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Kinetochore structural plasticity and its regulation in ***Saccharomyces cerevisiae***

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School of Life Sciences, 2010

SUBMITTED TO THE SCHOOL OF LIFE, HEALTH, AND CHEMICAL SCIENCES IN PARTIAL FULFILLMENT
OF THE REQUIREMENT FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

THE STOWERS INSTITUTE FOR MEDICAL RESEARCH,
an Affiliated Research Centre of The Open University
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Kinetochore structural plasticity and its regulation in *Saccharomyces cerevisiae*

by

Karthik Dhatchinamoorthy

Submitted to the School of Life, Health and Chemical Sciences, The Open University in partial fulfillment of the requirement for the degree of Doctor of Philosophy in Biology

ABSTRACT:

The kinetochore is a multi-protein complex that connects the chromosome with microtubule and pulls the chromosome during chromosome segregation. In yeast, a single kinetochore assembles on a Cse4 nucleosome and attaches to a single microtubule, making it an ideal system for studying the architecture of kinetochore-microtubule interaction, function, and regulation. The inner kinetochore assembles on the centromere and anchors the outer kinetochore that attaches to the dynamic microtubule. The interplay between the kinetochore structure and microtubule dynamics in a living cell has not been studied.

I have used a combination of in-house developed quantitative microscopy, genetic techniques and mathematical simulation in the budding yeast, *S.cerevisiae*, to elucidate a novel structural transition of the kinetochore. During anaphase, the microtubule undergoes rapid depolymerization. Kinetochore tracking of microtubule during this phase is critical for chromosome segregation, but how this tracking is achieved is not well understood. A combination of FRAP and photoconversion on kinetochore proteins have revealed that the kinetochore consists of highly dynamic sub-modules and stable sub-modules. I show that microtubule dynamics significantly affect the dynamic kinetochore sub-modules but not the stable ones. Mathematical modeling of microtubule dynamics and kinetochore attachment has revealed the importance of kinetochore structural transition for microtubule attachment. In addition, I have discovered that kinetochore structural transitions are regulated by known metaphase-anaphase specific pathways.

Scm3 is the chaperone responsible for assembling the centromeric nucleosome that contains a specific histone variant Cse4. I helped to identify and characterize a new chaperone for Cse4. I demonstrated that recombinant yCAF-1 can promote the assembly of Cse4 nucleosomes *in vitro* and at non-centromeric positions, causing problems for chromatin-based processes.

In summary, my thesis work has significantly contributed to our understanding of centromere and kinetochore function in budding yeast. I speculate that many of the findings will inform our perspectives on centromere and kinetochore function in higher eukaryotes.

Director of studies: Jennifer.L.Gerton

Title: Investigator

Dedicated to Mano and Sneha…

"Imagination is more important than knowledge."-*Albert Einstein*

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∞

CHAPTERS

Abstract 3

Personal Statement 5

Acknowledgements 7

Chapter 1 Introduction to yeast kinetochore structure, function and regulation in cell cycle 15

Chapter 2 Structural plasticity of the living kinetochore 71

Chapter 3 Regulation of kinetochore structural transitions 131

Chapter 4 Sequence-independent centromeric nucleosome assembly by CAF-1 complex 157

Chapter 5 Conclusion and future directions 183

LIST OF FIGURES

Figure 1.1: CDKs and cyclins over the cell cycle. 21
Figure 1.2: Kinetochore capture during metaphase. 25
Figure 1.3: Modes of kinetochore-microtubule attachments on the spindle. 26
Figure 1.4: Spindle assemble checkpoint recruitment and activation 28
Figure 1.5: FEAR and MEN pathways in S.cerevisiae. 30
Figure 1.6: Mitotic spindle in budding yeast 34
Figure 1.7: Centromere and centromeric nucleosome. 36
Figure 1.8: Kinetochore organization in budding yeast. 40
Figure 1.9: Budding yeast kinetochore-microtubule interaction site. 46
Figure 1.10: Microtubule is a dynamic structure and modulated by many factors. 53
Figure 1.11: Conformational wave model for the kinetochore function. 54
Figure 1.12: Biased diffusion model of kinetochore-microtubule attachment. 56
Figure 2.1: The intensity of some kinetochore sub-modules increases in anaphase. 81
Figure 2. 2: Subunits in the MIND and Ndc80 subcomplex increase in intensity during anaphase whereas the intensity of a subunit in the Dam1 subcomplex does not. 83
Figure 2.3: The kinetochore has higher copy numbers of Ndc80 and MIND subcomplexes in anaphase while the Dam1 complex has similar copy number throughout the cell cycle. 85
Figure 2.4: Quantification of FRAP shows subunits of the COMA, MIND and Ndc80 subcomplexes recover in anaphase but subunits of the Dam1 subcomplex do not. 88
Figure 2.5: Subunits of the Ndc80 and MIND subcomplexes recover in FRAP whereas subunits of the Dam1 subcomplex do not. 90
Figure 2.6: Quantification of FRAP shows subunits of the COMA, MIND and Ndc80 subcomplexes recover in anaphase but subunits of the Dam1 subcomplex do not. 92
Figure 2.7:Ndc80 and MIND subcomplexes add new copies during anaphase while Dam1 does not.94
Figure 2.8: Quantification of photoconversion experiments for outer kinetochore proteins. 96
Figure 2.9:Photoconversion of Dsn1-tdEos in the nucleoplasm reveals copy addition during anaphase. 98
Figure 2.10: A mutation in STU2 reduces the addition of Ndc80 subunits in anaphase. 99
Figure 2. 11: Motor proteins affect the kinetochore copy number in anaphase. 102
Figure 2.12: Simulations using Hill’s biased diffusion model 105
Figure 2.13: Ndc80 increase during anaphase is observed in S. pombe. 107
Figure 2.14: Quantification of kinetochore proteins in stu2-11, cnn1Δ mutants (S. cerevisiae) and in S. pombe. 108
Model 2.15: Kinetochore plasticity during cell cycle. 125
Figure 3.1: Metaphase tension does not affect the kinetochore copy number. 142
Figure 3.2: Ipl1 is important for chromosome segregation but it does not affect the Ndc80 copy number addition in anaphase. 144
Figure 3.3: Anaphase promoting complex does affect the Ndc80 addition in metaphase-anaphase transition. 145
Figure 3.4: FEAR and MEN pathways arrest the kinetochore in anaphase-state (high copy number) 147
Figure 3.5: Cytokinesis is not required for the anaphase-state kinetochore. 148
Model 3.6: Regulation of kinetochore structure by APC and MEN. 152
Figure 4.1: CAF-1 subunits interact with Cse4. 167
Figure 4.2: CAF-1 can assemble Cse4 nucleosomes in vitro. 169
Figure 4.3: When Scm3 is absent and Cse4 levels are high, CAF-1 may help target Cse4 to the centromere. 172

Figure 4.4: CAF-1 complex could be a potential chaperone for Cse4 in anaphase. 173
Model 4.5: CAF-1 complex assembles centromeric nucleosome without any sequence specificity. 176
Model 4.6: Proposed model for the CAF-1 complex in centromeric nucleosome assembly. 177
Figure 5.1: Anaphase kinetochore purification from cdc15-1. 194

LIST OF TABLES

Table 1.1: Evolutionary conservation of budding yeast kinetochore 40
Table.1.2. Copy number of kinetochore complex proteins per attachment (kinetochore). 58
Table 2.S1: Strains used in this study 120
Table 2.S2: Comparison of kinetochore copy number in anaphase. 122
Table 3.S1: Strains used in this study 154
Table 3.S2: List of mutants screened for the kinetochore structural transition defects...………… 154

Table:4.S1: Strains used in this study 179

CHAPTER 1

Introduction to yeast kinetochore structure, function, and regulation in cell cycle.

CHAPTER 1

Chapter 1 15
Introduction to yeast kinetochore structure, function and regulation in cell cycle. 15
Introduction 19
  Cell cycle 19
    G1 21
    S phase 21
    G2 22
    Metaphase 23
      Ipl1/Aurora B 25
      Spindle Assemble Checkpoint 27
      Anaphase Promoting Complex (APC) 27
    Anaphase/Telophase 28
      Mitotic exit 29
      Cdc Fourteen Early Anaphase Release (FEAR) 30
      Mitotic Exit Network 30
    Cytokinesis 31
  Cell cycle and kinetochore 32
  Kinetochore 33
    Centromeric region 37
      Cse4 37
      Cse4 chaperones 37
    Budding yeast kinetochore complex 38
    Inner kinetochore sub-modules 41
      Cbf3 41
      CCAN 42
      COMA complex 42
    Outer kinetochore 43
      Spc105 complex 44
      Mis12/Mtw1/MIND complex 44
      Ndc80 complex 45
      DAM1/DASH/DDD complex 46
      +TIPs 46
      Kinesins 47
  Kinetochore dynamics 51

  - Microtubule 51
  - Mode of kinetochore-microtubule interaction 53
    - Conformational wave model 53
    - Biased diffusion model 55
- Kinetochore architecture: 57
- Summary 59
- REFERENCES: 60

Introduction

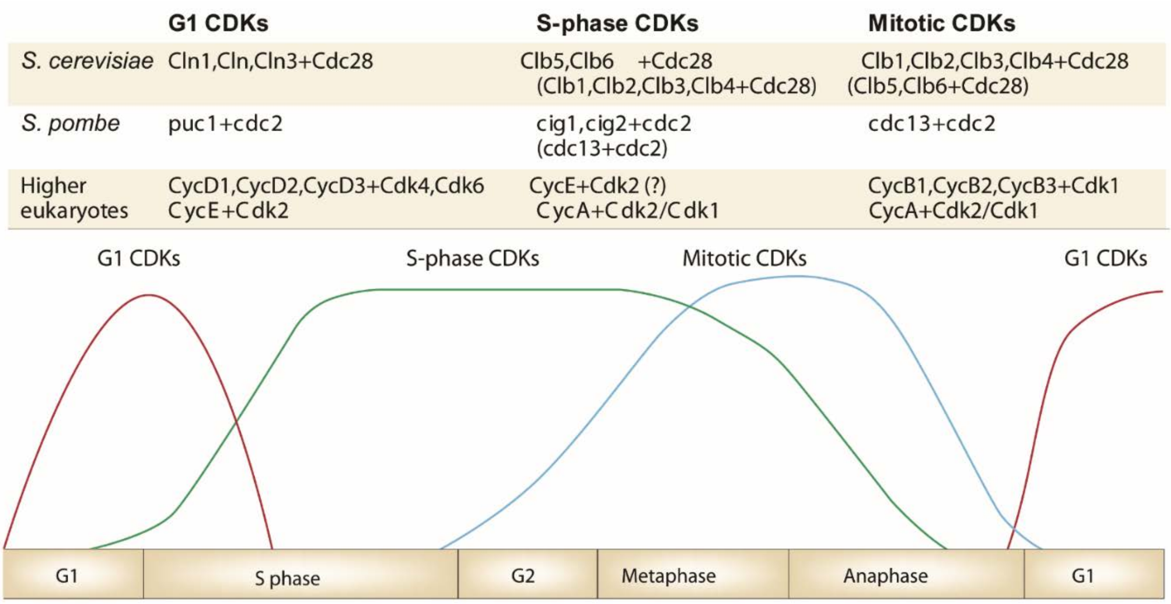
Successful propagation of living organisms depends on the faithful segregation of equal sets of chromosomes. Error in this process results in aneuploidy and birth defects (Pfau and Amon 2012). Understanding the chromosome segregation is critical. Microtubules (MT) emanate from the spindle pole body (SPB) (microtubule organizing center) and interact with the chromosomes through a mega-Dalton protein complex called the kinetochore (KT). Kinetochores, microtubules, and SPBs - along with the chromosomes - form a 'spindle-like' structure which is important for proper chromosome capture and alignment during segregation. The kinetochores assemble on a specialized region on the chromosome called the centromere. In *Saccharomyces cerevisiae* (budding yeast), the centromere sequence spans ~125 bp and is predefined. In yeast, each chromosome interacts with a single microtubule (Winey et al. 1995), making it an ideal system to study kinetochore behavior. In my thesis work, I investigated the structure of the kinetochore complex over the cell cycle, especially during G1, metaphase, and anaphase. In chapter 1, I'll start by introducing the major players in the cell cycle transition. Next, I will review what is known about the kinetochore and the centromere in budding yeast. I finish the chapter by discussing microtubule dynamics and propose a model for kinetochore-microtubule interaction. Chapters 2 and 3 present data to elucidate the structural plasticity of the yeast kinetochore. Chapter 4 presents experiments done to understand how the centromeric nucleosome is assembled by a histone chaperone. In the final chapter, I conclude my thesis work and present future directions based on my work.

Cell cycle

The cell cycle is an essential, complex, and well-choreographed event for equal partition of correctly duplicated sister chromatids. Several cyclins and cyclin dependent kinases (cdk) along with check point proteins make sure that genome is accurately duplicated and segregated into daughter cells. Cyclin dependent kinases are regulated by the quantities of activators (cyclins) or inhibitors and mark the different cell cycle phases. There are 4 major stages of the cell cycle, namely, G1 (Gap1- preparation phase), S (Synthesis phase- DNA duplication), G2 (Gap2- to complete the DNA replication) and M (Mitotic phase) (Morgan 2007).

Budding yeast has a major CDK called "Cdc28" that interacts with ~9 cyclins (3 Clns family and 6- b-type Clb cyclins) (Andrews and Measday 1998) to demark the different phases of the cell cycle (Fig.1.1) (Morgan 2007). In higher eukaryotes, multiple CDKs interact with a wide variety of cyclins over the cell cycle. CDK has different binding sites for the activators and inhibitors that regulate its function. CDK interacts with cyclins, and together it can phosphorylate multiple targets, controlling their functions. Most of the cyclins have redundant function, such that single deletion of a cyclin may still produce viable cells but multiple deletion of cyclins may make the yeast inviable (Richardson et al. 1989).

The kinetochore assembles on the centromeric region of a chromosome and connects to the microtubule. In human cells, during mitosis, the kinetochore assembles dynamically on the centromeric region after nuclear membrane break-down (Gascoigne and Cheeseman 2013). Mif2/CENP-C, an inner kinetochore protein, stably associates with the centromere throughout the cell cycle. In G2, Mis12/MIND binds to CENP-C to start the kinetochore assembly. During mitosis, nuclear membrane break-down allows the Ndc80 complex to bind the kinetochore. The kinetochore disassembles in mitotic exit and is regulated by Cdk1 (Gascoigne and Cheeseman 2013). Even though each phase has multiple CDK targets to start or stop the phase, little is known about their role in kinetochore dynamics. In this thesis work, I have shown evidence (Chapter 3) for regulation of kinetochore dynamics during cell cycle stages. Here I will review major cyclins and CDKs involved in the cell cycle transitions and their known roles in kinetochore assembly.



	G1 CDKs	S-phase CDKs	Mitotic CDKs
S. cerevisiae	Cln1,Cln,Cln3+Cdc28	Clb5,Clb6 +Cdc28 (Clb1,Clb2,Clb3,Clb4+Cdc28)	Clb1,Clb2,Clb3,Clb4+Cdc28 (Clb5,Clb6+Cdc28)
S. pombe	puc1+cdc2	cig1,cig2+cdc2 (cdc13+cdc2)	cdc13+cdc2
Higher eukaryotes	CycD1,CycD2,CycD3+Cdk4,Cdk6 CycE+Cdk2	CycE+Cdk2 (?) CycA+Cdk2/Cdk1	CycB1,CycB2,CycB3+Cdk1 CycA+Cdk2/Cdk1

Figure 1.1: CDKs and cyclins over the cell cycle.

Budding yeast has 3 G1-specific cyclins (Cln1, Cln2 and Cln3). Cln1 and Cln2 accumulate over the cell cycle by low-levels of Cln3. In late G1, S-phase CDKs begin to increase and are activated in S-phase. In G2 and mitosis, S-phase CDKs are inhibited by S-phase cyclins degradation. In S-phase, critical concentration of CDKs and cyclins initiate DNA replication. Clb5 and Clb6-associated CDKs are responsible for DNA replication in yeast. Mitotic cyclins can also support DNA replication, in the absence of S-phase cyclins. Mitotic CDKs can initiate chromosome condensation, spindle formation and nuclear breakdown (Bardin and Amon 2001).

G1

The cell cycle starts with a single copy of the genome in Gap1. G1 cells sense multiple inputs like nutrient availability, mating, and stress conditions before deciding to progress to the next phase (Andrews and Measday 1998; Mendenhall and Hodge 1998). First, Cln3 binds to Cdc28 and starts the transcription of Cln1 and Cln2 by promoting the nuclear import of phosphorylated transcription factor Whi5 (Polymenis and Schmidt 1999; Levine, Huang, and Cross 1996; Tyers et al. 1992; Costanzo et al. 2004; de Bruin et al. 2004). In late G1, accumulation of Cln2/3 activates Clb5 and Clb6 to start the processes of DNA replication and duplicated spindle pole bodies (SPB) separation (Andrews and Measday 1998). Budding yeast has a closed mitosis, meaning the nuclear membrane does not break during mitosis.

Because of this, the kinetochore can interact with the SPB throughout the cell cycle. In G1, the kinetochore cluster stays near the SPB and is disrupted with microtubule drugs, indicating that kinetochores are still attached to the SPB (Kitamura et al. 2007). Although kinetochores stay clustered, green fluorescent protein (GFP) tagged kinetochore proteins are slightly diffused but form a sub-diffraction limited spot, which can be ideal for quantitative microscopy like image calibration.

S phase

The presence of Sic1, a Cdc28-Clb5/Cdc28-Clb6 inhibitor, suppress the activation of the origin recognition complex (ORC) in G1 (Schwob et al. 1994). Towards the end of G1, degradation of Sic1 by Cln1/2-CDK mediated phosphorylation, relieves the inhibition of S phase cyclins and initiates the firing of the pre-replication complex (Pre-RC) (Toone et al. 1997). Deletion of CLB5 and CLB6 does not hinder the initiation of DNA replication as multiple B-type cyclins can initiate DNA replication (Schwob et al. 1994). CDKs with S-phase cyclins ensures the initiation of DNA replication once per cell cycle (Arias and Walter 2007; Toone et al. 1997).

During S-phase, the yeast kinetochore detaches from the microtubule for ~5 mins to complete the replication of the centromere (Kitamura et al. 2007). The kinetochore is then reassembled on the newly replicated centromeres to interact with microtubules from the duplicated SPB (microtubule organizing center). Microtubules from the SPB attach to the kinetochore through a ‘search and capture’ mechanism. First, the kinetochore attaches laterally and later is converted into end-on attachment for more stability as the microtubule depolymerizes (Tanaka 2010). To ensure segregation of identical sister chromatids, a ring-like structure known as the cohesin complex holds it together to create tension between sisters that facilitates bi-orientation on the spindle. Cohesin is loaded on to the chromosomes during G1 by Scc2-Scc4 complex (Fernius et al. 2013).

G2

In G2, the cell waits for the completion of DNA replication and repairs the DNA damage, if any, before entering mitosis. This is achieved by inhibitory phosphorylation on Tyrosine 15 (Tyr15) of Cdc2 in *S. pombe* (Gould and Nurse 1989) and humans (Stark and Taylor 2006; Krek and Nigg 1991; Heald, McLoughlin, and McKeon 1993). In *S. pombe,* mutation of Tyr15 prevents the cell from G2 arrest. However, in budding yeast, G2 arrest is not regulated by the phosphorylation of the Tyr15 (Keaton and Lew 2006), rather, budding yeast lacks typical G2 and G2/M transitions.

Metaphase

In higher organisms, metaphase is preceded by prophase where condensin I, another ring-like complex, condenses the chromosome and assembles the Spindle Assembly Checkpoint proteins (SAC) (Morgan 2007). Followed by prophase, the nuclear membrane breaks down and the kinetochore is captured by a microtubule, marking the prometaphase. In metaphase, oscillating chromosomes congress to the cell equator or middle of the cell. Budding yeast lacks most of the pre-metaphase stages as it has smaller chromosomes and the cell cycle is within a closed nuclear membrane without forming the metaphase plate.

Before entering a checkpoint-free anaphase, metaphase kinases and phosphatases make sure that each chromosome is correctly connected to the microtubule. Spindle assembly checkpoint (e.g. Mps1) and Aurora B/Ipl1 modulate the kinetochore affinity by increasing the affinity for a correct connection and reducing it for an incorrect connection. A major deciding factor for appropriate kinetochore-microtubule connection is the tension between the sister chromatids. Sister chromatids need to attach to the microtubules from two oppositely placed SPBs (centrosome equivalent in yeast) to bi-orient (amphitelic) and create tension (Fig.1.2). Error in this process can be syntelic where sister chromatids interact with microtubules from the same SPB, monotelic where only one sister chromatid attaches to one SPB, or merotelic (higher eukaryotes) where one of the sister chromatids attaches to both SPBs. Aside from merotelic attachment, the rest of the attachments can lead to spindle assembly checkpoint activation (Fig.1.3) (Biggins 2013).

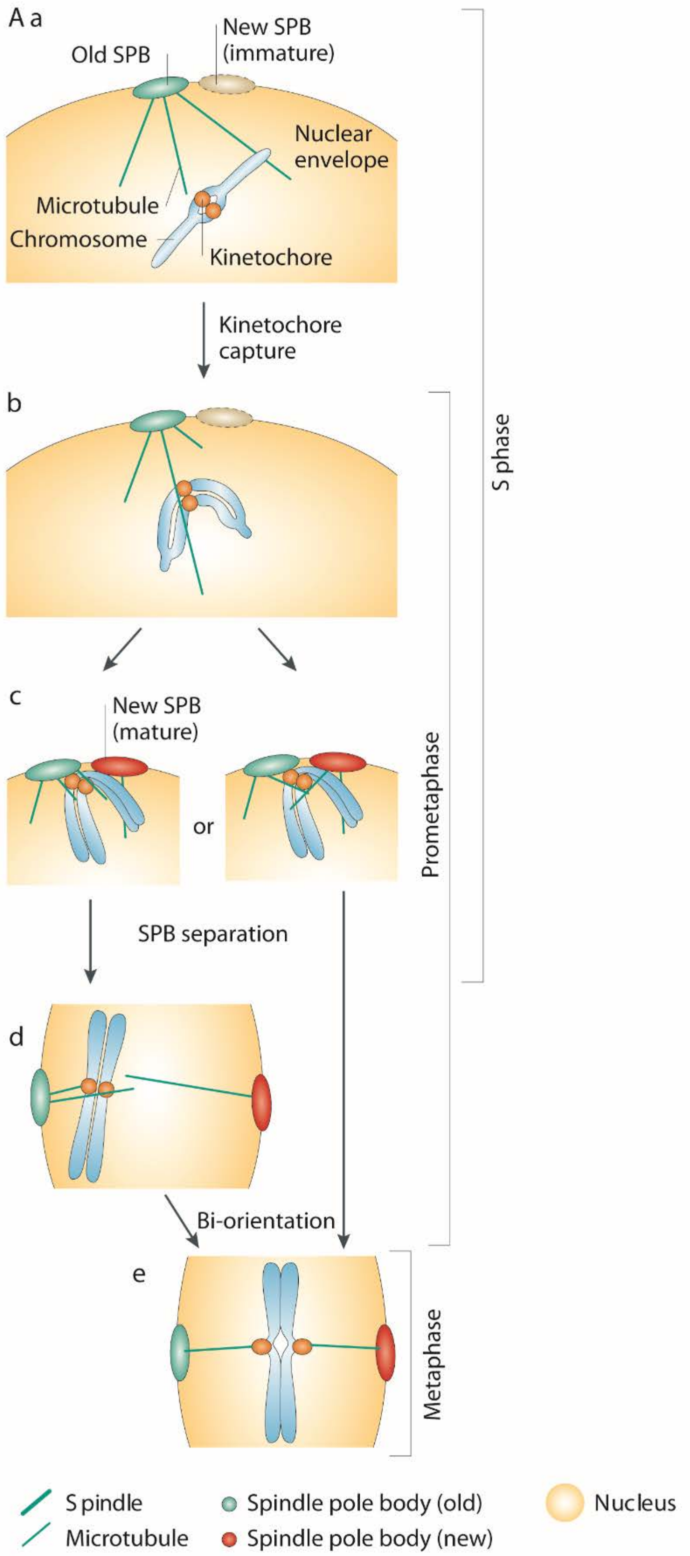

A a
Old SPB
New SPB (immature)
Nuclear envelope
Microtubule
Chromosome
Kinetochore
Kinetochore capture
b
c
New SPB (mature)
or
SPB separation
d
Bi-orientation
e
S phase
Prometaphase
Metaphase
Spindle
Microtubule
Spindle pole body (old)
Spindle pole body (new)
Nucleus

Figure 1.2: Kinetochore capture during metaphase.

In S-phase old mature SPB duplicates and produces immature SPB. In early S-phase, the centromere region replicates and disassembles the kinetochore. Soon after the centromere replication, the kinetochore assembles on the old and newly replicated centromere. Both the kinetochores interact with the old mature SPB. At the end of S-phase, the new SPB matures and initiates the microtubule polymerization. Now the kinetochores interact with the microtubules from both SPBs. Initially the kinetochore attaches to the microtubule through lateral attachment and it changes into end-on, a more stable attachment. Maturation of the new SPB subsequently separates the SPBs to form a bipolar spindle and bi-orients the chromosomes (Tanaka, Stark, and Tanaka 2005).

Ipl1/Aurora B

For the proper segregation of the sister chromatids in anaphase, the kinetochore must be attached to the microtubules and generate tension through that interaction. Ipl1/Aurora B kinase along with Bir1 (Survivin) and Sli15 (INCENP), localizes to the kinetochore from early S phase to the metaphase-anaphase transition and play a major role in detecting and detaching the syntelic kinetochore attachment (He et al. 2001; Tanaka et al. 2002). Ipl1 and Sli15 temperature-sensitive mutants have mono-oriented attachments with single SPBs. Inactivation of the kinetochore protein Dam1 after bi-polar attachment, cannot establish and maintain the bi-orientation attachment (Tanaka et al. 2002). However, similar experiments on Ipl1 mutants could maintain the bi-polar attachment, suggesting that Ipl1 is important for establishing bipolarity and acts early in the cell cycle to attach the microtubule from the newly replicated SPB (Tanaka et al. 2002). Ipl1 phosphorylates Cse4, Ndc80, and Dam1 to reduce the affinity of the kinetochore for the microtubule and enables bipolar attachments to silence the SAC (Biggins et al. 1999; Cheeseman et al. 2002). It is not clear how Ipl1-mediated phosphorylation leads to the kinetochore detachment or what its other direct targets are.

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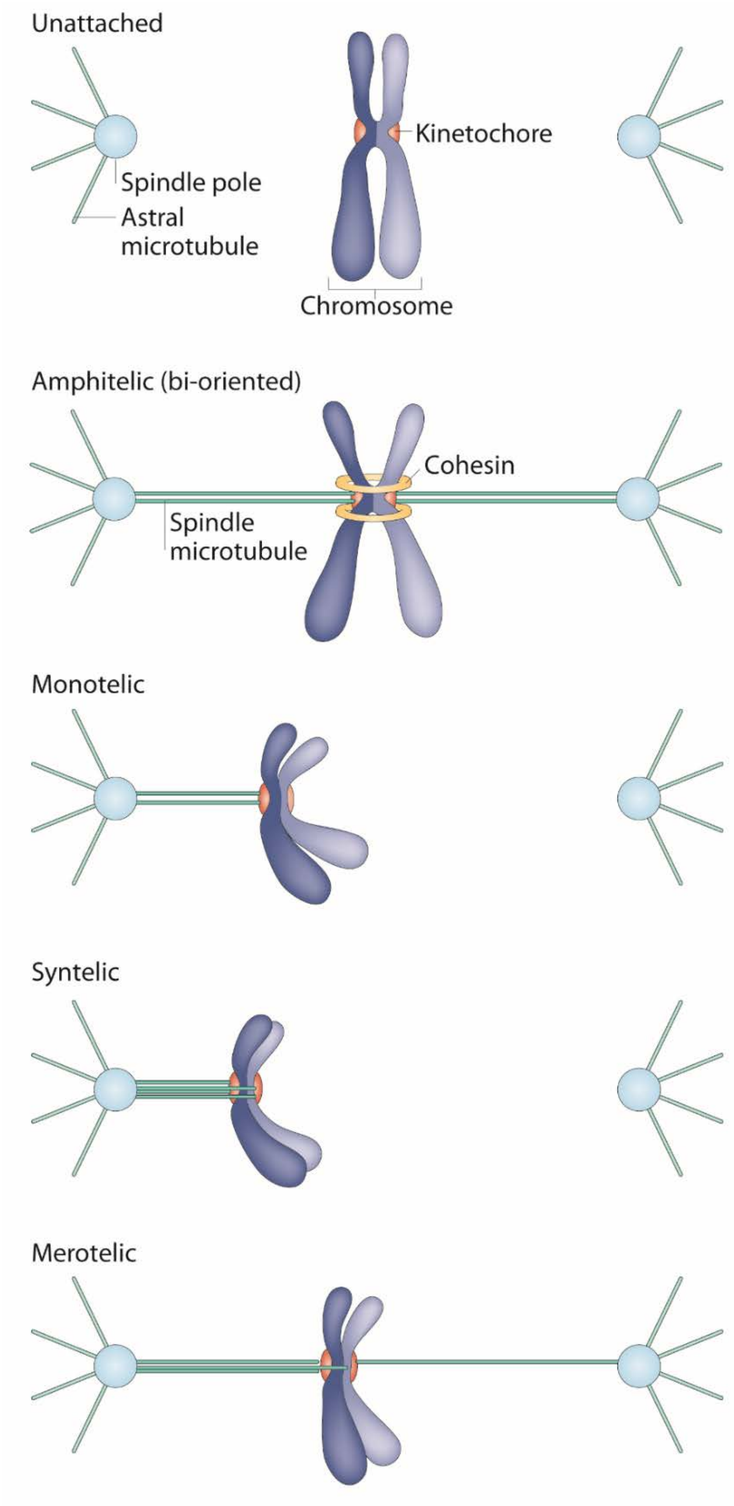
Unattached
Kinetochore
Spindle pole
Astral microtubule
Chromosome
Amphitelic (bi-oriented)
Cohesin
Spindle microtubule
Monotelic
Syntelic
Merotelic

Figure 1.3: Modes of kinetochore-microtubule attachments on the spindle.

Presence of two SPBs can create multiple attachment modes between microtubule and kinetochore. The constructive interaction is amphitelic or bi-orientation where microtubules from opposite SPBs interacts with sister chromatids. In monotelic attachment, the kinetochore only interacts with a microtubule from one SPB and the other kinetochore is unattached. In syntelic attachment, both kinetochores interact with a single SPB. In merotelic, a single kinetochore attaches to both SPBs. Syntelic and monotelic attachments can activate the spindle assembly checkpoint. However, merotelic attachment produces tension and can not necessarily activate the checkpoint (London and Biggins 2014).

.

Spindle Assemble Checkpoint

Although Ipl1 can resolve most of the syntelic attachment errors, other kinds of errors are detected by a second group of check point proteins called spindle assembly checkpoints that pause the metaphase-anaphase transition. A genetic screen in budding yeast identified several proteins involved in the SAC like Mps1, Mad1, Mad2, Mad3, Bub1 (Weiss and Winey 1996; Li and Murray 1991), and Bub3 (Hoyt, Totis, and Roberts 1991). The SAC is a very sensitive network as even a single unattached kinetochore can activate the checkpoint (Ault et al. 1991; Ault and Rieder 1992). Unlike in higher eukaryotes, most of the SAC proteins are non-essential in budding yeast. Bub1 and Bub3 are localized on the metaphase kinetochore while Mad1 binds only to the unattached kinetochore (Gillett, Espelin, and Sorger 2004; Hoyt, Totis, and Roberts 1991; Li and Murray 1991). The signal for Mad1 binding on the kinetochore is unknown. The SAC is best explained by the Mad2 template model, where the kinetochore bound Mad1 recruits Mad2 to the kinetochore causing it to undergo a conformational change to Mad2-C (Mad2-Closed). Kinetochore bound Mad2-C promotes the binding of Mad2-O (Mad2-open) and its conversion to Mad2-C for signal amplification (Fig.1.4) (London and Biggins 2014).

Anaphase Promoting Complex (APC)

Anaphase Promoting Complex/Cyclosome (APC/C) commits the highly regulated and irreversible metaphase-anaphase transition to segregate the genome equally into two daughter cells. APC/C is essential for cyclin destruction in telophase and was discovered as an ubiquitin ligase (Peters 2006; King et al. 1995; Sudakin et al. 1995). DNA damage and SAC inhibit the APC from initiating cohesin cleavage. Phosphorylation of Securin/Pds1 by the DNA checkpoint kinase Chk1, blocks it from APC-mediated proteolysis (Harrison and Haber 2006) and inhibits the metaphase-anaphase transition. Mutations in the phosphorylation site of Securin/Pds1 stabilizes the complex and increases the DNA damage sensitivity. In a parallel pathway, Rad53, a DNA damage kinase, inhibits the association of APC-C^{Cdc20} with Securin/Pds1 and blocks the metaphase-anaphase transition. Mec1, a downstream protein of Chk1 kinase pathway, activates PKA (cAMP-dependent kinase) which in turn phosphorylates Cdc20 preventing its association with Separase/Pds1 (Harrison and Haber 2006).

An unattached kinetochore activates the SAC by accumulating Mad2-C on the kinetochore (Hwang et al. 1998; Li and Murray 1991), that further can bind and sequester Cdc20 for stabilization. APC-C^{Cdc20} stabilization further stabilizes the Securin/Pds1 and stops the metaphase-anaphase transition. *In vitro* studies show that Cdc28-Clb2 phosphorylates multiple sites on most of the APC-C^{Cdc20} subunits, however, these sites are not important for APC function *in vivo* (Rudner and Murray 2000). APC-C^{Cdc20} blocks the metaphase-anaphase transition through cleavage of the cohesin ring. Cleaving the cohesin ring by inserting the TEV site within Scc1/Mcd1 and expressing TEV protease during metaphase, circumvents the Cdc20 requirement for the anaphase entry (Uhlmann et al. 2000).

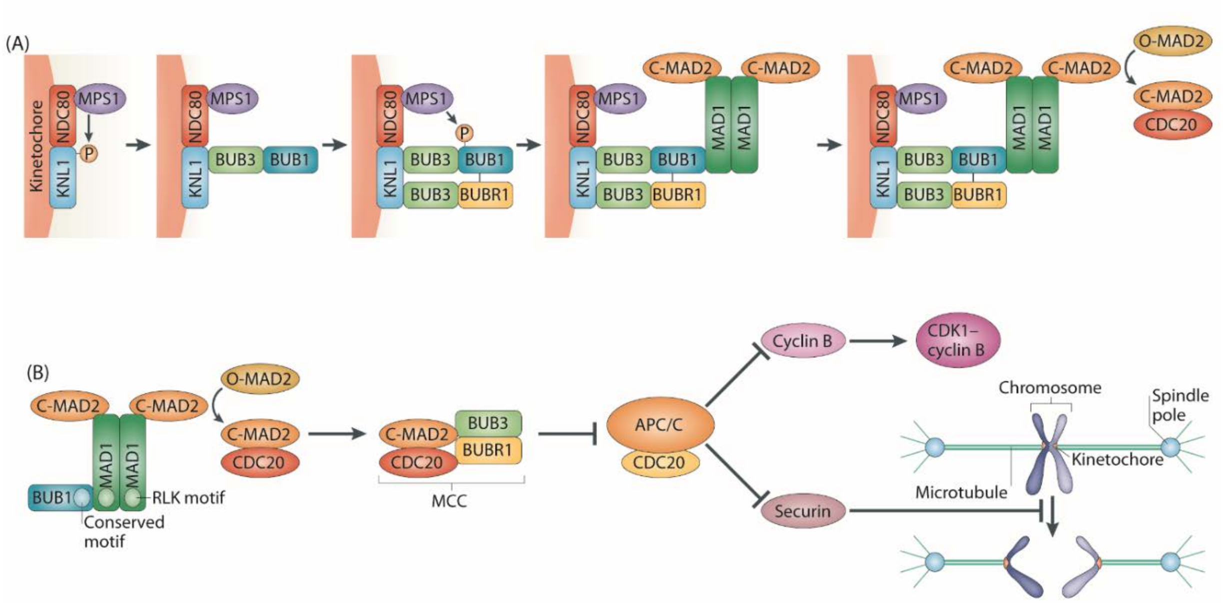
(A)
Kinetochore
NDC80
MPS1
P
KNL1
BUB3
BUB1
BUBR1
C-MAD2
MAD1
O-MAD2
CDC20
(B)
C-MAD2
O-MAD2
MAD1
BUB1
RLK motif
Conserved motif
BUB3
BUBR1
MCC
APC/C
CDC20
Cyclin B
CDK1–cyclin B
Securin
Chromosome
Spindle pole
Kinetochore
Microtubule

Figure 1.4: Spindle assemble checkpoint recruitment and activation

(A) The unattached kinetochore recruits the Mad1 through its kinetochore receptor, Nuclear division cycle 80 (Ndc80). Budding inhibited by benzimidazole 1-related receptor protein kinase (Bubr1) forms a complex with Bub1 and Bub3 on the kinetochore. Bub1-Bub3 complex is recruited by Monopolar spindle 1 (Mps1) to the kinetochore. Mad1 interacts with Bub1 to form a complex and recruits Mad1-Mad2 to the kinetochore. Mad1-Mad2 amplifies the checkpoint signal by recruiting more Mad2 to catalytically convert Mad2-(O) (open) to Mad2-(C) (closed) (London and Biggins 2014). (B) Initial assembly of checkpoint proteins on the kinetochore amplifies the signal to form the mitotic checkpoint complex (MCC). Mad2-(C) on the unattached kinetochore binds to the cell division control protein 20 (Cdc20) through an intermediate conformation. The APC/C^{cdc20} is inhibited by the MCC and stabilizes the Securin and mitotic cyclins (cyclin B) (London and Biggins 2014).

Anaphase/Telophase

When the SAC is cleared, the cell proceeds to anaphase to complete chromosome segregation . By this time the cell is committed to the irreversible step of the cell cycle, ending as two separated nuclear mass. Anaphase is divided into two parts called anaphase A and B. In yeast, anaphase A marks the separation of sister chromatids and anaphase B represents the spindle elongation (Straight et al. 1997). Multiple motor and MAPs work redundantly to complete anaphase. During anaphase, the kinetochore tracks on the depolymerizing microtubule. At the end of anaphase B, the cell must activate the mitotic exit pathway to complete the cell cycle after ensuring that mother and daughter cells have equally partitioned the genetic material. An error in this process activates the spindle position checkpoint (SPOC).

Mitotic exit

Before entering anaphase, the cell degrades most of the Clb5 and Clb2 through APC-C^{Cdc20} dependent ubiquitination (Yeong et al. 2000). To exit mitosis, the cell must get rid of all the CDKs and reverse all the CDK-mediated phosphorylation events. In budding yeast, release of Cdc14 from the nucleolus dephosphorylates the CDK substrates and stabilizes mitotic-CDK inhibitors (Stegmeier et al. 2004). Dephosphorylation of Cdh1 by Cdc14 triggers APC/C mediated cyclin destruction (Zachariae et al. 1998; Jaspersen, Charles, and Morgan 1999). Cdc14-mediated de-phosphorylation of Sic1 prevents its destruction and promotes the transport of the Sic1 transcription factor Swi5 into the nucleus (Visintin et al. 1998). Cdc14 is also important for rDNA segregation and to modulate the spindle midzone through microtubule dynamics (D'Amours, Stegmeier, and Amon 2004; Sullivan et al. 2004). Cdc14 is sequestered by Cfi1/Net1 in the nucleolus in the rest of the cell cycle (Shou et al. 1999; Visintin, Hwang, and Amon 1999). In anaphase, Cdc Fourteen Early Anaphase Release (FEAR) network and Mitotic Exit Network (MEN) are activated to release Cdc14 from the nucleolus (Stegmeier and Amon 2004). Early release of Cdc14 during early anaphase is important for timely cell cycle exit. On the other hand, MEN works in late anaphase (Stegmeier and Amon 2004).

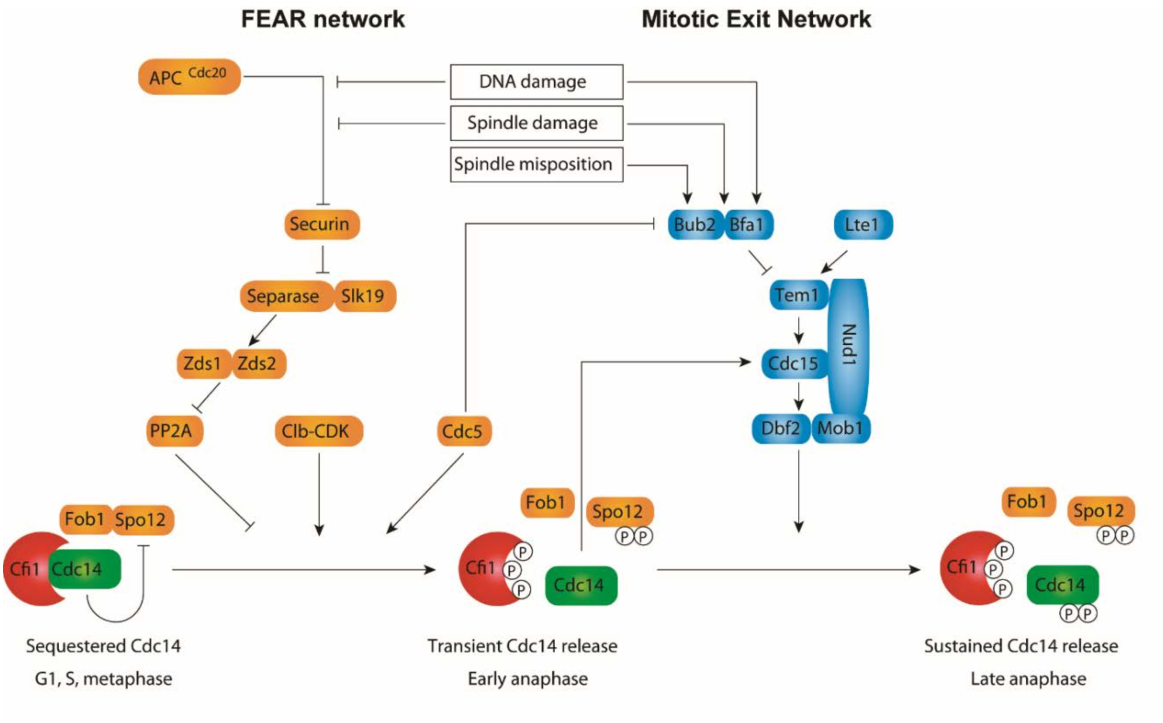
FEAR network
Mitotic Exit Network
APC Cdc20
DNA damage
Spindle damage
Spindle misposition
Securin
Bub2
Bfa1
Lte1
Separase
Slk19
Tem1
Zds1
Zds2
Nud1
Cdc15
PP2A
Clb-CDK
Cdc5
Dbf2
Mob1
Fob1
Spo12
Cfi1
Cdc14
P
Sequestered Cdc14
G1, S, metaphase
Transient Cdc14 release
Early anaphase
Sustained Cdc14 release
Late anaphase

Figure 1.5: FEAR and MEN pathways in *S.cerevisiae*.

Cdc14 is released from the nucleolus during early anaphase by FEAR network. Activation of MEN further releases Cdc14 from the nucleolus. Continuous presence of Cdc14 throughout anaphase, promotes the mitotic exit (Rock and Amon 2009).

Cdc Fourteen Early Anaphase Release (FEAR)

FEAR is not fully understood, but includes Separase/Esp1, Cdc5 (polo-like kinase), Slk19 (kinetochore protein), Spo12, Bns1 and Fob1 (replication fork blocking protein) (Stegmeier, Visintin, and Amon 2002; Stegmeier et al. 2004). Clb1/2-CDKs along with PP2A activates the FEAR network (Fig.1.5) (Azzam et al. 2004; Yellman and Burke 2006; Queralt et al. 2006; Wang and Ng 2006). There are two parallel pathways (Visintin, Stegmeier, and Amon 2003), one involving Spo12, Bns1 and Fob1, and another having Separase/Esp1, Slk19 (Visintin, Stegmeier, and Amon 2003). The Cdc5, polo-like kinase activates both pathways downstream, or in parallel with Esp1/Slk19 (Visintin, Stegmeier, and Amon 2003). Cdc5 has a role in MEN as well as in FEAR. In early anaphase, destruction of Securin/Pds1 triggers the Cdc14 release through the activation of Separase/Esp1 and sequesters PP2A. Spo12 or Cdc5 also phosphorylate Cfi1/Net1 to release Cdc14 (Azzam et al. 2004; Queralt et al. 2006). While the FEAR network needs MEN to complete the exit, the mechanism of inter-dependency is unknown.

Mitotic Exit Network

Tem1, a GTPase in Ras-like pathway activates downstream targets in the pathway (Stegmeier and Amon 2004; Bardin and Amon 2001; Seshan and Amon 2004). Lte1, a putative guanine nucleotide exchange factor (GEF) (Shirayama, Matsui, and Toh 1994; Shirayama, Matsui, and Toh-e 1996) and Bub2-Bfa1, a GTPase activating complex (GAP) (Krishnan et al. 2000; Alexandru et al. 1999; Bardin, Visintin, and Amon 2000; Fesquet et al. 1999) regulate the Tem1 GTPase function. Tem1 activation triggers the activation of downstream kinases: Cdc15 and Dbf2 (Fig.1.5). The mode of Cdc15 activation is not known, however activated Cdc15 phosphorylates and activates Dbf2 *in vitro* (Mah, Jang, and Deshaies 2001). Mob1, a Dbf2-associated factor, is required for Dbf2 activation (Mah, Jang, and Deshaies 2001; Komarnitsky et al. 1998; Luca et al. 2001). Cdc5 inhibits the Bub2-Bfa1 complex to promote MEN (Bardin, Visintin, and Amon 2000; Seshan, Bardin, and Amon 2002; Lew and Burke 2003).

Tem1 activation is not fully understood, but the movement of the daughter SPB into the daughter cell cortex is thought to activate it. Kin4 kinase specifically localizes on the mother cell cortex and misaligned spindle in the mother cell activates the SPOC through Kin4

(Lew and Burke 2003; D'Aquino et al. 2005). Kin4 phosphorylates Bub2-Bfa1 to inhibit MEN, preventing Cdc14 release (D'Aquino et al. 2005; Pereira and Schiebel 2005). Until the spindle enters the daughter cell, the cell does not exit from the cell cycle.

Cytokinesis

Cytokinesis is a poorly understood process in yeast. It ends the cell cycle by physically cleaving the cell into mother and daughter cells. Cytokinesis is coordinated by the actin-myosin ring and the septum formation. During anaphase, Cdc14 localizes on the putative septum site, although its function is not clear. The acto-myosin assembly includes septins, myosins, and formins to complete the cytokinesis in late telophase. Myosin II plays a major role in animal cells during the acto-myosin ring assembly (Moseley and Goode 2006). Myo1 (type II myosin) deletion does not have any effect, but mutation in the motor domain results in cytokinesis defect. There are four septins, Cdc3, Cdc10, Cdc11 and Cdc12 that are essential for septum formation during cytokinesis. Temperature sensitive mutants of these septins results in a cytokinesis defect. The septum is a chitin-rich disk that separates the mother and daughter cells.

Cell cycle and kinetochore

In budding yeast, the kinetochore attaches to the microtubule throughout the cell cycle. In higher eukaryotes, the inner kinetochore stably attaches to the centromere in G1 and adds the Mis12/MIND complex in G2. During mitosis, the kinetochore adds the Ndc80 complex and disassembles in mitotic exit. Cdk1 has been shown to affect the binding of CENP-T, an outer kinetochore protein, to the kinetochore. Kinetochore regulation of other subunits is not known and is crucial for understanding kinetochore dynamics during cell cycle stages. Whether the kinetochore structure is dynamic over the cell cycle and its role in microtubule tracking, are not known. Here I review the structural organization of the kinetochore and its attachment with the microtubule.

Kinetochore

The kinetochore (KT), an evolutionarily conserved multi-complex structure, connects the microtubule with the chromosome. The kinetochore assembles on the centromeric region, which is specified by the centromere specific histone 3 (H3) variant called Cse4 in budding yeast. In yeast, a single centromeric nucleosome assembles the whole kinetochore. Binding of the Cbf3 complex to the centromeric DNA along with Mif2 marks the assembly site of the future kinetochore complex.

The kinetochore is essential for error correction, tracking on the microtubule, and to generate force to pull the chromosome during anaphase. The kinetochore assembles in a hierarchical order, starting with Cbf3 complex, which is necessary for outer kinetochore assembly, and ends at the microtubule with the Dam1 complex. Each layer of the kinetochore provides the possibility for its regulation on microtubule attachment. Genetic screens, affinity purifications, chromatin immunoprecipitations, mass spectrometry analyses along with microscopy have identified >65 proteins to assemble the whole organization of the kinetochore (De Wulf, McAinsh, and Sorger 2003; Tytell and Sorger 2006; Miranda et al. 2005; Euskirchen 2002). Biophysical studies and electron microscopy on purified recombinant expression of protein complexes have shown their functions in kinetochore organization. Though the composition of kinetochore complexes has been studied,how each complex contribute to kinetochore function is unknown.

Chromosome segregation is chiefly governed by properties of the mitotic spindle. In haploid yeast (16 chromosomes), the mitotic spindle has ~42 microtubules (kMT) (32 kinetochore microtubules-one microtubule per kinetochore and ~8 interpolar kinetochore microtubule (ipMT)-overlap each other for sliding) arranged in a cylindrical structure, forming a bipolar microtubule bundle (Fig.1.6; Yeh et al., 2008; Stephens et al., 2011). Kinetochores on the centromeric region form a DNA-protein interface between the centromeric nucleosome and the microtubule. Kinetochores consist of more than 8 subcomplexes with ~65 different proteins (Cheeseman and Desai, 2008; Santaguida and Musacchio, 2009)). Major subcomplexes in the budding yeast are Dam1, Ndc80, Mtw1, Spc105, Ctf19, and Cbf3. The Dam1 complex forms a ring-like structure with ten proteins and it stabilizes the association of the Ndc80 complex with microtubules by helping during sliding. The Ndc80 complex forms a hetero-tetramer with Spc24, Spc25, Nuf2, and Ndc80. Phosphorylation of the N terminal of Ndc80 by Ipl1 and Mps1 controls its binding to microtubules (Cheeseman et al., 2006). Nnf1, Nsl1, Dsn1, and Mtw1 form the MIND complex and connect the Ndc80 complex to the inner kinetochore. Mis12 is connected to the Spc24-25 complex through the Nsl1-Dsn1 heterodimer. Ctf19 is the CCAN complex in human. Cbf3 interacts with Cse4 and is necessary for the establishment of the centromeric nucleosome. Mis-regualtion of kinetochore proteins and proteins involved in kinetochore-microtubule interaction lead to cancer development and aneuploidy (Yuen, Montpetit, and Hieter 2005; Meng et al. 2015). Understanding the kinetochore function is important for finding novel therapeutic targets for cancer.

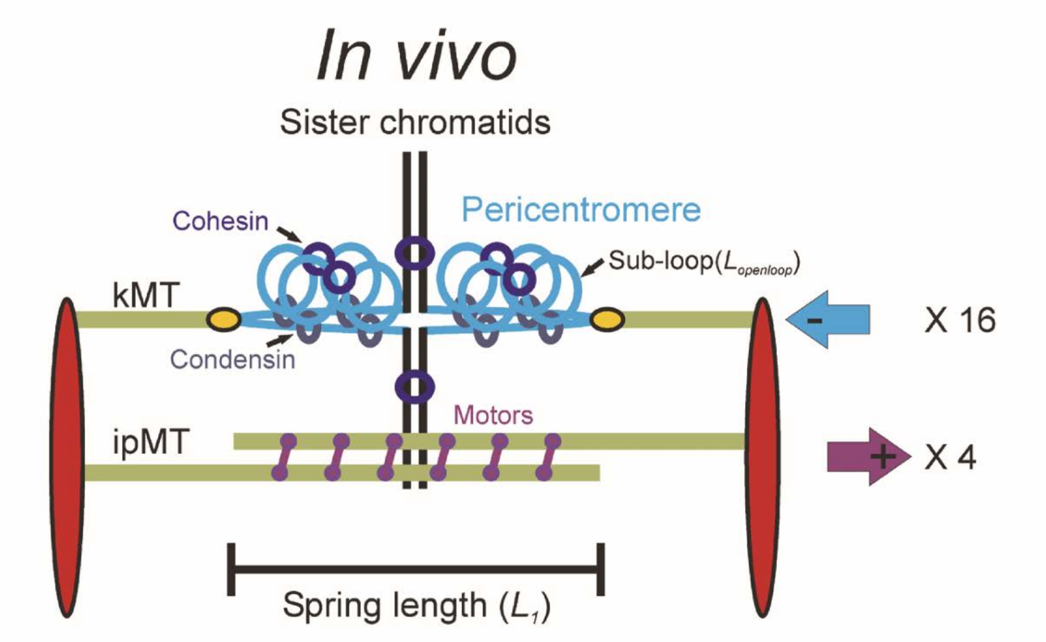

Figure 1.6: Mitotic spindle in budding yeast.

The kinetochore microtubule (kMT, green) emanating from the spindle pole body (SPB, Red) is attached to the centromere. Pericentromeric region form loops with cohesin (purple). Sister chromatids extend perpendicularly from the spindle axis. ipMT is interpolar microtubule.

The proper segregation of chromosomes depends on amphitelic attachment where sister kinetochores are attached to MTs from opposite poles creating tension. The physical connection between sister chromatids by the cohesin complex and ipMT sliding generates tension. To equalize the tension, pericentromeric chromatin is stretched and recoiled back by the ‘microtubule rescue’. This movement accounts for the oscillation of sister chromatids at the spindle equator and, due to high tension, nucleosome turnover is also high (Verdaasdonk et al., 2012). The Ndc80, MIND, and Dam1 complexes are essential for MT tracking.

The mitotic spindle and pole separation require kinesin-related motor proteins (Hagan and Yanagida, 1990; Hoyt et al., 1992). The presence of these motor proteins on MTs helps in the sliding of the anti-parallel midzone microtubules and the separation of spindle poles (Hagan and Yanagida, 1992; Hoyt et al., 1992). Cin8 and Kip1 are functionally redundant proteins predominantly working during metaphase to separate the spindle poles. Deletion of both motors collapses the spindle (Saunders and Hoyt, 1992). Kar3 (kinesin-14), localized near the SPB, is important for capturing the kinetochore. Cin8 (kinesin-5), present in the ipMT, helps in the sliding of MTs. Kip1, Kip2, and Kip3 are involved in spindle assembly and positioning. During anaphase, Dyn1, a minus-end directed motor (cytoplasmic Dynein 1 heavy chain) dominates sister chromatids separation, pulling the cytoplasmic MT to the pole.

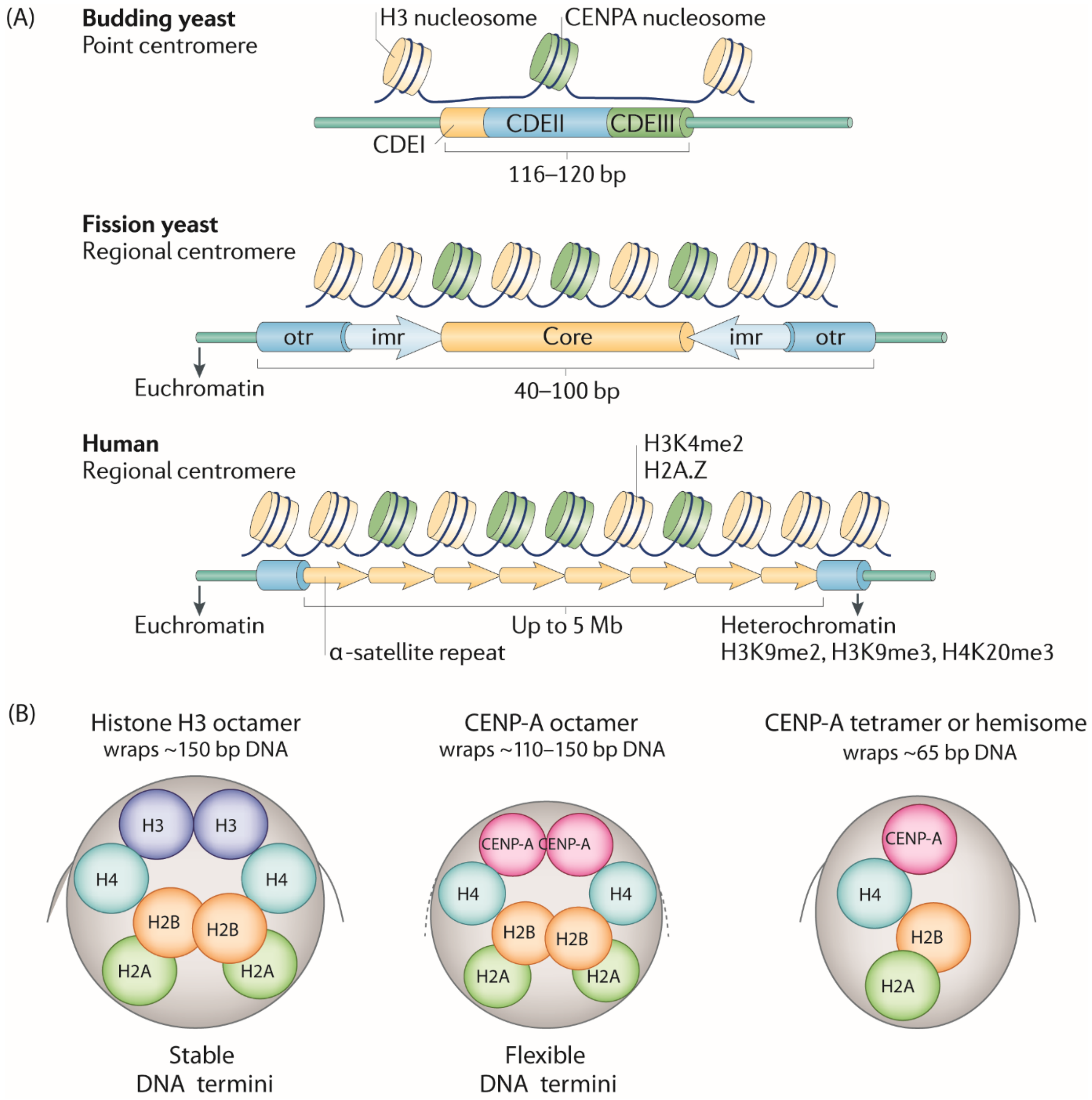
(A)
Budding yeast
Point centromere
H3 nucleosome
CENPA nucleosome
CDEI
CDEII
CDEIII
116–120 bp
Fission yeast
Regional centromere
otr
imr
Core
imr
otr
Euchromatin
40–100 bp
Human
Regional centromere
H3K4me2
H2A.Z
Euchromatin
Up to 5 Mb
α-satellite repeat
Heterochromatin
H3K9me2, H3K9me3, H4K20me3
(B)
Histone H3 octamer
wraps ~150 bp DNA
H3
H3
H4
H4
H2B
H2B
H2A
H2A
Stable
DNA termini
CENP-A octamer
wraps ~110–150 bp DNA
CENP-A
CENP-A
H4
H4
H2B
H2B
H2A
H2A
Flexible
DNA termini
CENP-A tetramer or hemisome
wraps ~65 bp DNA
CENP-A
H4
H2B
H2A

Figure 1.7: Centromere and centromeric nucleosome.

(A) Budding yeast has a point centromere with a single nucleosome per chromosome and it attaches to a single microtubule. It has 3 conserved DNA elements (CDEI, CDEII, and CDEIII). Higher eukaryotes have a much larger regional centromere which is epigenetically defined and contains centromeric protein A (CENPA). (B) The canonical nucleosome has 2 copies of H2A, H2B, H3, and H4 each forming an octamer. CENP-A octameric nucleosome comprising 2 copies of CENP-A, H4, H2A, and H2B. Proposed model for tetrameric centromeric nucleosome.

Centromeric region

The kinetochore assembles on a specialized region on the chromosome called the centromere, which is defined by sequence in budding yeast and epigenetically in higher eukaryotes. The budding yeast ‘point’ centromere was first identified by its plasmid stability in mitosis and meiosis. The ~125bp sequence has three distinct conserved regions called Centromere-Defining Elements (CDE): CDE1-an 8-bp palindrome, CDEII-a 78-bp AT-rich sequence and CDEIII-a conserved 26-bp element (Fitzgerald-Hayes 1987; Fitzgerald-Hayes, Clarke, and Carbon 1982; Clarke and Carbon 1985). Cbf1p of the inner kinetochore with its helix-loop-helix motif binds to the CDEI region and Cbf3 binds to the CDEIII region through its CCG motif. Deletion of CDE1 results in a 10-fold increase in chromosome missegregation (Bram and Kornberg 1987; Hegemann et al. 1988). CDEII and CDEIII are essential for kinetochore assembly (Fig.1.7). In higher eukaryotes, the centromeric region spans hundreds of kilobases of heterochromatic, repetitive α-satellite DNA.

Cse4

The centromere of a chromosome is important for the segregation of sister chromatids during anaphase. Centromeric regions form a connection with the spindle through the kinetochore. In budding yeast, the centromeric region is genetically defined (Clarke and Carbon, 1980) and well understood. But in higher eukaryotes, to some extent the centromere is epigenetically specified. Within yeast, *Saccharomyces cerevisiae* has a conserved centromeric sequence but *Schizosaccharomyces pombe* lacks it (Polizzi and Clarke, 1991). Even though the centromere is different in each organism, the centromeric nucleosome is universally marked by histone 3 variant called Cenp-A (in budding yeast-Cse4). The Cenp-A sequence is diverged in all species especially at the N-terminal region, and affects kinetochore formation, as yeast Cse4 is sufficient to build a functional kinetochore in human (Wieland et al., 2004).

A genetic screen for increased chromosome loss rate with CDEII sequence has identified Cse4 as one of the targets. Cse4 shares more than 70% homology with the H3 histone. A temperature-sensitive mutant form (*cse4-1*) activates the SAC with duplicated chromosomes. Centromeric nucleosome structure is still not clear because different experimental techniques suggest different structures, including hemisome and octasome (Fig.1.7). Our results suggest that the centromeric nucleosome exists in different structures depending on the cell cycle stages. In budding yeast, it forms a hemisome (Cse4/H4/H2A/H2B), switching to an octasome during anaphase (Shivaraju et al., 2012). In human, during S phase, the centromeric nucleosome changes to an octasome while forming a tetrasome during the rest of the cell cycle (Bui et al., 2012). Suppressor of Chromosome Missegregation (Scm3), a Cse4 histone chaperone (Shivaraju et al., 2011), is necessary for Cse4 deposition and regulation at the centromere (Camahort et al., 2007). Another histone chaperone called Chromatin assembly factor 1 (Caf1), an H3/H4 chaperone, also co-purifies with CENP-A *in vivo* and assembles the centromeric nucleosome *in vitro*. The CAF-1 complex's functions in assembling the Cse4 nucleosome on a non-centromeric region are not well studied. Here I review the known Cse4 chaperones.

Cse4 chaperone

Histone chaperones assemble the nucleosome in a step-wise manner to produce a functional nucleosome. They can prevent charge-based interactions, transport histones and disassemble nucleosomes.

Scm3

Centromeric nucleosomes are assembled by centromere specific chaperones called Scm3 in budding yeast (Camahort et al. 2007) and HJURP in human (Dunleavy et al. 2009; Foltz et al. 2009). Scm3 recruits Cse4 with Ndc10 to the centromere. Temperature sensitive mutant of Scm3also get arrested in metaphase like Cse4 mutants (Camahort et al. 2007). Scm3 is localized to the centromere throughout the cell cycle with reports showing Scm3 leaves the centromere during metaphase-anaphase transition. Scm3 is not sequence specific, however, it is a Cse4-specific chaperone, as it lacks the ability to assemble H3 nucleosome *in vitro*.

Budding yeast kinetochore complex

The kinetochore complex is a molecular machine essential for tracking the microtubule end and pulls the chromosome during chromosome capture and segregation. It consists of multiple submodules with more than 60 proteins in budding yeast and most of them are evolutionarily conserved from yeast to human. In budding yeast, the kinetochore has multiple sub-modules namely: Cbf3, CCAN, CENP-W, CENP-T/Cnn1, COMA/Ctf19, Mis12/MIND/Mtw1, KNL1/Spc105, and Ndc80 (centromeric nucleosome to microtubule interacting module) along with microtubule associated factors like Stu2 and motor proteins (Fig.1.8). The inner kinetochore binds and assembles the kinetochore on the centromeric region. Each complex in the inner kinetochore has specific functions. For example, Mif2 and Cbf3 find the centromeric sequence to recruit further kinetochore complexes. The outer kinetochore, mainly interacts with the microtubule and recruits other check point proteins.

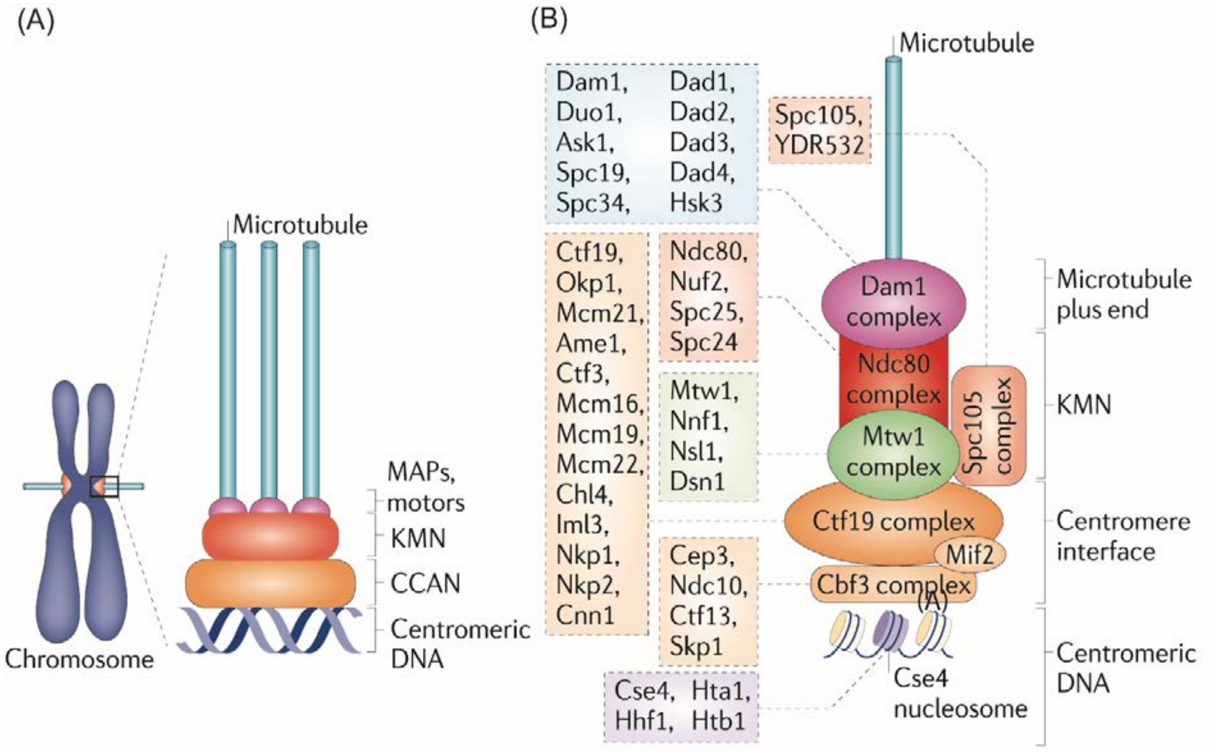
(A)
Microtubule
MAPs, motors
KMN
CCAN
Centromeric DNA
Chromosome
(B)
Microtubule
Dam1, Duo1, Ask1, Spc19, Spc34,
Dad1, Dad2, Dad3, Dad4, Hsk3
Spc105, YDR532
Ctf19, Okp1, Mcm21, Ame1, Ctf3, Mcm16, Mcm19, Mcm22, Chl4, Iml3, Nkp1, Nkp2, Cnn1
Ndc80, Nuf2, Spc25, Spc24
Mtw1, Nnf1, Nsl1, Dsn1
Cep3, Ndc10, Ctf13, Skp1
Cse4, Hta1, Hhf1, Htb1
Dam1 complex
Ndc80 complex
Spc105 complex
Mtw1 complex
Ctf19 complex
Mif2
Cbf3 complex
(A)
Cse4 nucleosome
Microtubule plus end
KMN
Centromere interface
Centromeric DNA

Figure 1.8: Kinetochore organization in budding yeast. (A) The human kinetochore consists of KMN as a core which is made up of the MIND and Ndc80 complexes and interacts with multiple microtubules. (B) Yeast kinetochore organization. The centromeric nucleosome is marked by Cse4. Mif2, and Cbf3 complexes and binds to CEN DNA. The CCNA complex interacts with Mis12/Mtw1 complex. The 'linker complex' Mtw1/Mis12 connects the inner kinetochore to the outer kinetochore. The Ndc80 and Dam1 complexes are microtubule interacting sub-modules (Lampert and Westermann 2011).

Table 1.1: Evolutionary conservation of budding yeast kinetochore

	S.cerevisiae			***H.sapiens***	**Features**
DNA-binding	Cse4			CENP-A	Histone H3 variant, CEN DNA binding
	Cbf3 complex		Ndc10		
			Cep3		Zn-cluster motif
			Ctf13		F-box domain, activated by Skp1
			Skp1		SCF component
	Cbf1				
Inner kinetochore	CCAN	Mif2		CENP-C	AT-hook
		COMA	Ctf19	CENP-P	RWD domain
			Okp1	CENP-Q	
			Mcm21	CENP-O	Less cohesin at the centromere
			Ame1	CENP-U	
Outer kinetochore		Ctf3	Ctf3	CENP-I	Coiled-coil domain
			Mcm16	CENP-H	
			Mcm22	CENP-K	
			Iml3/Mcm19	CENP-L	
			Chl4	CENP-N	
			Mhf1	CENP-S	Histone fold domain
			Mhf2	CENP-X	Histone fold domain
			Nkp1		
			Nkp2		
			Ybp2		
		Cnn1	Cnn1	CENP-T	Histone fold domain
			Wip1	CENP-W	Histone fold domain
		Spc105	Spc105	KNL-1	Mps1, Bub1 and Bub3 recruitment
			Ydr532/Kre28	Zwint	Mps1, Bub1 and Bub3 recruitment
		Mis12/MIND/ Mtw1	Mtw1	MIS12	Heterodimer with Nnf1
			Dsn1	Dsn1	Heterodimer with Nsl1
			Nnf1	Nnf1	
			Nsl1	Nsl1	
	Microtubule-binding	Ndc80	Spc24	Spc24	Spindle assembly checkpoint protein recruitment
			Spc25	Spc25	
			Nuf2	Nuf2	

			Ndc80	Ndc80	Positively charged calponin-homology domain
		Dam1	Ask1		
			Dad1		
			Dad2		
			Dad3		
			Dad4		
			Dam1		Positively charged N-terminal
			Duo1		MT binding
			Spc19		
			Spc34		
			Hsk1		
		MAPs	Stu2	Ch-TOG	Ndc80 binding, TOG domain
			Bik1	CLIP-170	MT plus-end binding
			Slk19		
			Stu1	CLASP	
			Bim1	EB1	
		Motors	Kip1	BimC-family	
			Kip3	Kinesin-8	
			Cin8	Kinesin-5	
			Kar3	Kinesin-14	
		Chromosome Passenger Complex	Ipl1	Aurora B	Protein kinase
			Sli15	INCENP	
			Nbl1	Borealin	
			Bir1	Survivin	
			Glc7	PP1	
		Spindle Assembly Checkpoint	Mad1	Mad1	
			Mad2	Mad2	
			Bub1	Bub1	Ser/Thr protein kinase
			Bub3	Bub3	WD40 domain
			Mps1	hMps1	Ser/Thr protein kinase

Inner kinetochore sub-modules

Here, inner kinetochore refers to complexes close to the chromatin and outer kinetochore refers to complexes which mediate the microtubule attachment. Constitutive Centromere Associated Network (CCAN) was identified during CENP-A purification (Obuse et al. 2004; Foltz et al. 2006; Okada et al. 2006). In higher eukaryotes, CCAN consists of

CENP-C, CENP-H/I/K, CENP-L/M/N, CENP-O/P/Q/R/U, CENP-T/W (histone-fold containing complexes) (McAinsh and Meraldi 2011; Perpelescu and Fukagawa 2011; Takeuchi and Fukagawa 2012), and CENP-S/X. Yeast has most of the CCAN orthologues along with yeast-specific Cbf3 complex (Table 1.1).

Cbf3

The centromere binding factor complex (Cbf3) was initially purified through its specific CEN DNA binding (Ng and Carbon 1987; Lechner and Carbon 1991). The CBF3 complex has four proteins: Ndc10 (CBF3A/CTF14), Cep3 (CBF3B), Ctf13 (CBF3C), and Skp1 (Cbf3/p19). Each protein from the CBF3 complex is essential for viability, and mutations either in CBF3 or CDEIII result in loss of Cbf3 binding *in vitro* and increased chromosome loss *in vivo* (Doheny et al. 1993; Goh and Kilmartin 1993; Strunnikov, Kingsbury, and Koshland 1995; Connelly and Hieter 1996; Jehn, Niedenthal, and Hegemann 1991). All of the proteins from the CBF3 complex can bind to CEN DNA. However, Cep3 has a zinc figure motif for DNA binding which specifically recognizes the CCG-sequence of CDEIII (Espelin, Kaplan, and Sorger 1997). Even though Ndc10 has a similarity to tyrosine DNA recombinases (Perriches and Singleton 2012; Cho and Harrison 2011), it does not have catalytic activity. A mutation in Ndc10 (*ndc10-1*) at non-permissive temperature disrupts kinetochore assembly (Poddar et al. 2004; Stoler et al. 1995) and does not activate the spindle assembly checkpoint.

CCAN

Mif2, a component of CCAN, was identified in a screen for mutants with a high rate of chromosome loss under overexpression. The low sequence similarity between yeast and human proteins delayed the identification of CCAN components in yeast. CENP-C is the orthologue of Mif2 in human. Mif2 mutant cells are inviable with G2/M arrest and activate the spindle assembly checkpoint (Meeks-Wagner et al. 1986; Brown, Goetsch, and Hartwell 1993; Meluh and Koshland 1995, 1997). Mif2 dimerizes and binds as a single dimer to the

CDEIII region. Synthetic lethality of Mif2 with Ndc10 suggest that their interaction is essential for kinetochore function (Meluh and Koshland 1997, 1995). Mif2 has an eight-amino acid sequence called ‘A-T hook’ that binds to the minor groove of AT-rich DNA (Cohen et al. 2008). Cse4 and CBF3 are necessary for Mif2 localization at the centromere. Mis12/MIND mutants also show mislocalization of Mif2 (Westermann, Cheeseman, et al. 2003).

COMA complex

Ctf19 or COMA complex has 4 major proteins (Ctf19, Okp1, Mcm21 and Ame1) and other additional proteins (De Wulf, McAinsh, and Sorger 2003). Only Okp1 and Ame1 are essential for cell growth, however Mcm21 deletion increases chromosome loss with reduced cohesin at the centromere (Ng et al. 2009). Four proteins of CCAN form two sub-complexes (Cnn1/Wip1 and Mhf1/Mhf2) with Histone fold domain (Bock et al. 2012; Schleiffer et al. 2012). Orthologues of these sub complexes in human (CENP-T/W, CENP-S/X) form a hetrotetramer and interact with DNA to form a nucleosome-like structure (Nishino et al. 2012; Hori et al. 2008). Cnn1, like CENP-T has an Ndc80 interacting domain and in yeast, it anchors the Ndc80 complex only during anaphase (Malvezzi et al. 2013; Bock et al. 2012; Schleiffer et al. 2012). Quantitative microscopy suggests that 2 to 3 COMA complexes associate with a single kinetochore in yeast (Joglekar et al. 2006). CTF3 complex is part of the COMA complex and contains proteins Ctf3, Mcm22 and Mcm16 which are non-essential and localize to the kinetochore. Okp1 and Ame1 anchor the Mis12/MIND complex along with Mif2. Temperature sensitive mutants of Okp1 and Ame1 at non-permissive temperature have declustered kinetochores.

Inner kinetochore complexes are essential for CPC (Chromosome Passenger Complex) which has Ipl1/Aurora B, Bir1 (Survivin), Sli15 and Nbl1 (Borealin) and localize to the kinetochore for proper spindle assembly checkpoint. The CPC complex binds to the G1 kinetochore and localize to the spindle midzone during anaphase (Widlund et al. 2006; Carmena et al. 2012).

Outer kinetochore

Most of the outer kinetochore submodules are important for the microtubule binding activity and tracking the microtubule without losing the interaction. All of the outer kinetochore complexes are essential for cell growth. Mtw1/MIs12/MIND complex act as a ‘linker complex’ between inner and outer kinetochore. Ndc80 complex interacts with the MT. Dam1 complex also interacts with MT with its ring-like structure and Spc105/Knl-1/Blinkin interacts with MT and brings the Mps1 kinase to the kinetochore (Aravamudhan, Goldfarb, and Joglekar 2015).

Spc105 complex

Spc105 (KNL1orthologue) is an essential complex consisting of two proteins called Spc105 and Kre28 in a 1:2 ratio (Nekrasov et al. 2003; Pagliuca et al. 2009). Mutation in this complex results in chromosome loss. Purified yeast Spc105 has a weak MT binding activity (Pagliuca et al. 2009) and is important for recruiting the Mps1, Bub1 and Bub3 (Aravamudhan, Goldfarb, and Joglekar 2015; Kiyomitsu, Obuse, and Yanagida 2007; Kiyomitsu, Murakami, and Yanagida 2011; Rosenberg, Cross, and Funabiki 2011; Liu et al. 2010). Spc105 was first identified during Mis12 complex purification. Its C-terminal interacts with the Mis12/MIND (Maskell, Hu, and Singleton 2010) while N-terminal of Spc105 has MT binding activity (Cheeseman et al. 2006; Kiyomitsu, Obuse, and Yanagida 2007; Pagliuca et al. 2009; Welburn et al. 2009). Spc105 is highly phosphorylated by Mps1 which recruits Bub1 to kinetochore and is dephosphorylated by Glc7/PP1 to silence the checkpoint (Kiyomitsu, Obuse, and Yanagida 2007; Liu et al. 2010; Rosenberg, Cross, and Funabiki 2011). The Spc105 interaction with MT is not fully understood. Purified kinetochore from *spc105-15* at non-permissive temperature showed reduced Ndc80 complex with defective MT attachment (Akiyoshi et al. 2010).

Mis12/Mtw1/MIND complex

Mis12 complex has four proteins (Mtw1, Nnf1, Nsl1, and Dsn1) in 1:1:1:1 stoichiometry (Fig.1.9; (Euskirchen 2002; De Wulf, McAinsh, and Sorger 2003; Nekrasov et al. 2003; Hornung et al. 2011; Pinsky et al. 2003; Westermann, Wiedemann, et al. 2003). Mtw1 was initially identified as a homolog of *S. pombe* Mis12^{+} (Goshima and Yanagida 2000). Mtw1 localizes to the kinetochore and mutations in Mtw1 results in loss of tension at the centromere (Goshima and Yanagida 2000). Mis12 complex in *S. pombe* and higher eukaryotes have similar kinetochore localization and depletion of any one of the subunit results in chromosome loss (Goshima et al. 2003; Obuse et al. 2004). The Mtw1/Nnf1 and Dsn1/Nsl1 form a heterodimeric globular domain (Maskell, Hu, and Singleton 2010; Hornung et al. 2011). The Mis12 complex lacks the microtubule binding activity but purified Mis12 complex with Ndc80 increase the microtubule attachment cooperatively (Cheeseman et al. 2006; Hornung et al. 2011; Kudalkar et al. 2015). C-terminal region of Dsn1 anchors the Ndc80 complex through Spc25/Spc24 heterodimer (Hornung et al. 2011). In human, (heterochromatin protein 1) HP1 is needed for the Mis12 recruitment to kinetochore though its function is not understood (Obuse et al. 2004).

Microtubule interacting submodules:

Ndc80 complex

Spc24, Spc25, Nuf2, and Ndc80 form a 200kDa complex called Ndc80 with 1:1:1:1 stoichiometry (Janke et al. 2001; Wigge and Kilmartin 2001; Wei, Sorger, and Harrison 2005). Electron microscopy and X-ray studies of the purified Ndc80 complex show a rod-like structure with two globular head domains (Ciferri et al. 2008; Wei, Al-Bassam, and Harrison 2007; Wei, Sorger, and Harrison 2005; Cheeseman et al. 2006). Mutations in the Ndc80 complex cause microtubule detachment. Loss of Spc24 or Spc25 inactivates the spindle assembly checkpoint with a detached kinetochore, suggesting Spc24/Spc25-mediated recruitment of checkpoint proteins (Janke et al. 2001; He et al. 2001). Dam1 complex and microtubule associated proteins (MAPs) localization also depends on the Ndc80 complex (Maure, Kitamura, and Tanaka 2007; Hsu and Toda 2011). Ndc80 has an unstructured N-terminal tail which is important for microtubule binding and has a loop region which recruits the Dam1 complex *in vivo* (Hsu and Toda 2011). In *S.pombe*, the loop region also recruits the Dis1 complex and in higher organism it interacts with the Ska1 complex, Dis/TOG/Stu2, and Cdt1 (Hsu and Toda 2011). Mutation in the Ndc80 complex disrupts the microtubule attachment (Wei, Sorger, and Harrison 2005; Ciferri et al. 2008; Wei, Al-Bassam, and Harrison 2007; Cheeseman et al. 2006), like the CBF3 complex.

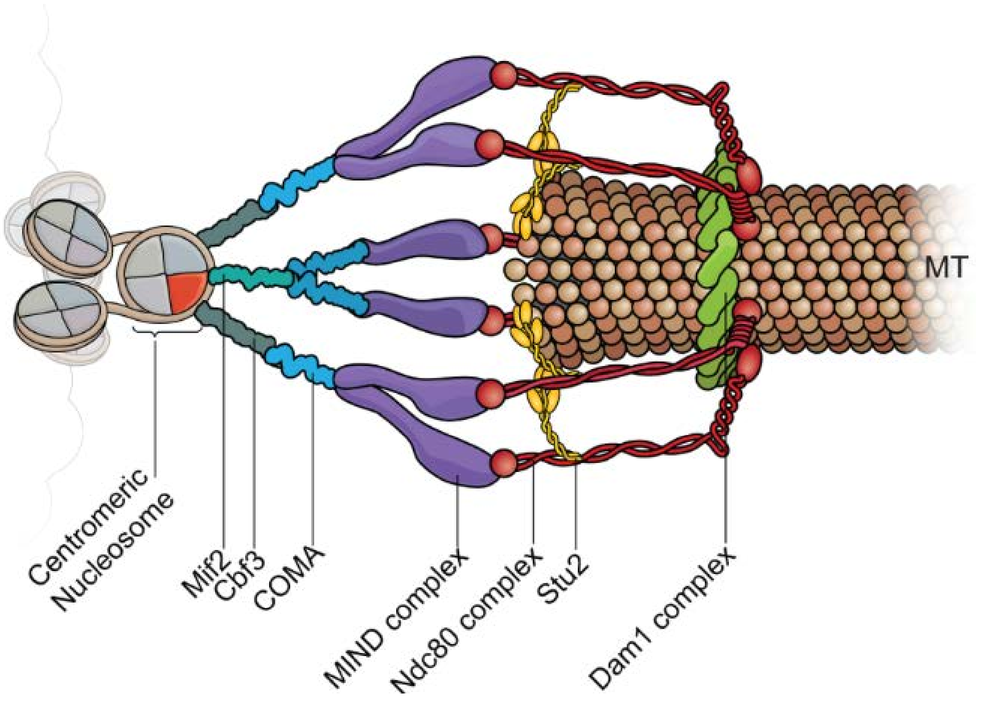

Figure 1.9: Budding yeast kinetochore-microtubule interaction site. The Ndc80 complex interacts with the microtubule through its N-terminal domain. The Dam1 complex forms a ring-like structure surrounding the microtubule. MAPs (like Stu2) localize on the plus-end microtubule and affect the microtubule dynamics. (Original drawing by Mark Miller)

DAM1/DASH/DDD complex

The Dam1 complex is a yeast-specific complex consisting of 10 essential proteins (Ask1, Dad1, Dad2, Dad3, Dad4, Dam1, Duo1, Hsk3, Spc19, and Spc34) that form a ring-like structure *in vitro* (Hofmann et al. 1998; Jones et al. 1999; Westermann et al. 2005). The Dam1 complex localizes on the spindle as well as on the kinetochore and mutations in Dam1 arrest the cell in M-phase with a broken or short spindle (Cheeseman and Boon 2001). The *dam1-1* mutant at non-permissive temperature shows defective monopolar attachment and directional instability on the spindle pole (Jones et al. 1999). The *spc34-3* mutant shows equal segregation of individual sisters, suggesting that this mutant does not have a strong kinetochore-microtubule interaction (He et al. 2001; Janke et al. 2001).

Bacterially expressed and purified Dam1 forms a 210 kDa complex and 16 Dam1 complexes forms a ring around the microtubule with 50 nm diameter (Miranda et al. 2005; Westermann et al. 2005; Wang et al. 2007; Ramey et al. 2011). The Dam1 ring binds to the GTP-tubulin and stabilizes the microtubule through polymerization *in vitro*. Different degrees of the oligomeric Dam1 complex interacts with microtubule and oligomerization of the Dam1 complex is necessary for its function (Gestaut et al. 2008; Grishchuk et al. 2008). The Dam1 complex at low concentration contacts the tubulin E-hook with its C-terminal (Westermann et al. 2005; Ramey et al. 2011). The Dam1 ring interacts with the microtubule through the electrostatic interaction of the Duo1 N-terminal similar to the Ndc80 complex (Hofmann et al. 1998; Cheeseman and Boon 2001; Rayala et al. 2007; Ramey et al. 2011). The Dam1 complex has known phosphorylation sites for Ipl1/Aurora B kinase and phosphorylation of the Dam1 complex reduces the Ndc80 association with the microtubule *in vitro*. Similar to Dam1 mutants, *ipl1-2* and *ipl1-321* mutants show monopolar spindles (He et al. 2001; Cheeseman et al. 2002). The Dam1 complex acts as microtubule force-coupler and moves along the depolymerizing microtubule.

+TIPs

Proteins associated with the plus-end of the microtubule are known as +TIPs or MAPs and they physically interact to regulate the microtubule dynamics during mitosis. There are at least eight known +TIPs in higher eukaryotes with known homologs in budding yeast. Most of the +TIPs are cytoplasmic in yeast, but a few localize to the kinetochore. Bik1, Bim1, and Stu2 localize to the kinetochore near the Ndc80 complex. Along with MAPs, there are motor proteins like Kip3, Kar3, Kip1, and Cin8 that also associate with the kinetochore.

Stu2:

Stu2 has been implicated in microtubule destabilization (van Breugel, Drechsel, and Hyman 2003; Kosco et al. 2001). Stu2 has a vertebrate orthologue known as XMAP215/DIS in Xenopus and ch-TOG in humans. It is localized on the kinetochore, cortical tips, the plus-end microtubule, and the spindle mid-zone (He et al. 2001; Wang and Huffaker 1997). Stu2 is transported to a newly captured kinetochore to promote microtubule polymerization and coincide with microtubule rescue (Kitamura et al. 2010). *In vivo*, Stu2 stabilizes the inter-polar microtubule and mutation in *STU2* does not affect the bi-orientation but does affect the transient separation. Along with reduced oscillation, the Stu2 mutant exhibits reduced kinetochore velocity by regulating the microtubule dynamics (Pearson et al. 2003). Stu2 dimerizes to bind the microtubule and relay on Ndc80 for kinetochore localization (Miller, Asbury, and Biggins 2016). Studies on kinetochore proteins localization have placed Stu2 near the Ndc80 complex (Aravamudhan et al. 2014).

Bik1:

Bik1, like Stu2, also localizes near the kinetochore. Deletion of BIK1 has no obvious phenotype in haploid or diploid. However, it is essential in polyploid cell to generate tension at the sister kinetochore (Lin et al. 2001). Bik1 binds in a Kip2 dependent manner to the shrinking and growing microtubule and stabilizes the growing microtubule (Perez et al. 1999; Carvalho et al. 2004). Kip2 is very specific to cytosol and its kinetochore receptor is not yet known. Bik1 specifically localizes on the kinetochore attached to the microtubule (Tanaka et al. 2005). Bik1 is not conserved as it cannot substitute the Clip-170 function in higher eukaryotes (Wieland et al. 2004).

Other kinetochore +TIPs:

Bim1, one of the MAPs, has a homolog in humans called EB1. Similar to Bim1, EB1 also associates with the kinetochore and stabilizes the microtubule (Kerres et al. 2004; Tirnauer et al. 2002). Depletion of EB1 leads to reduced tension at the kinetochore in human cells (Tirnauer et al. 1999; Draviam et al. 2006). Bik1 in yeast is not studied very well. Stu1 and Pac1 also interact with the plus-end of the microtubule. Stu1 is important for kinetochore capture and localizes on the spindle mid-zone as Stu1 mutants have a collapsed spindle. Stu1 genetically interacts with Dam1 and Cin8 but their association is not well studied in yeast (Jones et al. 1999; Yin et al. 2002).

Kinesins

Kinesins are motor proteins which use ATP hydrolysis (chemical energy) for directional motion (mechanical energy) to move a cargo along the MT (Asbury 2005). Kinesins have coiled-coil stalk for the cargo-binding and globular domain with ATPase function. Most of the kinesins take 8nm discrete steps after each ATP hydrolysis. The kinesin functions differ from moving the cargo to destabilizing the microtubule ends (Lawrence et al. 2004). Budding yeast has six kinesins and one dynein kinesin. The nuclear region has four kinesins (Cin8, Kip1, Kip3, and Kar3) that localize to the kinetochore and/or spindle mid-zone. The kinesins are classified based on structure, function, and directionality on the MT. Kinesin-5 motors (Cin8 and Kip1) form a homotetramer with plus-end direction and play a major role in cross-linking parallel, anti-parallel MT (Tytell and Sorger 2006). Even though Cin8 and Kip1 are not essential, deletion of CIN8 increases chromosome loss with frequent spindle collapse at 37°C. Deletion of KIP1 and CIN8 is synthetically lethal, however overexpression of Kip1 can suppress the spindle collapse in *cin8*Δ. The *cin8*Δ strain has synthetic lethality with deletion of MAD2, but the *kip1*Δ strain does not (Hoyt et al. 1992).

Kip3 (kinesin-8 or -13 family) has a functionally similar MCAK in mammals; XKCM1 in *Xenopus* and Klp5 and Klp6 in *S. pombe*. The kinesin-13 family of motors destabilize the microtubule end by using mechanical force to pull the protofilament and enhance the microtubule to curve away (Su et al. 2011). In yeast, the *kip3*Δ strain is resistant to benomyl (microtubule drug), suggesting the role of Kip3 in destabilizing microtubule. Both kinesin families are important for metaphase alignment of unattached kinetochore and de-polymerization of microtubule during anaphase.

Kar3, a kinesisn-14 family member, localizes on the SPB, the kinetochore, and at the cortical microtubule based on its interaction with non-motor proteins. Kar3s specifically destabilize the minus-end MT and cytoplasmic MT *in vitro (Maddox et al. 2003).* It is important for karyogamy during mating and depends on non-motor proteins for localization. Interaction with Vik1 localizes Kar3 on the nuclear site of the SPB and interaction with Cik1 localizes Kar3 on ipMT. Kar3/Cik1 is essential for nuclear migration during cytoplasmic microtubule mediated karyogamy (Sproul et al. 2005; Maddox et al. 2003).

Dynein and dynactin

Dynein is a cytoplasmic minus-end directed motor that localizes on the cortical. Dyn1, a 471kDa polypeptide, has a 4 ATP binding site (P-loops) flanked by a conserved MT binding coiled-coil domain. Cortically localized dynein-dynactin motor exerts force on the minus-end of SPB to pull it towards the periphery of the cell (Markus, Punch, and Lee 2009). In budding yeast, dynein, a non-essential motor, has overlapping function with another non-essential motor family, Cin8. Deletion of both the motors is inviable, probably because of failed anaphase spindle elongation.

Kinetochore dynamics

During metaphase, dynamic instability of the microtubules oscillates the sister chromatids front and back along the spindle axis. Tension between the sister chromatids stretches the chromatin region around the centromere (He et al. 2001). However, the tensile (spring) nature of the stretched chromatin comes back to the rest state by promoting microtubule polymerization (Gardner et al. 2005). Microtubule instability promotes the proper attachment and movement along the microtubule. Depolymerization coupled centromeric movement transiently disperses about 10kb of DNA and this transient separation is mediated by enrichment of cohesion at the peri-centromeric region (He et al. 2001). The kinetochore interaction with the microtubule imposes a structural constraint on the kinetochore. How the kinetochore maintains its interaction with the microtubule without losing the attachment is unknown. I have addressed this question in chapter 2.

Microtubule

The microtubule (MT) is a polymer of the tubulin monomer and is made up of α and β tubulin subunits. It is a self-assembled hollow structure with 25nm diameter, consisting of almost always 13 protofilaments (Desai and Mitchison 1997; Nogales 1999; Nogales et al. 1999). Tubulin heterodimer is asymmetrically assembled into MTs with α-tubulin at the stable minus-end and β-tubulin present on the unstable plus-end of the MT. Microtubule stochastically switches between polymerization (recue) and depolymerization (catastrophe) and it is referred to as dynamic instability. Four states characterize microtubule dynamic instability: growth (polymerization), shrinkage (depolymerization), rescue (switch from depolymerization to polymerization), and catastrophe (rapid depolymerization) (Mitchison and Kirschner 1984, 1984; Desai and Mitchison 1997). Even though GTP can bind to α and β tubulin, GTP is only hydrolyzed by β tubulin. MT only incorporates the GTP bound αβ-tubulin dimer and on incorporation GTP is hydrolyzed to form a microtubule lattice. The

GDP-bound tubulin is more unstable than the GTP-bound form promoting rapid depolymerization and release of mechanical force to move the attached kinetochore (Fig.1.10). The GDP-bound tubulin have curvature conformation and store potential energy in the microtubule lattice (Desai and Mitchison 1997).

In vitro studies of microtubule dynamics greatly differ from *in vivo* studies, suggesting that other factors might be influencing the dynamics. Kinesins and MAPs are known to influence the microtubule dynamic as these proteins specifically localize on GTP-bound plus-end of the microtubule.

Recently a new member of the tubulin superfamily was discovered through molecular genetic studies called γ–tubulin which is required for the microtubule nucleation and acts as a microtubule organizing center. In vivo studies have shown that gamma-tubulin can control the microtubule nucleation and it has independent role to control the cell cycle. The Gamma-tubulin concentration increases at the centrosome in the beginning of mitosis and decreases at the end of mitosis.

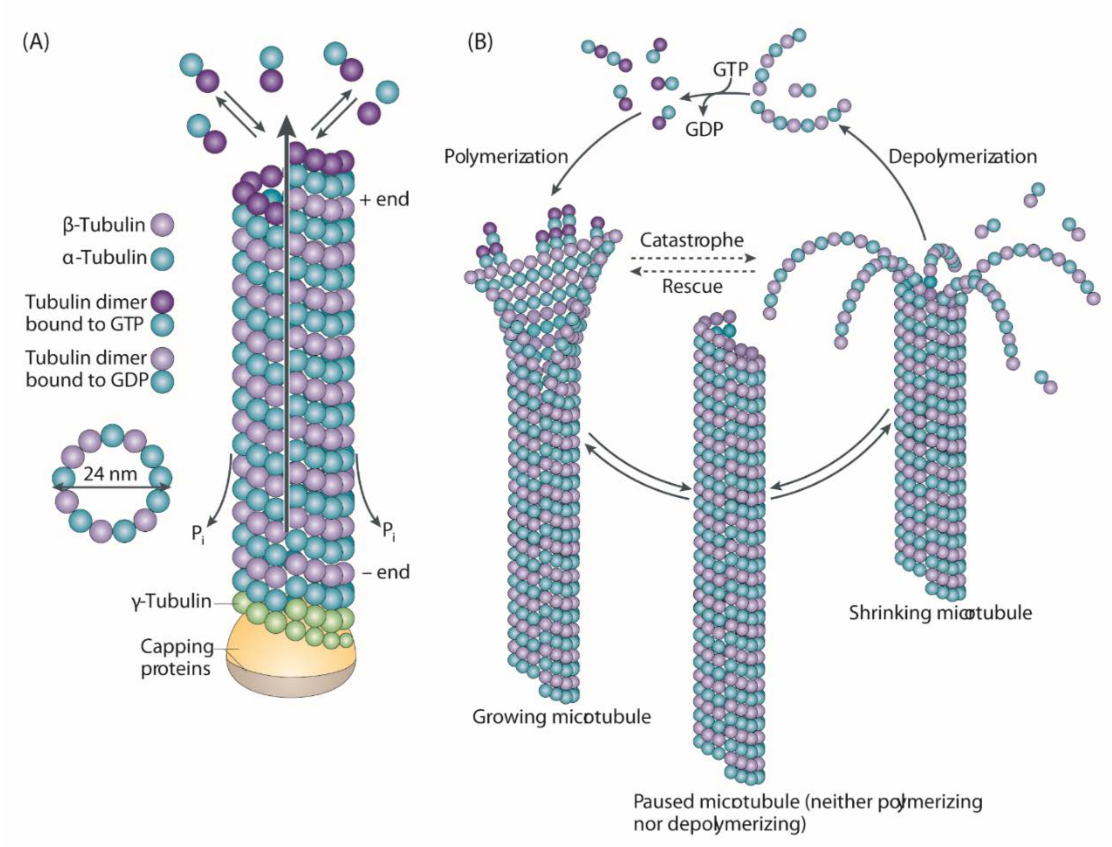
(A)
β-Tubulin
α-Tubulin
Tubulin dimer bound to GTP
Tubulin dimer bound to GDP
24 nm
+ end
– end
P_i
P_i
γ-Tubulin
Capping proteins
(B)
GTP
GDP
Polymerization
Depolymerization
Catastrophe
Rescue
Growing microtubule
Paused microtubule (neither polymerizing nor depolymerizing)
Shrinking microtubule

Figure 1.10: Microtubule is a dynamic structure and modulated by many factors.

(A) Microtubule is a polymer of α and β tubulin, a heterodimer. (B) Addition of GTP-bound tubulin dimer is called polymerization or growth phase. Removal of the tubulin dimer results in depolymerization or 'shrinkage'. Microtubule stochastically switch between growth and shrinkage. Kinetochore has to interact with this unstable structure without losing the attachment (Conde and Caceres 2009).

Mode of kinetochore-microtubule interaction

Based on the proteins involved in the kinetochore-MT interaction, three types of models were proposed to explain the kinetochore attachment on disassembling MT. ATP-powered microtubule depolymerizing motors are assumed to play an important role in the chromosome segregation but deletion of these motors does not affect the kinetochore attachment. Recently, two models have gained favor over other models namely: the conformational wave model and the biased diffusion model (Koshland, Mitchison, and Kirschner 1988; Hill 1985).

Conformational wave model

The conformational wave model is based on the movement driven by the conformation (power stroke) change of MT protofilaments to move the kinetochore (Fig.1.11). The power stroke gains energy from the bending strain of GDP-bound tubulin. During depolymerization, MT protofilaments peels away from the tip, producing enough energy to pull the kinetochore continuously (Mandelkow, Mandelkow, and Milligan 1991). Based on the kinetochore proteins interaction with the MT, two modes of interactions were proposed: 1) ring-based interaction and 2) fibril-based interaction. In both modes, curling microtubule protofilaments produce force to move the kinetochore. The purified yeast Dam1 complex, which forms a ring-like structure, tracks progressively on the depolymerizing microtubule, favoring the conformational wave model (Miranda et al. 2005; Westermann et al. 2005). The non-ring form of the oligomeric Dam1 complex can also track the depolymerizing microtubule and still validates the model. High-resolution EM showed curled protofilaments *in vivo* (McIntosh et al. 2008; VandenBeldt et al. 2006). Regulation of the kinetochore-microtubule interaction in the conformational wave model is achieved by changing the contacts with the protofilament (Asbury, Tien, and Davis 2011; Desai et al. 1999; Kerssemakers et al. 2006).

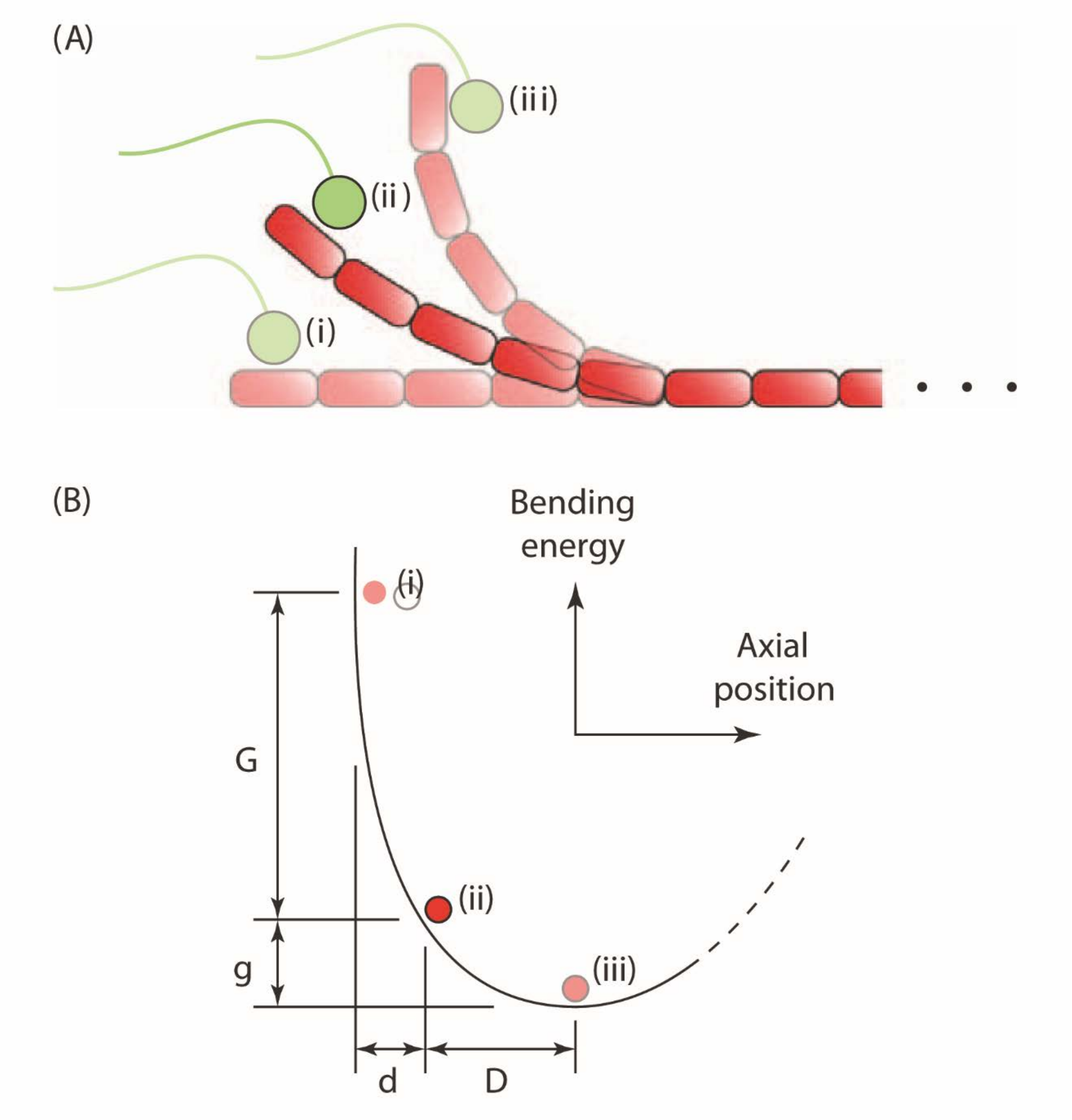

Figure 1.11: Conformational wave model for the kinetochore function.

(A) Kinetochore component (green) interacts with the curling microtubule protofilament (red) to drive the movement. From position (i) to (ii), kinetochore components make a small angle and little displacement. Development of larger angle (ii) to (iii) displaces the kinetochore for movement. (B) Bending energy versus kinetochore component position. Red dots mark the position of the kinetochore component from (a). When protofilament comes to its naturally favorable bent-position (iii) releases g+G total energy, results in d+D displacement. In (ii) initial bending, it releases G amount total energy with less axial displacement.

Biased diffusion model

The biased diffusion model was proposed first by Hill in 1985. The kinetochore has multiple binding sites and they make transient diffusive attachment with the MT (Fig.1.12). More attachments by motion favor the additional unidirectional attachment on the microtubule. In other words, depolymerization promotes additional attachment by Brownian motion. Hill’s theoretical model showed that the kinetochore can do mechanical work on the disassembling microtubule. The presence of multiple copies of Ndc80 and Dam1 complexes favors the biased diffusion model as purified the Ndc80 and Dam1 complex form a load-bearing attachment during assembly (Asbury et al. 2006; Franck et al. 2007; Powers et al. 2009; Tien et al. 2010). If the kinetochore follows a conformational wave model, then this state is least favored because protofilament lacks the curved feature. Interestingly, assembly state is most favored in biased diffusion. In terms of force production at the kinetochore, the Monte Carlo simulation of biased diffusion produces very close approximation to *in vivo* calculation of the pulling force (10pN) (Powers et al. 2009). The biased diffusion is also favored by the ultrastructure observation of the kinetochore, which reveals a web-like mat contacting the microtubule with parallel projections (Dong et al. 2007; McEwen and Dong 2010).

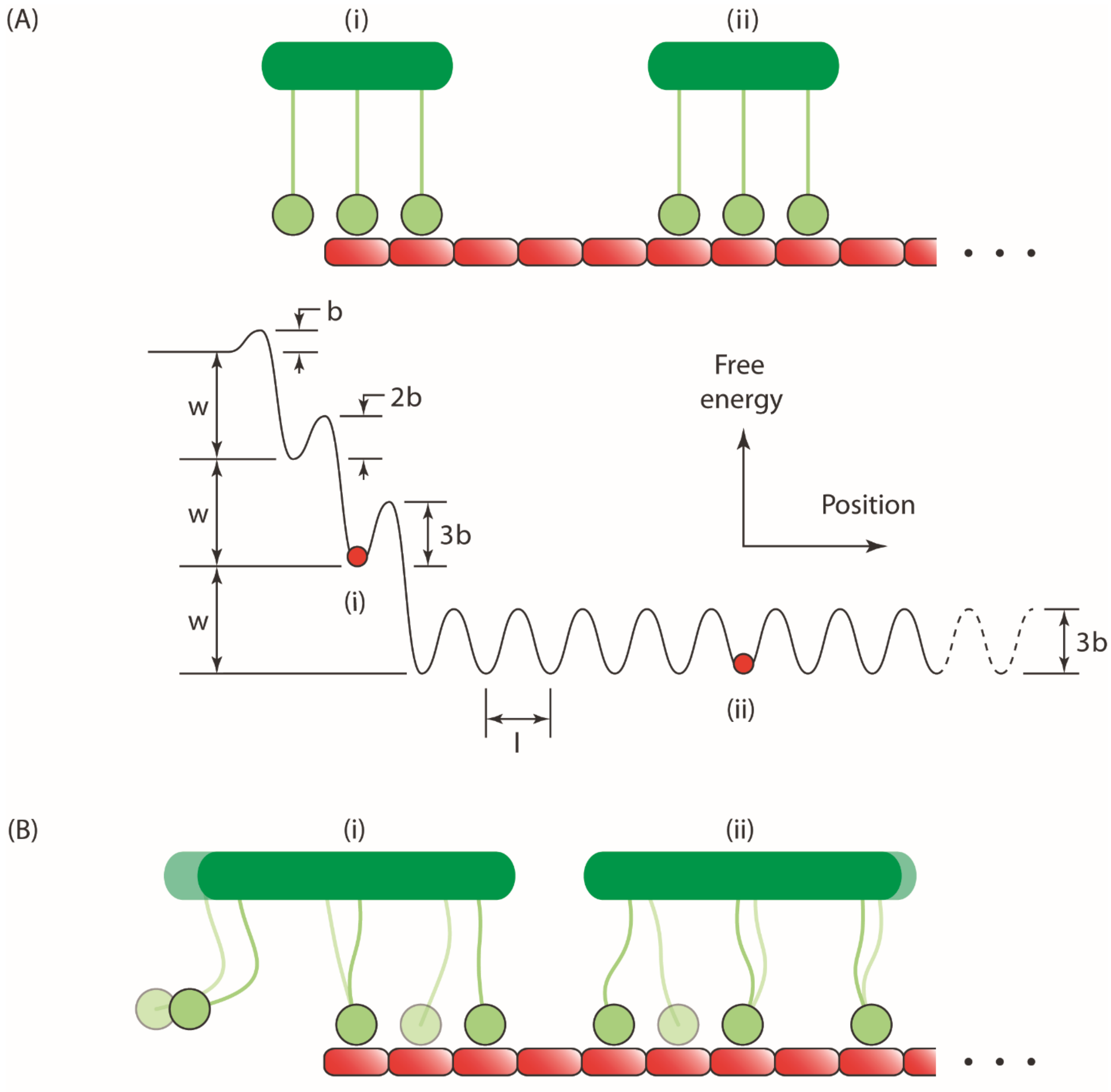

Figure 1.12: Biased diffusion model of kinetochore-microtubule attachment. Free energy versus kinetochore position with (*M=3*) microtubule-binding component. Free energy change for the detachment is W and b is the energy change needed for a single element to adopt transition between sites. Red dots represent energies corresponding to the position of the tip-bound and lattice–bound microtubule-binding element. (A) The energy landscape of the rigid microtubule is represented by the heights of the corrugations, *b, 2b, 3b* and increase in number of microtubule-binding elements will result in *M.b* energy. The effective step, *l*, by the microtubule-binding element is constant. (B) The energy landscape for a flexible array, the effective step size, *l, 1/2l, 1/3 l* and decrease as more elements are bound with minimum energy of *l/M*. In the case stated above, the Corrugated height, *b*, is constant. Experimental evidences show that transition energy for detachment (*w+b* for the landscapes shown here) is much larger than the corrugated height, b. Ndc80, a potential microtubule-binding element, has a lattice diffusion rate of D_o= 0.17μm^2 s^{-1}, implying fast hopping rate from site to site, k_{hop}= 2,600 s^{-1}(=D_o/l^2, *l*= 8nm (tubulin spacing)). k_{off}= 1.2 s^{-1} is for the Ndc80 complex detachment rate with transition energy of at least 7.7 *kgT* larger than *b*. It follows the Boltzmann's law, where ΔU is the

energy difference, $k_{hop/}\ k_{off}=exp\ (\Delta U/k_BT)$ represents ratio of rates. Here k_BT is thermal energy (4.1 pN nm at 25° C) (Asbury, Tien, and Davis 2011).

Kinetochore architecture:

Advancements in fluorescent microcopy have helped to quantify the number of proteins in each complex of the kinetochore and built the architecture of the kinetochore-microtubule site. Green fluorescence protein was widely used to genetically tag a protein *in vivo* to study the kinetochore function. Internal, well defined reference intensity has been used to define the kinetochore architecture. Budding yeast has a single centromeric nucleosome where the whole kinetochore assembles. The single centromeric nucleosome has 2 Cse4 molecules in anaphase. By using this as a reference, initial studies showed that the kinetochore has ~5 Mis12 complexes, ~ 8 Ndc80 complexes and 1-4 copies of other complexes (Table.1.2) (Joglekar et al. 2006; Joglekar et al. 2008). By choosing the dynamic reference, the results were misleading. In order to circumvent the problem, in my thesis work I have used a stable, ubiquitously expressed GFP with normal folding time as a reference to calculate most of the kinetochore complexes. By using Ndc80 intensity as a reference, quantitative microscopy of the kinetochore on point and regional centromere revealed the similar kinetochore architecture. Vertebrate kinetochores have multiple microtubules (~20-25 microtubules in humans) on their dynamic kinetochore, making it difficult to study the kinetochore architecture. Recent advances in tagging the kinetochore protein of chicken cell line DT40 have shown that they have similar copy number of kinetochore proteins per microtubule (Johnston et al. 2010). These studies provide evidence for the conservation of the basic kinetochore structure from yeast to human. Even though the architecture of the kinetochore-microtubule site is known, dynamics nature of the kinetochore over the cell cycle and how it tracks on the microtubule is not known.

Table.1.2. Copy number of kinetochore complex proteins per attachment (kinetochore).

Complex	Budding yeast	Fission yeast	Vertebrate homologue	*S.cerevisiae*		Chicken (DT40)	*S. pombe*
				Metaphase	Anaphase		
Ref. protein		Budding yeast Ndc80 (anaphase)		Cse4 in metaphase	Cse4 in anaphase	Budding yeast Ndc80 (anaphase)	
	Cse4	Cnp1	CENP-A	2	2		2
Cbf3	Ndc10			4	2-3		
	Cep3			2	1-2		
	Mif2	Mif2	CENP-C	1-2	1-2	9	1
COMA	Ctf19		CENP-F	3	2		
CCAN	Ctf3			-	1		
	Chl4			-	<1		
	Nkp2			-	1		
	Cnn1		CENP-T	-	-	7	
	Spc105		KNL-1	5	5	7	
MIND	Mtw1	Mis12	Mis12	6-7	4-5	9	5
Ndc80	Nuf2	Ndc80	Nuf2	8	7	9	7
Dam1	Ask1		Dam1	16-20	10-11		1

(Joglekar et al.2006)

Summary

The kinetochore is a molecular machine that pulls chromosomes by tracking on the depolymerizing microtubule during anaphase. Purification of submodules and the whole kinetochore have made a significant progress in understanding the kinetochore function and its organization. The biophysical studies on these kinetochore complexes have shown their unique properties under tension but their function *in vivo* is not clear. Due to the lack of available methods to study the kinetochore *in vivo*, it has been a mystery for years. In this thesis, I have elucidated the novel structural transition of the kinetochore over the cell cycle (Chapter 2). This structural plasticity is important for their tracking and proper chromosome segregation. Further I investigated the role of tension in the structural transition. Chapter 3 provides further evidence for how the kinetochore structural transitions in metaphase-anaphase are regulated and in late anaphase. In chapter 4, I present my studies on the role of chaperones on assembly of Cse4 nucleosomes by *in vitro* analysis and live-cell imaging. Chapter 5 contains speculations and future directions. Over all, in this thesis work, I have explained the novel conserved structural plasticity of the kinetochore during cell cycle which will further broaden our understanding of kinetochore biology and chromosome biology *in vivo*.

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CHAPTER 2

Structural plasticity of the living kinetochore

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CHAPTER 2

Chapter 2 71

Structural plasticity of the living kinetochore 71

Abstract 75

Introduction 77

Results 80

Sub-modules of the yeast kinetochore increase in intensity during anaphase 80

Some kinetochore subcomplexes increase during anaphase 84

MIND and Ndc80 complexes recover in FRAP but Dam1 complex does not 89

MIND and Ndc80 complexes add copies during anaphase while the Dam1 complex remains stable 92

MAPs affect kinetochore copy number 100

Motor proteins affect the kinetochore copy number. 103

Kinetochore copy number increase is predicted to improve chromosome attachment 104

Kinetochore intensity increase during anaphase is evolutionarily conserved 105

Discussion 110

Kinetochore submodules are adjustable and stoichiometric during anaphase 110

Kinetochore plasticity may be influenced by microtubule dynamics and may be evolutionarily conserved 113

How might structural plasticity promote chromosome segregation? 114

Experimental Procedures 115

Yeast Strains 115

Microscopic Techniques 115

Fluorescence correlation Spectroscopy (FSC) with image calibration 116

Fluorescence Recovery after Photobleaching 116

Photoconversion of kinetochore proteins at metaphase 117

Simulation of kinetochore-microtubule interaction with Hill's equation 118

Supplemental method 123

Simulation of microtubule depolymerization on kinetochore structure predicts the requirement of additional couplers for kinetochore-microtubule interaction in anaphase. 123

Graphical abstract: 125

REFERENCES: 126

Abstract

The kinetochore is a large evolutionarily conserved protein structure that connects chromosomes with microtubules. During chromosome segregation, outer kinetochore components track the depolymerizing end of a microtubule to facilitate separation of chromosomes into two cells. In budding yeast, each chromosome has a point centromere upon which a single kinetochore is built which attaches to a single microtubule. This defined architecture facilitates quantitative examination of kinetochores during the cell cycle. Using three independent measures, calibrated imaging, FRAP, and photoconversion, we find that the Dam1 submodule is unchanged during anaphase whereas MIND and Ndc80 submodules add copies. Stu2-dependent microtubule depolymerization contributes to copy addition. Mathematical simulations indicate that the addition of microtubule attachments facilitates tracking during rapid microtubule depolymerization. We speculate that the minimal kinetochore allows for correction of mis-attachments. Our study provides insight into the dynamics and plasticity of the kinetochore structure during chromosome segregation in living cells.

Introduction

Pairs of sister chromatids must be precisely divided into two cells during cell division to prevent missegregation. Chromosome missegregation results in aneuploidy which is associated with cancer and birth defects (Pfau and Amon 2012; Yuen, Montpetit, and Hieter 2005). Therefore, understanding the mechanisms of chromosome segregation is critical to understand the fidelity of chromosome transmission. Microtubules attach to the chromosome via kinetochores and pull them to the poles during chromosome segregation. The kinetochore is a several megadalton sized protein structure assembled on a specialized region of the chromosome called the centromere. The centromeric region is a ~130-bp sequence in budding yeast and is epigenetically defined in higher organisms. Most of the kinetochore proteins and their functions are evolutionarily conserved. In higher eukaryotes, each kinetochore interacts with multiple microtubules (Walczak, Cai, and Khodjakov 2010; Chan, Liu, and Yen 2005; McAinsh, Tytell, and Sorger 2003), whereas in *S. cerevisiae* each chromosome interacts with only one microtubule (Winey et al. 1995), making it an ideal defined system for studying the kinetochore-microtubule interaction.

The yeast kinetochore (Fig.2.1A) consists of more than 60 proteins that assemble into sub-modules, constituting the inner and outer kinetochore. The inner kinetochore consists of CBF3/Ndc10, Mif2, and COMA complexes that connect the centromeric region of the chromosome to the outer kinetochore. The MIND/Mis12, Spc105/CeKNL-1, Cnn1, Dam1, and Ndc80 complexes form the outer kinetochore, and link the inner kinetochore to the microtubule (De Wulf, McAinsh, and Sorger 2003; Lampert and Westermann 2011). The Ndc80 complex is a heterotetramer of Spc25, Spc24, Nuf2, and Ndc80. Ndc80 binds to the tubulin subunits of a microtubule using an unstructured N-terminus as a finger-like projection (Powers et al. 2009; Guimaraes et al. 2008). The MIND/MIS12 complex, a heterotetramer of Nsl1, Nnf1, Dsn1, and Mtw1, links the Ndc80 complex to the inner kinetochore (Hornung et al. 2011; Obuse et al. 2004). The Dam1 complex, a heterodecamer, is the functional analogue of the Ska1 complex in higher eukaryotes and forms a ring-like structure around the microtubule *in vitro* that may slide along the microtubule as it disassembles (Wang et al. 2007; Schmidt et al. 2012; Welburn et al. 2009). Ndc80 and Dam1 complexes use different means of microtubule contact but work cooperatively to track the disassembling end of the microtubule during chromosome segregation (Umbreit et al. 2014; Tien et al. 2010; Powers et al. 2009), resulting in the movement of chromosomes to the poles (Grishchuk and McIntosh 2006). Although some structural details of the kinetochore submodules that provide attachments to microtubules have been determined, how the submodular structure for kinetochore-microtubule attachments functions in a living cell is not clear.

A microtubule is a polymer of α and β tubulin dimers and interacts with the outer kinetochore to pull the chromosome. A microtubule is a highly dynamic structure, as it can add or lose tubulin dimers in processes called 'rescue' and 'catastrophe', respectively (as reviewed in (Desai and Mitchison 1997)). Growing and shrinking microtubule tip structures differ by their degree of microtubule protofilament curvature which can influence the interaction with the Ndc80 complex (as reviewed in (Foley and Kapoor 2013; Asbury, Tien, and Davis 2011)). Microtubule Associated Proteins (MAPs) can alter the microtubule dynamics by interacting with the microtubule tip and the tubulin dimers. MAPs have been implicated in chromosome capture, spindle stability, and chromosome movement (Pearson et al. 2003; Gandhi et al. 2011). Stu2, one of the XMAP215 family proteins, specifically interacts with the Ndc80 complex {Miller, 2016 #285; and affects microtubule behavior. Loss of Stu2 results in loss of bi-orientation, leading to missegregation of chromosomes (Miller, Asbury, and Biggins 2016). Although the function of Stu2 has been studied in metaphase with purified kinetochores, the function of MAPs during anaphase, when a kinetochore must track a microtubule with prolonged rapid disassembly, has not been examined.

Microtubule associated proteins also include motor proteins. Kip3 (kinesin-8 motor) is a non-essential protein that depolymerizes microtubules at its plus-end (Niederstrasser et al. 2002). Deletion of *KIP3* is lethal in the absence of *DYN1* (Dynein 1) and exhibits shorter spindle with unseparated nuclear mass (Miller et al. 1998). Cin8, a kinesis-5, localizes on the spindle midzone and has redundant functions with Kip1 (Hildebrandt and Hoyt 2001; Hoyt et al. 1992). Deletion of both the motors result in shortened spindles. In *cin8*Δ strain at 25°C shows chromosome loss with frequent broken spindle and is inviable at 37°C (Hoyt et al. 1992). Kar3 is a kinesin-14 motor protein that is important for karyogamy and depends on non-motor proteins for its localization. Kar3 binds to Vik1 to localize on SPB and interacts with Cik1 for its midzone localization. Deletion of the non-motor protein partners, forces Kar3 to its respective location. However, importance of motor protein for kinetochore attachment is not well understood.

We present data consistent with structural plasticity of the kinetochore during chromosome segregation. Calibrated imaging, Fluorescence Recovery after Photobleaching (FRAP), photoconversion, and genetic studies suggest that the outer kinetochore complexes, especially the MIND and Ndc80 complexes, add new copies of proteins during anaphase. However, the Dam1 complex remains unchanged. The MAP Stu2 influences the copy number increase of MIND and Ndc80 subcomplexes, suggesting these kinetochore submodules can adjust their addition based on the Stu2-determined rate of microtubule depolymerization. Simulations of kinetochore function using Hill's kinetochore attachment model (Hill 1985) predict that the addition of each coupler (or copy) decreases the detachment rate ~4 fold. Therefore, addition of the Ndc80 submodule could improve attachment during anaphase. We observe similar structural changes in kinetochores in fission yeast, suggesting structural plasticity is an evolutionarily conserved property of kinetochores in higher eukaryotes. Taken together, our results suggest that parts of the kinetochore structure can sense and adapt to microtubule dynamics while other parts remain constant. Overall, the design of the kinetochore may accommodate structural plasticity that promotes accurate segregation of chromosomes.

Results

Sub-modules of the yeast kinetochore increase in intensity during anaphase

Budding yeast has 16 chromosomes with centromeres that form a cluster (Jin et al. 1998; Jin, Fuchs, and Loidl 2000), and eGFP-tagged kinetochore proteins form a sub-diffraction-limited spot (Joglekar, Salmon, and Bloom 2008). Kinetochores form a single cluster from G1 through late S phase. When sister kinetochores bi-orient and start to separate, the single cluster divides into two equal clusters with half as many kinetochores. To investigate the kinetochore structure during the cell cycle, we used endogenously expressed kinetochore proteins tagged with eGFP at the C-terminus to examine fluorescence intensity. In an asynchronous culture, cells having no bud are categorized as G1 phase while anaphase cells exhibit the maximum distance between kinetochore clusters or spindle pole bodies. The fluorescence intensity of kinetochore clusters, represented by a heat map, shows subunits of the Ndc80 complex have higher intensity in anaphase cells than in G1 cells (Fig.2.1B). However, Dam1p of the Dam1 complex has similar intensity in both anaphase and G1 cells (Fig. 2.1C).

We further quantified the intensity of the kinetochore cluster through the cell cycle by arresting cells in G1 using alpha factor and subsequently releasing them for imaging. As expected, the intensity of Nuf2 (Ndc80 complex) increases during S phase and then drops as the cell progresses through metaphase (Fig.2.1D). However, the fluorescence intensity of the cluster increases again gradually as the chromosomes separate during anaphase (Fig. 2.1D and 2.2A). At the end of anaphase, the fluorescence intensity of a cluster (16 chromosomes) is on par with the intensity in S phase (32 chromosomes), suggesting more proteins are present in each kinetochore structure (Fig. 2.1D). We examined all proteins from the Ndc80 and the MIND complexes (not shown), finding similar intensity profiles to Nuf2 and Dsn1 (MIND complex, Fig.2.2B-C). In contrast, similar experiments with Dam1 complex proteins show little intensity increase during anaphase. As an example, we show Ask1, the intensity of which doubles from G1 to S phase as expected but does not increase during anaphase (Fig.2.1E, 2.2D).

A kymograph heat map analysis shows Nuf2 increases in intensity during anaphase whereas Ask1 does not (Fig. 2.1F, G). Our analyses indicate that the increase is specific to anaphase, since the intensity drops in the subsequent G1 stage.

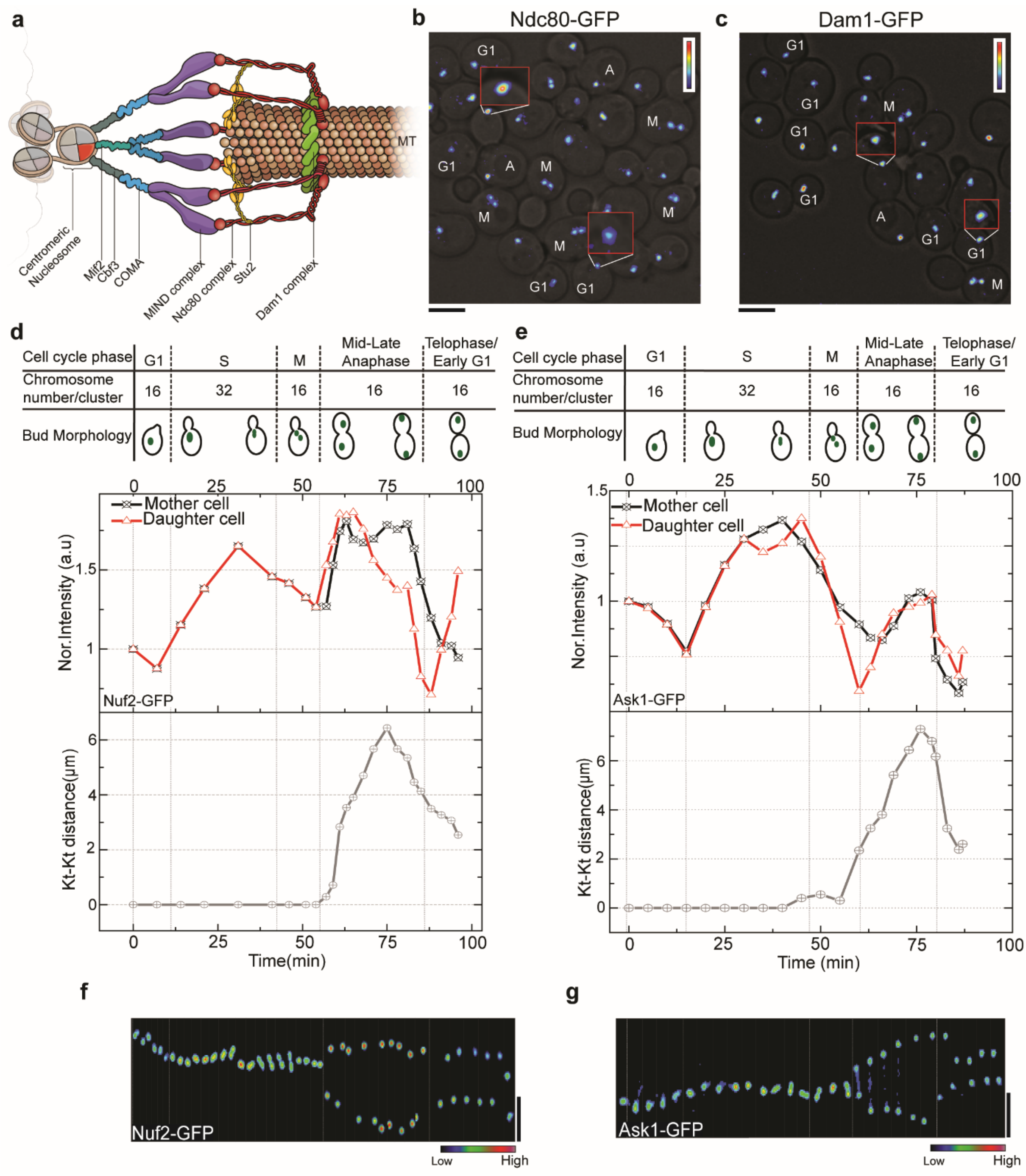


Figure 2.1. The intensity of some kinetochore sub-modules increases in anaphase.

(a) Model of a yeast kinetochore, showing inner and outer kinetochore complexes. The Ndc80 complex interacts with microtubules with finger-like projection; the Dam1 complex can form a ring-like structure. Heat map analyses of Ndc80-eGFP (b) and Dam1-eGFP (c) intensities in an asynchronous culture. Cell cycle stages have been marked near the cell. Red inserts show enlarged intensity heat map.

Ndc80 is much brighter in anaphase than G1. The intensity of Dam1 is similar in G1 and anaphase. Scale bar, 5µm. Intensity profiles of Nuf2-Egfp which is normalized to the intensity of the first-time point “0” (d) and Ask1-eGFP (e) over the cell cycle are shown. The distance between the two kinetochore clusters (Kt-Kt distance, bottom graph) helps to define the cell cycle stage, in addition to the bud morphology (top). (f) Kymograph of Nuf2-eGFP heat map over the cell cycle shows an increase in intensity as kinetochore clusters separate in anaphase. (g) Kymograph of Ask1-eGFP heat map shows G1 and anaphase kinetochore clusters have similar intensity. Scale bar, 5µm.

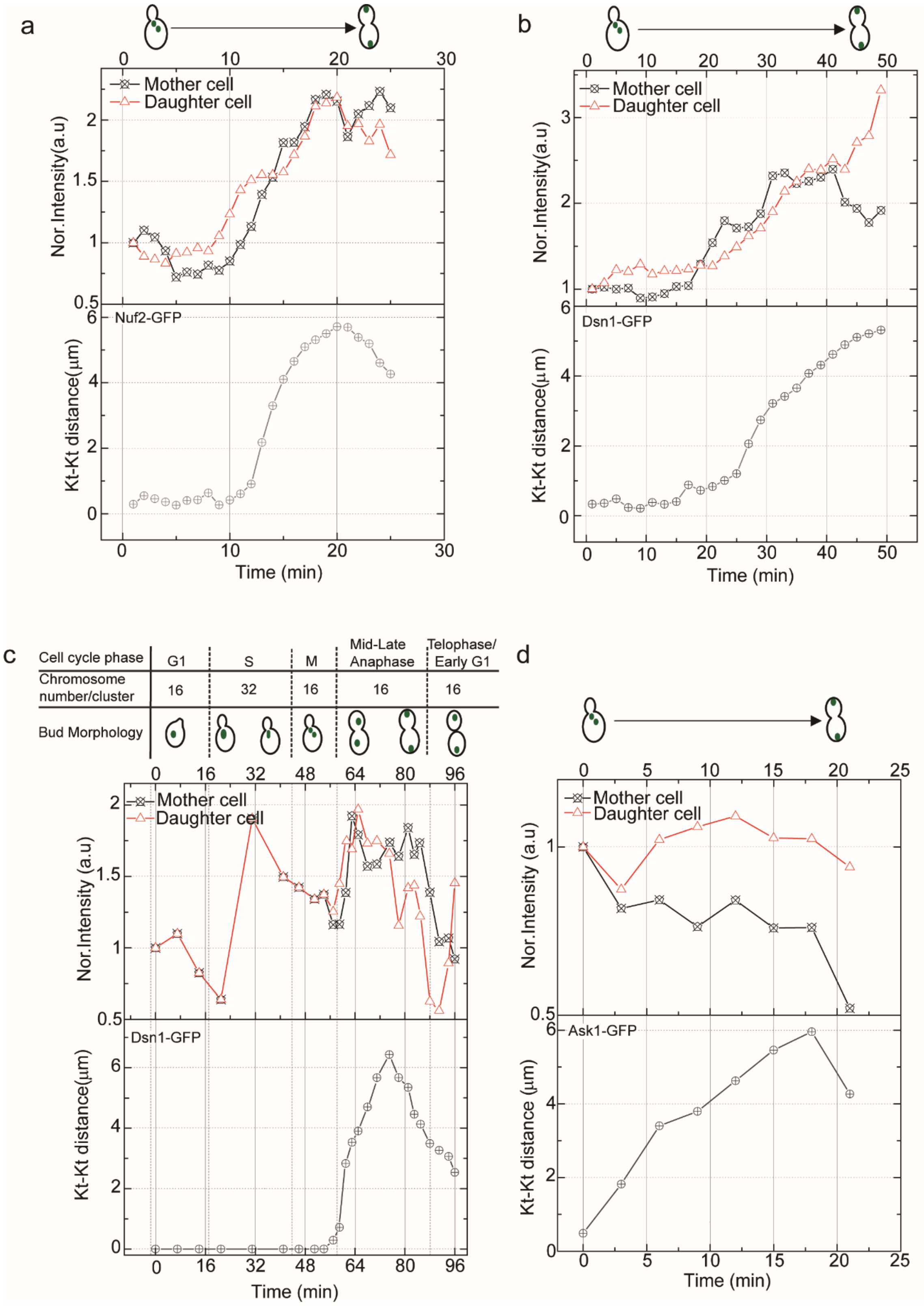


Figure 2. 2. Subunits in the MIND and Ndc80 subcomplex increase in intensity during anaphase whereas the intensity of a subunit in the Dam1 subcomplex does not.

(a)Intensity of Nuf2-GFP and Dsn1-GFP (b) were followed from metaphase (kinetochore cluster distance <0.8μm) to anaphase/telophase. In this experiment intensity measurements were collected at very short intervals. The intensity of Nuf2 and Dsn1 increases gradually as the kinetochore clusters separate from each other. (c) The intensity of Dsn1-GFP was followed over the cell cycle. α-factor arrested cells were released to image the complete cell cycle with more widely spaced timepoints to prevent photobleaching. The intensity of Dsn1 increases during anaphase like the intensity of subunits of the Ndc80 complex. (d) The intensity of Ask1 stays constant or decreases from metaphase to anaphase. For the metaphase to anaphase quantification the time point ”0” is defined as the first timepoint where kinetochore clusters separate apart by <0.8µm and for the whole cell cycle, the time point “0” is defined by the first timepoint of the movie at G1 (cells with Schmoo).

Some kinetochore subcomplexes increase during anaphase

To further quantify the fluorescence increase, we used calibrated imaging, which is based on measuring the absolute intensity of eGFP in live yeast using Fluorescence Correlation Spectroscopy (FCS). Nuclear pore complex proteins have been used extensively to validate this method (Shivaraju et al. 2012). Strains with eGFP epitope tagged proteins of Ndc10, Ctf19/COMA (hereafter COMA), Cnn1, Spc105, MIND, Ndc80, and Dam1 kinetochore complexes were used for quantification in G1 and anaphase. All strains were karyotyped to validate normal ploidy (Table 2. S1, see Materials and Methods). Although data was collected for calibrated imaging from an asynchronously growing culture, we compared calibrated imaging results for Dsn1 during G1 using an asynchronous culture versus alpha-factor arrest (Fig.2.4A). The copy number was independent of the method used to obtain G1 cells. This data also demonstrates the reproducibility of the calibrated imaging method between two independent experiments.

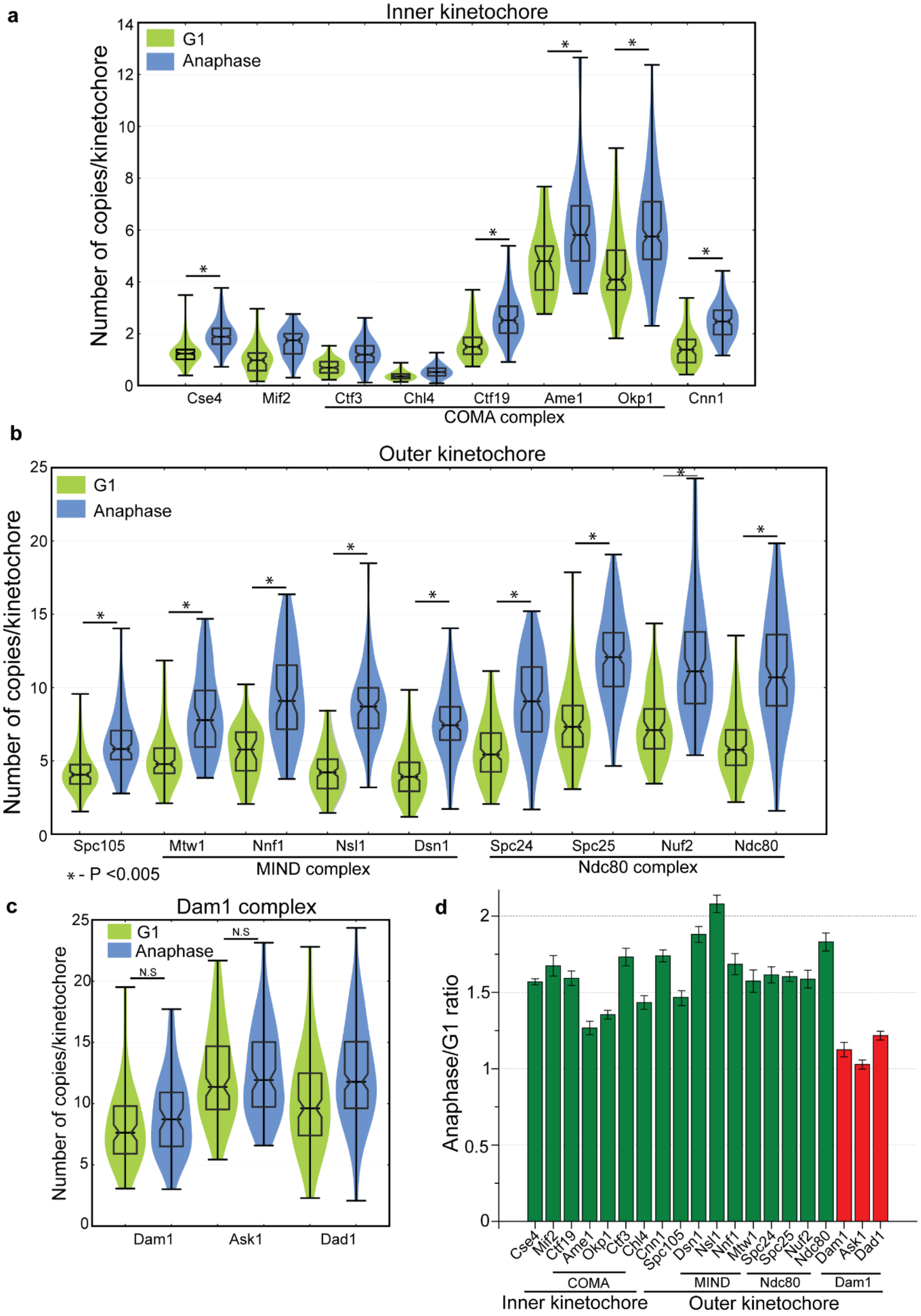


Figure 2.3. The kinetochore has higher copy numbers of Ndc80 and MIND subcomplexes in anaphase while the Dam1 complex has similar copy number throughout the cell cycle.

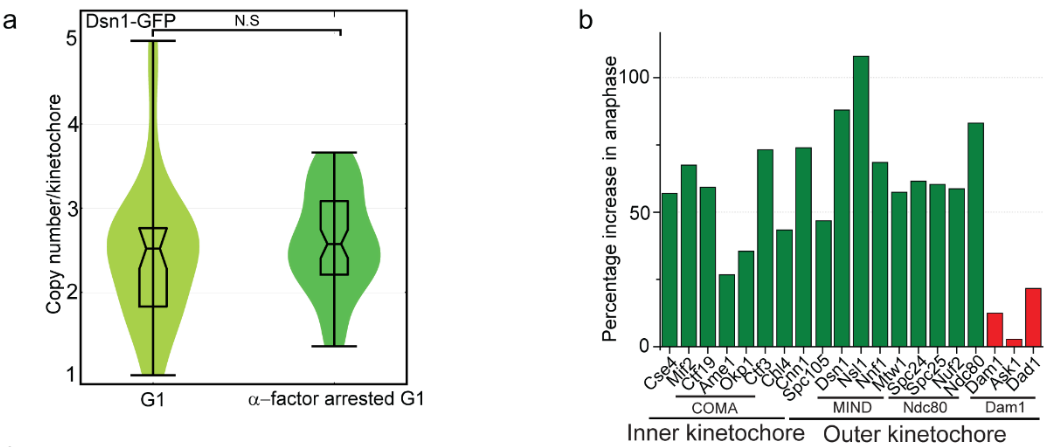
(a) Violin plot of copy numbers of the inner kinetochore proteins in G1 and anaphase. The horizontal middle line is the mean and the box represents the standard deviation. 50-173 clusters were used to calculate the copy number. (b) Violin plot showing copy number of the outer kinetochore proteins. Subunits in the MIND and Ndc80 subcomplexes nearly double in copy number in anaphase. 50-150 clusters were used to calculate the copy number. (c) Violin plot showing the copy number of the subunits of the Dam1 subcomplex, with similar counts in G1 and anaphase. 50-280 clusters were used to calculate the copy number. For a-c, a two-tailed t-test was used to test for statistical significance. N.S. indicates not significant. (d) The ratio of kinetochore proteins in anaphase to G1 show the subunits of the MIND and Ndc80 subcomplexes have higher copy number in anaphase as compared to the Dam1 subunits. Error bars represent standard error.

Several of the inner kinetochore proteins display a significant increase in their copy number during anaphase (Fig.2.3A). Outer kinetochore proteins also display an increase in copies during anaphase (Fig.2.2B). These include proteins from the MIND and Ndc80 complexes which increase in copy number in anaphase by 50-100% (Fig. 2.3D,2.4B). Subunits of the COMA subcomplex, such as Okp1 and Ame1, also increase in copy number in anaphase (Fig. 2.3A). The MIND complex is joined to the inner kinetochore by COMA (Hornung et al. 2014) and associates with the microtubule associated Ndc80 complex of the outer kinetochore. These three subcomplexes all display increases as chromosomes are separating during anaphase, consistent with the idea that they operate together structurally.

The subunits of the Dam1 complex, in contrast to subunits of COMA, MIND and Ndc80 complexes, have a similar copy number in G1 and anaphase (Fig. 3C, D). Subunits of the Dam1 complex show only a slight increase in copy number between G1 and anaphase (Fig. 3C). The mean distribution of 12±4 (SEM) correlates reasonably well with the modeling of 16-fold symmetry of a Dam1 ring encircling a microtubule, assembled *in vitro* (Westermann et al. 2006; Wang et al. 2007). Overall, inner complex proteins tend to be present at lower copy number relative to the outer complex proteins, consistent with a structure with an initial anchoring point to the centromere with amplification moving toward the microtubule side.

Debate in the past over calibrated imaging of centromere/kinetochore proteins in yeast has centered on the size of the cluster, which undergoes compaction in anaphase (Joglekar et al. 2006). Simulations of centromere compaction demonstrated an observed increase in the peak intensity of the cluster of centromeric Cse4 by ~40% (Aravamudhan, Felzer-Kim, and Joglekar 2013). It is worth noting that the 40% estimate (Aravamudhan, Felzer-Kim, and Joglekar 2013) for differences in amplitude assumed a 1.4 NA objective, and thus is inaccurate when applied to the data presented here and in a previous publication (Shivaraju et al. 2012), as this data was acquired with a 1.2 NA objective. The difference in amplitude expected based on spot size is highly dependent on the resolution of the system and given our resolution, the maximum difference in amplitude we could expect based on compaction is 20 to 25% (Smith, Slaughter, and Unruh 2014). We took specific steps to ensure that the data presented here are not clouded by this complication. First, we include data only on spots whose fit width is under a threshold (set as a standard deviation of 182 nm for the Gaussian fit) (Shivaraju et al. 2012). The increase by 50% or more by the end of anaphase for many of the kinetochore proteins after selecting similarly sized clusters surpasses the 25% increase that may occur due to compaction. Furthermore, we do not observe an increase in the Dam1 complex, despite the similar extent of compaction observed for it relative to other kinetochore submodules. These factors strongly suggest that the intensity increase we observe is not due to the compaction of the kinetochore.

Nonetheless, we repeated our analysis with the integrated intensity method (Joglekar et al. 2006), which is immune to artifacts from compaction. After applying the same size filter as above to eliminate spots that are disperse, we integrated the total intensity of each spot in G1 and anaphase. We normalized the kinetochore protein intensities to the average integrated intensity of Cse4 during anaphase (which we set as 2 Cse4/kinetochore) (Fig. 2.4C). While noise increases, as expected based on integration, we obtain consistent results, with many proteins in the inner and outer kinetochore increasing in copy number in anaphase, while the Dam1 complex does not.



c Copy number calculation by integrated intensity method:

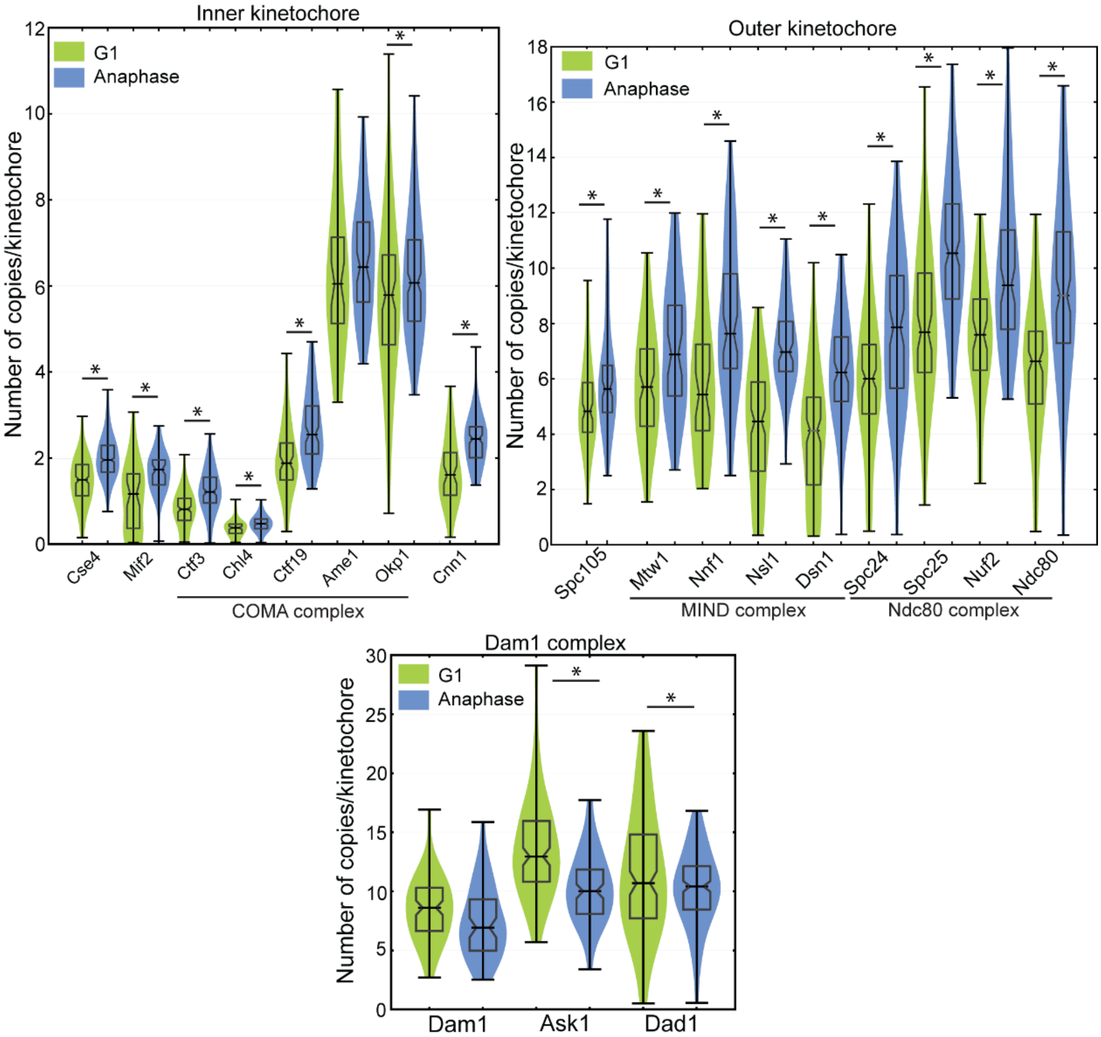


Figure 2.4. Copy number of kinetochore proteins in G1 and anaphase calculated by the integrated intensity method.

(a) The copy number of Dsn1-GFP as calculated by calibrated imaging from 100-150 clusters (n=3) is similar for cells in G1 whether derived from an asynchronous culture or α-factor arrest. (b) The percent change in kinetochore proteins from G1 to anaphase is shown (n=3).

Subunits of the MIND and Ndc80 subcomplexes typically display >50% increase in anaphase. (c) An alternative method for data processing to that shown in Figure 2, known as integrated intensity (Joglekar et al., 2008b), in which Cse4 levels from anaphase are used as a reference to calculate the copy number, also reveals an increase in subunits of the MIND and Ndc80 subcomplexes in anaphase as compared to G1. A two-tailed t-test was used to test for statistical significance. * indicates statistical significance ($p<0.005$). N.S. indicates not significant.

MIND and Ndc80 complexes recover in FRAP but the Dam1 complex does not

If copies of a kinetochore protein are added in anaphase, fluorescence recovery after photobleaching (FRAP) experiments should reveal this addition. We bleached the kinetochore cluster in metaphase (when kinetochore clusters are <1µm apart) and followed it through the cell cycle to quantify the percent recovery in the subsequent anaphase. As the kinetochore clusters move towards the pole, proteins from COMA, MIND and Ndc80 complexes recover to varying degrees (Fig. 2.5A, B; Fig. 2.6A-C). Quantification of subunits of the bleached MIND and Ndc80 complexes shows a recovery of >80% in late anaphase (Fig. 2.5E). The recovery of a few subunits of the MIND and Ndc80 subcomplexes in FRAP beyond the starting value (>100%) suggests that these complexes might have more protein in anaphase than metaphase. However, Ask1 and other subunits of the Dam1 complex fail to recover in anaphase (Fig. 2.5C, D; Fig.2.6D). While we see some experimental variation of the percent recovery with subunits of the same complex, we attribute this in part to variable bleaching of the nucleoplasm. Overall, however, our FRAP experiments reveal that all of the Ndc80 and MIND subunits examined recover more fluorescence than the Dam1 subunits (Fig. 2.5E; Fig.2.6). Similar FRAP experiments performed on metaphase clusters by ourselves (not shown) and others (Joglekar, Salmon, and Bloom 2008; Suzuki et al. 2016), resulted in no recovery within metaphase, suggesting that recovery is specific to progression from metaphase through anaphase. Furthermore, bleaching in mid-late anaphase did not result in recovery within anaphase as determined by ourselves (not shown) and others (Joglekar, Salmon, and Bloom

2008; Suzuki et al. 2016). Taken together, the FRAP data are consistent with the addition of MIND and Ndc80 subunits from early to late anaphase and little addition of Dam1 complex subunits.

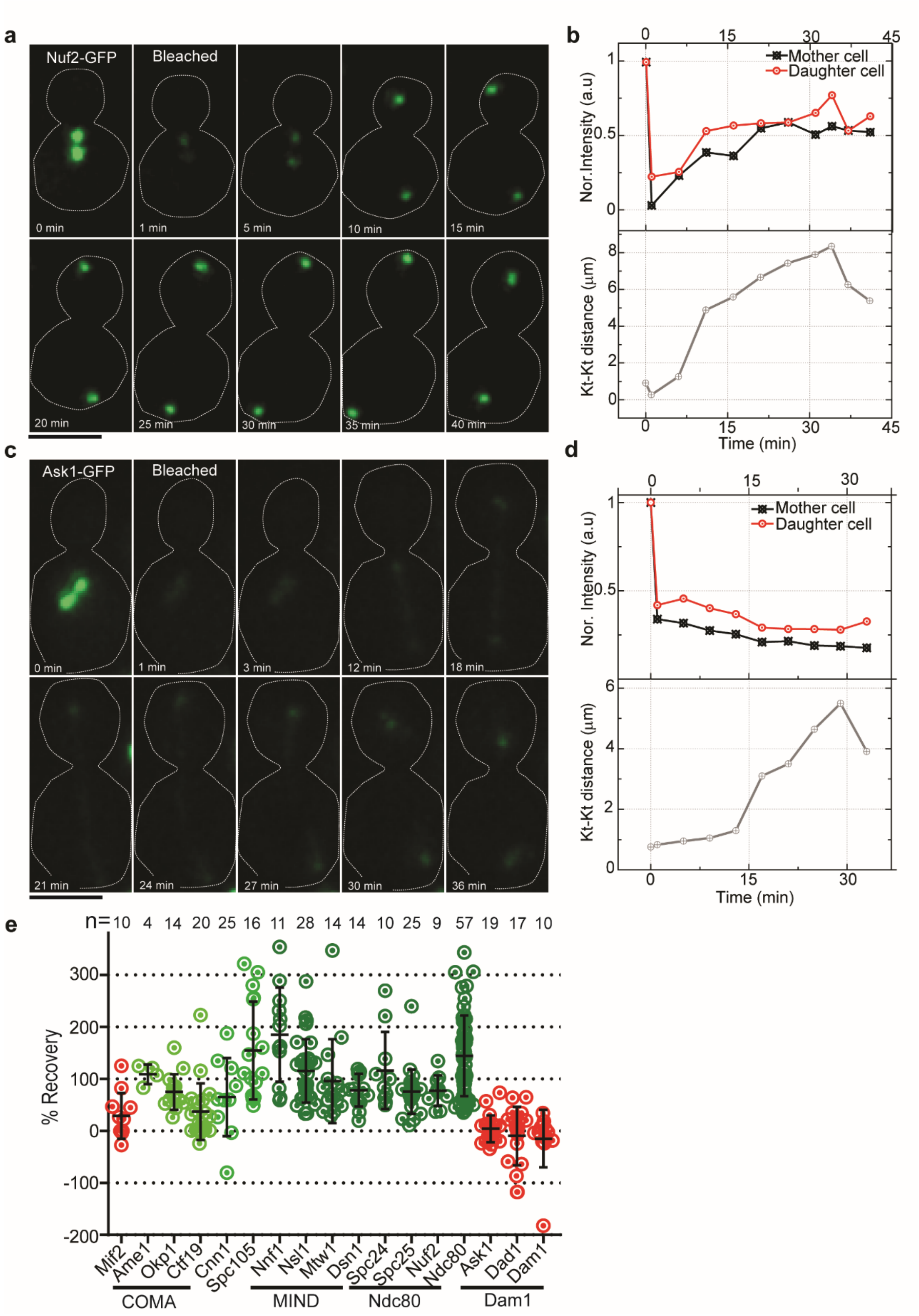


Figure 2.5. Subunits of the Ndc80 and MIND subcomplexes recover in FRAP whereas subunits of the Dam1 subcomplex do not.

(a-b) Kinetochore clusters in metaphase with Nuf2-eGFP were bleached once they were separated by <2µm (1 min) and recovery was monitored, and the intensities were quantified. For A, C the scale bar indicates 5µm. For both the mother and daughter clusters, intensity was normalized to 1 at 0 min. Second time point represents photobleach step. (c-d) Kinetochore clusters with Ask1-eGFP were followed as in (a-b). Quantification of the Ask1-eGFP kinetochore cluster intensity (d) shows no recovery. (e) Percent recovery after photobleaching for kinetochore proteins is shown; each dot represents an individual cluster. Subunits of the COMA (Ame1, Okp1), MIND, and Ndc80 complexes show >80% recovery in anaphase (green) whereas Mif2 and subunits of the Dam1 subcomplex show little recovery (red).

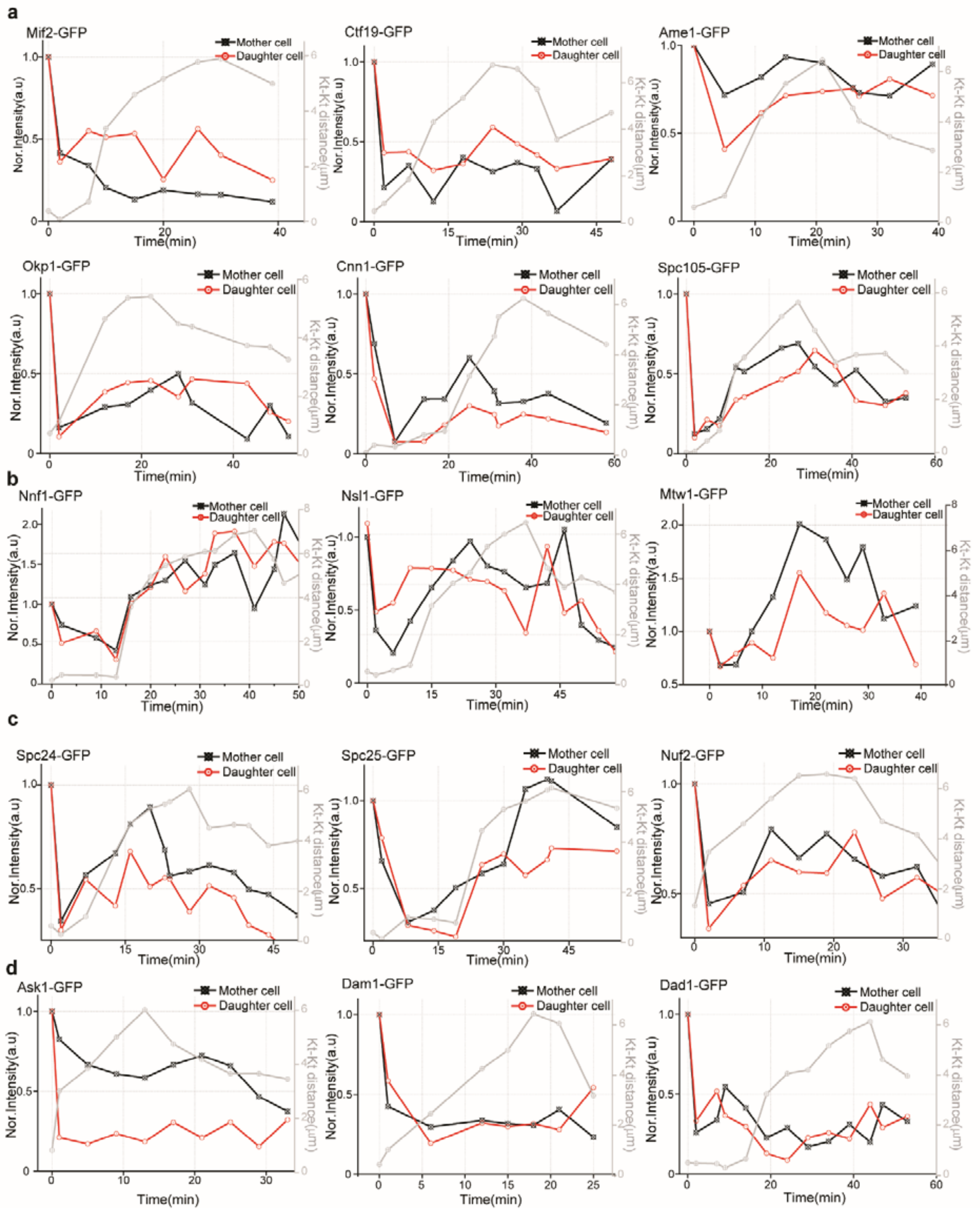


Figure 2.6. Quantification of FRAP shows subunits of the COMA, MIND and Ndc80 subcomplexes recover in anaphase but subunits of the Dam1 subcomplex do not.

(a) FRAP results are shown for centromere-proximal kinetochore proteins Mif2, Ctf19, Okp1 and Ame1. Metaphase cells were used to bleach the kinetochore cluster. Bleached kinetochore clusters were followed through anaphase. Intensity was normalized to 1 at 0 min. The second-time point represents the photobleach moment. Mif2 and Ctf19 do not recover in anaphase, suggesting some of the centromere proximal kinetochore proteins form a structure that does not turnover. However, essential proteins in the COMA subcomplex that anchor the MIND subcomplex do recover in anaphase (Ame1, Okp1). Cnn1 has some recovery. Spc105, which can interact with microtubules, shows recovery in anaphase after bleaching. (b) The MIND subcomplex subunits (Nnf1, Nsl1 and Mtw1) were subjected to similar FRAP experiments. These display >80% recovery in anaphase. (c) Proteins from the Ndc80 subcomplex (Spc24, Spc25 and Nuf2) recover in photobleaching experiments in anaphase. Similar recovery of MIND and Ndc80 subunits suggest they are both added as chromosomes are moving toward the poles in anaphase. (d) Proteins from the Dam1 subcomplex (Ask1, Dam1 and Dad1) do not recover in anaphase from photobleaching during metaphase. Anaphase was determined by the distance between the kinetochore clusters.

MIND and Ndc80 complexes add copies during anaphase while the Dam1 complex remains stable

Our FRAP studies indicate that sub-modules of the kinetochore, especially the MIND and Ndc80 complexes, are dynamic during anaphase. However, FRAP cannot distinguish between complete protein turnover vs. retention of old subunits plus the addition of new subunits. To address this issue, we used kinetochore proteins tagged with a photoconvertible epitope (tdEos) that can be converted from green to red fluorescence with a brief exposure to ultraviolet light (405 nm) (Wisniewski et al. 2014; McKinney et al. 2009). We selectively photoconverted the kinetochore cluster in metaphase and followed it through anaphase. Pre-existing protein will be red following photoconversion, and protein in the nucleoplasm will remain green (with the exception of a small amount of off-axis photoconversion, which we estimated to be less than 5%). Photoconversion of the cluster was >70% in our experiments. We then monitored the addition of protein to the kinetochore during anaphase. Addition of green fluorescence is unmistakable evidence for addition of protein subunits. In the case of Ndc80-tdEos, we observed a >40% increase in green intensity at the kinetochore cluster during anaphase, suggesting that new copies are being added (Fig. 2.7A, B). Red fluorescence at the kinetochore cluster was similar throughout the experiment, implying that the pre-existing protein is maintained, although in some experiments a slight increase in red fluorescence was observed, possibly due to photoconversion of nearby nucleoplasm (Fig. 2.8A).

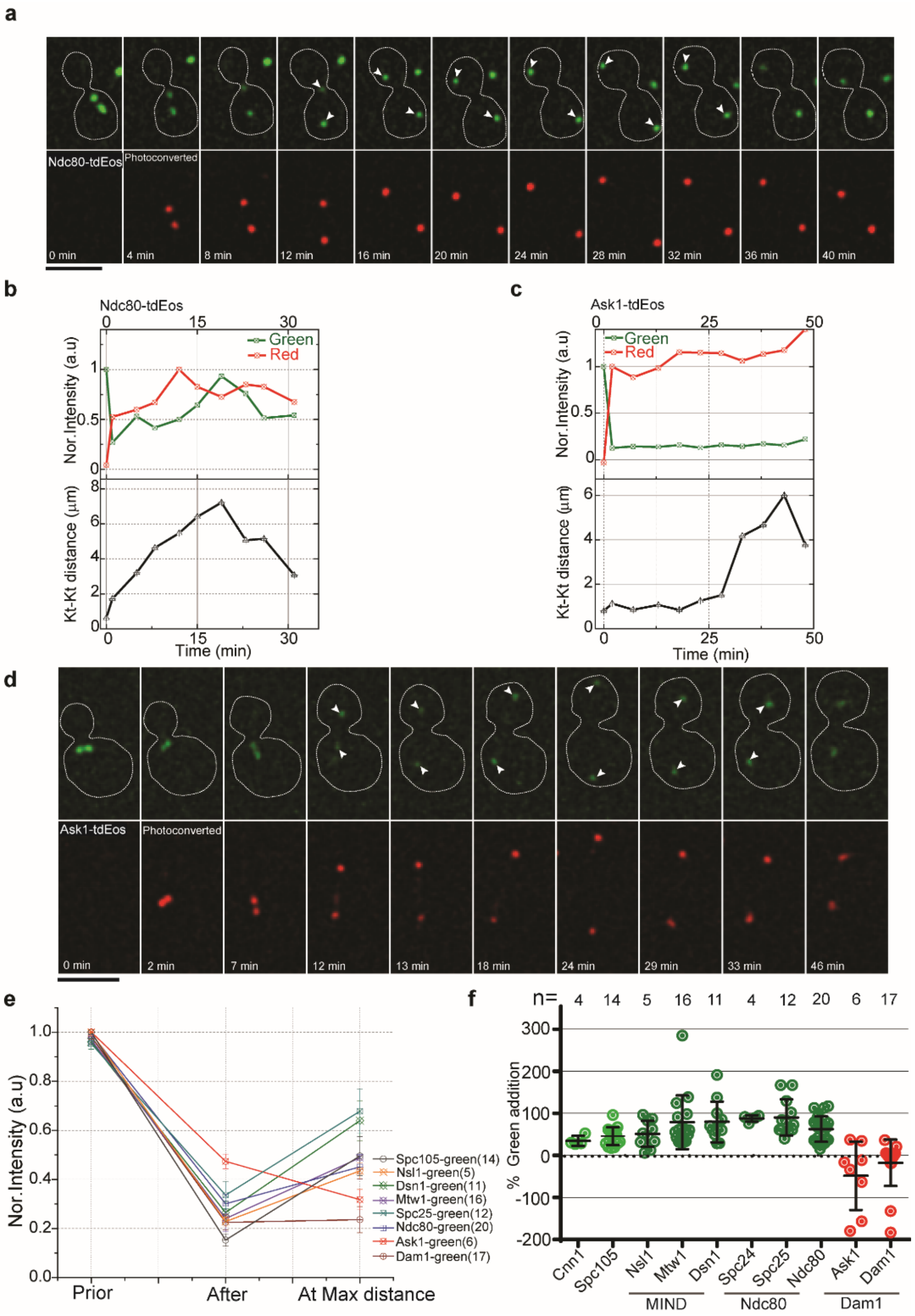


Figure 2.7. Ndc80 and MIND subcomplexes add new copies during anaphase while Dam1 does not.

(a) Ndc80-tdEos is photoconverted in metaphase (4 min) and followed through anaphase. The increase in green fluorescence (white arrows) suggests that new copies are added. Scale bar, 5µm. (b) Quantification of normalized green and red fluorescence intensities at the Ndc80-tdEos spot is plotted over time. (c and d) A similar analysis was performed for Ask1-tdEos. Scale bar, 5µm. The normalized green fluorescence plot shows that new protein is not added during anaphase. (e) The average change in the normalized green fluorescence intensity is shown for each kinetochore protein during anaphase. Error bars represent standard error. (f) Graph showing percent of green fluorescence addition during anaphase by kinetochore protein for the number of individual clusters indicated. MIND and Ndc80 subcomplexes (green) have more green fluorescence at the maximum kinetochore distance than Dam1 subunits (red).

A similar photoconversion experiment using Ask1-tdEos from the Dam1 complex does not show any addition of new protein in anaphase (Fig. 2.7c, d; Fig. 2.8e), suggesting this complex is more stable. Cnn1 and Spc105 are added during anaphase (Fig. 2.8b). Photoconversion experiments suggest MIND and Ndc80 complexes add >50% new protein in anaphase (Fig. 2.7e, f; Fig. 2.8c, d) while the Dam1 complex adds little if any new protein in anaphase.

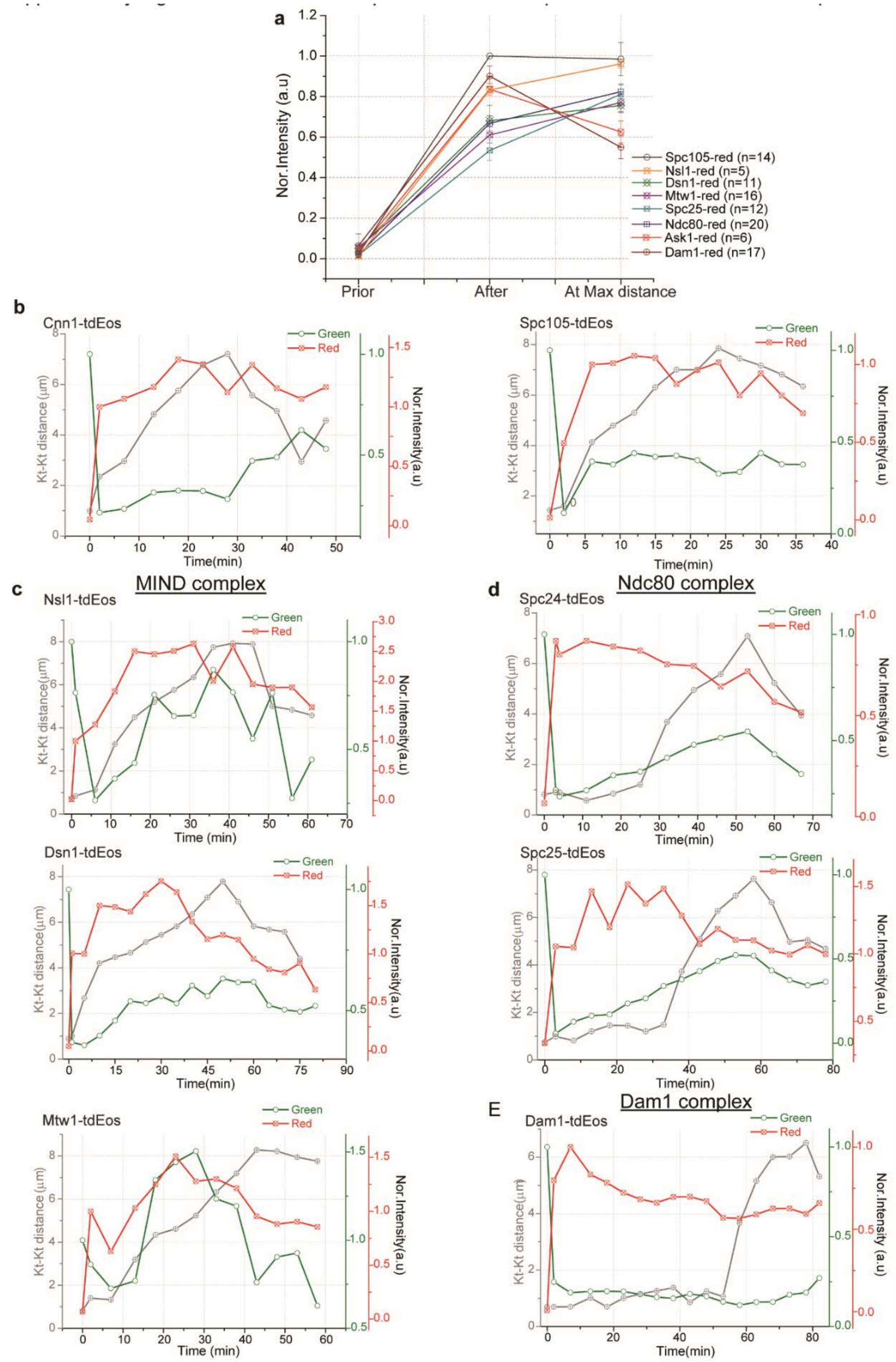


Figure 2.8. Quantification of photoconversion experiments for outer kinetochore proteins.

(a) Summary graph showing the retention of red fluorescence at the kinetochore in photoconversion experiments with number of experiments indicated in brackets. Most of the proteins retain a similar level of red fluorescence at anaphase, suggesting the kinetochore keeps its old copies. Dam1 and Ask1 subunits show a slight loss of red fluorescence, suggesting either a few copies are lost or there is some minor photobleaching. (b) In metaphase, a kinetochore cluster was photoconverted from green to red and followed through anaphase. Quantification of Cnn1-tdEos and Spc105-tdEos shows addition of new copies in anaphase. (c) Quantification of Nsl1-tdEos, Dsn1-tdEos and Mtw1-tdEos following photoconversion. Proteins from the MIND subcomplex show addition of new protein in anaphase. (d) Photoconversion of Ndc80 subcomplex subunits (Spc24 and Spc25) in metaphase reveals addition of copies (green) in anaphase. (e) Photoconversion of Dam1 shows no increase in green fluorescence, indicating that no new protein is added during anaphase. Percent green addition correlates well with the percent FRAP recovery.

To rule out the possibility that the increase in green fluorescence might be due to the compaction of the kinetochore, or that folding dynamics of tdEos are a factor, we photoconverted the surrounding nucleoplasm during metaphase, changing the protein to its red form, without photoconverting the kinetochore cluster (leaving it green). As these cells progressed into anaphase, red fluorescence was added to the kinetochore cluster, strongly suggesting that new copies were added from the nucleoplasm (Fig. 2.9a-c). Taken together, the independent observations from FRAP, photoconversion, and calibrated imaging experiments suggest that additional copies of the MIND and Ndc80 subcomplexes are added to kinetochores as cells progress from metaphase to anaphase while the Dam1 complex remains constant.

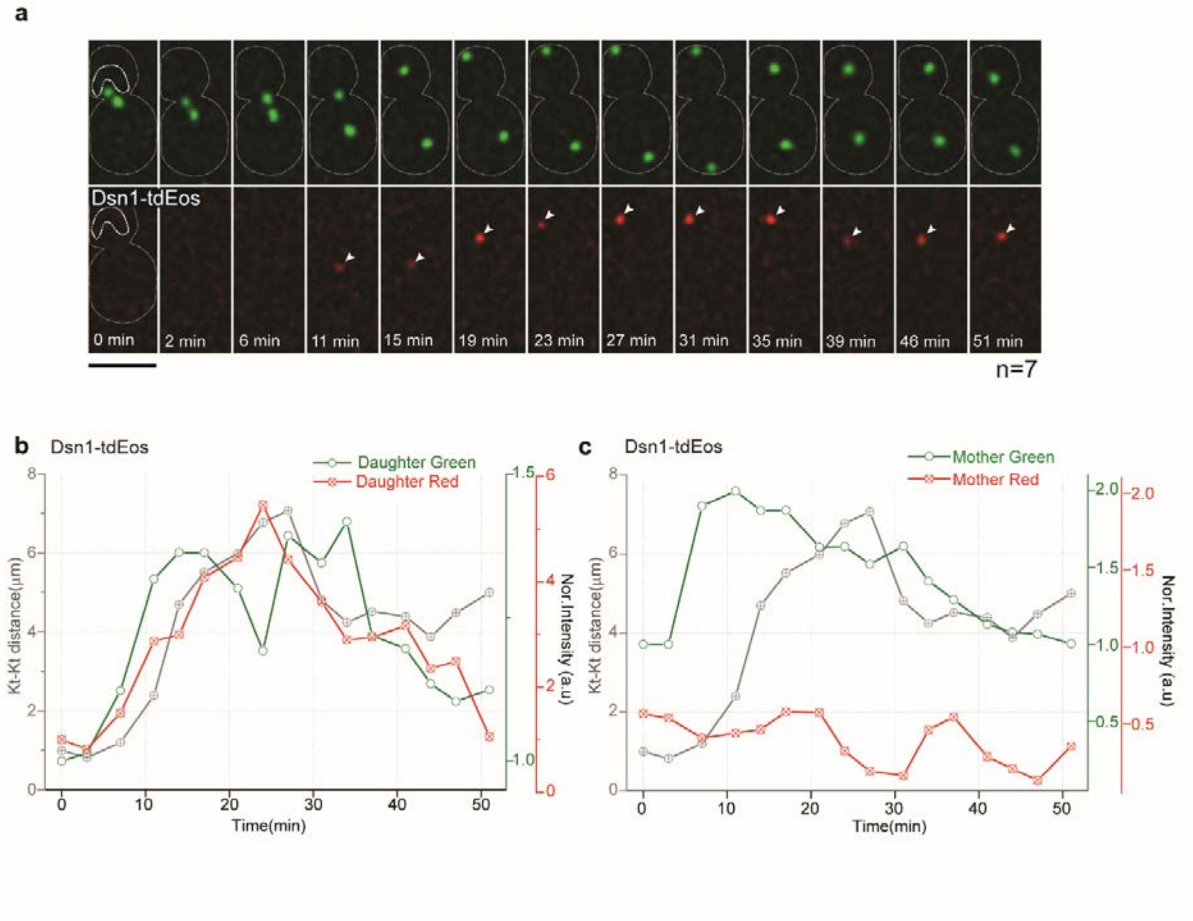


Figure 2.9. Photoconversion of Dsn1-tdEos in the nucleoplasm reveals copy addition during anaphase.

(a and b) Snapshots with quantification of nucleoplasm photoconverted (marked as a white dashed line) cell from metaphase through anaphase. Protein from photoconverted nucleoplasm (red) in the daughter cell (upper cluster) was added to the kinetochore during anaphase. The addition of new protein is demonstrated by the increase in the red fluorescence. (c) The mother cell kinetochore (lower cluster), without nucleoplasm photoconversion, shows the expected increase in green fluorescence but no increase in red fluorescence during anaphase. Scale bar, 5μm. This experiment was repeated 7 times with similar results.

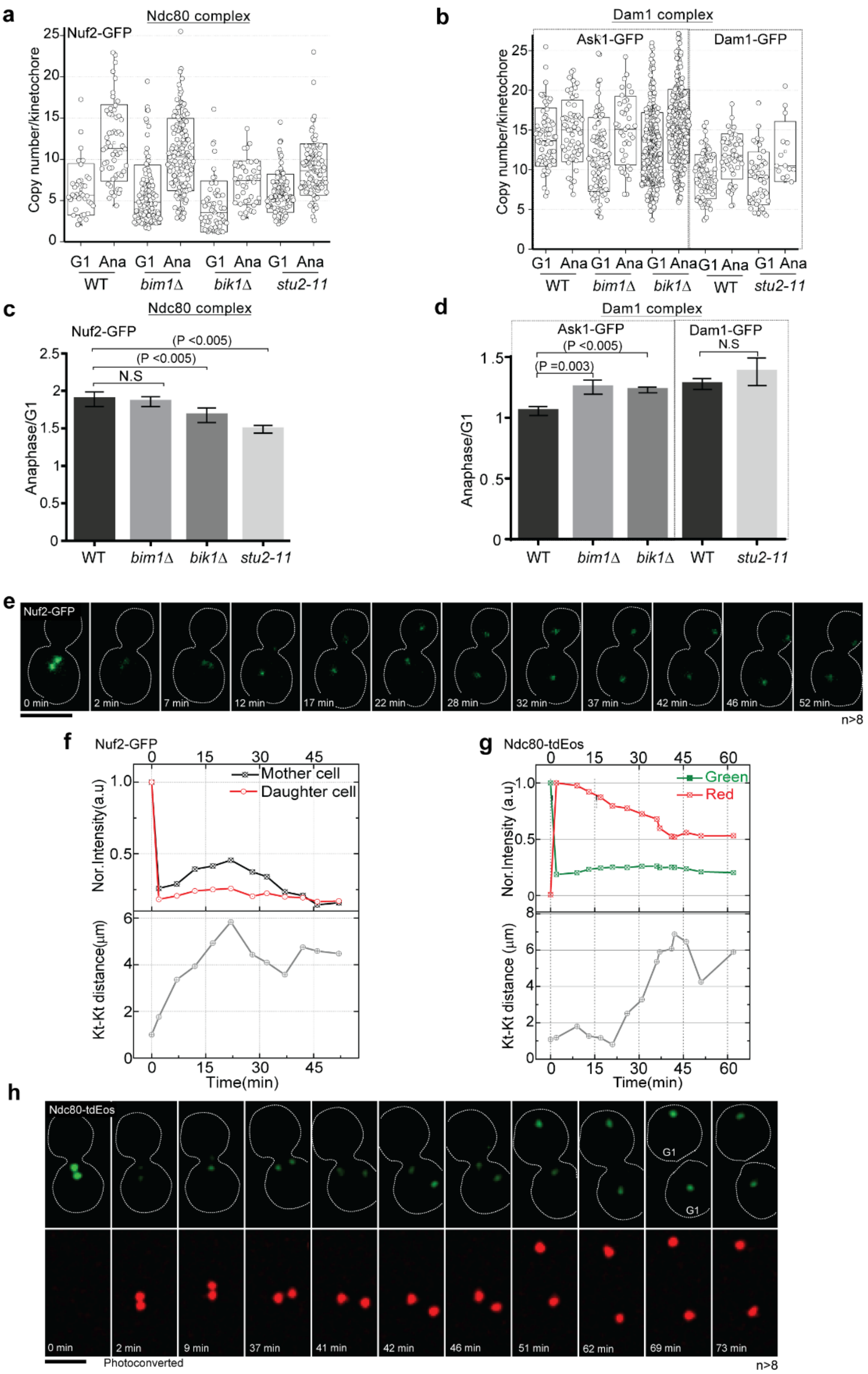


Figure 2.10. A mutation in *STU2* reduces the addition of Ndc80 subunits in anaphase.

(a) Box-plot analysis of Nuf2 copy number of wildtype and mutants. (b)box-plot analysis of Dam1 and Ask1 copy number in wildtype and mutants. (c) Calibrated imaging was used to calculate copy number for the protein indicated in each MAP mutant during G1 and anaphase, similar to Figure 2, and then plotted as in Figure 2D. The *stu2-11* mutant has a lower anaphase to G1 ratio than WT for Nuf2 whereas Dam1 is unaffected. 50-130 clusters were considered for the copy number calculation. (e and f) Snapshots and quantification of

FRAP shows that Nuf2 does not recover during anaphase in the *stu2-11* background. (g and h) Photoconversion of Ndc80-tdEos in the *stu2-11* background shows that green fluorescence does not increase in anaphase. Scale bar, 5µm.

MAPs affect kinetochore copy number

As the kinetochore tracks the depolymerizing microtubules during anaphase, chromosomes move to the poles. Microtubule Associated Proteins (MAPs) affect microtubule function and dynamics (Wolyniak et al. 2006; Mallavarapu, Sawin, and Mitchison 1999). Bik1, Stu2, and Bim1 are MAPs that locate near the kinetochore-microtubule attachment site (Aravamudhan et al. 2014; He et al. 2001). Stu2/XMAP215 (*STU2* homologue in human) has been implicated as a microtubule destabilizer and in some cases as a microtubule polymerase (van Breugel, Drechsel, and Hyman 2003; Al-Bassam et al. 2006; Podolski, Mahamdeh, and Howard 2014). Stu2 is important for spindle elongation and stabilization during anaphase (van Breugel, Drechsel, and Hyman 2003; Severin et al. 2001; Kosco et al. 2001). Stu2 mutants have slower microtubule dynamics with a longer paused state and shorter spindles (Pearson et al. 2003; He et al. 2001). Ndc80 complexes physically interact with Stu2 (Miller, Asbury, and Biggins 2016). A yeast strain bearing a temperature sensitive *stu2-11* allele has slower spindle elongation and takes longer to complete anaphase than wildtype cells (~10 mins slower). Mutations that affect MAPs can therefore affect microtubule dynamics.

To test the effect of microtubule dynamics on kinetochore structure, we used strains with compromised MAP function, deleting *BIK1*, *BIM1*, or using a temperature sensitive allele of the essential *STU2*, *stu2-11*. Calibrated imaging revealed that the *stu2-11* mutant had fewer copies of the Ndc80 complex in anaphase compared to the WT strain or the *bim1*Δ or *bik1*Δ strains, suggesting microtubule dynamics controlled by Stu2 could affect anaphase addition (Fig. 2.10a). In contrast, the Dam1 complex was relatively unaffected by compromising MAP function, suggesting that microtubule dynamics more specifically affect the Ndc80 complex (Fig. 2.10b). FRAP experiments show poorer recovery of Nuf2-GFP in the *stu2-11* mutant compared to WT (Fig. 2.10C, D, and Fig. 2.14A). Furthermore, photoconversion experiments suggest Ndc80-tdEos fails to add copies during anaphase in the *stu2-11* strain (Fig. 2.10E, F). Taken together, these data suggest that microtubule destabilization with slower spindle elongation in the *stu2-11* mutant specifically affects the number of Ndc80 complexes added during anaphase. We speculate that the addition of the Ndc80 submodule can adjust based on the Stu2-determined rate of microtubule depolymerization during anaphase, while the Dam1 submodule structure is relatively immune to microtubule dynamics during anaphase.

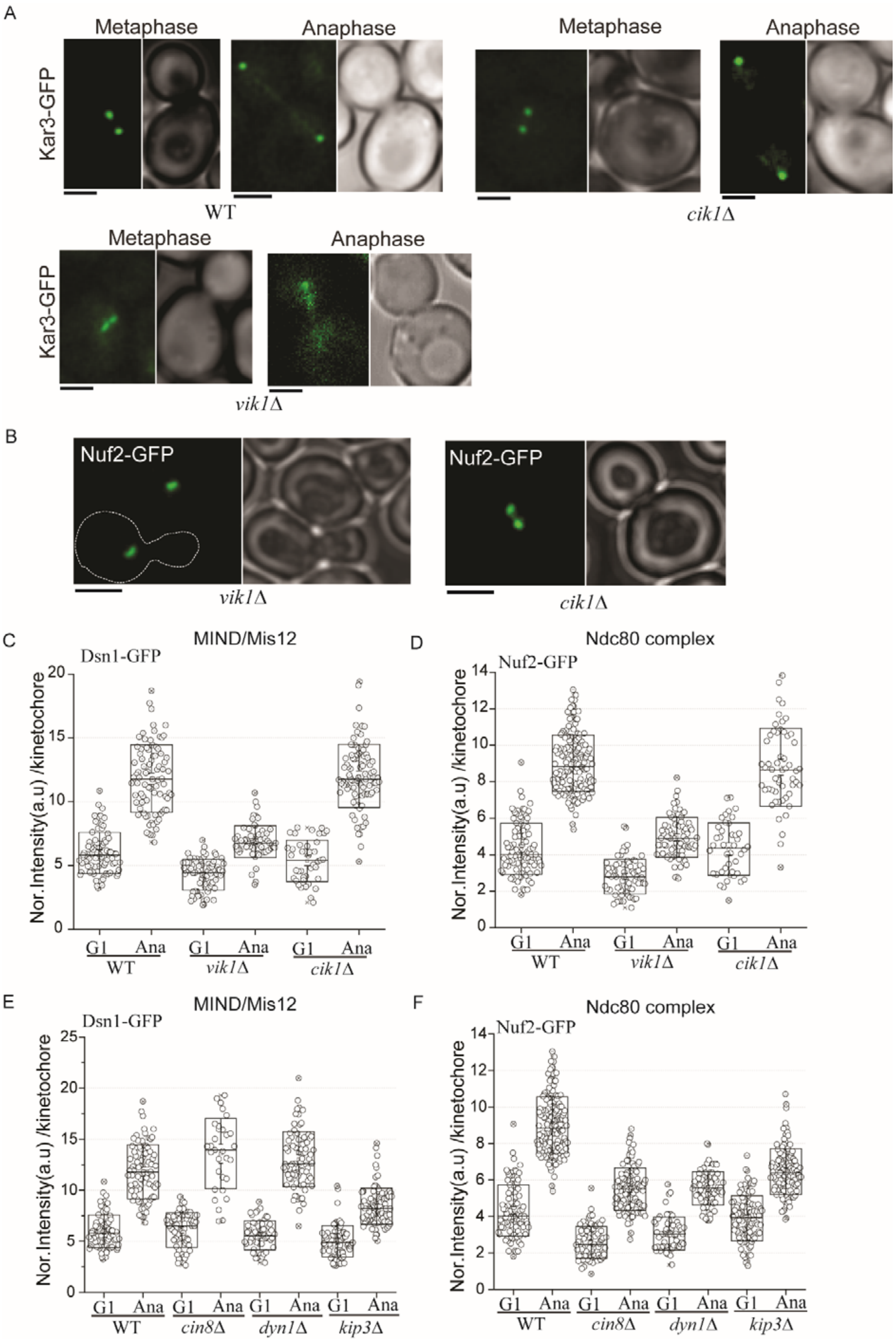


Figure 2. 11. Motor proteins affect the kinetochore copy number in anaphase.

(a) Kar3 associates with non-motor proteins Vik1 and Cik1 to localize on the SPB and spindle midzone respectively. In *vik1*Δ strain, Kar3 localizes on spindle midzone and deletion of *CIK1* localizes Kar3 to SPB. (b) Kar3 association with non-motor proteins have different effect on the spindle. In *vik1*Δ, Nuf2 clusters are not separated well in metaphase. Deletion of *CIK1* shows similar Nuf2 cluster separation in metaphase. (c, d) Kar3 association with non-motor proteins impact the kinetochore differently. (e, f) Deletion of motor proteins have differing effect on kinetochore. Kip3, a known microtubule depolymerase, affects the kinetochore copy number. The motor proteins have more profound effect on the Ndc80 complex than the MIND complex, suggesting that motor proteins may play a critical role in the addition of the Ndc80 sub complex.

Motor proteins affect the kinetochore copy number.

Kar3, a minus-end directed nonprocessive kinesin-14 motor, associates with non-motor proteins Vik1 and Cik1 for their location specific function and localization (Tytell and Sorger 2006). Vik1 interaction with Kar3 localize on SPB and Vik1 is also required for efficient cohesion (Mayer et al. 2004). On the other hand, Kar3 binds to Cik1 for midzone localization (Fig.2.11A). Cik1 binding to spindle midzone depends on another microtubule binding protein, Bim1 (Mieck et al. 2015). The kinetochore protein Nuf2 forms a bi-lobed structure in *cik1*Δ strain. However, in deletion of *VIK1*, the kinetochore forms a collapsed single lobe structure. In the absence of Cik1, Kar3 interacts with Vik1 to localize on the spindle and does not affect the kinetochore localization (Fig.2.11 A, B). Cik1-Kar3 can bind to the Ndc80 complex to localize on microtubule (Mieck et al. 2015). Our image calibration on these mutants showed that Kar3-Vik1 (SPB localized form) has reduced copy number of MIND and Ndc80 complexes (Fig.2.11a). However, both the complexes are not affected in *cik1*Δ strain (Fig.2.11b). Together, Kar3 affects the kinetochore based on its interaction with non-motor protein. Vik1 may be required for the structural transition.

Kip3 (MCAK in mammals) is a microtubule depolymerizing motor that localizes on the kinetochore (Su et al. 2011). *kip3*Δ strain is resistant to microtubule depolymerizing drug benomyl, consistent with depolymerizing activity of Kip3 (Su et al. 2011). Kip3 has been implicated in correcting improper kinetochore-microtubule attachment and aligning the chromatids at metaphase plate. Image calibration of MIND and Ndc80 complexes in *kip3*Δ strain shows that the kinetochore has anaphase-specific reduction of copy number (Fig.2.11c, d), consistent with reduced microtubule depolymerization the mutant has reduced copy number. Deletion of other motor proteins affect the Ndc80 complex both in G1 and anaphase, making it harder to interpret their role in MIND and Ndc80 regulation over the cell cycle. Taken together, some motor proteins differentially regulate the copy number of MIND and Ndc80 complexes. While others do not affect. Kip3 which has a pronounced effect on the structural transition may work via its effects on microtubule depolymerization, similar to Stu2.

Kinetochore copy number increase is predicted to improve chromosome attachment

In anaphase, the kinetochore must persistently track the rapidly depolymerizing microtubule. Hill's model for kinetochore-microtubule interaction has been extensively used to mathematically describe kinetochore movement. The number of proximal coupler microtubule attachments can change based on microtubule dynamics, with depolymerization favoring motion and polymerization favoring a stable attachment (Hill 1985; Joglekar et al. 2006; Asbury, Tien, and Davis 2011; Joglekar and Hunt 2002). Polymerization of the microtubule changes the kinetochore binding site from N to N+1, promoted by random thermal motion, while depolymerization promotes the movement of the microtubule out of the kinetochore "sleeve" (Fig.2.12A)(Joglekar and Hunt 2002). Given our experimental observations, we simulated kinetochore-microtubule attachment during prolonged depolymerization with different numbers of couplers, with the speculation that more couplers might prevent detachment of the kinetochore from the disassembling microtubule. Our simulation studies using Hill's equation show that the addition of couplers decreases the probability of detachment exponentially in a given anaphase time (Fig. 2.12B). If we simulate a 10 min anaphase, addition of a single coupler decreases the probability of a lost attachment by ~4 fold (Fig.2.12C). Based on our experimental evidence, the coupler that could be added to prevent a lost attachment is the Ndc80 submodule. The simulations demonstrate that adding even a single coupler is predicted to have a significant effect on persistence of attachment.

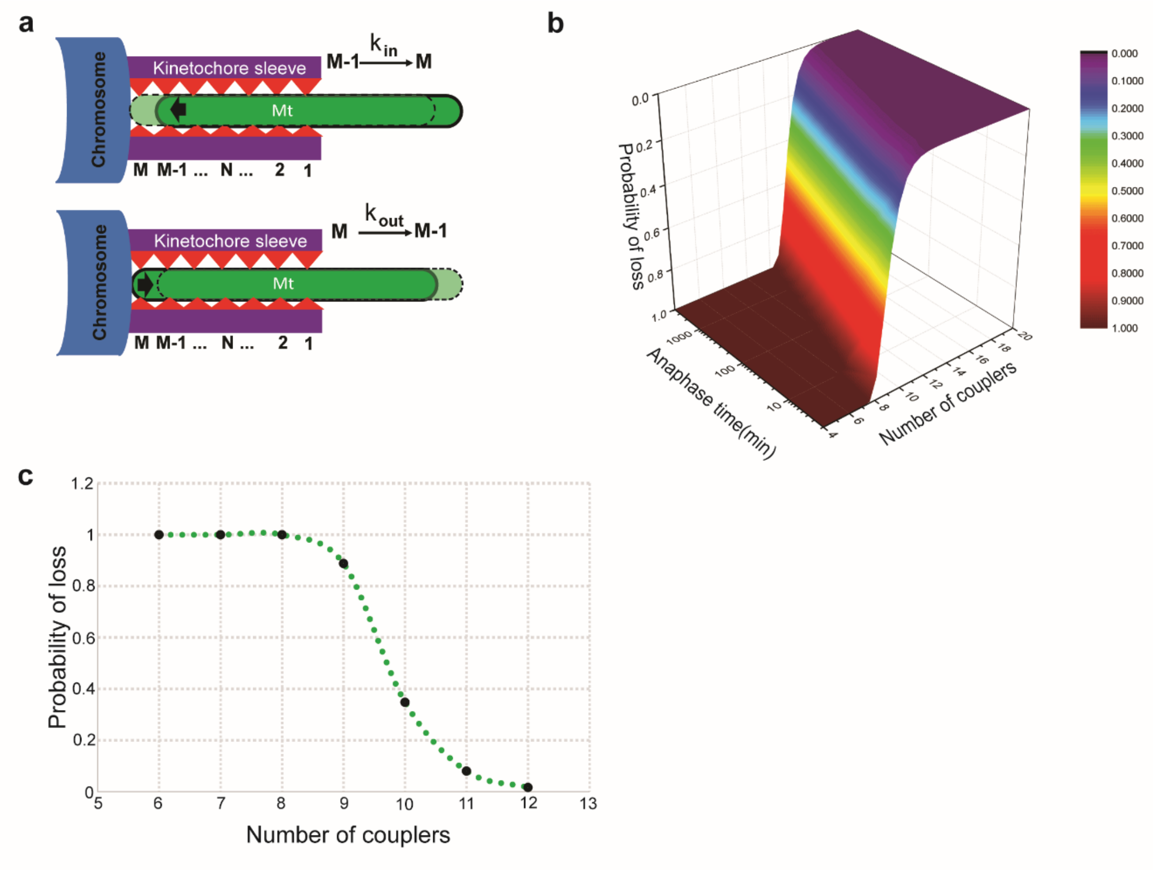


Figure 2.12. Simulations using Hill's biased diffusion model

(a) Hill's equation for the microtubule-kinetochore interaction is shown with a cartoon to depict each variable. During microtubule polymerization, the microtubule moves inside the kinetochore sleeve from $M-1$ to M, for calculating the k_{in}. During depolymerization of the microtubule, interactions are broken and reformed in a new position by moving from M to $M-1$, for calculating the k_{out}. (b) The number of couplers is plotted against the probability of detachment (shown by heat map) as a function of anaphase length. Addition of each coupler increases the probability of remaining attached exponentially. (c) The probability of an attachment breaking is plotted against the number of couplers for a 10-min anaphase, revealing that the addition of each coupler exponentially decreases the loss of kinetochore-microtubule interaction.

Kinetochore intensity increase during anaphase is evolutionarily conserved

Budding yeast and fission yeast diverged ~600 million years ago from a common ancestor (Hedges 2002). Fission yeast has a regional centromere but forms a sub-diffraction-limited kinetochore cluster like budding yeast (Liu et al. 2005) and the kinetochore-microtubule architecture is also conserved (Joglekar et al. 2008). We tagged kinetochore proteins with eGFP to study their intensity during the cell cycle in *S. pombe*. Dsn1-eGFP and Ndc80-eGFP intensities increase in anaphase compared to G2 (Fig.2.13A, B), similar to our observations in budding yeast (Fig.2.2, 3). We used the kinetochore distance to identify the cell cycle stages. For technical reasons, it was easier to quantify the intensity in G2 (rather than metaphase) and anaphase and normalize for the number of chromosomes. The intensity of the kinetochore cluster drops as the cell transitions from G2 to metaphase and then increases again during anaphase (Fig. 2.13C). Anaphase cells have higher fluorescence intensity per chromosome for subunits of the MIND and Ndc80 subcomplexes, suggesting more copies per chromosome in anaphase (Fig.2.13a-d; Fig.2.14b, c). The Dam1 complex in *S. pombe* localizes along the microtubule with multiple puncta (Gao et al. 2010), making it difficult to assess kinetochore-associated Dam1. Altogether, our data suggests that the Ndc80 and MIND subcomplexes also increase during anaphase in fission yeast, suggesting that plasticity of the kinetochore structure may be evolutionarily conserved.

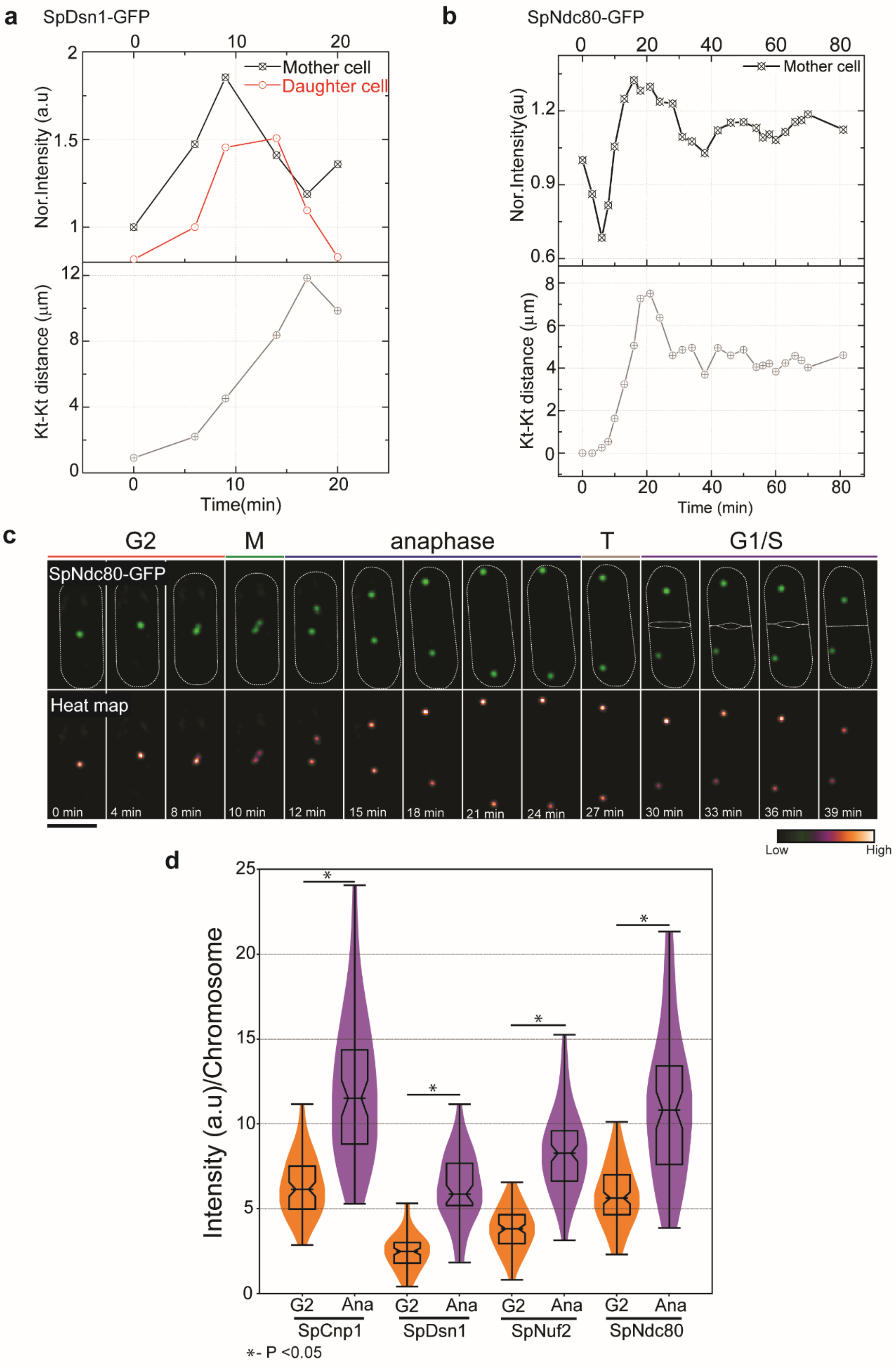


Figure 2.13. Ndc80 increase during anaphase is observed in *S. pombe*.

(a) Quantification of intensity of SpDsn1-GFP (MIND/MIS12 complex) from metaphase to anaphase shows an increase during anaphase. Quantification (b) and snapshots (c) showing an increase in intensity of SpNdc80-GFP from metaphase to anaphase in fission yeast. Scale bar, 5µm. (d) Quantification of kinetochore protein intensity per chromosome in G2 and anaphase is represented as a violin plot. Kinetochore clusters in anaphase have significantly higher intensity than kinetochore clusters in G2. 50-130 clusters were used for the violin plot. A two-tailed t-test was used to test for statistical significance.

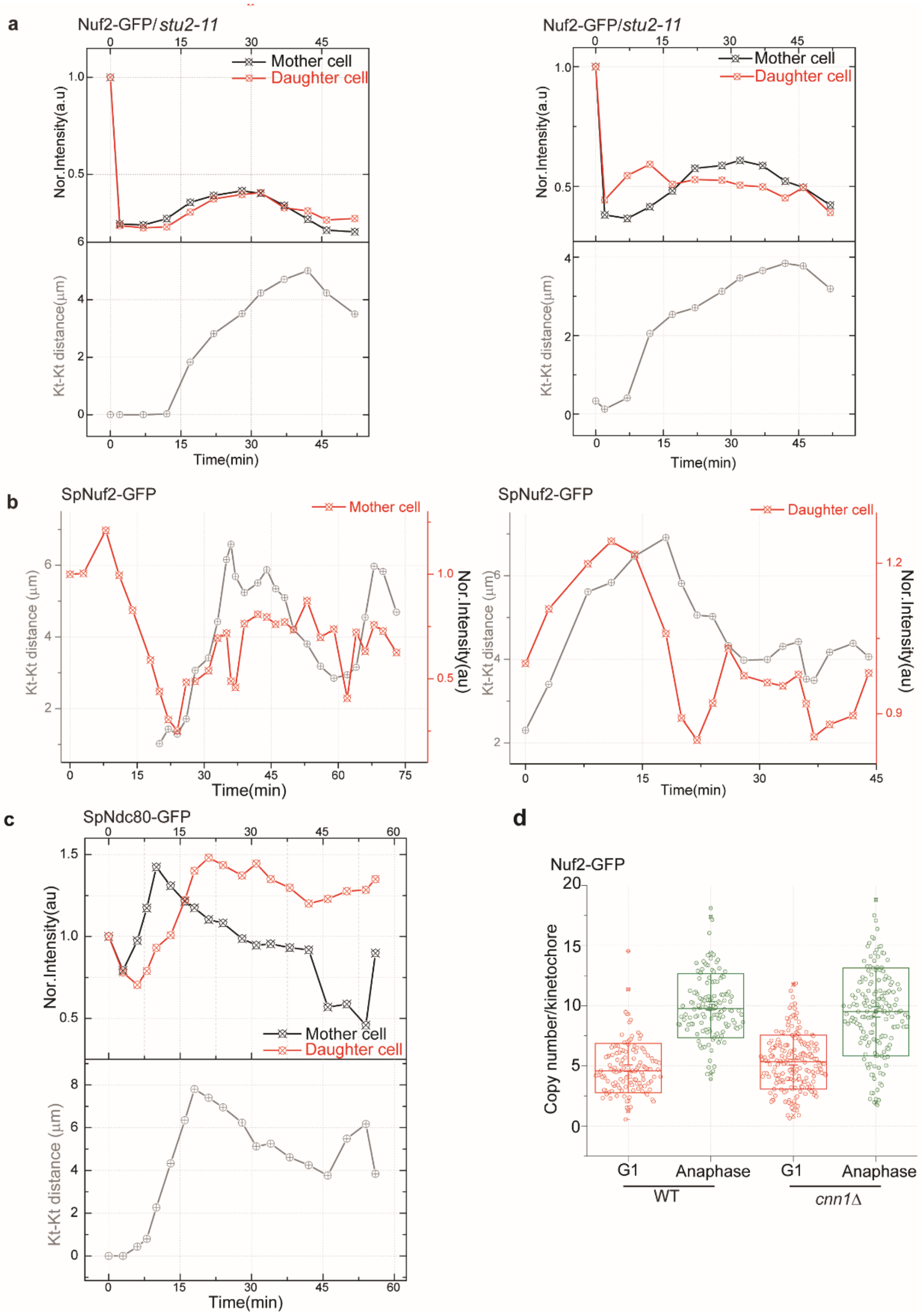


Figure 2.14. Quantification of kinetochore proteins in *stu2-11*, *cnn1Δ* mutants (*S. cerevisiae*) and in *S. pombe*.

(a) Additional FRAP quantification graphs of Nuf2-GFP during metaphase to anaphase in the *stu2-11* mutant background are shown. FRAP was performed as described in Figure 3 at 25°C. Nuf2 recovers less in the *stu2-11* mutant compared to the wildtype strain. (b) Kinetochore clusters with SpNuf2-GFP were followed from G2 to anaphase in *S. pombe*. The intensity of SpNuf2 increased from metaphase to anaphase. (c) A similar experiment using SpNdc80 also shows an increase in intensity during the metaphase to anaphase transition. The intensity increase in anaphase is similar to that observed for the budding yeast kinetochore, suggesting the structural change might be conserved through evolution. (d) Calibrated imaging was used to calculate and compare the copy number for Nuf2 in WT and *cnn1*Δ strains in G1 and anaphase. Deletion of *CNN1* does not affect the copy number of Nuf2 in either G1 or anaphase.

Discussion

Error-free chromosome segregation is essential for successful progression of life. Experiments using purified yeast kinetochores have revealed that microtubule depolymerization makes kinetochores highly susceptible to detachment (Akiyoshi et al. 2010) which in turn could be deleterious for chromosome transmission. Our work suggests that the kinetochore structure, particularly the MIND and Ndc80 subcomplexes, but not the Dam1 subcomplex, is modified during the rapid microtubule depolymerization of anaphase in living cells (Graphical abstract-model 2.15). The increase in Ndc80 copies has the potential to increase contacts between the microtubule and the kinetochore during anaphase, presumably to facilitate efficient tracking. We speculate that fewer copies of the Ndc80 submodule during G1 and metaphase relative to anaphase could facilitate correction of errors in microtubule attachment. Our results suggest that the kinetochore is an adjustable structure, which may contribute to robust functionality.

Kinetochore submodules are adjustable and stoichiometric during anaphase

The kinetochore interacts with the assembling and disassembling microtubule tip throughout the cell cycle. During anaphase, however, the kinetochore must track on a rapidly disassembling microtubule without losing the attachment. The Ndc80 complex makes this connection to the microtubule tip through its N-terminal unstructured finger-like projections. The Ndc80 complexes align parallel to each other with highest FRET in anaphase (Aravamudhan et al. 2014). As a microtubule disassembles, it is proposed that the Ndc80 complex breaks the interaction with the microtubule near the tip and forms a new more internal interaction (as reviewed in (Asbury, Tien, and Davis 2011; Foley and Kapoor 2013)). Oligomerization of the Ndc80 complex is necessary for it to track the microtubule *in vitro*. Dam1, an essential complex for kinetochore function in budding yeast, forms a ring-like structure and tracks the disassembling microtubule. The presence of these two different types of microtubule contacts may facilitate robust attachment. However, the type of attachments made by the Ndc80 submodule may be more amenable to adjustment. We observe that the Ndc80 complex gradually adds copies as the spindle pole bodies separate during anaphase, and this addition is accompanied by the addition of copies of the MIND complex and the Okp1 and Ame1 subunits of the COMA complex (Hornung et al. 2014), which anchors the MIND complex to the centromere. Together Okp1/Ame1-MIND-Ndc80 may form a working structural module, stretching from the inner kinetochore to the microtubule. The addition of Okp1/Ame1-MIND-Ndc80 kinetochore submodules could promote chromosome attachment to microtubules as chromosomes move to opposite poles during anaphase.

The relative stoichiometry of proteins within Ndc80 and MIND submodules remains similar in G1 and anaphase, reinforcing the idea that they function as structural submodules within the kinetochore. Because there are more copies of Ndc80 than MIND, and more copies of MIND than Okp1/Ame1, our results would suggest a single Okp1-Ame1 submodule may associate with more than one MIND submodule, and one MIND submodule may associate with more than one Ndc80 submodule. Although Okp1 and Ame1 may exist in the COMA submodule with Ctf19, they have significantly higher copy number than Ctf19. However, overall stoichiometry of the COMA submodule is roughly maintained from G1 to anaphase. In contrast to the subunits of the MIND and Ndc80 submodules, which all exhibit similar behavior in FRAP and photoconversion experiments, Okp1 and Ame1 show more recovery than Ctf19 after photobleaching, suggesting different structural dynamics for the subunits of this submodule. Cnn1 has been reported to function as a receptor for the Ndc80 complex in anaphase (Schleiffer et al. 2012; Malvezzi et al. 2013), and may associate with added copies of the Ndc80 subcomplex. However, deletion of *CNN1* did not affect the copy number or addition of Ndc80 in our hands, suggesting it is not required for recruitment of Ndc80 (Fig. S6D). Overall, our results start to reveal the behavior of subunits and submodules within the structure of the living kinetochore.

The Dam1 subcomplex tends to form an oligomeric structure and at high protein concentrations forms a complete ring with 16-fold symmetry *in vitro* (Miranda, King, &

Harrison, 2007; Ramey et al., 2011; Wang et al., 2007; Westermann et al., 2005). The Dam1 subcomplex can interact with the microtubule as a monomer, partial ring, complete ring or double ring *in vitro* (Gestaut et al. 2008; Miranda, King, and Harrison 2007; Kim et al. 2017), suggesting multiple modalities of interaction are possible. All forms of the Dam1 subcomplex form a load bearing contact with the microtubule and track the depolymerizing microtubule processively (Umbreit et al. 2014; Gestaut et al. 2008). Previous imaging estimated the Dam1 subcomplex at 10-20 copies per kinetochore *in vivo* (Joglekar et al., 2006), similar to our estimate of 12±4 copies. A partial Dam1 ring has been observed by tomography (McIntosh et al., 2013), suggesting that the Dam1 complex could form an incomplete ring *in vivo*. Our data are consistent with the speculation that the Dam1 subcomplex can exist as an incomplete ring *in vivo*.

Previous studies on kinetochore protein copy number specifically focused on metaphase or anaphase and typically used centromeric histone protein Cse4 as a reference (Joglekar et al. 2006; Joglekar et al. 2008). The relative copy number of kinetochore proteins was given in metaphase and anaphase relative to Cse4 in metaphase, and Cse4 in anaphase, respectively, with Cse4 unchanged over the cell cycle. However, a later study argued that Cse4 fluorescence changes between metaphase and anaphase (Shivaraju et al. 2012), suggesting these previous measurements may be reinterpreted. With the unchanging Cse4 reference used in the previous study, the anaphase values calculated relative to metaphase showed a slight relative decrease in copy number for the MIND complex member Mtw1p, a slight relative decrease for COMA complex member Ctf19p, and a large (~ factor of 2) relative decrease in copy number for Dam1 complex member Ask1p (Joglekar et al. 2006). Based on our collection of calibrating imaging, FRAP, and photoconversion data, we instead argue that subunits of the Dam1 subcomplex remain constant from metaphase to anaphase, while subunits of the MIND and COMA submodules are added during anaphase. Our data is therefore consistent with the previous report, but simply differs in the calibration reference. In fact, if we use Cse4 as a reference at 2 copies/kinetochore in anaphase, our anaphase numbers are strikingly similar to the previous report (Table 2. S2). Our FRAP results are also consistent with prior reports. Previous FRAP experiments were performed on a handful of kinetochore proteins for ~5 mins (300s) and did not reveal any recovery (Joglekar, Salmon, and Bloom 2008; Joglekar et al. 2006; Suzuki et al. 2016). However, these experiments differed from ours in that they were performed either in metaphase or in anaphase. In contrast, we followed recovery from metaphase to anaphase, and typically for a longer period. Furthermore, the critique that compaction of the cluster can affect the intensity of the spot by 40%, and therefore may explain the differences we observe in anaphase, is refuted by both the FRAP and photoconversion data, where intensity changes are not the readout – but rather the absence (Dam1 complex) or presence (Ndc80 complex, for example) of intensity in anaphase following photobleaching or photoconversion in metaphase. In sum, our results are compatible with the published literature.

Kinetochore plasticity may be influenced by microtubule dynamics and may be evolutionarily conserved

MAPs can influence the rate of microtubule assembly and disassembly during the cell cycle (Kosco et al. 2001). The Stu2/XMAP215 family is important for spindle elongation and kinetochore-microtubule interaction (Usui et al. 2003; Miller, Asbury, and Biggins 2016). Loss of Stu2 severely reduces the microtubule dynamics, makes spindles shorter and arrests cells in metaphase (Pearson et al. 2003; Kosco et al. 2001). The reduced microtubule dynamics reported in a *stu2* mutant (McAinsh, Tytell, and Sorger 2003) is associated with less addition of Ndc80 subunits in our experiments, revealing a correlation between a lower disassembly rate and the number of Ndc80 submodules added. Stu2 may help to directly recruit or stabilize additional Ndc80 complexes via its interaction with Ndc80. Given that Stu2 is evolutionarily conserved (human ortholog ch-TOG) and its interaction with Ndc80 is evolutionarily conserved (Miller, Asbury, and Biggins 2016), this protein could play a similar role in higher eukaryotes. In contrast, the Dam1 complex was unaffected by compromising MAP function. We speculate that the Ndc80 complex may be able to sense the rate of microtubule depolymerization and adjust the number of copies added for attachment while the Dam1 subcomplex may not vary its attachment by depolymerization rate. The kinetochore structure in the *stu2-11* mutant during anaphase is an intermediate between the normal G1 and anaphase state, suggesting fewer microtubule couplers are present under conditions of reduced depolymerization speed and reinforcing the idea of structural plasticity.

A purified kinetochore complex detaches from a disassembling microtubule ~100-fold faster than a polymerizing microtubule *in vitro* (Akiyoshi et al. 2010; Sarangapani et al. 2014); detachment in a cell could result in aneuploidy. Our results suggest that the living kinetochore enhances its interaction with the depolymerizing microtubule by increasing the copy number of the Ndc80 subcomplex. These structural changes would be difficult to observe *in vitro* since they depend on a soluble pool of subunits. Simulation of kinetochore interactions with microtubules predicts that the addition of each coupler decreases the probability of losing the microtubule attachment by 4-fold. We propose that the addition of the Ndc80-MIND-Okp1-Ame1 modules could serve this role. In yeast, as each chromosome is attached to a single microtubule, this addition may be especially important for faithful completion of chromosome segregation. Our results in fission yeast support addition of Ndc80 subcomplexes during anaphase as a conserved feature of a living kinetochore structure.

How might structural plasticity promote chromosome segregation?

The structural plasticity of the kinetochore may contribute to its functionality. We speculate that in metaphase, fewer copies of Ndc80 might facilitate error-correction by Aurora B kinase (Biggins et al. 1999; Cheeseman et al. 2002; Tanaka et al. 2002). Increased Ndc80 levels, as reported in cancer, may compromise error correction, leading to the increased aneuploidy observed (Meng et al. 2015; Yuen, Montpetit, and Hieter 2005; Pfau and Amon 2012). Once chromosomes start moving to the poles during anaphase, the addition of Ndc80-MIND-Okp1-Ame1 submodules may provide extra attachments that facilitate robust microtubule tracking. Interestingly, kinetochores revert to the lower copy structure in G1. Many kinases and phosphatases regulate mitosis, and the kinetochore in particular. We speculate that one or more of these kinases and/or phosphatases may contribute to the regulation of the kinetochore structure. In the future, it will be important to pursue how the living kinetochore structure is regulated, and how the structural plasticity contributes to its functionality.

Experimental Procedures

Yeast Strains

The *S. cerevisiae* and *S. pombe* strains used in this study are listed in Table 2.S1. Karyotyping by qPCR was done as previously described (Pavelka et al., 2010) to examine the ploidy level of strains used in microscopy studies. Briefly, genomic DNA was isolated from saturated culture qPCR primers were designed from the non-coding region on each arm of the 16 chromosomes. 500 bp fragment is chosen near the centromeric sequence, avoiding repetitive sequences. Standard qPCR protocol was followed and analysis for copy number was followed as in (Pavelka et al.,2010)

Microscopic Techniques

All microscope data for GFP counting, FCS, and FRAP were acquired as previously described (Shivaraju et al. 2012) by using Carl Zeiss LSM-510 Confocal microscope (Jena, Germany), outfitted with a ConfoCor 3 module and two single-photon counting avalanche photodiodes (APD’s). A C-Apochromat 40x 1.2 NA water objective was used. A HFT 488/561 main dichroic allowed excitation of GFP (488 nm laser line) and mCherry (561 nm laser). A secondary NFT 565 beam splitter was used as an emission dichroic. After passage through a 505–550 nm BP or LP 580 filter for GFP and mCherry, respectively, photon counts were collected on APDs in single-photon counting mode. Pinhole was set to 1 airy unit.

Fluorescence correlation Spectroscopy (FSC) with image calibration

Fluorescence Correlation Spectroscopy and image calibration were done as previously described. For details see (Shivaraju et al. 2012). Briefly, using the FCS module of the Zeiss confocor 3, FCS of an endogenously expressed monomeric-GFP in live cells was used to calculate the intensity of a single GFP. As with imaging, the 488 nm laser line was used to excite GFP, and emission was collected through a 505-550 nm BP filter. Pinhole was set to 1 airy unit. To calculate the copy number of a kinetochore protein, a Z series was taken with 0.4 µM step size and 6.4 µs pixel dwell time. The kinetochore cluster was fit to a 2-dimensional Gaussian, and peak amplitude of the fit was divided by single GFP intensity, after correcting for differences in laser power. For integrated intensity, we followed the protocol as previously described (Joglekar, Bloom, and Salmon 2009). We multiplied the spot intensity amplitude by (standard deviation)^2 to calculate the normalized intensity.

A Python script with matplotlib was used to plot the kinetochore copy number as a violin plot.

Origin 9.1 was used for box plots and statistical analysis.

Fluorescence Recovery after Photobleaching

FRAP measurements were performed to examine recovery of kinetochore protein during anaphase. Yeast cells expressing kinetochore proteins tagged with eGFP were grown to mid-log phase in synthetic complete media, harvested, and sandwiched between a slide and coverslip in a 1% agarose solution made with medium. Long-time lapse imaging demonstrated yeast cells were alive and divided at a normal rate in the agar pad for up to 4 hr. We took time points with 5 minute intervals to minimize bleaching. Prior to photobleaching, a Z series was taken with 0.4 µM step size and 6.4 µs pixel dwell time. Acquisition of a Z stack was essential due to the mobility of the kinetochore cluster in living yeast cells and to ensure proper quantitation of kinetochore intensity. After the initial acquisition, a kinetochore cluster labeled with eGFP was irreversibly photobleached by 4 rapid scans with high 488 nm laser power. The ability of the cells to continue to grow and divide ensured that photobleaching did not grossly damage the cells. After photobleaching, movies were acquired to examine recovery of the kinetochore cluster during anaphase. In most cases, cells were used that also expressed Spc42-mCherry from a centromeric plasmid to mark the cell cycle. Recovery of kinetochore proteins was observed as the reappearance of a punctate spot centered in the nucleus. ImageJ software (https:imagej.nih.gov/ij/) with custom written plugins was used to calculate the intensity and distance between the kinetochore clusters.

$$\%\ Recovery = \left(\frac{(Max.Intensity\ in\ anaphase - Post_{bleach}.Intensity)}{(Pre_{bleach}.Intensity - Post_{bleach}.Intensity)}\right) * 100$$

Photoconversion of kinetochore proteins at metaphase

For cell cycle time series and photoconversion studies, a Perkin Elmer Ultraview VoX spinning disc system with a Yokagawa CSU-10 spinning disc was used. The system was attached to a Carl Zeiss 200m inverted microscope. Images were acquired with a 100x 1.46 □-plan Apochromat oil objective, onto an EMCCD, using Volocity software. GFP and mcherry, or the green and red forms of tdEos, were excited with the 488 nm and 561 nm laser lines, respectively, using a 405/488/561/640 dichroic. Emission filter for green was a 500-550 nm BP, and for red it was a 415-475 nm / 580-650 nm dual BP filter. Data was acquired with alternative excitation, and was verified to be free of spectral cross-talk.

Photoconversion experiments were used to measure the addition of new protein at the kinetochore cluster in anaphase. Yeast cells expressing tdEos or mEos were grown and the experiment proceeded as in FRAP – with the exception that the kinetochore cluster was photoconversion with four iterations of 405 nm laser and subsequently imaged with the 488nm (Green)/561 nm (Red) laser line with spinning disk microscopy, using the system detailed above.

$$\% \, Green\ addition = \left(\frac{(Max.Green.Intensity\ in\ anaphase - Post_{activation}.Green.Intensity)}{(Pre_{activation}.Green.Intensity - Post_{activation}.Green.Intensity)}\right) * 100$$

For cell-cycle series/movies the cells were maintained on 2% agar pads at room temperature.

Simulation of kinetochore-microtubule interaction with Hill's equation

Simulations of kinetochore microtubule tracking for depolymerizing microtubules were performed according to the Hill model (Hill 1985) as described by Joglekar and Hunt (Joglekar and Hunt 2002). A full derivation is provided in supplementary information. Briefly, the rate of transition from position *N*-1 to position *N* (out of the kinetochore sleeve composed of *M* binding sites or couplers) is described by the following equation:

$$k_{out} = S\left[\frac{\mathrm{K}\, r^{M-N}}{\mathrm{f}_{(load)}} + \beta\right] \text{(Microtubule depolymerization)}$$

Here Ꝁ is the constant describing the sleeve thermal motion between tubulin binding sites, *s* is the exponent incorporating the loss of free energy due to movement out of the sleeve, *r* is the potential barrier associated with bond breaking in the sleeve, *β* is the rate of microtubule depolymerization, and *f* is the Boltzmann load factor coupling the tension force (*F*) on the chromosome to the distance traveled, *l*, as follows:

$$f = e^{-F\cdot l/_{2kT}}$$

Likewise the transition from position *N* to *N*-1 is described as follows:

$$k_{in} = \frac{\mathrm{K}\, r^{M-N}\, \mathrm{f}}{s} \quad \text{(Microtubule polymerization)}$$

We used a tension value of 20 pN for all simulations reported here. We used the recommended values from Joglekar and Hunt for all other parameters except in the case of the number of couplers, *M*, which we vary to observe the changes in dynamics of kinetochore loss.

Simulations of kinetochore tracking were initiated with the microtubule fully inserted in the kinetochore sleeve($N = M$). Simulations were run with a time step of 10 µs and for a maximum length of 10000 s. At each time step, a random number was generated between 0 and 1. If that random number was less than k_{in} (scaled to the time step increment) the microtubule was inserted one binding site further into the sleeve with a maximum insertion of M binding sites. Otherwise if the random number was greater than 1-k_{out}, the microtubule was pulled out of the sleeve by one binding site. If at any time this resulted in $N = 0$, the kinetochore was considered lost by the microtubule. 1000 simulations were performed, and the average loss time was found for each set of coupler number. The time required to lose the kinetochore was exponentially distributed for all parameter sets studied here. Therefore, the probability of loss within a particular anaphase could be estimated by integrating the exponential distribution up to the anaphase length time:

$$P_{loss} = 1 - e^{-\tau_{loss}/\tau_{anaphase}}$$

We mapped the average kinetochore loss time as a function of the number of couplers up to 9 couplers. Above that value, the simulations become prohibitively long, but the trend of average loss time is perfectly exponential, following the equation: $\tau_{loss} = e^{-13.1789+1.6331\cdot M}$. We then used this formula to extrapolate loss times (and probabilities) for higher numbers of couplers.

Author Contributions

KD, MS and BS conducted the experiments. BR and JU conducted simulations. JU, JL, and BS assisted KD with image quantification. KD and JG designed the experiments and wrote the paper.

Table 2.S1: Strains used in this study

Figure	Strain Name	Genotype
	Budding yeast (*Saccharomyces cerevisiae)*	
1B, 2B,2D,3E,S2C	KDH363	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Ndc80-GFP::URA3*
1C,2C,2D,3E,5B,S2C,S3D	KDH362	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Dam1-GFP::URA3*
1D,1F.3A,2B,5A,S1A,S2C,S6D	KDH180.1	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Nuf2-GFP::URA3*
1E,1G,3C,3D,S1D,S2C	KDH233	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Ask1-GFP::HIS3*
2A,2D,S2C	KDH181.1	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Cse4-GFP::URA3*
2A,2D,3E,S2C,S3A	KDH364	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Mif2-GFP::HIS3*
2A,2D,S2C	KDH368	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Ctf3-GFP::HIS3*
2A,2D,S2C	KDH370	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Chl4-GFP::HIS3*
2A,2D,S2C,S3A	KDH366	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Ctf19-GFP::HIS3*
2A,2D,3E,S2C,S3A	KDH324	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Ame1-GFP::HIS3*
2A,2D,3E,S2C,S3A	KDH325	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Okp1-GFP::HIS3*
2A,2D,3E,S2C,S3A	KDH367	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Spc105-GFP::HIS3*
2A,2D,S2C,S3A	KDH318	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Cnn1-GFP::URA3*
2B,2D,3E,S2C,S3B	KDH235	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Mtw1-GFP::HIS3*
2B,2D,3E,S2C,S3B	KDH371	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Nnf1-GFP::HIS3*
2B,2D,3E,S2C,S3B	KDH372	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Nsl1-GFP::HIS3*
2B,2D,3E,S1B,S1C,S2A,S2C	KDH179.1	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Dsn1-GFP::URA3*
2B,2D,3E,S2C,S3C	KDH374	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Spc24-GFP::HIS3*
2B,2D,3E,S2C,S3C	KDH373	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Spc25-GFP::HIS3*
2B,2D,3E,S2C,S3C	KDH180.1	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Nuf2-GFP::URA3*
2C,2D,3E,S2C,S3D	KDH233	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Ask1-GFP::HIS3*

2C,2D,3E,S2C,S3D	KDH375	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Dad1-GFP:HIS3*
4A,4B,4E,4F,5F	KDH199.1	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Ndc80-tdEos::URA3*
4C,4D,4E,4F	KDH226.1	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Ask1-tdEos::URA3*
4E,4F,S4E	KDH229.1	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Dam1-tdEos::URA3*
4E,4F,S4C,S5A,S5B,S5C	KDH407.1	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Dsn1-tdEos::URA3*
4E,4F,S4C	KDH220.1	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Mtw1-tdEos::URA3*
S4D	KDH277	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Spc24-tdEos::URA3*
4E,4F,S4D	KDH223.1	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Spc25-tdEos::URA3*
4E,4F,S4C	KDH198.1	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Nsl1-tdEos::URA3*
4E,4F,S4B	KDH404.1	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Spc105-tdEos::URA3*
S4B	KDH217	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Cnn1-tdEos::URA3*
5A	KDH189.1	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Nuf2-GFP::URA3,bik1Δ:: KANMX6*
5A	MMA196	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Nuf2-GFP::URA3,bim1Δ:: KANMX6*
5A,5C,5D,S6	KDH193.1	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Nuf2-GFP::URA3,stu2-10:: KANMX6*
5B	KDH538	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Ask1-GFP::URA3,bik1Δ:: KANMX6*
5B	KDH537	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Ask1-GFP::URA3,bim1Δ:: KANMX6*
5B	KDH399.1	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Dam1-GFP::URA3,stu2-10:: KANMX6*
5E,5F	KDH323.1	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Ndc80-tdEos::URA3,stu2-10:: KANMX6*
S6D	KDH143.1	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Nuf2-GFP::URA3,cnn1Δ:: KANMX6*
	Fission yeast *(Schizosaccharomyces pombe)*	

7D	KDH384	h^+ ade6-M210 leu1-32 ura4-D18 his3-D1 *Cnp1-GFP::NATMX6*
7C,7D	KDH385	h^+ ade6-M210 leu1-32 ura4-D18 his3-D1 *Dsn1-GFP::NATMX6*
7A,7B,7D,S6C	KDH386	h^+ ade6-M210 leu1-32 ura4-D18 his3-D1 *Ndc80-GFP::NATMX6*
7D,S6B	KDH387	h^+ ade6-M210 leu1-32 ura4-D18 his3-D1 *Nuf2-GFP::NATMX6*

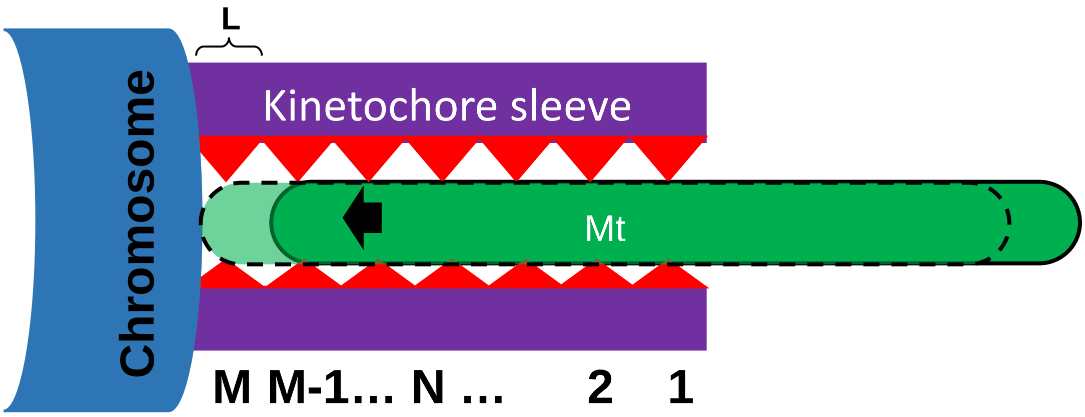
Table 2.S2: Comparison of kinetochore copy number in anaphase.

Complex	Protein	Anaphase number(Joglekar et al. 2006)	Anaphase number (this study)
Nucleosome	Cse4p	2	2
Cbf3	Ndc10p	2-3	3
	Mif2p	1-2	2
COMA	Ctf19p	2	3
	Spc105	5	5
MIND	Mtw1p	4-5	7
NDC80	Nuf2p	7	10
DAM-DASH	Ask1p	10-11	10
CTF3	Ctf3p	1	1
CHL4-IML3	Chl4p	<1	<1

Joglekar, A. P., Bouck, D. C., Molk, J. N., Bloom, K. S., & Salmon, E. D. (2006). Molecular architecture of a kinetochore-microtubule attachment site. *Nat Cell Biol, 8*(6), 581-585. doi: 10.1038/ncb1414

Supplemental method

Simulation of microtubule depolymerization on kinetochore structure predicts the requirement of additional couplers for kinetochore-microtubule interaction in anaphase.



The model follows the derivation of Hill (Hill 1985) and Joglekar and Hunt (Joglekar and Hunt 2002) with some minor modifications outlined below. The Microtubule (Mt) movement inside the sleeve is performed by discrete steps of the size $L = 0.615\ nm$, and the size of the sleeve is $M * L$. The position N of Mt is determined by its left tip $1 \leq N \leq M$ (see Fig. 6A)

The following physical mechanism are incorporated into the model:

1. Random thermal motion of the sleeve at a rate κ.
2. Loss of tubulin monomers at the Mt tip.

Each additional interaction of Mt with the tubulin binding site (corresponding to the increasing N) reduces the free energy by W (it has negative value), so that the insertion of the Mt into the sleeve is promoted. This movement requires first to break all existing binding between Mt and binding sites, which creates a potential energy barrier B for each occupied binding site, so that this barrier is equal to $N * B$.

We set free energy equal to zero when the Mt detaches from the sleeve. Thus, at position $N = 1$, we have it equal to $W < 0$, at $N = 2$, it is $2W$, and at arbitrary N, this value is $N * W$. Consider first the transition from N to $(N - 1)$ corresponding to outward movement. It can happen by both reasons mentioned above.

The first contribution to the rate k_{out} reads $\mathrm{K} * e^{\left[\frac{W}{k*T}\right]} * e^{\left[\frac{-(M-N)*B}{k*T}\right]}/f$, where k is the Boltzman constant, $T = 300K$ is the temperature, and f is the Boltzman factor representing the effect of the load on the kinetics. $f = e^{\frac{-F*L}{2kB*T}}$, where the numerator represents the mechanical work performed in shifting the sleeve against the tension F. We use $f < 1$, corresponding to the case when the tension pulls the Mt out of the sleeve.

The second contribution due to tubulin loss is estimated as $\beta * e^{[W/(k*T)}$, where β is the rate of tubulin depolymerization.

The reverse transition from $(N - 1)$ to N corresponding to inward movement is due to the sleeve motion only and its rate $k_{in} = \mathrm{K} * (e^{[-W/(k*T)}) * (e^{\frac{-(M-N)*B}{(k*T)}}) * f$.

Now we introduce the quantities $s = e^{\left[\frac{W}{k*T}\right]} < 1\ and\ r = e^{[\frac{(-B)}{k*T}]} < 1$. Using them we find $k_{out} = {}^{\kappa * s * r^{M-N}}/_{f} + \beta * s$ and $k_{in} = {}^{\kappa * r^{M-N} * f}/_{s}$. Note that the factor of s in the denominator of the kin term is different from previous derivations. This does not change the overall behavior of the system except to scale the tension required for tracking upwards by a factor of approximately 5.

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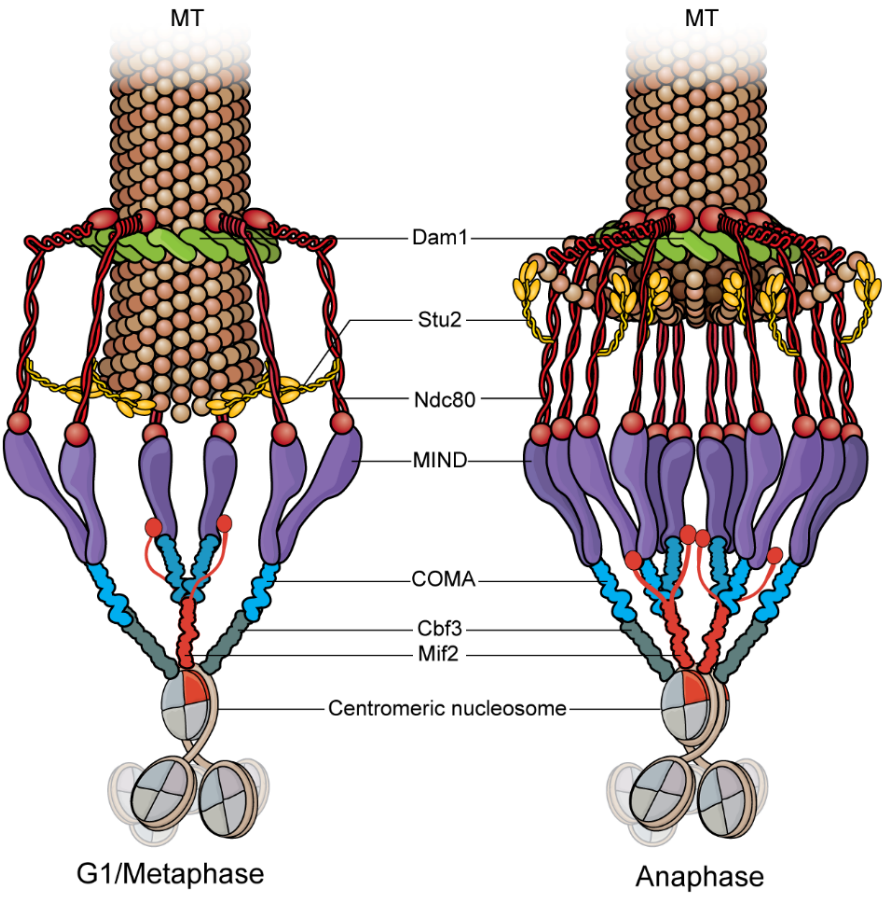
Graphical abstract:


MT
MT
Dam1
Stu2
Ndc80
MIND
COMA
Cbf3
Mif2
Centromeric nucleosome
G1/Metaphase
Anaphase

Model 2.15: Kinetochore plasticity during cell cycle.

The kinetochore in G1 has minimal-number of MIND and Ndc80 complexes. The kinetochore persists in the same structure until metaphase and during anaphase it gradually adds inner and outer kinetochore complexes except Dam1 complex. The anaphase kinetochore drops the copies added in telophase.

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CHAPTER 3

Regulation of kinetochore structural transitions

CHAPTER 3

Chapter 3 131
Regulation of kinetochore structural transition during cell cycle stages 131
  Abstract 135
  Introduction 137
  Results 140
    Tension between sister chromatids is not required for the addition of Ndc80 complex. 140
    Kinetochore copy number is not controlled by Ipl1/Aurora kinase or SAC. 143
    APC is required for initiating the Ndc80 addition during metaphase-anaphase transition 145
    Inactivation of MEN and FEAR arrest cells with anaphase-state kinetochore. 146
    Cytokinesis is not required for reducing the Ndc80 copy number. 148
  Discussion 149
    Tension between the sister chromatids at metaphase is not required for the anaphase kinetochore addition. 149
    Mitotic Exit Pathway mutants arrest the cells in anaphase-state kinetochore 150
    MEN might be required for reducing the copy number of kinetochore. 151
  Experimental Procedures 135
    Yeast Strains 153
    Microscopic Techniques 153
    REFERENCES: 155

Abstract

Accurate segregation of chromosomes during mitosis depends on regulation of multiple proteins. The kinetochore is a multi-protein complex that connects chromosome to dynamic microtubule. In the previous chapter I demonstrated that part of the kinetochore is a plastic structure which adapts minimal copy number state or ‘G1-state’ from G1 to metaphase and high copy number state or ‘anaphase-state’ in anaphase. The G1-state kinetochore persists until metaphase and transitions gradually to anaphase-state during anaphase. However, this kinetochore structural transition is regulated not known. Many regulators of the metaphase-anaphase transition and anaphase have been described. Spindle assembly checkpoint (SAC) and Cdc Fourteen Early Anaphase Release (FEAR) is activated in the beginning metaphase-anaphase transition. Cdc14 modulates the kinetochore and spindle associated factors to execute faithful segregation. The anaphase promoting complex/Cyclosome (APC/C) initiates anaphase by releasing separase. Mitotic Exit Network regulates mitotic exit and cytokinesis. I investigated the role of these known regulators in the kinetochore structural transition using imaging methods. The APC/C is critical for the increase in copies during anaphase while MEN and FEAR might be important for the transition back to the G1-state kinetochore in telophase. Therefore, the structural transitions of the kinetochore are coupled to known regulators of the cell cycle.

Introduction

Equal segregation of chromosomes into two daughters is essential for survival and propagation. Missegregation results in aneuploidy which leads to birth defects and cancer (Weaver and Cleveland 2006; Pfau and Amon 2012). Cell cycle is precisely controlled to make sure accurate segregation happens. During cell division chromosomes interact with microtubule through a multi-protein complex called the kinetochore. The kinetochore structure is dynamic with structural transitions occurring in the COMA, MIND and Ndc80 complexes. During anaphase, the kinetochore gradually adds copies important for kinetochore-microtubule interaction that are then lost to recreate the G1-state kinetochore. The anaphase structural transition depends on Stu2 controlled microtubule dynamics. However, how this structural transition regulated is unknown and what regulators affect the kinetochore directly or indirectly is also not clear.

There are a number of pathways involved in metaphase-anaphase transition, anaphase entry and mitotic exit to G1. Metaphase-anaphase transition is mainly controlled by spindle assembly checkpoint (SAC), anaphase promoting complex/Cyclosome (APC/Cdc20) and cyclin activity. After the cell enters anaphase, the MEN and FEAR pathways regulate the spindle alignment and initiate cytokinesis. COMA, MIND and Ndc80 complexes are added copies during anaphase and then lost in telophase. The kinetochore regulation in metaphase-anaphase transition and in anaphase is not known. However, these pathways have known kinetochore protein targets, making them potential regulators of the kinetochore structure. The Cdc28 is a solo Cdk1 that controls the whole cell cycle, including MEN and FEAR pathways (Surana et al. 1991). Before entering the next cell cycle, all the cyclins must be degraded. APC/C, one the cyclin regulator, interacts with two activators to degrade the cyclins.

Cohesin, a ring-like structure, is loaded onto the chromosome during G1 to hold the sister chromatids together (Losada 2014). During late S-phase, microtubules from the newly duplicated SPB attach to the sister chromatids (Tanaka 2010). In metaphase, sister chromatids attach to the SPBs from opposite pole to make a ‘bi-orientated’ interaction. Bi-orientation creates tension between sister centromeres, which is important for proper segregation (Goshima and Yanagida 2000). During metaphase, kinetochore microtubule dynamic instability pulls the centromere towards SPB. Recoiling nature of peri-centromeric chromatin pulls the kinetochore towards the centromere and promotes microtubule polymerization, these movements creates a centromere oscillation which is important for error-free attachment to the kinetochore (Pearson et al. 2004). Tension at the centromere from microtubule pulling on sister chromatids produces enough force to evict nucleosomes from the peri-centromeric region (Verdaasdonk et al. 2012). During metaphase-anaphase transition, separase cleaves the cohesin ring to release the sister chromatids for them to segregate to opposite poles (Uhlmann et al. 2000). Condensin, another ring-like structure, condenses chromosomes before metaphase (Vas et al. 2007). Even though most of the cohesins are cleaved during metaphase, residual cohesins and condensins localized on the chromosome arms may enable recoil the rest of the chromosomes (Renshaw et al. 2010). Theknetochore can withstand metaphase tension without losing the grip with the microtubule. Whether tension modulates kinetochore structure is unknown.

In budding yeast, metaphase-anaphase transition is controlled by the SAC. Unattached kinetochore and/or lack of tension at the sister chromatids activates the SAC and delays anaphase entry (Gillett, Espelin, and Sorger 2004). Ipl1/aurora B kinase, an essential kinase, localizes near the inner kinetochore to detect the tension-less kinetochore and reduces the affinity of the Ndc80 complex for microtubule through Ndc80 phosphorylation (Kudalkar et al. 2015). Syntelic, monotelic and merotelic attachments all are detected by Ipl1 (Pinsky et al. 2006). On anaphase entry, Ipl1 binds inter-polar microtubules of the spindle (Kotwaliwale et al. 2007). Mad1-mediated SAC detects the errors which are not sensed by the Ipl1 kinase (Pinsky and Biggins 2005). Mad1 specifically binds to the unattached kinetochore, suggesting its role in the spindle assembly checkpoint. Glc7, phosphatase reverses the Ipl1-mediated phosphorylation and promotes the kinetochore attachment to microtubule. Proper bi-orientation satisfies the SAC and signals anaphase entry. The kinetochore has known Ipl1/Aurora B targets which regulates their interaction with microtubule. However, Ipl1/Aurora B function on kinetochore transition is not known.

Anaphase promoting complex/Cyclosome (APC/C) is an E3 ubiquitin ligase that controls the anaphase entry by tagging securin/Pds1 for degradation and releasing separase/Esp1(Peters 2006; Zachariae et al. 1998; Yu et al. 1996). Cdc20 and Cdh1 are adapters for APC/C depending on the cell cycle. Cdc20, an essential factor, associates with APC/C for the metaphase-anaphase transition and Cdh1 binds to Cdc20 during anaphase. APC/C $^{\text{Cdh1}}$ gets degraded in mitotic exit /G1. APC/C $^{\text{Cdc20}}$ targets the securin/Pds1, Clb5 and other cyclins for the degradation. Inviability of *pds1*Δ and *clb5*Δ strains is rescued by the deletion of *CDC20*, suggesting that Clb5 and Pds1 are the unique substrates that control the metaphase-anaphase transition. APC/C$^{\text{Cdh1}}$ is activated on anaphase entry. Cdh1 is a non-essential substrate as the Cdc28 activity is antagonized by Clb-CDK inhibitor Sic1. Degradation of the Cyclins triggers the mitotic exit and subsequent cell cycle. The APC/C is another pathway which controls the metaphase-anaphase transition and has downstream regulators like Cdc14 with known kinetochore targets. APC/C might be a potential pathway which regulates the kinetochore addition of subcomplexes to the kinetochore during anaphase.

The FEAR network component, Cdc14 is released by the APC/C$^{\text{Cdc20}}$ to trigger the downstream pathway. A *cdc14-1* strain at non-permissive temperature has rDNA segregation problem and arrest cells, with elongated spindle (D'Amours, Stegmeier, and Amon 2004). Cdc14 also affects the microtubule dynamics at the beginning of anaphase (Higuchi and Uhlmann 2005). Cdc14 dephosphorylates the substrates that are phosphorylated by Cdks. Cdc14 also plays a role in Mitotic Exit Network pathway (MEN). MEN is only activated when the spindle enters the daughter cell and are aligned properly. Bub2-Bfa1 is a MEN inhibitor that gets inactivated on a perfectly aligned spindle by Cdc14 dephosphorylation and activation of Lte1 (Ras-GTPase) (Seshan, Bardin, and Amon 2002; Caydasi and Pereira 2009). Cdc15, a kinase of the MEN pathway, phosphorylates downstream targets for initiating the exit and cytokinesis. Since Cdc14 and Cdc15 regulate other anaphase events, these proteins are potential regulators of the kinetochore structure during anaphase.

In this chapter I elucidate the functions of these pathways in regulation of kinetochore structural transitions. The results presented are preliminary. One or two mutants are used for each pathway to study their role. APC/C, one of the metaphase-anaphase transition regulators is required for initiating the kinetochore structural transition. However Ipl1 and SAC are not required for anaphase kinetochore transition from metaphase to anaphase. After the initial additions, MEN and FEAR mutants arrest with the kinetochore in a high copy number state. My results suggest that the kinetochore loses COMA, MIND and Ndc80 copies after MEN activation and before cytokinesis.

Results

Tension between sister chromatids is not required for the addition of Ndc80 complex.

Tension at the centromere during metaphase pulls the kinetochore towards the centromere by promoting microtubule polymerization. This process fails when cohesin is cleaved and starts the segregation of sister chromatids during anaphase. Condensin and cohesin along with microtubule are the major factors that create tension between sisters during metaphase. To investigate whether tension plays a major role in transition to the anaphase kinetochore structure, I used temperature sensitive cohesin and condensin mutants to study the addition of Nuf2-GFP, a subunit of Ndc80 complex. Mcd1 (cohesin), Smc2 and Ycg1 (condensin) mutants showed similar intensity increase as in wild-type (Fig.3.1C), suggesting tension is not required for the copy number increase in anaphase.

Deletion of *MCM21*, an inner kinetochore protein, reduces cohesin at the centromere and peri-centromeric region (Ng et al. 2009). In *mcm21*Δ strain, the spindle is longer, and the kinetochore cluster has reduced oscillation as compared to wild-type in metaphase (Stephens et al. 2011). MIND and Ndc80 complexes have similar copy number as in wild-type (Fig.3.1A), suggesting reduced tension does not affect the kinetochore structural transitions. Cdc6 is a replication-licensing factor for DNA replication initiation. In the absence of Cdc6, cell fails to replicate the DNA. By deleting *MAD2*, the cell enters anaphase without the sister chromatids, creating a tension-less condition (Stern and Murray 2001). In this context, the MIND complex still showed an increase as the cell entered anaphase (Fig.3.1B), albeit attenuated, suggesting that tension is not required for addition of the MIND subcomplex to the kinetochore.

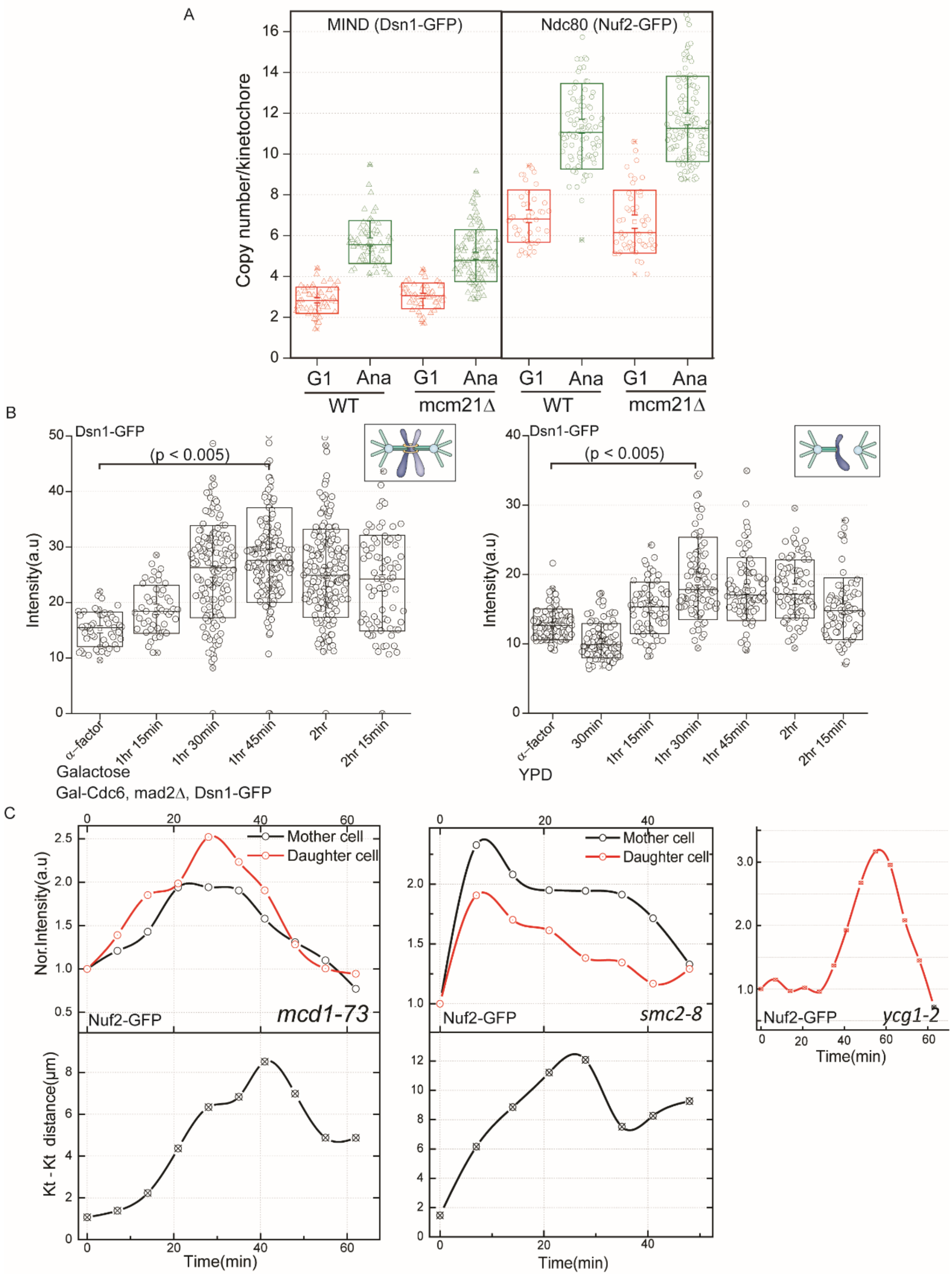


Figure 3.1: Metaphase tension does not affect the kinetochore copy number.

(A) Deletion of Mcm21 reduces the cohesin concentration at the centromere and affect the tension during metaphase. MIND and Ndc80 complexes are not affected in *mcm21*Δ, suggesting that tension does not affect the Ndc80 addition in the kinetochore. (B) Absence of sister chromatid creates tension-less environment in Gal-Cdc6 mutant (in YPD) and still shows addition of the MIND complex during anaphase. (C) Cohesin (*mcd1-73*) and condensin (*smc2-8* and *ycg1-2*) mutants show intensity increase from metaphase to anaphase at non-permissive temperature, suggesting that tension provided by these chromosomal proteins is not required for kinetochore copy number change. A two-tailed t-test was used to test for statistical significance.

Kinetochore copy number is not controlled by Ipl1/Aurora kinase or SAC.

To study whether Ipl1/Aurora B is required for structural transition in the kinetochore, I tagged Nuf2 of the Ndc80 complex in a temperature sensitive mutant (*ipl1-2)*. Alpha-factor arrested cells were washed with warm media and placed on an agar pad for live-cell imaging at 37°C. Mutant cells were mixed with wild-type cells (also having mCherry tag on another kinetochore subunit) to monitor whether this condition affects the wild-type. Based on their bud morphology and kinetochore clustering, both mutant and wild-type cells went through the cell cycle normally. As cells entered anaphase, mutant cells increased Ndc80 intensity gradually (Fig.3.2A). As reported before, *ipl1-2* showed severe chromosome loss and lagging chromosome defects. However, the intensity of the chromosome cluster increased during anaphase (Fig.3.2B). Dsn1 of the MIND complex is one of the targets for Ipl1-mediated phosphorylation. I used phosphomimetic (*dsn1-S240D*, *S250D*, *S264D*) and phosphodeficient (*dsn1-S240A*, *S250A*, *S264A*) (Akiyoshi, Nelson, and Biggins 2013) mutants; both showed increase in Dsn1 intensity similar to wild-type, suggesting that Ipl1 is not required for the structural changes in the kinetochore. To investigate the spindle assembly checkpoint protein, I used deletion of *MAD1* to check whether the addition of Nuf2-GFP was affected. The *mad1*Δ strain showed increased copy number of Nuf2-GFP during anaphase, suggesting the spindle assembly checkpoint to achieve the anaphase kinetochore structure.

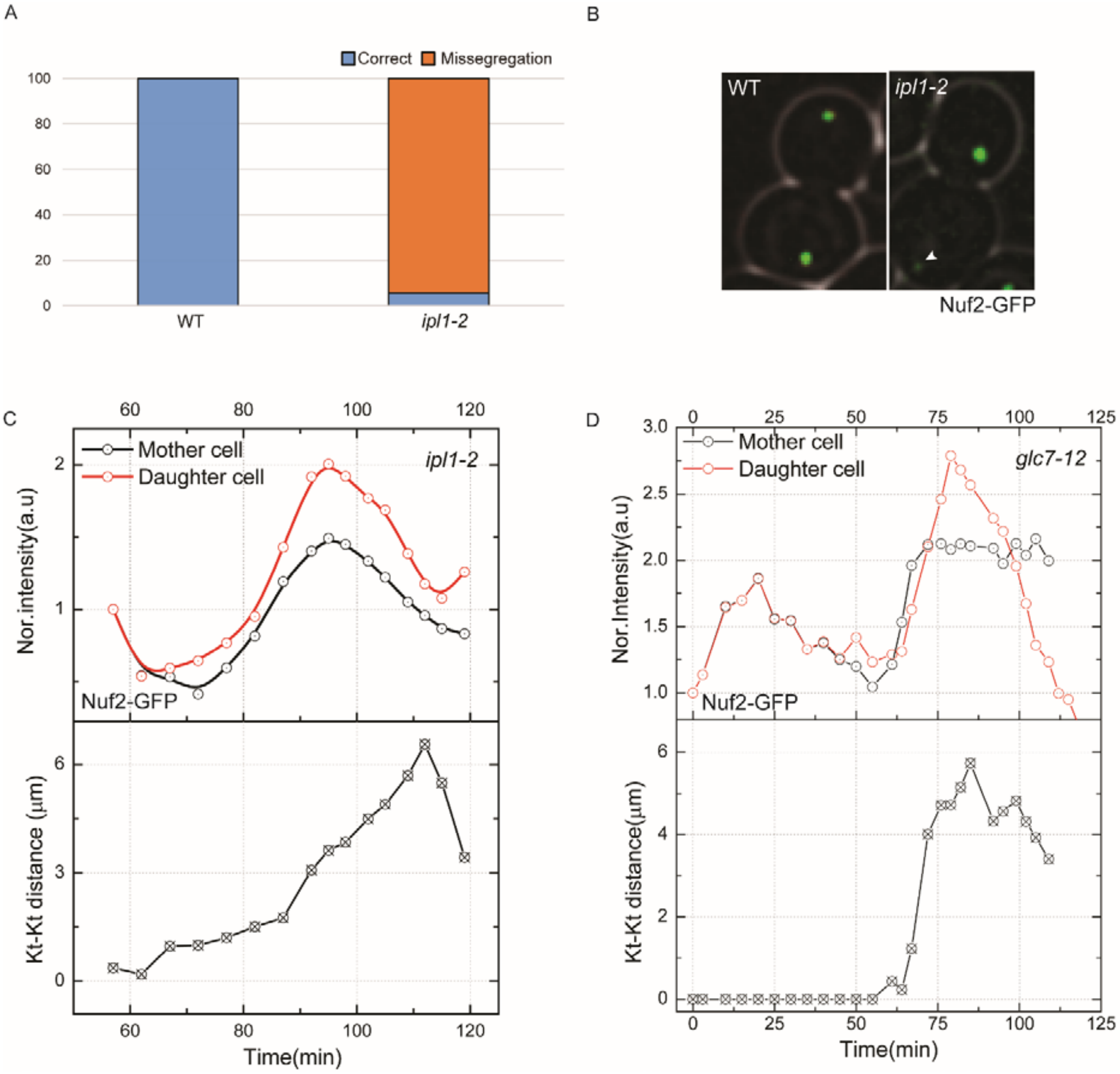


Figure 3.2: Ipl1 is important for chromosome segregation but it does not affect the Ndc80 copy number addition in anaphase.

(A) Live cell imaging of Nuf2-GFP is used quantify the intensity. Mutation in Ipl1 (*ipl1-2*) exhibits >95% of the cells with missegregated chromosomes. (B) Intensity of Nuf2-GFP in wild-type and *ipl1-2* mutant strain shows *ipl1-2* mutant has unequal segregation of the kinetochore (white arrow-head) (C) α-factor arrested *ipl1-2* mutant cell released from G1 for live-cell microscopy. Intensity of Nuf2-GFP was quantified as cell entered metaphase. The quantification shows addition of the Ndc80 complex as the cell enters anaphase (indicated by kinetochore-kinetochore distance). (D) Similar experiment on *glc7-12* (phosphatase), an antagonist of Ipl1 shows increase in intensity from metaphase to anaphase. This indicates Glc7 is not required for kinetochore structural transition.

APC is required for initiating the Ndc80 addition during metaphase-anaphase transition

To investigate kinetochore regulation by APC/C, I deleted a gene encoding one of the non-essential subunits called *CDH1* and tagged Nuf2 with GFP. I arrested the cells using alpha-factor and released them at 37°C to aggravate the phenotype. Cells proceeded through the cell cycle normally, but after metaphase took longer to finish anaphase. The intensity of Nuf2-GFP indicates that Ndc80 copies were not added in anaphase (Fig.3.3A). The APC/C targets securin/Pds1 for proteolysis and release of separase/Esp1. A strain bearing a temperature sensitive mutation in *PDS1* does not add Nuf2-GFP in anaphase, suggesting that the APC/C is required for initiating the addition of Ndc80 complexes to the kinetochore (Fig.3.3B). Most of the APC components are essential and mutant strains arrest at metaphase, making it difficult to study kinetochore structure in these mutant backgrounds. However, the lack of structural transition in *CDH1* and *PDS1*, suggests APC/C is required to achieve the anaphase kinetochore structure.

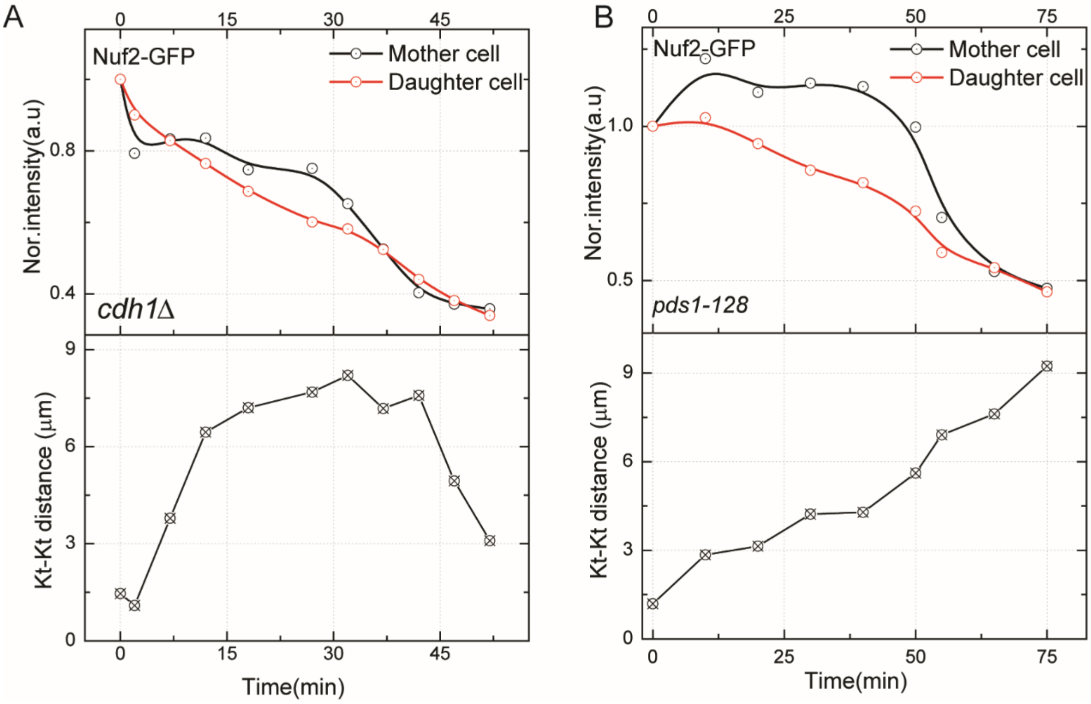


Figure 3.3: Anaphase promoting complex does affect the Ndc80 addition in metaphase-anaphase transition.

(A) Cells having deletion of CDH1, a component of APC, takes longer to finish anaphase at 37°C. It fails to add Ndc80 copies in metaphase. (B) Deletion of downstream APC component PDS1 interrupts the APC-mediated signaling. G1 arrested cells were released for live-cell microscopy at 37°C. Similar to *cdh1*Δ strain, a mutation in PDS1 (*pds1-128*) shows defect in Ndc80 addition at non-permissive temperature.

Inactivation of MEN and FEAR arrest cells with anaphase-state kinetochore.

Next, I checked the pathways involved in reducing the copy number during telophase. I used a number of FEAR network components. At restrictive temperature a *cdc14-3* strain is arrested in anaphase with the anaphase-state kinetochore. The intensity of the Ndc80 complex was similar to the intensity in a wild-type strain in anaphase. Other FEAR pathway mutations do not affect the copy number of the Ndc80 complex. After FEAR network activation, MEN is activated for the exit from cell cycle. Hence, I used mutations in MEN pathway components, *cdc15-1* and *tem1-3,* to check whether Ndc80 addition or reduction is affected by these mutations. The *tem1-3* and *cdc15-1* strains arrest with the anaphase-state kinetochore (Fig.3.4A-C). Cdc5, a common effector molecule of FEAR and MEN, activates the MEN pathway in the absence of Cdc14. Overexpression of Cdc5 does not require Cdc15 to execute the MEN. A similar arrest and release experiment in a strain bearing a mutation in Cdc5, a gene encoding a polo-like kinase, shows cells are arrested with an anaphase-state kinetochore, like the FEAR and MEN mutants. Together these experiments demonstrate that without the FEAR or MEN pathways, the kinetochore cannot transition back to the G1-state.

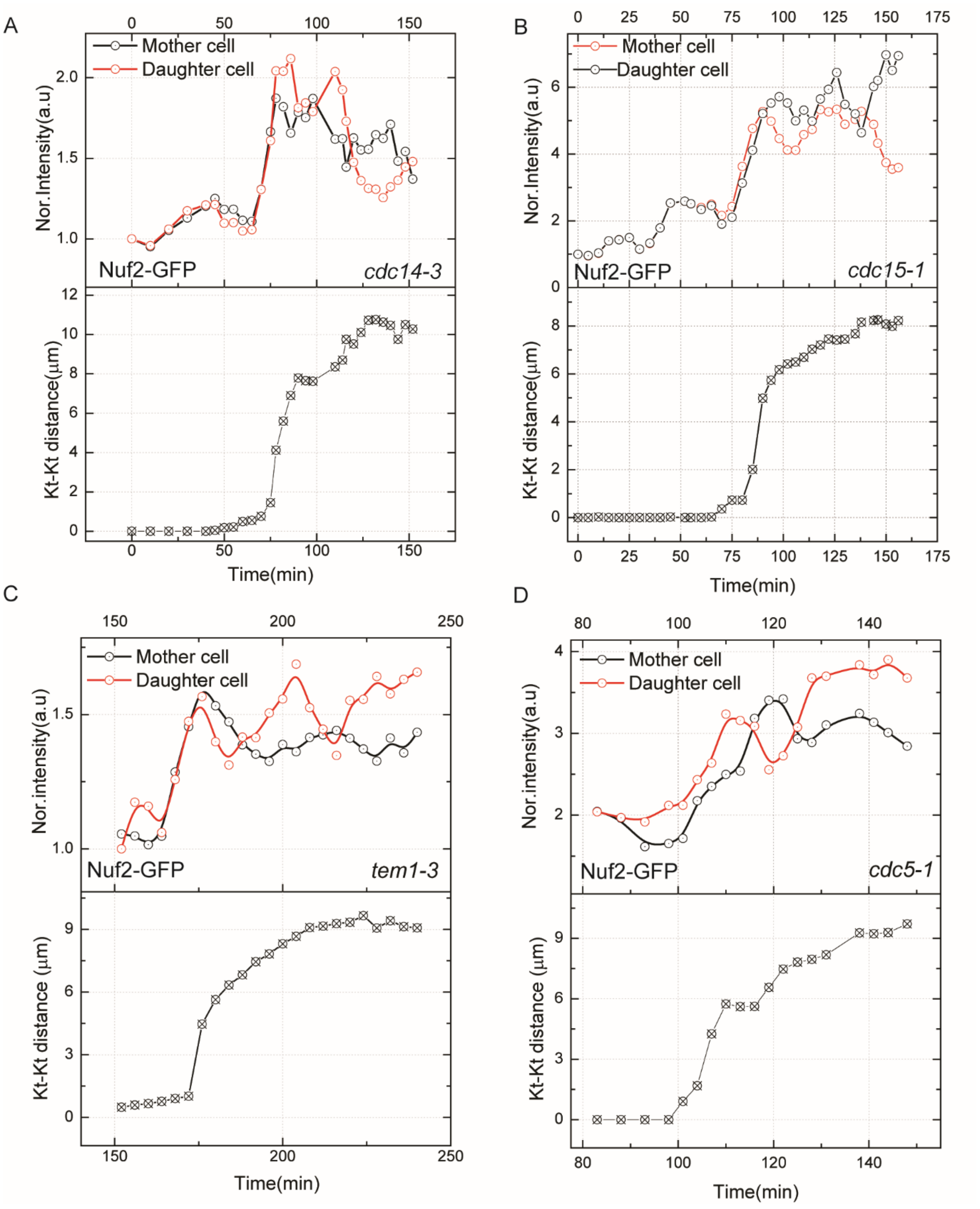


Figure 3.4: FEAR and MEN pathways arrest the kinetochore in anaphase-state (high copy number)

(A, B) α-factor arrested cells were released from G1 at 37° C for live-cell microscopy. FEAR mutant *cdc14-3* arrests at early anaphase with the anaphase kinetochore. Similar experiment on MEN mutants, *cdc15-1* and *tem1-3*, also exhibit an anaphase arrested kinetochore. (D) Cdc5, a common component of FEAR and MEN, arrest the cell cycle in late anaphase. At non-permissive temperature, *cdc5-1* released from G1 arrest showed Nuf2 intensity increase in anaphase, suggesting that Cdc5 does not control the Ndc80 addition in anaphase (Fig.3.4D).

Cytokinesis is not required for reducing the Ndc80 copy number.

Cytokinesis is marked by septum formation and acto-myosin contraction. Cdc11, Cdc12 and Cdc13 are septins which are important for proper cytokinesis (Juanes and Piatti 2016). A *cdc11-1* strain is used to study the kinetochore copy number addition. Temperature sensitive mutation in *CDC12* or *CDC13* allow cells to proceed to next the G1 without cytokinesis. In these mutants, the Ndc80 copy number goes back to the G1-state after anaphase (Fig.3.5A, B). These results suggest that mitotic exit pathways have role in bringing the copy number back to G1 state, but the structural transition is not coupled to cytokinesis per se. I speculate that Cdc14 mediated dephosphorylation and Cdc15- mediated phosphorylation are important for bringing the kinetochore back to the G1 state. In the future, it will be informative to know the specific targets of these kinases in the kinetochore.

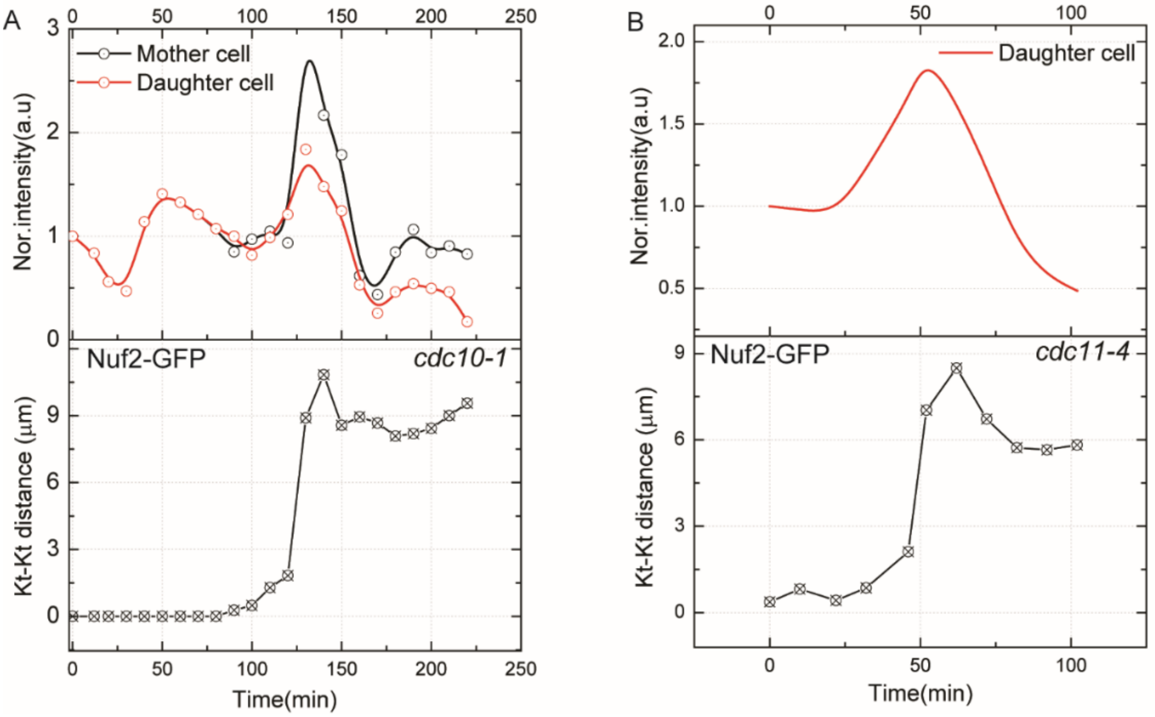


Figure 3.5: Cytokinesis is not required for the anaphase-state kinetochore.

(A, B) Temperature sensitive *cdc10-1* and *cdc11-4* mutants fail to complete cytokinesis at non-permissive temperature. The Ndc80 complex is added during anaphase and the copy number drops after the cell finishes anaphase, even without cytokinesis.

Discussion

Metaphase-anaphase transition plays a crucial role for the proper chromosome segregation. Before entering anaphase, a cell has to make sure that each chromosome has attached to the microtubule in a proper orientation. The kinetochore complex gradually adds COMA, MIND and Ndc80 complexes during anaphase. The metaphase-anaphase transition is controlled by a number of pathways including the spindle assembly checkpoint (SAC) and anaphase promoting complex/Cyclosome (APC/C). After finishing anaphase, cell initiates the exit pathway to complete cytokinesis. Our result suggests APC/C promotes the addition of the Ndc80 complex at the metaphase-anaphase transition and MEN is required for the transition back to the G1-state in telophase. This kinetochore structural transition occurs independently of tension and SAC.

Tension between the sister chromatids at metaphase is not required for the anaphase kinetochore addition.

Spindle assembly checkpoint proteins check the kinetochore-microtubule attachment and bi-orientation. Mutations in the spindle assembly checkpoint cause increased chromosome loss. I tried multiple approaches to check the role of tension in kinetochore structural transition. First, I utilized cohesin and condensin temperature sensitive mutants to inactivate these complexes and found that the kinetochore still adds the Ndc80 complex during anaphase. Second, I used image calibration method on a deletion of *MCM21*, an inner kinetochore protein which depletes cohesin at the centromere, to demonstrate normal Ndc80 increase in anaphase. Finally, I used imaging method to calculate the intensity of the MIND complex in a strain which lacks sister chromatid to create a tension-less condition during cell cycle. In the absence of tension, the MIND complex is still added during anaphase. Taken together, my results suggest tension is not required for the kinetochore transition during anaphase. SAC proteins mainly detect unattached kinetochores and loss of tension at the centromere. Results from the calibrated imaging on the SAC mutants also suggests that tension is not required for kinetochore transition in anaphase. Even though SAC mutants are not required for the anaphase kinetochore transition, SAC mutants show increased chromosome loss phenotype, suggesting that kinetochore structural transition and error-correction mechanisms might be working independently.

Mitotic Exit Pathway mutants arrest the cells in an anaphase-state kinetochore

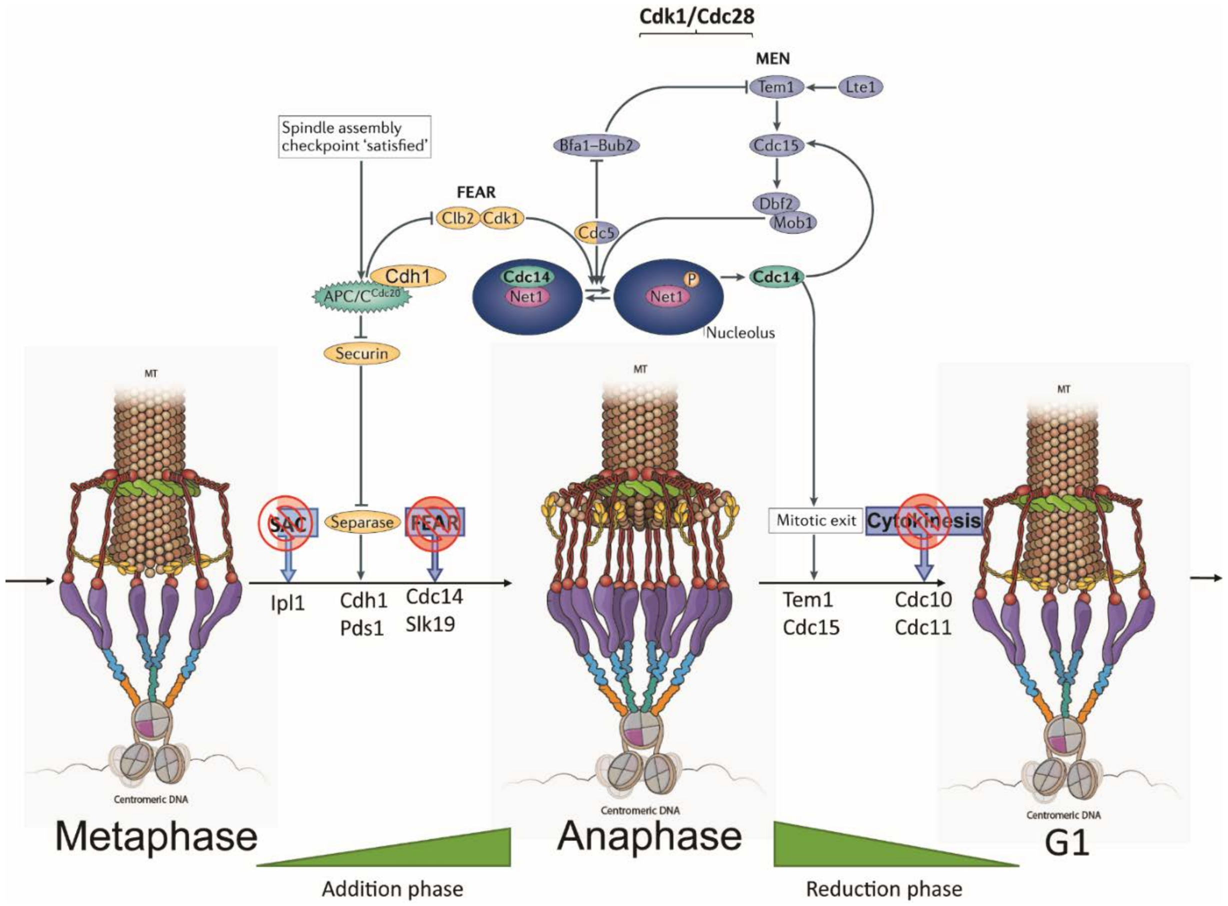
The APC/C activates the FEAR by releasing the Cdc14 and released Cdc14 subsequently activates the downstream proteins, particularly Cdc15. In late anaphase, MEN is initiated by the correct alignment of spindle between mother and daughter cell. Activated MEN further activates the downstream cytokinesis pathway. FEAR network mutants except Cdc14 have similar intensity profile of the kinetochore over the cell cycle like wild-type. However, lack of Cdc14 function results in arrest the kinetochore in the anaphase-state in late anaphase. Loss of MEN pathway function similar results in late anaphase arrest with the anaphase-state kinetochore. It is not clear whether cell is stuck with the high copy number because of cell cycle arrest or these enzymes are need for direct kinetochore regulation. Cdc5 and Cdc15 localizes on the kinetochore during anaphase and on the other hand, Cdc14 localizes on SPB (Faust et al. 2013). Cdc14 is a known phosphatase, dephosphorylates Dsn1 of the MIND complex (Akiyoshi and Biggins 2010) and possibly more targets. Cdc14, apart from dephosphorylating its targets, also affects the microtubule dynamics in anaphase (Higuchi and Uhlmann 2005).The kinetochore might be regulated directly or indirectly by these pathways. Kinetochore targets of Cdc15 are unknown and MudPIT analysis of affinity purified kinetochore complexes from *cdc15-1* strain could be used to find potential targets.

MEN might be required for reducing the copy number of the kinetochore.

Activation of MEN and Cdc5 leads to signal the cytokinesis pathway. Copies of the Ndc80 complex are added to the kinetochore in anaphase and then dropped prior to cytokinesis. This process is unaffected in the absence of cytokinesis, suggesting is not tethered directly to septation, but rather other cell cycle regulation. The mutants which affect the MEN pathway cause arrest with the anaphase-state kinetochore while mutations that affect cytokinesis have a normal Ndc80 profile, suggesting MEN could be an upstream pathway for the telophase drop of Ndc80 complex.

Higher eukaryotes lack Cdc15. however, Cdc15-like kinase plays similar function. Cdc5 (Plk1), a polo-like kinase, is evolutionarily conserved and have similar function as in yeast, suggesting these proteins might have a similar function in higher eukaryotes.

In conclusion, APC/C-mediated pathway initiates the Ndc80 addition in metaphase-anaphase transition and MEN might be helping to reduce the added Ndc80 copies in telophase (Model.3.6). The added Ndc80 copies enable the kinetochore to track on the depolymerizing microtubule. Reducing the Ndc80 complex copy number in telophase might be essential for error correction in the subsequent cell cycle. In late anaphase, most of the cyclins are degraded to start a new cycle(Endicott and Noble 1998). APC/C and MEN play major role in degrading the cyclins and activating the CDKs. In many cancers, APC and kinetochore proteins are mis-regulated, suggesting their role in cancer development (McKinley and Cheeseman 2017).


Cdk1/Cdc28
MEN
Tem1
Lte1
Spindle assembly checkpoint 'satisfied'
Bfa1–Bub2
Cdc15
FEAR
Clb2
Cdk1
Dbf2
Mob1
Cdc5
Cdh1
APC/C^Cdc20
Cdc14
Net1
P
Net1
Cdc14
Nucleolus
Securin
MT
MT
MT
SAC
Separase
FEAR
Mitotic exit
Cytokinesis
Ipl1
Cdh1
Pds1
Cdc14
Slk19
Tem1
Cdc15
Cdc10
Cdc11
Centromeric DNA
Centromeric DNA
Centromeric DNA
Metaphase
Anaphase
G1
Addition phase
Reduction phase

Model 3.6: Regulation of kinetochore structure by APC and MEN.

APC initiates the kinetochore complex addition during metaphase-anaphase transition. The anaphase-state of the kinetochore facilitates the kinetochore tracking on depolymerizing microtubule. Mutation in MEN pathway arrest the kinetochore in anaphase-state. Activation of MEN promotes cytokinesis. Before cytokinesis, the kinetochore loses the copied added during anaphase and goes back to G1-state. Cdc28/Cdk1 is the prime kinase that controls the most of the pathways involved in metaphase-anaphase transition and MEN (Modified from [33]).

Experimental Procedures

Yeast Strains

The *S. cerevisiae* and *S. pombe* strains used in this study are listed in Table 3.1. Karyotyping by qPCR was done as previously described (Pavelka et al., 2010) to examine the ploidy level of strains used in microscopy studies.

Microscopic Techniques

All microscope data were acquired as previously described (Shivaraju et al. 2012) by using Carl Zeiss LSM-510 Confocal microscope (Jena, Germany), outfitted with a ConfoCor 3 module and two single-photon counting avalanche photodiodes (APD's). A C-Apochromat 40x 1.2 NA water objective was used. A HFT 488/561 main dichroic allowed excitation of GFP (488 nm laser line) and mCherry (561 nm laser). A secondary NFT 565 beam splitter was used as an emission dichroic. After passage through a 505–550 nm BP or LP 580 filter for GFP and mCherry, respectively, photon counts were collected on APDs in single-photon counting mode. Pinhole was set to 1 airy unit.

Yeast cells expressing kinetochore proteins tagged with eGFP were grown to mid-log phase in synthetic complete media, harvested, and sandwiched between a slide and coverslip in a 1% agarose solution made with medium. Long-time lapse imaging demonstrated yeast cells were alive and divided at a normal rate in the agar pad for up to 4 hr. We took time points with 5 minute intervals to minimize bleaching.

Table: 3.1: Strains used in this study

Strain Name	**Genotype**
Budding yeast (*Saccharomyces cerevisiae)*	
KDH23	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Nuf2-GFP::HIS3,Mtw1-mcherry::HYG*
KDH313	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Gal-cdc6::URA3,mad1Δ :: KANMX6,Nuf2-GFP::His3*
KDH377	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 cdc14-3:: KANMX6,Nuf2-GFP::URA3*
KDH381	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 glc7-14:: KANMX6,Nuf2-GFP::URA3*
KDH383	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 cdc15-1:: KANMX6,Nuf2-GFP::URA3*
KDH477	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 tem1-1:: KANMX6,Nuf2-GFP::URA3*
KDH485	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 cdc5-1:: KANMX6,Nuf2-GFP::URA3*
KDH503	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 smc2-8:: KANMX6,Nuf2-GFP::URA3*
KDH504	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 mcd1-73:: KANMX6,Nuf2-GFP::URA3*
KDH505	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ycg1-2::KANMX6,Nuf2-GFP::URA3*
KDH641	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Nuf2-GFP::URA3,cdh1Δ:: KANMX6*
KDH701	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 cdc11-4:: KANMX6,,Nuf2-GFP::URA3*
KDH702	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 cdc10-5:: KANMX6,Nuf2-GFP::URA3*
KDH736	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 pds1-128:: KANMX6,Nuf2-GFP::URA3*

Table 3.2: List of mutants screened for kinetochore structural transition defects.

slk19Δ (normal transition)	*kip1Δ* (normal transition)
bub1Δ (normal transition)	*cin8Δ* (normal transition)
cdc20-3(metaphase arrest)	*mps1-1*
cks1-35	*esp1-1*(metaphase arrest)
ask1-2(metaphase arrest)	*spc105-15*(normal transition)
mad2Δ (normal transition)	*ydr532/kre28Δ*
kar3Δ (normal transition)	*clb2Δ* (normal transition)
dyn1Δ (normal transition)	*bub2Δ*
cdc23-4	*cdc15-2, gal-Spo12*

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CHAPTER 4

Sequence-independent centromeric nucleosome assembly by CAF-1 complex

Manuscript is under revision in Nucleic Acid Research:

Chromatin assembly factor-1 (CAF-1) chaperone regulates Cse4 deposition at active promoter regions in budding yeast

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Western blots in Figure 4.1A, B by Geetha Hewawasam.

Western blots in Figure 4.1C by Mark Mattingly.

CHAPTER 4

Chapter 4 157
Sequence-independent centromeric nucleosome assembly by CAF-1 complex 157
  Abstract 161
  Introduction 163
  Results 165
    CAF-1 subunits interact with Cse4 165
    CAF-1 can assemble Cse4 nucleosomes *in vitro* 168
    CAF-1 – a potential histone chaperone for Cse4 in anaphase. 169
    CAF-1 and centromeric localization of Cse4 170
  Discussion 174
    Graphical abstract: 16176
    Proposed model for CAF-1 role on centromeric nucleosome. 177
  Experimental Procedures 178
    REFERENCES: 180

Abstract

Accurately duplicated sister chromatids are equally segregated into two daughter cells in each division. Error in this process leads to aneuploidy and ultimately results in cancer development, birth defects. For proper segregation, chromosome must interact with a DNA-protein complex called the kinetochore. The iknetochore recognizes a specialized region on chromosome called centromere. The unique composition of nucleosome present at the centromere marks the future assembly of the kinetochore. Scm3, a centromere nucleosome specific chaperone, assembles and deposits Cse4 containing nucleosome at the centromere in budding yeast (Shivaraju et al. 2011; Camahort et al. 2007). In humans, HJURP, orthologue of Scm3, deposits Cse4 equivalent CENP-A nucleosome to its megabases long centromere. Centromeric nucleosome, depending on the cell cycle stages can adopt two different structure, hemisome and octasome in human and budding yeast (Shivaraju et al. 2012; Bui et al. 2012). CENP-A is mislocalized on chromosome arm in many human cancers and alters the chromatin structure which leads to defect in DNA replication, transcription and chromosome stability (Lacoste et al. 2014). Factors involved in deposition of centromeric histone variant on non-centromeric region is not clear. Here we show that CAF-1 interacts with Cse4 *in vivo* and loss of yCAF-1 can rescue the growth defects. Loss of yCAF-1 with overexpression of Scm3 delays the Cse4 transition to 2 copies/centromere in anaphase. Furthermore, reconstitution of purified yCAF-1–mediated nucleosome assembly on a plasmid showed that yCAF-1 can assemble Cse4 nucleosome on centromeric and non-centromeric sequence *in vitro*. Our results show that Cse4 nucleosomes can be assembled by CAF-1. The CAF-1 could be a potential Cse4 chaperone and might be responsible for the ectopic localization by Cse4.

Introduction

One of the unique features of eukaryotic centromeres is a specific histone H3 variant, CenH3. CenH3 is known as CENP-A in human and Cse4 in budding yeast. Nucleosomes containing this histone variant serve as the foundation for kinetochore assembly and attachment of chromosomes to spindle microtubules for faithful segregation. Eukaryotes have evolved stringent regulatory mechanisms to promote exclusive centromeric localization of CenH3/Cse4 (Choy et al. 2012).

Histone chaperones are indispensable for their role in regulation of histone deposition into chromatin. They facilitate proper nucleosome assembly as well as nucleosome disassembly (Das, Tyler, and Churchill 2010). In addition, histone chaperones play roles in nuclear import, storage, and stability of histones. Many chaperones regulate the canonical histones, creating functional redundancy. Most histone variants have dedicated chaperones (Das, Tyler, and Churchill 2010). CenH3 assembly into centromeric nucleosomes is regulated by the evolutionarily conserved histone chaperone known as Scm3 in budding yeast (Mizuguchi et al. 2007; Stoler et al. 2007; Camahort et al. 2007; Shivaraju et al. 2011), and HJURP in humans (Dunleavy et al. 2009; Foltz et al. 2009).

Regulating the deposition of Cse4 is critical to prevent mislocalization of Cse4 to non-centromeric regions. In addition to being regulated by the Cse4-specific chaperone Scm3, Cse4 levels are controlled by multiple mechanisms, including ubiquitin mediated proteolysis (Collins, Furuyama, and Biggins 2004). An E3 ubiquitin ligase Psh1 in conjunction with Casein Kinase 2 (CK2) facilitates ubiquitylation and proteasomal degradation of Cse4 (Hewawasam et al. 2010; Ranjitkar et al. 2010; Hewawasam et al. 2014). In addition, several other factors play roles in ubiquitin-mediated proteolysis of Cse4 (Au et al. 2013; Cheng, Bao, and Rao 2016; Ohkuni et al. 2016; Ohkuni, Abdulle, and Kitagawa 2014; Mishra et al. 2015; Canzonetta et al. 2016). Additional chromatin regulating proteins, including remodelers, have been identified that assist in efficient removal of mislocalized Cse4 nucleosomes (Deyter and Biggins 2014; Choi et al. 2012; Gkikopoulos et al. 2011).

Chaperones aside from Scm3 may play a role in the dynamics of Cse4 deposition, especially under conditions of overexpression when the balance of H3 and Cse4 levels is dramatically altered. Elevated expression and misincorporation of CenH3/CENP-A is reported in many human cancers (Zink and Hake 2016; Athwal et al. 2015; Tomonaga et al. 2003; Li et al. 2011; Lacoste et al. 2014). In human cells the transcription-coupled histone H3.3 chaperones DAXX/ATRX target CENP-A to ectopic locations (Lacoste et al. 2014; Athwal et al. 2015). Mislocalization of CenH3 could disrupt chromatin structure and lead to ectopic centromere/kinetochore formation, chromosome instability and missegregation (Au et al. 2008; Amato et al. 2009; Collins et al. 2007). Mislocalized CenH3 could also affect chromatin-based processes like DNA replication and transcription (Hildebrand and Biggins 2016). Therefore, it is important to identify and characterize factors that regulate promiscuous CenH3 localization.

Centromeric Cse4 nucleosome can adapt different nucleosome structure; hemisome-where Cse4/H4/H2A/H2B forms a nucleosome, octasome-where two copies of H4/H4/H2A/H2B forms a nucleosome. In budding yeast, Cse4 has one copy per centromere in interphase and 2 copies in anaphase. Scm3, Cse4 chaperone associates with Cse4. Scm3 assembled Cse4 favors the hemisome (Unpublished data). However, the chaperone which favors the octasome in anaphase is not known.

We present evidence that the evolutionarily conserved histone H3/H4 chaperone Chromatin Assembly Factor-1 (CAF-1) can function as a Cse4 chaperone in budding yeast. CAF-1 had previously been reported to be important for building functional kinetochores (Sharp et al. 2002) and in regulating Cse4/H3 exchange kinetics (Lopes da Rosa et al. 2011). We report that recombinant CAF-1 can assemble Cse4 nucleosomes *in vitro* and binds to Cse4 *in vivo*. Absence of Scm3 with *CAC2* deletion results in poor growth and overexpression of Scm3 in *cac2*Δ strain delays the Cse4 transition in anaphase. Overall our findings help to establish how the histone H3/H4 chaperone CAF-1 binds and assembles the nucleosome with Cse4.

Results

CAF-1 subunits interact with Cse4

We took a candidate approach to identify potential chaperones for Cse4. Known histone chaperones Vps75, Nap1, and the Cac2 subunit of CAF-1 were GFP tagged. Whole cell extracts were used to perform co-immunoprecipitation (co-IP) with anti-GFP antibody followed by detection of Cse4 in Western blots with anti-Cse4 antibody (Figure 1A, left panel). Scm3, the chaperone that targets Cse4 to the centromere, was a positive control. Of these three candidates, only Cac2 pulled down Cse4. Hir1 (Histone regulator 1) is another histone H3/H4 chaperone whose biological role significantly overlaps with CAF-1 (Lopes da Rosa et al. 2011). Hir1-FLAG was also tested for interaction with Cse4. Cac2-FLAG served as a control.Hir1-FLAG did not pull down Cse4, suggesting Hir1 does not associate with Cse4 *in vivo*, Cac2-FLAG pulled down Cse4-Myc, further confirming the interaction between Cac2 and Cse4 (Fig.4.1A, right panel).

CAF-1 is a heterotrimeric protein complex. In human, the three subunits are p150, p60 and p48 (also named RbAp48). The budding yeast subunits are named Cac1 (p90), Cac2 (p60) and Msi1/Cac3 (p50) (Ramirez-Parra and Gutierrez 2007). The Cac3 ortholog RbAp48 (also named p55) in *Drosophila* co-purified with CenH3/CID and assembled CID nucleosomes *in vitro* (Furuyama, Dalal, and Henikoff 2006; Furuyama and Henikoff 2006). The human CAF-1 subunits p48/RbAp48 and p150 also co-purified with CENP-A (Dunleavy et al. 2009; Foltz et al. 2009). Taken together, these findings suggest CAF-1 may serve as an evolutionarily conserved CenH3 chaperone.

To identify the subunits of CAF-1 important for physical interaction with Cse4, WT and *cac1Δ*, *cac2Δ* or *cac3Δ* strains carrying epitope tagged Cse4-Myc/Cac1-FLAG, Cse4-Myc/Cac2-FLAG and Cse4-Myc/Cac3-FLAG were used to perform co-immunoprecipitation and Western blotting (Figure 4.1B). All three subunits of CAF-1 pulled down Cse4-Myc. However, Cac1 and Cac3 subunits pulled down noticeably more Cse4-Myc (Figure 4.1B, upper panel). Furthermore, the interaction between Cse4 and Cac3, and to a lesser extent Cac1, was dependent on *CAC2*. Cse4 interaction with Cac2 was completely abolished without *CAC1* or *CAC3* (Figure 4.1B, lower panel). These observations suggest that Cac1 and Cac3 subunits are required for Cse4-CAF-1 interaction, while loss of Cac2 reduces the interaction. Cse4 likely interacts with the entire CAF-1 complex in budding yeast.

CAF-1 deposits histone H3/H4 into nucleosomes during DNA synthesis, although a previous report argued that the exchange of Cse4 for H3 mediated by CAF-1 was replication independent (Lopes da Rosa et al. 2011). We investigated whether the Cse4-CAF-1 interaction was cell cycle dependent using co-immunoprecipitation and Western blotting. Whole cell extracts were prepared from cells growing asynchronously or arrested in G2/M (nocodazole treated). Scm3 was used as a positive control. Similar to Scm3, Cac2 interacted with Cse4 in extracts generated from both asynchronously grown cells as well as G2/M-arrested cells, suggesting the interaction between Cse4 and CAF-1 is not confined to S phase (Figure 4.1C). Together, these experiments suggest that CAF-1 can interact with Cse4 outside of S phase and may therefore be able to assemble and disassemble Cse4 nucleosomes outside of S phase.

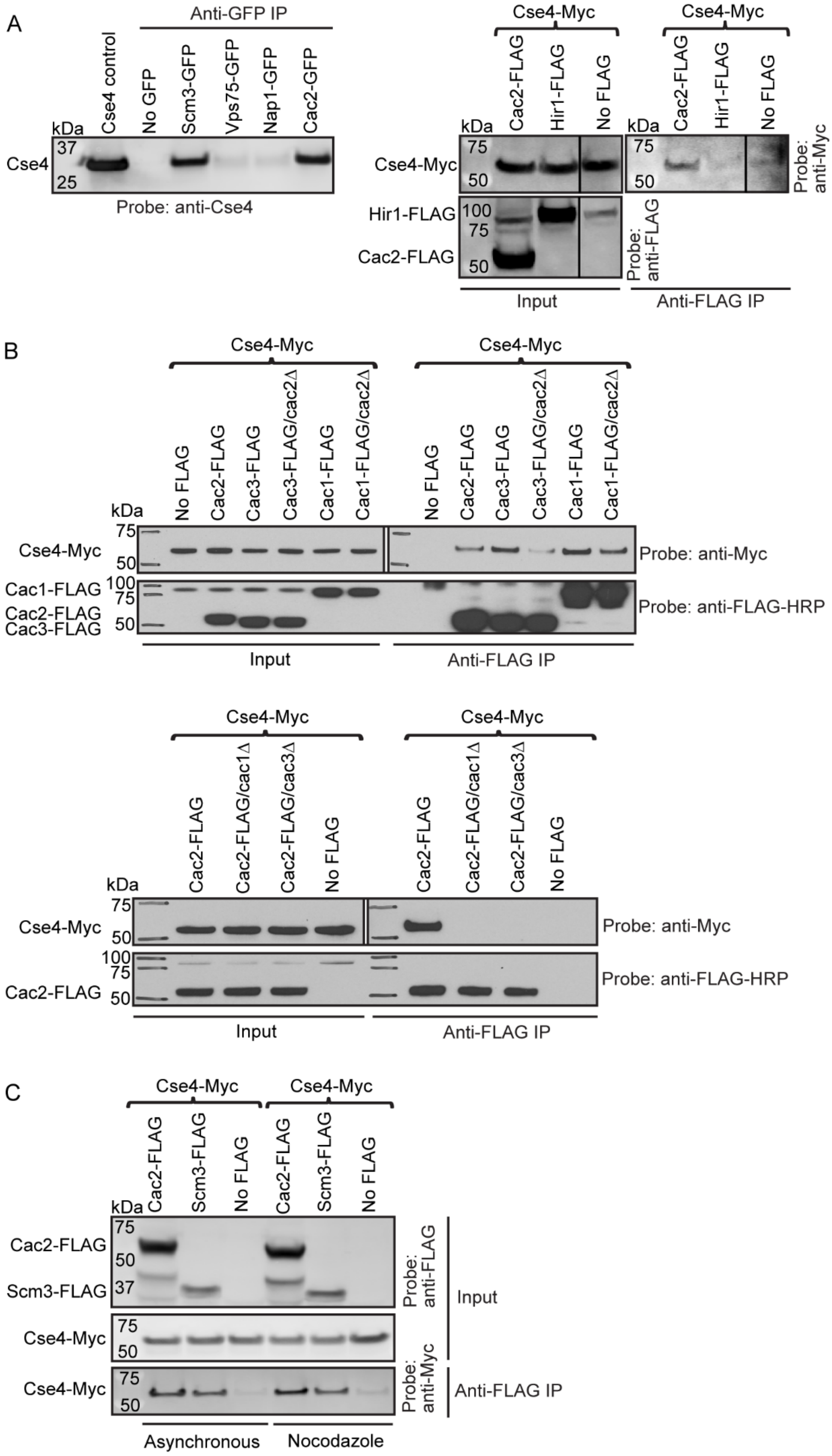

A
Anti-GFP IP
Cse4 control
No GFP
Scm3-GFP
Vps75-GFP
Nap1-GFP
Cac2-GFP
kDa
37
25
Cse4
Probe: anti-Cse4
Cse4-Myc
Cse4-Myc
Cac2-FLAG
Hir1-FLAG
No FLAG
Cac2-FLAG
Hir1-FLAG
No FLAG
kDa
75
50
Cse4-Myc
Probe: anti-Myc
Hir1-FLAG
Cac2-FLAG
100
75
50
Probe: anti-FLAG
Input
Anti-FLAG IP
B
Cse4-Myc
Cse4-Myc
No FLAG
Cac2-FLAG
Cac3-FLAG
Cac3-FLAG/cac2Δ
Cac1-FLAG
Cac1-FLAG/cac2Δ
kDa
75
50
100
75
50
Cse4-Myc
Cac1-FLAG
Cac2-FLAG
Cac3-FLAG
Probe: anti-Myc
Probe: anti-FLAG-HRP
Input
Anti-FLAG IP
Cse4-Myc
Cse4-Myc
Cac2-FLAG
Cac2-FLAG/cac1Δ
Cac2-FLAG/cac3Δ
No FLAG
kDa
75
50
100
75
50
Cse4-Myc
Cac2-FLAG
Probe: anti-Myc
Probe: anti-FLAG-HRP
Input
Anti-FLAG IP
C
Cse4-Myc
Cse4-Myc
Cac2-FLAG
Scm3-FLAG
No FLAG
kDa
75
50
37
Cac2-FLAG
Scm3-FLAG
Probe: anti-FLAG
Input
Cse4-Myc
Cse4-Myc
Probe: anti-Myc
Anti-FLAG IP
Asynchronous
Nocodazole

Figure 4.1: CAF-1 subunits interact with Cse4.

Whole cell extracts were used to carry out co-IPs in A, B and C. A. Left panel; Strains carrying GFP tagged chaperones, Scm3, Vps75, Nap1 and Cac2 subunit of CAF-1 were used in co-IP. Only Cac2-GFP pulled down Cse4. A control IP was performed from a strain lacking the GFP tag. Scm3-GFP IP was the positive control. Right panel; Affinity tagged strains (Cse4-Myc/Cac2-FLAG and Cse4-Myc/Hir1-FLAG) were used in co-IP. Control IP was performed from a strain lacking the FLAG tag. In anti-FLAG co-IP, Hir1-FLAG did not pull down Cse4, only Cac2-FLAG pulled down Cse4-Myc. B. Cac1 and Cac3 subunits of CAF-1 are required for interaction with Cse4. Upper panel; WT or *cac2*Δ strains carrying FLAG tagged Cac1 or Cac3 were used in co-IP. A strain lacking the tag on Cac1 and Cac3 was used as the negative control. In anti-FLAG co-IP, both Cac1 and Cac3 pulled down noticeably more Cse4-Myc. These interactions became weaker with deletion of *CAC2*. Lower panel; WT, *cac1*Δ or *cac3*Δ strains carrying FLAG tagged Cac2 were used in anti-FLAG co-IP. A strain lacking the tag was used as the control. Cac2/Cse4 interaction was completely abolished in *cac1*Δ or *cac3*Δ strains. C. CAF-1 can interact with Cse4 outside of S phase. Affinity tagged strains (Cse4-Myc/Cac2-FLAG and Cse4-Myc/Scm3-FLAG) were used in anti-FLAG co-IP using asynchronously grown cells and G2/M-arrested cells. A strain lacking the FLAG tag served as a negative control. Scm3 was used as a positive control. Cac2 interacted with Cse4 in both asynchronously grown cells as well as G2/M-arrested cells.

CAF-1 can assemble Cse4 nucleosomes *in vitro*

The Cac3 ortholog of RbAp48 in *Drosophila* can assemble CID nucleosomes *in vitro* (Furuyama, Dalal, and Henikoff 2006; Furuyama and Henikoff 2006). We tested whether recombinant budding yeast CAF-1 could facilitate the assembly of Cse4 nucleosomes *in vitro* using a plasmid supercoiling assay (Shivaraju et al. 2011). In this assay, wrapping of DNA around the histone core particle induces supercoiling in relaxed, closed, plasmid DNA. Following the assembly reaction, DNA is deproteinized, and plasmid topoisomers are resolved by agarose gel electrophoresis. We tested nucleosome assembly using a plasmid containing 10 copies of a 5S nucleosome positioning sequence (pG5E4-5S) as well as one containing 10 tandem copies of a yeast centromere 1 (CEN1) repeat unit (pCEN1-10X). Incubation of purified CAF-1 (Winkler et al. 2012) and Cse4 octamers with either pG5E4-5S or pCEN1-10X resulted in the induction of supercoiling in a dose dependent manner (Figure 4.2), demonstrating that CAF-1 can assemble Cse4 containing nucleosomes on both plasmids. Nap1 was used as a positive control. Although Nap1 does not co-immunoprecipitate with Cse4 in whole cell extracts, the high concentrations of purified proteins used in the assembly assay *in vitro*, combined with the absence of H3, may allow Nap1 to promote the assembly of

Cse4 nucleosomes. These experiments demonstrate that CAF-1 can assemble Cse4 nucleosomes irrespective of DNA sequence.

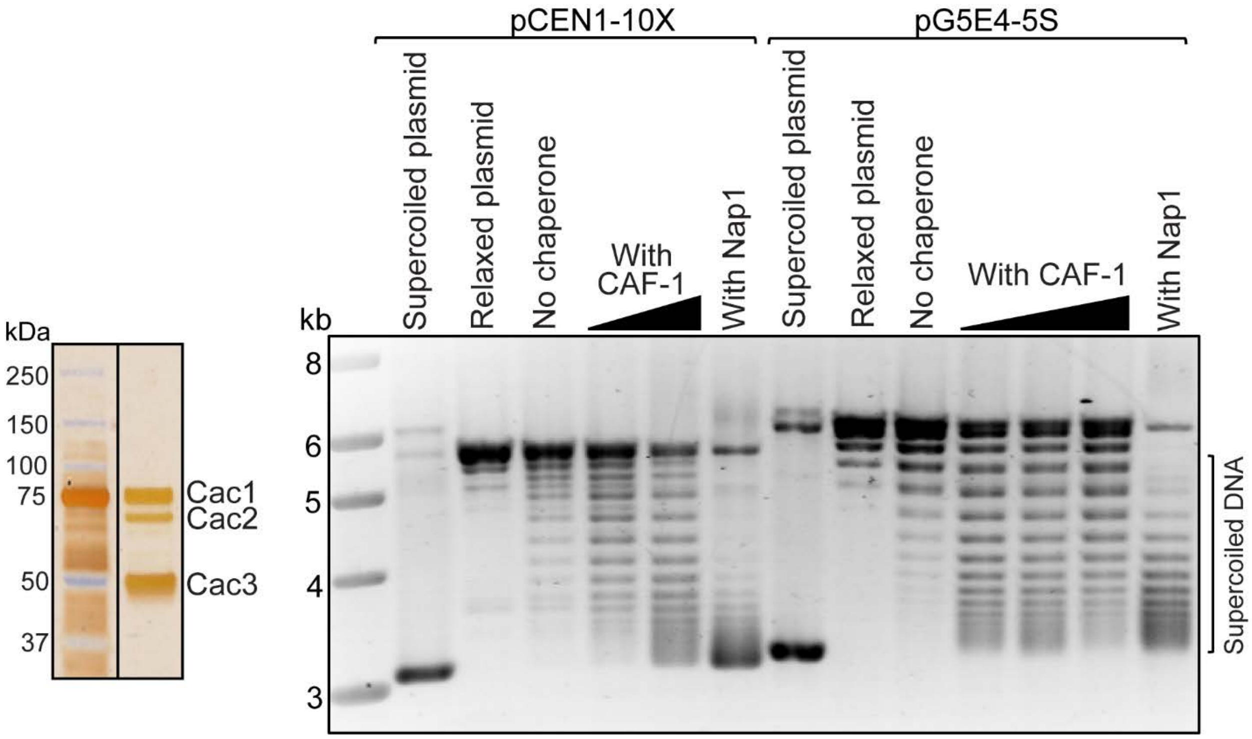

kDa
250
150
100
75
50
37
Cac1
Cac2
Cac3
pCEN1-10X
pG5E4-5S
Supercoiled plasmid
Relaxed plasmid
No chaperone
With CAF-1
With Nap1
Supercoiled plasmid
Relaxed plasmid
No chaperone
With CAF-1
With Nap1
kb
8
6
5
4
3
Supercoiled DNA

Figure 4.2: CAF-1 can assemble Cse4 nucleosomes in vitro. Nucleosome assembly activity of CAF-1 was studied using a plasmid supercoiling assay. Supercoiled plasmids were purified from *E. coli* and relaxed by addition of topoisomerase I. supercoiled plasmid, relaxed plasmids and no chaperone samples were included as controls for each assembly experiment. Chromatin assembly was performed by incubating the relaxed plasmids with increasing amounts of purified CAF-1 and Cse4 octamers. DNA and Cse4 octamer amounts are held constant. Nucleosome assembly was performed using a plasmid (pCEN1-10X) containing 10 tandem copies of a yeast centromere 1 (CEN1) repeat unit (left side), as well as a plasmid (pG5E4-5S) containing 10 copies of a 5S nucleosome positioning sequence (right side). Histone chaperone Nap1 was used as a control. CAF-1 resulted in the induction of supercoiling on both plasmids in a dose dependent manner.

CAF-1 – a potential histone chaperone for Cse4 in anaphase.

In yeast, centromeric region has conserved ~125bp sequence divided into 3 regions: CDEI, CDEII and CDEIII(Carbon 1984). The canonical nucleosome is containing around by ~147 bp of DNA which is longer than the centromeric sequence. Initial *in vitro* MNase digestion and FRET (Forster resonance energy transfer) studies on yeast centromeric nucleosomes suggests that it can form a hemisome (Furuyama, Codomo, and Henikoff 2013). Our lab used image calibration method to study the centromeric Cse4 nucleosome *in vivo* and found that centromeric nucleosome can have 2 different structures depending on the cell cycle stage. FRAP (Fluorescence recovery after photobleaching), FRET and immunoprecipitation studies eloquently show that in anaphase the centromeric Cse4 nucleosome has 2 copies of Cse4 per nucleosome (Shivaraju et al. 2012). But speculations from the FRAP recovery data suggested that it could happens in G1(Biggins 2013). To eliminate that possibility, I used the FRAP technique with telophase specific bud-neck marker (Myo1-mCherry) and confirmed that the switch to 2 Cse4 per centromere happens in anaphase, prior to cytokinesis.

CAF-1 and centromeric localization of Cse4

Previous findings suggest that CAF-1 may facilitate centromeric localization of CenH3. The Cac3 orthologs, Mis16 in fission yeast and RbAp46/RbAp48 in human, are required for efficient Cnp1 and CENP-A loading at centromeres, respectively (Hayashi et al. 2004; Mouysset et al. 2015). Interestingly, HJURP (human ortholog of Scm3) contains five highly conserved tryptophan residues resembling the tryptophan-aspartate (WD40) repeats found in CAF-1 subunits p60 and RbAp48/RbAp46 (Foltz et al. 2009). In budding yeast CAF-1 subunits are recruited to centromeres and CAF-1 and Hir1 are required for proper centromere/kinetochore structure and function (Sharp et al. 2002). We tested whether the localization of CAF-1 at centromeres is cell-cycle-dependent. We used asynchronously growing cells and also G1, S and G2/M arrested cells to perform Cac1 ChIP followed by qPCR to detect CEN3 enrichment (Fig.4.3A). We detected CEN3 enrichment in all four samples, indicating that CAF-1 localizes to centromeres regardless of the phase of the cell cycle.

Although Scm3 is the main chaperone responsible for assembly of Cse4 nucleosomes at centromeres in budding yeast, we wondered whether CAF-1 could in principle facilitate the assembly of Cse4 at centromeres. CAF-1 can assemble Cse4 nucleosomes *in vitro* on a plasmid containing centromere sequence (Fig.4.2B). Although deletion of Scm3 is lethal, Scm3 can be deleted or turned off (Scm3^{off}) if Cse4 is overexpressed, and Cse4 still localizes to the centromere (Camahort et al. 2009), arguing other chaperones can mediate the assembly of Cse4 nucleosomes. To test whether CAF-1 might be able to assemble Cse4 at centromeres, we used the Scm3$^{on/off}$ strain, which can be toggled by galactose, along with copper-inducible Cse4 overexpression. The level of Cse4 can be controlled by the concentration of copper. In this strain background we deleted a number of different chaperones, including *CAC2*. A growth assay was performed to compare growth under Scm3^{on} and Scm3^{off} conditions (Fig.4.3B). Three independent transformants with *CAC2* deletion were examined for growth. When Scm3 is on (Scm3^{on}), there is no difference in growth between WT and *cac2*Δ strains. However, when *CAC2* is deleted and Scm3 is turned off, the rescue of growth by Cse4 overexpression is poor (Fig.4.3B, compare WT+CSE4 and *cac2*Δ+CSE4). Deletion of *CAC2* from the Scm3^{off} strain does not completely abolish growth when Cse4 is overexpressed, suggesting either a partially functioning CAF-1 complex or some other chaperone might target Cse4 to centromeres under these conditions. Deletion of the chaperones Nap1, Vps75, Rtt106, and Hir1 did not compromise growth in Scm3^{off} conditions (Fig.4.3C). These results suggest that when Scm3 is absent and Cse4 levels are high, CAF-1 may be the primary chaperone targeting Cse4 to the centromere.

Scm3 localizes on the centromere throughout the cell cycle and is important for kinetochore assembly (Camahort et al. 2007; Luconi et al. 2011). Over expression of Scm3 or the orthologue HJURP have increased chromosome loss with reduced level of Cse4 at the centromere (Mishra et al. 2011). Overexpression of Scm3 also changes its association with centromere during different cell cycle stages (Mishra et al. 2011). Reduction of Cse4 at centromere in Scm3 overexpression can be correlated with the Cse4 transition delay in our experiment (Fig.4.4 A). Overexperssion of Scm3 has reduced Cse4 at the centromere. However image calibration on kinetochore protein shows that Scm3 overexpression does not affect the kinetochore assembly (Fig.4.4 B). The CAF-1 complex localizes on centromere,assembles centormeric nucleosome and pulls down with CENP-A; suggesting that the CAF-1 complex might be a secondary histone chaperone for Cse4. We speculate that Scm3 favours the hemisome structure as evident from Cse4 transition delay in Scm3 overepression and association of CAF-1 with centromere inhibits the Scm3 chaprone activity to promote octasome structure (Model.4.5).

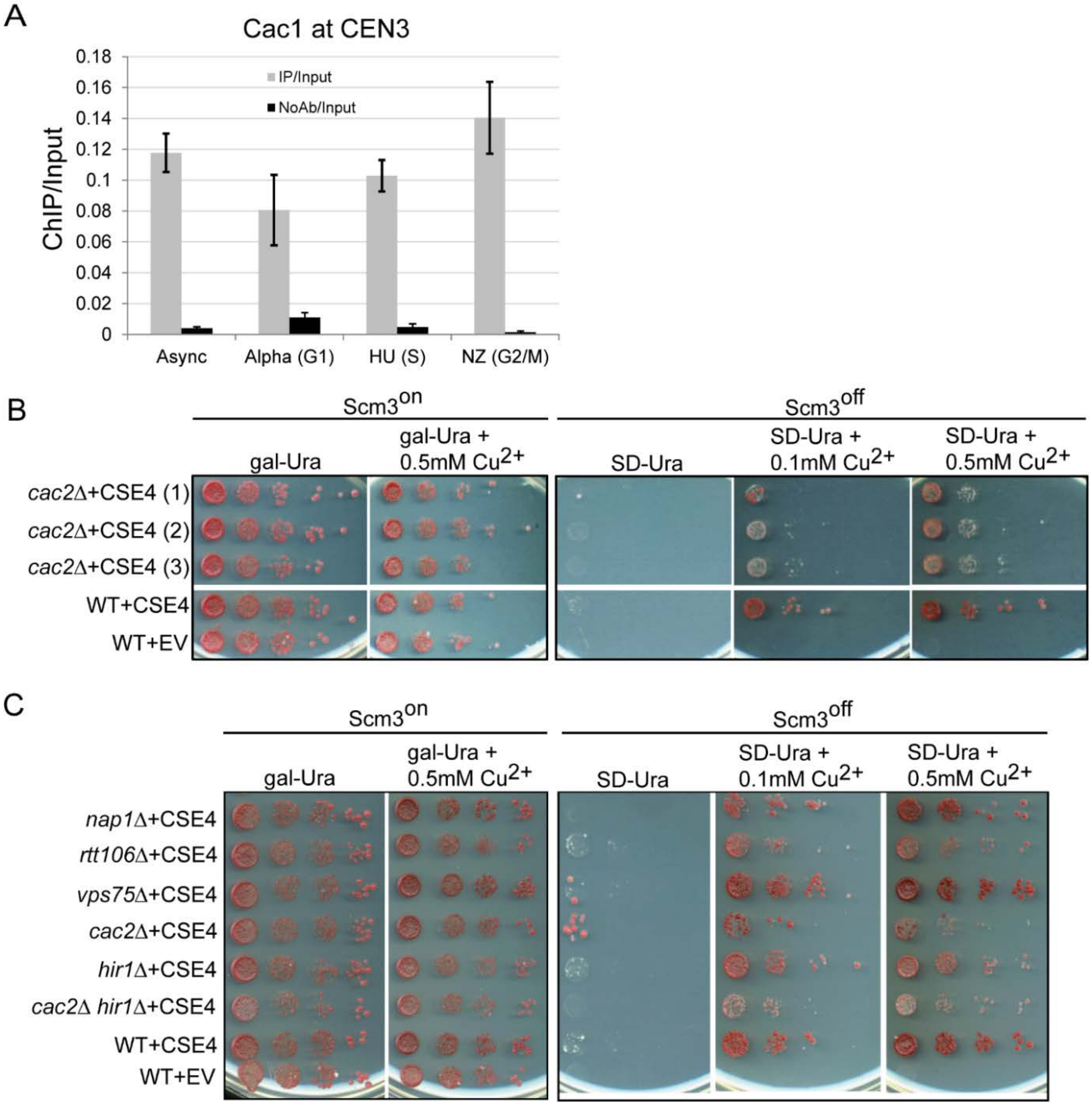


Figure 4.3: When Scm3 is absent and Cse4 levels are high, CAF-1 may help target Cse4 to the centromere. A. Cac1 localization to centromeres is cell-cycle-independent. ChIP was performed for Cac1 using a FLAG tagged strain using asynchronously growing cells and also G1, S and G2/M arrested cells. qPCR was used to detect Cac1 levels at CEN3. The y axis indicates arbitrary units representing the Cac1 enrichment at CEN3 from ChIP performed with and without antibody, with respect to the signal for total chromatin for each sample. The error bars represent the standard

deviation for ChIP performed in triplicate. B. Rescue of Scm3^{off} strain by overexpression of Cse4 is less efficient when *CAC2* is deleted. Strains with a single copy of Scm3 under the control of the gal promoter, allowing the expression of Scm3 to be controlled with glucose/galactose, were used in the spotting assay. Cse4 was under the control of a copper-inducible promoter on a plasmid. “EV” indicates empty vector. When Scm3 is expressed (Scm3^{on}), no growth differences are observed in 10-fold serial dilutions. Induction of Cse4 does not efficiently rescue Scm3^{off} strain when *CAC2* is deleted as compared to WT. Three independent isolates of the *cac2*Δ strain (1, 2 and 3) were tested. C. Growth of the Scm3^{off} strain is compromised by deletion of *CAC2* but not genes encoding other chaperones. A growth assay similar to figure 4.3 B was performed using gal inducible Scm3 strains. Cse4 was under the control of a copper-inducible promoter on a plasmid. “EV” indicates empty vector. When Scm3 is expressed (Scm3^{on}) no growth differences are observed in 10-fold serial dilutions. Induction of Cse4 does not efficiently rescue Scm3^{off} strain when *CAC2* is deleted (*cac2*Δ) as compared to WT. Deletion of genes encoding other chaperones (*NAP1*, *RTT106*, *VPS75*, *HIR1*) did not compromise growth.

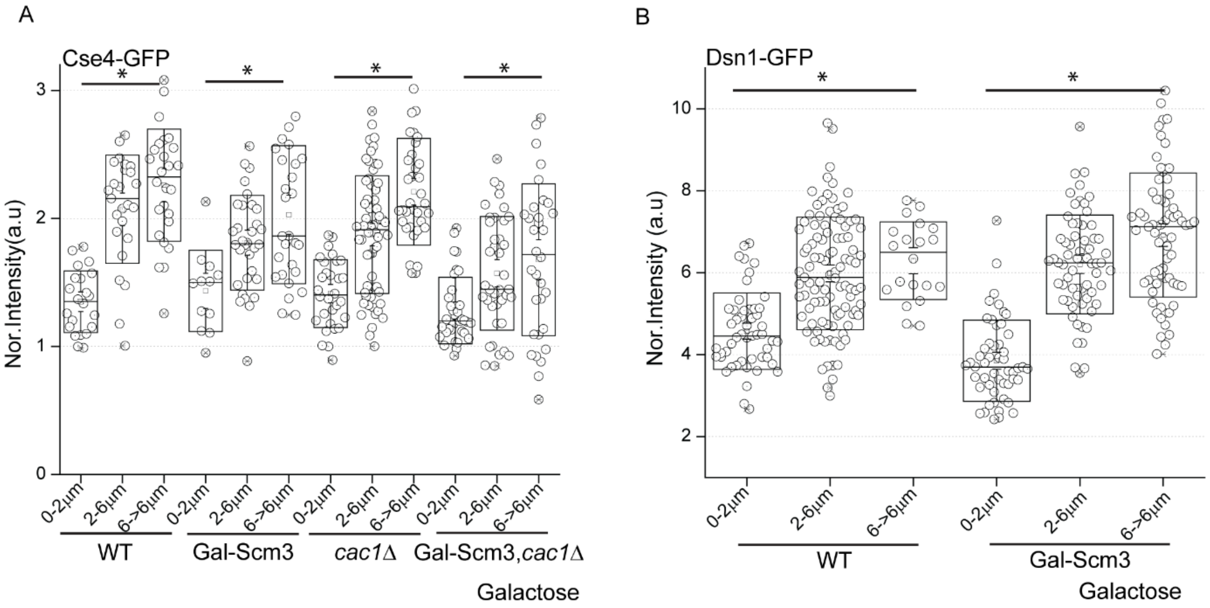


Figure 4.4: CAF-1 complex could be a potential chaperone for Cse4 in anaphase.

(A) Strains containing a single copy of Scm3 under the control of the gal promoter, allowing the expression of Scm3 to be controlled with glucose/galactose. Image calibration of Cse4-GFP on Scm3 overexpression strain (Gal-Scm3) shows the Cse4-GFP increase in anaphase is delayed. In *cac1*Δ strain, overexpression of Scm3 further delays the Cse4 transition in anaphase. (B) However, Scm3 overexpression does not impact the kinetochore transition in anaphase. A two-tailed t-test was used to test for statistical significance. * indicates statistical significance ($p<0.005$).

Discussion

Our data are consistent with CAF-1 being the main H3 chaperone in yeast that can interact with Cse4. CAF-1 deposits Cse4 at promoter nucleosomes experiencing high rates of turnover. We examined multiple other chaperones for physical interaction with Cse4 and for growth effects under Cse4 overexpression conditions and in both respects CAF-1 was unique. Although Cse4 is misincorporated into chromatin in the absence of CAF-1 when Cse4 is overexpressed, suggesting other chaperones can incorporate Cse4 when Cse4 levels are high, CAF-1 still may be special with respect to Cse4 deposition, eviction, and proteolysis. We speculate that the ability of Cse4/CenH3 to physically interact with CAF-1 in yeast, flies, and human is unlikely to be an evolutionary accident, and that CAF-1 plays an evolutionarily conserved role in Cse4 biology that has been difficult to discern since CAF-1 is also an H3/H4 chaperone.

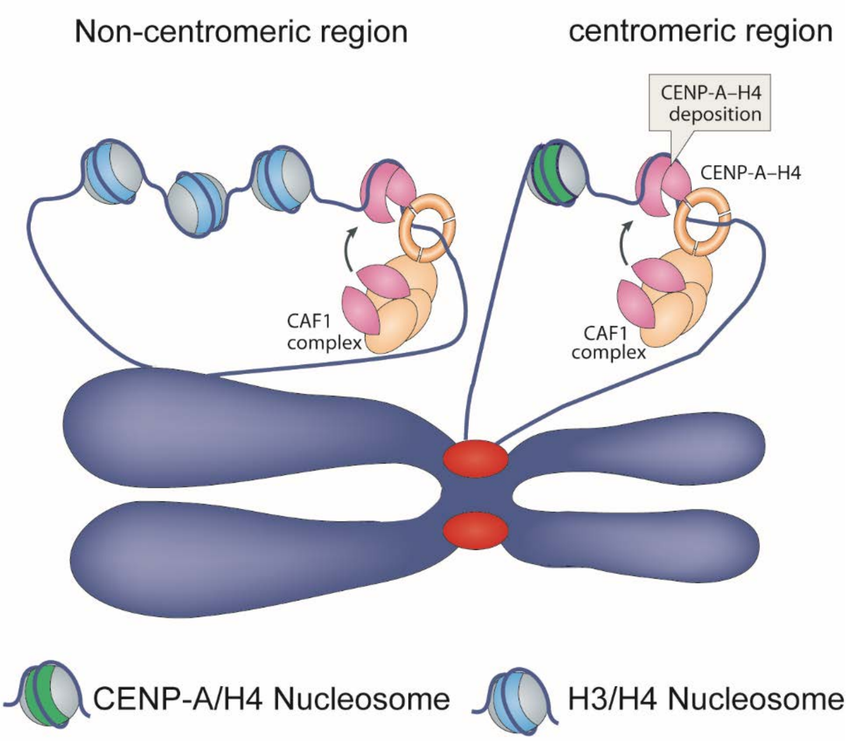
Misregulation of CENP-A and the factors that regulate CENP-A localization are hallmarks of many human cancers (Zink and Hake 2016). Proper stoichiometric balance between CenH3 and its canonical counterparts is critical for exclusive centromere targeting of CenH3 by chaperones. Overexpressed CenH3 may lead to the unintended deposition of CenH3 by H3 chaperones with deleterious consequences. Chaperones that misincorporate CenH3 into non-centromeric sites are beginning to be identified. In human cancer cells, overexpressed CENP-A occupies transcription factor binding sites and subtelomeric locations, potentially chaperoned in part by the transcriptionally coupled histone variant H3.3 chaperones DAXX and ATRX (Athwal et al. 2015; Lacoste et al. 2014).

In this study we present evidence that the evolutionarily conserved histone H3/H4 chaperone CAF-1 physically interacts with Cse4 and can assemble Cse4 nucleosomes *in vitro*. Under normal cellular conditions, histone H3 is present in large excess over Cse4. CAF-1 is normally a replication and repair coupled histone H3 chaperone.

Analysis of ectopic CENP-A nucleosomes from colorectal cancer cells reveals a subpopulation of structurally distinct hybrid nucleosomes containing CENP-A and H3.3 (Athwal et al. 2015; Lacoste et al. 2014). This CENP-A/H3.3 nucleosome forms a highly stable structure compared to CENP-A nucleosomes (Arimura et al. 2014). In budding yeast Cse4 nucleosomes are highly enriched at sites of high nucleosome turnover genome-wide (Krassovsky, Henikoff, and Henikoff 2012). We speculate CAF-1 may facilitate assembly of heterotypic (Cse4/H3) octasomes at gene promoters and active subtelomeric regions when Cse4 levels are high and proteolytic removal of Cse4 by Psh1 is compromised. These unusual nucleosomes might pose difficulties for expression or replication.

Consistent with a recent report (Hildebrand and Biggins 2016), our work indicates that one means by which Cse4 misincorporation can negatively affect cellular function is altered gene expression. We highlight one molecular mechanism by which this can occur by identifying and characterizing an evolutionarily conserved histone chaperone CAF-1 that can incorporate Cse4 into non-centromeric region.

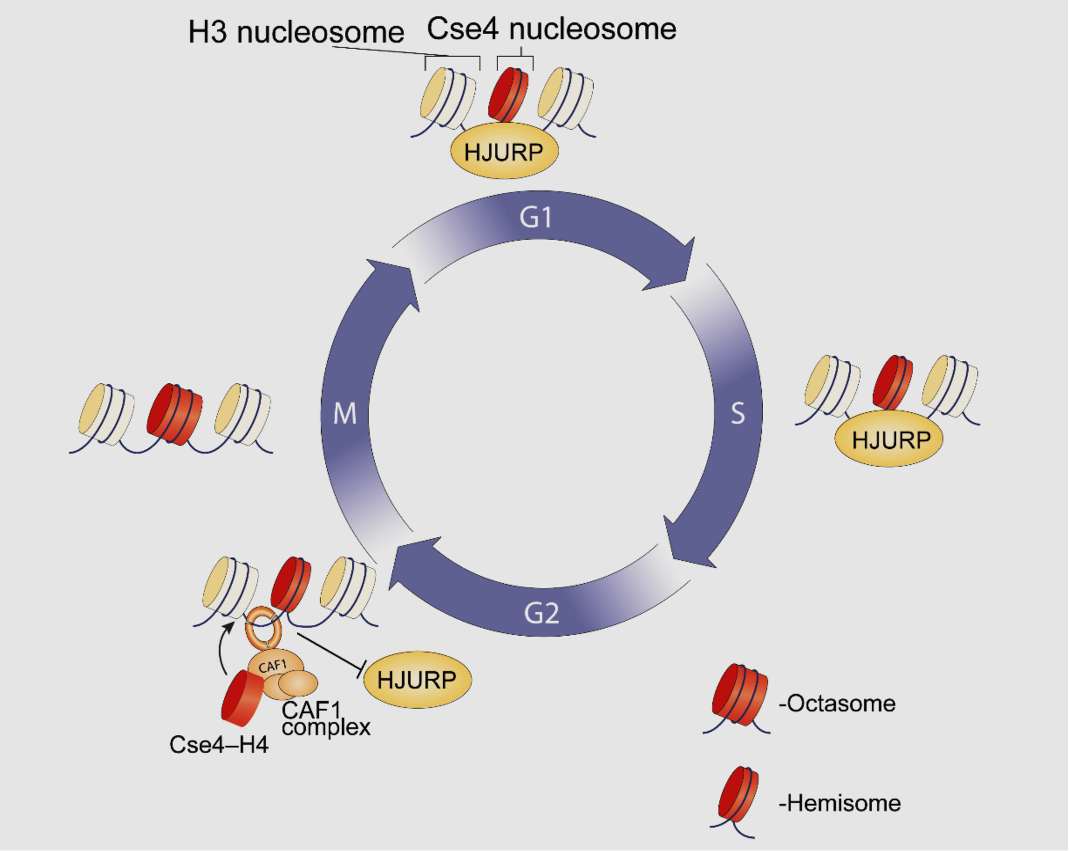
Graphical abstract:


Non-centromeric region
centromeric region
CENP-A–H4 deposition
CENP-A–H4
CAF1 complex
CAF1 complex
CENP-A/H4 Nucleosome
H3/H4 Nucleosome

Model 4.5: CAF-1 complex assembles centromeric nucleosome without any sequence specificity.

The CAF-1 complex is a secondary chaperone which interacts with Cse4 *in vivo* and assembles centromeric nucleosome on non-centromeric region *in vitro* (Modified from (Hammond et al. 2017; McKinley and Cheeseman 2016)).

Proposed model for CAF-1 role on centromeric nucleosome.


H3 nucleosome
Cse4 nucleosome
HJURP
G1
S
G2
M
HJURP
CAF1
CAF1 complex
Cse4–H4
HJURP
-Octasome
-Hemisome

Model 4.6: Proposed model for the CAF-1 complex in centromeric nucleosome assembly.

Scm3, binds to the centromeric region in anaphase. We speculate association of Scm3 to centromere converts the octasome to hemisome in G1. From G1 to metaphase, Scm3 association keeps the centromeric nucleosome in hemisome-structure. During metaphase-anaphase transition, the CAF-1 complex might be associated with Cse4 to assemble octasome. Overexpression of Scm3 delays Cse4 transition and deletion of *CAC1* (CAF-1 complex) further delays the transition, supports our hypothesis (Modified from (McKinley and Cheeseman 2016; Hammond et al. 2017)).

Experimental Procedures

Yeast strains

All the yeast strains are listed in Table 4.S1.

Whole cell extract Co-IP

Cultures grown to mid-log phase in appropriate media were used to prepare cell extracts in lysis buffer (50mM Tris (pH 7.5), 150mM NaCl, 0.1% NP-40, 1mM DTT, 10% glycerol and protease inhibitors). Protein concentration was determined using Bradford assay. Cell extracts were diluted with dilution/wash buffer (50mM Tris (pH 7.5), 150mM NaCl, 0.1% NP-40). Diluted cell extracts were incubated with the antibody overnight followed by 2h with protein G dynabeads (Invitrogen-10004D) at 4°C. Some co-IPs was performed using antibody conjugated beads. The beads were washed three times with dilution/wash buffer and proteins were eluted with SDS buffer (10mM Tris pH 7.5, 1mM EDTA and 1% SDS). Immunoprecipitates were subjected to SDS-PAGE and Western blotting.

CAF-1 complex purification:

CAF-1 complex expression and purification was performed as described previously (Winkler et al. 2012). Briefly, optimum level of Cac1, Cac2 and Cac3 viruses were used to infect the sf9 cells for 72hrs. After infection, cells were collected for the nuclei isolation and subsequent purification. The extraction buffer (15mM Tris-HCl, pH 7.5, 400mM NaCl, 10% sucrose, 1mM EDTA ,1mM DTT, 0.1Mm PMSF) was used to resuspend the nuclear pellet. Supernatant with the CAF-1 complex was collected after spinning at 33000rpm for 40min and 15% of ammonium sulfate was added to precipitate the protein. After the clarification at 12,000rpm for 30min, the supernatant was adjusted to 65% saturation with ammonium sulfate to precipitate the remaining proteins. After spinning at 12,000rpm for 30min, the precipitated proteins containing CAF-1 complex, were dissolved in buffer B0 (20mM HEPES, pH7.5, 10% glycerol, 1mM EDTA, 1mM DTT, 0.01% Triton X-100). After final clarification, the supernatant was loaded onto a HiTrap SP HP column and proteins were eluted between gradient of B100 to B1000. Fractions with the CAF-1 complex were detected by western and pooled for the overnight binding with the M2-FLAG agarose beads. Elution buffer (B100+1mg/mL 3X FLAG) was added to elute the CAF-1 complex. Purified CAF-1 complex was analyzed by silver staining and dialyzed against a storage buffer (20mM HEPES, pH7.5, 1mM EDTA, 1mM DTT, 10% glycerol, 25mM NaCl).

In vitro chromatin assembly with purified CAF-1 complex

Purified CAF-1 complex with Cse4-containing histone octamer was used for the *in vitro* nucleosome assembly. Briefly, Topoisomerase I relaxed 200ng of plasmid was incubated with CAF-1 complex or Nap1 for 2hr at 30°C in the presence of Topoisomerase I with 8.3 mM HEPES pH7.4, 0.5 mM EGTA, 0.65 mM $MgCl_2$, 1.7% glycerol, 0.005% NP-40, 33 mM KCl, 0.33 mM DTT and 0.02 mg/mL BSA. Topoisomers of the plasmids were deproteinized and isolated by standard protocol and resolved in 0.8% agarose gel. Topoisomerase I was a kind gift from S. Venkatesh, Stowers Institute. Two plasmids were used: 1) pG5E4-5S containing five repeats of 5S flanking each side of an E4 core promoter downstream of five Gal4- binding sites (gift from the Workman lab, Stowers Institute) and 2) pCEN1-10X containing 10 tandem repeats of the centromere 1 sequence.

Table: 4.S1: Strains used in this study

Strain	Genotype	Used in Figure
GH329	*Cac1-FLAG::KANMX6/cac2Δ:: NATMX6*	4.1B
GH330	*Cac3-FLAG:: KANMX6/cac2Δ:: NATMX6*	4.1B
GH358	*Cac2-FLAG:: KANMX6/cac1Δ:: NATMX6*	4.1B
GH359	*Cac2-FLAG:: KANMX6/cac3Δ:: NATMX6*	4.1B
JG1924	*Cac2-FLAG:: KANMX6*	4.1A, 1B, 1C,
JG1925	*Hir1-FLAG:: KANMX6*	4.1A
JG1999	*Cac1-FLAG:: KANMX6*	4.1B
JG2000	*Cac3-FLAG:: KANMX6*	4.1B
JG2001	*Cac1-FLAG:: KANMX6*	4.8A
JG595	*Scm3-FLAG:: KANMX6*	4.1C
MS210	*Cac2-GFP*	4.1A
MS227	*Vps75-GFP*	4.1A
MS228	*Nap1-GFP*	4.1A
RC122	*Scm3-GFP*	4.1A
SBY617	MATa *ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1Δ* CSE4::CSE4-12myc::*URA3*	4.1A,4. 1B, 4.1C

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CHAPTER 5

Conclusion and future directions

CHAPTER 5

Chapter 5 183

Conclusion and future directions 183

- Key conclusions 187
  - Kinetochore is a highly dynamic structure in anaphase. 187
  - Ndc80 complex adds more copies during anaphase. 188
  - Dam1 complex is a stable complex in anaphase. 189
  - Microtubule dynamics affect the kinetochore copy number. 189
  - Biased diffusion to explain kinetochore function. 189
  - Regulation of the kinetochore structure. 190
  - Evolutionary conservation of kinetochore complex. 191
- Future direction: 192
  - How does kinetochore add more copies during anaphase? 192
  - What are the direct targets signaling the kinetochore to add copies? 193
  - How anaphase kinetochore is different from G1 kinetochore? 193
  - Centromeric Cse4 nucleosome: 194
- Summary: 196
  - REFERENCES: 197

Key conclusions

The kinetochore is a molecular machine that connects chromosome to microtubule. Proper kinetochore-microtubule interaction is essential for the faithful segregation of chromosomes. When I started my thesis work, the yeast kinetochore had been purified and mechanically studied *in vitro* (Akiyoshi et al. 2010). But our knowledge about its function *in vivo* was not fully understood. Advancement of fluorescence microscopy had started being used in biological systems. I took advantage of this to study the kinetochore function *in vivo*.

In my thesis work, I have utilized in-house developed quantitative microscopy to study the structure of the kinetochore (Shivaraju et al. 2012). I have used a combination of quantitative microscopy, genetic techniques and mathematical simulation to understand the kinetochore structural dynamics and elucidate their structural transition in cell cycle stages. Furthermore, I have studied the regulation of kinetochore structure during the cell cycle. In the later part of the thesis, I have focused on a new Cse4 chaperone, CAF-1 which might be a potential chaperone for Cse4 in anaphase. CAF-1 can interact with Cse4 *in vivo* and assembles Cse4 nucleosome *in vitro*. In addition, the CAF-1 complex can assemble the Cse4 nucleosome on non-centromeric region. Overexpression of Scm3 with deletion of *CAC1* has delayed the Cse4 transition in anaphase *in vivo*.

Kinetochore is a highly dynamic structure in anaphase.

The kinetochore is a multi-protein complex that connects the chromosome to the microtubule and the structure is highly dynamic during anaphase. Part of the kinetochore gradually adds copies during metaphase-anaphase transition and some of the subcomplexes stably associate with the kinetochore. The sub-complexes that change during anaphase, particularly the Ndc80 complex, have shown to diffuse along the microtubule *in vitro*. On the other hand, the stable complex, the Dam1 complex, slides along the microtubule *in vitro*. Taken together, the *in vitro* studies suggest the kinetochore depends on different mode of microtubule-interacting sub-complexes for their function. How the kinetochore uses these two modes interaction to achieve this kinetochore structural transition is not known. In my thesis work I have addressed the kinetochore structural transition and factors that influences their dynamics in a living cell.

Ndc80 complex adds more copies during anaphase.

The Ndc80 complex attaches to the negatively charged microtubule through its positively charged N-terminal unstructured calponin-domain. Mutation in *NDC80* prevents microtubule-kinetochore attachment and activates the checkpoint. However, mutation in Spc24 and Spc25, lacks Bub1, Bub3 and Mad1 association and prevents attachment of the kinetochore without activating the spindle assembly checkpoint (Janke et al. 2001; Wigge and Kilmartin 2001; Gillett, Espelin, and Sorger 2004). Intensity analysis on GFP tagged proteins from Ndc80 and MIND/Mis12 complexes revealed unique dynamics of the kinetochore in anaphase. As discussed in Chapter 2, Ndc80 and MIND complexes show increase in intensity in anaphase. To study the intensity change, I used image calibration to count their copy numbers in G1 and anaphase. The analyses of these intensities showed that kinetochores approximately double the copy number of Ndc80 and MIND/Mis12 complexes in anaphase. Our method can reproduce the near stoichiometry of proteins from these complexes. The increase in Ndc80 and MIND complexes reflect addition of more complexes in near stoichiometry. In anaphase, microtubule shows prolonged depolymerization (Koshland, Mitchison, and Kirschner 1988) and makes the purified kinetochore to detach frequently (Akiyoshi et al. 2010). Increase in the Ndc80 complex might be crucial for the kinetochore attachment during prolonged microtubule depolymerization. Ndc80 is essential for Dam1 and Stu2 localization on the kinetochore (He et al. 2001; Janke et al. 2002). However, the importance of Dam1 complex for Ndc80 addition is unknown.

Dam1 complex is a stable complex in anaphase.

The Ndc80 complex localizes the Dam1 complex on the kinetochore. Mutations in Dam1 complex arrest cells in metaphase with monopolar spindle (He et al. 2001; Janke et al. 2002). Cell cycle analysis of a GFP tagged Dam1 complex showed similar intensity profile across the cell cycle, suggesting Dam1 complexes have same amount of protein at the kinetochore at all times. This claim is further supported by the FRAP, where the Dam1 complex does not recover in anaphase, and by photoactivation, where the kinetochore does not add any new copies during anaphase. The Dam1 complex forms a ring-like structure around the microtubule, but our studies show that kinetochore can have a non-ring oligomeric form and still it could be functional. Purified recombinant Dam1 complex can form a complete ring or partial ring and still can track the depolymerizing microtubule *in vitro*, further supporting our result that the Dam1 complex could function in all forms.

Microtubule dynamics affect the kinetochore copy number.

Microtubule, tubulin dimer of α and β tubulin, stochastically switch between polymerization and depolymerization. Mutation in tubulin subunits arrest with large budded cell, making it difficult to study their role on kinetochore attachment directly (Oakley et al. 1985). MAPs can influence the microtubule dynamics, particularly, Stu2 which interacts with the Ndc80 complex and localizes on the microtubule (Miller, Asbury, and Biggins 2016). Temperature sensitive Stu2 mutant has reduced the Ndc80 complex and the unaffected Dam1 complex (Chapter 2), suggesting that reduced microtubule depolymerization might reduce the Ndc80 complex requirement. Stu2 is the prime target for Cdc28 (Cdk1) and might be regulated during anaphase to sense the depolymerization to add the Ndc80 complex (Holt et al. 2009).

Biased diffusion to explain kinetochore function.

Conformational wave model and biased diffusion model gained popularity to explain the kinetochore function. In conformation wave model, curved protofilament favors kinetochore movement. In biased diffusion model, transition interaction along the microtubule is favored by Brownian motion (Asbury, Tien, and Davis 2011). Recent studies on the purified Ndc80 complex favored the biased diffusion model (Powers et al. 2009). Simulation of biased diffusion model shows that increase in coupler or attachment increase the attachment with microtubule by 4 fold in a given anaphase time (10 mins).

In conformational wave model, microtubule protofilament curling pulls the kinetochore by releasing the energy stored in the microtubule lattice. The purified Dam1 complex slides or diffuses along the depolymerizing microtubule (Gestaut et al. 2008; Cheeseman et al. 2002; Tien et al. 2010), favors the conformational wave model. The Dam1 complex can enhance the binding of the Ndc80 complex on the microtubule, promotes their tracking distance and forms a load-bearing attachment (Tien et al. 2010). *In vitro* studies on microtubule interacting kinetochore proteins suggest that part of the kinetochore sub-complexes favor biased diffusion (Ndc80, MIND/Mis12)(Powers et al. 2009; Kudalkar et al. 2015) and the Dam1 complex favors both the models(Asbury et al. 2006; Westermann et al. 2006). Based on our study and published results, we propose that the kinetochore might be rely on both type of interactions to function.

Regulation of the kinetochore structure.

Before the cell enters anaphase, the metaphase-anaphase transition is highly regulated. APC ubiquitinylates and activates a number of targets for degradation and further activation, respectively. By inactivating the number of APC components through deletion or temperature sensitive mutants, we observe a failure to add the Ndc80 complex with prolonged anaphase. This might be due to longer anaphase that does not require to add more copies in anaphase or difficulty in Ndc80 addition might signal to slow-down the depolymerization. Association of APC on the kinetochore is not known yet. We speculate that APC indirectly control the kinetochore transition in metaphase. APC releases the separase and promotes the Cdk1/Cdc28 targets for degradation. APC controls the anaphase-G1 transition by inhibiting Clb2. From our experiments, we cannot rule out the possibility of APC effect on the kinetochore in anaphase.

Mitotic exit pathway mutants arrest with the kinetochore in the anaphase state. However, without cytokinesis, the kinetochore still converts back to the G1-state. We speculate that MEN might have a direct and indirect targets on the kinetochore. It might be possible that APC along with MEN transitions anaphase-state kinetochore to G1-state kinetochore. MEN prepares the cell to complete the cell cycle. MEN might be a potential pathway, important for transitioning the anaphase-state kinetochore to G1-state.

Recent bioinformatics studies on cancer samples from patients showed that kinetochore proteins are mis-regulated as a complex than a single protein. Overexpression of theNdc80 complex is also found in pancreatic cancer. If overexpression of Ndc80 promotes the anaphase-state kinetochore, then kinetochores might be stably attached to microtubule and less-prone to SAC-mediated detachment for error-correction. Tension at metaphase can increase the number of microtubules attached to the kinetochore in higher organism (King and Nicklas 2000). However, number of microtubule has an upper limit. Further supporting our result that there is a variation in microtubule attachment. Reduced copy number of the kinetochore complex might reduce tracking and attachment, leading to lost attachment and chromosome loss. My thesis work suggest that kinetochore structure is regulated by these known pathways but kinetochore-specific targets are unknown.

Evolutionary conservation of kinetochore complex.

In higher eukaryotes, the centromeric sequence is not conserved as in yeast and is mainly marked epigenetically with CENP-A nucleosome. Most of the yeast kinetochore proteins, organization, and their regulators are evolutionarily conserved. Studies on vertebrate kinetochore with multiple microtubule attachment indicate that number of simple identical units are spread along the centromere to construct the kinetochore (Zinkowski, Meyne, and Brinkley 1991). This multi-identical-subunit structure may be constructed by replicating individual attachment sites, similar to what found in budding yeast. The human kinetochore region has ~24 microtubules (Takeuchi and Fukagawa 2012) ,interacting with possibly ~24 individual unit (similar to yeast kinetochore) kinetochores. Quantitative measurement studies using budding yeast, fission yeast and chicken cell line DT40 show similar copy number per microtubule per kinetochore, suggesting basic structural conservation of kinetochore organization. Interestingly, my work shows kinetochore structural transitions found in budding yeast are also conserved in *S. pombe*, suggesting a possibility that kinetochore dynamics is evolutionarily conserved at the level of individual microtubule attachment site.

Future direction:

Part of the yeast kinetochore is highly dynamic in anaphase and this dynamic nature may be controlled by microtubule dynamics. But there are number questions which are not clear like how does the kinetochore add more copies during anaphase? What are the direct targets signaling the kinetochore to add more copies? And how does the kinetochore drop these copies at the end of anaphase and why? How the anaphase kinetochore is different from the G1 kinetochore?

How does kinetochore add more copies during anaphase?

Copy number increase of the kinetochore in anaphase is reflected by the increase of the inner and outer kinetochore. Cnn1 /CENP-T is proposed to be anaphase-specific receptor which anchors the Ndc80 complex (Schleiffer et al. 2012; Bock et al. 2012) and the anaphase specific addition of the Ndc80 complex could be explained by Cnn1. However, deletion of it does not affect the copy number change in anaphase. One future focus will be to find anaphase-specific receptor by finding proteins only enriched in an anaphase-purified kinetochore in MudPIT analysis. Preliminary studies indicate that the inner and outer kinetochores add copies simultaneously. Mif2, COMA and MIND/Mis12 complexes rely on number of metaphase kinases and phosphatases for their interaction with other proteins. In future, contribution from these pathways on kinetochore regulation are necessary to understand the chromosome segregation.

What are the direct targets signaling the kinetochore to add copies?

Cdk1, Ipl1 and Mps1 have different targets on the kinetochore, particularly inner, MIND, Ndc80, Dam1 complexes and MAPs (Jones et al. 1999; Shimogawa et al. 2006; Vigneron et al. 2004; Cheeseman et al. 2002; Pinsky et al. 2003; Pinsky et al. 2006; Akiyoshi et al. 2009). N-terminal of Ndc80 is highly phosphorylated by Ipl1 for microtubule detachment (Akiyoshi et al. 2009). Cdc14 phosphatase dephosphorylate Dsn1 on anaphase entry. Mps1 also phosphorylates outer kinetochore proteins. Stu2 has number of sites for Cdk1-mediated phosphorylation. Even though we know the sites of modification, the function of these modification is not known and might be important for kinetochore regulation.

How the anaphase kinetochore is different from the G1 kinetochore

The G1-state kinetochore has fewer copies whereas the anaphase-state kinetochore has a high-copy number state. The kinetochore drops the added copies from the anaphase-state kinetochore back to G1 state. This process happens after telophase. We reasoned that by purifying kinetochore complexes from mutants that arrest in anaphase and G1 cells will shed light on copy number addition and reduction. I used α-factor arrested cell and *cdc15-1* strain to purify G1 and anaphase kinetochores respectively (Fig.5.1), for MudPIT mass spectrometry and I am analyzing the data to identify the stoichiometry and phosphorylation sites of different associated proteins.

A number of kinases and phosphatase target kinetochore and regulate the protein-protein interaction. Phosphorylation of the Ndc80 complex and the Dam1 complex reduces the affinity for the microtubule and the Ndc80 complex respectively. By comparing the protein abundance in G1 and the Ndc80 complex will elucidate their stable interaction with other kinetochore complexes. Anaphase-specific protein might be found which might have a role in kinetochore transition. Purification of cell cycle specific purification has not been done before. Stability of these complexes in solution is not known.

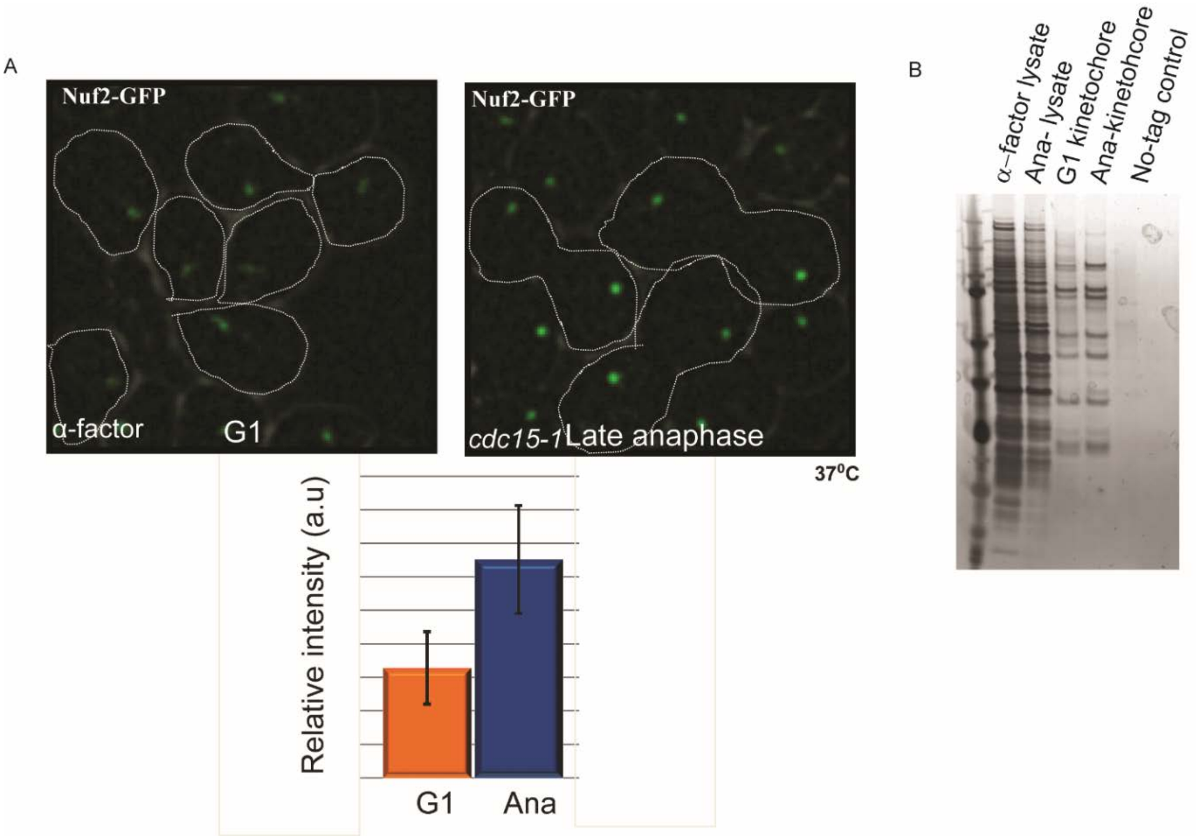

Figure 5.1: Anaphase kinetochore purification from *cdc15-1*.

(A) Intensity quantification of Nuf2-GFP in G1 arrested cell and *cdc15-1* (bottom) with images (Top) show that anaphase arrested cells have higher intensity than G1 arrested cell. (B) Flag-tag affinity immunoprecipitation of kinetochore proteins using Dsn1-his-flag protein. Silver stained gel showing the purified kinetochore complex.

Centromeric Cse4 nucleosome:

Cse4, a H3 variant containing nucleosome presents at the centromeric region. Quantitative microscopy of GFP tagged Cse4 has revealed that centromeric Cse4 nucleosome can adapt hemisome during interphase and octasome in anaphase. Scm3, a Cse4-specific chaperone is important for Cse4 assembly. Size exclusion chromatography of Scm3 assembled Cse4 nucleosome is smaller than the H3 containing nucleosome. Nucleosome assembly with fluorescent dye conjugated-H4 by Scm3 shows presence of single copy of Cse4 per nucleosome. Taken together, Scm3 might be favoring the hemisome structure of Cse4 nucleosome.

We found that Cse4 can interact with the CAF-1 complex *in vivo*. The CAF-1 complex can mediate the Cse4 nucleosome assemble *in vitro*. The CAF-1 complex is a H3/H4 chaperone predominately assembles octameric nucleosome. Quantitative microscopy on Cse4-GFP in Scm3 overexpression strain shows delay in Cse4 transition in anaphase. This transition is further delayed in deletion of *CAC1*. Taken together, CAF-1 could be a potential chaperone for Cse4 in anaphase. Whether CAF-1-mediated Cse4 nucleosome forms a hemisome or octasome is not known. To further characterize the Cse4 nucleosome structure, we can use size-exclusion chromatography, cryo-EM and fluorescent dye conjugated H4 assembled Cse4 nucleosome.

Summary:

In summary, the data presented in this thesis provide evidence for a novel structural change of the kinetochore complex. This structural plasticity is cell cycle-specific particularly in anaphase and is essential for kinetochore-microtubule attachment. The structure of centromeric Cse4 nucleosome also transition in anaphase but is not coupled with kinetochore transition. I have helped to identify a new Cse4 chaperone, CAF-1 which mediates Cse4 nucleosome assembly on centromeric and non-centromeric regions. The Kinetochore adds the Ndc80 complex in anaphase and loses it in telophase. MAPs control the microtubule dynamics in turn microtubule dynamics control the copy number addition. I also present data for the regulation of kinetochore addition in metaphase and drop in telophase. This novel structural dynamics is new to biology and will extend our knowledge in chromosome biology.

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