Title
Mechanism of the mitotic kinesin CENP-E in tethering kinetochores to spindle microtubules

Permalink
https://escholarship.org/uc/item/3x82w9cf

Author
Kim, Yumi

Publication Date
2009

Peer reviewed|Thesis/dissertation
Mechanism of the Mitotic Kinesin CENP-E in Tethering Kinetochores to Spindle Microtubules

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Biological Sciences by Yumi Kim

Committee in charge:

Professor Don W. Cleveland, Chair
Professor Arshad Desai
Professor Douglass J. Forbes
Professor Lawrence S. Goldstein
Professor Martin W. Hetzer
Professor Amy Kiger

2009
Copyright

Yumi Kim, 2009

All rights reserved.
The dissertation of Yumi Kim is approved, and it is acceptable in quality and form for publication of microfilm and electronically:

_______________________________________________

_______________________________________________

_______________________________________________

_______________________________________________

_______________________________________________

Chair

University of California, San Diego

2009
Dedication

This work is dedicated to my friend Christina Chung (1980~2009) who very much deserved to live a full life.

This work is also dedicated to my father Jongman Kim who had dreamed to become a scientist himself and has inspired me ever since I remember.
# Table of Contents

Signature Page........................................................................................................................................... iii
Dedication.................................................................................................................................................. iv
Table of Contents...................................................................................................................................... v
List of Figures........................................................................................................................................... ix
Acknowledgements................................................................................................................................. xi
Vita............................................................................................................................................................ xiii
Abstract of the Dissertation...................................................................................................................... xiv

Chapter 1: Introduction .............................................................................................................................. 1

1.1 Chromosome movements during mitosis ....................................................................................... 1

1.2 The kinetochore................................................................................................................................. 2

1.3 Dynamic kinetochore-microtubule interface ................................................................................. 3

1.4 Contribution of kinetochore motors to chromosome movement .................................................. 8

1.4.1 Cytoplasmic dynein ...................................................................................................................... 8

1.4.2 MCAK .......................................................................................................................................... 11

1.4.3 Kif18a .......................................................................................................................................... 12

1.5 Identification and initial characterization of CENP-E ................................................................... 13

1.6 CENP-E as an essential component for stable microtubule capture at kinetochore ................... 16

1.7 Role of CENP-E in the mitotic checkpoint ..................................................................................... 20

1.8 Post-translational modifications of CENP-E ................................................................................. 23

1.8.1 Phosphorylation .......................................................................................................................... 24
Chapter 2: CENP-E combines a slow, processive motor and a flexible coiled-coil to produce an essential motile kinetochore tether

2.1 Introduction

2.2 Purification of recombinant full-length Xenopus CENP-E

2.3 230 nm-long and highly flexible coiled-coil structure of CENP-E

2.4 Hydrodynamic properties of CENP-E

2.5 CENP-E motility assay with a single molecule imaging

2.6 CENP-E is a slow, processive motor that maintains microtubule attachment for long periods

2.7 CENP-E motility contains one-dimensional diffusion

2.8 Full-length CENP-E is a slow, plus end-directed kinetochore motor

2.9 CENP-E motor activity is essential for metaphase chromosome alignment

2.10 Discussion

2.11 Methods

Chapter 3: Phosphorylation-dependent regulation of CENP-E by Aurora kinases is essential for chromosome congression

3.1 Introduction
3.2 Highly conserved CENP-E T422 positioned near the kinesin neck domain is phosphorylated by Aurora A and B in vitro

3.3 Generation of phosphoT422-specific antibodies

3.4 Phosphorylation of CENP-E occurs at kinetochores

3.5 Aurora kinases, both A and B, contribute to phosphorylation of CENP-E at T422

3.6 Aurora-mediated phosphorylation of CENP-E reduces the affinity of CENP-E for microtubules

3.7 Phosphorylation of CENP-E reduces the run length

3.8 Phosphorylation of CENP-E T422 is essential for chromosome congression

3.9 CENP-E phosphorylation is required downstream of Aurora activation for the congression of incorrectly attached chromosomes that are moved to the spindle pole

3.10 Discussion

3.11 Methods

Chapter 4: Conclusions and future directions

4.1 Properties and phospho-regulation of CENP-E

4.1.1 CENP-E as a motile kinetochore tether

4.1.2 Direct control of CENP-E by Aurora kinases to promote chromosome biorientation while keeping the kinetochores error free

4.2 Potential of CENP-E in regulating microtubule dynamics

4.2.1 CENP-E forms a nucleotide-sensitive complex with free tubulin
4.2.2 CENP-E does not affect the overall microtubule poleward flux in spindles assembled in *Xenopus* extracts .................................................................................................................. 100

4.2.3 Implication from the interaction of CENP-E with microtubule plus end-tracking proteins................................................................................................................................. 102

4.3 Properties of CENP-E under load ........................................................................ 104

References....................................................................................................................... 109
List of Figures

Figure 2.1 Purification of recombinant full-length *Xenopus* CENP-E ......................... 48
Figure 2.2 Electron micrographs of individual CENP-E molecules .................................... 49
Figure 2.3 CENP-E is a highly flexible, dimeric kinesin with a 230-nm discontinuous coiled-coil ......................................................................................................................... 50
Figure 2.4 Hydrodynamic properties of CENP-E .............................................................................. 51
Figure 2.5 CENP-E motility assays with a single molecule imaging ........................................... 52
Figure 2.6 CENP-E is a slow, processive motor that maintains microtubule attachment for long periods ....................................................................................................................................... 53
Figure 2.7 CENP-E motility contains one-dimensional diffusion .................................................. 54
Figure 2.8 Full-length CENP-E is a slow, plus end-directed motor ............................................. 55
Figure 2.9 CENP-E motor activity is essential for metaphase chromosome alignment .... 56
Figure 2.10 CENP-E localization in the *Xenopus* extract spindles ............................................. 57
Figure 2.11 A model for CENP-E as a motile, flexible tether for kinetochore microtubule capture and maintenance of linkage to dynamic spindle microtubules .................................................... 58
Figure 3.1 Phosphorylation sites of CENP-E ............................................................................... 82
Figure 3.2 Highly conserved CENP-E Threonine 422 is phosphorylated by Aurora A and Aurora B kinases in vitro .......................................................................................................................... 83
Figure 3.3 Generation of polyclonal phosphoT422-specific CENP-E antibody ......................... 84
Figure 3.4 Phosphorylation of CENP-E T422 occurs at kinetochores .......................................... 85
Figure 3.5 PhosphoT422 signal is enriched at the leading kinetochore ....................................... 86
Figure 3.6 Phosphorylation of CENP-E at T422 is sensitive to Aurora kinase inhibitor . 87
Figure 3.7 Phosphorylation of CENP-E T422 is contributed by both Aurora A and B in vivo ................................................................. 88

Figure 3.8 Phosphorylation of CENP-E by Aurora kinase reduces its affinity for microtubules in the ADP state ................................................................. 89

Figure 3.9 Single molecule assays................................................................................................................................. 90

Figure 3.10 Phosphorylation of CENP-E reduces the run length ................................................................. 91

Figure 3.11 Phosphorylation of CENP-E Threonine 422 is essential for chromosome congression ................................................................. 92

Figure 3.12 CENP-E phosphorylation is required downstream of Aurora activation for congression of incorrectly attached chromosomes that are moved to the spindle pole .... 93

Figure 3.13 Model................................................................................................................................. 94

Figure 4.1 CENP-E forms a nucleotide-sensitive complex with free tubulin.................. 107

Figure 4.2 Inhibition of CENP-E does not affect the flux machinery in general ......... 108
Acknowledgements

I am indebted to my thesis advisor Don Cleveland for taking a chance on me and giving me the opportunity to work on one of the most exciting problems in cell biology. He has never underestimated the potential of a ‘deceptively quiet’ graduate student, and amazingly knew how to motivate her in the best possible way. From him I have learned passion for science, critical mind, patience, optimism, sense of humor, and many others. If, one day, I be considered as a good scientist, it must have been because I was once a student of his.

My sincere gratitude is extended to all the past and current members of the Cleveland lab for tremendous support, encouragement, a company during countless coffee breaks, laughter, and friendship over the years. Especially, I would like to thank Geert Kops, Lars Jansen, and Beth Weaver, who shared the bay with me, for their guidance in early days, and Andrew Holland for our exciting collaboration on later work.

I have been very fortunate to have several other mentors and friends close by in Oegema and Desai labs. Especially, I am deeply grateful to Arshad Desai and Paul Maddox for their patience, generosity, many advices, exciting discussions, and again encouragement.

Lastly, I would like to pay tribute to my family: my father, who had dreamed to become a scientist himself, for true inspiration and relentless support, my mom, the strongest woman I know, for teaching me music, life, and love, and my little brother for fun we shared growing up together.
Chapter 2, in part, is a reprint of the material as it appears in *Journal of Cell Biology*, 2008 181, 411-419, Kim Y., Heuser J.E., Waterman C.M., and Cleveland D.W. I was the primary researcher and the first author of this paper.

Chapter 3, in part, is in preparation for publication of the material, Kim Y., Holland, A.J., Lan, W., and Cleveland, D.W. The dissertation author and Andrew Holland contributed equally to this work.
Vita

2003 Bachelor of Science, Seoul National University, Seoul, Korea

2009 Doctor of Philosophy, University of California, San Diego

Publications


Abstract of the Dissertation

Mechanism of the Mitotic Kinesin CENP-E in Tethering Kinetochore to Spindle Microtubules

by

Yumi Kim

Doctor of Philosophy in Biological Sciences
University of California, San Diego, 2009
Professor Don W. Cleveland, Chair

The mitotic kinesin CENP-E is an essential kinetochore motor that directly contributes to the capture and stabilization of spindle microtubules by kinetochores. Although it has been well established that CENP-E is essential for metaphase chromosome alignment and reduction of CENP-E leads to high rates of whole
chromosome missegregation in cells, its properties as a microtubule-dependent motor, the mechanism by which CENP-E contributes to the dynamic linkage between kinetochores and spindle microtubules, and how its motility is regulated are unknown.

In this dissertation, using single molecule assays, CENP-E has been shown to be a processive, plus end-directed motor with one-dimensional diffusion, which allows microtubule attachment for long periods. Direct visualization of recombinant full-length *Xenopus* CENP-E by electron microscopy reveals a highly flexible, 230 nm-long coiled-coil separating its kinesin motor and kinetochore targeting domains. Furthermore, full-length CENP-E is a plus end-directed motor whose activity is essential for metaphase chromosome alignment. Based on these findings, I propose that the highly processive microtubule-dependent motor activity of CENP-E serves not only to power chromosome congression, but also to provide a flexible, motile tether linking kinetochores to dynamic spindle microtubules.

CENP-E is highly phosphorylated during mitosis, which raises the possibility that phosphorylation may regulate the function of CENP-E at individual kinetochores. Strikingly, blocking phosphorylation of a single conserved site close to the motor domain of CENP-E prevents chromosome alignment with few chromosomes remaining close to spindle poles. Using purified components and a phospho-specific antibody, I demonstrate that Aurora kinases, both A and B, phosphorylate this site both in vitro and in vivo. Phosphorylation occurs while CENP-E is bound to the kinetochore and reduces the affinity of CENP-E for microtubules in vitro. Moreover, CENP-E phosphorylation is shown to be required, downstream of Aurora activation, for the congression of incorrectly
attached chromosomes that are moved to the spindle pole. My findings demonstrate that CENP-E is under direct control of Aurora kinases to promote chromosome biorientation.
Chapter 1: Introduction

1.1 Chromosome movements during mitosis

Mitosis is the process in which a cell partitions its duplicated genome, equally into two daughter cells. This vital event was noted as early as the late 19th century by Walther Flemming, who coined the term mitosis for the process of cell division from the Greek word *mitos*, a thread, referring to the thread-like appearance of chromosomes (Flemming, 1882). Replicated DNA molecules, held together by cohesion of sister chromatids, reach their most compacted state in mitosis, and the shortening of chromosome length is essential to effectively segregate chromosomes to opposite poles. Chromosome segregation is accompanied with cleavage of the cohesion between sisters and is beautifully choreographed by a complex cytoskeletal machine made of microtubules, or the mitotic spindle. Oriented with their minus ends anchored at the spindle pole and their plus ends extending away from the opposite poles, the spindle microtubules exert forces to move chromosomes during mitosis.

Mitotic chromosome movement is a slow, but precisely directed process (Nicklas, 1988). Breakdown of the nuclear envelope marks the beginning of prometaphase and allows highly condensed chromosomes to be captured by spindle microtubules nucleated from the centrosomes. Initial attachment is often mediated via an interaction with the lateral surface of a single microtubule, and kinetochores attached in this manner undergo a rapid motion toward the spindle pole (Rieder and Alexander, 1990). Accurate chromosome segregation requires that all the chromosomes are properly attached by
microtubules emanating from the opposite poles and lined up at the spindle equator before anaphase onset. Metaphase chromosome alignment is achieved as result of bi-orientation of the chromosomes (Rieder and Salmon, 1994), or as mono-oriented chromosomes near the spindle pole congress to the equator along the microtubule bundles that are connected to already bi-oriented chromosome (Kapoor et al., 2006). Once all the chromosomes are aligned at metaphase, cohesion between sister chromatids is simultaneously resolved in anaphase and each set of chromatids is segregated to opposite poles of the cell. Chromosomes then decondense, the nuclear envelope reforms around two sets of daughter nuclei, and mitosis is completed by cytokinesis.

1.2 The kinetochore

Connection between chromosomes and the spindle microtubules takes place at the centromere, which is discernibly recognized by a primary constriction on condensed chromosomes. Each time a cell divides, a specialized proteinaceous structure called the kinetochore assembles on the surface of each centromere, and it is the kinetochore that binds to spindle microtubules and directs chromosome motion during mitosis (Cleveland et al., 2003). Classically, the kinetochore has been viewed by conventional electron microscopy as a tri-laminar structure, consisting of electron-dense outer plate, lightly-staining middle layer, inner plate, and inner centromere adjacent to the underlying heterochromatin (Brinkley and Stubblefield, 1966). Although the plate-like appearance becomes less pronounced when kinetochores are well preserved by high-pressure freezing methods (Dong et al., 2007; McIntosh et al., 2008), such stereotypical micrographs of vertebrate kinetochores have lead to the widely held image of
kinetochore-microtubule interface in which the plus ends of microtubules embed into the kinetochore outer plate.

1.3 Dynamic kinetochore-microtubule interface

Unlike the impression of fixed images, the mitotic spindle is, in fact, labile and dynamic. First observed by polarized light microscopy, spindle microtubules undergo a series of polymerization and depolymerization events during mitosis (Inoue and Sato, 1967). Discovery of their dynamic nature has led to the important hypothesis that microtubule dynamics can generate force to support mitotic chromosome movements. Direct observation of spindle dynamics using labeled tubulins has identified the kinetochore as site of microtubule assembly and disassembly, with kinetochore microtubules adding and losing subunits during chromosome movement (Gorbsky et al., 1987, 1988; Mitchison et al., 1986). Moreover, spindle microtubules were observed to translocate poleward in a manner coupled to minus-end disassembly at the spindle poles (Mitchison, 1989). Although the rate of microtubule poleward translocation (flux) varies significantly among different organisms, both microtubule poleward flux and kinetochore-based motility contribute to chromosome movements during mitosis.

The dynamic nature of spindle microtubules originates from the intrinsic properties of microtubules called dynamic instability. Originally discovered by the length distribution of individual microtubules at steady state in vitro, dynamic instability describes the coexistence of growing and shrinking microtubules which interconvert rather infrequently (Mitchison and Kirschner, 1984). This unique polymerization dynamics is due to GTP hydrolysis by β-tubulin after it becomes incorporated into the
microtubule. Binding of GTP to β-tubulin straightens the subunit so as to allow lateral contacts into the sheet of protofilaments at growing ends of microtubules (Wang and Nogales, 2005). Shortly after polymerization, β-tubulin hydrolyzes its bound GTP, which in turn induces a conformational change to increase the bending between tubulin monomers in their GDP-bound state (Muller-Reichert et al., 1998). However, GDP-tubulin is constrained, unable to adopt its fully curved form in the straight microtubule lattice; therefore, the free energy from GTP hydrolysis is stored in microtubule as a mechanical strain. The strain energy is released only when GDP-tubulin is exposed to the ends, fueling microtubule depolymerization.

Dynamic microtubules can provide forces to do mechanical work (Howard and Hyman, 2003; Inoue and Salmon, 1995). Direct measurements in vitro have shown that a single polymerizing and depolymerizing microtubule can generate 3-4 pN of pushing (Dogterom and Yurke, 1997) and 30~65 pN of pulling forces (Grishchuk et al., 2005), respectively. The magnitude of force produced by dynamic microtubules is comparable to the stall force of a single kinesin molecule (5~7 pN) (Visscher et al., 1999), or even significantly larger, up to the opposing force per kinetochore microtubule that completely stops anaphase chromosome movement (50 pN) (Nicklas, 1983, 1988). Chromosome movement during mitosis is tightly coupled to the dynamics of kinetochore microtubules. By simply maintaining a linkage with spindle microtubules, kinetochores exploit the energy liberated during microtubule assembly or disassembly to power mitotic chromosome movement (Coue et al., 1991; Koshland et al., 1988).
An important question is then how the kinetochores maintain their dynamic linkage to spindle microtubules while allowing subunit addition or loss. An initial insight came from in vitro experiments using kinesin-coated beads or purified chromosomes that were attached by plus ends of microtubules. Surprisingly, when microtubules were induced to disassemble, the kinesin-coated beads moved, despite kinesin’s opposite polarity, toward the minus end following the depolymerizing microtubule ends (Lombillo et al., 1995b). In addition, microtubule depolymerization-driven chromosome movement was inhibited by either pan kinesin antibodies or those specific for CENP-E (Centromere-associated protein E) (Lombillo et al., 1995a), suggesting a role of CENP-E in coupling microtubule dynamics to chromosome motility.

Recent discovery of ring structure of the yeast Dam1/DASH complex on microtubules has generated ample excitement (Miranda et al., 2005; Westermann et al., 2005), provoking the idea that the microtubule-encircling ring would provide an ideal coupling device for kinetochores. Indeed, it was shown that the Dam1 complex can move processively on depolymerizing microtubule ends and harness microtubule dynamics to produce force and movement (Asbury et al., 2006; Westermann et al., 2006). While direct evidence supporting the presence of a ring-like structure at the yeast kinetochore is missing (McIntosh, 2005), a recent study has shown that the rings may not be necessary for the ability of the Dam1 complex to move processively with disassembling microtubule tips (Gestaut et al., 2008).

Core microtubule attachment sites at kinetochores are believed to be formed by the conserved network of KNL-1, Mis12 complex, and Ndc80 complex (KMN network)
Homologues of the KMN network are found in all eukaryotes (Cheeseman et al., 2004), and it has been established that the Ndc80 complex is essential for kinetochore-microtubule interaction in various organisms (DeLuca et al., 2002; McCleland et al., 2003; Wigge and Kilmartin, 2001). Singly, Ndc80 complex and KNL-1 bear low affinity for microtubules; however, formation of the complete KMN network synergistically increase the microtubule binding activity (Cheeseman et al., 2006). This observation has led to the proposal that the KNL network provides multiple binding sites with relatively weak microtubule affinity, which would allow a robust, yet dynamic connection between kinetochores and spindle microtubules. Indeed, Ndc80 complex-coated beads have been shown to move with depolymerizing microtubules and make a load-bearing attachment to dynamic microtubule tips in vitro (McIntosh et al., 2008; Powers et al., 2009).

In fact, the outer kinetochore contains a number of other proteins with microtubule-binding activities. The Ska1 complex, composed of Ska1, Ska2 and Ramal (Ska3), has most recently been shown to bind to microtubule directly and to be required for stable kinetochore-microtubule interaction (Gaitanos et al., 2009; Welburn et al., 2009). Depletion of the Ska1 complex did not displace Ndc80 from the kinetochore and yet resulted in severe chromosome alignment defects, suggesting that this complex might form a separate branch from the KMN network for microtubule attachment. Interestingly, Ska1 complex-coated beads, just like the Ndc80 complex, can couple microtubule depolymerization to cargo movement in vitro (Welburn et al., 2009).

Dynamic linkage of kinetochores to microtubules can be explained by a biased diffusion reaction. First proposed by Hill, this model proposes that a “sleeve” with
multiple binding sites for the microtubule lattice will provide a stable attachment for a shortening microtubule by exploiting thermal diffusion, as long as it is biased to favor more interactions between microtubules and the kinetochore sleeve (Hill, 1985). The Hill sleeve model is not restricted to so-called core attachment sites at kinetochores, and the model can be applied to any kind of array of microtubule-interacting proteins. Most likely, it is the collective action by the meshwork of proteins with varying degrees of microtubule interaction at the outer kinetochore that mediates the linkage between each kinetochore and dynamic spindle microtubules.

Additional proteins known to directly interact with microtubules include CENP-F, a large coiled-coil protein, which is required for stable microtubule capture at kinetochore (Bomont et al., 2005; Feng et al., 2006), and a group of microtubule plus end tracking proteins (+TIPs), most of which are localized to the outer kinetochores and have roles in regulating dynamics of kinetochore microtubules (Akhmanova and Steinmetz, 2008; Cheeseman and Desai, 2008). Among them, XMAP215/TOG1, a microtubule associated protein (MAP) that promotes microtubule polymerization, has been shown to tip-track both polymerizing and depolymerizing microtubule plus ends (Brouhard et al., 2008). Also CLASP1 (Clip-associated protein) promotes polymerization specifically of kinetochore microtubules (Maiato et al., 2005), and has been proposed to tether dynamic microtubule plus ends to kinetochores possibly via an interaction with CENP-E (Hannak and Heald, 2006).
1.4 Contribution of kinetochore motors to chromosome movement

In addition to microtubule dynamics, microtubule-dependent motors positioned at kinetochores also contribute to aspects of mitotic chromosome movement. Motor proteins convert chemical energy released by ATP hydrolysis to directional movement along microtubules. The presence of such enzymes at kinetochore has been inferred from the studies using isolated chromosomes, and has been demonstrated that kinetochores contain both minus and plus end-directed motilities capable of supporting microtubule translocation in ATP-dependent manner in vitro (Hyman and Mitchison, 1991; Mitchison and Kirschner, 1985). The existence of kinesin-related proteins at kinetochores was further hinted at by continued efforts to identify mitotic kinesins using antibodies raised against the conserved sequences in the kinesin motor domain (Sawin et al., 1992). Real excitement then came from the discovery that, indeed, cytoplasmic dynein (Pfarr et al., 1990; Steuer et al., 1990), and three kinesin family members, CENP-E (Yen et al., 1992), MCAK (Wordeman and Mitchison, 1995), and Kif18a, are localized to kinetochores during mitosis.

1.4.1. Cytoplasmic dynein

Cytoplasmic dynein supports a fast minus end-directed movement (Paschal and Vallee, 1987; Schroer et al., 1989), and for this reason it has been widely assumed that this multi-subunit motor protein is involved in poleward chromosome motions during mitosis. However, studying the function of kinetochore-localized dynein has been difficult due to its multiple roles throughout mitosis. Only recently, contribution of
kinetochore dynein to the mitotic chromosome movements has begun to be tested by selectively inhibiting dynein localization to the kinetochores.

Dynein is largely responsible for the rapid poleward chromosome motion in prometaphase that is mediated through the lateral binding to a single astral microtubule (Rieder and Alexander, 1990; Yang et al., 2007). This dynein-mediated chromosome transport to the pole is believed to be an important mechanism to collect chromosomes to a common microtubule-dense region, which would greatly increase the chance for a mono-oriented chromosome to be encountered by neighboring kinetochore microtubules and to be efficiently aligned at the spindle equator (Kapoor et al., 2006).

Although controversial, dynein has also been implicated in anaphase chromosome movement. In *Drosophila* embryos and in human cells, depletion of the Rod/Zw10/Zwilch (RZZ) complex, conserved proteins required for the recruitment of dynein/dynactin to the kinetochores (Starr et al., 1998), was reported to result in a significantly attenuated poleward chromosome movement during anaphase A (Savoian et al., 2000; Yang et al., 2007). Similar conclusions were also reached by more direct methods in *Drosophila* embryos by microinjecting the p50 subunit of dynactin or anti-dynein heavy chain (DHC) antibodies (Sharp et al., 2000). However, as dynein mostly dissociates from the kinetochores once they are stably attached by microtubules (Hoffman et al., 2001), it is hard to envision how dynein would generate force for chromosome movement during anaphase A. A later report, again in fly embryos, has suggested that the microtubule poleward flux, rather than dynein, is a dominant mechanism for anaphase chromosome-to-pole movement in this system (Maddox et al.,
Consistently, dynein inhibition in mammalian cells, by microinjecting either p50 or 70.1 monoclonal anti-dynein intermediate chain (DIC) antibody, was reported not to block chromosome congression or anaphase chromosome segregation (Howell et al., 2001; Vorozhko et al., 2008). 

Recent studies on Spindly, a novel protein which acts downstream of the RZZ complex to recruit dynein to kinetochores (Griffis et al., 2007), have revealed new aspects of dynein function in chromosome segregation. In *C. elegans* embryos, Spindly depletion mimicked the lack of stable end-on attachments, giving rise to significantly more severe defects in chromosome segregation than those found in embryos inhibited of the RZZ complex. Surprisingly, co-depletion of Spindly with RZZ subunits were found to rescue the phenotype to the degree of RZZ inhibition alone (Gassmann et al., 2008). This interesting observation has led to the proposal that the RZZ complex without dynein/dynactin inhibits the formation of stable end-on attachments, and the inhibition is relieved by the dynein/dynactin recruited via Spindly (Gassmann et al., 2008). Also recently, displacing endogenous DHC by over-expressing the tail fragments, without affecting other dynein-associated proteins, has been shown to cause problems in the stability of kinetochore-microtubule attachment (Varma et al., 2008). It seems that the kinetochore-localized dynein promotes strong end-coupled, load-bearing attachment, a prerequisite for faithful chromosome separation, and probably does so by generating tension using its minus end-directed motility which, in turn, stabilizes the interaction between kinetochore and spindle microtubule (Gassmann et al., 2008; Varma et al., 2008).
In budding yeast, instead of cytoplasmic dynein, the minus end-directed motor Kar3 (Kinesin-14) is known to drive rapid poleward chromosome motion during prometaphase (Tanaka et al., 2005). The members of this kinesin family (Ncd in *Drosophila*; XCTK2 in *Xenopus*; HSET in human), however, are not found at mammalian kinetochores. Interestingly, in fission yeast, none of the minus end-directed motors (dynein and two Kinesin-14 members, Pkl1p and Klp2p) are needed for prometaphase chromosome movement towards the spindle pole; instead, this process appears to depend purely on microtubule dynamics (Grishchuk and McIntosh, 2006). Therefore, in yeasts, the typical task of dynein found in higher eukaryotes seems to have been replaced by the redundant mechanisms to bring the chromosomes to the spindle poles for efficient congression.

1.4.2 MCAK

MCAK (Mitotic centromere-associated kinesin, Kinesin-13) is a unique kinesin member whose motor domain is located internally in the middle of the protein. Unlike other kinesins with a directed motility, MCAK targets microtubule plus and minus ends for destabilization by means of random diffusion along the microtubule lattice (Desai et al., 1999b; Helenius et al., 2006). During mitosis, MCAK is dynamically localized to the inner centromere (Wordeman and Mitchison, 1995), and its activity is under direct control of the mitotic kinase Aurora B (Andrews et al., 2004; Lan et al., 2004). Because of its enrichment at merotelically attached kinetochores and its role in regulating microtubule dynamics, MCAK has been proposed to play a role in correcting attachment errors (Knowlton et al., 2006). However, it is not yet clear how phosphorylation of
MCAK can selectively target aberrant microtubules for detachment to promote chromosome biorientation, as Aurora B-mediated phosphorylation inhibits the microtubule depolymerizing activity of MCAK, resulting in more stable microtubule attachment (Andrews et al., 2004; Lan et al., 2004).

Besides MCAK, two additional members of the Kinesin-13 family (Kif2a and Kif2b) are present in human genome and play distinct roles during mitosis. Localized to the spindle pole, Kif2a is required for bipolar spindle assembly and for microtubule poleward flux by disassembling minus ends of microtubules at the spindle poles (Gaetz and Kapoor, 2004; Ganem and Compton, 2004; Ganem et al., 2005). Kif2b is localized predominantly to centrosomes, spindle microtubules, and transiently to the kinetochores in early prometaphase (Manning et al., 2007). Depletion of Kif2b causes delay in bipolar spindle assembly and lagging chromosomes in anaphase, and Kif2b has been shown to be required for the correction of attachment errors (Bakhoum et al., 2009). Recent evidence now suggests that Kif2b and MCAK promote turnover of kinetochore microtubule at temporally distinct periods in mitosis to keep the chromosomes error free (Bakhoum et al., 2009).

1.4.3. Kif18a

Recently added to the list of kinesin motors localized to kinetochores, Kif18a (Kinesin-8) in human cells is recruited to the kinetochores and the plus ends of kinetochore microtubules, using its motor activity in a microtubule-dependent manner (Mayr et al., 2007; Stumpff et al., 2008). Members of this kinesin family are found at kinetochores from prometaphase until the anaphase onset in various organisms (Kip3p in
budding yeast; Klp5/6 in fission yeast; Klp67A in Drosophila; Kif18a in human), and depletion of Kinesin-8 results in mitotic cells with abnormally long spindles with misaligned chromosomes (Garcia et al., 2002; Goshima and Vale, 2003; Mayr et al., 2007; Savoian et al., 2004; Tytell and Sorger, 2006; West et al., 2002). In yeast, deletion alleles of Kinesin-8 show elongated cytoplasmic microtubules, increased resistance to microtubule poisons, and defects in spindle positioning, suggesting a role of Kinesin-8 in regulating microtubule dynamics (Cottingham and Hoyt, 1997; Garcia et al., 2002; West et al., 2001).

Kinesin-8 is a dual enzyme that contains both plus end-directed motility and a microtubule depolymerizing activity (Gupta Jr et al., 2006; Mayr et al., 2007; Varga et al., 2006). Kip3p in budding yeast has been demonstrated to be a slow kinesin with a remarkable processivity whose run length by single molecules corresponds to 1500 steps before dissociating from the microtubule (Varga et al., 2006). Distinct from MCAK, Kinesin-8 uses its motility to accumulate at the plus end of microtubule and destabilizes exclusively the plus ends in a length-dependent manner (Varga et al., 2006). By dampening kinetochore oscillations at the spindle equator, Kif18a has been shown to facilitate the chromosome alignment during mitosis (Stumpff et al., 2008). Although Kif18a has been proposed to regulate the plus end dynamics of kinetochore microtubules, whether and how Kif18a modulates the microtubule dynamics is not known.

1.5 Identification and initial characterization of CENP-E

CENP-E was first discovered in human cells with a monoclonal antibody raised against chromosome scaffold proteins enriched for kinetochore components (Yen et al.,
1991), and later CENP-E was found to be a kinesin family motor (Kinesin-7) that localizes to the kinetochores (Yen et al., 1992). Homologues of CENP-E have been subsequently identified in higher eukaryotes including mouse (Putkey et al., 2002), *Xenopus laevis* (Wood et al., 1997), *Drosophila melanogaster* (Yucel et al., 2000), *Dictyostelium discoideum* (Nag et al., 2008), and also in the plant kingdom (ten Hoopen et al., 2002).

CENP-E is a large protein (312 kDa in human) with a kinesin motor domain at the N-terminus (~38% sequence identity with a human Kinesin-1), followed by a long discontinuous α-helical coiled-coil and C-terminus globular domain. Consistent with its function during mitosis, expression of CENP-E is regulated in a cell cycle-dependent manner. CENP-E is largely absent in interphase cells, and its protein level begins accumulated at late G2/M and is rapidly reduced during late mitosis (Brown et al., 1994). The kinetochore targeting domain of CENP-E has been mapped to its C-terminus by transiently expressing fragments of CENP-E in human cells (Chan et al., 1998), and this has proven to be the case also in *Xenopus* extracts after immunodepleting endogenous protein and complementing with recombinant CENP-E tail fragment (Mao et al., 2005). CENP-E is recruited to the kinetochores right after nuclear envelope breakdown and immunoelectron microscopy showed CENP-E is a constituent of the corona fibers that extend from the surface of the kinetochores from prometaphase to anaphase A (Cooke et al., 1997; Yao et al., 1997). CENP-E is especially enriched at unattached kinetochores and, although significantly reduced upon microtubule capture, remains at kinetochores
until anaphase A. Then, CENP-E relocates to spindle midbody in late anaphase and telophase, after which it is gradually degraded (Brown et al., 1996).

CENP-E was initially reported to be associated with a minus end-directed motility (Thrower et al., 1995). Partially purified fractions enriched in CENP-E from mitotic HeLa extracts supported relatively fast minus end-directed motility, and the motility was shown to be depleted with antibodies against human CENP-E. The biochemical purification, however, was simply based on microtubule affinity, followed by a sucrose gradient sedimentation, which could lead to a contamination by other microtubule-dependent motors with a similar size to CENP-E. While the initial description of CENP-E motility had remained questionable, Wood et al. showed that CENP-E is a slow plus end-directed motor using recombinant *Xenopus* CENP-E motor domain (Wood et al., 1997). The minus end-directed motility initially co-purified with CENP-E was most likely from contaminating cytoplasmic dynein, which also localizes to the kinetochore surface together with CENP-E. It is generally accepted that a kinesin with its motor domain at N-terminus powers movement toward microtubule plus ends. Therefore, it would have been a big surprise if CENP-E was truly responsible for the minus end-directed motility. However, controversy on the directionality of CENP-E motility continued for some time, as the native CENP-E purified from mitotic HeLa cells was once more reported not to support any motility (DeLuca et al., 2001).

Recent structural studies on CENP-E motor domain also support the plus end-directed motility of CENP-E. Motor domain of human CENP-E (aa 1-342) has been crystallized at 2.5 Å resolution (Garcia-Saez et al., 2004), revealing its neck linker
docked to the motor core as found in other kinesins with a plus end-directed motility (Kikkawa, 2008). Atomic models of a CENP-E-microtubule complex derived by fitting the crystal structure into the cryo-electron microscopy density map further confirmed the preferential position of the neck linker toward the microtubule plus end (Neumann et al., 2006). Interestingly, despite considerable functional differences, the binding interface of the CENP-E motor to microtubules was found to share some similarity with the fast monomeric kinesin Kif1a (Kinesin-3) and the minus end-directed kinesin Ncd (Kinesin-14). However, due to the disordered loops not seen in the crystal structure (L1 and L10), image reconstruction into the electron density map was not clear enough to distinguish between the up and down orientations when CENP-E is docked onto the microtubule. Additionally, as it is the monomeric motor construct containing only a short neck linker without any of coiled-coil sequence that was used in the study, interesting information on the CENP-E-specific neck and loops in regard to their interaction with microtubule is missing. Further characterization on CENP-E-microtubule interface at higher resolution using dimeric motor construct under different nucleotide conditions would be extremely valuable to understand the mechanism of CENP-E motility and its functions during mitosis.

1.6 CENP-E as an essential component for stable microtubule capture at kinetochore

Given that CENP-E is a microtubule-dependent motor, it is not surprising to find CENP-E as one of the components directly responsible for stable capture of kinetochore by spindle microtubules. In various cell types and organisms, removal or inhibition of
CENP-E invariably led to failure in metaphase chromosome alignment and problems in kinetochore-microtubule attachment. Suppression of protein synthesis by antisense oligonucleotides (Yao et al., 2000) or RNA interference (Tanudji et al., 2004), and inhibition of CENP-E recruitment to kinetochore by antibody microinjection (McEwen et al., 2001; Schaar et al., 1997) in human cells all lead to problems in chromosome congression. When CENP-E is removed from the kinetochore, tension between sister chromatids, as manifested by interkinetochore distance, is significantly reduced, and a few chromosomes are found close to the spindle poles (McEwen et al., 2001; Putkey et al., 2002; Yao et al., 2000).

Germline deletion of the *Drosophila* CENP-E homolog, CENP-meta, leads to lethality in early fly embryos resulting from defects in metaphase chromosome alignment, and, consequently, chromosome missegregation and aneuploidy (Yucel et al., 2000). Complete genetic knock-out of CENP-E is also lethal in mouse, with no embryos surviving passed implantation. When cultured at the blastocyst stage (E3.5), CENP-E null embryos displayed various abnormal mitotic figures, predominantly misaligned chromosomes clustered around spindle poles (Putkey et al., 2002). Without CENP-E, one or few chromosomes are found abnormally close to the spindle poles in primary murine embryo fibroblasts (MEFs), and this has been considered as a typical “CENP-E phenotype” (Putkey et al., 2002; Weaver et al., 2003). Despite the dense microtubule population near spindle poles, those centrophilic chromosomes lack associated kinetochore microtubules and appear to be virtually stuck without exhibiting typical oscillatory movement during chromosome congression. Serial section electron
microscopy revealed that the kinetochores of those chromosomes are juxtaposed to a centriole within a distance of 430 nm, and the chromosome is often V-shaped with its arms dragging away from the spindle pole (Putkey et al., 2002). This strongly suggests the presence of dominating poleward force on few CENP-E null kinetochores, consistent with the observation that inhibition of CENP-E does not affect the dynein-mediated, fast poleward chromosome motion along a laterally-attached, single astral microtubule (Rieder and Alexander, 1990; Yang et al., 2007). Removal of CENP-E from the kinetochore also reduces the number of microtubules bound by each kinetochore on the chromosomes that are bioriented (McEwen et al., 2001; Putkey et al., 2002). Overall, the combined data suggest that CENP-E is essential for chromosome alignment by making a stable kinetochore attachment to spindle microtubules and by providing an anti-poleward force to drive the chromosome movement toward the metaphase plate.

Immunodepletion of CENP-E or inhibitory antibody addition in Xenopus extracts causes massive chromosome misalignment, demonstrating again that CENP-E is essential for metaphase chromosome alignment (Wood et al., 1997). In the Xenopus extracts, artificial chromosomes made by plasmid DNA-coated beads are sufficient to drive bipolar spindle assembly (Heald et al., 1996). Taking advantage of this unique opportunity to study mitotic spindle formation in the absence of centrosomes and kinetochores, it was further shown that inhibition of CENP-E is specific to kinetochores as CENP-E antibody did not affect bipolar spindle assembly around those chromatin beads (Wood et al., 1997). Interestingly, alignment defects from CENP-E depletion in Xenopus extracts seem to be much stronger than those of most mammalian cells where,
without CENP-E, only one or a few chromosomes are displaced from the metaphase plate. The difference may be attributed to a special requirement of CENP-E motor activity for maintaining a dynamic linkage between kinetochore to microtubules in meiosis, where spindle dynamics are predominantly manifested as rapid microtubule poleward flux (Rogers et al., 2005).

Recently, CENP-E has been proposed to facilitate chromosome congression by powering movement of a mono-oriented chromosome pair toward the spindle equator by attachment to, and translocation along, the kinetochore fiber of another chromosome pair that had already bi-oriented (Kapoor et al., 2006). The evidence for this came from experiments using reversible chemical inhibitors to enrich syntelic chromosomes, which, after inhibitor washout, would eventually be corrected by disassembly of kinetochore fibers, coupled with chromosome movement to the pole (Lampson et al., 2004). With combined use of CENP-E siRNA and the same chemical inhibitors, Kapoor et al. have shown that without CENP-E chromosomes still remain close to the pole after drug removal, thereby concluding that CENP-E is responsible for the transport of monooriented chromosomes to the metaphase plate. However, as CENP-E inhibition alone would give rise to the same typical “CENP-E phenotype” regardless of complicated treatment scheme of chemical inhibitors, it cannot be simply reasoned that CENP-E is required for this congression mechanism of drug-derived monooriented chromosomes. Nevertheless, the Kapoor model quite nicely explains why inhibition of CENP-E results in few chromosomes positioned aberrantly close to the spindle poles, and further opens up important questions regarding the motor properties of CENP-E.
1.7 Role of CENP-E in the mitotic checkpoint

Besides its apparent role at the kinetochore-microtubule interface, CENP-E has also been implicated in the signaling cascade of the mitotic checkpoint. The mitotic checkpoint ensures faithful chromosome segregation into daughter cells by producing a “wait anaphase” signal from unattached kinetochores until all chromosomes are properly attached by spindle microtubules. The mitotic checkpoint inhibits Cdc20-activated anaphase-promoting complex/cyclosome (APC/C), an E3 ligase, which targets key mitotic regulators, cyclin B and securin, for ubiquitin-mediated degradation. The molecules involved in the mitotic checkpoint are conserved from yeast to human, and these include Mad1, Mad2, Mad3/BubR1, Bub1, Bub3, and Mps1 (Musacchio and Salmon, 2007).

CENP-E was first reported as an essential component for establishment and maintenance of the mitotic checkpoint from the immunodepletion and inhibitory antibody addition experiments in *Xenopus* egg extracts. CENP-E depleted extracts are unable to produce a high level of Cdk1 activity in response to disassembly of spindle microtubules, accompanied by loss of Mad1 and Mad2 from unattached kinetochores (Abrieu et al., 2000). Regarding its function in the mitotic checkpoint, CENP-E is best known as a binding partner of the checkpoint kinase BubR1. Initially from yeast two-hybrid screen using a C-terminus kinetochore targeting domain of human CENP-E (Chan et al., 1998), CENP-E has been shown to interact with BubR1 in human tissue cultures as well as in *Xenopus* extracts (Mao et al., 2003; Yao et al., 2000). When reconstituted in vitro, recombinant CENP-E and BubR1 form a stoichiometric complex, and the presence of
CENP-E greatly enhances the kinase activity of BubR1 toward an exogenous substrate histone H1 or BubR1 itself (Mao et al., 2003; Weaver et al., 2003). Interestingly, addition of microtubules resulted in a ternary complex consisted of BubR1, CENP-E and microtubules, and the presence of microtubules, presumably captured by CENP-E, silences the BubR1 kinase activity (Mao et al., 2005). Based on these findings, CENP-E has been proposed to be a “cyclin-like” activator of BubR1 kinase and also a signal transducing linker that senses the status of microtubule capture at kinetochores to turn off the “wait anaphase” signal produced by BubR1.

The role of CENP-E in BubR1-dependent mitotic checkpoint signaling is further supported by a weakened checkpoint in CENP-E depleted primary MEFs, which permit to enter anaphase even in the presence of few unattached kinetochores (Weaver et al., 2003). With reduced recruitment of BubR1 to the kinetochore, a dramatic increase in the number of polar chromosomes is found in anaphases of CENP-E depleted MEFs, which then leads to high rates of whole chromosome missegregation. As seen from complete loss of CENP-E, CENP-E heterozygous cells also rapidly develop aneuploidy compared to wild-type (Weaver et al., 2007). Having mitosis-specific, random chromosome segregation errors in the absence of DNA damage or growth defects, CENP-E heterozygous mice have been studied as an animal model for investigating the contribution of aneuploidy in tumorigenesis (Weaver et al., 2007).

The fact that CENP-E is needed to protect the loss of one or few chromosomes at every cell division seems consistent with its role in the mitotic checkpoint. However, careful distinction has to be made between the aneuploidy resulting from checkpoint
defects versus one from failures to make proper microtubule attachment. In human cells, depletion of CENP-E does not accelerate mitosis unlike cells depleted of Mad2 or BubR1 (Meraldi et al., 2004). Instead, reduction of CENP-E leads to a chronic mitotic arrest caused by a few misaligned chromosomes positioned near at the spindle poles (Yao et al., 2000), suggesting that in human cells the checkpoint machinery is functional without CENP-E. CENP-E null MEFs are also able to arrest in mitosis when all the kinetochores are unattached in the presence of microtubule depolymerizing drug (Weaver et al., 2003). An argument on a weakened checkpoint in a CENP-E null mouse is nonetheless tempered by the reality that rodent cells are naturally resistant to spindle poisons (Kung et al., 1990; Midgley et al., 1959; Orsini and Pansky, 1952) and tend to spend less time, slipping through mitosis relatively easily when mitosis is delayed by the checkpoint (Rieder and Maiato, 2004). Overall, whether CENP-E is a *bona fide* checkpoint protein seems to depend on the BubR1 kinase activity that is possibly involved in formation of the APC/C$^{Cdc20}$ inhibitors and robust signal amplification, especially when only few kinetochores are signaling.

Whether the kinase activity of BubR1 is required for the checkpoint, however, is a matter of controversy. In some experimental settings, BubR1 kinase activity seems to be dispensable for inhibiting APC/C$^{Cdc20}$. Earlier experiment in *Xenopus* egg extracts has reported that the kinase activity of BubR1 is not required for maintaining high Cdk1 activity in response to spindle damage (Chen, 2002). Similarly, kinase-dead BubR1 as well as the BubR1 fragment lacking kinase domain are shown to inhibit APC/C$^{Cdc20}$ with equal efficiency as the wild-type BubR1 in an in vitro ubiquitination assay (Tang et al.,
An increasing number of reports now indicate that BubR1 kinase might have an additional role in microtubule attachment at kinetochores (Harris et al., 2005; Lampson and Kapoor, 2005; Zhang et al., 2007). The microtubule plus end-binding protein adenomatous polyposis coli (APC) has been shown to be phosphorylated by BubR1, somehow contributing to kinetochore-microtubule interaction (Kaplan et al., 2001; Zhang et al., 2007). However, how BubR1 kinase activity contributes to mitotic checkpoint signaling and kinetochore microtubule attachment still remain largely unexplored, and identifying its downstream substrates would be the obvious next step to pursue. Furthermore, how CENP-E binding to BubR1 activates its kinase activity and how microtubule capture by the CENP-E motor domain transduces the signal through the very long, discontinuous coiled-coil (Kim et al., 2008) and silences BubR1 kinase activity at the opposite end of the molecule await future structural studies.

1.8 Post-translational modifications of CENP-E

Post-translational modification regulates the properties of proteins after synthesis from their RNA transcripts. Due to its reversible nature (protein prenylation and protein degradation are a few of the exceptional, irreversible modifications), protein modifications are widely used in modulating protein location, activity, interaction and its abundance, in response to various environmental cues. Cell cycle progression is one great example that is tightly coordinated by post-translational modifications of key targets, namely phosphorylations by cyclin-dependent kinases (Cdks), whose activities are controlled by ubiquitin-mediated protein degradation of cyclins and Cdk inhibitors. Being a large protein, CENP-E is subjected to a battery of post-translational modifications
including phosphorylation, farnesylation, SUMO-modification, and ubiquitination. The following overview summarizes the current knowledge on the post-translational modifications of CENP-E and how they may regulate the function of CENP-E during mitosis.

1.8.1 Phosphorylation

Identified from mass spectrometry-based phosphoproteome analysis on the human mitotic spindle, CENP-E contains at least 12 residues known to be phosphorylated in vivo (Figure 3.1) (Dephoure et al., 2008; Nousiainen et al., 2006; Zecevic et al., 1998). Among those, 6 sites reside in the C-terminus tail of CENP-E, in which the last 99 amino acids of CENP-E were shown to be necessary and sufficient for binding microtubules in ATP-independent manner (Liao et al., 1994). Surprisingly, phosphorylation of the CENP-E tail by Cdk1 was able to abolish the microtubule binding through the C-terminus of CENP-E (Liao et al., 1994). Having overlapping substrate specificity with Cdk1 and reported to be recruited to the kinetochores during mitosis, MAP kinase is also implicated in phosphorylation of the same sites on the C-terminus of CENP-E (Zecevic et al., 1998).

Unlike other conventional microtubule-associated proteins (MAPs), binding to microtubules through this secondary microtubule binding domain of CENP-E does not seem to involve an electrostatic interaction. The binding has been demonstrated to be resistant to salt extraction of up to 2 M NaCl and insensitive to microtubules treated with subtilisin, a bacterial protease that selectively cleaves the acidic C-termini of α- and β-tubulin (Liao et al., 1994). Although previous studies have speculated that the phospho-regulation of microtubule cross-linking activity of CENP-E would be important for anti-
parallel bundling of microtubules at spindle midzone and anaphase spindle elongation, whether CENP-E functions in later stages of mitosis is still unclear. Moreover, the significance of the secondary microtubule binding domain present in the C-terminus of CENP-E remains to be determined.

Recently, phosphorylation of the C-terminal CENP-E tail by Mps1 or Cdk1, but not by MAP kinase, has been shown to activate the motor activity by relieving the auto-inhibition by the CENP-E tail (Espeut et al., 2008). Regulation of a kinesin by adopting a folded conformation through interaction between the motor catalytic core and the cargo-binding tail has been established in conventional kinesins (Kinesin-1) (Cai et al., 2007; Friedman and Vale, 1999; Hackney et al., 1992). Different from Kinesin-1, however, CENP-E does not seem to undergo a dramatic conformational change, as only a slight change in sedimentation coefficient occurs upon phosphorylation in vitro (Espeut et al., 2008). As the inhibitory tail region of CENP-E differs from its kinetochore targeting domain, the relief mechanism of CENP-E auto-inhibition may not simply involve a binding to its cargo, the kinetochore. However, important information on the sites of phosphorylation by Mps1 or Cdk1 in the C-terminus of CENP-E has not yet been reported. Phospho-mapping in the CENP-E tail and the functional studies using phospho-mutants in living cells should be undertaken to test the in vivo significance of those phosphorylation events by Mps1, Cdk1, or other mitotic kinases. Also importantly, evaluating other phosphorylation sites that are scattered throughout CENP-E would help to understand how this essential kinetochore motor is regulated during mitosis.
1.8.2 Farnesylation

Farnesylation is a relatively rare post-translational modification, catalyzed by the protein farnesyl transferase, that involves an addition of a 15-carbon isoprenoid lipid moiety to the cysteine residue within a C-terminal CaaX motif (Zhang and Casey, 1996). The fact that mutations in Ras are commonly found in tumors and the farnesylation is critical for transforming activity of Ras (Kato et al., 1992), has made farnesyl transferase inhibitor (FTI) an attractive anti-cancer agent. Unexpectedly, treating human cells with FTI causes accumulation of cells in G2/M phase, suggesting the presence of previously uncharacterized farnesyl substrates involved in mitotic progression (Ashar et al., 2000; Crespo et al., 2001). A search for mitotic proteins containing a conserved CaaX motif at their C-termini has led to the finding that CENP-E and CENP-F are farnesylated in human cells (Ashar et al., 2000; Hussein and Taylor, 2002).

Although the significance of CENP-E farnesylation and its processing still remains unclear, FTI treatment has been shown to deplete CENP-E from metaphase kinetochores, causing chromosome misalignment with few polar chromosomes and prolonged prometaphase (Schafer-Hales et al., 2007). Furthermore, blocking farnesylation of CENP-E has been reported to affect its association with microtubules. A significantly reduced amount of CENP-E is found in the microtubule pellet fraction from FTI-treated cells after two rounds of polymerization and depolymerization (Ashar et al., 2000). It is worthy of note that the assay was performed in the presence of an ATP-regenerating system, so in this condition the motor domain of CENP-E would not have a strong affinity to microtubules. Given that the farnesylation occurs at the very C-terminus
of CENP-E, it is likely that adding a farnesyl group would affect the secondary microtubule binding domain present in the CENP-E tail. A hypothetical structural docking study suggests that the farnesyl group could potentially bind to hydrophobic patches on the microtubule lattice (Schafer-Hales et al., 2007). The proposed hydrophobic nature of interaction seems consistent with the previous observation that microtubule binding through the C-terminus of CENP-E is resistant to high salt extraction (Liao et al., 1994). Classically, farnesylation is used in targeting proteins to the cellular membranes or in specifying protein-protein interaction. Although unlikely, it is not known whether CENP-E associates with membrane structures through its farnesyl moiety added in the C-terminus. It remains to be determined through which specialized farnesyl-binding partners CENP-E is recruited to the kinetochore and how farnesylation affects microtubule affinity in the secondary microtubule binding domain of CENP-E.

1.8.3. SUMO-modification and ubiquitination

Unlike the small molecule modifications such as phosphorylation, acetylation, methylation etc., small ubiquitin-like modifier (SUMO) modification involves covalent attachment of entire protein moiety (Ulrich, 2008). Distantly related to ubiquitin, SUMO was first identified as a small protein that is covalently linked to RanGAP1 (Ran GTPase-activating protein) and that is required for it to be targeted to RanBP2 at the nuclear pore complex (Mahajan et al., 1997; Matunis et al., 1996). In contrast to ubiquitination which prominently initiates protein degradation by the 26S proteosome, reversible SUMO modification regulates protein localization and protein-protein interactions or acts as an ubiquitin antagonist by blocking other lysine-dependent modifications (Hay, 2005).
Global inhibition of SUMO modification by overexpressing SENP2, a SUMO-specific isopeptidase which reverses a SUMO conjugation from targets, has shown to cause a prometaphase arrest with few misaligned chromosomes near at the spindle poles (Zhang et al., 2008). Consistent with this typical “CENP-E phenotype” in SENP2 expressing cells, CENP-E turned out to be the one missing from the kinetochores, among other centromere/kinetochore components investigated. CENP-E seems to directly interact with polymers of SUMO-2, but not with monomeric forms, and its kinetochore targeting was shown to depend on the internal SUMO-interacting motif in CENP-E (Zhang et al., 2008). Although CENP-E itself is also subjected to covalent SUMO-2/3 modification and so are CENP-E interacting partners BubR1 and Nuf2, whether SUMO modifications of these proteins are physiologically relevant in recruiting CENP-E to the kinetochore has not been tested.

In addition to the SUMO modification, some aspects of CENP-E recruitment to kinetochore may also be regulated by ubiquitination. Microinjecting recombinant Cdc34/Ubc3, an E2 ubiquitin-conjugating enzyme, but not the enzymatically inactive form, into PtK1 cells at early mitotic stages has been reported to cause prometaphase arrest with misaligned chromosomes remaining close to the spindle poles (Topper et al., 2001). Interestingly, elevating the level of Cdc34 specifically inhibits association of CENP-E with kinetochores, and this is not because of the premature degradation of CENP-E. Cdc34 is the E2 enzyme for SCF (Skp1-Cul1-F-box-protein) family ubiquitin ligases that maintains a stable interaction to the SCF complex throughout cell cycle (Mathias et al., 1998). However, whether Cdc34 interacts directly with CENP-E or
whether CENP-E is targeted by Cdc34 to be destroyed by the SCF complex at the end of mitosis is not known. Although CENP-E was implicated in interacting with Skp1 via central coiled-coil region of CENP-E for degradation at the spindle midzone (Liu et al., 2006), the biochemical evidence for such an interaction was poorly demonstrated, and the significance of this interaction still remains open to question.

The dire consequences of missing CENP-E from kinetochores are now clear. However, important questions, including 1) the key roles of this essential kinetochore kinesin on the motor properties of CENP-E, 2) by which mechanism CENP-E contributes to the dynamic linkage of kinetochores to spindle microtubules, and 3) how its motility is regulated in cells, are unanswered. Here I have answered aspects of each of these questions.
Chapter 2: CENP-E combines a slow, processive motor and a flexible coiled-coil to produce an essential motile kinetochore tether

2.1 Introduction

Mitotic chromosome motions are driven by microtubule-based motors positioned at kinetochores as well as the dynamic properties of spindle microtubules. CENP-E (Centromere-associated protein E) and cytoplasmic dynein are the two microtubule motors in metazoans known to localize at kinetochores (Pfarr et al., 1990; Yen et al., 1992). Each uses chemical energy released from ATP hydrolysis to direct movement toward plus or minus ends of microtubules, respectively (Schroer et al., 1989; Wood et al., 1997). MCAK (Mitotic centromere-associated kinesin), a nonmotile kinesin, is also found at the inner centromere and its microtubule depolymerizing activity plays a role in error correction so as to generate proper kinetochore microtubule attachment (Desai et al., 1999b; Ohi et al., 2003). Besides the energy-consuming motors, however, energy stored in the microtubule lattice from cleavage of GTP during microtubule assembly can generate sufficient force to power aspects of chromosome movement (Coue et al., 1991; Grishchuk et al., 2005; Inoue and Salmon, 1995; Koshland et al., 1988). By simply maintaining a linkage with spindle microtubules, kinetochores can exploit the energy liberated from the microtubule lattice during disassembly to drive chromosome movement, independent of a power stroke generated by motors and additional chemical energy (Howard and Hyman, 2003).
An important unsolved question is how the kinetochore holds onto both growing and shrinking microtubules. Using purified chromosomes attached to dynamic microtubules, either pan kinesin antibodies or those specific for CENP-E were shown to inhibit microtubule depolymerization-dependent motion of chromosomes in vitro (Lombillo et al., 1995a). Moreover, beads coated with kinesin were able to follow the end of disassembling microtubules (Lombillo et al., 1995b), suggesting a role of kinesin family members in coupling kinetochores to dynamic microtubules. Discovery of the ring structure of the yeast Dam1/DASH complex (Miranda et al., 2005; Westermann et al., 2005) has introduced such rings as plausible coupling devices for chromosome attachment and movement. In vitro, the ring complex can move processively on depolymerizing microtubule ends (Westermann et al., 2006), and harness microtubule dynamics to produce force (Asbury et al., 2006). The Dam1/DASH complex, however, has not been found outside fungi. Conversely, motor enzymes, such as dynein, CENP-E, and MCAK are missing from yeast kinetochores (McIntosh, 2005).

Initially found to be a kinesin family member that localizes to kinetochores (Yen et al., 1992), CENP-E is a mitosis-specific kinesin with a cyclin-like accumulation and degradation, reaching its peak during G2 and early M, followed by rapid degradation during completion of mitosis (Brown et al., 1994). An essential kinetochore component whose loss leads to high rates of whole chromosome missegregation (Weaver et al., 2003), CENP-E is one of the components directly responsible for stable capture of spindle microtubules by kinetochores (Putkey et al., 2002; Schaar et al., 1997; Wood et al., 1997; Yao et al., 2000). Inhibition or removal of CENP-E not only leads to failure of metaphase chromosome alignment resulting in unattached chromosomes that are
abnormally close to a spindle pole, it also reduces the number of microtubules bound by
kinetochores even on chromosomes that are bi-oriented (McEwen et al., 2001; Putkey et
al., 2002; Weaver et al., 2003). Most recently, CENP-E has been proposed to facilitate
chromosome congression by powering movement of a mono-oriented chromosome pair
toward the spindle equator by attachment to, and translocation along, the kinetochore
fiber of another chromosome pair that had already bi-oriented (Kapoor et al., 2006).
Although simple microtubule gliding assays with the CENP-E motor domain have shown
it to be a plus end-directed motor (Wood et al., 1997), the motor properties of CENP-E
and the mechanism by which CENP-E contributes to kinetochore attachment to spindle
microtubules are not known.

Using purified recombinant CENP-E, I found that CENP-E is a very slow, highly
processive motor that maintains microtubule attachment for long periods with a highly
flexible 230 nm long contour length. Metaphase chromosome alignment is shown to
require the plus end-directed motor activity, rather than simple microtubule binding by
CENP-E. Based on our findings, I propose that CENP-E combines a slow, processive
motor and a highly flexible coiled-coil not only to power chromosome congression, but
also to provide an essential, motile kinetochore tether.

2.2 Purification of recombinant full-length Xenopus CENP-E

To assess the biochemical and biophysical properties of CENP-E, I purified
untagged, 340 kDa recombinant CENP-E from insect cells infected with baculovirus
expressing full-length Xenopus CENP-E cDNA. The protein was purified using two-step
ion exchange chromatography with a final Superose 6 gel filtration step from which
CENP-E was excluded. This purification yielded CENP-E very close to homogeneity as shown both in coomassie and silver-stained gels (Figure 2.1).

2.3 230 nm-long and highly flexible coiled-coil structure of CENP-E

Immediately after purification, the CENP-E was adsorbed to mica and individual molecules were visualized by electron microscopy using the quick-freeze, deep-etch technique and platinum replication (Heuser, 1989). Consistent with its predicted long α-helical coiled-coil domain, CENP-E was seen to be highly elongated (Figure 2.2). The two kinesin motor heads, as indicated by arrows, were found separated from by a long coiled-coil, followed by globular tails (Figure 2.3A), directly demonstrating CENP-E is a homodimer. The average contour length of CENP-E was measured to be 230 nm ± 25 nm (n = 20) (Figure 2.3B), which is almost three times longer than the overall length of conventional kinesin (Hirokawa et al., 1989).

A striking feature of CENP-E was the high flexibility of the α-helical coiled-coil as inferred from the wide variety of configurations that it adopted. Conventional kinesins mainly exist as either extended or folded conformations with a hinge located in the middle of the coiled-coil (Hirokawa et al., 1989). In contrast, CENP-E molecules were never uniformly straight, but rather displayed a myriad of diverse conformations, none of which could be categorized by frequency as preferred conformations. Throughout the long coiled-coil domain, CENP-E seemed to contain multiple hinges and local distortions that accommodated sharp bends. Analysis of CENP-E sequence showed that its predicted coiled-coil structure is disrupted more than 20 times, almost always by segments containing proline or glycine residues (Figure 2.3C). Although it is unknown whether all
the predicted coiled-coil disruptors actually loop out from the coiled-coil axis, the discontinuities in the coiled-coil are the mostly likely source of the high flexibility of CENP-E.

2.4 Hydrodynamic properties of CENP-E

Measurement of hydrodynamic properties of full-length CENP-E in solution confirmed a highly elongated, flexible coiled-coil domain. A native molecular weight of 590 kDa for recombinant *Xenopus* CENP-E was calculated from the measured values determined by sucrose gradient sedimentation and gel filtration, respectively, of sedimentation coefficient (8.6 S) and Stokes radius (16 nm) (Figure 2.4A, B). This value was close to the predicted molecular weight of a CENP-E homodimer (680 kDa), consistent with our evidence from electron microscopy that CENP-E is dimeric. Calculating frictional ratio ($f/f_o$) using the mass of the dimer (680 kDa) and the measured Stokes radius gave a value of 2.8 (Figure 2.4C). If CENP-E were a rigid prolate ellipsoid with axial ratio of ~100 (230 nm contour length/ 2 nm coiled-coil diameter), the $f/f_o$ would be > 4. With the $f/f_o$ of 2.8, assuming no hydration and a prolate ellipsoid shape, the maximum possible axial ratio (a/b) of the CENP-E dimer was calculated (Harding and Colfen, 1995) to be 45, with an estimated length of 148 nm, which is obviously much shorter than CENP-E’s actual contour length as determined by electron microscopy. Therefore, CENP-E is substantially elongated on average, but not rigidly extended, consistent with the high flexibility observed by electron microscopy.
2.5 CENP-E motility assay with a single molecule imaging

To characterize the motor properties of CENP-E, I purified the motor plus neck domain of *Xenopus* CENP-E (aa 1-473) with GFP and a hexahistidine tag fused at the C-terminus (CE473-GFP thereafter) (Figure 2.6A). The presumed dimeric state of this CENP-E fragment was tested by measuring hydrodynamic parameters in solution. A sedimentation coefficient (6 S) and Stokes radius (5.2 nm) were obtained by sucrose gradient sedimentation and gel filtration chromatography, respectively (Figure 2.5A, B). This produced a calculated native molecular weight (Siegel and Monty, 1966) for CE473-GFP of ~130 kDa, close to the predicted molecular weight of the dimer (160 kDa), demonstrating that the preponderance of CENP-E motor domain exists as a dimer in solution.

I also verified CE473-GFP to be dimeric by using total internal reflection fluorescence (TIRF) microscopy to measure fluorescence intensity of single molecules immobilized on coverslips. Freshly prepared protein was nonspecifically adhered on a coverslip surface, and the fluorescence intensity of CE473-GFP spots was measured and compared to that of K560-GFP, the well-characterized dimeric motor head of human Kinesin-1 (Case et al., 1997). The fluorescence intensity distributions between CE473-GFP (n = 432) and K560-GFP (n = 347) were indistinguishable (Figure 2.5D), confirming that most imaged CENP-E spots were dimers. When the fluorescent CENP-E molecules were followed over time to examine their photobleaching behavior, most CE473-GFP and K560-GFP spots disappeared in either two steps or a single step (Figure 2.5E), as expected if each spot consisted of either two GFPs or a single GFP. Further, the
initial fluorescence intensity of moving spots was measured to confirm that the majority of moving CENP-E was indeed dimeric (Figure 2.5F). Therefore, both the hydrodynamic measurements in solution and the fluorescence analysis of single molecules viewed microscopically demonstrate that CE473-GFP is dimeric.

2.6 CENP-E is a slow, processive motor that maintains microtubule attachment for long periods

I then tested the processivity of CENP-E in vitro by directly observing single molecules of CE473-GFP moving along microtubules using TIRF microscopy. X-rhodamine-labeled GMPCPP microtubules were immobilized on a coverslip surface using an anti-tubulin antibody, and subsequently a low concentration (0.5 - 1 nM) of CE473-GFP was introduced into the flow chamber (Figure 2.6B). CENP-E single molecules moved processively and unidirectionally along microtubules (Figure 2.6C, D) with a median velocity of 8 nm/sec (n = 320, 4 independent preparations) (Figure 2.6E). Contemporaneous measurement of K560-GFP, on the other hand, yielded an average velocity of 330 nm/sec, consistent with speeds reported by others (Friedman and Vale, 1999). Substantial variation in speed (ranging from 1.7 to 48 nm/sec) was observed for CENP-E. However, this was not due to heterogeneity in motor activity among different protein preparations, as large variation in the velocity was consistently observed in the assays performed on the same day. Furthermore, often the velocity of movement differed by a factor of two even between the molecules running on same microtubule (compare particles 1 and 2 in Figure 2.6C).
### 2.7 CENP-E motility contains one-dimensional diffusion

Processive movement of CENP-E motor single molecules was not smooth, and the CENP-E changed instantaneous velocity with frequent pauses within a single run (see kymographs in Figure 2.6D). Frequent changes in instantaneous velocity with large variations in the speed and even reversals in direction observed when frames were taken more frequently in our single molecule assays (Figure 2.7B, C), suggested that CENP-E motility has a one-dimensional diffusion component. A mean square displacement (MSD) plot of CENP-E was well fitted with biased Brownian movement (Figure 2.6F). Polynomial regression to MSD \( \rho = \nu^2 t^2 + 2Dt \) \( (\nu, \text{mean drift velocity}; D, \text{diffusion coefficient}) \) produced a mean velocity of \( 9.7 \pm 0.6 \) nm/sec \( (n = 90) \) and a diffusion coefficient of \( 690 \pm 94 \) nm\(^2\)/sec \( (n = 90) \), larger than expected from the fluctuation of a kinesin with an 8 nm step size and \( \sim 10 \) nm/sec speed \( (D \approx 80 \) nm\(^2\)/sec\); (Svoboda et al., 1994). Similar mean velocities and diffusion coefficient for CENP-E were found in the experiments performed using more frequent frame rates and quantum dot-labeled CENP-E motor domain (Figure 2.7D).

The presence of a diffusional component in motility has been reported for other microtubule-dependent motors (Furuta and Toyoshima, 2008; Helenius et al., 2006; Kwok et al., 2006; Okada and Hirokawa, 1999; Vale et al., 1989), and suggests that CENP-E contains an additional electrostatic binding mode within the head-neck domain, in addition to its force-generating strong microtubule binding state. This weak binding of CENP-E to a microtubule might prevent dissociation of CENP-E even when both motors are unbound, thus facilitating efficient reattachment. Consistent with this, CENP-E was
able to maintain microtubule attachment for long periods, producing remarkably processive movement despite its slow velocity. The run time, defined as the time interval between the start and the end of each processive run, was distributed exponentially and the average run time was determined by fitting the data into a cumulative distribution function. The average duration of CENP-E processive runs was $195 \pm 13$ sec ($= 3.3$ min) (Figure 2.6G), a value 50 times longer than the reported association time of K560-GFP single molecules (Friedman and Vale, 1999). The longest run we observed (shown in kymograph 2 of Figure 2.6 D) lasted > 20 min. The slow velocity and longer association time of CENP-E motor domain resulted in an average run length of $1.5 \pm 0.1$ μm (Figure 2.6H), which is comparable to the processivity of conventional kinesin. Therefore, unlike fast-moving organelle transport kinesins, CENP-E is a slow motor, but its high processivity maintains an interaction with a microtubule for long periods.

### 2.8 Full-length CENP-E is a slow, plus end-directed kinetochore motor

The characteristics of full-length CENP-E as a motor were determined using polarity-marked fluorescent microtubules and GFP tagged full-length CENP-E (366 kDa) tethered to a coverslip (Figure 2.8A). Directionality of CENP-E has been controversial over the years. Partial purification of CENP-E was initially reported to contain minus end-directed motility (Thrower et al., 1995), while later recombinant motor domain was shown to be a plus end-directed motor (Wood et al., 1997). It was also reported that the native CENP-E purified from mitotic HeLa cells did not support any motility (DeLuca et al., 2001). Here I unambiguously show that full-length CENP-E powers motility toward plus ends of microtubules, with microtubule gliding seen solely with the bright minus end.
leading. The average velocity of gliding was 30 ± 7.6 nm/sec (n=112) (Figure 2.8B). While the speed difference between the motor domain and the full-length CENP-E could reflect autoinhibition of the C-terminal tail binding to the motor domain as recently proposed by (Espeut et al., 2008), none of our electron micrographs (Figure 2.2) offered support for motor binding to the tail of CENP-E.

2.9 CENP-E motor activity is essential for metaphase chromosome alignment

It has been well established that CENP-E is essential for stable microtubule capture at the kinetochore and is required for chromosome alignment (Putkey et al., 2002; Schaar et al., 1997; Weaver et al., 2003; Wood et al., 1997; Yao et al., 2000). To test whether it is the motor activity of CENP-E that is essential for chromosome alignment, we used the Xenopus egg extract system in which mitotic spindles can be assembled in vitro. When added to Xenopus extracts prior to spindle assembly (Figure 2.8C), CENP-E-GFP localized to kinetochores and remained kinetochore-bound throughout metaphase (Figure 2.8D) and at least until the early stage of anaphase (not shown).

The contribution of CENP-E motor activity to chromosome alignment and its maintenance was tested by assembling spindles in CENP-E depleted Xenopus extracts that were supplemented with either wild-type CENP-E or motor-dead CENP-E proteins (Figure 2.9A, B). To make a motor-dead CENP-E, I introduced a single point mutation in the ATP-binding P loop (T91N mutation) in the highly conserved kinesin motor domain of CENP-E (Nakata and Hirokawa, 1995). This mutation provided a rigor binding to microtubules that cannot be dissociated even in the presence of ATP, and the motor
domain of CENP-E\textsuperscript{T91N} failed to support microtubule gliding even in the presence of saturating ATP concentrations (not shown). As reported previously (Wood et al., 1997), chromosomes failed to align properly in majority of bipolar spindles in CENP-E depleted extracts (Figure 2.9C). When wild-type CENP-E was added back into CENP-E depleted extracts prior to spindle assembly, chromosome alignment defects were rescued in part with the CENP-E localized to kinetochores (Figure 2.9C, D; Figure 2.10A). Although still bound to kinetochores, rigor CENP-E\textsuperscript{T91N}, however, failed to rescue chromosome alignment, with the chromosomes instead spread along the spindle axis and located close to the spindle poles (Figure 2.9C, D). Accumulation at the broad region of spindle poles in CENP-E\textsuperscript{T91N} added extracts (Figure 2.10A) presumably reflected initial rigor binding all along spindle microtubules followed by poleward transport by flux.

Thus, CENP-E motor activity rather than simple microtubule binding is essential for accurate chromosome congression. This conclusion is tempered by realization that in *Xenopus* extract spindles chromosomes are pre-positioned at the center (pre-congressed) during spindle assembly and the process of chromosome alignment may differ from the typical prometaphase congression in somatic cells (Sawin and Mitchison, 1991). Nevertheless, it is clear that the plus end-directed motility of CENP-E, at a minimum, is required for maintenance of chromosome alignment even in a system with fewer required chromosome movements for establishing an initial alignment. I believe that the strong misalignment phenotype from CENP-E depletion in *Xenopus* extracts (Figure 2.9C), in which fast microtubule poleward flux dominates forces acting on kinetochores (Maddox
et al., 2003a), further supports CENP-E as an essential component for powering plus end directed chromosome movement to counteract flux.

2.10 Discussion

Use of electron tomography has shown that the vertebrate kinetochore is composed of a fibrous protein network with multiple microtubule interactions (Dong et al., 2007). CENP-E is certainly part of the fibrous network for kinetochore attachment [as demonstrated with immunogold electron microscopy (Cooke et al., 1997; Yao et al., 1997)], with its 230 nm-length likely to be one of the longest fibers extending out from the kinetochore to capture microtubules. From my evidence it is now clear that the long coiled-coil of CENP-E could work advantageously for the initial capture of kinetochores to spindle microtubules by searching a large radial volume in cells (Figure 2.11E). After initial capture, combination of slow processive movement along with one-dimensional diffusion would yield translocation of CENP-E to the plus ends of microtubules, potentially damping dynamics there (Figure 2.11A). An important novel aspect is that the high flexibility would allow versatile configurations of CENP-E binding to microtubules, permitting kinetochore attachment to microtubules approaching from a wide range of angles (Figure 2.11D, F). This would be in contrast to the highly conserved Ndc80 complex, which has been implicated as a key non-motor microtubule binding component of kinetochores (Ciferri et al., 2007) and whose ~55 nm stiff rod (Wei et al., 2005) apparently binds with a consistent polarity at a fixed angle relative to the microtubule lattice (Cheeseman et al., 2006).
Finally, the 230 nm-long coiled-coil of CENP-E is long enough to cover the entire kinetochore region and could even reach over to kinetochore fibers of other chromosomes passing close by (Figure 2.11F). Our evidence supports that CENP-E, as a highly processive kinesin, is capable of driving the congression of a mono-oriented chromosome to the center along the neighboring kinetochore fibers (Kapoor et al., 2006). Indeed, the speed of microtubule gliding driven by multiple CENP-E molecules immobilized on a coverslip is similar to what has been observed for the velocities of kinetochore motility during chromosome congression in cells (Figure 2.8B) (Skibbens et al., 1993). Furthermore, the high flexibility of the CENP-E would allow multiple molecules to work together along the kinetochore fibers without forcing each other into unproductive conformations.

The CENP-E family kinesin (Kinesin-7) has a longer family-specific neck domain than conventional kinesins (Endow, 1999). I hypothesize that the longer neck and highly flexible, discontinuous coiled-coil domain of CENP-E are specialized for three roles during mitosis: efficient microtubule capture by kinetochores, towing mono-oriented chromosomes through viscous cytoplasm (especially those initially far from the spindle equator), and perhaps most importantly, as a motile kinetochore tether maintaining linkage to dynamic kinetochore microtubule plus ends. The last of these properties couples the slow, processive motility with one dimensional diffusion that together offer a molecular explanation for how relatively few CENP-E molecules (~50 dimers per human kinetochore – Brown et al., 1994) can sustain kinetochore attachment to individual, dynamic (growing and shrinking) microtubules without losing connection. Combined
with the highly flexible, 230 nm-long coiled-coil, and the slow plus end-directed motility with the ability of single molecules to remain microtubule bound for minutes, I propose that CENP-E is a part of the kinetochore ‘slip-clutch’ that is engaged on fluxing kinetochore microtubules (Maddox et al., 2003a). In effect, CENP-E combines a slow but processive motor, flexibility, reach, and stable microtubule binding to produce a motile, molecular Velcro at kinetochores (Figure 2.11).

2.11 Methods

2.11.1 Cloning, expression and purification of CENP-E

Full-length *Xenopus* CENP-E (340 kDa) and CENP-E-GFP (366 kDa) was expressed and purified from High Five cells (Invitrogen) as previously described (Abrieu et al., 2000), followed by a final purification with a Superose 6 column. To make rigor CENP-E, the Thr at residue 91 was mutated to Asn (ACG → AAC) using site-directed mutagenesis. Fresh proteins were used in all experiments with exception of the *Xenopus* extracts experiment.

cDNA encoding *Xenopus* CENP-E residues 1-473 fused at the C terminus to a GFP-6His tag was cloned into pET23d (Novagen), and protein expression was induced at 13°C for ~12 hr with 10 nM IPTG in Rosetta (DE3). Bacterial pellets were suspended in lysis buffer (25 mM K-PIPS [pH 6.8], 300 mM KCl, 40 mM Imidazole, 2 mM MgCl₂, 0.5 mM EGTA, 10 mM β-mercaptoethanol, 0.1 mM ATP, 1 mM PMSF and protease inhibitors (from cocktail tablet (Roche)) and lysed by sonication following the lysozyme treatment (1 mg/ml) on ice for 30 min. After centrifugation at 15,000 rpm (Sorvall, SA-600) for 30 min, the supernatant was incubated with Ni-NTA (Qiagen) for 1 hr at 4°C.
The protein was further purified by HiTrap SP HP (GE Healthcare) using 100 mM - 1M KCl gradient elution in 25 mM PIPES [pH 6.8], 2 mM MgCl₂, 1mM DTT, 0.1 mM ATP. The peak fraction from the SP column was then loaded onto Superose 6 10/300 (GE Healthcare) pre-equilibrated with the same buffer containing 100 mM KCl, and the peak fraction was used in the assay.

2.11.2 Sucrose gradient sedimentation and gel filtration chromatography

To measure the S value of CENP-E, 100 µl of protein was centrifuged through 2 ml of 5 – 40 % sucrose gradients at 50,000 rpm for 6 hr at 4 °C (TLS 55 rotor, Beckman). The sedimentation coefficient of CENP-E was determined by linear interpolation of a standard curve (R² = 0.96) using proteins of known S values (BSA, 4.6 S; aldolase, 7.3 S; catalase, 11.3 S; thyroglobulin, 19 S). To measure the Stokes radius of full-length CENP-E, Sephacryl S-500 HR (GE Healthcare) was packed into XK 26/70 column (GE Healthcare) calibrated with protein standards (myosin II, 20 nm; IgM, 12.5 nm; thyroglobulin 8.5 nm; catalase, 5.2 nm; aldolase, 4.8 nm) (R² = 0.99). 500 µl of partially purified protein was loaded, and CENP-E was detected by immunoblot. The partial specific volume (υ) of CENP-E was calculated to be 0.735 cm³/g from the average partial specific volumes of the individual amino acids as described by (Perkins, 1986). Native molecular weight and frictional ratio (f/f₀) were calculated as described (Siegel and Monty, 1966). The maximum possible axial ratio was calculated as described in Harding and Cölfen (1995) assuming no hydration and a prolate ellipsoid. The dimensions for CENP-E were calculated from the volume of a spherical mass of 680 kDa and the partial specific volume, with V = υ·M/A = (4/3) π a b², where M represents molecular weight, A represents Avogadro’s number (6.02 x 10²³), and a = 45b.
2.11.3 Single molecule assays

CENP-E single molecule assays were performed at room temperature using a digital TIRF imaging system described in detail in (Mc Adams et al., 2004). Briefly, this consisted of a TE2000-U microscope equipped with a custom-modified TIRF epi-illuminator and a 100X 1.45A TIRF objective (Nikon). Illumination for TIRF imaging of GFP and X-rhodamine was provided by the 488 and 568 nm lines of a 50mW KrAr laser, with individual lines selected by a polychromatic acousto-optic modulator (Neos Technologies). TIRF images using an evanescent field illumination depth of 150 nm were captured using a Cascade II EMCCD camera (Photometrics). Microscope automation, image acquisition and processing were performed using Metamorph Software (Molecular Devices). Flow cells were constructed with a slide and 22 x 22 mm square coverslip separated by two strips of double-stick tape. For measuring fluorescence intensity and photobleaching, ~0.5 nM of motors were incubated for 10 min. Fluorescence intensity was measured by integrating the signal of a fluorescent spot per frame and subtracting the background (integrated area = 49 pixel^2). For motility assays, a rat monoclonal tubulin antibody (50 µg/ml, Serotec) was incubated for 5 min, followed by BRB80 (80 mM K-PIPES [pH 6.8], 1 mM MgCl_2, 1 mM EGTA) wash, X-rhodamine-labeled GMPCPP microtubules for 10 min, and 0.5 mg/ml casein blocking solution for 10 min. Then, 0.5 ~ 1 nM of CENP-E motor in motility buffer with an oxygen scavenging system (BRB80, 1 mM DTT, 3 mM ATP, 4.5 mg/ml glucose, 0.2 mg/ml glucose oxidase, 35 ug/ml catalase) was flowed into the chamber. Frames were captured every 5 sec with 120 ms exposure (three frames with 40 ms exposure were averaged to increase the signal to noise ratio), and the duration of imaging was 5 min ~ 20 min, which results in a total
exposure of 7.2 ~ 28.8 sec. Events that lasted more than two frames (> 10 sec) were tracked and non-moving molecules were excluded from our analysis. CENP-E processive runs were analyzed by drawing kymographs with Metamorph, and for mean square displacement (MSD) analyses, the intensity centroid of moving CENP-E spots was tracked using Metamorph Track Object tool. Particle coordinates \( (x_n = x(n\Delta T), y_n = y(n\Delta T)) \) were used to calculate MSD \( \rho(n) = \rho(n\Delta T) = \sum_{i=0}^{N}(x_{i+n} - x_i)^2 / (N+1) + \sum_{i=0}^{N}(y_{i+n} - y_i)^2 / (N+1) \), where \( \Delta T \) is the data acquisition time interval (Qian et al., 1991). MSD was fitted with the equation for biased Brownian movement: MSD \( \rho = \nu^2 t^2 + 2Dt \) (\( D \), diffusion coefficient; \( \nu \), mean velocity).

2.11.4 Microtubule gliding assays

Microtubule gliding assays were performed as described (Wood et al., 1997) with use of GFP antibody to coat CENP-E-GFP onto a coverslip. Time-lapse image acquisition was done at room temperature using a Nikon Eclipse TE 300 inverted microscope with a 60X 1.4A objective and the images were captured with a CoolSNAP HQ camera (Photometrics) controlled by Metamorph.

2.11.5 Electron microscopy

Freshly prepared CENP-E was maintained on ice overnight and processed for freeze-drying the following morning. CENP-E proteins were adsorbed to a suspension of mica flakes, followed by freeze-drying and platinum replication (Heuser, 1989).

2.11.6 Spindle assembly, live imaging and immunofluorescence in Xenopus extract
The preparation of *Xenopus* CSF arrested egg extracts and the cycled spindle assembly *in vitro* was done as described (Desai et al., 1999a). For live imaging, ~5 ug/ml of X-rhodamine tubulin and 25 nM of recombinant CENP-E-GFP were added in the *Xenopus* extracts before spindle assembly, and squashed extract was imaged using spinning-disk confocal mounted on a TE2000e microscope (Nikon) with a 100X 1.4A objective (Nikon), and 2 x 2 binning as described in (Maddox et al., 2003b). CENP-E immunodepletion and immunofluorescence were performed as described (Abrieu et al., 2000). >95 % of CENP-E was consistently depleted using this method. For rescue experiments, either wild-type or rigor CENP-E$^{T91N}$ protein was added to CENP-E depleted extract at endogenous level (~25 nM) after completion of DNA replication.
Figure 2.1 Purification of recombinant full-length *Xenopus* CENP-E

(A) Purification scheme of recombinant full-length *Xenopus* CENP-E. Untagged full-length *Xenopus* CENP-E (340 kDa) was expressed in High Five insect cells using Bac-to-Bac baculovirus expression system, and was purified by two-step ion exchange and gel filtration chromatographies. (B and C) Cation exchange (HiTrap SP) and the following anion exchange (Source 15Q) steps enriched CENP-E over other proteins as shown in coomassie-stained 8% SDS-PAGE gels. (D) Final Superose 6 gel filtration excluded CENP-E from entering the column, eluting CENP-E at a void volume. This step separates CENP-E from majority of smaller proteins, yielding CENP-E very close to homogeneity. Approximately 100 µg of recombinant full-length CENP-E protein was consistently purified using this method from 100 ml of cultured High Five cells. Lys, Lysates; FT, flow-through.
Figure 2.2 Electron micrographs of individual CENP-E molecules

Scale bar, 100 nm.
**Figure 2.3** CENP-E is a highly flexible, dimeric kinesin with a 230-nm discontinuous coiled-coil

(A) Electron micrographs of individual CENP-E molecules. Two motor heads are clearly visible as indicated by yellow arrows. Scale bar, 100 nm. (B) Length distribution of CENP-E. Contour lengths of molecules were measured, and the average length of CENP-E was 230 ± 25 nm (mean ± s.d., n=20). (C) Coiled-coil prediction of *Xenopus* CENP-E. Coiled-coil scores were generated using software Protean (DNAsstar) and graphed below the amino acid scale bar. The number 1.3 is the default value indicating the minimum score for known coiled-coils, and resulting predicted coiled-coil domains are shown as solid orange rectangles.
Figure 2.4 Hydrodynamic properties of CENP-E

(A) Purified CENP-E was sedimented through a 5% - 40% sucrose gradient and fractions were visualized by silver-staining. S value of CENP-E was determined to be 8.6 S from a standard curve with protein standards of known S values. (B) Partially purified CENP-E was passed through a Sephacryl S-500 gel filtration column calibrated with protein standards of known Stokes radii. Stokes radius of CENP-E was 16 nm. The results are summarized in (C).
Figure 2.5 CENP-E motility assays with a single molecule imaging

CE473-GFP forms a dimer. (A, B) *Xenopus* CENP-E motor (aa 1-473)-GFP (80 kDa) is a dimer in solution. S value (6 Svedberg) and Stokes radius (5.2 nm) were measured by sucrose gradient sedimentation and gel filtration chromatography, respectively, allowing calculation of a native molecular weight (130,000) for CENP-E (aa 1-473)-GFP. (C) K560-GFP and CE473-GFP were nonspecifically adhered on a coverslip and the fluorescence intensity was measured using TIRF microscopy. (D) Fluorescence intensity distribution of K560-GFP and CE473-GFP immobilized on a coverslip. a.u., arbitrary unit. (E) Two-step photobleaching behavior of K560-GFP and CE473-GFP molecules. (F) Initial fluorescence intensity distribution of moving CE473-GFP. The mean intensity was measured to be 4.5 ± 0.2 a.u. (mean ± SEM, n =108).
Figure 2.6 CENP-E is a slow, processive motor that maintains microtubule attachment for long periods

(A) Purified CE473-GFP. (B) Experimental setup for imaging CENP-E single molecules moving along microtubules with TIRF microscopy. X-rhodamine-labeled GMPCPP microtubules were immobilized on a coverslip using anti-tubulin antibody, and a low concentration of CE473-GFP (0.5-1 nM) was flowed into the flow chamber. (C) Frames from time-lapse imaging of CENP-E motor-GFP moving along microtubules. A fluorescent speckle on the microtubule is indicated with a red line. Two CENP-E molecules moving at different speeds are pointed to with arrows. Green, CE473-GFP; Red, X-rhodamine labeled GMPCPP microtubules; Scale bar, 2 μm. (D) Kymographs showing processive movements of CENP-E motor domain. X-rhodamine speckles on stable microtubules produced vertical red lines. Single molecules of CE473-GFP are shown in green. Vertical yellow arrowheads indicate starts and stops of processive movements. Actual durations of kymographs 1, 2, and 3 were 1205, 1190 and 1205 seconds, respectively. Scale bar, 2 μm. (E) Velocity distribution of single CENP-E motor molecules. The median velocity is 8 nm/sec (equal to 0.48 μm/min, n=320; 4 independent preparations). (F) MSD ($\rho(t)$) of CENP-E fitted with $\rho(t) = v^2 t^2 + 2Dt$ (n = 90). Error bars are standard error of the mean (SEM). (G) Run time of CENP-E motor was distributed exponentially. The mean run time was determined by fitting the data into a cumulative distribution function. The inset shows the ‘1- cumulative probability’ of CENP-E run time plotted on a log scale. The mean run time is 195 ± 13 seconds (mean ± SEM, n=320). (H) Run length of CENP-E motor was determined by fitting the data into a cumulative distribution function. The inset shows the ‘1- cumulative probability’ of CENP-E run length plotted on a log scale. The mean run length is 1.5 ± 0.1 μm (mean ± SEM, n=320).
Figure 2.7 CENP-E motility contains one-dimensional diffusion

(A) One dimensional diffusion of CENP-E confirmed with Quantum dot (Qdot)-labeled CENP-E. (F) Purification of CENP-E (aa 1-473) SNAP-6His (76 kDa). The SNAP tag (Keppler et al., 2003; Jansen et al, 2007) was reacted with biotin tagged benzylguanine, and ~ 0.1 nM of biotin-labeled CENP-E was prebound to microtubules in the absence of ATP in flow chambers (with 4 U/ml apyrase for 5 min). Subsequently, 1 nM of streptavidin conjugated Qdot 525 (Invitrogen) was flowed in to tag the biotin-labeled CENP-E. Unbound Qdots were washed out and the motility of CENP-E was initiated by flowing in buffer containing 3mM ATP. (G) Frames from time-lapse imaging of Qdot-labeled CENP-E motor domain moving along microtubules. Backward movement was observed with frequent frame rate (1 sec⁻¹). Green, CENP-E motor-Qdot525; Red, X-rhodamine microtubule; Scale bar, 2 µm. (H) Kymographs showing occasional reversals in direction as indicated with arrowheads. Scale bar, 2 µm. (I) MSD (ρ(t)) of Qdot-labeled CENP-E fitted with ρ(t) = v²t² + 2Dt (v, mean drift velocity; D, diffusion coefficient). Error bars on the graph represent SEM. Mean velocity (v, 15 ± 1.5 nm/sec) and diffusion coefficient (D, 830 ± 170 nm²/sec) of Qdot-labeled CENP-E (aa 1-473) (n =44) were comparable to the values of CENP-E-GFP as shown in Figure 2.5.
Figure 2.8 Full-length CENP-E is a slow, plus end-directed motor

(A) Coomassie-stained full-length CENP-E-GFP (366 kDa) purified from baculovirus-induced insect cells. 
(B) Microtubule gliding assay with full-length CENP-E. CENP-E-GFP proteins were tethered to a GFP antibody-coated surface of a flow chamber and polarity-marked microtubules were introduced subsequently. Minus ends of the microtubules are brightly marked. Colored arrowheads indicate the starting positions of three microtubules, and colored dots point to the minus ends. The average gliding velocity was 30 ± 7.6 nm/sec (mean ± s.d., n=112). Scale bar, 2 μm.
Figure 2.9 CENP-E motor activity is essential for metaphase chromosome alignment

(A) Experimental scheme for *Xenopus* extract manipulation. (B) Immunoblot of CENP-E (340 kDa) and Rod (220 kDa, a loading control) in mock-depleted, CENP-E depleted, wild-type CENP-E supplemented, and rigor CENP-E T91N supplemented *Xenopus* extracts. (C) Recombinant full-length CENP-E partially rescued chromosome alignment, whereas rigor CENP-E failed at rescue. Red, X-rhodamine tubulin; Green, DAPI; Scale bar, 10 µm. (D) Quantification of structures formed in *Xenopus* extracts. More than 200 spindles were scored each in 3 independent depletion/add-back experiments. Error bars represent standard deviations.
Figure 2.10 CENP-E localization in the *Xenopus* extract spindles

(A) Red, X-rhodamine tubulin; Green, Alexa488-labeled anti-CENP-E antibody; Blue, DAPI; Scale bar, 10 μm. Addition of rigor CENP-E<sup>T91N</sup> accumulated in large amounts at spindle poles, presumably the result of transport of rigor motors initially bound randomly to spindle microtubules, with subsequent poleward translocation by microtubule flux. This is consistent with our similar observation that a rigor CENP-E motor fragment (aa1-473)-GFP lacking the kinetochore targeting domain also ended up accumulating at the spindle pole region when added into the preassembled spindles in *Xenopus* extracts (not shown). In addition, the polar accumulation of rigor CENP-E motor fragment was not affected with dynein inhibition by 70.1, the monoclonal antibody against dynein intermediate chain, while dynein inhibition gave rise to the longer spindle with splayed poles as previously reported (not shown). Addition of rigor CENP-E<sup>T91N</sup> also induced numerous microtubule bundles and small asters that did not contain DNA, but those structures were not included in our analysis.
Figure 2.11 A model for CENP-E as a motile, flexible tether for kinetochore microtubule capture and maintenance of linkage to dynamic spindle microtubules

(A) Using its slow processive motor activity and a weak diffusive binding mode to microtubules, CENP-E walks toward the plus ends of kinetochore microtubules or diffuses along the lattice without dissociating for extended periods. (B) The 230 nm-long coiled-coil of CENP-E functions as a safety catch for disassembling microtubules detached from the core kinetochore attachment components, thereby stabilizing the microtubule and enabling rescue. (C) CENP-E is likely to be a part of kinetochore ‘slip-clutch’ that is engaged on fluxing kinetochore microtubules with its slow plus end-directed motility (Maddox et al., 2003a). CENP-E bound to the microtubule surface may affect kinetochore microtubule plus ends, thereby promoting growth and allowing recapture. (D) Unlike other shorter and more rigidly structured kinetochore capture components, multiple CENP-E molecules are likely to work together by allowing simultaneous attachment at many different microtubule orientations relative to the kinetochore axis without forcing each other into unproductive conformations. Lastly, (E) the highly flexible, extended coiled-coil of CENP-E mediates initial capture of microtubules by searching a large volume in cells, and (F) its slow, processive motility powers mono-oriented chromosomes to congress using an adjacent kinetochore fiber (Kapoor et al., 2006).
The text of Chapter 2, in part, is a reprint of the material as it appears in *Journal of Cell Biology*, 2008 181, 411-419, Kim Y., Heuser J.E., Waterman C.M., and Cleveland D.W. The dissertation author was the primary researcher and the first author of this paper.
Chapter 3: Phosphorylation-dependent regulation of CENP-E by Aurora kinases is essential for chromosome congression

3.1 Introduction

Accurate chromosome segregation during mitosis requires the bipolar attachment of duplicated chromosomes to microtubules emanating from the opposite poles of the spindle. Each time a cell divides, a specialized proteinaceous structure called the kinetochore assembles on the surface of centromeres, and it is the kinetochore that binds to spindle microtubules and directs chromosome motion during mitosis (Cleveland et al., 2003). Microtubule capture by the kinetochore is a stochastic process. Initial kinetochore attachment is often mediated via an interaction with the lateral surface of a microtubule, and kinetochores attached in this manner undergo rapid, dynein-mediated poleward motion (Rieder and Alexander, 1990; Yang et al., 2007). Although some chromosomes are able to achieve biorientation without being transported to the spindle pole, dynein-mediated transport is an important mechanism to collect chromosomes to a common microtubule-dense region, where kinetochores have a greater chance of promoting efficient chromosome alignment.

Congression of polar localized, mono-oriented chromosomes to the metaphase plate is powered by a processive, plus end-directed kinetochore motor CENP-E (Centromere-associated protein E) (Kapoor et al., 2006; Kim et al., 2008). In various cell types and organisms, removal or inhibition of CENP-E leads to a failure in complete metaphase chromosome alignment, with a few chromosomes found unusually close to the
spindle poles (McEwen et al., 2001; Putkey et al., 2002; Wood et al., 1997; Yao et al., 2000; Yucel et al., 2000). CENP-E is one of the components that is directly responsible for stable capture of kinetochore by spindle microtubules (Putkey et al., 2002). However, my finding of the highly flexible and very long coiled-coil of CENP-E (Kim et al., 2008) raises the possibility that, while it can work advantageously for initial capture by searching a large volume within cells, CENP-E may also contribute, in part, to inappropriate attachments of kinetochore.

Indeed, the process of capturing spindle microtubules by kinetochores is prone to errors. Undesirable attachment does occur in early prometaphase, such that occasionally a single kinetochore captures microtubules from both spindle poles (merotelic attachment), or both sister kinetochores are attached to the same pole (syntelic attachment) (Cimini and Degrassi, 2005). These improper kinetochore attachments, if not resolved, can lead to chromosome missegregation and aneuploidy (Cimini et al., 2001; Thompson and Compton, 2008).

Error correction of aberrant kinetochore attachment is carried out by the conserved Ser/Thr kinase Aurora/Ipl1 (Lampson et al., 2004; Tanaka et al., 2002). Like Ipl1 in budding yeast, Aurora B is a component of the four protein chromosome passenger complex together with INCENP, Survivin, and Borealin/Dasra, and is targeted to the inner centromere from prophase to metaphase (Ruchaud et al., 2007). Aurora B is thought to aid chromosome biorientation by destabilizing the kinetochore-microtubule interaction of improperly attached chromosomes (Cimini et al., 2004; Pinsky et al., 2006; Tanaka et al., 2002). Several key Aurora substrates, including Dam1 and Ndc80, are directly involved in microtubule capture at the kinetochore, and phosphorylation by
Aurora has been shown to decrease the affinity of these proteins for microtubules (Cheeseman et al., 2002; Cheeseman et al., 2006; DeLuca et al., 2006; Gestaut et al., 2008).

CENP-E is highly phosphorylated during mitosis (Nousiainen et al., 2006), raising the possibility that phosphorylation may regulate the function of CENP-E at individual kinetochores. Although phosphorylations of the C-terminal tail domain of CENP-E by Cdk1, MAPK, or Mps1 have been previously implicated in regulation of CENP-E (Espeut et al., 2008; Liao et al., 1994; Zecevic et al., 1998), the significance of these and other phosphorylation events have not been established in cells. Here we have investigated the effects of preventing CENP-E phosphorylation in human cells. Surprisingly, preventing phosphorylation at a single, conserved site close to the CENP-E kinesin neck domain inhibits chromosome alignment, with a few chromosomes remaining unusually close to the spindle poles. Aurora kinases, both A and B, phosphorylate this site in vitro and in vivo, and phosphorylation reduces the affinity of CENP-E for microtubules in vitro. This demonstrates that CENP-E is under direct control of Aurora kinases so as to promote chromosome biorientation while preventing kinetochores from making microtubule attachment errors.

3.2 Highly conserved CENP-E T422 positioned near the kinesin neck domain is phosphorylated by Aurora A and B in vitro

While searching for the origin of one-dimensional diffusion in CENP-E motility that was observed in my previous study (Kim et al., 2008), I noticed a highly conserved stretch of basic residues downstream of the CENP-E coiled-coil neck domain (Figure
3.2A). Consisting of 4~7 consecutive arginines or lysines, this basic stretch and the following threonine residue (422 in human and 424 in Xenopus laevis) are conserved in almost all the eukaryotes in which CENP-E is found. The last two residues of the basic stretch are invariably arginines, and most interestingly, the conserved threonine has been previously mapped as a phosphorylation site in a mass spectrometry-based proteomic screen (Nousiainen et al., 2006). Together with the highly conserved double arginines and the hydrophobic residue in between, CENP-E threonine 422 resides in the consensus motif for phosphorylation by Aurora kinase (Cheeseman et al., 2002).

To test whether CENP-E T422 (424 in Xenopus laevis) is indeed phosphorylated by Aurora kinases, in vitro kinase assays were performed using purified Aurora kinases and various fragments of recombinant Xenopus CENP-E as substrates. Xenopus Aurora B, together with its activator INCENP, was able to phosphorylate the full-length and the motor fragment (aa 1-473) of CENP-E (CE473), but failed to phosphorylate the mutant fragment in which Xenopus CENP-E T424 was converted to alanine (Figure 3.2B). CENP-E T424 was also phosphorylated by Aurora A (Figure 3.2C). Therefore, CENP-E can be a substrate for both Aurora A and B in vitro, and the highly conserved threonine located near the CENP-E motor domain is the phosphorylation site by Aurora kinases.

### 3.3 Generation of phosphoT422-specific antibodies

To examine the phosphorylation of CENP-E T422 in vivo, a rabbit polyclonal antibody was generated against the phosphopeptide of human CENP-E surrounding T422 (Figure 3.3A). Affinity purified antibody (anti-pT422) was demonstrated to be specific for the phosphorylated form of CENP-E, as it recognized the recombinant human CENP-
E (aa1-429) (hCE429) only when it was phosphorylated by Aurora A kinase, but not by itself or when it was mixed with the kinase dead mutant of Aurora A (Figure 3.3A). The antibody also recognized the wild-type CENP-E when it was immunoprecipitated from nocodazole-arrested DLD-1 cells, but not full-length CENP-E with the T422A mutation (Figure 3.3C). Moreover, the pT422 signal disappeared when bead-bound CENP-E was treated with  λ-phosphatase (Figure 3.3C), again supporting the phospho-specificity of the anti-pT422 hCE antibody.

Interestingly, pT422 antibody also recognized the recombinant Xenopus CENP-E motor fragment in a phosphorylation-dependent manner (Figure 3.3B). The corresponding Xenopus sequence differs from that of human by two amino acids at two residues downstream of T422 (Figure 3.3B). Even a Xenopus CENP-E mutant, in which five amino acids at 3 residues upstream of T422 are mutated to alanines (5A), was recognized by the anti-pT422 antibody at a level that is consistent with the degree of 32P-γATP incorporation into XCE4285A compared to the wild-type (data not shown). This antibody also recognized XCE428RR→KK but only at an extremely low level when it was mixed with the Aurora kinase, consistent with minimal 32P-γATP incorporation into this mutant CENP-E (Figure 3.10B; Figure 3.3B). Together, the combined data suggest that anti-pT422 antibody is phospho-specific and its major epitope requires phosphorylated T422.

### 3.4 Phosphorylation of CENP-E occurs at kinetochores

By immunofluorescence using anti-pT422 antibody, we then tested where in cells the phosphorylation occurs. The pT422 signal was found clearly at kinetochores on
metaphase chromosome spreads, as recognized by the centromere marker ACA (anti-centromere antibodies) (Figure 3.4A). Consistently, pT422 signal was found at individual kinetochores in early prometaphase in HeLa cells, and the phospho-signal overlapped nicely with that of total CENP-E (Figure 3.4B). Interestingly, in one example the pT422 signal was preferentially associated with the leading kinetochore of a congressing chromosome, with a stronger signal asymmetrically stretched and shifted inward to the inner centromere (Figure 3.5A). Although there was no evidence to support the view that the kinetochore closer to the spindle equator was actually leading the chromosome congression, the increased immunoreactivity of CENP-E on the leading kinetochore has previously been reported by immunogold electron microscopy (Yao et al., 1997). The stretched pT422 signal closer to the inner centromere at the leading kinetochore suggests that CENP-E T422 might be phosphorylated by Aurora B, and the phosphorylation may play an important role in powering chromosome congression to the metaphase plate. We conclude that phosphorylation of CENP-E T422 occurs at individual kinetochores in early prometaphase, and the phosphorylation may occur preferentially at the leading kinetochore of congressing chromosome.

Besides kinetochores, however, the phospho-CENP-E antibody also recognized a broad region at the spindle poles, spindle microtubules, (to a lesser degree) condensed chromosomes from prometaphase to metaphase, and spindle midzone from late anaphase to early G1 (data not shown). However, the antibody binding to structures other than kinetochores did not disappear with depletion of CENP-E by siRNA and therefore are not specific to CENP-E (data not shown).
3.5 Aurora kinases, both A and B, contribute to phosphorylation of CENP-E at T422

In nocodazole-treated HeLa cells, pT422 staining completely overlapped with the signal for conventional CENP-E antibodies, identifying a large crescent around individual kinetochores, again demonstrating that CENP-E is phosphorylated at kinetochores (Figure 3.6A). Inhibition of Aurora kinases by VX-680, a potent inhibitor of both Aurora A and B (Harrington et al., 2004), diminished the total amount of CENP-E at kinetochores, consistent with a prior report that Aurora B is required for the recruitment of CENP-E to kinetochores (Figure 3.6A). Nevertheless, VX-680 treatment almost completely abolished the pT422 signal when it was normalized to the level of remaining CENP-E at kinetochores, establishing that CENP-E T422 is indeed a substrate of Aurora kinases in vivo (Figure 3.6B).

In mammals, there are three members of the conserved Aurora kinase family (Aurora A, B, and C). While Aurora C is only present in germ cells, Aurora A and B are widely expressed in most cell types and localized to different cellular structures, playing distinct roles during mitosis. Given that the phosphorylation occurs at kinetochores, we initially hypothesized it was likely to be Aurora B at inner centromeres that phosphorylated CENP-E T422. However, to unambiguously distinguish which Aurora kinase phosphorylates CENP-E in cells, we took advantage of a series of Aurora kinase inhibitors. MLN8054 and ZM447439 are small molecule inhibitors that preferentially inhibit Aurora A and Aurora B, respectively (Ditchfield et al., 2003; Manfredi et al., 2007). Using antibodies against the Aurora A-specific phospho-TACC3
(Transforming acidic coiled-coil) (pTACC3) and Aurora B-specific phospho-Histone H3 (p-Histone H3), we then assessed which Aurora kinases were actually inhibited under different drug treatments.

As expected, treating cells with VX-680 abolished both pTACC3 and p-Histone H3 signals in mitotic lysates, consistent with inhibition by VX-680 of both Aurora kinases in cells. In agreement to our results from immunofluorescence (Figure 3.6), the pT422 antibody binding to CENP-E immunoprecipitates completely disappeared when the cells were treated with VX-680 (Figure 3.7). However, while MLN8054 or ZM447439 treatment alone inhibited their specific targets and left others uninhibited, either of these treatments alone failed to abolish the pT422 signal from CENP-E immunoprecipitates, indicating that the inhibition of either Aurora kinase alone is not sufficient to prevent the phosphorylation of CENP-E T422. Indeed, when cells were treated with both MLN8054 and ZM447439 together, CENP-E recognized by pT422 antibody completely disappeared, just as it did when both Aurora kinases were inhibited by VX-680 (Figure 3.7). Surprisingly therefore, we conclude that both Aurora A and B contribute to the phosphorylation of CENP-E at T422 in vivo, even under conditions where spindle microtubules are completely disassembled (by nocodazole).

3.6 Aurora-mediated phosphorylation of CENP-E reduces the affinity of CENP-E for microtubules

The fact that the highly conserved T422 is located right next to the consecutive basic residues that are close to the CENP-E motor domain raises an interesting possibility that the Aurora-mediated phosphorylation of T422 may regulate aspects of CENP-E-
microtubule interaction. To determine how phosphorylation affects the motor properties of CENP-E, we first tested its motility in microtubule gliding assays using fluorescently labeled GMPCPP microtubules. *Xenopus* CENP-E motor fragment (aa 1-473) fused to a myc-6His tag (CE473) was used as previously described (Wood et al., 1997), with or without a prior phosphorylation by Aurora A. After tethering to a coverslip using anti-His antibody, the speed of microtubule gliding was measured by time-lapse fluorescence microscopy. I chose to use Aurora A rather than Aurora B to phosphorylate CENP-E, because the requirement of INCENP for Aurora B kinase activity obscures observing the gliding by a single microtubule due to its own microtubule bundling activity. I determined that the gliding speeds of CENP-E remained unchanged regardless of its phosphorylation status, as the mean microtubule gliding speed by CE473 and phospho-CE473 were 4.4 ± 0.7 µm/min (mean ± SD; n = 57) and 4.6 ± 0.7 µm/min (mean ± SD; n = 119), respectively (Figure 3.8A). I conclude that it is unlikely that the phosphorylation affects the rate-limiting steps in the CENP-E enzymatic cycle.

To further test whether and if so how phosphorylation affects CENP-E motility, microtubule-stimulated ATPase activity of CENP-E was measured in the presence of increasing concentration of microtubules (Figure 3.8B). Consistent with the results from microtubule gliding assays, the maximal ATP turnover rate ($k_{cat}$) was not affected by phosphorylation [$k_{cat}$ of CE473 and phospho-CE473 were 13 ± 0.6 s$^{-1}$ (best fit value ± SE; n=3) and 14 ± 1.3 s$^{-1}$ (best fit value ± SE; n=3), respectively]. However, the apparent $K_{mMT}$ (the concentration of microtubules required to reach half maximal ATPase rate) was significantly increased upon phosphorylation by Aurora A (0.17 ± 0.03 µM for CE473, and 0.64 ± 0.15 µM for phospho-CE473). $K_{mMT}$ reflects an affinity of kinesins
like CENP-E for the microtubules. Without microtubules, kinesins are tightly bound to ADP in solution, and the rate of ADP release is extremely low (Hackney, 1988). Binding of ADP-bound kinesin to microtubules, however, greatly accelerates the rate of ADP release (~1000-fold), and kinesin proceeds to complete its enzymatic cycle (Gilbert and Johnson, 1993). Since phosphorylation of CENP-E increased $K_m$MT over 3-fold without significantly affecting $k_{cat}$ and the gliding speed, it is most likely that the phosphorylation of CENP-E reduces its affinity for microtubules primarily in the ADP state.

To test this hypothesis, the binding affinity of CENP-E to microtubules, with or without prior phosphorylation by Aurora kinase, was tested in the presence of 2 mM MgADP (Figure 3.8C). 2 µM of CE473 or phospho-CE473 were mixed with varying concentrations of taxol-stabilized microtubules and the microtubule bound CENP-E was examined by pelleting the microtubules through a glycerol cushion. Indeed, less CE473 co-sedimented with microtubules when CENP-E was phosphorylated by Aurora A prior to incubation with microtubules, indicating that phosphorylation by Aurora kinase reduces the affinity of CENP-E for microtubules in the ADP state. The microtubule pelleting experiments will be carefully repeated and expanded to CE473$^{T424A}$ with or without Aurora kinase to confirm that the reduced affinity by phosphorylation is in fact specific to T424. The same set of experiments will also be performed in different nucleotide and salt conditions.

### 3.7 Phosphorylation of CENP-E reduces the run length

How phosphorylation affects CENP-E motor properties was also investigated at a single molecule level using TIRF microscopy. CENP-E motor fragment (aa1-473) was
tagged with the monomeric, photostable red fluorescent protein TagRFP-T (CE473-RFP) (Shaner et al., 2008) (this was required because of changes to the TIRF microscope). Oregon Green 488-labeled GMPCPP microtubules were tethered to a coverslip in a flow chamber and a 0.5~1 nM concentration of CE473-RFP was added (Figure 3.9A) in the presence of apyrase to generate rigor binding. As expected, CE473-RFP was stably bound without any nucleotides, and the fluorescence signals were photobleached in one or two steps 89% of the time (75 double steps and 68 single step; n=160), consistent with the dimeric state of CE473 (Figure 3.9B). When the motors were introduced into the flow chamber in a buffer containing 3 mM MgADP, both CE473 and the phosphorylated CE473 remained loosely bound to microtubules without displaying directional motility (Figure 3.10A), supporting my previous observation that CENP-E motility contains a diffusive mode (Kim et al., 2008). Additionally, this experiment demonstrates that the diffusion along microtubules does not require ATP hydrolysis. In the presence of ADP, the duration of CE473-RFP binding to microtubules was shortened by 30% upon phosphorylation (t = 17 ± 0.13 sec for CE473, n = 231; t = 12 ± 0.07 sec for phospho-CE473, n = 240) (Figure 3.10B), consistent with my finding that phosphorylation of CENP-E reduces its affinity to microtubules in the ADP state.

Processivity of CENP-E was also reduced when the motor was phosphorylated by Aurora kinase. When the movements by individual molecules were observed in the presence of 3 mM MgATP, the run length of phosphorylated CENP-E was shorter than that of the unphosphorylated CENP-E (1.6 µm for CE473, n=337; 1.2 µm for phospho-CE473, n=294) (Figure 3.10C). Since the motility assay was performed at an elevated temperature (33 °C), the speed of movement was much faster than the speed measured at
room temperature (Chapter 2). I conclude that phosphorylation of CENP-E by Aurora kinase modestly reduces its run length.

3.8 Phosphorylation of CENP-E T422 is essential for chromosome congression

To examine the significance of Aurora-mediated phosphorylation at CENP-E T422 in vivo, stable DLD-1 human colon cancer cell lines were generated in which siRNA resistant transgenes encoding CENP-E tagged with myc and GFP at the N-terminus were integrated into a unique Flp recombination target (FRT) site in the genome. Expression of CENP-E transgenes was induced by addition of Tetracycline. CENP-E transgenes, both wild-type and phosphomutant (T422A) were normally recruited to kinetochores from prometaphase to early anaphase and then relocated to the spindle midzone in late mitosis (data not shown). siRNA-mediated depletion of endogenous CENP-E and expression of resistant transgenes was used to determine the effects of preventing CENP-E phosphorylation at T422 in vivo.

Surprisingly, replacing endogenous CENP-E with a phosphomutant (CENP-E<sup>T422A</sup>) prevented complete metaphase chromosome alignment with a few chromosomes remaining close to the spindle poles (Figure 3.11C). Highly reminiscent of complete CENP-E inhibition, failure of alignment demonstrates that the phosphorylation of CENP-E at T422 is essential for chromosome congression. It is remarkable given that a point mutation at a single phosphorylation site gave rise to the phenotype that is seen when CENP-E is completely depleted in cells. To corroborate this finding, another mutant of CENP-E was generated, in which two arginines in the Aurora consensus motif were
converted to lysines (CENP-E$^{RR\rightarrow KK}$) to prevent the phosphorylation at T422 while preserving the overall charge of this region (Figure 3.11A). In vitro, recombinant *Xenopus* CENP-E (aa1-428) carrying this mutation could not be efficiently phosphorylated by either Aurora kinase in the time scale we have tested (1 hour) (Figure 3.11B). Replacing endogenous CENP-E with CENP-E$^{RR\rightarrow KK}$ similarly caused a mitotic delay with a few chromosomes found close to the spindle poles (Figure 3.11C). Thus, by using two alternative mutations to block phosphorylation at T422, these effects have eliminated possibilities other than preventing phosphorylation for the phosphomutant (CENP-E$^{T422A}$) and have convincingly shown that phosphorylation of CENP-E T422 is essential for chromosome congression.

3.9 CENP-E phosphorylation is required downstream of Aurora activation for the congression of incorrectly attached chromosomes that are moved to the spindle pole

To test whether phosphorylation of CENP-E is required for congression of polar chromosomes in metaphase, we have adopted a method to enrich monooriented chromosomes using reversible chemical inhibitors (Kapoor et al., 2006; Lampson et al., 2004). Cells were first treated with monastrol, a small molecule inhibitor of Eg5 (Kinesin-5), to generate a monopolar spindle with high frequency of syntelically attached chromosomes (Figure 3.12A and B). Cells were then released from monastrol into Aurora kinase inhibitor ZM447439 and a proteosome inhibitor MG132, which is permissive for bipolar spindle assembly but preserves syntelic attachments without exiting mitosis (Figure 3.12B). Reactivation of Aurora kinase induced a poleward movement of
improperly attached chromosomes and subsequent congression to metaphase (Figure 3.12B). Examination of chromosome alignment in cells where the endogenous CENP-E was replaced by CENP-E\(^\text{WT}\) or CENP-E\(^{T422A}\) revealed that as expected, the majority of cells expressing CENP-E\(^{T422A}\) (\(\sim 85\%\)) were predisposed to the presence of polar chromosomes, even without drug treatment (Figure 3.12D). However, the number of polar chromosomes was significantly increased in CENP-E\(^{T422A}\) cells when cells were challenged to accumulate monooriented chromosomes. In contrast, cells expressing CENP-E\(^{\text{WT}}\) exhibited a low, similar level of polar chromosomes with or without drug treatments, indicating that the initially monooriented chromosomes congress successfully in a CENP-E dependent manner (Figure 3.12F). Thus, Aurora-mediated phosphorylation of CENP-E is required for congression of maloriented chromosomes that are initially transported to a spindle pole. Intriguingly, accumulation of chromosomes at spindle poles is a common feature seen in both an error correction process (Lampson et al., 2004) and a part of general chromosome behavior during early prometaphase (Rieder and Alexander, 1990) (Figure 3.13). I speculate that CENP-E will reach its fully phosphorylated state when chromosomes are closest to the spindle pole with phosphorylation mediated by Aurora A. The consequent reduction in affinity for microtubules may be an important mechanism to permit CENP-E to preferentially glide along bundles of kinetochore microtubules (Figure 3.13).
3.10 Discussion

Here I demonstrate that phosphorylation of CENP-E by Aurora kinases at a single conserved site close to the motor domain regulates its intrinsic motor properties and is essential for the role of CENP-E in chromosome congression. Phosphorylation of CENP-E lowers its affinity for microtubules and makes the motor dissociate more readily during its processive runs. Intriguingly, the phosphorylation site (T422) is located next to positively charged amino acids, and the phosphorylation is likely to oppose the basic charge of this conserved region which may directly be involved in CENP-E-microtubule interaction. In conventional kinesin (Kinesin-1), mutations that affect the overall charge of the neck coiled-coil have been shown to modulate its processivity (Thorn et al., 2000). Although the basic stretch found in CENP-E is not part of the neck coiled-coil, a disordered 20-amino acid sequence linking to the neck domain may provide flexibility for this region to interact with acidic tubulin C-terminus.

An electrostatic interaction between a positively charged region of a kinesin and the negatively charged microtubule surface has been shown to confer important properties to several kinesin motors. The monomeric kinesin Kif1a (Kinesin-3) has a positively charged K-loop in the motor domain which permits diffusion along the microtubule lattice (Okada and Hirokawa, 1999). Similarly, the non-motile microtubule depolymerizing enzyme, MCAK (Kinesin-13), possesses positively charged residues in its neck region, which extend out vertically to microtubules to interact with the acidic tubulin C-terminus (Okada et al., 2003). I propose that the conserved basic stretch found in CENP-E similarly functions as an electrostatic tether, and the interaction with microtubules is under direct control of Aurora kinases. Given that a rare amino acid
tryptophan is curiously conserved following T422, however, I do not exclude possibilities that this bulky and hydrophobic residue has a significant effect on the protein structure and contributes to the protein interaction mediated through this region. I am currently investigating the contribution of this tryptophan to aspects of CENP-E and how it may be modulated by T422 phosphorylation.

Unexpectedly, both Aurora A and B were found to contribute to the phosphorylation of CENP-E T422 in cells. Interestingly, Aurora A inhibition has been reported to cause chromosome misalignment with a few chromosomes found close to the spindle poles (Hoar et al., 2007; Kunitoku et al., 2003; Marumoto et al., 2003), a phenotype highly reminiscent of that observed when the function of CENP-E is impaired. Although Aurora A-mediated phosphorylation of centromere-specific histone H3 CENP-A has previously been proposed to promote chromosome congression (Kunitoku et al., 2003), it remains unclear how phosphorylation of CENP-A aids chromosome alignment or whether additional kinetochore localized Aurora A substrates exist. Here I provide a mechanistic explanation for the chromosome misalignment in Aurora A inhibited cells, by demonstrating that Aurora A directly targets CENP-E to promote chromosome congression.

The correction of mal-oriented kinetochore attachment shares a common feature with that of normal chromosome congression: laterally attached prometaphase chromosomes and syntelically attached chromosomes both move to the spindle pole (Lampson et al., 2004; Rieder and Alexander, 1990) (Figure 3.13). Therefore, collecting chromosomes at the spindle pole and subsequent congression powered by CENP-E is an efficient mechanism to achieve chromosome biorientation. Here, we show that
phosphorylation of CENP-E by Aurora kinases is essential for this process (Figure 13.3). Aurora A is concentrated at the spindle poles during mitosis and is ideally positioned as a spatial cue to phosphorylate CENP-E on the kinetochores of chromosomes that are collected at the pole (Figure 13.3). The reduced tension of mono-oriented kinetochores and the juxtaposed position of sister kinetochores on syntelically attached chromosomes (Loncarek et al., 2007), brings Aurora B in close proximity to kinetochore substrates allowing it to also contribute to the phosphorylation of CENP-E T422. I speculate therefore, that CENP-E reaches its fully phosphorylated state when chromosomes are near to the spindle poles. Decreasing the microtubule affinity of CENP-E by phosphorylation at the pole may reduce its binding to the densely populated, dynamic astral microtubules and provide selectivity toward bundles of kinetochore microtubules for CENP-E to glide along. This would help prevent futile chromosome movements along labile astral microtubules nucleated at the spindle pole.

If not regulated, the large reach and flexibility of CENP-E (Kim et al., 2008) could contribute to erroneous attachment by allowing CENP-E to promiscuously grab microtubules in the proximity of the kinetochore. Indeed, the frequency of lagging chromosomes has been reported to be significantly increased in cells recovering from a nocodazole block (Cimini et al., 2001), possibly as a result of the elevated levels of CENP-E recruited to kinetochores during a long-term blockage of microtubule assembly. The depletion of CENP-E from the kinetochores of congressed chromosomes may reduce attachment errors by dampening the ability of kinetochores to form lateral contacts with microtubules once chromosomes have achieved a stable end-on attachment.
Dephosphorylation of the remaining molecules of CENP-E at aligned chromosomes may also help kinetochores to sustain attachment to dynamic spindle microtubules.

3.11 Methods

3.11.1 Cloning and protein purification

TagRFP-T (Accession No. ACD03281), a gift from Roger Tsien (UC, San Diego), was amplified using the forward primer with a BamHI recognition site (5’-cggggatcccccaggtgtctagggcggc-3’) and the reverse primer (5’-cgcctcctaggtgctggtggggcgtggtgtgctgtacagctgc-3’) with an XhoI site, a 6 histidine tag and a termination codon by PCR, and cloned into pET23d vector containing *Xenopus* CENP-E (aa1-473) using BamHI and XhoI at C-terminus to CENP-E. This cloning strategy generates a 16 amino acid-long linker (MASMTGGGGMGRLR) between CENP-E and TagRFP-T.

Recombinant CE473-TagRFP-T protein was expressed in *E.coli* [strain Rosetta (DE3)] and purified as described for CE473-GFP in Chapter 2.

3.11.2 In vitro kinase assays

In vitro kinase assays were performed at room temperature in 20 mM Tris pH 7.5, 25 mM KCl, 1 mM MgCl₂, 1mM DTT in the presence of 1 mM MgATP and 50 µCi/ml ³²P γ-ATP using 2 µM of CENP-E fragments and 0.2 µM of bacterially purified Aurora A or bacterially purified Aurora B together with full-length INCENP co-expressed from a polycistronic vector. Samples were incubated at room temperature for 30 min, with the exception of kinetics studies. The kinase reaction is stopped with SDS-PAGE sample buffer and is analyzed by 10% SDS-PAGE. Each coomassie-stained gel was dried and
exposed to film (Kodak) for autoradiography. For quantification, individual bands were excised and the amount of incorporated $^{32}$P was quantified using a scintillation counter (Beckman).

3.11.3 Phosphopeptide antibody production and affinity purification

Phosphorylated peptide KRKRRVpTWCLGK whose C-terminus was modified by amidation was synthesized (Biomatik) and coupled to KLH (keyhole limpet hemacyanin, Sigma) using EDC (1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride) zero-length crosslinker (Pierce). Rabbit polyclonal antibodies were generated (Covance), and the immune serum was affinity purified using HiTrap NHS-activated HP column (GE Healthcare) coupled with the phosphopeptide. After the serum has been passed the phosphopeptide-coupled column, the column was intensively washed with PBS containing 1 M NaCl before elution with 0.2 M Glycine, pH 2.5.

3.11.4 Immunofluorescence

For use in immunofluorescence (IF), affinity purified anti-pT422 antibody was directly labeled with Rhodamine Red-X (Invitrogen). DLD-1 cells were adhered on polylysine-coated coverslips for at least 24 hours and pre-extracted for 5 min at 37°C with pre-warmed MTSB (100 mM K-PIPES, pH 6.9, 30% glycerol, 1 mM EGTA, 1 mM MgSO$_4$) containing 0.5% Triton X-100 and phosphatase inhibitors 1 mM NaF, 500 nM microcystin-LR (EMD) before fixing with 2% formaldehyde in MTSB containing the above phosphatase inhibitors for 10 min at room temperature. Fixed cells were blocked either for 1 hr at room temperature or overnight at 4°C with Triton Block (0.2 M Glycine, 2.5% FBS, 0.1% Triton X-100 in PBS). All the primary antibodies used were diluted in Triton Block and incubated at room temperature for 1 hr. Dilutions used for primary
antibodies are: 1:1000 for anti-pT422 antibody (2.5 mg/ml stock); 1:500 1H12 CENP-E mouse monoclonal antibody (Abcam). The coverslips were washed 3 times at 5 min intervals with PBST (PBS + 0.1% Triton X-100), and the secondary antibody (Jackson ImmunoResearch Laboratories, FITC-conjugated goat anti-mouse antibody to stain 1H12) was used at 1:200 in Triton Block incubated at room temperature for 45 min. After washing, DNA was stained with 1 ug/ml of DAPI for 2 min and the coverslips were mounted using Prolong Gold (Invitrogen).

For chromosome spreads, DLD-1 cells were treated with 0.2 µg/ml of nocodazole for 6 hours to arrest cells in mitosis and swelled in pre-warmed hypotonic buffer (10 mM PIPES, pH 7.0, 55 mM KCl, 0.5 mM EGTA, 2 mM MgCl₂) containing phosphatase inhibitors 1 mM NaF, 500 nM microcystin-LR (EMD) for 20 min in a 37°C water bath. Cells were spun onto microscope slides using a Cytospin at 1500 rpm for 5 min and immediately pre-extracted for 5 min with pre-warmed MTSB containing 0.5% Triton X-100 and above phosphatase inhibitors. Then the cells were fixed with 2% formaldehyde in MTSB containing 0.1% Triton X-100 and the phosphatase inhibitors for 10 min at room temperature and preceded to following steps as regular IF.

3.11.5 ATPase assays

Steady-state ATPase rate was measured at room temperature in a coupled enzyme system based on 2-amino-6-mercapto-7-methyl-purine riboside (MESG) and purine nucleoside phosphorylase (PNP) (EnzChek Phosphate Assay Kit, Invitrogen). 2 µM of freshly prepared CE473 was pre-incubated with or without 0.2 µM of active Aurora A for 30 min at room temp in the above kinase buffer used for phosphorylation reaction, and
later the reaction was kept on ice until use. To measure ATPase rate, the kinase reaction was diluted to have final concentration of 20 nM CENP-E with varying concentrations of microtubules (0 – 4 µM) in assay buffer (10 mM Imidazole, pH 6.8, 10 mM MgCl₂, 5 mM K-acetate, 4 mM Mg-acetate, 2 mM EGTA, 0.1 mM bovine serum albumin, 2 mM ATP) and 0.2 mM MESG and 1 U/ml of PNP. Software Prism (Graphpad) was used for hyperbola curve fitting to derive $k_{cat}$ and $K_m$MT, and the CENP-E concentration was determined by quantifying the coomassie staining on SDS-PAGE compared to BSA standards (Pierce) using ImageJ.

3.11.6 Microtubule pelleting assays

Assembly of taxol-stabilized microtubules and measurement of polymerized tubulin concentration were performed as described (http://mitchison.med.harvard.edu/protocols.html). 2 µM of WT or T424A *Xenopus* CENP-E¹⁴⁷³ was incubated at room temperature with or without 0.2 µM of Aurora A for 15 min in BRB80 buffer (80 mM K-PIPES, pH 6.8, 1 mM MgCl₂, 1 mM EGTA) with 0.1 mM MgATP. CENP-E¹⁴⁷³ was subsequently mixed with varying concentrations of microtubules in the presence of 2 mM MgADP and incubated for 15 mins at room temperature. 40 µl of the reaction was layered onto 100 µl of BRB80 + 40% glycerol and sedimented for 5 min at 90,000 rpm in a TLA100 rotor. 20 µl was removed from the top for the supernatant sample and the remaining supernatant aspirated and 40 µl of BRB80 containing 5 mM CaCl₂ was added to the pellet for 10 min on ice. Equivalent volumes of supernatant and pellet fractions were analyzed by SDS-PAGE.

3.11.7 Single molecule assays
CENP-E single molecule assays were performed as previously described in Chapter 2 with following modifications. Slides and 22 x 22-mm square coverslips were cleaned and silanized as described (Helenius et al., 2006). A flow chamber was incubated with 50 µg/ml of a rat monoclonal tubulin antibody (YL1/2, Serotec) for 5 min, followed by 1% Pluronic F-127 (Invitrogen) in BRB80 for 15 min and Oregon Green 488-labeled GMPCPP microtubules for 10 min. ~0.2 mg/ml of *Xenopus* CENP-E<sup>1-473</sup>-RFP was incubated with 50 µg/ml of Aurora A in 20 mM Tris pH 7.5, 25 mM KCl, 1 mM MgCl<sub>2</sub>, 1 mM DTT, 1 mM MgATP for 15 min at room temperature to allow phosphorylation and diluted to ~0.5 nM just before imaging in motility buffer (25 mM K-PIPES, pH 6.9, 1 mM MgCl<sub>2</sub>, 1 mM EGTA, 1 mM DTT, 0.25% Brij35, 0.5 mg/ml casein, 4.5 mg/ml glucose, 0.2 mg/ml glucose oxidase, 0.35 mg/ml catalase, 0.5% bME) containing either 3 mM MgATP or 3 mM MgADP. Frames were captured every 500 ms with 200 ms exposure, and the typical duration of imaging was 2~3 min. Note, that since the imaging was performed at an elevated temperature (33 °C) and in higher MgCl<sub>2</sub>, the speed of CENP-E movement was faster than that measured at room temperature in Chapter 2.
Figure 3.1 Phosphorylation sites of CENP-E

(A) Previously reported phosphorylation sites of human CENP-E are shown on the diagram of domain structures. Asterisks (*) indicate the phosphorylation residues that are conserved from *Drosophila* to human. (B) 12 phosphoresidues of CENP-E and their surrounding sequences. All positional information refers to Swiss-Prot Q02224, the longest sequence of human CENP-E.
Figure 3.2 Highly conserved CENP-E Threonine 422 is phosphorylated by Aurora A and Aurora B kinases in vitro

(A) CENP-E sequences from different organisms are aligned by ClustalW algorithm. Highly conserved threonine 422 (424 in *Xenopus*) is within Aurora consensus motif following consecutive basic sequences, and is located near the kinesin neck domain. Although CENP-meta is used for sequence alignment above for *Drosophila* CENP-E homolog, the conserved basic patch and the threonine residue are also found in CENP-ana. Note that tryptophan 423 (the least frequent amino acid in CENP-E, 0.3% in human) with bulky side chain is also highly conserved among species. (B) Purified full-length *Xenopus* CENP-E, and motor fragments (aa 1-473) (CE473) with or without T424A mutation were used as substrates for in vitro kinase assay using *Xenopus* Aurora B and its activator INCENP. Coomassie stained gel showing protein purification and autoradiogram showing the incorporation of $^{32}$P-$\gamma$ATP. (C) The conserved threonine of CENP-E can be phosphorylated by both Aurora A and B in vitro. Recombinant CE473 and a shorter fragment *Xenopus* CENP-E (aa1-415) (CE415) not containing the phosphothreonine were used for in vitro kinase assay using bacterially purified *Xenopus* Aurora A or Aurora B with INCENP.
Figure 3.3 Generation of polyclonal phosphoT422-specific CENP-E antibody

(A) A rabbit polyclonal antibody was generated using phosphopeptide encompassing human CENP-E residue 416-427, in which threonine 422 is modified to have a phosphate at its side chain. Affinity purified anti-pT422 antibody was used to blot recombinant human CENP-E motor fragment (aa 1-429) (hCE429) with or without phosphorylation by active Aurora B. Kinase dead (KD) Aurora B was also used for kinase control. (B) Anti-pT422 antibody was tested against various mutants of recombinant Xenopus CENP-E (aa1-428) in vitro. Kinase reactions by Aurora A were taken at 0, 10, 20 minute time points and blotted using anti-pT422 hCENP-E antibody. Suggested epitope of anti-pT422 antibody is diagramed in and highlighted with gray. (C) CENP-E was immunoprecipitated using anti-myc antibody from nocodazole-arrested DLD-1 cells which stably express either myc-GFP-CENP-EWT or myc-GFP-CENP-E422A, and the immunoprecipitates were blotted with anti-pT422 antibody. The pT422 signal disappears upon λ-phosphatase treatment, corroborating the specificity of this antibody.
Figure 3.4 Phosphorylation of CENP-E T422 occurs at kinetochores

(A) Metaphase chromosome spreads from DLD-1 cells. Green, ACA; Red, pT422 CENP-E; Blue, DAPI (B) Asynchronous cycling HeLa cells were fixed after pre-extraction (0.5% Triton X-100 for 5 min) and stained with CENP-E (Green; 1H12, mouse monoclonal; FITC-conjugated anti-mouse antibody), pT422 (Red; rabbit polyclonal directly conjugated to Rhodamine Red-X), and DNA (Blue; DAPI).
Figure 3.5 PhosphoT422 signal is enriched at the leading kinetochore

(A) Asynchronously cycling DLD-1 cells were fixed after pre-extraction and stained for CENP-E (1H12 mouse monoclonal, FITC-conjugated anti-mouse antibody), pT422 CENP-E (rabbit polyclonal directly labeled with Rhodamine Red-X), ACA (human monoclonal, Cy5-conjugated anti-human antibody), and DNA (DAPI). (B) The kinetochore pair of the last congressing chromosome was subjected to a line scan analysis (Metamorph). Red, pT422; Green, CENP-E; Blue, ACA.
Figure 3.6 Phosphorylation of CENP-E at T422 is sensitive to Aurora kinase inhibitor

(A) Mitotically arrested HeLa cells (with nocodazole) were treated with Aurora kinase inhibitor VX-680 (0.5 µM) for 2 hr. Green, CENP-E (1H12 mouse monoclonal); Red, pT422 CENP-E (rabbit polyclonal); Blue, DNA (DAPI). (B) The pT422 fluorescence intensity was quantified and normalized to the total CENP-E.
Figure 3.7 Phosphorylation of CENP-E T422 is contributed by both Aurora A and B in vivo

DLD-1 cells expressing myc-GFP tagged wild-type CENP-E were arrested with nocodazole for 12 hours and treated with different Aurora kinase inhibitors for 2 hours before harvest. Whole cell lysates were blotted with pTACC3 (an Aurora A substrate) and pHistone H3 (an Aurora B substrate) antibodies to examine which Aurora kinase was inhibited by each drug treatment regime. CENP-E was immunoprecipitated using anti-myc antibody and blotted with anti-pT422 antibody. Concentrations of small molecule inhibitors used: VX-680 (0.5 µM), MLN8054 (0.25 µM), ZM447439 (3 µM).
Figure 3.8 Phosphorylation of CENP-E by Aurora kinase reduces its affinity for microtubules in the ADP state

(A) Microtubule gliding assays with CE473 and phospho-CE473 by Aurora A kinase. CE473 was phosphorylated in vitro by incubating with Xenopus Aurora A for 15 min at room temperature (the reaction is saturated by 10 min in this condition – data not shown). The mean gliding velocity for CE473 was 4.4 ± 0.7 \( \mu \)m/min (mean ± SD; \( n = 57 \)) and 4.6 ± 0.7 \( \mu \)m/min (mean ± SD; \( n = 119 \)) for phospho-CE473. The gliding speeds were not significantly different between wild-type and phosphorylated CE473. (B) ATPase rates were measured at room temperature in a coupled enzyme system, using 20 nM of CE473 or phospho-CE473 with increasing concentration of microtubules (n=3). The \( k_{cat} \) and \( K_{m,MT} \) values were derived by fitting the data to hyperbolic curve (one site binding) using the software Prism (GraphPad). Error bars represent SE. (C) Equilibrium microtubule binding of CE473 in the ADP state was tested in the microtubule pelleting assay. CE473 or phospho-CE473 was incubated with varying concentration of taxol-stabilized microtubules (0–5 \( \mu \)M) in BRB80 with 2 mM MgADP for 15 min at room temperature, and the microtubules were sedimented through 40% glycerol cushion to separate the unbound in supernatant. S, supernatant; P, pellet. A graph quantifying the bound CE473 is shown on the right.
Figure 3.9 Single molecule assays

(A) *Xenopus* CENP-E motor fragment (aa1-473) was tagged with the newest version of monomeric red fluorescent protein TagRFP-T (CE473-RFP). The experimental setup for imaging CENP-E single molecules with TIRF microscopy. Oregon Green 488-labeled GMPCPP microtubules were immobilized on a coverslip using tubulin antibody (YL1/2, Serotec), and a low concentration (0.5~1 nM) of CE473-RFP was flowed into the chamber. (B) Kymograph showing microtubule-bound CE473-RFP in the presence of apyrase. CE473-RFPs were photobleached in two or one steps in 89% of the time (75 double and 68 single steps, out of 160). (C) Initial fluorescence intensity of moving dots were measured (n=197).
Figure 3.10 Phosphorylation of CENP-E reduces the run length

(A) Kymographs showing diffusive motion of CE473-RFP in the presence of 3 mM MgADP. (B) The duration of binding was distributed exponentially, and the mean binding time was determined by fitting the data into a cumulative distribution function. The graph on the right shows cumulative probability of CE473-RFP binding time plotted on a log scale. (C) Kymographs showing processive motion of CE473-RFP in the presence of 3 mM MgATP at 33 °C. CENP-E accumulates at the end of microtubule once it reaches there using its processive motility. The run length of CE473-RFP was determined by fitting the data into a cumulative distribution function.
Figure 3.11 Phosphorylation of CENP-E Threonine 422 is essential for chromosome congression

(A) CENP-E mutants used in the study. Xenopus CENP-E was mutated to produce a mutant which cannot be phosphorylated at T424 in Xenopus CENP-E (T424A) and RR→KK mutation which would significantly reduce the phosphorylation at T424. (B) In vitro kinase assays were performed in the presence of $^{32}$P-$\gamma$ATP using Aurora B/INCENP and various recombinant Xenopus CENP-E motor fragments bearing above mutations as substrates. Samples from the kinase reaction were taken, and the reaction was stopped by sample buffer at indicated time points. The phosphorylation kinetics were examined by running SDS-PAGE ($^{32}$P, autoradiography; C, Coomassie blue staining). Each band was excised based on the Coomassie staining, and the incorporation of $^{32}$P-$\gamma$ATP was quantified using scintillation counting. (C) Stable DLD-1 cell lines were generated in which siRNA resistant CENP-E transgenes were integrated via Flp-In recombination into a single FRT (Flp recombination target) site in the genome. Endogenous CENP-E was reduced by siRNA and the expression of wild-type or mutant CENP-E tagged with myc-GFP was induced by adding Tetracycline to the medium to produce transgenes expresssing at a level similar to endogenous (data not known). Cells were then fixed for immunofluorescence. Green, GFP-tagged CENP-E; Red, tubulin.
Figure 3.12 CENP-E phosphorylation is required downstream of Aurora activation for congression of incorrectly attached chromosomes that are moved to the spindle pole

(A) Experimental scheme to enrich monooriented chromosomes near spindle poles. (B) Diagram for expected chromosome configuration from above drug treatment regime. (C,E) Immunofluorescence after release into MG132. Green, KNL-1; Red, DNA (D) Graph quantifying the percentage of cells with polar chromosomes in DLD-1 cells in which the endogenous has been replaced by either CENP-EWT or CENP-E<T422A> transgene (F) Graph showing the number of polar chromosomes.
Figure 3.13 Model

Accumulation of chromosomes at the spindle pole is a common mechanism to efficiently achieve chromosome biorientation: (1) Chromosomes laterally associated with a single astral microtubule are rapidly transported toward the spindle pole in dynein-dependent manner, (2) Activated by Aurora B kinase, a process of correcting attachment errors also involves poleward chromosome movement coupled to selective disassembly of kinetochore microtubules. (3) CENP-E is fully phosphorylated by Aurora A (and possibly Aurora B) when chromosomes are near the spindle pole. (4) Phosphorylation of CENP-E may be required for releasing chromosomes from the densely populated microtubules around each spindle pole and may be an important mechanism to select bundles of microtubules to power chromosome congression to the metaphase plate.
Chapter 3, in part, is in preparation for publication of the material, Kim Y., Holland A.J., Lan, W., and Cleveland D.W. The dissertation author and Andrew Holland contributed equally to this work.
Chapter 4: Conclusions and future directions

4.1 Properties and phospho-regulation of CENP-E

4.1.1 CENP-E as a motile kinetochore tether

Using purified recombinant CENP-E, I have established that CENP-E is a highly processive, plus end-directed kinesin. The motor properties of CENP-E support the idea that CENP-E can tow mitotic chromosomes along adjacent kinetochore fibers so as to deposit them at the metaphase alignment (Kapoor et al., 2006). While duplicated chromosome pairs already near the cell center can align without CENP-E, it is essential especially for chromosomes initially far from spindle equator. CENP-E motility also contains an element of one-dimensional diffusion, which may increase the residence time of CENP-E on the microtubule lattice by maintaining a weak binding state to microtubules. The ability to diffuse along the microtubule lattice is shared by a broad class of microtubule-binding proteins and probably plays an important role in keeping the dynamic linkage between kinetochore and spindle microtubules.

Perhaps the most unexpected finding was the unusually long (230 nm on average), highly flexible coiled-coil domain of CENP-E. Previously, CENP-E has been conceptualized as a rigid rod that signals the attachment status of the kinetochore to the mitotic checkpoint machinery. Indeed, the biochemical demonstration that presence of microtubules abolished the kinase activity of BubR1, which is supposedly activated by CENP-E, has led to a very attractive model that microtubule capture by CENP-E silences the checkpoint signaling produced by BubR1 (Mao et al., 2005). With the discovery of
highly flexible, discontinuous coiled-coil of CENP-E, however, it remains unclear whether and how the microtubule capture by CENP-E motor domain can transduce such a signal through its very long and floppy coiled-coil to the opposite end of the molecule.

Nevertheless, the shape of CENP-E has shed light on multiple aspects of its function in capturing spindle microtubule by kinetochores. First of all, CENP-E can work advantageously for the initial capture of kinetochores by searching a large radial volume in cells. Also, the highly flexible coiled-coil allows versatile configurations of CENP-E binding to microtubules, thereby permitting kinetochore attachments to microtubules approaching from a wide range of angles. Recent use of electron tomography has shown that the vertebrate kinetochore is composed of a fibrous protein network with multiple microtubule interactions (Dong et al., 2007; McIntosh et al., 2008). Interestingly, the outer kinetochore seems to contain slender fibrils directly connecting to curved microtubule protofilaments (McIntosh et al., 2008). Although the molecular identity of those kinetochore fibrils is not known, it is worth pointing out that most of the outer kinetochore proteins implicated in microtubule-binding do have elongated shapes with abundant coiled-coil structures. CENP-E, with its 230-nm length, is likely to be one of the longest fibers extending out from kinetochore to capture microtubules, and my evidence supports the proposal that CENP-E functions as a motile, flexible, molecular Velcro at the kinetochores.
4.1.2 Direct control of CENP-E by Aurora kinases to promote chromosome biorientation while keeping the kinetochores error free

I have demonstrated that Aurora-mediated phosphorylation of CENP-E at a single conserved site close to the motor domain regulates its intrinsic motor properties, and is essential for chromosome congression during mitosis. Phosphorylation of CENP-E at T422 reduces its affinity for microtubules, and phosphorylated CENP-E requires a higher concentration of microtubules to be activated to its maximum ATPase rate. This is counterintuitive since the phosphorylation of CENP-E, which makes CENP-E less productive as a transport motor, is essential for powering chromosome congression to the metaphase plate. However, the exact same properties of CENP-E which can facilitate microtubule capture by kinetochores (its reach and flexibility), if not regulated, may also contribute to erroneous attachment by promiscuously grabbing microtubules in the proximity of any kinetochore. Indeed, the frequency of lagging chromosomes has been reported to be significantly increased in cells recovering from a mitotic block (by nocodazole treatment), possibly due to abnormal kinetochore expansion and the curved kinetochore morphology that is induced by a long term blockage of microtubule assembly (Cimini et al., 2001).

Intriguingly, the error correction process for syntelic attachment shares a common feature with that of normal chromosome congression. Activated by Aurora B, mal-oriented chromosomes initially move to the spindle pole by selective disassembly of kinetochore microtubules (Lampson et al., 2004). Thus, collecting chromosomes to the spindle pole seems to be an important mechanism to achieve chromosome biorientation
not only for normal chromosome congression but also for correcting errors. To that, I have provided evidence that phosphorylation of CENP-E is essential for congression of polar localized chromosomes to the spindle equator. Since the phosphorylation of CENP-E is contributed to both by Aurora A and B, I speculate that CENP-E reaches its fully phosphorylated state when chromosomes are near at the spindle poles, most likely as a target of Aurora A. Perhaps reducing microtubule affinity of CENP-E by phosphorylation is an important mechanism to release chromosomes from the densely populated microtubules around each spindle pole and also to preferentially select the bundles of kinetochore microtubules to glide along.

4.2 Potential of CENP-E in regulating microtubule dynamics

4.2.1 CENP-E forms a nucleotide-sensitive complex with free tubulin

Besides powering unidirectional movement along microtubules, several kinesin motors play critical roles in regulating microtubule dynamics. The best example is immotile microtubule depolymerizing enzyme Kinesin-13 which targets both ends for destabilization (Desai et al., 1999b). A common feature of those kinesins is that they form a nucleotide-sensitive complex with free α- and β-tubulin heterodimers, a biochemical property that is also shared by XMAP215, which promotes microtubule polymerization (Al-Bassam et al., 2006; Brouhard et al., 2008). Interestingly, the majority of motors known to regulate microtubule dynamics is mitotic kinesins and localized to specialized structures facing dynamic microtubules, such as kinetochores, spindle poles and chromosome arms.
The observation that CENP-E stays at the plus end of microtubules once it reaches there using its plus end-directed motility (Figure 3.10C) suggests a potential role of CENP-E in regulating microtubule dynamics. As an initial effort to test the hypothesis that CENP-E directly affects microtubule dynamics, I used gel filtration chromatography to examine the interaction between CENP-E and free tubulin in different nucleotide conditions. Surprisingly, CENP-E motor domain (aa 1-473) was found to form a 1:1 stoichiometric complex with free tubulin in the presence of AMP-PNP, but not in the presence of ATP (Figure 4.1). Although preliminary, this result suggests that CENP-E has a potential to regulate aspects of microtubule dynamics possibly by binding free tubulin during its ATPase cycle while interacting with microtubule lattice as a kinesin motor. I am now in the early stage of determining whether CENP-E affects microtubule dynamics and, if it does, how CENP-E regulates growth or shrinkage of microtubules in collaboration with Dr. Benjamin Vitre. Experiments to measure parameters of dynamic instability, using centrosome-nucleated microtubules with or without CENP-E in vitro will provide answers to these questions. Furthermore, visualizing individual molecules of CENP-E interacting with dynamic microtubules by TIRF microscopy will give great mechanical insight into how CENP-E regulates plus end microtubule behavior once it reaches there by using its processive motility.

4.2.2 CENP-E does not affect the overall microtubule poleward flux in spindles assembled in Xenopus extracts

I originally attempted to determine whether CENP-E has roles in regulating microtubule dynamics in the context of mitotic spindle assembly using Xenopus extracts.
Displacing endogenous CENP-E from the kinetochores of pre-assembled spindles (Figure 4.2A and B) by antibody addition resulted in chromosome misalignment, a result suggesting that CENP-E is required for maintenance of the metaphase chromosome alignment in this system (Figure 4.2C). Interestingly, the spindle was also found to be elongated in CENP-E inhibited extracts along with the amount of antibody added (Figure 4.2D; n = 30 spindles each). Spindle length is known to be controlled by multiple mechanisms, most of which are probably still unknown, but it must reflect a force balance within its complex molecular machine. A longer spindle seen in CENP-E inhibited extracts suggests that disruption of CENP-E tips the balance of forces, mostly likely toward the reduction of pulling forces by the kinetochore microtubules. With reduced pulling force, less inward tension is generated between two sister kinetochores, thereby failing to maintain a tight spindle structure.

To test this hypothesis, I imaged microtubule poleward flux in the spindle assembled in *Xenopus* extracts with fluorescence speckle microscopy. The speckles on microtubules were generated by introducing a low concentration of X-rhodamine-labeled tubulin (5-8 μg/ml) and kinetochores were labeled with Alexa 488-labeled anti-human CENP-F antibody to differentiate the kinetochore microtubules from bunches of non-kinetochore microtubules (Figure 4.2E). Spindle length clearly expanded and collapsing sister kinetochores were frequently observed in time-lapse series of CENP-E inhibited spindles. However, inhibition of CENP-E did not affect the flux machinery in general (Figure 4.2F) with the average velocity of tubulin speckles to be $1.92 \pm 0.78 \mu\text{m/min}$ for control and $1.91 \pm 0.80 \mu\text{m/min}$ for CENP-E inhibited spindles [measured by Matlab-based software which can track individual tubulin speckles (Yang et al., 2008)].
My attempt to determine the role of CENP-E in microtubule plus end dynamics at kinetochores was not successful, unfortunately. Since only 5-10% of the microtubules in a *Xenopus* extract spindle are kinetochore fibers, I was unable to follow these amid the much more abundant non-kinetochore microtubules. Nevertheless, I have shown that depletion of CENP-E from kinetochore does not affect the flux machinery in general, an expected outcome. Thus, an important question - whether CENP-E affects the polymerization dynamics at kinetochore - still remains to be answered.

### 4.2.3 Implication from the interaction of CENP-E with microtubule plus end-tracking proteins

A group of microtubule plus end tracking proteins (+TIPs), as their names imply, accumulate at the plus ends of growing microtubules and have been shown to regulate aspects of microtubule dynamics and microtubule interaction with various cellular structures and signaling factors (Akhmanova and Steinmetz, 2008). During mitosis, most of the +TIPs - APC, CLASP1, CLIP-170, EB1, Lis1, dynactin and MAP215/TOG1, are localized to the outer kinetochores and contribute to establishing kinetochore-microtubule attachment. Recruited to the kinetochore regardless of microtubule attachment status, CLIP-associated protein, CLASP1 regulates microtubule dynamics, particularly, at kinetochores (Maiato et al., 2003). CLASP1 was shown to promote polymerization of kinetochore-bound microtubules in *Drosophila* S2 cells, thus regulating the length of kinetochore microtubules and the position of chromosomes within mitotic spindle (Maiato et al., 2005). CLASP1 physically interacts with HCP-1/2, two CENP-F related proteins in *C. elegans* (Cheeseman et al., 2005), whose counterpart in human has weak
microtubule-binding activity on its own and is required for stable microtubule capture at kinetochore (Bomont et al., 2005; Feng et al., 2006). Interestingly, the C-terminus of CLASP1 was also shown to interact with CENP-E in Xenopus extracts (Hannak and Heald, 2006), suggesting that CLASP1 and CENP-E might function together to tether chromosomes to spindle microtubules.

Several motor proteins, which include cytoplasmic dynein, yeast Kinesin-7s (Kip2 in S. cerevisiae; Tea2 in S. pombe), a minus end-directed Kar3 (S. cerevisiae Kinesin-14) and microtubule-depolymerizing Kinesin-13s (Klp10A and Klp59C in Drosophila), are also found to be enriched at the microtubule plus end (Wu et al., 2006). These motors accumulate at the plus end via direct interaction with +TIPs or through plus end-directed kinesin whose intrinsic motility delivers them to the microtubule plus end (Browning et al., 2003; Maddox et al., 2003c; Mennella et al., 2005; Vaughan et al., 1999). An interesting example is yeast kinesins Kip2/Tea2, which have been shown to target CLIP170 (Cytoplasmic linker protein) homologs (Bik1 in S. cerevisiae; Tip1 in S. pombe) to the plus end using their plus end-directed motilities (Busch et al., 2004; Carvalho et al., 2004). In fission yeast, unlike in budding yeast, accumulation of CLIP-170 at the growing microtubule tip requires one additional component, the EB1 homolog Mal3 (Busch and Brunner, 2004). Indeed, a stable ternary complex consisting of Tea2, Tip1 and Mal3 has recently been reconstituted in vitro, and Mal3 has been shown to facilitate loading of kinesin motor Tea2 and its cargo, the CLIP-170 homolog Tip1, to the growing ends of microtubules (Bieling et al., 2007).
Although a CENP-E homologue is missing in yeast, the fact that the yeast kinesins Kip2/Tea2 belong to the same kinesin family (Kinesin-7) as CENP-E is very intriguing. CENP-E is significantly larger than Kip2/Tea2 with abundant coiled-coil structures, and Kip2/Tea2 contains an N-terminal extension before the motor domain that is not present in CENP-E. However, members of the Kinesin-7 family share a long family-specific neck (Miki et al., 2005) and possess similar motor properties – both of them are slow and processive (Browning and Hackney, 2005; Kim et al., 2008). As CENP-E has recently been identified in a complex with CLASP1 and its binding partner CLIP-170 in *Xenopus* extracts (Hannak and Heald, 2006), it is probable that, similar to yeast plus tip tracking system, CENP-E may cooperate with +TIPs to regulate microtubule dynamics and contribute to dynamic linkage between kinetochore and spindle microtubules. Dr. Benjamin Vitre in the lab has initiated an effort to investigate the interaction between CENP-E and +TIPs with special focus on CLASP1, CLIP-170, and EB1. He will determine the CLASP1-interacting domain in CENP-E using purified components, and examine how CENP-E, together with CLASP1 and other +TIPs, affects microtubule dynamics in vitro.

4.3 Properties of CENP-E under load

As chromosomes are captured by microtubules emanating from opposite poles, tension is developed across sister centromeres against the poleward force that is generated by attached kinetochore microtubules. Two sister kinetochores become stretched as a result of such forces, and therefore, interkinetochore distance has been used as the classical readout for such kinetochore tension. Recent studies suggest, however,
that kinetochores monitor a stretch *within* each kinetochore rather than the distance between two sisters for silencing the mitotic checkpoint to initiate anaphase onset (Maresca and Salmon, 2009; Uchida et al., 2009). Although structural rearrangement at the outer kinetochore has been seen in response to microtubule attachment using electron tomography (Dong et al., 2007), we are far from understanding the nature of molecular rearrangement(s) occurring at kinetochores following microtubule capture.

In recent high resolution fluorescence microscopy-based measurement of the kinetochore protein architecture, CENP-E was one of the proteins that underwent the most dramatic conformational change following taxol treatment at human kinetochores (Wan et al., 2009). Using two antibodies directed against different regions of CENP-E (one against the coiled-coil close to the motor domain (aa 663-973), and the other against the middle of coiled-coil (aa 1571-1859)), Wan et al. have shown that CENP-E changes from a bent configuration in control metaphases, with both its tail and motor domains located near the Ndc80 complex, to an extended conformation in taxol-treated cells. Although the epitopes of these two antibodies are not exactly at the very ends of the CENP-E, this observation seems consistent with the 230 nm-long, highly flexible coiled-coil of CENP-E which allows a myriad of configurations, as I have shown in electron micrographs (Kim et al., 2008). Yet, it is not clear what triggers the tension-dependent conformational change of CENP-E, if any, and more importantly, whether the unusually long and flexible coiled-coil of CENP-E has any functional role in a tension-dependent regulation of kinetochores.
Analogous to a cargo-moving conventional kinesin, CENP-E is connected to its cargo, the kinetochore, through its tail domain and it then moves along the kinetochore-captured microtubule(s). When tension is created between sister kinetochores upon biorientation, CENP-E would experience both impeding and assisting loads, as kinetochores oscillate with abrupt switches between persistent phases of poleward and anti-poleward movement (Skibbens et al., 1993). [As an additional note, since CENP-E is a plus end-directed motor, the poleward tension developed cannot be generated by CENP-E.] To determine the contribution of CENP-E to the load-bearing, kinetochore coupling machinery to dynamic spindle microtubules, I have started a collaboration with Dr. Ekaterina Grishchuk and J. Richard McIntosh at the University of Colorado, Boulder. Using their expertise on optical tweezers and force measurement of polystyrene beads associated with dynamic microtubules (Grishchuk et al., 2005), the biophysical properties of CENP-E elucidating how it behaves under opposing and assisting load will be determined. I have provided purified the GFP-tagged motor fragment (aa 1-473) and full-length CENP-E over the past several months. As expected from a plus end-directed processive kinesin, our initial attempt has demonstrated that CENP-E-coated beads moved along microtubules in the presence ATP (data not shown). A force clamp and a motion controlled stage will now be used to trace the bead and stage motions at different forces applied. We are in the process of collecting data to establish the force-velocity relationship for CENP-E. Continued efforts to investigate the biophysical properties of this essential kinetochore motor will provide clues to unravel the long-standing question of how chromosome movements are coordinated to faithfully transmit genetic information to the next generation.
Figure 4.1 CENP-E forms a nucleotide-sensitive complex with free tubulin

(A) Mixture of 5 µM of CENP-E (aa1-473)-SNAP and 10 µM of tubulin heterodimer in BRB80 buffer containing either 1 mM ATP or 1 mM AMP-PNP was loaded onto Superose 6 gel filtration column. (B) Mixture of 5 µM of CENP-E (aa1-392)-GFP and 10 µM of tubulin heterodimer in BRB80 buffer containing either 1 mM ATP or 1 mM AMP-PNP was loaded onto Superose 12 gel filtration column. The shift for both CENP-E fragments in the presence of AMP-PNP is a change in Stokes radius of approximately 1.1 nm. These are preliminary results.
Figure 4.2 Inhibition of CENP-E does not affect the flux machinery in general

(A) Experimental scheme to measure microtubule flux in *Xenopus* extract spindles. (B) Anti-CENP-E antibody displaced CENP-E from the kinetochores. Scale bar, 10 μm. (C) CENP-E is required for maintenance of chromosome alignment in *Xenopus* extracts. Green, DAPI; Red, X-rhodamine tubulin. (D) Spindle length is increased along with the amount of antibody added (n = 30 spindles each). Error bars are s.d. (E) Speckles on microtubules were generated by introducing low amount of X-rhodamine tubulin and kinetochores were labeled with Alexa488-hCENP-F antibody. Flux rate at kinetochore microtubules measured by analyzing kymographs. (F) CENP-E inhibition does not affect the flux machinery during metaphase in *Xenopus* extract spindles. Automatic speckle tracking showed that the average flux velocity in control was 1.92 ± 0.78 μm/min (number of tracks, 5168) and 1.91 ± 0.80 μm/min (number of tracks, 4036) in CENP-E inhibited spindles.
References


