of perturbing the CLASP2 phosphorylation state on the dynamics of chromosome segregation, we used spinning disk confocal microscopy to monitor chromosome segregation in cells expressing EGFP-CLASP2 constructs and Histone H2B-mCherry to mark chromosomes (Figure 2.7). Cells expressing wildtype EGFP-CLASP2 behaved similarly to control cells and somewhat surprisingly, the majority of cells expressing EGFP-CLASP2 phosphomutants proceeded through mitosis. We quantified both the timing of chromosome alignment, using the time from nuclear envelope breakdown (NEBD) to metaphase, and the subsequent time to anaphase onset. Overall, expression of either CLASP2 phosphomutant did not affect congression of chromosomes, instead, expression partially delayed anaphase onset. These data suggest that overexpression of EGFP-CLASP2 constructs does not lead to

complete mitotic arrest, delay, or failure, but rather plays a more subtle role during chromosome segregation, specifically after chromosome alignment.

H. Regulation of CLASP2 phosphorylation is required for proper mitotic spindle formation

To determine if regulation of the CLASP2 phosphorylation state is required for proper formation of a bipolar spindle, we tested the ability of CLASP2 phosphomutants to rescue mitotic defects of CLASP2 knockdown cells. We generated HaCaT cell lines stably expressing either a control non-targeting shRNA or a shRNA targeting CLASP2, which resulted in specific reduction of CLASP2 protein levels (Figure 2.8). Cells depleted of both CLASP isoforms displayed severe growth defects, likely due to gross mitotic phenotypes. Following depletion, we transiently expressed EGFP-tagged CLASP2 constructs and arrested cells at metaphase using a short incubation with the proteasome inhibitor, MG132, and fixed and stained for DNA. (Figure 2.9 A). We categorized EGFP-expressing cells as having either normal bipolar spindles, with a tight metaphase chromosome arrangement, or abnormal spindles, which included misaligned chromosomes, wider metaphase plates, tilted spindles, or a completely disorganized chromosome arrangement. Consistent with previous reports, depletion of CLASP2 resulted in a large decrease in the number of cells with normal metaphase chromosome arrangements (~20%) (Figure 2.9 B) (Mimori-Kiyosue et al., 2006). Roughly 40% of CLASP2 depleted cells exhibited completely disorganized bipolar spindles. Expression of wildtype EGFP-CLASP2 can partially rescue mitotic defects in CLASP2 knockdown cells, and we predict that variations in knockdown and EGFP-CLASP2 expression level account for the lack of full rescue. Interestingly, expression of either EGFP-CLASP

phosphomutant fails to rescue mitotic defects of CLASP2 depleted cells, strongly suggesting that the phosphorylation state of CLASP2 must be tunable during mitosis in order for formation of a proper bipolar mitotic spindle. Notably, expression of phosphomimetic EGFP-CLASP2α(8xS/D), which shows decreased microtubule-end binding, results in more cells with misaligned chromosomes. In addition, we observed spindle rotation defects in CLASP-depleted cells.

I. Deregulation of CLASP2 phosphorylation results in increases lagging chromosomes

Because dynamic regulation of CLASP2 phosphorylation is required for stable kinetochore-microtubule attachments and the formation of proper mitotic spindles, we tested if these phenotypes correlated with an increase in chromosome missegregation events. We expressed EGFP-CLASP2 constructs and fixed and stained cells for DNA during anaphase and quantified the number of cells with one or more lagging chromosomes (Figure 2.9, C and D). To increase the percentage of cells in anaphase, we released cells from a G2/M block, as detailed in the Experimental Procedures. We observed ~8% of control HaCaT cells or cells expressing wildtype EGFP-CLASP2α with one or more lagging chromosomes. Expression of cells with either EGFP-CLASP2α(9xS/A) or (8xS/D) results in an increased percentage of cells with lagging chromosomes (~15% and ~13%, respectively). These data suggest that either locking CLASP2 phosphorylation, and thus microtubule-binding, in an ‘on’ or ‘off’ state has a downstream effect on the segregation of chromosomes during anaphase.

III. Live-cell imaging of kinetochore dynamics and analysis using computational image analysis software

A. Rationale

In an effort to apply an unbiased approach to determine if CLASP2 phosphorylation regulates the dynamics of kinetochore microtubules, we employed the use of computational image analysis software written by Khuloud Jaqaman, in collaboration with the Danuser lab at Harvard University (currently at UT Southwestern) (Jaqaman et al., 2010). This Matlab-based software builds upon expertise in the Danuser lab in spot detection and tracking algorithms for the analysis of dynamic cellular processes (Jaqaman et al., 2008; Matov et al., 2010). There are many problems with using traditional tracking algorithms in the detection and tracking of kinetochores, and Khuloud's software tries to overcome some of these issues by imposing on the software known parameters of kinetochore behavior. Because mitosis is a three-dimensional (3D) process, kinetochores move a great deal in 3D and therefore, it is necessary to track data in 3D and be able to visualize results in 3D to verify the software's output. Khuloud's software identifies kinetochores and tracks their movements over time uses a Matlab plugin for Imaris, 3D data visualization software, to view the results. Secondly, because kinetochores exist in pairs, their dynamics are linked and much can be learned about studying their coordination and movements relative to one another. However, traditional tracking software packages have no way of knowing which kinetochores to pair together. After individual kinetochores are tracked, Khuloud's software pairs kinetochores using known behaviors of sister kinetochores; sisters are more likely to be close to and follow one another and their movements in relation to the metaphase plate tend to be parallel with each other. The ability to pair

kinetochores correctly allows for dynamic parameters of kinetochore oscillations to be measured. Lastly, many tracking algorithms fail to generate longs tracks of kinetochore movements because kinetochores exist in close proximity to many other kinetochores and they are constantly moving in 3D. To circumvent these issues, Khuloud's software has an adjustable search radius in which two spots in time can be linked with a track. The closer the kinetochores are in a given cell, the smaller the search radius is set. In addition, there is a built-in gap closing function, which fills in kinetochore track breaks caused by kinetochores moving out of the focal plane or having signal below the limit of detection.

B. Imaging and analysis of metaphase kinetochore dynamics in HaCaT cells

In order to visualize kinetochores, I created a cell line that stably expresses CENPA-mCherry to mark kinetochores, using lentiviral constructs followed by antibiotic selection and FACs (Stehbens et al., 2012). Following 24 hrs. of expression of EGFP-CLASP2 constructs, I imaged metaphase cells used spinning disk confocal microscopy. Specifically, I used a Nikon TI inverted microscope stand equipped with a Yokogawa CSU-X1 spinning disk confocal head, which I used at near-maximum speed of ~4,500 rpm to allow for complete sampling with short exposure times. Using a 100X 1.49 NA TIRF objective lens (Nikon) and an Andor iXon electron-multiplying charge-coupled device (EMCCD) camera, I was able to achieve fast acquisition with low illumination intensities. To capture as many kinetochores as possible while still maintaining temporal resolution needed for tracking, I imaged 15 z-planes through the metaphase plate (Figure 2.10 A), for a total of 8-10 minutes. After image acquisition, I processed the images for Khuloud's Matlab software, which first detected kinetochore spots using 3D

Gaussian functions to precisely localize kinetochores (Figure 2.10 C). After kinetochore detection, a metaphase plane was fit to the center of all spots, which allowed for the determination of oscillation parameters because it gives a plane of reference that is independent of cell or spindle movement. Using the metaphase plane fit, Khuloud's software detects outlier kinetochores, which are those that are localized 2.5 or more standard deviations away from the plate. Individual spots are then tracked over time in 3D. Kinetochore tracks were optimized using the search radius and gap closing functions. Lastly, kinetochores were paired together based on the fact that movements of sister kinetochores are more coordinated than movements of non-sister kinetochores.

C. Results

We tested this software on a subset of CENPA-mCherry HaCaT cells expressing EGFP-CLASP2 or depleted of endogenous CLASP2 protein. The first measurement we made was interkinetochore distance. We observed a wide range of interkinetochore distances in both control and CLASP-depleted cells (Figure 2.10 D). Because the spot detection worked quite well, the accuracy of this measurement depended largely on the ability of the software to successfully pair sister kinetochores. Unfortunately, this is not always feasible to confirm for every pair at every time point, because of the difficulty of seeing all of the kinetochores in 3D. In addition to interkinetochore distance, we used this software to determine metaphase plate angle relative to the coverslip in cells expressing EGFP-CLASP2 constructs (Figure 2.10 A). In control cells, majority of metaphase plates are perpendicular to the coverslip, and thus exhibit metaphase plate angles near 90°. We detected a small decrease in metaphase plate angle in cells expressing EGFP-CLASP2(8xS/D) (Figure 2.10 E).

D. Limitations

This software is indeed an unbiased approach to analyze movements of a large set of kinetochores. The spot detection works very well and this is easily verified using Imaris visualization software. The tracking and pairing, however, are much more difficult to verify, especially in 3D. In mammalian cells, the kinetochores are very dense within the metaphase plate making it challenging to be certain about the track of one single or one pair of kinetochores. Because of the need to image with high temporal resolution, it was impossible to capture all kinetochores in a given cell, making it near impossible to verify that the sister kinetochore pairs generated by the software were accurate. The main issue with utilizing this software for my project is that all of the data is pooled for a given cell, obscuring subtle phenotypes or drastic phenotypes that occur to a small subset of kinetochores. The relevant data is further diluted because normal metaphase cells already have a high degree of variability of kinetochore behaviors, which are partially dependent on exactly where the cell is in metaphase and the location of kinetochores within the spindle. In addition, the software was designed based on assumptions of normal, known kinetochore behaviors, and therefore is automatically biased against any phenotypes, let alone subtle changes in kinetochore dynamics. Because this is a complex software package composed of multiple algorithms written and optimized by different people, it was too difficult to change the software parameters to adjust for all of the issues mentioned above. Therefore, the advantage of generating large datasets of tracked kinetochores masked subtle defects in CLASP2 phosphomutant-expressing cells and ultimately, made this approach far from optimal for the purpose of this dissertation research.

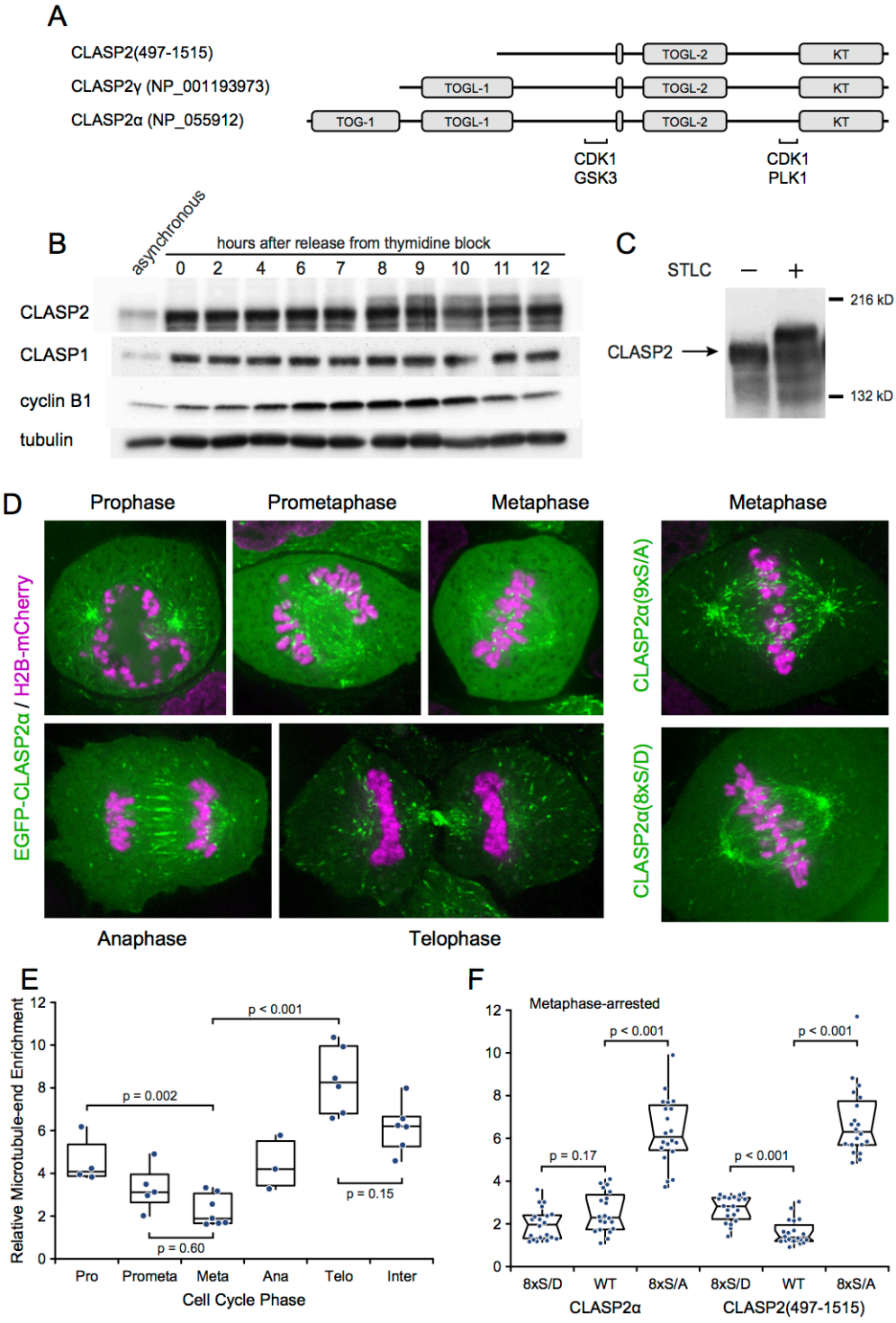

Figure 2.1 Phosphorylation of full-length CLASP2α inhibits its microtubule end-binding activity during mitosis. (A) Domain structure of human CLASP2 constructs used in this paper and our previous study, depicting TOG and TOG-like (TOGL) domains and regions of phosphorylation by CDK, GSK, and PLK (Maia et al., 2012; Kumar et al., 2009; 2012). Amino acid numbering for short construct is based on the most recent NCBI reference sequence NP_055912.2 and is different from numbering

we used previously. (B) Immunoblot of CLASPs, cyclin B1, and α-tubulin from HeLa cell lysates at various timepoints following release from double thymidine block. (c) Immunoblot of endogenous CLASP2α from lysates of HaCaT cells growing asynchronously or arrested in mitosis with S-trityl-L-cysteine (STLC). In this and subsequent figures, data is obtained from HaCaT cells only. (D) Spinning disk confocal images of HaCaT cells expressing H2B-mCherry (magenta) and EGFP-CLASP2α WT, 9xS/A, or 8xS/D (green) in different mitotic phases. (E-F) Quantification of enrichment of EGFP-CLASP2 on microtubule ends relative to cytoplasmic EGFP-CLASP2 signal from HaCaT cells in different mitotic phases (E) or arrested in metaphase with the proteasome inhibitor, MG132 (F). The box-and-whisker plot shows median, first and third quartile (box) and 95% confidence intervals (notches). Each dot represents the mean of three microtubule ends from one cell. P values were calculated by non-parametric Kruskal-Wallis analysis of variance.

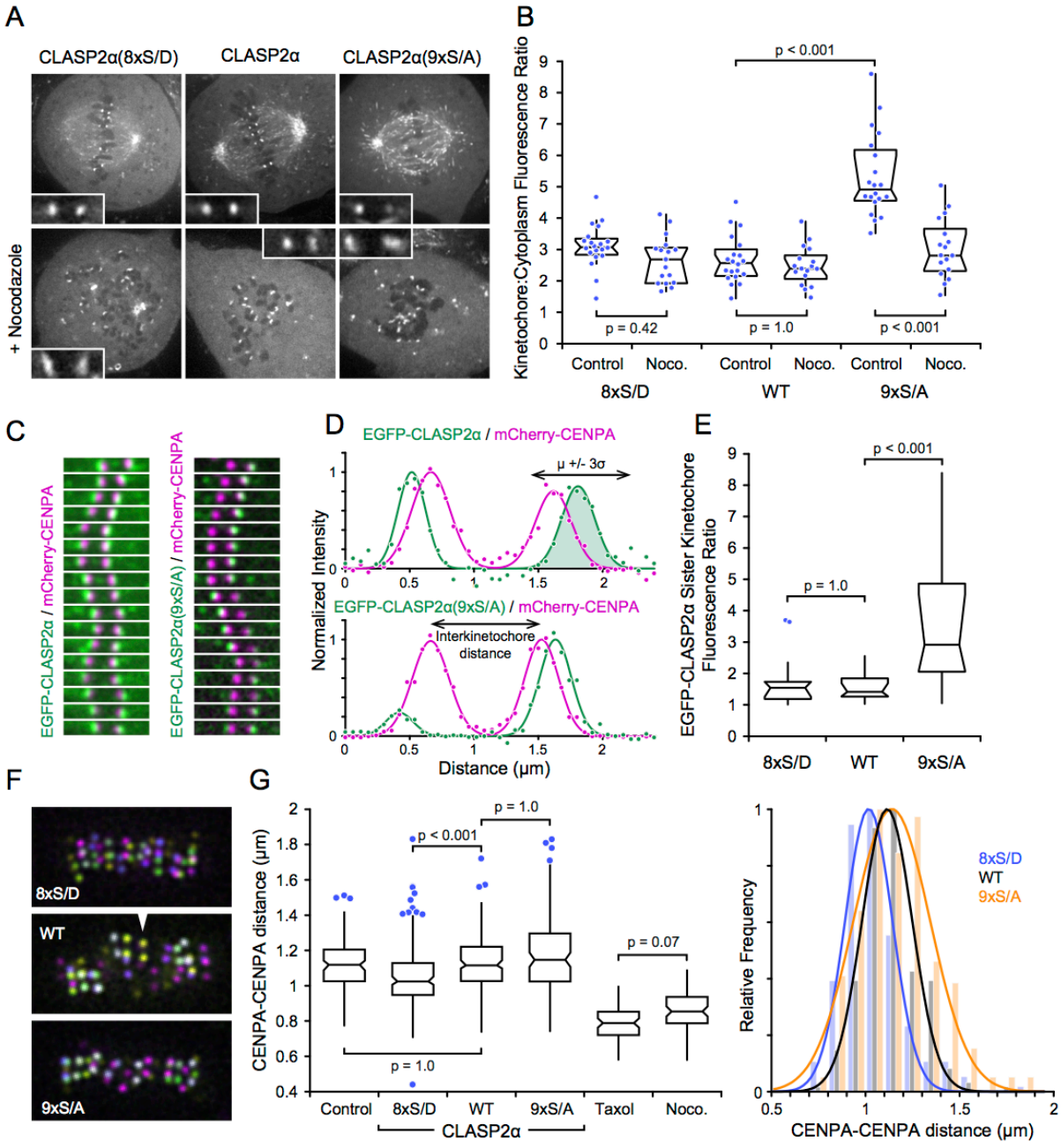

Figure 2.2 CLASP2 kinetochore binding is not regulated by phosphorylation. (A) Confocal images of metaphase-arrested HaCaT cells expressing EGFP-CLASP2α and treated with 1 uM nocodazole (bottom row). Insets are from one kinetochore pair. (B) Quantification of relative enrichment of EGFP-CLASP2α on kinetochores compared to cytoplasmic EGFP-CLASP2α signal. Each dot represents the mean of three kinetochores from one cell. n=20 cells (without nocodazole); n=17 cells (with nocodazole) from two independent experiments. The box-and-whisker plot shows median, first and third quartile (box) and 95% confidence intervals (notches). P values were calculated non-parametric Kruskal-Wallis analysis of variance. (C) Time-lapse sequences of sister kinetochore oscillations in HaCaT cells at metaphase expressing

CENPA-mCherry and EGFP-CLASP2α. (D) Example linescans across sister kinetochore pairs showing exponentially modified Gaussian fits (solid line) of intensity profiles of EGFP-CLASP2α (green line) and CENPA-mCherry (red line). (E) Quantification of maximum EGFP-CLASP2α sister kinetochore fluorescence ratio calculated using area under the curve +/- three standard deviations from the mean (shaded green curve). Ratios were normalized to CENPA-mCherry to correct for focal plane differences between sister kinetochores. (F) Maximum intensity projection images of five z-planes of CENPA-mCherry signal in HaCaT cells expressing EGFP-CLASP2α sister and color coded by z-position. (G) Quantification of interkinetochore distances in HaCaT cells expressing EGFP-CLASP2α constructs or treated with nocodazole or taxol. Measurements were made using the distance between the peaks of Gaussian fits to CENPA-mCherry intensity profiles. Each dot represents one interkinetochore measurement. n=175 (control); n=210 (WT); n=198 (9xS/A); n=198 (8xS/D); n=120 (nocodazole); n=108 (taxol). Representative data from three independent experiments. Six measurements were taken per cell. (H) Data from (G) plotted as a histogram, showing smaller interkinetochore distances for EGFP-CLASP2α(8xS/D)-expressing cells and larger than WT interkinetochore distances for EGFP-CLASP2α(9xS/A)-expressing cells.

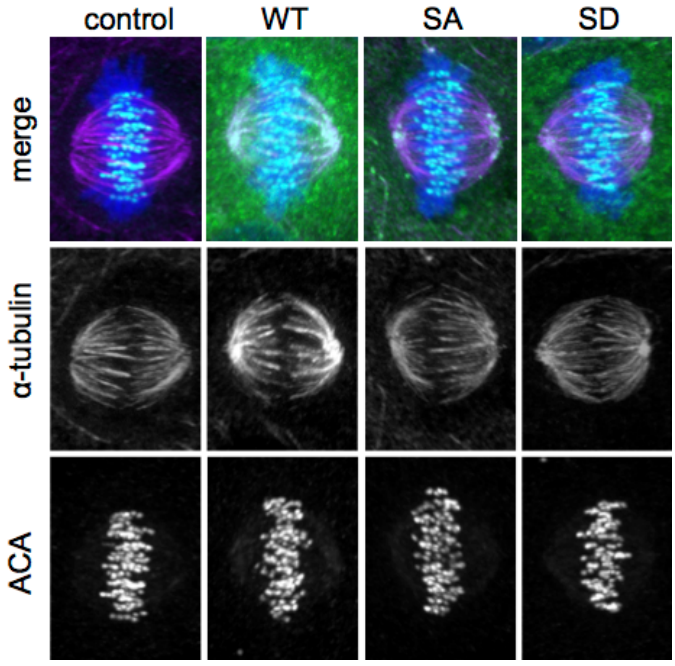

Figure 2.3 CLASP2 phosphorylation does not regulate the stability of cold stable kinetochore fibers. Immunofluorescence images of HaCaT cells expressing EGFP-CLASP2α constructs, arrested in metaphase for 1 hr. with proteasome inhibitor treatment (MG132), fixed, and stained for microtubules (α-tubulin), kinetochores (ACA), and DNA (DAPI).

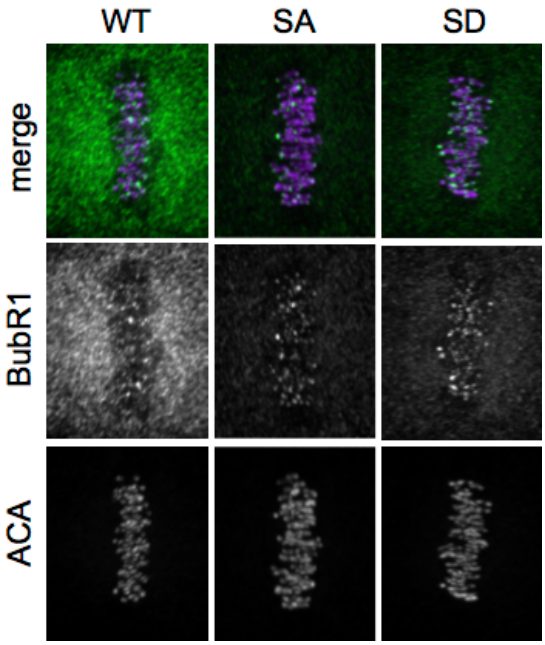

Figure 2.4 CLASP2 phosphorylation does not affect BubR1 levels at metaphase kinetochores. Immunofluorescence images of metaphase HaCaT cells expressing EGFP-CLASP2α constructs, fixed with paraformaldehyde, and stained for kinetochores (ACA) and BubR1 (EGFP-CLASP2α expression shown in merge image).

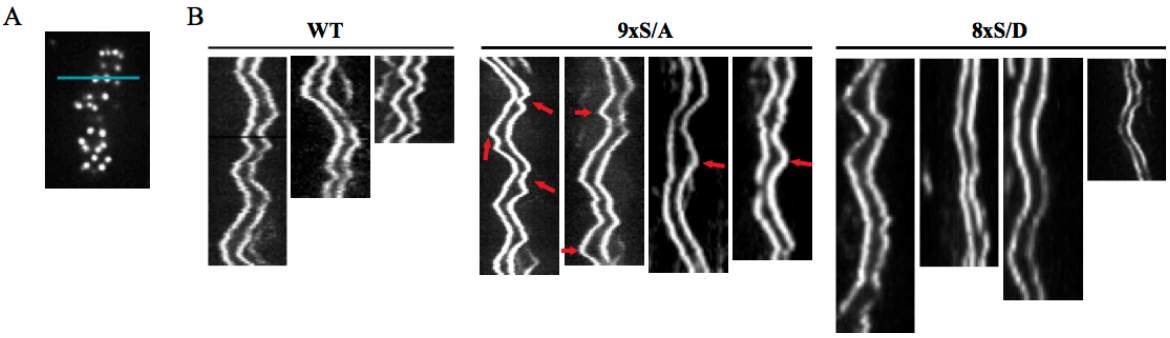

Figure 2.5 Sister kinetochore dynamics kymographs. (A) HaCaT cell at metaphase expressing CENPA-mCherry to mark kinetochores. Turquoise line depicts an example region for two-dimensional kymograph of a sister kinetochore pair. (B) Two-dimensional kymographs of cropped sister kinetochore pairs in HaCaT cells expressing EGFP-tagged CLASP2α constructs. EGFP-CLASP2 wildtype-expressing cells exhibit metaphase oscillations with coordinated sister kinetochore movements. EGFP-CLASP2(9xS/A)-expressing cells have sister kinetochores that exhibit abnormal dynamics at points of kinetochore direction reversal (red arrows).

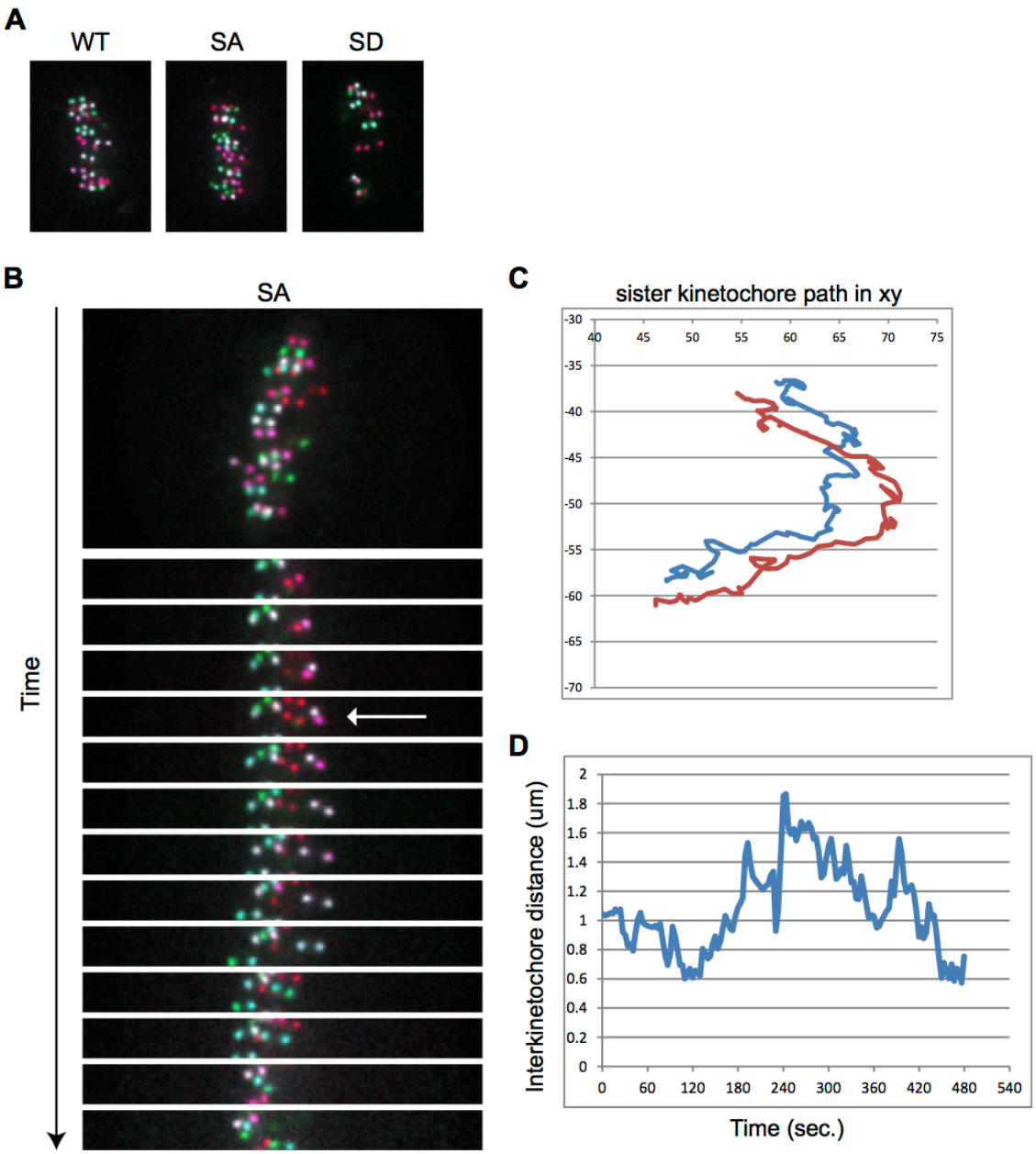

Figure 2.6 Deregulation of CLASP2 phosphorylation induces abnormal kinetochore dynamics. (A) Maximum intensity projection images of metaphase HaCaT cells stably expressing CENPA-mCherry and transiently expressing EGFP-CLASP2α constructs. Only the mCherry channel is shown and kinetochores are pseudo-colored based on z-position (middle of z-stack is colored white and boundaries are red and green). (B) Example timelapse sequence of a metaphase cell expressing EGFP-CLASP2α(9xS/A) showing a sister kinetochore pair moving out of the metaphase plate and flipping orientation (arrow). At the time of flipping, the sister kinetochores are in different z-planes as noted by their different colors. (C) Path of sister kinetochore highlighted in (B) showing flipping behavior. (D) Interkinetochore distance over time of sister kinetochore pair highlighted in (B), which ranges from 0.6 – 1.8 μm over the timecourse.

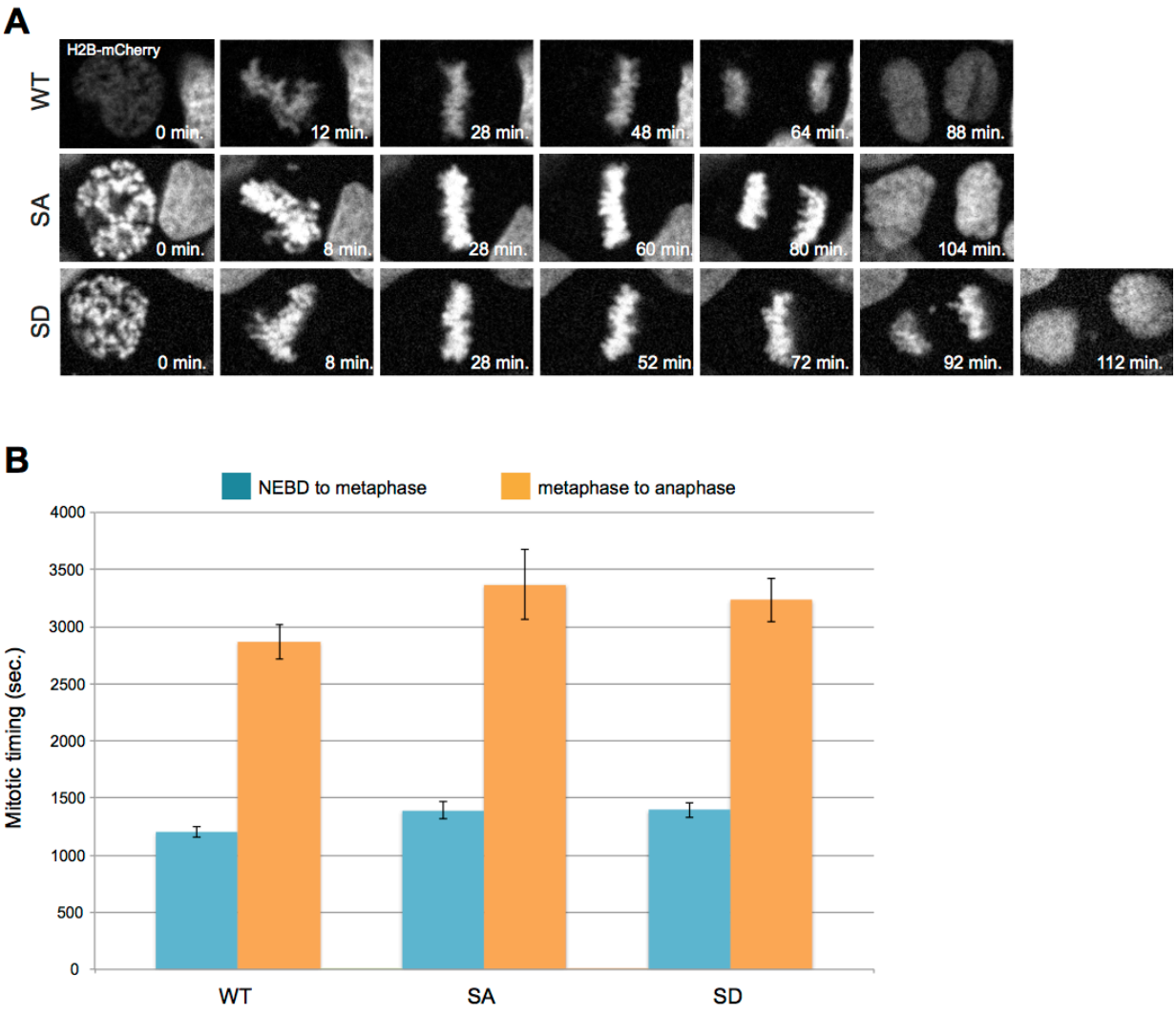

Figure 2.7 Regulation of CLASP2 phosphorylation is required for timely chromosome segregation. (A) Images from timelapse sequences of HaCaT cells stably expressing H2B-mCherry and transiently expressing EGFP-CLASP2α constructs during mitosis. Cells were synchronized following release from 16 hr. arrest using the Cdk1 inhibitor, RO-3306 (EMD BioScience 9 μM). (B) Quantification of mitotic time, including NEBD to metaphase (blue bars) and metaphase to anaphase (orange bars) from cells in (A) expressing EGFP-CLASP2α constructs. Data is from three independent experiments and >100 cells per condition. Error bars, SEM.

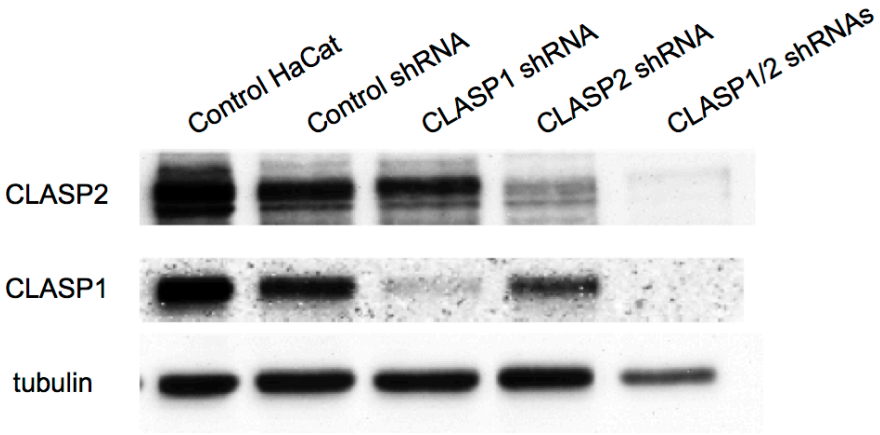

Figure 2.8 Depletion of CLASPs in HaCaT cells. Western blot of CLASPs from HaCaT cell lysates 6 days following infection with lentivirus-mediated CLASP1 and CLASP2 specific shRNAs. Knockdown of both CLASPs results in severe growth defects. Tubulin was used as a loading control.

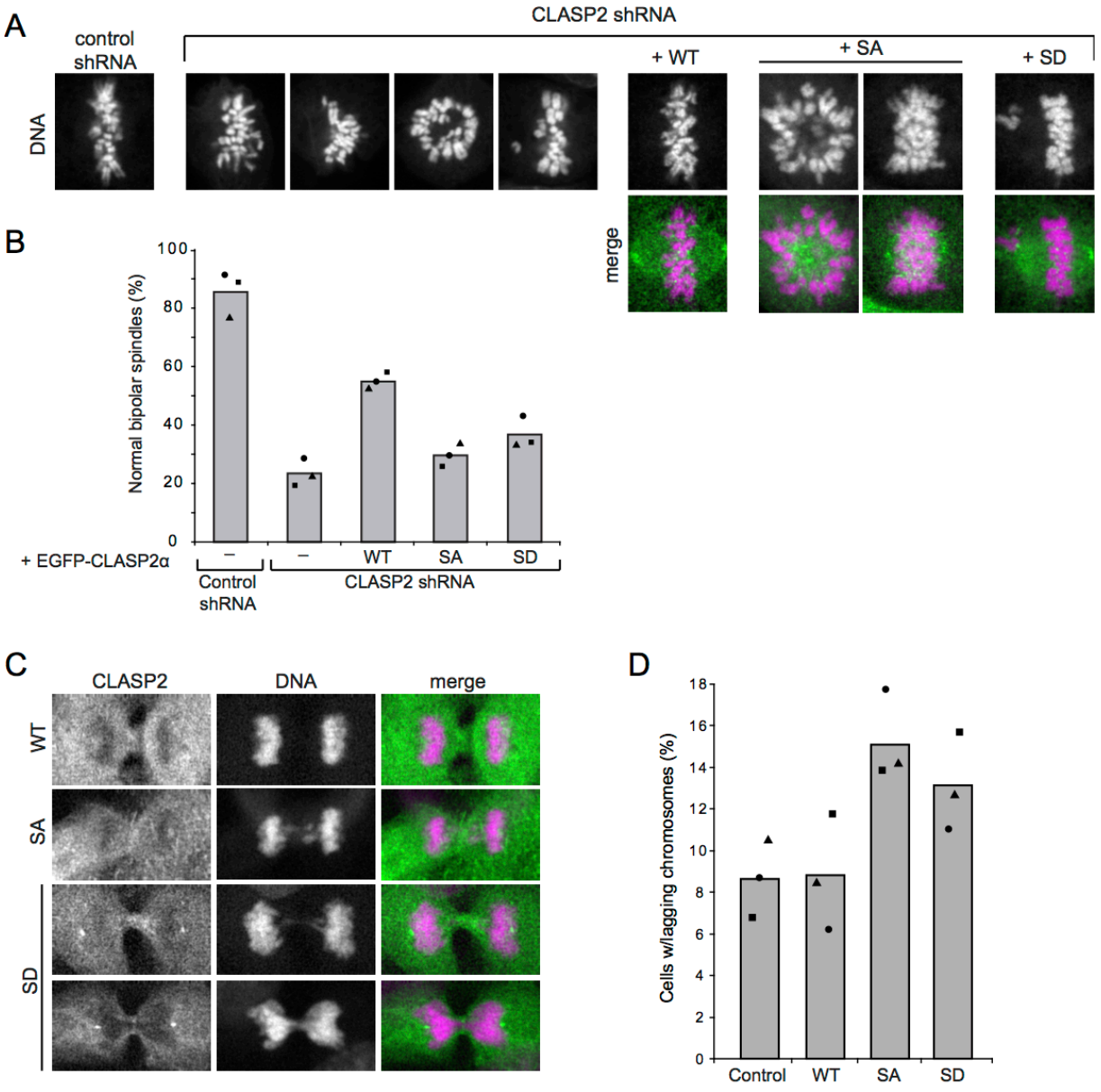

Figure 2.9 Regulation of the CLASP2 phosphorylation state is required for proper mitotic spindle formation and fidelity of chromosome segregation. (A) Images of HaCaT cells arrested a metaphase with the proteasome inhibitor, MG132, and stained for DNA with propidium iodide. Representative examples of metaphase chromosome arrangements from cells treated with control shRNAs or CLASP2 shRNAs and expressing EGFP-CLASP2 constructs. (B) Quantification of normal bipolar spindles from three independent experiments (different symbols) in control and CLASP-depleted cells rescued with EGFP-CLASP2 constructs. Normal bipolar spindles classified as a compact, linear bipolar spindle as in the control shRNA cell in (A). (C) Images of HaCaT cells expressing EGFP-CLASP2 constructs and stained for DNA in anaphase. (D) Quantification of three independent experiments (different symbols) of the percent of cells in control and EGFP-CLASP2-expressing cells with one or more lagging chromosomes in anaphase.

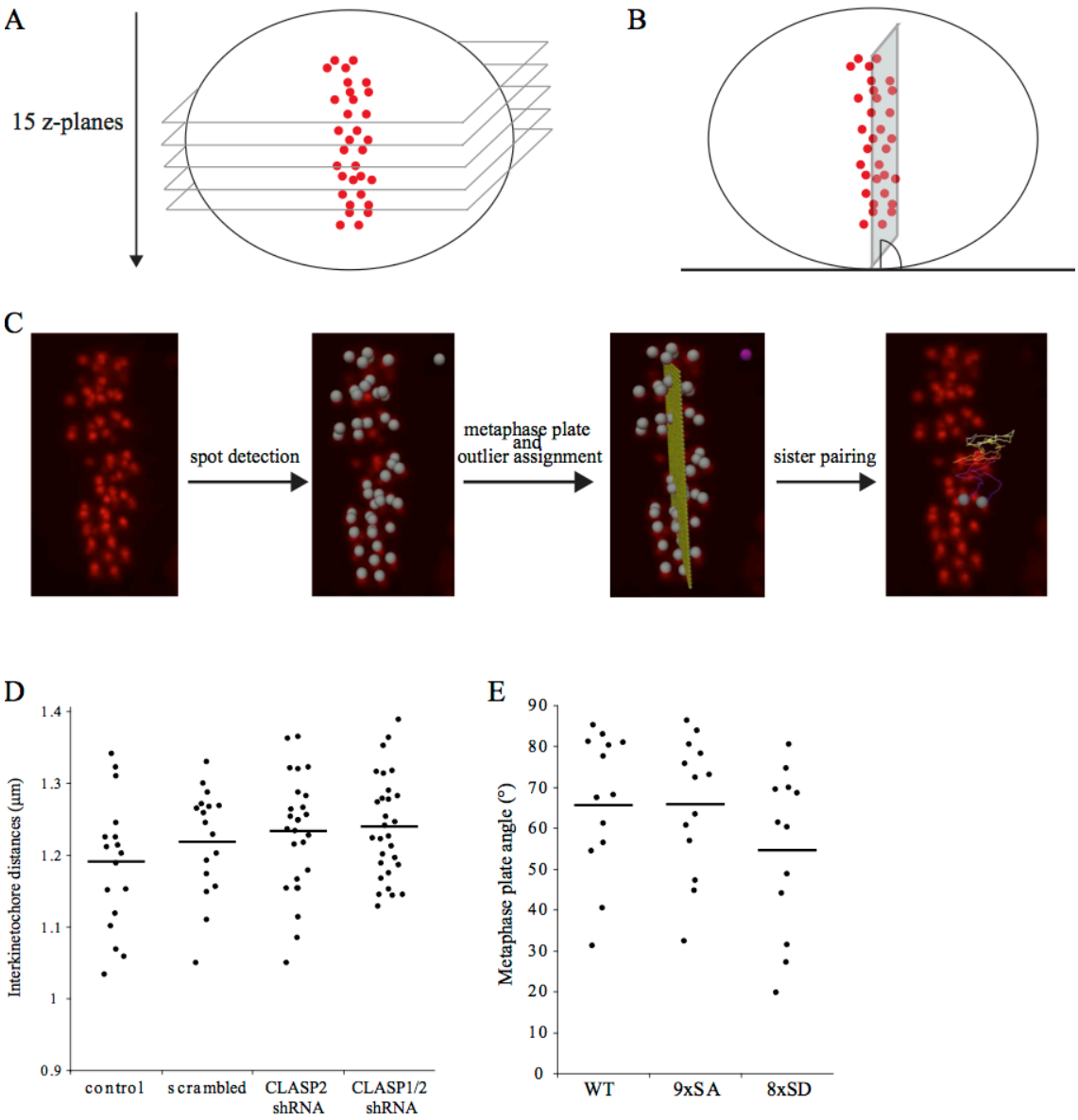

Figure 2.10 Analysis of kinetochore dynamics using computational image analysis software. (A) Schematic representation of image acquisition, showing 15 z-sections acquired through a metaphase cell expressing CENPA-mCherry (red dots). (B) Schematic representation of how analysis software computes metaphase plate angle shown in (E). (C) Maximum intensity projection images of metaphase cells expressing CENPA-mCherry to mark kinetochores (red) and showing spot detection (grey), metaphase plate fitting (yellow), outlier detection (magenta), and sister pairing (with corresponding tracks) functions of the software. (D, E) Quantification of interkinetochore distance and metaphase plate angle in CENPA-mCherry HaCaTs expressing EGFP-CLASP2 constructs or depleted of CLASP2. For interkinetochore distances in (D), each dot represents all interkinetochore distance or measurement from all sister kinetochores in one cell. For metaphase plate angles in (E), each dot represents the average of all metaphase plate angles from all timepoints in one cell. Lines denote the mean.

Chapter 3: Function of CLASP2 phosphorylation during other cell processes

The follow published papers represent co-authorships and collaborations based largely on reagents I developed to study CLASP2 phosphorylation. Specifically, these include adenoviruses that allow for expression of full-length EGFP-CLASP2α (WT, 9xS/A, and 8xS/D), which is amenable for difficult to transfect cells or to test functional rescue. Together, they represent a body of work that highlight the importance of precise regulation of CLASP2 phosphorylation during many different cellular processes, including focal adhesion disassembly during cell migration, acetylcholine receptor clustering necessary for signal transduction at neuromuscular junctions, and axonal regeneration.

I. CLASP association with focal adhesions is independent of microtubules and required for focal adhesion disassembly and ECM degradation

A. Introduction

Focal adhesions are protein complexes that link the inside of the cell to the extracellular matrix (ECM) (Plotnikov and Waterman, 2013). During cell migration, focal adhesion complexes assemble in the front of the cell, and become mature when they are linked to the contractile actin cytoskeleton (Stehbens and Wittmann, 2012). In order for cells to move forward, focal adhesions must disassemble and detach from the ECM. It has been known for decades that microtubules are required for focal adhesion turnover, as addition of the microtubule-depolymerizing drug, nocodazole, prevents focal adhesion disassembly and cell migration (Etienne-Manneville, 2013). However,

the molecular mechanism of microtubule-mediated focal adhesion disassembly is not well understood.

B. Results

A postdoctoral fellow in the Wittmann lab, Samantha Stehbens, studied the role of CLASPs during cell migration, specifically their function in focal adhesion disassembly. She showed that CLASPs associate with focal adhesions, in both clusters and along microtubules (Figure 3.1 A). Using adenovirus expressing EGFP-CLASP2(497-1515)8xS/D, which I generated, she showed that CLASP cluster formation around focal adhesions is independent of microtubules (Figure 3.1 B, C). In addition, she demonstrated that CLASPs are required for focal adhesion disassembly and degradation of the ECM during cell migration. To determine if the matrix metalloprotease, MT1-MMP, was implicated in CLASP-dependent ECM degradation, fast time lapse imaging of MT1-MMP-EGFP was used. I, along with another postdoc in the lab, Andreas Ettinger, helped Samantha gather imaging data for this experiment. While we were able to visualize MT1-MMP exocytic events near focal adhesions, we were unable to robustly quantitate this exocytic behavior. Instead, we imaged Rab6, a GTPase involved in targeting and fusing vesicles, dynamics as a readout for exocytic vesicles (Martinez and Goud, 1998). We quantified Rab6-positive vesicle fusion events near focal adhesions in control and CLASP-depleted cells. There were significantly less Rab6 exocytosis events around focal adhesions in CLASP knockdown cells as compared to control cells, suggesting that CLASP is required for localized exocytosis and focal adhesion disassembly. The results described above and reprinted here in

Figure 3.1 were published in a Nature Cell Biology article, for which I was a co-author (Stehbens et al., 2014).

C. Conclusion

Since microtubules are required for focal adhesion disassembly (Etienne-Manneville, 2013; Stehbens and Wittmann, 2012) and CLASP microtubule lattice-binding activity is spatially activated in the lamella through inhibition of GSK3beta (Kumar et al., 2009), it is tempting to speculate that there is local regulation of microtubule binding at focal adhesions to allow for confined microtubule capture during cell migration. While CLASPs and another +TIP, ACF7, associate with focal adhesions independently of microtubules, albeit in clusters, it is unclear if these microtubule-independent complexes at focal adhesions are able to facilitate focal adhesion disassembly (Wu et al., 2008; 2011). It will be interesting in the future to determine if microtubule-binding activities of CLASP and other microtubule-associated proteins at focal adhesions are required for disassembly. In particular, it will be interesting to test if plus-end binding or lattice binding of CLASP2 is required for focal adhesion disassembly. Future experiments will also be critical to determine if microtubule binding is locally activated around focal adhesions through dephosphorylation of focal adhesion-associated microtubule-binding proteins. Intriguingly, protein phosphatase 1 (PP1) associates with focal adhesions and interacts biochemically with focal adhesion kinase (FAK) (Fresu et al., 2001).

II. Localized inhibition of GSK3β at the neuromuscular junction (NMJ) activates CLASP2 microtubule binding and capture to promote acetylcholine receptor (AChR) clustering

A. Introduction

Neuromuscular junctions (NMJs) form between motor neurons and skeletal muscle cells and are used to transmit information from the brain to the skeletal system through the action of the neurotransmitter, acetylcholine (ACh) (Witzemann et al., 2013). Motor neurons contain presynaptic membranes, which serve as sites of ACh release and conversely, the skeletal cells form postsynaptic synapses, which have a high density of ACh receptors (AChRs) to transduce signals from the motor neuron (Wu et al., 2010). A major question in the field is how NMJs develop, and in particular what signaling pathways direct formation of a functional postsynaptic synapse. Agrin stimulates the differentiation of postsynaptic synapses and induces clustering of AChRs (Cohen et al., 1997; Jones et al., 1997). In muscle cells, microtubule capture is required for delivery of ACh to postsynaptic membranes and agrin has been shown to inactivate GSK3β (Schmidt et al., 2012b). The authors wanted to test if agrin is inhibiting GSK3β activity at the NMJ to activate CLASP2 microtubule-binding activity in a spatial manner to promote clustering of AChRs.

B. Results

In this paper, the authors used a myotube culture system in which they extracted primary muscle cells from mice and plated them on a laminin substrate (Basu et al., 2014). They added COS-1 cells that are transfected with an agrin expression cassette, which causes local secretion of agrin and thus promotes delivery and clustering of

AChRs to the NMJ (Schmidt et al., 2012b). Treatment of the myotubes with constitutively active GSK3β (mRFP- GSK3β-S9A) decreased the area of AChR staining at the NMJ. This decrease in AChR clustering due to increased GSK3β activity could be rescued if myotubes were treated with adenovirus expressing nonphosphorylatable EGFP-CLASP2(9xS/A). Since CLASP2(9xS/A) microtubule-binding constitutively active, this suggests that localized dephosphorylation of CLASP2 can induce AChR clustering at NMJs. To test the consequences of perturbing CLASP2 microtubule-binding activity at the NMJ, the authors infected the myotubes with phosphomimetic EGFP-CLASP2(8xS/D) and stained for AChRs. They saw a decrease in the size, but not the density of AChRs, strongly suggesting that microtubule capture by CLASP2 is required for AChR delivery and clustering at the NMJ.

C. Conclusion

This group used our adenoviral reagents expressing EGFP-CLASP2 phosphomutants to determine if local regulation of the CLASP2 phosphorylation state is required for the clustering of AChRs, and thus development of NMJs. They observed that CLASP2 microtubule-binding activity is required for the clustering of AChRs, and they suggest that GSK3β is locally inhibited through agrin at the NMJ. These results strongly suggest another mechanism of localized regulation of CLASP2 microtubule binding through GSK3β phosphorylation in order to activate microtubule capture in a spatially restricted manner. The results described here resulted in co-authorship in an article published in the Journal of Biological Chemistry (Basu et al., 2014).

III. Regulation of CLASP2 phosphorylation does not play a role in axonal regeneration

To investigate the cytoskeletal mechanisms underlying axonal regeneration, the authors used dorsal root ganglia (DRG) from rats with spinal cord injuries or lesions and perturbed GSK3β signaling (Liz et al., 2014). They showed an increase in microtubule dynamics in the growth cone following injury that is mediated by inactivation of GSK3β. Using adenoviral constructs I generated that express EGFP-CLASP2 phosphomutants, they showed that growth cones overexpressing wildtype or nonphosphorylatable EGFP-CLASP2 contained stabilized microtubules. Since microtubules become more dynamic during axonal regeneration, the authors suggest that regeneration is not mediated through localized regulation of the CLASP2 phosphorylation state. Our contributions to this study resulted in co-authorship in an article published in BMC Biology (Liz et al., 2014).

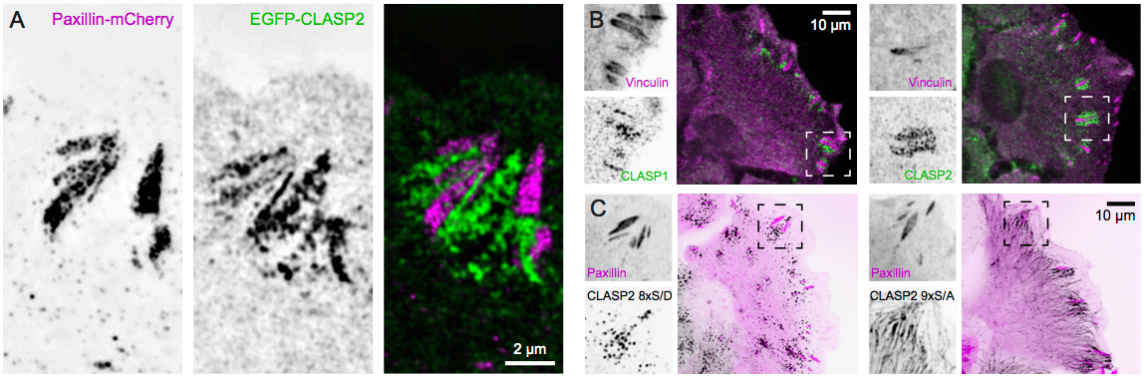

Figure 3.1 CLASP clusters around focal adhesions do not depend on microtubules. (A) Structured illumination super-resolution microscopy of EGFP–CLASP2 and paxillin–mCherry-expressing nocodazole-treated HaCaT cells, illustrating close intercalation of CLASP clusters and focal adhesions. (B) Localization of endogenous CLASP1 (left) and CLASP2 (right) around focal adhesions in nocodazole-treated HaCaT cells. (C) HaCaT cells expressing paxillin–mCherry and either phosphomimetic EGFP–CLASP2(8xS/D) (left) or nonphosphorylatable EGFP–CLASP2(9xS/A) (right).

CONCLUSIONS

Microtubule-binding proteins are involved in many dynamic cellular processes, yet the mechanisms by which they are regulated are not completely understood. During mitosis, kinetochore-microtubule interactions must be precisely regulated in space and time to ensure accurate chromosome segregation. In particular, kinetochore-microtubule attachments must be stable to power chromosome movements during both alignment and separation, yet dynamic to allow for error correction and proper dynamics of attached microtubules. The Ndc80 protein complex is a major contributor to kinetochore-microtubule attachments and is regulated through phosphorylation, but how exactly other kinetochore proteins contribute to kinetochore-microtubule interactions remains unclear (Cheeseman and Desai, 2008). In particular, the list of microtubule +TIP proteins continues to expand and many of these proteins localize to kinetochores during mitosis. I initiated the research in this dissertation to investigate whether the microtubule +TIP protein, CLASP2, and its hyperphosphorylation during mitosis serves as a mechanism to regulate kinetochore-microtubule interactions during mitosis.

In Chapter 1, I describe the work that I contributed to on the characterization of CLASP2 hyperphosphorylation during mitosis. We showed that the previously identified GSK3β phosphorylation sites in the plus-end tracking domain of CLASP2 are hyperphosphorylated during mitosis (Kumar et al., 2009). Further, we showed that Cdk1 priming phosphorylation is required for subsequent GSK3β phosphorylation. While GSK3β has been shown to be required for mitosis, its mitotic substrates have remained elusive (Tighe et al., 2007; Ong Tone et al., 2010; Wakefield, 2002). This research identifies one such mitotic GSK3β substrate and provides insight into the role of

CLASP2 phosphorylation during mitosis. Interestingly, a recently identified Cdk1 phosphorylation site in the c-terminal kinetochore-binding region of CLASP2 was shown to be required for mitotic spindle assembly, suggesting multiple mechanisms by which CLASP2 is phosphoregulated during mitosis (Maia et al., 2012). In addition, I describe our attempts to use a commercially generated phospho-specific CLASP2 antibody to assay for local regulation of the CLASP2 phosphorylation state at kinetochores. While this approach did not yield kinetochore-specific results by immunofluorescence experiments, we showed that it is indeed specific for phosphorylated CLASP2 by immunoblotting, which may prove useful for future biochemical experiments. Lastly, I contributed to experiments that demonstrated that CLASP2 plus-end-tracking *in vitro* is EB1-dependent.

Chapter 2 is a manuscript we are submitting for publication describing the role of CLASP2 phosphorylation in the regulation of kinetochore-microtubule interactions. The research in Chapter 1 describing CLASP2 hyperphosphorylation in mitosis and its inhibitory effect on CLASP2 microtubule binding led to the hypothesis that there is local regulation of the CLASP2 phosphorylation state at kinetochores to activate CLASP2 microtubule binding in a localized fashion. We quantitatively showed that mitotic phosphorylation of CLASP2 inhibited its microtubule end-binding activity during mitosis, yet had no effect on kinetochore binding. We further showed that mimicking phosphorylation of CLASP2 weakens kinetochore-microtubule interactions significantly, indicating dominant negative effects of perturbing CLASP2 phosphoregulation at kinetochores. We demonstrated that weaker kinetochore-microtubule interactions were not a result of less stable kinetochore fibers and instead characterized abnormal

kinetochore dynamics following perturbation of CLASP2 phosphorylation. We utilized sophisticated live-cell imaging and quantitation techniques to visualize precise kinetochore movements. I showed that neither nonphosphorylatable nor phosphomimetic CLASP2 could rescue mitotic defects of CLASP2 knockdown cells. Furthermore, perturbed CLASP2 phosphorylation increased the incidence of lagging chromosomes, suggesting a significant downstream consequence of the abnormal kinetochore-microtubule interactions I observed. While we are still in the process of publishing this work, it describes the importance of precise regulation of the phosphorylation state of a kinetochore component for proper kinetochore-microtubule interactions and chromosome segregation fidelity.

Chapter 3 refers to a set of published papers that represent co-authorships and collaborations based largely on reagents I developed to study CLASP2 phosphorylation. In particular, I generated adenoviruses that allow for expression of full-length EGFP-CLASP2α (WT, 9xS/A, and 8xS/D), which is amenable for studying CLASP2 phosphoregulation in difficult to transfect cells or to test functional rescue of CLASP2 phosphomutants. While many previously published papers utilized a shorter construct of CLASP2 lacking N-terminal TOG domains, I found that this shorter construct failed to rescue mitotic defects of CLASP2 depletion to levels following rescue with full-length CLASPα (data not shown). While this strongly suggests a role for the N-terminal TOG domains in CLASP2 mitotic function, this remains an interesting and outstanding question in the field. Because of our observations with shorter CLASP2 constructs, we provided our co-authors in Basu et al. and Liz et al. with full-length CLASPα adenoviruses. Together, these papers represent a body of work that highlight the

importance of precise regulation of CLASP2 phosphorylation during many different cellular processes, including focal adhesion disassembly during cell migration, acetylcholine receptor clustering necessary for signal transduction at neuromuscular junctions, and axonal regeneration.

This dissertation research characterizing the role of CLASP2 phosphorylation in kinetochore-microtubule interactions brings up many outstanding research questions. Exactly how does CLASP2 phosphorylation regulate kinetochore microtubule dynamics? Work in other organisms suggests that CLASP can promote microtubule rescue, which begs the question of whether CLASP2 is mediating rescue of microtubule ends at kinetochores during chromosome movements (Al-Bassam and Chang, 2011). Does phosphorylation play a role in regulating CLASP2-mediated microtubule rescue? This question could be addressed by comparing microtubule dynamics following incubation of purified CLASP2 phosphomutants *in vitro*. Interestingly, Ndc80 phosphomutants have recently been shown to directly affect microtubule dynamics *in vitro* (Umbreit, 2012). Unfortunately, CLASP2 remains a difficult protein to purify, making these experiments not feasible currently. Is EB1 binding necessary for CLASP2 kinetochore function? EB1 is attached to polymerizing microtubules at kinetochores and thus begs the question of whether CLASP2 has EB1-independent functions at kinetochores. We have previously characterized CLASP2 constructs in the Wittmann lab that contain SxIP motif mutations to abolish EB1 binding. Experiments using these mutants during mitosis are necessary to test this question. If there are EB1-independent CLASP2 kinetochore functions, are they mediated through other microtubule binding domains within CLASP2? Are the additional microtubule-binding TOG domains in

CLASP2 required for its kinetochore function? This could easily be addressed by comparing mitotic function in cells expressing TOG domain mutants, which have been characterized in CLASP homologs. Does CLASP2 phosphorylation regulate CLASP2 kinetochore-binding partners? It has been shown that as cells progress from prometaphase to metaphase, CLASP2 forms a complex with astrin to promote stable kinetochore-microtubule interactions (Manning et al., 2010). An interesting hypothesis is that at prometaphase CLASP2 is highly phosphorylated, promoting its interaction with Kif2b and favoring weak kinetochore-microtubule interactions, and as cells enter metaphase CLASP2 dephosphorylation favors its interaction with astrin and functions to stabilize kinetochore-microtubule attachments.

How is CLASP2 dephosphorylated? It is tempting to speculate that PP1 at kinetochores, which has been shown to oppose Aurora B phosphorylation at kinetochores to stabilize-microtubule attachments, may serve as the phosphatase for CLASP2. However, there are no obvious PP1 docking motifs in CLASP2. Interestingly, I observed differential phosphorylation of CLASP1 and CLASP2 during mitosis by immunoblotting. While CLASP2 was hyperphosphorylated during mitosis, CLASP1 was much less phosphorylated. Does CLASP1 bind more strongly to microtubule ends during mitosis? Intriguingly, CLASP2, but not CLASP1, contains an Aurora B consensus motif in between the two SKIP domains in the central plus-end tracking domain. Does Aurora B phosphorylate this site in CLASP2 during mitosis and if so, does this determine further downstream posttranslational modifications of CLASP2? It will be interesting in the future to further determine the mechanisms by which kinetochore-microtubule interactions are precisely regulated spatially and temporally during mitosis.

EXPERIMENTAL PROCEDURES

Cell Culture and Drug Treatments

Immortalized HaCaT cells, HEK-293FT, HEK-293A, and HeLa cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen), 10% fetal bovine serum (Invitrogen), 100 i.u. ml^{-1} penicillin, 100 µg/ml streptomycin (Invitrogen, 15070063), 2 mM L-glutamine (Invitrogen, 25030), and 0.1 mM MEM non-essential amino acids (Invitrogen, 11140-050). According to NIH recommendations, the identity of HaCaT cells was established by short tandem repeat profiling (DDC Medical), and confirmed as described previously (Stehbens et al., 2014).

For metaphase arrest, 10 µM MG132 was used (Calbiochem) for imaging assays and 5 µM STLC (Tocris Bioscience) was used for immunoblotting. For synchronization in chromosome segregation experiments, cells were incubated O/N (16 hr.) with a CDK1 inhibitor, RO-3306, (EMD BioScience, 9 µM). Prior to imaging, cells were released from G2/M arrest by washing 3X with PBS. To synchronize cells using a double thymidine block, cells were incubated for 18 hrs. in DMEM containing 2mM thymidine, released into DMEM for 9 hrs., and incubated again in DMEM/thymidine for 16 hrs. Following release from the second thymidine block, cells were lysed at the indicated time points and processed for immunoblotting, as described below. To perturb microtubule function, taxol (1 µM) and nocodazole (1 µM for kinetochore-binding assays and 80 nM for interkinetochore distance measurements, Sigma) were used.

Immunofluorescence and Immunoblotting

For imaging, HaCaT cells were grown on 35 mm, #1.5 thickness glass-bottom dishes (Mattek) or seeded on #1.5 thickness pre-washed glass coverslips. For cold-

stable microtubule assays, cells were treated with 10 μM MG132 for 1 hr. and incubated with cold medium containing MG132 on ice for 10 min. and fixed with 0.25% gluteraldehyde as described previously (Gierke and Wittmann, 2012). Primary antibodies used for microtubule staining were rat-anti-tubulin (1:250, Serotec clone YL1/2), human-ACA (1:200, Antibodies Inc. 15-235), and DNA was stained using Hoescht (1:1000 in PBS, Molecular Probes H-3570). For BubR1 staining, cells were treated with MG132 for 1 hr. and fixed with 4% paraformaldehyde, as described previously (Stehbens et al., 2014). Mouse anti-BubR1 (1:200, Abcam ab4637) was used. For knockdown/rescue assays, cells were infected with lentiviral non-targeting shRNAs or shRNAs targeting CLASP2 and selected for 3 days with puromycin (Invitrogen), 48 hrs. after infection. Following selection, cells were infected with adenoviral EGFP-CLASP2alpha constructs for 24 hrs, synchronized with 9 μM RO-3306, as described above, arrested in metaphase with MG132, and fixed with 4% paraformaldehyde. For lagging chromosome assays, cells were infected with adenoviral EGFP-CLASP2 alpha constructs for 24 hrs, treated with RO-3306 for 8 hrs. to arrest cells at G2/M transition, and fixed with 4% paraformaldehyde 90 min. following release from RO-3306 arrest to enrich for anaphase cells. Since CLASP2 rescue constructs were tagged with GFP, DNA was stained with propidium iodide (500 nM, Invitrogen P3566), which produced less cytoplasmic staining than Sytox (Invitrogen, data not shown).

Primary and secondary antibodies were diluted in blocking buffer and samples were mounted using Mowiol, as described previously (Stehbens et al., 2014). For immunoblotting, cells were lysed in NP40 lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM

NaCl, 1% NP40) containing protease inhibitors and phosphatase inhibitors (50 mM NaF, 1 mM NaVO4, 1 mM EGTA, 10 mM sodium pyrophosphate, 1 mM β-glycerol phosphate, and 10 nM calyculin A). Lysates were centrifuged for 5 min. at 13,000 rpm and supernatants were boiled for 5 min. following addition of sample buffer. Metaphase-arrested lysates were obtained by mitotic shake off following 16 hr. treatment with STLC. Protein separation using SDS-polyacrylamide gel electrophoresis and immunoblotting was performed as described previously (Stehbens et al., 2014). Blots were imaged using a FluorChem Q documentation system (Alpha Innotech). Primary antibodies used for immunblotting include rat-anti-CLASP1 (1:1000, Absea Bioscience, KT66), rabbit-anti-CLASP2 (1:1000, (Stehbens et al., 2014)), mouse-anti-cyclin B1 (1:400, Santa Cruz Biotechnology sc-245), and mouse-anti-tubulin (1:10,000, Sigma, T9026). Secondary Alexa Fluor antibodies (Invitrogen) were used at 1:10,000 in blocking buffer.

Microscopy and Image Analysis

For live cell microscopy, the growth medium was supplemented with 20 mM HEPES, pH 7.5, and sealed with vacuum grease, as described previously (Gierke and Wittmann, 2012). Spinning disk confocal microscopy was done on an environmentally controlled Nikon TI microscope equipped with a Borealis-modified Tokogawa CSU-XI confocal head (Spectral Applied Research) and a Clara cooled scientific grade interline CCD camera (Andor). For kinetochore dynamics imaging, a iXon electron-multiplying CCD camera (Andor) and increased spinning disk speed (3500 rpm) was used. Details regarding this microscope setup have been previously described (Stehbens et al., 2012). Objectives used were 100X NA 1.49 (CFI APO TIRF, Nikon) for EGFP-CLASP2,

CENPA-mcherry, H2B-mCherry, and fixed cells stained for DNA, BubR1, microtubules, and kinetochores; 40X NA 0.6 (CFI Plan Fluor ELWD DM, Nikon) for H2B-mCherry chromosome segregation assays and immunofluorescence assays for lagging chromosomes and spindle morphologies. NIS Elements software was used to control microscope hardware and perform image processing and analysis. Statistical analysis was done using Excel and the Analyse-it plugin (Microsoft). Least square curve fitting was done using the Solver function in Excel. Videos were formatted using Quicktime Pro (Apple).

DNA, Adeno- and Lentiviral Constructs

Cells stably expressing H2B-mCherry or CENPA-mCherry were generated by transient transfection and selection of pH2B-mCherry-IRES-neo3 (Addgene, plasmid #21044) or pCENPA-mCherry-IRES-neo3. Alternatively, stable cell lines were generated by lentivirus-mediated stable expression using pLenti6/V5-DEST-H2B-mCherry or pLenti6/V5-DEST-CENPA-mCherry (Invitrogen Gateway cloning (Invitrogen, K2400) and Virapower Lentiviral Packaging Mix (Invitrogen, K4975)) as previously described (Stehbens et al., 2012). Partially truncated CLASP2 wildtype and (9xS/A) constructs (497-1515) were generated previously (Kumar et al., 2012). CLASP2(8xS/D) (497-1515) was generated via insertion of mutations from XbaI/BamH1 digest of CLASP2 wildtype (497-1515). Full-length CLASP2 alpha was a gift from Irina Kaverina (Mimori-Kiyosue et al., 2005), and cloned by PCR amplification into pENTR/D-TOPO (Invitrogen) and subsequently pAd/CMV/V5-DEST (Invitrogen). A validated shRNA pLKO.1 targeting CLASP2 was purchased from Open Biosystems (CLASP2 RHS4533-NM_015097). A non-targeting shRNA sequence that has no known target in the

mammalian genome was used as a control. All shRNA sequences and lentiviral constructs were produced and used to infect HaCaT cells as described previously (Stehbens et al., 2014). HaCaT cells infected with lentiviral shRNAs were maintained in puromycin selection media for 3 days before experimental use.

PCR primer title	**Sequence**	**Plasmid**
oHP27_H2B_TOPO_fwd	5' caccatgccagagccagcgaagtc 3'	- pENTR/D-TOPO-H2B-mCherry - pLenti6/V5-DEST-H2B-mCherry
oHP28_mCherry_rev	5’ gcgcttacttgtacagctcgtccatg 3’	- pENTR/D-TOPO-H2B-mCherry - pLenti6/V5-DEST-H2B-mCherry - pENTR/D-TOPO-CENPA-mCherry - pLenti6/V5-DEST-CENPA-mCherry
oHP31_CENPA_TOPO_fwd	5’ caccatgggcccgcgccgccggagc 3’	- pENTR/D-TOPO-CENPA-mCherry - pLenti6/V5-DEST CENPA-mCherry

REFERENCES

Akhmanova, A., and M.O. Steinmetz. 2008. Tracking the ends: a dynamic protein network controls the fate of microtubule tips. *Nat Rev Mol Cell Biol*. 9:309–322. doi:10.1038/nrm2369.

Akhmanova, A., C.C. Hoogenraad, K. Drabek, T. Stepanova, B. Dortland, T. Verkerk, W. Vermeulen, B.M. Burgering, C.I. De Zeeuw, and F. Grosveld. 2001a. Clasps are CLIP-115 and-170 associating proteins involved in the regional regulation of microtubule dynamics in motile fibroblasts. *Cell*. 104:923–935.

Akhmanova, A., C.C. Hoogenraad, K. Drabek, T. Stepanova, B. Dortland, T. Verkerk, W. Vermeulen, B.M. Burgering, C.I. De Zeeuw, F. Grosveld, and N. Galjart. 2001b. Clasps are CLIP-115 and -170 associating proteins involved in the regional regulation of microtubule dynamics in motile fibroblasts. *Cell*. 104:923–935.

Akiyoshi, B., K.K. Sarangapani, A.F. Powers, C.R. Nelson, S.L. Reichow, H. Arellano-Santoyo, T. Gonen, J.A. Ranish, C.L. Asbury, and S. Biggins. 2010. Tension directly stabilizes reconstituted kinetochore-microtubule attachments. *Nature*. 468:576–579. doi:10.1038/nature09594.

Al-Bassam, J., and F. Chang. 2011. Regulation of microtubule dynamics by TOG-domain proteins XMAP215/Dis1 and CLASP. *Trends in Cell Biology*. 21:604–614. doi:10.1016/j.tcb.2011.06.007.

Al-Bassam, J., H. Kim, G. Brouhard, A. van Oijen, S.C. Harrison, and F. Chang. 2010. CLASP Promotes Microtubule Rescue by Recruiting Tubulin Dimers to the Microtubule. *Developmental Cell*. 19:245–258. doi:10.1016/j.devcel.2010.07.016.

Alushin, G.M., V.H. Ramey, S. Pasqualato, D.A. Ball, N. Grigorieff, A. Musacchio, and E. Nogales. 2010. The Ndc80 kinetochore complex forms oligomeric arrays along microtubules. *Nature*. 467:805–810. doi:10.1038/nature09423.

Amaro, A.C., C.P. Samora, R. Holtackers, E. Wang, I.J. Kingston, M. Alonso, M. Lampson, A.D. Mcainsh, and P. Meraldi. 2010. Molecular control of kinetochore-microtubule dynamics and chromosome oscillations. *Nat Cell Biol*. 12:319–329. doi:10.1038/ncb2033.

Andrews, P.D., Y. Ovechkina, N. Morrice, M. Wagenbach, K. Duncan, L. Wordeman, and J.R. Swedlow. 2004. Aurora B regulates MCAK at the mitotic centromere. *Dev Cell*. 6:253–268.

Archuleta, A.J., C.A. Stutzke, K.M. Nixon, and M.D. Browning. 2011. Optimized Protocol to Make Phospho-Specific Antibodies that Work. *In* Methods in Molecular Biology. Humana Press, Totowa, NJ. 69–88.

Asbury, C.L., D.R. Gestaut, A.F. Powers, A.D. Franck, and T.N. Davis. 2006. The Dam1 kinetochore complex harnesses microtubule dynamics to produce force and

movement. *Proceedings of the National Academy of Sciences*. 103:9873–9878.

Asbury, C.L., J.F. Tien, and T.N. Davis. 2011. Kinetochoresâ€™ gripping feat: conformational wave or biased diffusion? *Trends in Cell Biology*. 21:38–46. doi:10.1016/j.tcb.2010.09.003.

Bakhoum, S.F., G. Genovese, and D.A. Compton. 2009a. Deviant Kinetochore Microtubule Dynamics Underlie Chromosomal Instability. *Current Biology*. 19:1937–1942. doi:10.1016/j.cub.2009.09.055.

Bakhoum, S.F., S.L. Thompson, A.L. Manning, and D.A. Compton. 2009b. Genome stability is ensured by temporal control of kinetochore–microtubule dynamics. *Nat Cell Biol*. 11:27–35. doi:10.1038/ncb1809.

Basu, S., S. Sladecek, H. Pemble, T. Wittmann, J.A. Slotman, W. van Cappellen, H.R. Brenner, and N. Galjart. 2014. Acetylcholine receptor (AChR) clustering is regulated both by glycogen synthase kinase 3 (GSK3)-dependent phosphorylation and the level of CLIP-associating protein 2 (CLASP2) mediating the capture of microtubule plus-ends. *Journal of Biological Chemistry*. doi:10.1074/jbc.M114.589457.

Bieling, P., I.A. Telley, C. Hentrich, J. Piehler, and T. Surrey. 2010. Fluorescence Microscopy Assays on Chemically Functionalized Surfaces for Quantitative Imaging of Microtubule, Motor, and. 95. First edition. Elsevier. 26 pp.

Bieling, P., L. Laan, H. Schek, E.L. Munteanu, L. Sandblad, M. Dogterom, D. Brunner, and T. Surrey. 2007. Reconstitution of a microtubule plus-end tracking system in vitro. *Nature*. 450:1100–1105. doi:10.1038/nature06386.

Bieling, P., S. Kandels-Lewis, I.A. Telley, J. Van Dijk, C. Janke, and T. Surrey. 2008. CLIP-170 tracks growing microtubule ends by dynamically recognizing composite EB1/tubulin-binding sites. *The Journal of Cell Biology*. 183:1223–1233. doi:10.1038/nsmb1291.

Biggins, S., F.F. Severin, N. Bhalla, I. Sassoon, A.A. Hyman, and A.W. Murray. 1999. The conserved protein kinase Ipl1 regulates microtubule binding to kinetochores in budding yeast. *Genes & Development*. 13:532–544.

Bird, S.L., R. Heald, and K. Weis. 2013. RanGTP and CLASP1 Cooperate to Position the Mitotic Spindle. *MBOC*. 1–25. doi:10.1091/mbc.E13-03-0150.

Bloom, K., and A. Joglekar. 2010. Towards building a chromosome segregation machine. *Nature*. 463:446–456. doi:10.1038/nature08912.

Bomont, P., P. Maddox, J.V. Shah, A.B. Desai, and D.W. Cleveland. 2005. Unstable microtubule capture at kinetochores depleted of the centromere-associated protein CENP-F. *EMBO*. 24:3927–3939. doi:10.1038/sj.emboj.7600848.

Böhm, K.J., W. Vater, H. Fenske, and E. Unger. 1984. Effect of microtubule-associated

proteins on the protofilament number of microtubules assembled in vitro. *Biochimica et Biophysica Acta (BBA)-General Subjects*. 800:119–126.

Brinkley, B.R. 2001. Managing the centrosome numbers game: from chaos to stability in cancer cell division. *Trends in Cell Biology*. 11:18–21.

Brinkley, B.R., and J. Cartwright. 1975. Cold-labile and cold-stable microtubules in the mitotic spindle of mammalian cells. *Annals of the New York Academy of Sciences*. 253:428–439.

Bulinski, J.C., J.E. Richards, and G. Piperno. 1988. Posttranslational modifications of alpha tubulin: detyrosination and acetylation differentiate populations of interphase microtubules in cultured cells. *JCB*. 106:1213–1220.

Buttrick, G.J., and J.G. Wakefield. 2008. PI3-K and GSK-3: Akt-ing together with microtubules. *Cell Cycle*. 7:2621–2625.

Campbell, C.S., and A. Desai. 2013. Tension sensing by Aurora B kinase is independent of survivin-based centromere localization. *Nature*. 497:118–121. doi:10.1038/nature12057.

Caplow, M. 1992. Microtubule dynamics. *Current Opinion in Cell Biology*. 4:58–65.

Cassimeris, L. 2002. The oncoprotein 18/stathmin family of microtubule destabilizers. *Current Opinion in Cell Biology*. 14:18–24.

Chan, G., S.A. Jablonski, V. Sudakin, J.C. Hittle, and T.J. Yen. 1999. Human BUBR1 is a mitotic checkpoint kinase that monitors CENP-E functions at kinetochores and binds the cyclosome/APC. *JCB*. 146:941–954.

Chan, K.-S., C.-G. Koh, and H.-Y. Li. 2012a. Mitosis-targeted anti-cancer therapies: where they stand. 3:e411–11. doi:10.1038/cddis.2012.148.

Chan, Y.W., A.A. Jeyaprakash, E.A. Nigg, and A. Santamaria. 2012b. Aurora B controls kinetochore-microtubule attachments by inhibiting Ska complex-KMN network interaction. *The Journal of Cell Biology*. 196:563–571. doi:10.1083/jcb.201109001.

Cheeseman, I.M. 2014. The Kinetochore. *Cold Spring Harbor Perspectives in Biology*. 6:a015826–a015826. doi:10.1101/cshperspect.a015826.

Cheeseman, I.M., and A. Desai. 2008. Molecular architecture of the kinetochore–microtubule interface. *Nat Rev Mol Cell Biol*. 9:33–46. doi:10.1038/nrm2310.

Cheeseman, I.M., I. MacLeod, J.R. Yates Iii, K. Oegema, and A. Desai. 2005. The CENP-F-like Proteins HCP-1 and HCP-2 Target CLASP to Kinetochores to Mediate Chromosome Segregation. *Current Biology*. 15:771–777. doi:10.1016/j.cub.2005.03.018.

Cheeseman, I.M., J.S. Chappie, E.M. Wilson-Kubalek, and A. Desai. 2006. The conserved KMN network constitutes the core microtubule-binding site of the kinetochore. *Cell*. 127:983–997. doi:10.1016/j.cell.2006.09.039.

Cheeseman, I.M., S. Anderson, M. Jwa, E.M. Green, J.S. Kang, J.R. Yates, C.S.M. Chan, D.G. Drubin, and G. Barnes. 2002. Phospho-regulation of kinetochore-microtubule attachments by the Aurora kinase Ipl1p. *Cell*. 111:163–172.

Cheng, L. 2011. mDia3-EB1-APC. *Communicative and Integrative Biology*. 1–3. doi:10.4161/cib.4.4.15655.

Cheng, L., J. Zhang, S. Ahmad, L. Rozier, H. Yu, H. Deng, and Y. Mao. 2011. Aurora B Regulates Formin mDia3 in Achieving Metaphase Chromosome Alignment. *Developmental Cell*. 20:342–352. doi:10.1016/j.devcel.2011.01.008.

Ciferri, C., S. Pasqualato, E. Screpanti, G. Varetti, S. Santaguida, G. Dos Reis, A. Maiolica, J. Polka, J.G. De Luca, and P. De Wulf. 2008. Implications for kinetochore-microtubule attachment from the structure of an engineered Ndc80 complex. *Cell*. 133:427–439.

Cimini, D. 2008. Merotelic kinetochore orientation, aneuploidy, and cancer. *Biochimica et Biophysica Acta (BBA) - Reviews on Cancer*. 1786:32–40. doi:10.1016/j.bbcan.2008.05.003.

Cimini, D., B. Howell, P. Maddox, A. Khodjakov, F. Degrassi, and E.D. Salmon. 2001. Merotelic kinetochore orientation is a major mechanism of aneuploidy in mitotic mammalian tissue cells. *JCB*. 153:517–527.

Clarke, P.R., and C. Zhang. 2008. Spatial and temporal coordination of mitosis by Ran GTPase. *Nat Rev Mol Cell Biol*. 9:464–477. doi:10.1038/nrm2410.

Cleveland, D.W., Y. Mao, and K.F. Sullivan. 2003. Centromeres and kinetochores: from epigenetics to mitotic checkpoint signaling. *Cell*. 112:407–421.

Cohen, I., M. Rimer, T. Lømo, and U.J. McMahan. 1997. Agrin-induced postsynaptic-like apparatus in skeletal muscle fibers in vivo. *Molecular and Cellular Neuroscience*. 9:237–253. doi:10.1006/mcne.1997.0623.

Deluca, J.G. 2002. hNuf2 inhibition blocks stable kinetochore-microtubule attachment and induces mitotic cell death in HeLa cells. *The Journal of Cell Biology*. 159:549–555. doi:10.1083/jcb.200208159.

Deluca, J.G., and A. Musacchio. 2012. Structural organization of the kinetochore. *Current Opinion in Cell Biology*. 24:48–56. doi:10.1016/j.ceb.2011.11.003.

Deluca, J.G., W.E. Gall, C. Ciferri, D. Cimini, A. Musacchio, and E.D. Salmon. 2006. Kinetochore microtubule dynamics and attachment stability are regulated by Hec1. *Cell*. 127:969–982.

Deluca, J.G., Y. Dong, P. Hergert, J. Strauss, J.M. Hickey, E.D. Salmon, and B.F. Mcewen. 2005. Hec1 and nuf2 are core components of the kinetochore outer plate essential for organizing microtubule attachment sites. *MBOC*. 16:519–531. doi:10.1091/mbc.E04-09-0852.

DeLuca, K.F., S.M.A. Lens, and J.G. Deluca. 2011. Temporal changes in Hec1 phosphorylation control kinetochore-microtubule attachment stability during mitosis. *Journal of Cell Science*. 124:622–634. doi:10.1242/jcs.072629.

Desai, A. 1997. Microtubule Polymerization Dynamics. *Annual Reviews Cell Dev. Biology*. 1–37.

Desai, A., S. Rybina, T. Muller-Reichert, and A. Shevchenko. 2003. KNL-1 directs assembly of the microtubule-binding interface of the kinetochore in C. elegans. *Genes & Development*. 17:2421–2435. doi:10.1101/gad.1126303.

Diamantopoulos, G.S., F. Perez, H.V. Goodson, G. Batelier, R. Melki, T.E. Kreis, and J.E. Rickard. 1999. Dynamic localization of CLIP-170 to microtubule plus ends is coupled to microtubule assembly. *JCB*. 144:99–112.

Dimitrov, A., M. Quesnoit, S. Moutel, I. Cantaloube, C. Poüs, and F. Perez. 2008. Detection of GTP-tubulin conformation in vivo reveals a role for GTP remnants in microtubule rescues. *Science*. 322:1353–1356.

Dixit, R., B. Barnett, J.E. Lazarus, M. Tokito, Y.E. Goldman, and E.L.F. Holzbaur. 2009. Microtubule plus-end tracking by CLIP-170 requires EB1. *PNAS*. 106:492–497. doi:10.1073/pnas.0807614106.

Dong, Y., K.J. Vanden Beldt, X. Meng, A. Khodjakov, and B.F. Mcewen. 2007. The outer plate in vertebrate kinetochores is a flexible network with multiple microtubule interactions. *Nat Cell Biol*. 9:516–522. doi:10.1038/ncb1576.

Draviam, V.M., I. Shapiro, B. Aldridge, and P.K. Sorger. 2006. Misorientation and reduced stretching of aligned sister kinetochores promote chromosome missegregation in EB1- or APC-depleted cells. *EMBO J*. 25:2814–2827. doi:10.1038/sj.emboj.7601168.

Dunsch, A.K., E. Linnane, F.A. Barr, and U. Gruneberg. 2011. The astrin-kinastrin/SKAP complex localizes to microtubule plus ends and facilitates chromosome alignment. *The Journal of Cell Biology*. 192:959–968. doi:10.1083/jcb.201008023.

Etienne-Manneville, S. 2013. Microtubules in Cell Migration. *Annu. Rev. Cell Dev. Biol.* 29:471–499. doi:10.1146/annurev-cellbio-101011-155711.

Foley, E.A., M. Maldonado, and T.M. Kapoor. 2011. Formation of stable attachments between kinetochores and microtubules depends on the B56-PP2A phosphatase. *Nat Cell Biol*. 13:1265–1271. doi:10.1038/ncb2327.

Fresu, M., M. Bianchi, J.T. Parsons, and E. Villa-Moruzzi. 2001. Cell-cycle-dependent association of protein phosphatase 1 and focal adhesion kinase. *Biochem. J.* 358:407–414.

Galjart, N. 2005. CLIPs and CLASPs and cellular dynamics. *Nat Rev Mol Cell Biol*. 6:487–498. doi:10.1038/nrm1664.

Gardner, M.K., M. Zanic, and J. Howard. 2013. Microtubule catastrophe and rescue. *Current Opinion in Cell Biology*. 25:14–22. doi:10.1016/j.ceb.2012.09.006.

Gascoigne, K.E., and I.M. Cheeseman. 2013. CDK-dependent phosphorylation and nuclear exclusion coordinately control kinetochore assembly state. *The Journal of Cell Biology*. doi:10.1083/jcb.201301006.

Gierke, S., and T. Wittmann. 2012. EB1-Recruited Microtubule +TIP Complexes Coordinate Protrusion Dynamics during 3D Epithelial Remodeling. *Curr Biol*. 22:753–762. doi:10.1016/j.cub.2012.02.069.

Gonen, S., B. Akiyoshi, M.G. Iadanza, D. Shi, N. Duggan, S. Biggins, and T. Gonen. 2012. The structure of purified kinetochores reveals multiple microtubule-attachment sites. *Nature Publishing Group*. 19:925–929. doi:10.1038/nsmb.2358.

Gönczy, P. 2012. Towards a molecular architecture ofcentriole assembly. *Nat Rev Mol Cell Biol*. 13:425–435. doi:10.1038/nrm3373.

Green, R.A., R. Wollman, and K.B. Kaplan. 2005. APC and EB1 function together in mitosis to regulate spindle dynamics and chromosome alignment. *MBOC*. 16:4609–4622. doi:10.1091/mbc.E05.

Gregan, J., S. Polakova, L. Zhang, I.M. Tolić-Nørrelykke, and D. Cimini. 2011. Merotelic kinetochore attachment: causes and effects. *Trends in Cell Biology*. 21:374–381. doi:10.1016/j.tcb.2011.01.003.

Hanisch, A. 2006. Timely anaphase onset requires a novel spindle and kinetochore complex comprising Ska1and Ska2. 1–12.

Hanks, S., K. Coleman, S. Reid, A. Plaja, H. Firth, D. FitzPatrick, A. Kidd, K. Méhes, R. Nash, N. Robin, N. Shannon, J. Tolmie, J. Swansbury, A. Irrthum, J. Douglas, and N. Rahman. 2004. Constitutional aneuploidy and cancer predisposition caused by biallelic mutations in BUB1B. *Nat Genet*. 36:1159–1161. doi:10.1038/ng1449.

Hannak, E. 2006. Xorbit/CLASP links dynamic microtubules to chromosomes in the Xenopus meiotic spindle. *The Journal of Cell Biology*. 172:19–25. doi:10.1083/jcb.200508180.

Hauf, S. 2013. The spindle assembly checkpoint: progress and persistent puzzles. *Biochem. Soc. Trans*. 41:1755–1760. doi:10.1042/BST20130240.

Holland, A.J., and D.W. Cleveland. 2009. Boveri revisited: chromosomal instability, aneuploidy and tumorigenesis. 1–10. doi:10.1038/nrm2718.

Honnappa, S., C.M. John, D. Kostrewa, F.K. Winkler, and M.O. Steinmetz. 2005. Structural insights into the EB1-APC interaction. *EMBO*. 24:261–269. doi:10.1038/sj.emboj.7600529.

Honnappa, S., S.M. Gouveia, A. Weisbrich, F.F. Damberger, N.S. Bhavesh, H. Jawhari, I. Grigoriev, F.J.A. van Rijssel, R.M. Buey, A. Lawera, I. Jelesarov, F.K. Winkler, K. Wüthrich, A. Akhmanova, and M.O. Steinmetz. 2009. An EB1-Binding Motif Acts as a Microtubule Tip Localization Signal. *Cell*. 138:366–376. doi:10.1016/j.cell.2009.04.065.

Howard, J., and A.A. Hyman. 2003. Dynamics and mechanics of the microtubule plus end. *Nature*. 422:753–758.

Inoue, Y.H., M. do Carmo Avides, M. Shiraki, P. Deak, M. Yamaguchi, Y. Nishimoto, A. Matsukage, and D.M. Glover. 2000. Orbit, a novel microtubule-associated protein essential for mitosis in Drosophila melanogaster. *JCB*. 149:153–166.

Jansen, L.E.T., B.E. Black, D.R. Foltz, and D.W. Cleveland. 2007. Propagation of centromeric chromatin requires exit from mitosis. *The Journal of Cell Biology*. 176:795–805. doi:10.1083/jcb.200701066.

Jaqaman, K., D. Loerke, M. Mettlen, H. Kuwata, S. Grinstein, S.L. Schmid, and G. Danuser. 2008. Robust single-particle tracking in live-cell time-lapse sequences. *Nat Meth*. 5:695–702. doi:10.1038/nmeth.1237.

Jaqaman, K., E.M. King, A.C. Amaro, J.R. Winter, J.F. Dorn, H.L. Elliott, N. Mchedlishvili, S.E. McClelland, I.M. Porter, M. Posch, A. Toso, G. Danuser, A.D. McAinsh, P. Meraldi, and J.R. Swedlow. 2010. Kinetochore alignment within the metaphase plate is regulated by centromere stiffness and microtubule depolymerases. *The Journal of Cell Biology*. 188:665–679. doi:10.1083/jcb.200909005.

Jones, G., T. Meier, M. Lichtsteiner, V. Witzemann, B. Sakmann, and H.R. Brenner. 1997. Induction by agrin of ectopic and functional postsynaptic-like membrane in innervated muscle. *Proceedings of the National Academy of Sciences*. 94:2654–2659.

Jope, R.S., and G.V.W. Johnson. 2004. The glamour and gloom of glycogen synthase kinase-3. *Trends in Biochemical Sciences*. 29:95–102. doi:10.1016/j.tibs.2003.12.004.

Kaplan, K.B., A.A. Burds, J.R. Swedlow, S.S. Bekir, P.K. Sorger, and I.S. Nathke. 2001. A role for the Adenomatous Polyposis Coli protein in chromosome segregation. *Nat Cell Biol*. 3:429–432. doi:10.1038/35070123.

Kaverina, I., and A. Straube. 2011. Seminars in Cell & Developmental Biology. *Seminars in Cell & Developmental Biology*. 22:968–974. doi:10.1016/j.semcdb.2011.09.017.

Kern, D.M., and I.M. Cheeseman. 2012. Kinetochore Structure: Pulling Answers from Yeast. *Curr Biol*. 22:R842–R844. doi:10.1016/j.cub.2012.08.001.

Khodjakov, A. 2010. Centromere tension: a divisive issue. *Nature*. 1–5.

Kim, S., and B.D. Dynlacht. 2013. Assembling a primary cilium. *Current Opinion in Cell Biology*. 25:506–511. doi:10.1016/j.ceb.2013.04.011.

Kim, Y., A.J. Holland, W. Lan, and D.W. Cleveland. 2010. Aurora Kinases and Protein Phosphatase 1 Mediate Chromosome Congression through Regulation of CENP-E. *Cell*. 142:444–455. doi:10.1016/j.cell.2010.06.039.

Kim, Y., J.E. Heuser, C.M. Waterman, and D.W. Cleveland. 2008. CENP-E combines a slow, processive motor and a flexible coiled coil to produce an essential motile kinetochore tether. *The Journal of Cell Biology*. 181:411–419. doi:10.1038/359536a0.

Kline-Smith, S.L., and C.E. Walczak. 2004. Mitotic spindle assembly and chromosome segregation: refocusing on microtubule dynamics. *Mol. Cell*. 15:317–327. doi:10.1016/j.molcel.2004.07.012.

Kops, G.J.P.L., B.A.A. Weaver, and D.W. Cleveland. 2005. On the road to cancer: aneuploidy and the mitotic checkpoint. *Nat Rev Cancer*. 5:773–785. doi:10.1038/nrc1714.

Kumar, P., and T. Wittmann. 2012. +TIPs: SxIPping along microtubuleends. *Trends in Cell Biology*. 22:418–428. doi:10.1016/j.tcb.2012.05.005.

Kumar, P., K.S. Lyle, S. Gierke, A. Matov, G. Danuser, and T. Wittmann. 2009. GSK3 phosphorylation modulates CLASP-microtubule association and lamella microtubule attachment. *The Journal of Cell Biology*. 184:895–908. doi:10.1083/jcb.200901042.

Kumar, P., M.S. Chimenti, H. Pemble, A. Schönichen, O. Thompson, M.P. Jacobson, and T. Wittmann. 2012. Multisite phosphorylation disrupts arginine-glutamate salt bridge networks required for binding of cytoplasmic linker-associated protein 2 (CLASP2) to end-binding protein 1 (EB1). *Journal of Biological Chemistry*. 287:17050–17064. doi:10.1074/jbc.M111.316661.

Lampson, M.A., and I.M. Cheeseman. 2011. Sensing centromere tension: Aurora B and the regulation of kinetochore function. *Trends in Cell Biology*. 21:133–140. doi:10.1016/j.tcb.2010.10.007.

Lemos, C. 2000. Mast, a conserved microtubule-associated protein required for bipolar

mitotic spindle organization. *EMBO*. 1–15.

Lesage, B., J. Qian, and M. Bollen. 2011. Spindle Checkpoint Silencing: PP1 Tips the Balance. *Curr Biol*. 21:R898–R903. doi:10.1016/j.cub.2011.08.063.

Liu, D., G. Vader, M.J.M. Vromans, M.A. Lampson, and S.M.A. Lens. 2009. Sensing chromosome bi-orientation by spatial separation of aurora B kinase from kinetochore substrates. *Science*. 323:1350–1353. doi:10.1126/science.1167000.

Liu, D., M. Vleugel, C.B. Backer, T. Hori, T. Fukagawa, I.M. Cheeseman, and M.A. Lampson. 2010. Regulated targeting of protein phosphatase 1 to the outer kinetochore by KNL1 opposes Aurora B kinase. *The Journal of Cell Biology*. 188:809–820. doi:10.1083/jcb.201001006.

Liu, Z., Q.P. Vong, and Y. Zheng. 2007. CLASPing microtubules at the trans-Golgi network. *Dev Cell*. 12:839–840. doi:10.1016/j.devcel.2007.05.007.

Liz, M.A., F.M. Mar, T.E. Santos, H.I. Pimentel, A.M. Marques, M.M. Morgado, S. Vieira, V.F. Sousa, H. Pemble, T. Wittmann, C. Sutherland, J.R. Woodgett, and M.M. Sousa. 2014. Neuronal deletion of GSK3beta increases microtubule speed in the growth cone and enhances axon regeneration via CRMP-2 and independently of MAP1B and CLASP2. *BMC Biol*. 12:47. doi:10.1186/1741-7007-12-47.

Logarinho, E., S. Maffini, M. Barisic, A. Marques, A. Toso, P. Meraldi, and H. Maiato. 2012. CLASPs prevent irreversible multipolarity by ensuring spindle-pole resistance to traction forces during chromosome alignment. *Nat Cell Biol*. 14:1–10. doi:10.1038/ncb2423.

Maddox, A.S., and K. Oegema. 2003. Deconstructing cytokinesis. *Nat Cell Biol*. 5:773–776. doi:10.1038/ncb0903-773b.

Maddox, P., A. Desai, K. Oegema, and T. Mitchison. 2002. Poleward Microtubule Flux Is a Major Component of Spindle Dynamics and Anaphase A in Mitotic …. *Current Biology*.

Maffini, S., A.R.R. Maia, A.L. Manning, Z. Maliga, A.L. Pereira, M. Junqueira, A. Shevchenko, A. Hyman, J.R. Yates III, and N. Galjart. 2009a. Motor-independent targeting of CLASPs to kinetochores by CENP-E promotes microtubule turnover and poleward flux. *Curr Biol*. 19:1566.

Maffini, S., A.R.R. Maia, A.L. Manning, Z. Maliga, A.L. Pereira, M. Junqueira, A. Shevchenko, A. Hyman, J.R. Yates Iii, N. Galjart, D.A. Compton, and H. Maiato. 2009b. Motor-Independent Targeting of CLASPs to Kinetochores by CENP-E Promotes Microtubule Turnover and Poleward Flux. *Curr Biol*. 19:1566–1572. doi:10.1016/j.cub.2009.07.059.

Magidson, V., C.B. O'connell, J. Lončarek, R. Paul, A. Mogilner, and A. Khodjakov. 2011. The Spatial Arrangement of Chromosomes during Prometaphase Facilitates

Spindle Assembly. *Cell*. 146:555–567. doi:10.1016/j.cell.2011.07.012.

Maia, A.R.R., Z. Garcia, L. Kabeche, M. Barisic, S. Maffini, S. Macedo-Ribeiro, I.M. Cheeseman, D.A. Compton, I. Kaverina, and H. Maiato. 2012. Cdk1 and Plk1 mediate a CLASP2 phospho-switch that stabilizes kinetochore-microtubule attachments. *The Journal of Cell Biology*. 199:285–301. doi:10.1083/jcb.201203091.

Maiato, H. 2003. How do Kinetochores CLASP Dynamic Microtubules? *Cell Cycle*. 1–4.

Maiato, H., A. Khodjakov, and C.L. Rieder. 2005. Drosophila CLASP is required for the incorporation of microtubule subunits into fluxing kinetochore fibres. *Nat Cell Biol*. 7:42–47. doi:10.1038/ncb1207.

Maiato, H., E.A. Fairley, C.L. Rieder, J.R. Swedlow, C.E. Sunkel, and W.C. Earnshaw. 2003. Human CLASP1 is an outer kinetochore component that regulates spindle microtubule dynamics. *Cell*. 113:891–904. doi:10.1016/S0092-8674(03)00465-3.

Malumbres, M. 2011. Physiological Relevance of Cell Cycle Kinases. *Physiological Reviews*. 91:973–1007. doi:10.1152/physrev.00025.2010.

Mandelkow, E.-M., E. Mandelkow, and R.A. Milligan. 1991. Microtubule dynamics and microtubule caps: a time-resolved cryo-electron microscopy study. *JCB*. 114:977–991.

Manning, A.L., S.F. Bakhoum, S. Maffini, C. Correia-Melo, H. Maiato, and D.A. Compton. 2010. CLASP1, astrin and Kif2b form a molecular switch that regulates kinetochore-microtubule dynamics to promote mitotic progression and fidelity. *EMBO J*. 29:3531–3543. doi:10.1038/emboj.2010.230.

Martinez, O., and B. Goud. 1998. Rab proteins. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*. 1404:101–112.

Matov, A., K. Applegate, P. Kumar, C. Thoma, W. Krek, G. Danuser, and T. Wittmann. 2010. Analysis of microtubule dynamic instability using a plus-end growth marker. *Nat Meth*. 7:761–768. doi:10.1038/nmeth.1493.

Maurer, S.P., P. Bieling, J. Cope, A. Hoenger, and T. Surrey. 2011. GTPγS microtubules mimic the growing microtubule end structure recognized by end-binding proteins (EBs). *Proceedings of the National Academy of Sciences*. 108:3988–3993. doi:10.1073/pnas.1014758108/-/DCSupplemental/pnas.201014758SI.pdf.

Mcewen, B.F., A.B. Heagle, G.O. Cassels, K.F. Buttle, and C.L. Rieder. 1997. Kinetochore fiber maturation in PtK1 cells and its implications for the mechanisms of chromosome congression and anaphase onset. *JCB*. 137:1567–1580.

McIntosh, J.R., E. O'Toole, K. Zhudenkov, M. Morphew, C. Schwartz, F.I. Ataullakhanov, and E.L. Grishchuk. 2013. Conserved and divergent features of kinetochores and

spindle microtubule ends from five species. *The Journal of Cell Biology*. 200:459–474. doi:10.1083/jcb.201209154.dv.

McIntosh, J.R., E.L. Grishchuk, M.K. Morphew, A.K. Efremov, K. Zhudenkov, V.A. Volkov, I.M. Cheeseman, A. Desai, D.N. Mastronarde, and F.I. Ataullakhanov. 2008. Fibrils Connect Microtubule Tips with Kinetochores: A Mechanism to Couple Tubulin Dynamics to Chromosome Motion. *Cell*. 135:322–333. doi:10.1016/j.cell.2008.08.038.

Miller, P.M., A.W. Folkmann, A.R.R. Maia, N. Efimova, A. Efimov, and I. Kaverina. 2009. Golgi-derived CLASP-dependent microtubules control Golgi organization and polarized trafficking in motile cells. *Nat Cell Biol*. 11:1069–1080. doi:10.1038/ncb1920.

Mimori-Kiyosue, Y., I. Grigoriev, G. Lansbergen, H. Sasaki, C. Matsui, F. Severin, N. Galjart, F. Grosveld, I. Vorobjev, S. Tsukita, and A. Akhmanova. 2005. CLASP1 and CLASP2 bind to EB1 and regulate microtubule plus-end dynamics at the cell cortex. *JCB*. 168:141–153. doi:10.1083/jcb.200405094.

Mimori-Kiyosue, Y., I. Grigoriev, H. Sasaki, C. Matsui, A. Akhmanova, S. Tsukita, and I. Vorobjev. 2006. Mammalian CLASPs are required for mitotic spindle organization and kinetochore alignment. *Genes Cells*. 11:845–857. doi:10.1111/j.1365-2443.2006.00990.x.

Mishra, R.K., P. Chakraborty, A. Arnaoutov, B.M.A. Fontoura, and M. Dasso. 2010. The Nup107-160 complex and γ-TuRC regulate microtubule polymerization at kinetochores. *Nat Cell Biol*. 12:164–169. doi:10.1038/ncb2016.

Mitchison, T., and M. Kirschner. 1984. Dynamic instability of microtubule growth. *Nature*. 312:237–242.

Musinipally, V., S. Howes, G.M. Alushin, and E. Nogales. 2013. The Microtubule Binding Properties of. *Journal of Molecular Biology*. 1–15. doi:10.1016/j.jmb.2013.07.027.

Müller-Reichert, T., D. Chrétien, F. Severin, and A.A. Hyman. 1998. Structural changes at microtubule ends accompanying GTP hydrolysis: information from a slowly hydrolyzable analogue of GTP, guanylyl (α, β) methylenediphosphonate. *Proceedings of the National Academy of Sciences*. 95:3661–3666.

Nicklas, R.B. 1988. The forces that move chromosomes in mitosis. *Annual review of biophysics and biophysical chemistry*. 17:431–449.

O'Connell, C.B., A. Khodjakov, and B.F. Mcewen. 2012. Kinetochore flexibility: creating a dynamic chromosome-spindle interface. *Current Opinion in Cell Biology*. 24:40–47. doi:10.1016/j.ceb.2011.12.008.

Ong Tone, S., B. Dayanandan, A.E. Fournier, and C.A. Mandato. 2010. GSK3

Regulates Mitotic Chromosomal Alignment through CRMP4. *PLoS ONE*. 5:e14345. doi:10.1371/journal.pone.0014345.s005.

Ookata, K., S. Hisanaga, M. Sugita, A. Okuyama, H. Murofushi, H. Kitazawa, S. Chari, J.C. Bulinski, and T. Kishimoto. 1997. MAP4 is the in vivo substrate for CDC2 kinase in HeLa cells: identification of an M-phase specific and a cell cycle-independent phosphorylation site in MAP4. *Biochemistry*. 36:15873–15883. doi:10.1021/bi971251w.

Palmer, D.K., K. O'Day, H.L. Trong, H. Charbonneau, and R.L. Margolis. 1991. Purification of the centromere-specific protein CENP-A and demonstration that it is a distinctive histone. *Proceedings of the National Academy of Sciences*. 88:3734–3738.

Palmer, D.K., K. O'Day, M.H. Wener, B.S. Andrews, and R.L. Margolis. 1987. A 17-kD centromere protein (CENP-A) copurifies with nucleosome core particles and with histones. *JCB*. 104:805–815.

Patel, K., E. Nogales, and R. Heald. 2012. Multiple domains of human CLASP contribute to microtubule dynamics and organization in vitro and in Xenopus egg extracts. *Cytoskeleton*. 69:155–165. doi:10.1002/cm.21005.

Pereira, A.L., A.J. Pereira, A.R.R. Maia, K. Drabek, C.L. Sayas, P.J. Hergert, M. Lince-Faria, I. Matos, C. Duque, T. Stepanova, C.L. Rieder, W.C. Earnshaw, N. Galjart, and H. Maiato. 2006. Mammalian CLASP1 and CLASP2 cooperate to ensure mitotic fidelity by regulating spindle and kinetochore function. *MBOC*. 17:4526–4542. doi:10.1091/mbc.E06-07-0579.

Perez, F., G.S. Diamantopoulos, R. Stalder, and T.E. Kreis. 1999. CLIP-170 highlights growing microtubule ends in vivo. *Cell*. 96:517–527.

Perpelescu, M., and T. Fukagawa. 2011. The ABCs of CENPs. *Chromosoma*. 120:425–446. doi:10.1007/s00412-011-0330-0.

Peters, J.-M. 2006. The anaphase promoting complex/cyclosome: a machine designed to destroy. *Nat Rev Mol Cell Biol*. 7:644–656. doi:10.1038/nrm1988.

Pines, J., and C.L. Rieder. 2001. Re-staging mitosis: a contemporary view of mitotic progression. *Nat Cell Biol*. 3:E3–E6.

Plotnikov, S.V., and C.M. Waterman. 2013. Guiding cell migration by tugging. *Current Opinion in Cell Biology*. 25:619–626. doi:10.1016/j.ceb.2013.06.003.

Powers, A.F., A.D. Franck, D.R. Gestaut, J. Cooper, B. Gracyzk, R.R. Wei, L. Wordeman, T.N. Davis, and C.L. Asbury. 2009. The Ndc80 Kinetochore Complex Forms Load-Bearing Attachments to Dynamic Microtubule Tips via Biased Diffusion. *Cell*. 136:865–875. doi:10.1016/j.cell.2008.12.045.

Prokop, A. 2013. The intricate relationship between microtubules and their associated motor proteins during axon growth and maintenance. *Neural Development*. 8:1–1. doi:10.1186/1749-8104-8-17.

Raaijmakers, J.A., M.E. Tanenbaum, A.F. Maia, and R.H. Medema. 2009. RAMA1 is a novel kinetochore protein involved in kinetochore-microtubule attachment. *Journal of Cell Science*. 122:2436–2445. doi:10.1242/jcs.051912.

Radtke, F. 2005. Self-Renewal and Cancer of the Gut: Two Sides of a Coin. *Science*. 307:1904–1909. doi:10.1126/science.1104815.

Rappaport, R. 1990. Role of the Mitotic Apparatus in Furrow Initiationa. *Annals of the New York Academy of Sciences*. 582:15–21.

Regnier, V., P. Vagnarelli, T. Fukagawa, T. Zerjal, E. Burns, D. Trouche, W. Earnshaw, and W. Brown. 2005. CENP-A Is Required for Accurate Chromosome Segregation and Sustained Kinetochore Association of BubR1. *Molecular and Cellular Biology*. 25:3967–3981. doi:10.1128/MCB.25.10.3967-3981.2005.

Rieder, C.L. 1981. The structure of the cold-stable kinetochore fiber in metaphase PtK1 cells. *Chromosoma*. 84:145–158.

Rogers, G.C. 2005. Spindle microtubules in flux. *Journal of Cell Science*. 118:1105–1116. doi:10.1242/jcs.02284.

Rusan, N.M., C.J. Fagerstrom, A.M. Yvon, and P. Wadsworth. 2001. Cell cycle-dependent changes in microtubule dynamics in living cells expressing green fluorescent protein-alpha tubulin. *MBOC*. 12:971–980.

Sacristan, C., and G.J.P.L. Kops. 2014. Joined at the hip: kinetochores, microtubules, and spindle assembly checkpoint signaling. *Trends in Cell Biology*. 1–8. doi:10.1016/j.tcb.2014.08.006.

Sammak, P.J., and G.G. Borisy. 1988. Direct observation of microtubule dynamics in living cells. *Nature*. 332:724–726.

Samora, C.P., B. Mogessie, L. Conway, J.L. Ross, A. Straube, and A.D. Mcainsh. 2011. MAP4 and CLASP1 operate as a safety mechanism to maintain a stable spindle position in mitosis. *Nat Cell Biol*. 13:1040–1050. doi:10.1038/ncb2297.

Sardar, H.S., V.G. Luczak, M.M. Lopez, B.C. Lister, and S.P. Gilbert. 2010. Mitotic Kinesin CENP-E Promotes Microtubule Plus-End Elongation. *Current Biology*. 20:1648–1653. doi:10.1016/j.cub.2010.08.001.

Schmidt, J.C., H. Arthanari, A. Boeszoermenyi, N.M. Dashkevich, E.M. Wilson-Kubalek, N. Monnier, M. Markus, M. Oberer, R.A. Milligan, M. Bathe, G. Wagner, E.L. Grishchuk, and I.M. Cheeseman. 2012a. The Kinetochore-Bound Ska1 Complex Tracks Depolymerizing Microtubules and Binds to Curved Protofilaments. *Dev Cell*.

1–13. doi:10.1016/j.devcel.2012.09.012.

Schmidt, J.C., T. Kiyomitsu, T. Hori, C.B. Backer, T. Fukagawa, and I.M. Cheeseman. 2010. Aurora B kinase controls the targeting of the Astrin-SKAP complex to bioriented kinetochores. *The Journal of Cell Biology*. 191:269–280. doi:10.1083/jcb.201006129.

Schmidt, N., S. Basu, S. Sladecek, S. Gatti, J. van Haren, S. Treves, J. Pielage, N. Galjart, and H.R. Brenner. 2012b. Agrin regulates CLASP2-mediated capture of microtubules at the neuromuscular junction synaptic membrane. *The Journal of Cell Biology*. 198:421–437. doi:10.1083/jcb.201111130.

Schroer, T.A. 2004. DYNACTIN. *Annu. Rev. Cell Dev. Biol.* 20:759–779. doi:10.1146/annurev.cellbio.20.012103.094623.

Shen, W.-W., M. Frieden, and N. Demaurex. 2012. Remodelling of the endoplasmic reticulum during store-operated calcium entry. *Biology of the Cell*. 103:365–380. doi:10.1042/BC20100152.

Shrestha, R.L., and V.M. Draviam. 2013. Lateral to End-on Conversionof Chromosome-Microtubule Attachment Requires Kinesins CENP-E and MCAK. *Curr Biol*. 1–13. doi:10.1016/j.cub.2013.06.040.

Silva, V.C., and L. Cassimeris. 2014. CAMSAPs Add to the Growing Microtubule Minus-End Story. *Dev Cell*. 28:221–222. doi:10.1016/j.devcel.2014.01.025.

Simon, J.R., and E.D. Salmon. 1990. The structure of microtubule ends during the elongation and shortening phases of dynamic instability examined by negative-stain electron microscopy. *Journal of Cell Science*. 96:571–582.

Skoufias, D.A., P.R. Andreassen, F.B. Lacroix, L. Wilson, and R.L. Margolis. 2001. Mammalian mad2 and bub1/bubR1 recognize distinct spindle-attachment and kinetochore-tension checkpoints. *Proceedings of the National Academy of Sciences*. 98:4492–4497.

Skoufias, D.A., S. DeBonis, Y. Saoudi, L. Lebeau, I. Crevel, R. Cross, R.H. Wade, D. Hackney, and F. Kozielski. 2006. S-Trityl-L-cysteine Is a Reversible, Tight Binding Inhibitor of the Human Kinesin Eg5 That Specifically Blocks Mitotic Progression. *Journal of Biological Chemistry*. 281:17559–17569. doi:10.1074/jbc.M511735200.

Slep, K.C. 2009. The role of TOG domains in microtubule plus end dynamics. *Biochem. Soc. Trans*. 37:1002. doi:10.1042/BST0371002.

Stehbens, S., and T. Wittmann. 2012. Targeting and transport: How microtubules control focal adhesion dynamics. *The Journal of Cell Biology*. 198:481–489. doi:10.1073/pnas.1000975107.

Stehbens, S., H. Pemble, L. Murrow, and T. Wittmann. 2012. Imaging intracellular

protein dynamics by spinning disk confocal microscopy. *Meth. Enzymol.* 504:293–313. doi:10.1016/B978-0-12-391857-4.00015-X.

Stehbens, S.J., M. Paszek, H. Pemble, A. Ettinger, S. Gierke, and T. Wittmann. 2014. CLASPs link focal-adhesion-associated microtubule capture to localized exocytosis and adhesion site turnover. *Nat Cell Biol*. 16:561–573. doi:10.1038/ncb2975.

Stumpff, J., G. von Dassow, M. Wagenbach, C. Asbury, and L. Wordeman. 2008. The Kinesin-8 Motor Kif18A Suppresses Kinetochore Movements to Control Mitotic Chromosome Alignment. *Developmental Cell*. 14:252–262. doi:10.1016/j.devcel.2007.11.014.

Stumpff, J., M. Wagenbach, A. Franck, C.L. Asbury, and L. Wordeman. 2012. Kif18A and Chromokinesins Confine Centromere Movements via Microtubule Growth Suppression and Spatial Control of Kinetochore Tension. *Developmental Cell*. 22:1017–1029. doi:10.1016/j.devcel.2012.02.013.

Suzuki, A., T. Hori, T. Nishino, J. Usukura, A. Miyagi, K. Morikawa, and T. Fukagawa. 2011. Spindle microtubules generate tension-dependent changes in the distribution of inner kinetochore proteins. *The Journal of Cell Biology*. 193:125–140. doi:10.1083/jcb.201012050.

Tamura, N., and V.M. Draviam. 2012. Microtubule plus-ends within a mitotic cell are “moving platforms” with anchoring, signalling and force-coupling roles. *Open Biology*. 2:120132–120132. doi:10.1098/rsob.120132.

Tanaka, K. 2012. Regulatory mechanisms of kinetochore–microtubule interaction in mitosis. *Cell. Mol. Life Sci.* doi:10.1007/s00018-012-1057-7.

Tanaka, T.U., M.J.R. Stark, and K. Tanaka. 2005. Kinetochore capture and bi-orientation on the mitotic spindle. *Nat Rev Mol Cell Biol*. 6:929–942. doi:10.1038/nrm1764.

Tanenbaum, M.E., N. Galjart, M.A.T.M. Van Vugt, and R.H. Medema. 2006. CLIP-170 facilitates the formation of kinetochore–microtubule attachments. *EMBO J*. 25:45–57. doi:10.1038/sj.emboj.7600916.

Thompson, S.L., S.F. Bakhoum, and D.A. Compton. 2010. Mechanisms of Chromosomal Instability Review. *Curr Biol*. 20:R285–R295. doi:10.1016/j.cub.2010.01.034.

Tighe, A., A. Ray-Sinha, O.D. Staples, and S.S. Taylor. 2007. GSK-3 inhibitors induce chromosome instability. *BMC Cell Biol*. 8:34. doi:10.1186/1471-2121-8-34.

Tirnauer, J.S., J.C. Canman, E.D. Salmon, and T.J. Mitchison. 2002. EB1 targets to kinetochores with attached, polymerizing microtubules. *MBOC*. 13:4308–4316. doi:10.1091/mbc.E02-04-0236.

Tulu, U.S., C. Fagerstrom, N.P. Ferenz, and P. Wadsworth. 2006. Molecular Requirements for Kinetochore-Associated Microtubule Formation in Mammalian Cells. *Current Biology*. 16:536–541. doi:10.1016/j.cub.2006.01.060.

Tulu, U.S., N.M. Rusan, and P. Wadsworth. 2003. Peripheral, Non-Centrosome-Associated Microtubules Contribute to Spindle Formation in Centrosome-Containing Cells. *Current Biology*. 13:1894–1899. doi:10.1016/j.cub.2003.10.002.

Umbreit, N.T. 2012. The Ndc80 kinetochore complex directly modulates microtubule dynamics. *PNAS*. 1–6. doi:10.1073/pnas.1209615109/-/DCSupplemental/pnas.201209615SI.pdf.

Umbreit, N.T., and T.N. Davis. 2012. Mitosis puts sisters in a strained relationship: Force generation at the kinetochore. *Exp Cell Res*. 318:1361–1366. doi:10.1016/j.yexcr.2012.04.008.

van der Vaart, B., C. Manatschal, I. Grigoriev, V. Olieric, S.M. Gouveia, S. Bjelic, J. Demmers, I. Vorobjev, C.C. Hoogenraad, M.O. Steinmetz, and A. Akhmanova. 2011. SLAIN2 links microtubule plus end-tracking proteins and controls microtubule growth in interphase. *The Journal of Cell Biology*. 193:1083–1099. doi:10.1091/mbc.E05-09-0892.

VandenBeldt, K.J., R.M. Barnard, P.J. Hergert, X. Meng, H. Maiato, and B.F. Mcewen. 2006. Kinetochores Use a Novel Mechanism for Coordinating the Dynamics of Individual Microtubules. *Current Biology*. 16:1217–1223. doi:10.1016/j.cub.2006.04.046.

Varma, D., P. Monzo, S.A. Stehman, and R.B. Vallee. 2008. Direct role of dynein motor in stable kinetochore-microtubule attachment, orientation, and alignment. *The Journal of Cell Biology*. 182:1045–1054. doi:10.1083/jcb.200710106.

Vasiliev, J.M., I.M. Gelfand, L.V. Domnina, O.Y. Ivanova, S.G. Komm, and L.V. Olshevskaja. 1970. Effect of colcemid on the locomotory behaviour of fibroblasts. *Journal of embryology and experimental morphology*. 24:625–640.

Vasquez, R.J., D.L. Gard, and L. Cassimeris. 1999. Phosphorylation by CDK1 regulates XMAP215 function in vitro. *Cell Motil. Cytoskeleton*. 43:310–321. doi:10.1002/(SICI)1097-0169(1999)43:4<310::AID-CM4>3.0.CO;2-J.

Wade, R.H. 2009. On and Around Microtubules: An Overview. *Mol Biotechnol*. 43:177–191. doi:10.1007/s12033-009-9193-5.

Wakefield, J.G. 2002. A role for glycogen synthase kinase-3 in mitotic spindle dynamics and chromosome alignment. *Journal of Cell Science*. 116:637–646. doi:10.1242/jcs.00273.

Walczak, C.E., and R. Heald. 2008. Mechanisms of mitotic spindle assembly and function. *International Review of Cytology*. 265:111–158. doi:10.1016/S0074-

7696(07)65003-7.

Walczak, C.E., S. Cai, and A. Khodjakov. 2010. Mechanisms of chromosome behaviour during mitosis. *Nat Rev Mol Cell Biol*. 1–12. doi:10.1038/nrm2832.

Walker, R.A., E.T. O'brien, N.K. Pryer, M.F. Soboeiro, W.A. Voter, H.P. Erickson, and E.D. Salmon. 1988. Dynamic instability of individual microtubules analyzed by video light microscopy: rate constants and transition frequencies. *JCB*. 107:1437–1448.

Wan, X., R.P.O. Quinn, H.L. Pierce, A.P. Joglekar, W.E. Gall, J.G. Deluca, C.W. Carroll, S.-T. Liu, T.J. Yen, B.F. Mcewen, P.T. Stukenberg, A. Desai, and E.D. Salmon. 2009. Protein Architecture of the HumanKinetochore Microtubule Attachment Site. *Cell*. 137:672–684. doi:10.1016/j.cell.2009.03.035.

Wang, X., X. Zhuang, D. Cao, Y. Chu, P. Yao, W. Liu, L. Liu, G. Adams, G. Fang, Z. Dou, X. Ding, Y. Huang, D. Wang, and X. Yao. 2012. Mitotic regulator SKAP forms a link between kinetochore core complex KMN and dynamic spindle microtubules. *Journal of Biological Chemistry*. doi:10.1074/jbc.M112.406652.

Watanabe, T., J. Noritake, M. Kakeno, T. Matsui, T. Harada, S. Wang, N. Itoh, K. Sato, K. Matsuzawa, A. Iwamatsu, N. Galjart, and K. Kaibuchi. 2009. Phosphorylation of CLASP2 by GSK-3 regulates its interaction with IQGAP1, EB1 and microtubules. *Journal of Cell Science*. 122:2969–2979. doi:10.1242/jcs.046649.

Weaver, B.A., and D.W. Cleveland. 2006. Does aneuploidy cause cancer? *Current Opinion in Cell Biology*. 18:658–667. doi:10.1016/j.ceb.2006.10.002.

Weaver, B.A.A., A.D. Silk, C. Montagna, P. Verdier-Pinard, and D.W. Cleveland. 2007. Aneuploidy Acts Both Oncogenically and as a Tumor Suppressor. *Cancer Cell*. 11:25–36. doi:10.1016/j.ccr.2006.12.003.

Wei, R.R., J. Al-Bassam, and S.C. Harrison. 2007. The Ndc80/HEC1 complex is a contact point for kinetochore-microtubule attachment. *Nat Struct Mol Biol*. 14:54–59. doi:10.1038/nsmb1186.

Welburn, J.P.I., E.L. Grishchuk, C.B. Backer, E.M. Wilson-Kubalek, J.R. Yates Iii, and I.M. Cheeseman. 2009. The Human Kinetochore Ska1 Complex Facilitates Microtubule Depolymerization-Coupled Motility. *Dev Cell*. 16:374–385. doi:10.1016/j.devcel.2009.01.011.

Welburn, J.P.I., M. Vleugel, D. Liu, J.R. Yates Iii, M.A. Lampson, T. Fukagawa, and I.M. Cheeseman. 2010. Aurora B Phosphorylates Spatially Distinct Targets to Differentially Regulate the Kinetochore-Microtubule Interface. *Molecular Cell*. 38:383–392. doi:10.1016/j.molcel.2010.02.034.

Wendell, K.L., L. Wilson, and M.A. Jordan. 1993. Mitotic block in HeLa cells by vinblastine: ultrastructural changes in kinetochore-microtubule attachment and in centrosomes. *Journal of Cell Science*. 104:261–274.

Westermann, S., A. Avila-Sakar, H.-W. Wang, H. Niederstrasser, J. Wong, D.G. Drubin, E. Nogales, and G. Barnes. 2005. Formation of a Dynamic Kinetochore- Microtubule Interface through Assembly of the Dam1 Ring Complex. *Molecular Cell*. 17:277–290. doi:10.1016/j.molcel.2004.12.019.

Wilson-Kubalek, E.M., I.M. Cheeseman, C. Yoshioka, A. Desai, and R.A. Milligan. 2008. Orientation and structure of the Ndc80 complex on the microtubule lattice. *The Journal of Cell Biology*. 182:1055–1061. doi:10.1083/jcb.200804170.

Winey, M. 1995. Three-Dimensional Ultrastructural Analysis of the Saccharomyces cerevisiae Mitotic Spindle. 1–15.

Wittmann, T. 2005. Spatial regulation of CLASP affinity for microtubules by Rac1 and GSK3 in migrating epithelial cells. *The Journal of Cell Biology*. 169:929–939. doi:10.1083/jcb.200412114.

Witzemann, V., F. Chevessier, P.G. Pacifici, and P. Yampolsky. 2013. The neuromuscular junction: Selective remodeling of synaptic regulators at the nerve/muscle interface. *Mechanisms of Development*. 130:402–411. doi:10.1016/j.mod.2012.09.004.

Wollman, R., E.N. Cytrynbaum, J.T. Jones, T. Meyer, J.M. Scholey, and A. Mogilner. 2005. Efficient Chromosome Capture Requires a Bias in the “Search-and-Capture” Process during Mitotic-Spindle Assembly. *Current Biology*. 15:828–832. doi:10.1016/j.cub.2005.03.019.

Wu, H., W.C. Xiong, and L. Mei. 2010. To build a synapse: signaling pathways in neuromuscular junction assembly. *Development*. 137:1017–1033. doi:10.1242/dev.038711.

Wu, X., A. Kodama, and E. Fuchs. 2008. ACF7 Regulates Cytoskeletal-Focal Adhesion Dynamics and Migration and Has ATPase Activity. *Cell*. 135:137–148. doi:10.1016/j.cell.2008.07.045.

Wu, X., Q.-T. Shen, D.S. Oristian, C.P. Lu, Q. Zheng, H.-W. Wang, and E. Fuchs. 2011. Skin Stem Cells Orchestrate Directional Migration by Regulating Microtubule-ACF7 Connections through GSK3&beta. *Cell*. 144:341–352. doi:10.1016/j.cell.2010.12.033.

Wu, X., X. Xiang, and J.A. Hammer III. 2006. Motor proteins at the microtubule plus-end. *Trends in Cell Biology*. 16:135–143. doi:10.1016/j.tcb.2006.01.004.