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 Regulation of the Mitotic Checkpoint

by

Wenbin Ji

Submitted to the Graduate Faculty as partial fulfillment of the requirements for the

Doctor of Philosophy Degree in

Biology

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An Abstract of

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The mitotic checkpoint is an evolutionarily conserved mechanism that preserves genomic integrity. The mitotic checkpoint can be viewed as a special signal transduction pathway. The signal amplification step for the mitotic checkpoint signaling involves the mitotic checkpoint protein MAD2 converting from open (O-MAD2) to closed (C-MAD2) conformation (MAD2 O-C conversion). In current models, the MAD2 O-C conversion is catalyzed by an unusual catalyst, the complex formed by MAD1 and C-MAD2 (the MAD1:C-MAD2 complex). Previously it was known that MAD1\(^{(485-584)}\) (a MAD1 fragment spanning 485-584 residues that contains MAD2 Interacting Motif, or MIM) is responsible for forming the complex with C-MAD2. MAD1\(^{(597-718)}\) (MAD1 C-terminal domain, CTD) also seemed essential for the mitotic checkpoint. Despite these findings, how MAD2 O-C conversion is catalyzed and regulated remains largely unclear.

We found that deleting MAD1\(^{(1-485)}\) (MAD1 N-terminal domain, NTD) or MAD1\(^{(597-718)}\) (MAD1-CTD) resulted in a defective mitotic checkpoint, suggesting that both MAD1-NTD and MAD1-CTD are required for efficient catalysis of MAD2 O-C conversion. We further demonstrated that MAD1-NTD and MAD1-CTD bind to both O-
MAD2 and C-MAD2, as expected for an enzyme to associate with its substrates and products. Moreover, our data showed that MAD1-NTD directly interacts with MAD1-CTD, suggesting possible coordination between different MAD1 domains.

Additionally, we discovered that MPS1 kinase, which promotes MAD2 O-C conversion during mitosis, regulates the MAD1:C-MAD2 complex activity through directly phosphorylating both MAD1-NTD and MAD1-CTD. Our data revealed that MPS1 kinase reduced the interaction between MAD1-NTD and MAD1-CTD. Phosphorylation of MAD1-CTD by MPS1 seems required for a functional mitotic checkpoint. The newly characterized protein-protein interactions and their modulation by MPS1 kinase may indicate a delicately controlled regulatory mechanism for the catalytic activity of the MAD1:C-MAD2 complex. Our work has led to an updated mechanistic model to understand how the mitotic checkpoint signal is amplified by the unusual catalyst---the MAD1:C-MAD2 complex.

In addition to MPS1 kinase, we also characterized other kinases that might regulate the mitotic checkpoint including Aurora B, BUB1, MELK and Abl. The results provided clues for better understanding the regulation of the mitotic checkpoint signal transduction pathway. We also investigated how a pseudokinase BUBR1 contributes to the mitotic checkpoint. We found that BUBR1(487-700) directly interacts with C-MAD2 and p31comet, which is a MAD2-binding protein and negative regulator of the mitotic checkpoint. Our study on BUBR1 will fill the knowledge gap on how the mitotic checkpoint complex (MCC), the effector of the mitotic checkpoint, assembles and functions.
In summary, our work has demonstrated the functions of currently little appreciated MAD1 domains in the mitotic checkpoint and advanced the mechanistic understanding about how different MAD1 domains coordinate to catalyze MAD2 O-C conversion. In addition, our work provided insights into how mitotic kinases regulate the MAD2 O-C conversion and other aspects of the mitotic checkpoint in order to maintain the high fidelity of chromosome segregation.
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List of Abbreviations

APC/C .......................Anaphase promoting complex/cyclosome
ATM ..........................Ataxia telangiectasia mutated

BSA ..........................Bovine serum albumin
BUB1/3 .......................Budding uninhibited by benzimidazoles 1/3
BUBR1 ........................Budding uninhibited by benzimidazoles related 1

CCAN ........................Constitutive centromere associated network
CDC16/20/27 .............Cell division cycle 16/20/27
CDC20_{APC/C}_{WD40} ......CDC20 in the APC/C
CDC20_{MCC}_{WD40} ......CDC20 in the MCC
CDK ...........................Cyclin dependent kinase
cDNA .........................Complimentary deoxyribonucleic acid
CENP-A/C/I ..................Centromere Protein A/C/I
CPC .............................Chromosomal passenger complex

DAPI .........................4’, 6’-diamidino-2-phenylindole
D box ..........................Destruction box
DMEM ..........................Dulbecco’s modified Eagle’s medium
DMSO ..........................Dimethyl sulfoxide

FBS ...........................Fetal bovine serum
FRAP ..........................Fluorescence recovery after photo bleaching

GFP ............................Green fluorescent protein
GST .............................Glutathione-S-Transferase

HA .............................Hemagglutinin
Hes .............................Hesperadin
hr ..............................hour
hrs ............................hours
INCENP .................. Inner centromeric protein  
IP .......................... Immunoprecipitation 

KMN .................. Knl1/Mis12 complex/Ndc80 complex  
KNL1 .................. Kinetochore null protein 1  

MAD1/2/3 .......... Mitotic arrest deficient 1/2/3  
MCC .................. Mitotic checkpoint complex  
MELK .................. Maternal embryonic leucine zipper  
mg .................. Milligram  
ml .......................... Milliliter  
mM .................. Millimolar  
µg .................. Microgram  
µl .................. Microliter  
µM .................. Micromolar  
MPS1 .................. Monopolar spindle 1  

Nek2A .............. NIMA-related kinase 2A  
ng .................. Nanogram  
nM .................. Nanomolar  
Noc .......................... Nocodazole  

O/N .................. Overnight  

PAGE .................. Polyacrylamide gel electrophoresis  
PBS .................. Phosphate buffered saline  
PCR .................. Polymerase chain reaction  
PEI .................. Polyethylenimine  
PLK1 .................. Polo-like kinase 1  
PP1 .............. Protein phosphatase 1  

Rev .......................... Reversine  
RZZ .................. Rod-Zw10-Zwilch  
RT .......................... Room temperature  

SAC .................. Spindle assembly checkpoint  
STA .................. Single thymidine arrest  

TEV .................. Tobacco etch virus  
TPR .................. Tetratricopeptide repeat  

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TRIP13 .....................Thyroid hormone receptor interacting protein 13
List of Symbols

α ........................................alpha
β ........................................beta
γ ........................................gamma
Chapter 1

Introduction

Aneuploidy—deviation of chromosome number from that in the normal haploid and diploid cells—is commonly associated with birth defects and solid tumors (Lengauer, Kinzler et al. 1998). Solid tumors characterized by aneuploidy torture and kill many people every year (Kops, Weaver et al. 2005). During the development of solid tumors, aneuploidy cells are likely generated from a tetraploidization event followed by gradual loss of individual chromosomes (Torres, Sokolsky et al. 2007).

Inaccurate cell division can lead to aneuploidy through chromosome mis-alignment and mis-segregation (Torres, Sokolsky et al. 2007). To maintain the genomic integrity, cells have evolved a surveillance system called the mitotic checkpoint, which prevents aneuploidy through delaying chromosome segregation until all kinetochores are properly attached to the microtubules (Musacchio and Salmon 2007). Understanding how the mitotic checkpoint is regulated during mitosis can provide insights into tumorigenesis and cancer treatment.
1.1. Mitosis is one important stage of the cell cycle

The cell cycle is an ordered sequence of events through which one cell divides to produce two daughter cells (Hartwell and Weinert 1989). A cell cycle can be broadly divided into interphase and mitosis. The interphase for an actively proliferating animal cell lasts for about 95% of the whole cell cycle. During interphase cells perform diverse activities that are essential to make next mitosis possible. The interphase can be further divided into three phases, which are Gap phase 1 (G1), DNA replication/synthesis phase (S) and Gap phase 2 (G2) (Figure 1-1).

Figure 1-1. Stages of the Cell Cycle.
The cell cycle is divided into interphase and mitosis. The interphase is composed of the first growth phase (G1), followed by DNA replication during S phase and the second growth phase (G2). After successful completion of interphase cells enter mitosis (M). Mitosis can be divided into prophase, prometaphase, metaphase, anaphase, telophase followed by cytokinesis (not drawn to scale).
Cells enter mitosis after completion of interphase. Cell division takes place during mitosis to make two genetically identical daughter cells. Despite of the fact that mitosis only lasts for about 5% of the whole cell cycle, it is a critical and elaborate process for keeping genomic integrity. Mitosis can be further divided into 6 phases including prophase, prometaphase, metaphase, anaphase, telophase and cytokinesis (Figure 1-1).

During prophase, DNA condenses into compacted chromosomes. Two centrosomes migrate to opposite ends of a cell before formation of the mitotic spindle. Nuclear envelope breakdown marks the beginning of the prometaphase, during which the bipolar spindle is fully established. Kinetochores form as approximately 200 proteins are recruited to the centromeres (Tipton, Wang et al. 2012). Spindle microtubules originating from opposite poles of the cell search out and attach kinetochores. During metaphase, all chromosomes are attached and bi-oriented by microtubules with tension and aligned at the metaphase plate. As cells progress to anaphase, two sister chromatids are pulled by microtubules to opposite poles of the cell following cleavage of cohesion between them. Telophase marks the decondensation of chromosomes and reformation of the nuclear envelope. Finally, cytokinesis results in the production of two identical daughter cells from one parent cell.

1.2. Regulations of the cell cycle

The cell-cycle control system works like a timer that triggers the events of a cell cycle in a set sequence (Figure 1-2). Cyclin-dependent kinases (Cdks) are the central components of the cell-cycle control system. The activities of Cdks increase and decrease during the cell cycle, leading to cyclical changes in the phosphorylation of intracellular
proteins, which are essential for regulating the major events of the cell cycle. Among all the Cdk regulators, cyclins are the most important. Different classes of cyclins and Cdks form cyclin-Cdk complexes to activate Cdks at different stages of the cell cycle (Figure 1-2).

Cell cycle checkpoints are additional control mechanisms besides Cdks. In most eukaryotic cells, there are three major cell cycle checkpoints including G1 checkpoint, G2/M checkpoint and mitotic checkpoint (Hartwell and Weinert 1989, Lara-Gonzalez, Westhorpe et al. 2012, Johnson and Skotheim 2013). The progression of the cell cycle will be blocked at each of these checkpoints if the control system detects problems inside or outside the cell. The control system blocks cells through G1 checkpoint to prevent cell division until cells have adequate energy reserves and reach appropriate size. At this point, the cells also check for DNA damage. The cell cycle is blocked at the G2/M checkpoint until any problems of DNA replication are resolved. The mitotic checkpoint blocks cells at metaphase until all the kinetochores are bi-oriented. Despite the undoubted importance of all the checkpoints in cell cycle regulation, this dissertation will focus specifically on the regulation of the mitotic checkpoint.
1.3. **The mitotic checkpoint**

To prevent aneuploidy, cells have a highly conserved mechanism termed the mitotic checkpoint, which delays chromosome segregation until all kinetochores are properly attached to the microtubules (Musacchio and Salmon 2007, Lara-Gonzalez, Westhorpe et al. 2012, Foley and Kapoor 2013). A single unattached kinetochore is enough to activate the mitotic checkpoint thus preventing premature anaphase onset (Rieder, Cole et al. 1995) (Figure 1-3).
Figure 1-3. The mitotic checkpoint detects unattached kinetochores. The mitotic checkpoint is activated to prevent anaphase onset until all kinetochores (green) are well attached by microtubules (red). Once all chromosomes (blue) achieve bipolar attachment to the mitotic spindle, the mitotic checkpoint is inactivated thus allowing the metaphase to anaphase transition.
1.4. Molecular components of the mitotic checkpoint

Previous studies have characterized a group of proteins that are essential for the mitotic checkpoint. The *MAD* (Mitotic Arrest Deficient) genes including *MAD1, MAD2* and *MAD3* and *BUB* (Budding Uninhibited by Benzimidazole) genes including *BUB1* and *BUB3* were identified through two independent genetic screens in *Saccharomyces cerevisiae* (Hoyt, Totis et al. 1991, Li and Murray 1991). The budding yeast cells with mutations in these genes failed to arrest in mitosis in response to spindle damage. Using similar technique, MPS1 (Monopolar Spindle 1) kinase was characterized as another key mitotic checkpoint protein, which is important for activating the mitotic checkpoint (Weiss and Winey 1996). Afterwards, additional mitotic checkpoint proteins such as Aurora B and RZZ complex have been identified (Williams, Karr et al. 1992, Chan and Botstein 1993, Biggins, Severin et al. 1999, Scaerou, Starr et al. 2001, Williams, Li et al. 2003, Kops, Kim et al. 2005, Lin, Chen et al. 2006, Famulski, Vos et al. 2008). The homologues of mitotic checkpoint proteins are found in most eukaryotic cells, suggesting that the mitotic checkpoint is evolutionarily conserved (Hardwick, Weiss et al. 1996, Musacchio and Salmon 2007, Sczaniecka and Hardwick 2008, Hardwick and Shah 2010, Vleugel, Hoogendoorn et al. 2012).
Figure 1-4. MCC is composed of BUBR1, CDC20, BUB3 and MAD2. 
(A). Schematic figures showing the components of the MCC. (B). The interactions of BUBR1 with CDC20 in MCC (CDC20\textsuperscript{MCC}) and CDC20 in APC/C (CDC20\textsuperscript{APC/C}). The 7 blades of CDC20 WD40 domain are numbered and the positions of BUBR1 inhibitory degrons (orange) are indicated. D=Destruction box; K=KEN box; A=ABBA motifs. The CRY degron mediates Cdc20\textsuperscript{MCC} interactions with Cdc20\textsuperscript{APC/C} (Alfieri, Chang et al. 2016). CDC20\textsuperscript{MCC,WD40} indicates the CDC20 in the MCC. CDC20\textsuperscript{APC/C,WD40} indicates the CDC20 in the APC/C.
1.5. The effector of the mitotic checkpoint-the mitotic checkpoint complex (MCC)

The mitotic checkpoint complex (MCC) is the effector of the mitotic checkpoint. MCC binds to and inhibits anaphase promoting complex/cyclosome (APC/C, more in section 1.6). Human MCC is composed of BUBR1, CDC20, BUB3 and MAD2 (Liu and Zhang 2016) (Figure 1-4 A). Although BUBR1 or MAD2 alone can inhibit APC/C, MCC inhibits APC/C much more efficiently (Sudakin, Chan et al. 2001).

BUBR1 is the homologue of yeast MAD3 in higher eukaryotes such as human. Human BUBR1 contains 1050 residues and is the largest MCC component. Different from its yeast homologue MAD3, BUBR1 contains a kinase domain. However, it remains controversial whether BUBR1 is a bona fide kinase or pseudo kinase (Mao, Abrieu et al. 2003, Guo, Kim et al. 2012, Suijkerbuijk, van Dam et al. 2012). BUBR1 contains two KEN (Lys-Glu-Asn) boxes, the first of which is required for interactions with MAD2 and CDC20 to nucleate MCC formation while the second functions to block substrate recruitment to APC/C (Lara-Gonzalez, Scott et al. 2011). The other important domains characterized on BUBR1 are three tetratricopeptide repeating (TPR) motifs immediately following the first KEN box which are required for MCC-APC/C interactions (D'Arcy, Davies et al. 2010), a GLEBS motif where BUB3 binds to form the cell cycle independent BUBR1-BUB3 complex (Larsen, Al-Bassam et al. 2007) and an ABBA motif (A1) and D box (D1), which are used to interact with the CDC20 (CDC20^{APC/C}) molecule as part of the APC/C^{CDC20} (Alfieri, Chang et al. 2016, Yamaguchi, VanderLinden et al. 2016) (Figure 1-4 B).

While BUBR1 N-terminal 486 amino acids are widely believed to be essential for mitotic checkpoint function (Tipton, Wang et al. 2011, Izawa and Pines 2015), recent
studies have also revealed the importance of BUBR1 C-terminal domains on mitosis regulation. BUBR1 KARD domain (664-681) was reported to be essential for stable kinetochore microtubule attachments (Elowe, Dulla et al. 2010, Suiskerbijijk, Vleugel et al. 2012). In addition, the second ABBA motif (A2) and the second D box (D2) are two functional motifs in the "second CDC20 binding domain" of BUBR1 identified for binding to CDC20 in the MCC (CDC20^{MCC}) and promoting MCC homeostasis in human cells (Figure 1-4B). However, A2 and D2 are dispensable for the mitotic checkpoint (Lischetti, Zhang et al. 2014, Di Fiore, Davey et al. 2015, Diaz-Martinez, Tian et al. 2015).

Cells with reduced BUBR1 levels tend to have more mis-aligned chromosomes (Kim, Murphy et al. 2005, Elowe, Hummer et al. 2007). Homozygous depletion of BUBR1 in mice is embryonic lethal while BUBR1^{+-} mice compared with wild type show early aging and have higher chance to get cancer under carcinogen treatment (Baker, Jeganathan et al. 2004, Dai, Wang et al. 2004).

CDC20 is another component of the MCC, containing 499 residues in humans. As part of the MCC, CDC20 binds to MAD2 via a lysine-isoleucine-leucine-arginine tetrapeptide (KILR) motif (Luo, Tang et al. 2002, Izawa and Pines 2012). Its interaction with BUBR1 has been detailed above (Figure 1-4B). Similarly to BUBR1, knocking out CDC20 in mice arrests embryos at metaphase thus leading to the failure of embryogenesis (Li, York et al. 2007).

CDC20 also serves as the coactivator of APC/C, which activates anaphase onset. CDC20 folds primarily as a WD40 β-propeller with amino- and carboxyl-terminal extensions (Yu 2007, Chao, Kulkarni et al. 2012, Tian, Li et al. 2012). As a substrate
specifying subunit of APC/C, CDC20 presents substrates to the APC/C through multiple degron-binding motifs on its β-propeller (Primorac and Musacchio 2013). The best characterized motifs are KEN box and D box (Primorac and Musacchio 2013). In addition to the motifs on β-propeller, the amino-terminal and carboxyl-terminal extensions of CDC20 also contain several regulatory domains, which are essential for binding to and activating APC/C, including the C-box, the C-terminal isoleucine-arginine (IR) tail and the lysine-isoleucine-leucine-arginine tetrapeptide (KILR) motif (Schwab, Neutzner et al. 2001, Vodermaier, Gieffers et al. 2003, Izawa and Pines 2011, Izawa and Pines 2012, Primorac and Musacchio 2013).

BUB3, which contains 328 amino acids, forms a constitutive complex with BUBR1 through the latter's GLEBS motifs. Similarly, BUB1, a BUBR1 paralog that is also an important mitotic checkpoint protein, also binds to BUB3 (Taylor, Ha et al. 1998). BUB3 targets BUB1 to kinetochores by binding to phosphorylated MELT repeats on KNL1, which is the receptor for many mitotic checkpoint proteins at kinetochores (Primorac, Weir et al. 2013). The BUB1:BUB3 complex then recruits BUBR1 to kinetochores (Overlack, Primorac et al. 2015, Zhang, Lischetti et al. 2015).

Whether BUB3 is required for a functional MCC remains controversial and needs further investigation. BUB3 is not present in the *S. pombe* MCC (Sczaniecka, Feoktistova et al. 2008, Chao, Kulkarni et al. 2012). Several studies have indicated that BUB3 is not necessary for BUBR1 to bind to MAD2-CDC20 or APC/C inhibition *in vitro* (Tang, Bharadwaj et al. 2001, Fang 2002, Kulukian, Han et al. 2009, Lara-Gonzalez, Scott et al. 2011). However, BUB3 was reported to increase the binding affinity of MCC for APC/C (Primorac and Musacchio 2013, Han, Vitre et al. 2014, Overlack, Primorac et al. 2015),
although the mechanism is unknown. Knocking out *BUB3* in mice causes the accumulation of mitotic errors, thus leading to the failure of embryogenesis (Kalitsis, Earle et al. 2000).

MAD2 contains 205 residues in humans and was the first mitotic checkpoint protein shown to be conserved in vertebrate cells (Li and Benezra 1996). MAD2 is a unique protein, which adopts two distinct conformations named as open and closed MAD2 (O-MAD2 and C-MAD2 respectively) (Mapelli, Massimiliano et al. 2007, Luo and Yu 2008). The major difference between O-MAD2 and C-MAD2 lies on the N-terminus and C-terminus. Two β-strands and some disordered segments in the C-terminal part of O-MAD2 refold to a new β-hairpin, which moves across the face of the protein to create the C-MAD2, while an N-terminal β-strand in O-MAD2 is replaced by an α-helix in C-MAD2 (Mapelli and Musacchio 2007, Luo and Yu 2008) (Figure 1-5).

MAD2 acts as the signaling molecule in the mitotic checkpoint signal transduction pathway by conducting the O-C conversion. During interphase when mitotic checkpoint is inactive, most endogenous MAD2 is O-MAD2, the inactive form of MAD2. C-MAD2, the active form of MAD2, peaks during mitosis when the mitotic checkpoint is most robust (Luo, Tang et al. 2004, De Antoni, Pearson et al. 2005). C-MAD2 is selectively incorporated into the MCC (Tipton, Tipton et al. 2011, Chao, Kulkarni et al. 2012). Overexpressing MAD2^{L13A}, a MAD2 mutant locked in C-conformer, but not MAD2^{AC10}, a MAD2 mutant locked in O-conformer, caused mitotic arrest in HeLa cells, suggesting that C-MAD2 but not O-MAD2 is required for active mitotic checkpoint (Tipton, Tipton et al. 2011).
C-MAD2 forms a cell cycle independent complex with MAD1 (Campbell, Chan et al. 2001, Luo, Tang et al. 2002, Tipton, Ji et al. 2013). The MAD1:C-MAD2 complex localizes at unattached kinetochores to recruit MAD2 and catalyze MAD2 O-C conversion during mitosis (De Antoni, Pearson et al. 2005, Kulukian, Han et al. 2009, Simonetta, Manzoni et al. 2009). It remains unclear how exactly the MAD1:C-MAD2 performs as a catalyst to convert O-MAD2 to C-MAD2. I will discuss more details about this catalysis later in this thesis.

Disruption of one MAD2 allele causes chromosome loss, defective mitotic checkpoint and may promote tumorigenesis (Dobles, Liberal et al. 2000, Michel, Liberal et al. 2001).
Figure 1-5. The structure of O-MAD2 and C-MAD2.
(A). Topology diagram of O-MAD2 and C-MAD2. In C-MAD2, the two β8'- β8" are extensions in opposite directions of the β8 strand of O-MAD2. (B). The crystal structure of O-MAD2. (C). The crystal structure of C-MAD2. (Mapelli, Massimiliano et al. 2007, Yang, Li et al. 2008).
1.6. The anaphase promoting complex/cyclosome (APC/C)

The target of the mitotic checkpoint is the anaphase promoting complex/cyclosome (APC/C). The APC/C is an E3 ubiquitin ligase that initiates anaphase by ubiquitylating securin and B-type cyclins for degradation by the 26S proteasome (Yu 2002, Peters 2006, Primorac and Musacchio 2013). B-type cyclins and securin are two inhibitors of the metaphase to anaphase transition. Securin inhibits separase, which is a protease that cleaves cohesin subunits thus allowing the separation of sister chromatids (Funabiki, Yamano et al. 1996, Michaelis, Ciosk et al. 1997, Ciosk, Zachariae et al. 1998, Nasmyth 2002, Primorac and Musacchio 2013). B-type cyclins activate CDK1, which phosphorylates and inhibits separase and proteins required for mitotic exit (Stemmann, Zou et al. 2001, Gorr, Boos et al. 2005, Holland and Taylor 2006).

CDC20 and CDH1 are two structurally related substrate-binding and co-activator subunits of APC/C (Sigrist, Jacobs et al. 1995, Schwab, Lutum et al. 1997, Sigrist and Lehner 1997, Visintin, Prinz et al. 1997, Ciosk, Zachariae et al. 1998, Fang, Yu et al. 1998, Kramer, Gieffers et al. 1998, Lim, Goh et al. 1998, Lorca, Castro et al. 1998, Shirayama, Zachariae et al. 1998, Kimata, Baxter et al. 2008). CDC20 and CDH1 have opposing activity profiles. CDC20 activates APC/C during early mitosis, when the APC/C is phosphorylated by CDK1 and PLK1 while CDH1 is inactivated (Kramer, Scheuringer et al. 2000). APC/C\textsuperscript{CDH1} is stimulated by APC/C\textsuperscript{CDC20} mediated reduction of CDK1 activity while in turn APC/C\textsuperscript{CDH1} contributes to inactivation of APC/C\textsuperscript{CDC20} through ubiquitylating CDC20 (Pfleger and Kirschner 2000). APC/C\textsuperscript{CDC20} and APC/C\textsuperscript{CDH1} have overlapping but distinct substrate specificities, thus allowing for ordered
cell cycle progression. Importantly, the target of the mitotic checkpoint is APC/C$^{CDC20}$ (Hagting, Den Elzen et al. 2002, Chang and Barford 2014).

1.7. The mitotic checkpoint signal transduction pathway—signal initiation

The mitotic checkpoint can be regarded as a signal transduction pathway, which has three steps including signal initiation, signal amplification and formation of the effector (Musacchio and Hardwick 2002) (Figure 1-6).

![Figure 1-6. The mitotic checkpoint signaling pathway.](image)

The mitotic checkpoint signaling is initiated at unattached kinetochores, which recruit many important mitotic checkpoint proteins such as BUBR1, BUB3, CDC20, MAD2, MAD1, BUB1 and MPS1. The signal is amplified through MAD2 conformational change from O-MAD2 to C-MAD2. C-MAD2 promotes the assembly of the MCC, which then binds and inhibits APC/C thus preventing the transition from metaphase to anaphase.
Mitotic checkpoint signaling is initiated at unattached kinetochores, which are complex multi-subunit structures located adjacent to centromeric chromatin on each sister chromatid (Liu 2014). Laser ablation of the last unattached kinetochore accelerates mitotic exit (Rieder, Cole et al. 1995), which indicates that unattached kinetochores activate mitotic checkpoint.

Kinetochores have an inner layer, which interfaces with the centromeric chromatin, and an outer layer, which is involved in microtubule binding and mitotic checkpoint control (Cheeseman 2014, Fukagawa and Earnshaw 2014). Centromere Protein A (CENP-A) containing chromatin recruits the constitutive centromere-associated network (CCAN) of proteins independently of cell cycle and form the inner layer (Foltz, Jansen et al. 2006, Okada, Cheeseman et al. 2006, Black and Cleveland 2011). The outer layer of kinetochores is built on the inner layer mostly after nuclear envelope breakdown (Jia, Kim et al. 2013). Within the outer layer, the 10-subunit KMN network formed by the 2-subunit KNL1 complex, the 4-subunit Mis12 complex and the 4-subunit Ndc80 complex acts as a receptor for microtubules (Cheeseman, Chappie et al. 2006, Cheeseman, Hori et al. 2008, Liu 2014).

In the KMN network, the Mis12 complex links the KNL1 and Ndc80 complexes through direct protein-protein interactions (Gascoigne and Cheeseman 2013, Petrovic 2014). The Mis12 complex also binds to Centromere Protein C (CENP-C) to bridge the KMN network to centromeric DNA (Foley and Kapoor 2013, Pesenti, Weir et al. 2016). In addition to microtubule binding, the KMN network is also essential for recruiting mitotic checkpoint proteins to unattached kinetochores during mitosis. For example, KNL1 recruits BUB1-BUB3 and BUBR1-BUB3 complexes (London, Ceto et al. 2012,

During prometaphase when mitotic checkpoint is robust, all mitotic checkpoint proteins are recruited to unattached kinetochores. The levels of mitotic checkpoint proteins at kinetochores decline with the progression of the cell cycle from prometaphase to metaphase when all the kinetochores are attached by microtubules, although a significant fraction of BUB1 and BUBR1 remains at kinetochores even in anaphase cells (Jablonski, Chan et al. 1998, Taylor, Hussein et al. 2001, Howell, Moree et al. 2004, Johnson, Scott et al. 2004, Famulski and Chan 2007). Physical tethering of certain mitotic checkpoint proteins, such as MAD1 and MPS1, to the KMN network is sufficient for maintaining a permanent metaphase arrest in some systems (Jelluma, Dansen et al. 2010, Maldonado and Kapoor 2011, Ito, Saito et al. 2012, Ballister, Riegman et al. 2014, Kruse, Larsen et al. 2014, Kuijt, Omerzu et al. 2014). In conclusion, unattached kinetochores initiate mitotic checkpoint signal transduction by recruiting mitotic checkpoint proteins.

1.8. Mitotic checkpoint signal transduction pathway--signal amplification

The signal amplification of the mitotic checkpoint is mediated by the MAD2 O-C conversion. As discussed in section 1.5, C-MAD2 is selectively incorporated into the MCC by direct interaction with BUBR1 and CDC20 (Tipton, Tipton et al. 2011, Chao, Kulkarni et al. 2012). During mitosis, O-MAD2 is converted to C-MAD2 to facilitate formation of the MCC, the effector of the mitotic checkpoint (Luo, Tang et al. 2004, De Antoni, Pearson et al. 2005). Fluorescence recovery after photobleaching (FRAP) revealed that MAD2 cycles on and off kinetochores rapidly (Howell, Hoffman et al. 2000,
Howell, Moree et al. 2004, Shah, Botvinick et al. 2004). Later use of *in vitro* FRAP confirmed the existence of two pools of MAD2 (Vink, Simonetta et al. 2006). One pool of rapidly exchangeable MAD2 is recruited by the other pool of C-MAD2 stably bound to MAD1. The results shown by the two FRAP assays are the foundation of current model for MAD2 O-C conversion, which is described in Section 1.8.1.

1.8.1. The MAD1:C-MAD2 complex catalyzes MAD2 O-C conversion

MAD1, which contains 718 residues, is essential for mitotic checkpoint activation (Figure 1-7 A). Knocking down MAD1 in HeLa cells led to defective mitotic checkpoint and mislocalized MAD2 from kinetochores during prometaphase (Luo, Tang et al. 2002, Martin-Lluesma, Stucke et al. 2002).

MAD1 can be divided into three domains: The N-terminal domain (NTD) spanning 1-485 residues, the MAD2 interaction motif domain (MIM) containing 485-596 residues and the C-terminal domain (CTD) including 597-718 residues. MAD1 binds to C-MAD2 to form the MAD1:C-MAD2 complex through the MAD2 interaction motif (MIM) (Campbell, Chan et al. 2001, Luo, Tang et al. 2002). The MAD1:C-MAD2 complex becomes activated when localized at kinetochores, catalyzing MAD2 O-C conversion to promote the MCC assembly (Kulukian, Han et al. 2009, Simonetta, Manzoni et al. 2009). Both MAD1-NTD and CTD are essential for MAD1 kinetochore localization (Jin, Spencer et al. 1998, Sironi, Melixetian et al. 2001, Chung and Chen 2002, Kim, Sun et al. 2012) (Figure 1-7 B). Whether MAD1-NTD and CTD are required for the MAD2 O-C conversion in addition to targeting the MAD1:C-MAD2 complex to kinetochores remains unknown.
Figure 1-7. The MAD1 structure and the MAD2 template model.

(A). Schematic diagram of MAD1. (B). The MAD1:C-MAD2 heterotetramer at an unattached kinetochore recruits O-MAD2 and converts the O-MAD2 to C-MAD2. As C-MAD2 is accumulated, MCC is formed. The MCC inhibits the metaphase to anaphase transition. (C). Structure of the MAD1-MIM:C-MAD2 complex. MAD2$^\alpha$ and MAD2$^\beta$ (yellow) are two MAD2 monomers in the complex. The MAD1 chains (MAD1$^\alpha$ and MAD1$^\beta$) interact via the N-terminal coiled-coil (cyan). The dark blue segments contain the MAD2 binding motif (MIM). A helical conformation is resumed in the C-terminal region (green). (Sironi, Mapelli et al. 2002). (D). Structure of the MAD1-CTD (Kim, Sun et al. 2012).
O-MAD2 can spontaneously convert to C-MAD2 \textit{in vitro}. However, the process is very slow since the topological change of MAD2 entails large activation energies, which suggests that the MAD2 O-C conversion may be the rate-limiting step for accumulating MCC during mitotic checkpoint activation (Luo, Tang et al. 2004, Simonetta, Manzoni et al. 2009). It is logical to predict that the MAD1:C-MAD2 complex, as the catalyst for MAD2 O-C conversion, determines the overall rate of MCC formation. Once the kinetochores are bi-orientated through microtubule attachment, the MAD1:C-MAD2 complex is removed from kinetochores by a complex of cytoplasmic dynein with the Rod-Zwilch-Zw10 (RZZ) complex and Spindly. As a result, the mitotic checkpoint is silenced allowing the cells to progress to anaphase (Howell, McEwen et al. 2001, Wojcik, Basto et al. 2001, Griffis, Stuurman et al. 2007, Chan, Fava et al. 2009, Barisic, Sohm et al. 2010, Gassmann, Holland et al. 2010). Constitutively targeting the MAD1:C-MAD2 complex at kinetochores can prevent mitotic checkpoint silencing thus maintaining cells at metaphase even when all the chromosomes are well aligned at the metaphase plates (Maldonado and Kapoor 2011, Ballister, Riegman et al. 2014, Kruse, Larsen et al. 2014, Kuijt, Omerzu et al. 2014), suggesting that the MAD1:C-MAD2 complex at kinetochores promotes the assembly of the MCC. The detailed mechanism of the MAD2 O-C conversion catalyzed by the MAD1:C-MAD2 complex is unclear. This is the focus of the thesis, which I will discuss in details later.

1.8.2. The regulation of the MAD1:C-MAD2 complex

Multiple kinetochore proteins such as CENP-I, Hec1, Nuf2, RZZ, BUB1, Nek2A and Cep57 were reported to be required for kinetochore recruitment of the MAD1:C-MAD2 complex as depleting these proteins decreased MAD1 kinetochore localization

The KMN network plays critical roles in recruiting the MAD1:C-MAD2 complex to kinetochores (Espeut, Cheerambathur et al. 2012, London, Ceto et al. 2012, Yamagishi, Yang et al. 2012). KNL1, as one component of the KMN network, recruits RZZ complex and BUB1 (Krenn, Wehenkel et al. , Burke and Stukenberg 2008, London, Ceto et al. 2012, Yamagishi, Yang et al. 2012). Both RZZ complex and BUB1 are reported to be required for MAD1 kinetochore localizations (Chan, Jablonski et al. 2000, Johnson, Scott et al. 2004, Karess 2005, Kops, Kim et al. 2005, Mora-Santos, Hervas-Aguilar et al. 2016). However, the direct binding partner of MAD1 at kinetochores remains unclear. BUB1 binds to MAD1 in *Caenorhabditis elegans* and budding yeast but the two organisms utilize totally different regions of BUB1 and MAD1 for interactions (London and Biggins 2014, Moyle, Kim et al. 2014, Mora-Santos, Hervas-Aguilar et al. 2016). It is unknown whether human BUB1 directly interacts with MAD1. A recent study revealed direct interaction between MAD1 and Cep57, which itself associates with the KMN network, is essential for MAD1 kinetochore localization (Zhou, Wang et al. 2016). However, deleting Cep57 could not completely remove MAD1 from kinetochores, which indicates that there are other kinetochore receptors for MAD1. In summary, multiple
proteins have been indicated to be required for MAD1 kinetochore localization in human cells, but the kinetochore receptor of MAD1 remains to be confirmed.

While people are trying hard to understand the minimal structural elements at kinetochores to recruit MAD1, the kinases that regulate MAD1 localization and activity are also under investigation. Monopolar spindle 1 (MPS1) kinase is crucial for the activation of the mitotic checkpoint (Weiss and Winey 1996). MPS1 regulates the kinetochore localization of many critical mitotic checkpoint proteins including MAD1 and MAD2 (Martin-Lluesma, Stucke et al. 2002, Liu, Chan et al. 2003). MPS1 localizes to kinetochores during prometaphase. Once all the kinetochores are properly attached by microtubules, MPS1 is removed from kinetochores. The recruitment of MPS1 to kinetochores depends on Aurora B, which is a serine/threonine protein kinase (Vigneron, Prieto et al. 2004, Santaguida, Tighe et al. 2010). Recent studies revealed that the direct interaction between Ndc80/Hec1 calponin homology (CH) domain and MPS1 is important for MPS1 localization to kinetochores (Aravamudhan, Goldfarb et al. 2015, Hiruma, Sacristan et al. 2015, Ji, Gao et al. 2015). Tethering MPS1 to kinetochores can extend the metaphase and maintain MAD1 and MAD2 at kinetochores regardless of microtubule attachment (Jelluma, Dansen et al. 2010), suggesting that MPS1 activity and kinetochore localization are both essential for recruiting the MAD1:C-MAD2 complex to kinetochores. On the other hand, MPS1 kinase activity is required for the metaphase arrest caused by tethering MAD1 to kinetochores (Maldonado and Kapoor 2011), which suggests that MPS1 functions beyond recruiting the MAD1:C-MAD2 complex to kinetochores. Our lab previously found that MPS1 activates the mitotic checkpoint by
promoting the production of C-MAD2 (Tipton, Ji et al. 2013). However, how MPS1 facilitates the MAD2 O-C conversion remains unclear.

Aurora B kinase is essential for responding to the state of kinetochore-microtubule attachment and correcting syntelic and merotelic attachment errors (Biggins and Murray 2001, Tanaka, Rachidi et al. 2002). In addition to acting as a tension sensor at centromeres and kinetochores, Aurora B also directly regulates the mitotic checkpoint. As described above, the recruitment of MPS1 to kinetochores is dependent on Aurora B (Vigneron, Prieto et al. 2004, Santaguida, Tighe et al. 2010, Saurin, van der Waal et al. 2011). Inhibiting Aurora B drives cells out of mitosis even when MAD1 is tethered to kinetochores, suggesting that Aurora B may also regulate the MAD1:C-MAD2 complex catalytic activity at kinetochores (Maldonado and Kapoor 2011). Aurora B may fulfil this regulation through either activating MPS1 or the MAD1:C-MAD2 complex directly. How Aurora B regulates the MAD1:C-MAD2 complex activity remains to be elucidated (See more in section 4.4).

1.9. The mitotic checkpoint signal transduction pathway--effector formation

During the signal amplification step described above, C-MAD2 is generated at unattached kinetochores by the MAD1:C-MAD2 complex to promote the formation of the MCC, which binds to and inhibits APC/C. The MCC assembles from the interactions between the components BUBR1, CDC20, BUB3 and MAD2 (Hwang, Lau et al. 1998, Kim, Lin et al. 1998, Hardwick, Johnston et al. 2000, Fraschini, Beretta et al. 2001, Sudakin, Chan et al. 2001). BUBR1, CDC20, BUB3 and MAD2 in the MCC isolated from mitotic HeLa cells were reported to be at a ratio of 1:1:1:1 (Sudakin, Chan et al. 2001). This MCC is called "core" MCC as later more evidence indicated that a second
CDC20 is incorporated into the core MCC via BUBR1 (Diaz-Martinez, Tian et al. 2015, Izawa and Pines 2015). The KILR of the second CDC20 is not required for interacting with the core MCC (Izawa and Pines 2015). The results suggested that the two CDC20 subunits associated with the MCC through different interactions. These seemingly complicated results could be explained by more recent studies using electron microscopy (EM). These structural studies revealed that the APC/C\(^{MCC}\) complex contains two CDC20 subunits (Alfieri, Chang et al. 2016, Yamaguchi, VanderLinden et al. 2016). The KEN1, A2, D2 boxes of BUBR1 are required for the first subunit of CDC20 to incorporate into the core MCC, while the KEN2, A1, D1 boxes of BUBR1 are essential for interacting the second CDC20, which is already bound to APC/C (Figure 1-4 B). This model provides great insights into how MCC is assembled and how MCC interacts with APC/C. However, there are still many points that cannot be totally explained. I will discuss more in the end of this thesis (section 4.8).

1.10. The mitotic checkpoint signal transduction pathway--silencing

Once all the kinetochores are attached by microtubules and bi-oriented, the mitotic checkpoint needs to be silenced thus allowing the anaphase onset. Silencing the mitotic checkpoint is regulated by multiple mechanisms, including removal of mitotic checkpoint proteins from kinetochores and disassembly of the MCC and the APC/C:MCC complexes (Vanoosthuyse and Hardwick 2009, Hardwick and Shah 2010, Liu and Zhang 2016).

The removal of the MAD1:C-MAD2 complex from attached kinetochores halts the MAD2 O-C conversion, thus extinguishing the mitotic checkpoint signal. There are at least two independent mechanisms that lead to the dissociation of the MAD1:C-MAD2
complex from kinetochores. One is the stripping of checkpoint proteins along microtubules mediated by dynein, a minus-end directed motor, and the other is the reversal of kinetochore phosphorylation by protein phosphatase 1 (PP1).

Dynein redistributes proteins from kinetochores to the poles. Blocking dynein activity prevented the removal of the MAD1:C-MAD2 complex and BUBR1 from kinetochores and caused cells to arrest at metaphase (Howell, McEwen et al. 2001). The localization of dynein to kinetochores requires the RZZ complex (Rod, Zw10 and Zwilch) and Spindly (Gassmann, Essex et al. 2008, Gassmann, Holland et al. 2010, Barisic and Geley 2011, Famulski, Vos et al. 2011). Depletion of Spindly does not completely prevent the MAD1:C-MAD2 removal, which suggests existence of additional conserved removal pathway (Gassmann, Holland et al. 2010, Barisic and Geley 2011).

PP1 silences the mitotic checkpoint by dephosphorylating mitotic checkpoint proteins (Kadura, He et al. 2005, Akiyoshi, Nelson et al. 2009, Pinsky, Nelson et al. 2009, Vanoosthuyse and Hardwick 2009, Rosenberg, Cross et al. 2011, London, Ceto et al. 2012). PP1 localizes to kinetochores through the direct interaction with KNL1, which is a component of the KMN (KNL1/Mis12 complex/ Ndc80 complex) network (Rosenberg, Cross et al. 2011). PP1 opposes the phosphorylation of kinetochore proteins by Aurora B to stabilize microtubule attachment, which is the prerequisite for anaphase onset (Liu, Vleugel et al. 2010). In addition, PP1 removes Aurora B from centromeres by counteracting the phosphorylation of Histone H3 T3 by Haspin (Wang, Dai et al. 2010, Qian, Lesage et al. 2011), which is required for Aurora B localization to centromeres. Interestingly, Aurora B prevents PP1 from localizing to kinetochores through the phosphorylation of KNL1, which disrupts the interaction between KNL1 and PP1 (Liu,
The phosphorylation and dephosphorylation of proteins during mitosis dynamically regulates the activation and silencing of the mitotic checkpoint.

The other important step to silence the mitotic checkpoint is to disassociate MCC from APC/C and disassemble MCC. APC15, which is a component of the APC/C, is essential for silencing the mitotic checkpoint (Mansfeld, Collin et al. 2011). Depletion of APC15 led to the accumulation of the MCC more stably bound to APC/C (Mansfeld, Collin et al. 2011), suggesting that APC15 may be essential for dissociating MCC from APC/C. APC15 was later reported to be required for MCC-dependent CDC20 autoubiquitylation, which promotes silencing of the mitotic checkpoint (Reddy, Rape et al. 2007, Jia, Li et al. 2011, Varetti, Guida et al. 2011, Foster and Morgan 2012, Uzunova, Dye et al. 2012).

Another important mitotic checkpoint silencer is p31\textsuperscript{comet}. p31\textsuperscript{comet}, first identified as a MAD2-binding protein, has very similar 3D structure to MAD2 (Habu, Kim et al. 2002). Knocking down p31\textsuperscript{comet} delays the metaphase to anaphase transition (Xia, Luo et al. 2004, Hagan, Manak et al. 2011, Jia, Li et al. 2011, Westhorpe, Tighe et al. 2011, Wang, Sturt-Gillespie et al. 2014), suggesting that p31\textsuperscript{comet} is required for silencing the mitotic checkpoint. p31\textsuperscript{comet} disassembling the MCC \textit{in vitro} depends on ATP (Teichner, Eytan et al. 2011). How exactly ATP hydrolysis couples with p31\textsuperscript{comet} to disassemble MCC remains unclear until our lab found that thyroid hormone receptor interacting protein 13 (TRIP13) AAA-ATPase functions together with p31\textsuperscript{comet} to disassemble the MCC (Eytan, Wang et al. 2014, Wang, Sturt-Gillespie et al. 2014).

The MCC disassembly was proposed to be accompanied by C-MAD2 converting to O-MAD2 (Wang, Sturt-Gillespie et al. 2014). Indeed, TRIP13 was later found to
catalyze MAD2 C-O conversion (Ye, Rosenberg et al. 2015). Although more and more evidence has demonstrated the roles of TRIP13 as a mitotic checkpoint silencer, TRIP13 was also reported to be required for mitotic checkpoint activation (Nelson, Hwang et al. 2015, Ma and Poon 2016). More investigation on the function of TRIP13 during mitosis is needed.

1.11. Questions to be addressed

As introduced above, our lab and many scientists have been trying to understand the mitotic checkpoint signal transduction pathway including signal initiation, signal amplification, effector formation and signal silencing. In this thesis, I will focus on understanding how the mitotic checkpoint signal is amplified by an unusual catalyst--the MAD1:C-MAD2 complex. I will also explore the possible regulators of MAD1:C-MAD2 complex during mitosis.
Chapter 2

Hypothesis

The mitotic checkpoint is a special signaling pathway that is essential for faithful chromosome segregation. MAD2 O-C conversion a key signal amplification mechanism for the checkpoint. The conversion is catalyzed by MAD1:C-MAD2 complexes localized at unattached kinetochores, but the underlying mechanisms remain obscure. Here we found that human MAD1 N-terminal and C-terminal domains (NTD and CTD), in addition to its well-known C-MAD2 interacting motif (MIM), are required for maintaining an efficient mitotic checkpoint. We therefore hypothesize that MAD1-NTD and CTD coordinate with MAD1-MIM to catalyze the MAD2 O-C conversion.

As we found that MPS1 kinase phosphorylates both MAD1-NTD and MAD1-CTD and a MAD1 phospho-mutant at MPS1 phosphorylation sites cannot maintain the mitotic checkpoint, we hypothesize that the catalytic activity of the MAD1:C-MAD2 complex is directly regulated by MPS1 kinase. In addition to MPS1, we also hypothesize that other mitotic kinases such as Aurora B, BUB1, MELK or certain tyrosine kinases regulate the mitotic checkpoint.
Finally, the "second CDC20 binding domain" of BUBR1 directly interacts with C-MAD2. We hypothesize that the BUBR1 second CDC20 binding domain is essential for assembling a functional mitotic checkpoint complex.
Chapter 3

Materials and Methods

3.1. Cell culture, synchronization and drug treatment

HeLaM, a subline of HeLa (Tiwari, Kusari et al. 1987), was maintained in DMEM with 10% fetal bovine serum at 37 °C in 5% CO₂. To block cells in prometaphase, HeLaM cells were treated with 2.5 mM thymidine (Sigma-Aldrich) for 24 hr and then directly released into medium containing 0.2 mM (60 ng/ml) nocodazole (Sigma-Aldrich) or 10 mM Taxol (Biomol International) for 12 hr. Some variations of cell synchronization protocols are described in more details in figure legends. Aurora B inhibitors ZM447439 (Cayman Chemical) and Hesperadin (Adooq bioscience) were used at 2.3 µM and 100 nM, respectively. The MPS1 kinase inhibitor reversine (Calbiochem), AZ3146 (Selleckchem) and proteasome inhibitor MG132 (Cayman Chemical) were used at 500 nM, 2 µM and 20 µM final concentrations respectively.

Sf9 cells were grown at 27 °C in SFX medium (Hyclone) supplemented with 10% fetal bovine serum.
3.2. DNA Constructs and Transfection

Full length p31\textsuperscript{comet}, BUBRI and MAD2 cDNAs were PCR amplified from a prostate cDNA library (Invitrogen) or freshly prepared from reverse transcribed cDNAs that were provided by Dr. Douglas Leaman (Wright State University). Full length or truncated human MAD1 cDNA were amplified and cloned into mCherry-Mis12 vector from Dr. Tarun Kapoor’s lab to express mCherry-Mis12-MAD1. pCS2-MAD1-GFP, BUBR1 phosphomutants BUBR1\textsuperscript{5A} (S543A, S574A, S670A, S720A, and S1043A) and BUBR1\textsuperscript{QA} (S435A, S543A, S670A, and S1043A) were from Ted Salmon (University of North Carolina), Sabin Elowe (Université Laval), and Tim Yen (Fox Chase Cancer Center) respectively. BUBR1\textsuperscript{11A} was made by adding T54A, S435A, S676A, T792A, T1008A and T1042A on BUBR1\textsuperscript{5A} based on known in vivo phosphorylation sites in PubMed Central. Full length human MELK cDNA was amplified using primers 5'-tccagatctATGAAAGATTATGATGAACTTCTCA-3' and 5'-caGGATCCAAGGATCCATCAATTATAC-3', digested with BglII and BamHI, and cloned into the BamHI site of a home-made eGFP vector (pWS-GFP) to express GFP-MELK. The MELK shRNA was purchased from Sigma-Aldrich with advice from Dr. Ichiro Nakano at the Ohio State University.

Mutagenesis was conducted using multi-site directed lightening mutagenesis kit (Agilent). Using the Gateway recombination reactions kit (Invitrogen), full-length cDNAs and fragments were cloned into pENTR-D/TOPO vector followed by gateway cloning into different Gateway destination vectors for expression in E. coli or mammalian cells. All DNA constructs were confirmed by DNA sequencing.
DNA transfection was carried out using TransIT-LT1 reagent (Mirus) following the manufacturer’s instructions or using polyethylenimine (PEI) following a modified protocol. Briefly, linear PEI (MW 25,000, from Polysciences) was dissolved in 0.2 N HCl at 5 mg/ml (final pH around 1.0) for long term storage at -80 °C. For transfection, the thawed PEI was neutralized to pH7.0 with NaOH and used within a month at a DNA: PEI mass ratio at 1:2.5. Cellfectin (Invitrogen) was used to transfact bacmids into SF9 cells. Oligofectamine (Invitrogen) was used for siRNA transfection according to the manufacturer's instructions.

The MPS1 shRNA and RNAi resistant pLAP-MPS1\textsuperscript{WT} (wildtype) or MPS1\textsuperscript{KD} (kinase-dead) constructs were gifts from Geert Kops (University of Utrecht) and transfected together with pBabe-puromycin at a ratio of 10:5:1. The shRNA-transfected cells were enriched 24 hr post-transfection by selection in puromycin (1 g/ml) for 48 hr. MELK shRNA was also transfected with pBabe-puromycin as described above.

3.3. Recombinant protein expression

Different fragments of MAD1 and BUBR1 were cloned into pENTR-D-TOPO vector using TOPO cloning kit (Invitrogen) and then the Gateway reaction kit (Invitrogen) was used to clone MAD1 fragments into pDEST15 or pDEST17 vectors. GST-tagged, His-tagged MAD1 and His-tev-tagged MAD2\textsuperscript{L13A} or His-tev-tagged MAD2\textsuperscript{AC10} were expressed in \textit{E. coli} BL21(DE3) -CodonPlus RIPL (Stratagene) at 37 °C or 25 °C or 16 °C. His-MPS1 was expressed in Sf9 cells using baculovirus. All expressed proteins were purified using GSH-agarose or Probond nickel beads (Invitrogen). The His-tagged tobacco etch virus protease variant, TEV(S219P), was prepared using a construct purchased from Addgene (Kapust, Tozser et al. 2001). The protease was used to cleave
His-tag to make untagged MAD2^{L13A} and MAD2^{AC10}. Concentrations of recombinant proteins were determined by comparing the target band with BSA standards on Coomassie blue stained gels.

3.4. Cell lysates, immunoblotting, immunoprecipitation and GST pulldown

Cell pellets were washed once with 1× PBS and then were lysed in cell lysis buffer (1×PBS, 10% glycerol, 0.5% NP-40) supplemented with protease inhibitors (Protease Inhibitor Mixture set III, EDTA-free; Calbiochem) and phosphatase inhibitors (10 mM NaF, 1 mM Na₃VO₄, 60 mM β-glycerophosphate). The supernatant was collected and the protein concentration of the lysates was measured using the BCA Protein Assay kit (Pierce). Immunoblotting was used to probe specific proteins in the cell lysates, immunoprecipitates and in vitro binding assays. Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) usually at 180 volts. Gels were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore) usually at 400 milliamperes. Membranes were blocked with blocking buffer 1 hr at room temperature followed by incubating with primary antibody at 4 °C overnight. Secondary antibodies conjugated with alkaline phosphatase (AP) were incubated with membranes for 1 hr at room temperature. The signals were detected by chemiluminescence. In some experiments the band intensities were quantified using Image J. For immunoprecipitation, 200 -300 ug of lysates were incubated with desired antibodies for 1 hr at 4 °C and then mixed with protein-A agarose beads (RepliGen) for another 1 hr. The beads were washed with cell lysis buffer four times before subjected to western blot analysis. For GST pulldown assays, cell lysates or protein mixtures were incubated with glutathione-agarose
(Pierce) at 4 °C for 1 hr before western blot analysis. A list of primary antibodies used in this study is summarized in Table B.1.

3.5. In vitro kinase assays

MPS1 Kinase was purified from Sf9 cell lysates. Myelin basic protein was purchased from Sigma. For kinase reactions, 4 µl of 5× kinase buffer (125 mM Tris-HCl, pH 7.5, 300 mM β-glycerophosphate, 50 mM MgCl₂) was mixed with recombinant kinase, substrates, 5 µCi ³²P –ATP or cold ATP. H₂O was added to make the final volume of 20 µl. The reactions were incubated at 30°C for 30 min and then terminated by adding 20 µl 2× SDS sample buffer. Samples were subjected to SDS-PAGE followed by Coomassie staining. After destaining, the SDS-PAGE gel was dried. Phosphorylation of the substrates was visualized by autoradiography.

GST-MELK (1-340) and His-Aurora B were purified from E.coli BL21(DE3)-CodonPlus RIPL (Stratagene). BUB1 was immunoprecipitated from mitotic cell lysates. The beads were washed 4 times with 1× kinase buffer. The kinase assay was performed as described above.

3.6. In vitro binding assays

Four microliters of 5×binding buffer (100 mM Tris-HCl, pH 8.0, 750 mM NaCl, 2.5% NP-40, 50 mM MgCl₂, 50% glycerol) was mixed with recombinant GST-tagged MAD1 fragments and His-MPS1 or MAD2 mutants or His-MAD1-CTD. H₂O was added to make the final volume of 20 µl. The reactions were incubated at 37 °C for 1 hr and then were rotated at 4 °C with 10 µl GSH agarose beads for 40 min. The beads were washed 4 times with wash buffer (1× PBS, pH 7.4, 150mM NaCl, 0.5% NP-40, 10% glycerol). 10 µl 2× SDS sample buffer was added to the beads. Samples were subjected to
SDS-PAGE followed by transferring to PVDF transfer membranes (Millipore). Proper primary and secondary antibodies were used to detect the proteins on the membrane.

3.7. Immunofluorescence and Live Cell Imaging

Cells grown on poly-lysine treated No. 1.5 coverslips were fixed in freshly prepared 3.5% paraformaldehyde containing 0.5% Triton X-100 for 10 min and then blocked with KB (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mg/ml BSA) for >5 min prior to immunofluorescence. The coverslips were incubated with each primary and secondary antibody sequentially, all at 37 °C for 30 minutes in a wet chamber. The secondary antibodies were from the Alexa Fluor series from Molecular Probes used at a 1:1000 dilution. The stained coverslips were mounted on slides using Vectashield mounting medium containing DAPI (Vector Laboratories) and imaged on a Leica TCS SP8 confocal microscope with a 63 × objective (numerical aperture = 1.40). Usually, z-stacks of 1.0 μm were collected and maximum projections or single focal planes are presented. Live cell imaging of HeLaM transfected with mCherry-Mis12-MAD1 was performed similarly as before on an automated Olympus IX-81 microscope to collect phase contrast and RFP images at 15 min intervals using a 60×objective while cells were maintained at 37 °C in a heating chamber. Single-plane images were acquired for 24 hr at multiple positions using a CoolSNAP HQ2 camera with 2×2 binning.

3.8. APC/C activity assay using concentrated mitotic extracts

The extracts were prepared following Braunstein et al. (Braunstein, Miniowitz et al. 2007) with minor modifications. Nocodazole-arrested HeLaM cells were harvested, washed with ice-cold PBS, and resuspended in 75% of pellet volume of hypotonic buffer (20 mM Hepes-NaOH, pH 7.6, 5 mM KCl, 1 mM DTT) containing protease inhibitors.
After repeated freeze-thawing, the cell lysates were centrifuged at 16,000 g for 1 h. The supernatants were collected, supplemented with glycerol to 10% (v/v), aliquoted, and stored in liquid nitrogen. The protein concentration of the extracts was 15–20 mg/ml. To assay for APC/C activity, 20 ul reaction mixtures contained 10ul of concentrated mitotic extract, 2 ul of 10 degradation mixture (100 mM Tris-HCl, pH 7.6, 50 mM MgCl₂, 10 mM DTT, 10 mg/ml ubiquitin, 100 mM phosphocreatine, 5 mM ATP, 0.1 mg/ml UbcH10), and 1 ul of 20 creatine phosphokinase (1mg/ml). Recombinant proteins were added in some experiments. Reactions were incubated at 30 °C, and 3 ul samples were collected at various times, and then rapidly quenched with SDS-PAGE sample buffer. Degradation of cyclin B and securin was followed by immunoblotting.

3.9. Statistics Analysis

Student’s t-test was used to compare the mean value of mitotic durations after different treatment.
Chapter 4

Results

4.1. MAD1-NTD, MIM and CTD coordinate to catalyze MAD2 O-C conversion

The mitotic checkpoint can be regarded as a special signal transduction pathway, which consists of three steps including signal initiation, signal amplification and the effector formation. The signal amplification is realized through the MAD2 O-C conversion (Figure 1-6). The MAD2 O-C conversion is catalyzed by a special enzyme known as the MAD1:C-MAD2 complex. In the current model, the MAD1:C-MAD2 complex localized at unattached kinetochores recruits cytoplasmic O-MAD2 through its heterodimerization with C-MAD2 in the MAD1:C-MAD2 complex and stimulates the MAD2 O-C conversion (Figure 1-7). The mechanism of the catalytic reaction remains unclear.

4.1.1. Both MAD1-NTD and CTD are required for maintaining a functional mitotic checkpoint

MAD1 can be divided into three different domains including NTD (1-485), MIM (485-596) and CTD (597-718) (Figure 1-7). Previous studies have solved the crystal structures of MIM and CTD domains (Sironi, Mapelli et al. 2002, Kim, Sun et al. 2012).
For functional analyses, most attention has been focused on the MIM domain. MIM binds to C-MAD2 to form the MAD1:C-MAD2 complex using MAD2 interaction motif (530-550) (Sironi, Mapelli et al. 2002). However, MIM itself is not enough to maintain mitotic arrest. Multiple studies indicated that MAD1 functions more than recruiting MAD2 to kinetochores. Such function of MAD1 in addition to recruiting MAD2 was surmised to depend on MAD1-CTD (Ballister, Riegman et al. 2014, Heinrich, Sewart et al. 2014, Kruse, Larsen et al. 2014).

To understand how the MAD1:C-MAD2 complex catalyzes MAD2 O-C conversion, we decided to investigate the function of MAD1-NTD and CTD on catalyzing MAD2 O-C conversion at kinetochores. The original motivation was based on a simple enzymology principle: any functional enzyme must have at least one active site, which not only binds to the substrate but also carries out the catalysis per se. In the case of the MAD1:C-MAD2 catalyst, if the substrate binding function indeed fell on the C-MAD2 moiety in the complex as stated in the current model, the catalytic sites have remained elusive but it is reasonable to hypothesize that MAD1 might contribute to the catalysis.

Kinetochore targeting and catalytic activity are two separable aspects of regulation of the MAD1:C-MAD2 catalyst (Maldonado and Kapoor 2011, Kuijt, Omerzu et al. 2014). To focus on the functions of the catalytic activity of the MAD1:C-MAD2 complex at kinetochores, we employed a separation-of-function construct, mCherry-Mis12-MAD1, developed by Maldonado and Kapoor (Maldonado and Kapoor 2011). MAD1 fused with Mis12 can constitutively localize at kinetochores. As a positive control, mCherry-Mis12-MAD1WT localized at kinetochores and maintained the cells at
metaphase for more than 12 hrs (Figure 4-1B). As a negative control, cells transfected with mCherry-Mis12-MAD1^{AA} mutant (K541A, L543A in MIM), which cannot form MAD1:C-MAD2 complex through the MIM domain, exited mitosis within about 50 min (Figure 4-1B). Using this system to investigate whether MAD1-NTD or CTD is required for the mitotic checkpoint, we made the MAD1 truncations mCherry-Mis12-MAD1^{ΔNTD} (with N-terminal 485 amino acids deleted) and mCherry-Mis12-MAD1^{ΔCTD} (with C-terminal 597-718 deleted). Cells transfected with either truncation construct cannot maintain mitotic arrest as well as mCherry-Mis12-MAD1^{WT} (mCherry-Mis12-MAD1^{WT}, mean=749.3min; mCherry-Mis12-MAD1^{ΔNTD}, mean=101.3min; mCherry-Mis12-MAD1^{ΔCTD}, mean=98.5min), which suggests that both MAD1-NTD and CTD are essential for maintaining the mitotic checkpoint. (Figure 4-1B).
Figure 4-1. Both MAD1-NTD and CTD are required for maintaining a functional mitotic checkpoint.

(A) The schematic figure showing different MAD1 truncations and mutants used in this study. NTD indicates N-terminal domain, the MIM indicates the MAD2 interaction motif domain and CTD indicates C-terminal domain. (B) Mitotic durations (from nuclear envelop breakdown to anaphase onset) of HeLa cells were recorded for 13 hrs after transfection with mCherry-Mis12-MAD1 constructs as shown in (A). Student's t-test was used, **** indicates p<0.0001. NS indicates no significant difference. (C) Cells in (B) were harvested and cell lysates were subjected to Mis12 IP followed by western blot. Mis12, MAD1 and MAD2 were probed.
Previously, to examine the roles of MAD1 phosphorylation during mitosis, we had made many phospho-mimic point mutations on the mCherry-Mis12-MAD1 construct based on the *in vivo* phosphorylation sites deposited in a database (phosphosite.org). We then used similar approach as in Fig 4-1B to screen for potential phosphorylation sites that are important for maintaining the mitotic checkpoint (Appendix B Table B.2). We identified one point-mutation Y634E that showed mitotic checkpoint defect. Since Y634 is located within the CTD, this result further supports the importance of MAD1-CTD in maintaining the mitotic checkpoint (I will discuss more about Y634 site later in section 4.7).

To exclude the concern that either the point mutants or the truncations may change the structure of the protein thus causing the defect in maintaining mitotic checkpoint, we performed immunoprecipitation with anti-Mis12 antibody using the lysates from HeLa cells transfected with mCherry-Mis12-MAD1 constructs. All the fusion proteins except the AA mutant associated with MAD2 through the MIM domain (Figure 4-1C). We also co-transfected mCherry-Mis12-MAD1 constructs with GFP-MAD2<sup>L13A</sup> (a well characterized MAD2 mutant locked in C-conformer (Mapelli and Musacchio 2007, Luo and Yu 2008)) into HeLa cells followed by live cell imaging. In the cells with no mCherry-Mis12-MAD1, MAD2<sup>L13A</sup> localizes at kinetochores during prometaphase but not metaphase. However, in the cells with mCherry-Mis12-MAD1, all the truncations and mutants can still recruit C-MAD2 to kinetochores even at metaphase (Figure 4-2). These data suggest that the MAD1 mutants still bind to MAD2 and localize to kinetochores, arguing against the possibility of severe structure disruption in the MAD1 mutants.
Figure 4-2. MAD1\textsuperscript{\triangle NTD} and MAD1\textsuperscript{\triangle CTD} recruit C-MAD2 to kinetochores. mCherry-Mis12-MAD1 constructs were transfected with GFP-MAD2\textsuperscript{L13A}. Twenty-four hours after transfection, live cells were imaged to check the localization of MAD1 and MAD2 at prometaphase and metaphase.
4.1.2. MAD1-NTD and CTD can interact with both O-MAD2 and C-MAD2

To explore how MAD1-NTD and CTD could facilitate the checkpoint responses, we hypothesized that these domains enhance the MAD2 O-C conversion. As described above, the MAD1:C-MAD2 complex acts as an enzyme to catalyze the MAD2 O-C conversion. To be more specific, the MAD1:C-MAD2 works as an isomerase, which binds to a substrate and catalyzes its conformational change to generate the product. Inspired by this notion, we reasoned that MAD1-NTD and CTD may interact with either the O-MAD2 (substrate) or C-MAD2 (product) to contribute to the MAD2 O-C conversion. Therefore, we tested whether MAD1-NTD and CTD can interact with MAD2 using *in vitro* binding assays. To do this, we purified recombinant proteins such as GST-MAD1-NTD, -MIM, -CTD, untagged MAD2^{L13A} (C-MAD2 mutant) and MAD2^{ΔC10} (O-MAD2 mutant) from *E.coli*. We found that MAD1-MIM only interacts with C-MAD2 but not O-MAD2 as reported by others (Luo, Tang et al. 2002, Sironi, Mapelli et al. 2002). Interestingly, MAD1-NTD and -CTD can interact with both O-MAD2 and C-MAD2 (Figure 4-3B). Extensive control experiments were performed to confirm the results (Figure A-1). The above results suggest that MAD1-NTD and CTD could facilitate the MAD2 O-C conversion through the interaction with the substrate O-MAD2 and the product C-MAD2.
4.1.3. The integrity of MAD1-NTD and CTD is required for interacting with MAD2.

MAD1-NTD is a fragment containing 485 amino acids. We attempted to further delimit the regions of MAD1-NTD that bind to MAD2. MAD1-NTD was believed for a long time to be a rigid coiled coil. However, the secondary structure prediction of NTD suggests that MAD1-NTD contains some segments that are unlikely to form coiled coils or even helical structures (ovals in Figure 4-4 A). To define the MAD2 binding region on MAD1-NTD, we made a series of truncations guided by the secondary structure prediction (Figure 4-4 B). GST tagged truncation proteins were purified and tested by in vitro binding assays. We found that MAD1(1-327) can still bind to MAD2, but at a much reduced level compared with full length NTD (1-485), while MAD1(327-423) and MAD1(327-488) are unable to bind to either O-MAD2 or C-MAD2 (Figure 4-4C). The results suggest that the integrity of MAD1-NTD is required for its binding to MAD2.

Figure 4-3. MAD1-NTD and CTD interact with both O-MAD2 and C-MAD2. (A) Coomassie stain after SDS-PAGE shows the purity of the recombinant proteins isolated from E. coli. (B) GST-NTD, -MIM, -CTD or GST alone were incubated with either MAD2L13A or MAD2ΔC10 at 37 °C for 1hr. GST pull-down assays were followed by western blot. GST and MAD2 were probed.
Figure 4-4. MAD2 binding domain mapping on MAD1-NTD.
(A) Secondary structure prediction of MAD1 indicates that multiple segments of MAD1 may form structures other than coiled coil (ovals interspersed on the shaded coiled coils). (B) Schematic figure showing the N-terminal linear truncations. (C) GST tagged N-terminal truncations were incubated with MAD2^{ΔC10} or MAD2^{L13A} followed by GST pull-down assay. The stars indicate the expected major bands.
Similarly, we also tried to map out the MAD2 binding domains on MAD1-CTD. Based on the crystal structure of MAD1-CTD, it contains a coiled coil region (597-638) and a C-terminal globular domain (639-718) (Kim, Sun et al. 2012). We prepared the recombinant proteins of GST-MAD1\(^{597-638}\) and GST-MAD1\(^{639-718}\) to test their interactions with either O-MAD2 or C-MAD2. The result showed that MAD1-CTD globular domain binds to more MAD2 compared with the coiled coil domain (Figure A-2). Further mapping is still required to find the MAD2 binding region or sites on MAD1-NTD and CTD.

4.1.4. The interactions between MAD1-NTD or CTD with MAD2 do not depend on MAD2 dimerization domain or N terminal 10 residues

There are two known protein-binding interfaces on MAD2: one is the "safety belt" and nearby segments that distinguish MAD2 O and C-conformations; and the other is the dimerization domain (Mapelli and Musacchio 2007, Luo and Yu 2008). Both O-MAD2 and C-MAD2 conformation locked mutants bind to MAD1-NTD and CTD, indicating the interactions are independent of MAD2 conformation determinants.

To test whether the interactions between MAD1-NTD or CTD with MAD2 depends on MAD2 dimerization domain, we employed the MAD2\(^{LARQ}\) mutant (L13A, R133E, Q134A), which is a C-MAD2 mutant that cannot form dimers (Mapelli, Massimiliano et al. 2007, Yang, Li et al. 2008). We performed \textit{in vitro} binding assays as described above to compare the interactions between MAD1 fragments and MAD2\(^{L13A}\) or MAD2\(^{LARQ}\). No difference was found between interactions of MAD1 fragments with MAD2\(^{L13A}\) and MAD2\(^{LARQ}\), suggesting that the MAD2 dimerization domain is not required for the interaction with MAD1-NTD or CTD (Figure 4-5A).
To further investigate how MAD2 interacts with MAD1-NTD or CTD, we also tested the interactions between MAD1-NTD or CTD with MAD2$^{\Delta N_{10}}$. Recent study indicated that MAD2$^{\Delta N_{10}}$ may mimic the intermediate conformer of MAD2 (I-MAD2, which is a transient MAD2 conformer during the conformational change from O-MAD2 to C-MAD2) (Hara, Ozkan et al. 2015). We found that MAD1-MIM can bind to MAD2$^{\Delta N_{10}}$ as reported (Sironi, Melixetian et al. 2001). However, interestingly, both MAD1-NTD and CTD can still interact with MAD2$^{\Delta N_{10}}$ (Figure 4-5B). The combined results suggested that MAD2 could bind to MAD1-NTD or CTD using a new interface that is distinct from previously characterized ones. This is in contrast to the previous knowledge that MAD1-MIM selectively binds to C-MAD2 through the "safety belt" (Sironi, Mapelli et al. 2002).
Figure 4-5. The interactions between MAD1-NTD or CTD with MAD2 are not dependent on MAD2 dimerization domain or N-terminal 10 residues. (A). GST tagged MAD1-NTD, -MIM, -CTD or GST alone were incubated with MAD2\textsuperscript{L13A} or MAD2\textsuperscript{LARQ} at 37 °C for 1 hr followed by GST pull-down assays. The samples were subjected to western blot and GST and MAD2 were probed. (B). GST tagged MAD1-NTD, -MIM, -CTD or GST alone were incubated with MAD2\textsuperscript{ΔN10} at 37 °C for 1 hr followed by GST pull-down assays. The samples were subjected to western blot and GST and MAD2 were probed.
4.1.5. MAD1-CTD is required for MAD1 dimerization

Both MAD1-MIM and CTD dimerize by forming coiled coils (Sironi, Mapelli et al. 2002, Kim, Sun et al. 2012). The current model on MAD2 O-C conversion suggests that MAD1 and C-MAD2 form a 2:2 heterotetramer (Figure 1-7). However, whether MAD1 dimerization is regulated or functionally important remains unknown. In Figure 4-1C, mCherry-Mis12-MAD1\(^{\Delta CTD}\) cannot precipitate endogenous MAD1, suggesting that MAD1-CTD is required for the dimerization of full-length MAD1 in cells. Similar results can be seen in Figure 4-14C. Whether MAD1-NTD dimerizes remains unclear, since mCherry-Mis12-MAD1\(^{\Delta NTD}\) and endogenous MAD1 migrate at similar position in the SDS-PAGE. We are working on tagging MAD1\(^{\Delta NTD}\) with a different tag that can be distinguished from endogenous MAD1 by size and then whether MAD1-NTD is required for MAD1 dimerization will be ascertained.

4.1.6. MAD1-NTD interacts with CTD

MAD1-NTD and CTD are both required for efficient mitotic checkpoint and they both bind to MAD2 \emph{in vitro} regardless of O-MAD2 or C-MAD2 conformations (Figure 4-1B, Figure 4-3). The results agree with the notion that MIM, which forms the stable complex with C-MAD2, is not enough to maintain mitotic checkpoint (Ballister, Riegman et al. 2014, Heinrich, Sewart et al. 2014, Kruse, Larsen et al. 2014). We reason that NTD, MIM and CTD may coordinate to catalyze MAD2 O-C conversion. This would be hard to imagine if the commonly held idea that MAD1 forms a long rigid coiled coil were true. Indeed, the secondary structure prediction indicates MAD1 contains some regions that are very unlikely to form coiled coils. To investigate whether and how different MAD1 domains coordinate with each other, we tested whether there are any
direct interactions between the three domains. To do that, we incubated GST tagged MAD1-NTD, MIM or CTD with His tagged CTD and performed the \textit{in vitro} binding assay. The result shows that CTD can interact with CTD as shown in the crystal structure (Kim, Sun et al. 2012) and MIM does not directly interact with CTD, but interestingly, MAD1-NTD also interacts with CTD (Figure 4-6).

Figure 4-6. MAD1-NTD interacts with CTD.
GST tagged MAD1-NTD, -MIM, -CTD, GST or beads alone were incubated with His tagged MAD1-CTD at 37 °C for 1 hr followed by GST pull-down assays. The samples were subjected to western blot. GST and His were probed. The stars indicate the expected major bands.

4.1.7. The integrity of MAD1-NTD compromises the interaction with MAD1-CTD

To investigate the binding region of MAD1-CTD on NTD, we tested the interactions between MAD1-CTD and MAD1 NTD truncations as used in Figure 4-4B. We found that MAD1\textsuperscript{(1-327)} maintains a very weak interaction with CTD compared with
MAD1(1-488) while MAD1(327-423) and MAD1(371-488) do not bind to CTD (Figure 4-7). It seemed that the integrity of MAD1-NTD is also required for the interaction with MAD1-CTD, similarly to the interactions between MAD1-NTD and MAD2 (Figure 4-4).

**Figure 4-7. MAD1-CTD binding domain mapping on MAD1-NTD.**
GST tagged NTD truncations were incubated with MAD1-CTD followed by GST pull-down assay. The stars indicate the expected major bands.

4.1.8. The integrity of MAD1-NTD is important for efficient mitotic checkpoint

To investigate whether the interactions between MAD1-NTD and CTD or MAD2 are important for the mitotic checkpoint, we made the construct mCherry-Mis12-MAD1ΔN327 with N-terminal 327 residues deleted. As the MAD1-NTD integrity seems critical for its association with CTD or MAD2 (Figures 4-4 and 4-7), we would expect that mCherry-Mis12-MAD1ΔN327 cannot maintain efficient mitotic checkpoint. Indeed, mCherry-Mis12-MAD1ΔN327 transfected cells cannot arrest at metaphase compared with MAD1WT, suggesting a less efficient mitotic checkpoint. We further checked the mitotic
duration of cells transfected with MAD1^{ΔN100} and found that MAD1^{ΔN100} maintains the mitotic arrest longer than MAD1^{ΔN327} (Figure 4-8), but still shorter than MAD1^{WT}. The results suggest that the integrity of MAD1-NTD is required for the mitotic checkpoint.

Figure 4-8. The integrity of MAD1-NTD is essential for efficient mitotic checkpoint. mCherry-Mis12-MAD1 WT, ΔN100, and ΔN327 were transfected to HeLa cells. The mitotic duration of the transfected cells was recorded using live cell imaging. ** indicates p<0.01, ***indicates p<0.001 (Student’s t-test).

4.2. MPS1 kinase promotes the production of C-MAD2

MPS1 kinase is essential for activating the mitotic checkpoint. However, the functioning mechanisms of MPS1 remain incompletely understood. Previous studies have indicated that MPS1 functions more than targeting the MAD1:C-MAD2 complex to unattached kinetochores (Maldonado and Kapoor 2011).
4.2.1. MPS1 kinase activity is required for the assembly of the MCC

The MCC is the effector of the mitotic checkpoint (section 1.9) (Liu and Zhang 2016). To examine whether MPS1 kinase activity is required for the MCC assembly, we arrested HeLa cells in mitosis with nocodazole or taxol and cells were then further treated with DMSO (as control) or reversine (a well characterized MPS1 inhibitor(Santaguida, Tighe et al. 2010)) with MG132 (a proteasome inhibitor) for 2 hrs before harvesting (Figure 4-9 A). The cell lysates were subjected to BUBR1 IP or MAD2 IP followed by western blot analysis. The MCC components such as BUBR1, CDC20, BUB3 and MAD2 were probed (Figure 4-9B, C, D). The result showed BUBR1 associated with less MAD2, while MAD2 associated with less BUBR1, CDC20 and BUB3 when MPS1 is inhibited. To confirm the result and especially, to address the potential specificity concerns over kinase inhibitors, we repeated the experiment using another MPS1 inhibitor called AZ3146 and got the same result (Figure 4-9E). In addition, the level of MAD2 coimmunoprecipitated with BUBR1 was also reduced when MPS1 was knocked down with shRNA. Furthermore, only wild-type but not kinase-dead MPS1 could restore the level of MAD2 in BUBR1 IP when endogenous MPS1 was knocked down (Figure 4-9F). The combined results demonstrate that MPS1 kinase activity is required for the assembly of the MCC.
Figure 4-9. MPS1 kinase activity is required for MAD2 incorporation into the MCC.
(A) Outline of cell synchronization protocol for experiments shown in (B-E). Following single thymidine arrest (STA) for 24 hr (numbers above arrows indicate time in hours), cells were released into either nocodazole (Noc) or Taxol for 12 hr and then treated with DMSO or reversine (Rev) or AZ3146 plus MG132 (to prevent mitotic exit). After 2 hr, cells were processed for immunoprecipitation (IP). (B-D) Lysates from nocodazole or Taxol arrested mitotic HeLa cells (with or without reversine, "+" or "-"+) were either analyzed directly (B) or first subjected to BUBR1 (C) or MAD2 (D) IP and then probed for MCC subunits. (E) Nocodazole and MG132 arrested cells were treated with DMSO or AZ3146, then cell lysates and BUBR1 IP were probed for MCC subunits. (F) mitotic HeLa cell lysates were harvested after transfection with MPS1 shRNA- and RNAi-resistant LAP-MPS1 constructs (see "Experimental Procedures" for details; KD, kinase dead; vec, vector) and then subjected to BUBR1 immunoprecipitation and Western blot analysis for MCC subunits. The asterisks denote possible LAP-MPS1 degradation products.

Our previous studies indicated that C-MAD2 is selectively incorporated into the MCC (Tipton, Tipton et al. 2011). We also found that C-MAD2 expression in reversine-treated cells bypasses the requirement for MPS1 kinase and rescues MCC assembly and
checkpoint responses (Tipton, Ji et al. 2013). Although MPS1 might phosphorylate BUBR1, a core subunit of the MCC, BUBR1 phosphorylation seems not essential for the assembly of the MCC (more in section 4.2.3). Taken all these discoveries together, we conclude that the major function of MPS1 kinase as a mitotic checkpoint regulator is to promote the production of C-MAD2, thus activating mitotic checkpoint during mitosis.

4.2.2. MPS1 kinase activity is essential for maintaining the MAD1:C-MAD2 complex at kinetochores

Tethering MPS1 to kinetochores recruits the MAD1:C-MAD2 complex to even bi-oriented chromosomes and delays anaphase onset and this recruitment depends on MPS1 kinase activity (Jelluma, Dansen et al. 2010). However, Hewitt et al. have shown that MPS1 activity is required for establishing but not for maintaining the MAD1:C-MAD2 complex at kinetochores (Hewitt, Tighe et al. 2010). To better understand the regulation of the MAD1:C-MAD2 complex localization by MPS1, we decided to further study kinetochore localization of the MAD2 conformers and MAD1 in the presence of MPS1 inhibitor reversine.
Figure 4-10. MPS1 kinase activity is required for targeting and maintaining MAD1:C-MAD2 complex at kinetochores.

(A) HeLa-mRFP-H2A cells transfected with GFP-MAD2\textsuperscript{L13A} were treated with MG132 for 1 hr, followed by nocodazole (Noc) addition for 1 hr. Reversine was then added, and cells were imaged live for 1 hr. Still images at critical time points are shown. For clarity, single-plane images are shown. (B) Like (A), except that cells were transfected with MAD1-GFP. Two still images after reversine addition are shown. (C) Like (B), except that the imaged cells were transfected with GFP-MAD2\textsuperscript{AC10}. (D) HeLa cells were cotransfected with mCherry-Mis12-MAD1 and GFP-MAD2 and then treated and imaged as in (A). (E) HeLa cells were cotransfected with mCherry-Mis12-MAD1 and GFP-MAD2 and then treated and imaged as in (A). In all panels, scale bar 10 µM. Time stamp, min:sec after the start of imaging.

Cells transfected with GFP-MAD2\textsuperscript{L13A} (C-MAD2) were treated with MG132 for 1 hr. The live cell imaging showed that cells with a well-aligned metaphase plate have no
GFP signals at kinetochores (Fig 4-10A, left panel). However, when the metaphase plates were disrupted by nocodazole (3.3 µM), GFP signal reappeared on chromosomes in the same cells shortly, indicating C-MAD2 recruitment as kinetochores lost microtubule attachment (Figure 4-10A, center panel). Interestingly, MPS1 inhibition by reversine removed GFP-MAD2L13A from unattached kinetochores very rapidly (<6 min under our experimental conditions) (Figure 4-10A right panel). This result, differently from what Hewitt et al reported, suggests that MPS1 activity is required for maintaining C-MAD2 at kinetochores.

We further tested MAD1 and MAD2ΔC10 (O-MAD2) kinetochore localization with reversine and nocodazole. We found that MPS1 inhibition also removed MAD1 and O-MAD2 from unattached kinetochores (Figure 4-10 B, C). The combined results suggest that MPS1 kinase activity is required for the maintenance of the MAD1:C-MAD2 complex at kinetochores. Therefore, when MPS1 is inhibited, failure to recruit O-MAD2 to kinetochores may be caused by the loss of the MAD1:C-MAD2 complex, the O-MAD2 receptor, from kinetochores. To further validate this interpretation, we examined the localization of GFP-MAD2L13A and GFP-MAD2ΔC10 in cells expressing mCherry-Mis12-MAD1, which constitutively localizes at kinetochores. We found that both GFP-MAD2L13A and GFP-MAD2ΔC10 localize at kinetochores regardless of the inhibition of MPS1 (Figure 4-10 D and E). In conclusion, MPS1 kinase activity is essential for maintaining the MAD1:C-MAD2 complex at unattached kinetochores, which in turn, functions as the O-MAD2 receptor.
4.2.3. BUBR1 phosphorylation by MPS1 and other mitotic kinases during mitosis is not essential for MCC assembly.

BUBR1 is hyper phosphorylated during mitosis (Chan, Jablonski et al. 1999) (Figure 4-11). It has been long thought that mitotic BUBR1 phosphorylation was essential for the mitotic checkpoint activation and MCC assembly. To test this, we treated the lysates prepared from cells arrested in mitosis by nocodazole and MG132 with different kinase inhibitors including SP600125 (JNK and MPS1 inhibitor) (Schmidt, Budirahardja et al. 2005), Reversine (MPS1 inhibitor) (Santaguida, Tighe et al. 2010), AZ3146 (MPS1 inhibitor) (Hewitt, Tighe et al. 2010), ZM447439 (Aurora B inhibitor) (Ditchfield, Johnson et al. 2003) and BI2536 (PLK1 inhibitor) (Lenart, Petronczki et al. 2007). We noticed the increase of a faster-migrating BUBR1 species after all treatments, indicating the increase of dephosphorylated or unphosphorylated BUBR1 (Figure 4-11A). These results suggest that BUBR1 phosphorylation is regulated directly or indirectly by MPS1 and other kinases, agreeing with others' reports (Chan, Jablonski et al. 1999, Elowe, Hummer et al. 2007, Matsumura, Toyoshima et al. 2007, Huang, Hittle et al. 2008, Elowe 2011).

To further investigate whether BUBR1 phosphorylation has any effects on the assembly of MCC, we created a GST tagged BUBR1 phospho-resistant mutant, BUBR1^{11A} with 11 known BUBR1 phosphorylation sites mutated to alanines. Cells transfected with GST-BUBR1 WT or 11A were synchronized in mitosis with nocodazole and then harvested. The cell lysates were subjected to GST pull-down assays followed by western blot. BUBR1^{11A} migrated faster than BUBR1^{WT}, suggesting that BUBR1^{11A} mutant cannot be phosphorylated during mitosis. BUBR1^{11A} can still pull down the same
level of other MCC components including CDC20, BUB3 and MAD2 compared with BUBR1\(^{WT}\), suggesting that BUBR1 phosphorylation is not required for the assembly of MCC (Figure 4-11B).

Figure 4-11. BUBR1 phosphorylation is not required for MCC assembly or stability. (A). Cells were synchronized and treated as in Fig. 4-9A using reversine or other small molecule inhibitors. Cell lysates were then analyzed for BUBR1. \(Noc\), nocodazole. (B). Cells were transfected with GST vector alone, GST-tagged BUBR1\(^{WT}\), or GST-tagged BUBR1\(^{11A}\). Mitotic cell lysates and GST pull-downs were probed for BUBR1, CDC20, BUB3, and MAD2. The bands corresponding to GST-BUBR1 and endogenous BUBR1 are indicated.
4.3. MPS1 kinase orchestrates different MAD1 domains to maintain the mitotic checkpoint

Based on the results above, MPS1 kinase activity is essential for activating the mitotic checkpoint through promoting C-MAD2 production. How MPS1 promotes MAD2 O-C conversion remains unclear. MPS1 activity is required for establishing and maintaining the kinetochore localization of the MAD1:C-MAD2 complex during mitosis. In addition, MPS1 kinase is also essential for the mitotic arrest caused by mCherry-Mis12-MAD1 (Maldonado and Kapoor 2011), which constitutively localizes at kinetochores. This suggests MPS1 activity is required beyond kinetochore recruitment of the MAD1:C-MAD2 complex, most likely empowering or enhancing the catalytic activity of the MAD1:C-MAD2 complex.

4.3.1. MPS1 kinase phosphorylates MAD1-NTD and CTD in vitro

To investigate how MPS1 regulates the MAD1:C-MAD2 complex, we first tested whether MPS1 as a kinase can phosphorylate MAD1 or MAD2. In vitro kinase assays were performed and we found that MPS1 phosphorylates MAD1-NTD (and its degradation products) and CTD but not MIM or any conformers of MAD2 in vitro (Figure 4-12A). To determine the phosphorylation sites on MAD1-NTD and CTD phosphorylated by MPS1, the in vitro phosphorylated MAD1 fragments were submitted for LC-MS/MS analyses (MSBioworks, Ann Arbor). A total of 8 putative phosphorylation sites were identified. Four sites are on MAD1-NTD (T8, S22, S62 and T323) while the other four are on MAD1-CTD (S598, S610, T624 and T716) (Figure 4-12B).
Figure 4-12. MPS1 kinase phosphorylates MAD1-NTD and CTD.

(A). *In vitro* kinase assays of GST-MPS1 with GST-MAD1-NTD, MIM or CTD. Phosphorylation of MAD1 domains by MPS1 was detected by autoradiography. The asterisks indicate MAD1-NTD, MIM and CTD. The arrow indicates GST-MPS1. Myelin basic protein (MBP) is an artificial substrate for MPS1. Rev=reversine, an MPS1 inhibitor. (B). MAD1 sites phosphorylated by MPS1 *in vitro* as determined by LC/MS/MS.

4.3.2. The putative MPS1 phosphorylation sites on MAD1-CTD are required for the mitotic checkpoint

To investigate the effects of MAD1 phosphorylation by MSP1 on the mitotic checkpoint, we mutated all the 8 phosphorylation sites revealed by mass spectrometry to alanines on the mCherry-Mis12-MAD1 to create the phospho-resistant mutant, mCherry-Mis12-MAD1^8A^. We found that mCherry-Mis12-MAD1^8A^ cannot maintain mitotic arrest
as efficiently as mCherry-Mis12-MAD1WT, suggesting that putative MPS1 phosphorylation sites on MAD1 are crucial for the checkpoint activity (Figure 4-13 A). We further tested mCherry-Mis12-MAD1NTD4A (mutating the N terminal 4 residues to alanines) and mCherry-Mis12-MAD1CTD4A (mutating the C terminal 4 residues to alanines). The results showed that mCherry-Mis12-MAD1CTD4A cannot maintain efficient mitotic arrest while mCherry-Mis12-MAD1NTD4A performs like WT, suggesting that the critical sites lie within the C terminal 4 residues (Figure 4-13 A). Additionally, MPS1 cannot phosphorylate recombinant MAD1-CTD4A in vitro, suggesting that our mass spectrometry covered all the major MPS1 phosphorylation sites on MAD1-CTD (Figure 4-13 B).
Figure 4-13. The putative MPS1 phosphorylation sites on MAD CTD are required for the mitotic checkpoint.

(A). mCherry-Mis12-MAD1 WT, 8A (T8, S22, S62, T323, S598, S610, T624, T716 to A), NTD 4A (T8, S22, S62, T323 to A) or CTD 4A (S598, S610, T624, T716 to A) were transfected to HeLa cells. 24 hours after transfection, the mitotic duration of transfected cells was recorded. Student’s t-test was used. **** indicates p<0.0001. (B). In vitro kinase assay was performed by incubating recombinant MPS1 with MBP, MAD1-CTD WT, MAD1-CTD 4A and GST with or without reversine (Rev). The samples were subjected to SDS-PAGE followed by Coomassie stain. The gel was then dried and autoradiography was used to detect the phosphorylation signal.
To investigate the molecular mechanism for the mitotic defects caused by MAD1-CTD\textsuperscript{4A}, we tested the interactions between MAD1-CTD\textsuperscript{4A} and MAD2. We found that MAD1-CTD\textsuperscript{4A} binds to much less O-MAD2 and C-MAD2 compared with MAD1-CTD\textsuperscript{WT} (Figure 4-14A). These results are surprising because we did not expect that the S/T to A mutants show defects in MAD2 binding in the absence of MPS1 kinase. It is possible that one or more sites among the four residues are essential for MAD1 function regardless of MPS1 phosphorylation (discussed more in Section 4.3.3). Nevertheless, the good correlation between imaging and \textit{in vitro} binding results further confirm that the interaction between MAD1-CTD and MAD2 is essential for the mitotic checkpoint.

GST-tagged MAD1-CTD\textsuperscript{4A} also shows the defect in interacting with His-tagged MAD1-CTD fragment \textit{in vitro} (Figure 4-14B). Compared to GST-CTD\textsuperscript{WT}, GST-CTD\textsuperscript{4A} binds less His-CTD\textsuperscript{WT} and nearly none of His-CTD\textsuperscript{4A}. mCherry-Mis12-MAD1\textsuperscript{CTD4A} (4A in the full length of MAD1) maintains the ability to interact with endogenous MAD1 in cells as mCherry-Mis12-MAD1\textsuperscript{CTD4A} can still immunoprecipitate endogenous MAD1 (Figure 4-14C), in contrast to mCherry-Mis12-MAD1\textsuperscript{ACTD} (Figure 4-1C, Figure 4-14C). The oligomerization level of MAD1-CTD\textsuperscript{4A} cannot be determined in these pulldown or immunoprecipitation experiments. To test this, gel filtration and cross-linking experiments can be used to check whether MAD1-CTD\textsuperscript{4A} exists as a monomer or dimer.
Figure 4-14. The putative MPS1 phosphorylation sites on MAD CTD are required for the interaction with MAD2.

(A). GST-MAD1-CTDWT, CTD4A or GST alone was incubated with MAD2L13A or MAD2∆C10 at 37 °C for 1 hr followed by GST pull-down assays. The samples were subjected to SDS-PAGE and western blot analysis. (B). Similarly, GST-MAD1-CTD and CTD4A were incubated with His-MAD1-CTDWT or 4A and GST pull-down was performed followed by SDS-PAGE and western blot analysis. (C). Cells transfected with mCherry-Mis12-MAD1 WT, AA, ∆CTD or full length CTD 4A were harvested. The cell lysates were used to perform the Mis12 immunoprecipitation. The samples were subjected to SDS-PAGE and western blot analysis.
4.3.3. MAD1-CTD\textsuperscript{4E} is also defective in maintaining the mitotic checkpoint

To further confirm that MPS1 phosphorylation of the four sites on MAD1-CTD is essential for MAD1 functions, we created a phospho-mimic mutant by mutating all four residues to glutamic acids (E). If MPS1 phosphorylation on CTD is sufficient for the mitotic checkpoint, we would expect that MAD1-CTD\textsuperscript{4E} retains the capability to maintain the checkpoint even when MPS1 is inhibited. Surprisingly, mCherry-Mis12-MAD1\textsuperscript{CTD4E} transfected cells showed no difference in mitotic duration compared with mCherry-Mis12-MAD1\textsuperscript{CTD4A} (Figure 4-15A). Furthermore, like MAD1-CTD\textsuperscript{4A}, MAD1-CTD\textsuperscript{4E} cannot bind to MAD2 \textit{in vitro}, either even though a mobility shift due to the 4E mutations is clearly visible (Figure 4-15B). These results suggest that MAD1-CTD\textsuperscript{4E} is also defective in maintaining the mitotic checkpoint.

It is possible that the 4E mutant disrupted the MAD1 structure, although that is hard to imagine based on known structure of MAD1-CTD (Kim, Sun et al. 2012). T716 is at the unstructured tail of MAD1 molecule (whose full length is 718 residues) while the other three S/T residues are localized in the coiled coil region and their polar side chains should face outwards of the coiled coil. None are expected to cause severe structural distortions of the MAD1-CTD. However, a reasonable alternative, as mentioned in Section 4.3.2, is that at least one of the four residues may be essential for interacting with other proteins such as MAD2 for the mitotic checkpoint. The sites phosphorylated by MPS1 kinase \textit{in vitro} might not be phosphorylated in cells. When mutated as either a phosphor-resistant or phosphomimic mutant, loss-of-function would occur. Nevertheless, the correlation of MAD2 binding activity \textit{in vitro} and the capability to maintain the mitotic checkpoint in cells of all MAD1 constructs strongly support the functional
importance of the newly identified protein-protein interactions between MAD1-CTD and MAD2.

Figure 4-15. MAD1-CTD^{4E} cannot reverse the defects caused by CTD^{4A}.
(A). mCherry-Mis12-MAD1 WT, CTD4A or CTD4E were transfected into HeLa cells. Twenty-four hours later, the mitotic duration of the transfected cells was recorded. The mitotic duration of cells staying in mitosis for more than 13 hrs was recorded as 780 min. Student's t-test was used. **** indicates p<0.0001, NS indicates "not significance" (B). GST-tagged MAD1-CTD WT, 4A or 4E were incubated with either MAD2^{L13A} or MAD2^{ΔC10} at 37 °C for 1 hr followed by GST pull down.
4.3.4. S610 and T716 of MAD1 are critical residues for the mitotic checkpoint

To better dissect the roles of potential MPS1 phosphorylation sites on MAD1-CTD, we constructed single site mutations for each of the four sites on mCherry-Mis12-MAD1. Both phospho-mimic (mutating S/T to E) and phospho-resistant (mutating S/T to A) mutants were created. The mitotic durations of HeLa cells transfected with the MAD1 mutants were recorded. mCherry-Mis12-MAD1S598A or MAD1S598E and mCherry-Mis12-MAD1T624A or MAD1T624E can maintain mitotic arrest as MAD1WT (Figure 4-16A), which suggests that S598 and T624 are not essential for the mitotic checkpoint. However, mCherry-Mis12-MAD1S610A and mCherry-Mis12-MAD1T716A both showed defects in maintaining mitotic arrest compared with MAD1WT. Interestingly, MAD1S610E behaved similarly as MAD1S610A while MAD1T716E showed extended mitotic delay as compared to MAD1T716A (T716E, 441.5±38.0 min; T716A, 242.3±48 min) (Figure 4-16A). The results suggest that both S610 and T716 are critical for MAD1 function. But more interestingly, T716 may be phosphorylated and required for the MAD1:C-MAD2 complex activity at kinetochores.

We further tested the interactions between MAD1-CTD716A or MAD1-CTD716E and MAD2. We found that MAD1-CTD716A binds to less MAD2 (both O-conformer and C-conformer) compared with MAD1-CTD716E and -CTDWT (Figure 4-16B). This provides the explanation for the mitotic checkpoint defects caused by mutating MAD1 T716 to A.
Figure 4-16. MAD1 T716 site is essential for the mitotic checkpoint.
(A). mCherry-Mis12-MAD1 mutants were transfected. Twenty-four hrs after transfection, the mitotic duration of the transfected cells was recorded. The mitotic duration of the cells that stayed at mitosis for more than 13 hrs was recorded as 780 min. (B). GST-tagged MAD1-CTD WT, T716A or T716E were incubated with either MAD2L13A or MAD2∆C10 at 37 °C for 1 hr followed by GST pull down assay. (C). Cells were transfected with mCherry-Mis12-MAD1 WT or T716E. The mitotic duration of the transfected mitotic cells after treating with 500 nM reversine was recorded. Student's t-test was used. ** indicates p<0.01.

If MPS1 phosphorylation at MAD1 T716 activates the MAD1:C-MAD2 complex at kinetochores to promote the production of C-MAD2 at least to a certain degree, it would be expected that mCherry-Mis12-MAD1T716E can stay in mitosis longer than mCherry-Mis12-MAD1WT when MPS1 is inhibited by reversine. However, our result showed that reversine still drove cells transfected with mCherry-Mis12- MAD1T716E out
of mitosis as well as mCherry-Mis12-MAD1\textsuperscript{WT} transfected cells (Figure 4-16C). As MPS1 may phosphorylate many targets to coordinate the mitotic checkpoint signaling, our results showed that the T716E mutant alone cannot override the inhibition of MPS1.

4.3.5. MPS1 kinase activity reduces the interaction between MAD1-NTD and CTD

Currently, how MPS1 promotes C-MAD2 production is unknown. We have uncovered several novel protein-protein interactions involving MAD1-NTD or CTD. The hypothesis is that MPS1 enhances MAD2 O-C conversion through regulating the newly characterized protein-protein interactions. In light of some surprising results with the phosphomutants, we decided to directly test the effects of MPS1 on the protein-protein interactions.

To do that, we added recombinant MPS1 into the \textit{in vitro} binding reactions and compared the interactions with or without active MPS1. It seems that MPS1 does not affect the interactions between MAD1-NTD or CTD and C-MAD2 or O-MAD2 (Figure A-3) however, MPS1 kinase activity reduces the interaction between MAD1-NTD and CTD. Omitting ATP from the reactions or adding MPS1 inhibitor reversine or AZ3146 relieved the MPS1 effect on the interaction between MAD1-NTD and MAD1-CTD (Figure 4-17A).

In addition, we also noticed that MPS1 directly interacts with MAD1-NTD and CTD (Figure 4-17B), and increasing concentrations of kinase dead MPS1 also gradually reduced the level of CTD associated with NTD (Figure 4-17C), suggesting MPS1 may regulate MAD1-NTD and CTD interaction through at least two mechanisms: direct competition and phosphorylation.
We have tried to test whether the NTD or CTD interacts in vivo by creating a CFP-MAD1-YFP construct for FRET. We also created a FKBP-MAD1-FRB construct aiming to test the functional consequence of artificially locking MAD1-NTD and MAD1-CTD together. However, all the constructs might not be able to differentiate intramolecular or intermolecular interactions. Initial tests also did not detect positive FRET signals or observable phenotype with the inducible dimerization construct, so we were forced to suspend the experiments due to technical difficulties.

Figure 4-17. MPS1 kinase reduces the interaction between MAD1-NTD and CTD. (A). Recombinant GST-Mad1-NTD or GST purified from E. coli were incubated with His-MAD1-CTD at 37 °C for 1 hr. MPS1, reversine (MPS1 inhibitor), AZ (another MPS1 inhibitor) and ATP were added as indicated and then GST pull down was performed. (B). GST tagged MAD1-NTD, MIM or CTD were incubated with His tagged MPS1 at 37 °C for 1 hr followed by GST pull down assay. (The asterisks indicate the major bands). (C). Similar with (A) and (C), in vitro binding assay was performed using GST-NTD and His-CTD by titrating His tagged MPS1 kinase dead mutant.
4.4. Aurora B does not phosphorylate MAD1 or MAD2 \textit{in vitro}.

In addition to MPS1, other kinases including Aurora B and BUB1 are also essential regulators of the mitotic checkpoint (Musacchio 2015). Inhibiting Aurora B abolishes the mitotic arrest caused by the expression of mCherry-Mis12-MAD1, suggesting that Aurora B is also essential for the activity of the MAD1:C-MAD2 complex at kinetochores (Maldonado and Kapoor 2011). However, in our \textit{in vitro} kinase assays, Aurora B autophosphorylates itself but does not directly phosphorylate either MAD1 or MAD2 (Figure 4-18), suggesting the effect of Aurora B on the mitotic checkpoint is not directly through affecting MAD1 or MAD2. It is possible that inhibiting Aurora B disrupts MPS1 recruitment to kinetochores thus leading to the mitotic checkpoint defect in mCherry-Mis12-MAD1 transfected cells.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{Figure_4-18.png}
\caption{Aurora B kinase does not phosphorylate either MAD1 or MAD2 \textit{in vitro}.}
\end{figure}

\textit{In vitro} kinase assays of His-Aurora B with GST-MAD1-NTD, MIM or CTD and MAD2 (L13A or ΔC10). Phosphorylation of proteins was detected by autoradiography. The asterisks indicate the expected bands of MAD1-NTD, MIM and CTD. The arrow indicates Aurora B. (Hes: hesperadin, an Aurora B inhibitor).
4.5. BUB1 is required for the mitotic arrest caused by mCherry-Mis12-MAD1

BUB1 is required for kinetochore localizations of many important mitotic checkpoint proteins such as BUBR1, MAD1 and MAD2 (Chan, Jablonski et al. 2000, Johnson, Scott et al. 2004, Karess 2005, Kops, Kim et al. 2005, Overlack, Primorac et al. 2015, Zhang, Lischetti et al. 2015). Direct interaction between BUB1 and MAD1 was discovered in yeast and *C. elegans*. Whether human BUB1 binds to MAD1 remains unclear (Moyle, Kim et al. 2014, Vleugel, Hoek et al. 2015). In addition, the recruitment of CDC20 by BUB1 is important for mitotic checkpoint activation (Di Fiore, Davey et al. 2015, Vleugel, Hoek et al. 2015). Although a lot of progress has been made during the past decade, the roles of BUB1 in mitotic checkpoint remains puzzling.

To test whether BUB1 regulates the MAD1:C-MAD2 complex catalytic activity at kinetochores, we co-transfected mCherry-Mis12-MAD1 with BUB1 siRNA and recorded the mitotic duration of the transfected cells. Interestingly, we found that BUB1 depletion by siRNA abolished the mitotic arrest caused by mCherry-Mis12-MAD1, which suggests that BUB1 may be important for the catalysis of MAD2 O-C conversion (Figure 4-19). Previous report has revealed that BUB1 phosphorylates human MAD1 *in vitro* (Seeley, Wang et al. 1999). It is reasonable to hypothesize that BUB1 phosphorylates MAD1 to facilitate the MAD2 O-C conversion. That will be one of our future directions.
Figure 4-19. BUB1 is required for the mitotic arrest led by mCherry-Mis12-MAD1. HeLa cells were treated with BUB1 siRNA. mCherry-Mis12-MAD1 was transfected 24 hrs later. Twenty-four hrs after the transfection, the mitotic duration of the transfected cells was recorded. The mitotic duration of the cells that stayed at mitosis for more than 13 hrs was recorded as 780min. Student's t-test was used. **** indicates p<0.0001. (Dr. Yibo Luo supplied the data for the BUB1 siRNA treated group.)

4.6. Explore the roles of maternal embryonic leucine zipper kinase (MELK) in mitosis

Maternal embryonic leucine zipper kinase (MELK, also called MRK38 or pEg3) is a serine/threonine protein kinase that belongs to the AMP-activated kinase (AMPK) related kinase family (Gil, Yang et al. 1997, Heyer, Warsowe et al. 1997, Blot, Chartrain et al. 2002, Jiang and Zhang 2013, Ganguly, Mohyeldin et al. 2015) (Figure 4-20). MELK protein level peaks during prometaphase (Badouel, Korner et al. 2006, Badouel, Chartrain et al. 2010). MELK was previously characterized to regulate G2/M transition although the exact functions of MELK on G2/M transition remain controversial (Davezac, Baldin et al. 2002, Gray, Jubb et al. 2005).
Figure 4-20. Diagram of MELK domains.
The kinase domain (11–268) is at the N-terminus, followed by a UBA domain that supports the folding and activity of the kinase domain. The functions of threonine-proline (TP) rich domain and kinase associated 1 domain (KA1) are not fully understood but may inhibit kinase activity.

We have found that MELK is co-transcribed with a group of 64 core centromere/kinetochore components, suggesting the potential roles of MELK in mitosis (Tipton, Wang et al. 2012). Interestingly, MELK also activates transcription factor FOXM1, which drives expression of multiple mitosis regulatory proteins (Joshi, Banasavadi-Siddegowda et al. 2013). Furthermore, MELK was also reported to regulate cytokinesis in *Xenopus* and in human cancer cells (Le Page, Chartrain et al. 2011, Du, Qu et al. 2014, Wang, Lee et al. 2014). Microarray profiling listed MELK as one of the top-ranking (#11) chromosomal instability (CIN) signature genes (Carter, Eklund et al. 2006). High level of MELK expression has been reported in cancers and cancer stem cells (Hebbard, Maurer et al. 2010, Ganguly, Hong et al. 2014, Ganguly, Mohyeldin et al. 2015). MELK is currently regarded as a promising target for novel cancer therapy, and several MELK small molecule inhibitors including OTSSP167 have been published (Mahasenan and Li 2012, Canevari, Re Depaolini et al. 2013, Chung and Nakamura 2013). However, it is still unclear whether MELK overexpression in cancer cells has any causal relationship with the CIN phenotype (Nakano, Paucar et al. 2005, Nakano, Masterman-Smith et al. 2008, Nakano and Kornblum 2009, Pickard, Green et al. 2009, Hebbard, Maurer et al. 2010, Kappadakunnel, Eskin et al. 2010, Kuner, Falth et al. 2013).
The mitotic effects of MELK inhibition at molecular and cellular level remain to be fully characterized. All the interesting clues mentioned above motivate us to investigate the roles of MELK during mitosis.

4.6.1. OTSSP167 causes defective mitotic checkpoint and cytokinesis

OTSSP167 was recently characterized as a potent inhibitor of MELK and is currently in Phase I clinical trials for solid tumors that have not responded to other treatment (ClinicalTrials.gov Identifier: NCT01910545) (Chung, Suzuki et al. 2012, Chung and Nakamura 2013, Ganguly, Hong et al. 2014). To test whether MELK regulates the mitotic checkpoint, we treated MCF7 cells arrested in prometaphase by nocodazole with OTSSP167 and found that it drove cells out of mitosis even in the presence of microtubule inhibitor (Figure 4-21B). This result suggests possible roles of OTSSP167 on regulating mitotic checkpoint. Previous reports indicated that MELK may regulate cytokinesis (Le Page, Chartrain et al. 2011, Wang, Lee et al. 2014). To test the effects of OTSSP167 on cytokinesis, MCF7 cells blocked in mitosis by nocodazole were released to fresh medium containing DMSO or OTSSP167. In the control where cells were released into DMSO, the majority aligned their chromosomes, entered anaphase and proceeded through cytokinesis within 2 hours. However, cells released into OTSSP167-containing medium failed to finish cytokinesis. Instead they flattened out and entered interphase rapidly without observable cleavage furrow formation (Figure 4-21A). This result agrees with earlier discovery that MELK may regulate cytokinesis.
4.6.2. OTSSP167 disrupts MCC and MCC-APC/C interaction

To further test whether OTSSP167 induces the mitotic checkpoint defects through disrupting the MCC assembly or the MCC-APC/C interaction, we treated HeLa cells arrested by nocodazole or taxol with MG132 and DMSO or OTSSP167. The cells were harvested and the lysates were subjected to BUBR1 IP. Endogenous MELK was dephosphorylated (indicated by a fast-migrating species of MELK in SDS-PAGE) with OTSSP167 treatment, which could suggest the inhibition of MELK. Similarly, dramatic mobility down-shift of CDC27 was also noticed in OTSSP167 treated cell lysates. In the presence of OTSSP167, we found that there was clear reduction of MAD2 in BUBR1 IP using either nocodazole or taxol arrested cells, suggesting that OTSSP167 disrupts MCC
assembly (Figure 4-22 B, C). Furthermore, the association between MAD2 or CDC20 with BUBR1, and the interaction between MCC and APC/C (indicated by CDC27 and CDC16 levels in the BUBR1 IP), were all significantly reduced when cells arrested in mitosis by taxol but by nocodazole (Figure 4-22C). This difference between nocodazole and taxol treated cells can be explained by previous results that mitotic checkpoint induced by taxol is weaker than that induced by nocodazole (Waters, Chen et al. 1998, Westhorpe, Tighe et al. 2011). The combined results indicated that OTSSP167 induces mitotic checkpoint defects through disrupting MCC assembly and the interaction between MCC and APC/C.
Figure 4-22. OTSSP167 causes loss of MAD2 from the MCC.

(A). Outline of cell synchronization protocol. Following single thymidine arrest (STA) for 24 hrs (numbers above arrows indicate time in hours), HeLa cells were released into nocodazole (Noc) or Taxol for 12 hrs and then treated with DMSO or OTSSP167 (100 nM) ("+") plus MG132 (to prevent mitotic exit). After 2 hrs, cells were processed for lysates and immunoprecipitation (IP). (B&C). Nocodazole (B) or Taxol (C) arrested cells were treated with DMSO ("-")) or OTSSP167 ("+")) and the lysates were subjected for nonimmune rabbit IgG or BUBR1 IP and Western blot. The MCC and APC/C components were probed together with MELK. Molecular weight markers (in kDa) were labeled.

4.6.3. MELK depletion by shRNA did not recapitulate the mitotic effects induced by OTSSP167

We acknowledge that many kinase inhibitors especially ATP analogs have off-target effects. To validate the results obtained with OTSSP167, we tested whether MELK depletion by shRNA can recapitulate the results with OTSSP167. We found that MELK knockdown cannot override mitosis in cells treated with microtubule inhibitors. Consistently, MELK shRNA disrupts neither MCC assembly nor the interaction between
MCC and APC/C using similar experiments described above (Figure 4-23). These results suggest that OTSSP167 effects on mitosis might not be caused by MELK inhibition.

**Figure 4-23. MELK knockdown does not compromise MCC assembly.**
HeLa cells were transfected with MELK shRNA for 24 hrs. Transfected cells were selected by puromycin while synchronized as described in Figure 4-22A. The nocodazole (A) and taxol (B) arrested cell lysates were subjected to IP and then probed for proteins as indicated.

**4.6.4. OTSSP167 inhibits Aurora B, BUB1 and Haspin kinases**

In the cell lysates treated with OTSSP167, we also noticed reduced autophosphorylation of Aurora B on T232 (detected using phospho-specific antibody anti Aurora B pT232), suggesting possible inhibition of Aurora B kinase activity (Figure A-4). To examine whether OTSSP167 caused mitotic defects through inhibiting Aurora B, we performed the *in vitro* kinase assay using recombinant Aurora B purified from *E. coli* and found that OTSSP167 indeed inhibits Aurora B kinase activity with IC$_{50}$ at about 25 nM when using substrates H3.3 or myelin basic protein (MBP) as substrates (Figure 4-24A). As a control, OTSSP167 inhibited MELK (1-340) kinase with IC$_{50}$ at about 8 nM under our experimental conditions (Figure 4-24B). The inhibition of Aurora B by OTSSP167 was further confirmed using Aurora B immunoprecipitated from mitotic cell lysates (Figure 4-24C). Little phospho-H3$_{S10}$ signal was detected after the IP kinase assay.
performed either using lysates treated with OTSSP167 or ZM447439, the latter a well characterized Aurora B inhibitor (Ditchfield, Johnson et al. 2003).

Figure 4-24. OTSSP167 inhibits Aurora B, BUB1, Haspin as well as MELK.

(A) In vitro kinase assays with ~40 nM of recombinant 6×His-Aurora B/INCENP and 1 μg each of histone H3.3 and myelin basic protein (MBP). OTSSP167 (OT) was added at different concentrations (nM) with DMSO as a control (lane "-"). The samples were separated by SDS-PAGE, blotted and then processed for autoradiography ("P"). The same blot was then stained shortly with Coomassie brilliant blue (CBB) to show equal loading of substrates, and probed by immunoblotting ("IB") for Aurora B. The numbers on the right indicate molecular weight markers (kDa). (B) Similar as in (A), except that ~40 nM of recombinant GST-MELK was used. (C) HeLa cells arrested with nocodazole and MG132 were further treated with DMSO ("ctrl"), ZM447439 (ZM, 2.5 μM final concentration) or OTSSP167 (OT, 100 nM final concentration) and the lysates were used for in vitro Aurora B IP kinase assays. Alternatively, the IPs from DMSO treated lysates
were used in kinase assays in the presence of ZM or OT. The kinase reactions were applied to SDS-PAGE followed by Western blot. The membranes were stained for recombinant histone H3.3 by Coomassie staining before blocking. The phosphorylated H3.3 was probed with anti-phospho-H3\textsuperscript{S10} antibody. (D) HeLa cells arrested with nocodazole and MG132 were further treated with DMSO("ctrl"), or OTSSP167(OT) and the lysates were used for \textit{in vitro} BUB1 IP kinase assays. In one lane OT was also added directly to the reactions containing the IP from DMSO treated lysates. The kinase reactions were applied to SDS-PAGE followed by Western blot. The membrane was stained for recombinant histone H2A before blocking. The phosphorylated H2A was probed with anti-phospho-H2A\textsuperscript{T120} antibody. (E) HeLa cells arrested in mitosis by nocodazole and MG132 were further exposed to DMSO ("ctrl"), 5-ITU (ITU1 and ITU2 are two batches) or OTSSP167 (OT), and the whole cell lysates prepared in 1×SDS sample buffer were separated for Western blot. The membrane was stained by Coomassie blue first before being distained and probed for MAD2 or phospho-H3\textsuperscript{T3}.

According to our results, OTSSP167 inhibits Aurora B, however, Aurora B inhibition cannot totally explain the phenotype caused by OTSSP167. Because Aurora B inhibition usually has more significant impact on taxol arrested cells but only caused very mild mitotic checkpoint defects in nocodazole arrested cells. OTSSP167 drove cells out of mitosis even in the presence of nocodazole (Figure 4-21B). This suggests additional kinases other than Aurora B can be the target of OTSSP167.

Using immunofluorescence, we found that mitotic cells treated with OTSSP167 showed significant reduction of phospho-H3\textsuperscript{T3}, phospho-H2A\textsuperscript{T120} and Sgo1 at the centromeres when compared with controls (Ji, Arnst et al. 2016). BUB1 is also mislocalized from kinetochores by OTSSP167 treatment. We reasoned that OTSSP167 may also inhibit BUB1 and Haspin kinases. To test that, we performed BUB1 IP kinase assay and found that OTSSP167 treated lysates or direct addition of OTSSP167 to the IPs abolished the phosphorylation of histone H\textsubscript{2}A at T120 (Figure 4-24D). Immunoblotting of the lysates harvested from mitotic cells treated with either OTSSP167 or known haspin kinase inhibitor 5-ITU both showed reduction of phospho-H3\textsuperscript{T3} signals compared with
untreated cell lysates (Figure 4-24E). All the results indicate that OTSSP167 inhibits multiple kinases that are important for the mitotic checkpoint such as Aurora B, BUB1 and Haspin kinases.

4.6.5. OTSSP167 promotes cell cortex localization of GFP-MELK in prometaphase cells

As reported previously (Chartrain, Blot et al. 2007, Le Page, Chartrain et al. 2011), GFP-MELK was primarily diffuse in the cytoplasm in prometaphase cells but a fraction clearly re-localized to the cortex or cytoplasmic membrane concomitantly with the metaphase-to-anaphase transition, with some enrichment at the cleavage furrow (Chartrain, Couturier et al. 2006, Tipton, Wang et al. 2012) (Figure 4-25A). OTSSP167 treatment resulted in premature cortex association of GFP-MELK in prometaphase indicated by the relocation of GFP-MELK from cytoplasm to the cortex or cytoplasmic membrane (Figure 4-25B). Inhibitors of other mitotic kinases such as Aurora B kinase, MPS1 and PLK1 kinase did not affect the timing of cortex association of GFP-MELK. Although the mitotic defects caused by OTSSP167 can be explained by the off-target effects we characterized above, OTSSP167 induced premature cortex association of GFP-MELK in prometaphase may be due to the inhibition of MELK. The localization of MELK to cortex and enrichment at the cleavage furrow may indicate the roles of MELK on cytokinesis. Further studies on MELK are still needed.
Figure 4.25. OTSSP167 causes GFP-MELK localization to cell cortex in prometaphase cells.
Single-plane images of GFP-MELK transfected HeLa cells were shown in all panels. Bar = 10 μm. (A). A metaphase and an anaphase cell in the same field were shown in the absence of drugs. Note the cortex association of GFP-MELK only appears obviously during anaphase. (B). Shown are three representative transfected prometaphase cells after treatment with OTSSP167 for 2 hr. (C). GFP-MELK transfected prometaphase cells do not show cortex association after exposure to hesperadin (Aurora B inhibitor), reversine (MPS1 inhibitor) or Plk1 inhibitor III.
4.7. Explore possible roles of tyrosine kinases on silencing the mitotic checkpoint

4.7.1. The MAD1$^{Y634E}$ mutant cannot maintain the mitotic checkpoint

The MAD1:C-MAD2 complex catalyzes MAD2 O-C conversion to activate the mitotic checkpoint during mitosis (Musacchio 2015). Interestingly, formation of the MAD1:C-MAD2 complex is cell cycle independent (Campbell, Chan et al. 2001). However, the MAD1:C-MAD2 complex only gains activity during mitosis, suggesting that the activity of the MAD1:C-MAD2 complex is cell cycle regulated. As discussed above, MPS1 kinase may activate the MAD1:C-MAD2 complex through phosphorylation. In addition to the mapped MPS1 sites on MAD1, according to phosphosite.org, MAD1 has 35 in vivo phosphorylated sites. To examine the roles of MAD1 phosphorylation on the MAD1:C-MAD2 complex activity, we made single site phospho-mimic mutants (replacing the S/T/Y residues with glutamic acids) on mCherry-Mis12-MAD1 and tested the mitotic duration of transfected cells (the summary of the screening data is shown on table in Table B.2 in the appendix).

By doing the screening, we found that MAD1$^{Y634E}$ mutant created on mCherry-Mis12-MAD1 did not maintain mitotic arrest (Figure 4-1B). The defect was not due to just any mutation at the Y634 residue as mCherry-Mis12-MAD1$^{Y634F}$ (phospho-resistant mutant) transfected cells were arrested in mitosis. The Mis12 IPs using the lysates transfected with either mCherry-Mis12-MAD1$^{Y634E}$ or mCherry-Mis12-MAD1$^{Y634F}$ showed that both mutants can still interact with endogenous MAD1 and MAD2 as efficiently as MAD1$^{WT}$ (Figure 4-1C), suggesting no substantial structural distortions of MAD1 mutants. Furthermore, overexpressing GFP-MAD2$^{L13A}$ restored mitotic arrest in the cells transfected with mCherry-Mis12-MAD1$^{Y634E}$ (Figure 4-26A). Based on the
results, we reasoned that MAD1<sup>Y634E</sup> or possibly MAD1 Y634 phosphorylation lost the activity to convert O-MAD2 to C-MAD2 therefore could not maintain the mitotic checkpoint.
Figure 4-26. mCherry-Mis12-MAD1\textsuperscript{Y634E} cannot maintain the mitotic checkpoint.

(A). HeLa cells were transfected with both mCherry-Mis12-Mad1\textsuperscript{Y634E} and GFP-Mad2\textsuperscript{L13A}, which is a Mad2 mutant locked in the "C" conformer. 24 hrs later, mitotic duration was recorded. As shown in the figure, C-Mad2 transfected cells can stay in mitosis for more than 9 hrs, even in the presence of mCherry-Mis12-Mad1\textsuperscript{Y634E}. (B). GFP-Mad1 WT, Y634E, Y634F were transfected to HeLa cells. Cells were fixed and stained with anti-Hec1 antibodies. (C). GST tagged MAD1-CTD WT, Y634E and Y634F were incubated with MAD2\textsuperscript{L13A} or MAD2\textsuperscript{AC10} at 37 °C for 1 hr followed by GST pull down assay. GST and MAD2 were probed.
To investigate whether mutating MAD1 Y634 to E disrupts the MAD1:C-MAD2 catalytic activity, we tested the interactions between MAD1-CTD$_{\text{Y634E}}$ and MAD2. *In vitro* binding assays show that MAD1-CTD$_{\text{Y634E}}$ binds to less either C-MAD2 or O-MAD2 when compared with MAD1-CTD$_{\text{WT}}$ and MAD1-CTD$_{\text{Y634F}}$. To address the concern that the MAD1$_{\text{Y634E}}$ mutant might disrupt the structure of the protein, we transfected MAD1$_{\text{Y634E}}$-GFP and MAD1$_{\text{Y634F}}$-GFP into HeLa cells and performed immunofluorescence on cells blocked in mitosis by nocodazole. The result shows that both MAD1$_{\text{Y634E}}$ and MAD1$_{\text{Y634F}}$ are localized to unattached kinetochores, suggesting the Y634E mutant is not dysfunctional in all aspects (Figure 4-26B). Our result suggests that phosphorylation on MAD1 Y634 or at least the Y634E mutant reduces the interaction with MAD2, which might underlie the loss of the catalytic activity of the MAD1:C-MAD2 complex (Figure 4-26C).

4.7.2. Inhibition of Abl kinases increases the mitotic index in HeLa cells

Despite our discovery on potential functional significance on MAD1 Y634, very little is known about tyrosine phosphorylation in the SAC signaling. Src or Abl inhibition was reported to lead to mitotic arrest (Moasser, Srerhapakdi et al. 1999, Wolanin, Magalska et al. 2010), suggesting that Src or Abl tyrosine kinases may promote mitotic checkpoint silencing. We tested the effects of several Src kinase inhibitors on mitotic duration using live cell imaging. Unfortunately, we did not get consistent results using different Src kinase inhibitors. However, when we treated cells with different Abl kinase inhibitors, especially PD166326 and GNF5, cells reproducibly showed higher mitotic indices, suggesting that Abl inhibition caused mitotic arrest (Figure 4-27). Conversely, Abl kinase activity may promote anaphase onset. This prediction agrees with our
MAD1\textsuperscript{Y634E} data. The function of Abl1 and Abl2 kinases in mitosis will be further studied, including investigating the possibility that Abl kinases phosphorylate MAD1 on Y634 to promote mitotic checkpoint silencing.

Figure 4-27. Abl kinase inhibitors increase mitotic indices in HeLa cells. HeLa cells were treated with DMSO (control) or five structurally distinct Abl kinase inhibitors for 16 hrs, and the mitotic indices were plotted. All inhibitors were used at 5 µM final concentration. The mitotic indices in all treatment groups are significantly different from the control cells. Student's t-test was used. * indicates that p< 0.05 when comparing drug treatments with control.

4.8. Characterize the roles of the second "CDC20 binding domain" of BUBR1 in regulating the mitotic checkpoint.

As the largest MCC component (1050 residues in human), BUBR1 acts as a scaffold for assembling the MCC. The crystal structure of the MCC from 
\textit{Schizosaccharomyces pombe} revealed how MCC assembles through direct interactions of BUBR1, CDC20 and C-MAD2 (Chao, Kulkarni et al. 2012). Although conserved, human
MCC is still quite different from the yeast MCC. For example, human MCC contains BUB3 while fission yeast MCC does not. In addition, human BUBR1 is double the size as yeast MAD3 and contains a second CDC20 binding domain. These facts indicate that human MCC assembly may be more complicated.

Recent studies using electron microscopy (EM) revealed how different BUBR1 segments interact with the two CDC20 molecules (Alfieri, Chang et al. 2016, Yamaguchi, VanderLinden et al. 2016). This is a significant step in understanding the MCC assembly and especially the interaction between MCC and APC/C. However, in the model some key segments of BUBR1 including the regions that bind to BUB3, the second CDC20 binding domain and the pseudo kinase domain are largely missing. BUB3 also cannot be detected in the electron density map.

4.8.1. **BUBR1**(487-700) interacts with CDC20

For a long time, it was believed that BUBR1(1-486) was enough for the mitotic checkpoint function. CDC20 and C-MAD2 associate with BUBR1(1-486) to assemble the core MCC (Musacchio 2015). However, several studies pointed that another BUBR1 fragment localized on 487-700 residues also binds to CDC20 (Tang, Bharadwaj et al. 2001, Davenport, Harris et al. 2006). BUBR1(487-700) is called the "second CDC20 binding domain" of BUBR1.

To better understand how BUBR1 second CDC20 binding domain associates with other MCC components and APC/C, we made 12 GST tagged BUBR1 constructs containing its various domains as shown in Figure 4-28A. HeLa cells transfected with BUBR1 constructs were blocked in mitosis using nocodazole. Cell lysates were subjected to GST pull-down assays followed by SDS-PAGE and western blot analysis. CDC27 (an
APC/C subunit), CDC20, BUB3 and MAD2 were probed (Figure 4-28B). Consistent with previous reports, the first KEN box in BUBR1 is essential for interacting with CDC20 and MAD2 as BUBR1\textsuperscript{K26A} (a KEN box mutation) binds to significantly less CDC20 and MAD2 compared with BUBR1\textsuperscript{WT}. The longer exposure indicated that BUBR1\textsuperscript{K26A} still interacts with some level of MAD2, suggesting residual interaction between BUBR1 and MAD2 independently of the first KEN box. Interestingly, we found that BUBR1\textsuperscript{K26A} pulled down similar levels of CDC20 and MAD2 as BUBR1\textsuperscript{(487-700)}. These results agree with previous reports on the second CDC20 binding domain locating between 560-700 (Tang, Bharadwaj et al. 2001, Davenport, Harris et al. 2006). It is noteworthy that the second CDC20 binding domain does not associate with CDC27, an APC/C subunit. This is consistent with recent studies on the association between MCC and APC/C (Alfieri, Chang et al. 2016, Yamaguchi, VanderLinden et al. 2016).
Figure 4-28. BUBR1\(^{487-700}\) associates with CDC20 and MAD2 independently of the first KEN box of BUBR1.
(A). Schematic figure showing different BUBR1 constructs. (B). GST tagged BUBR1 constructs were transfected into HeLa cells. Cells were synchronized in prometaphase and harvested. Cell lysates were subjected to GST pull down. Both the lysates and GST pulldowns were subjected to SDS-PAGE followed by western blot analysis. CDC27, CDC20, BUB3, MAD2 were probed first. The membranes were stripped and reprobed for GST. KEN26AAA = K26A.
4.8.2. BUBR1\textsuperscript{(487-700)} directly interacts with C-MAD2 to inhibit APC/C

Based on the results above, BUBR1\textsuperscript{(487-700)} can associate with both CDC20 and MAD2. BUBR1\textsuperscript{(487-700)} was known to directly bind to CDC20 (Tang, Bharadwaj et al. 2001, Davenport, Harris et al. 2006). However, whether BUBR1\textsuperscript{(487-700)} interacts with MAD2 directly remains unknown. To test that, we did \textit{in vitro} binding assay using recombinant GST-tagged BUBR1\textsuperscript{(487-700)} and MAD2\textsuperscript{L13A} purified from \textit{E. coli}. We found that BUBR1\textsuperscript{(487-700)} can interact with MAD2\textsuperscript{L13A} \textit{in vitro}, suggesting that the interaction between BUBR1\textsuperscript{(487-700)} and MAD2\textsuperscript{L13A} does not depend on CDC20 (Figure 4-29A).

To explore the function of the BUBR1 second CDC20 binding domain on the mitotic checkpoint, we examined the degradation of cyclin B in the mitotic extracts that faithfully reproduce APC/C activation or mitotic checkpoint silencing events \textit{in vitro} (Rape, Reddy et al. 2006, Braunstein, Miniowitz et al. 2007, Tipton, Wang et al. 2011), with or without addition of GST tagged BUBR1\textsuperscript{(487-700)}. The mitotic extracts were incubated at 30 °C; samples were collected at different time points as indicated in (Figure 4-29B). Cyclin B, the substrate of APC/C, is degraded as APC/C gets activated over time. Addition of GST-BUBR1\textsuperscript{(487-700)} delayed the degradation of cyclin B compared to the control: more than 80% of cyclin B was degraded at 30 min in control extract while in the mitotic extract with GST-BUBR1\textsuperscript{(487-700)} only about 20% of total cyclin B was degraded, suggesting that exogenous BUBR1\textsuperscript{(487-700)} can inhibit APC/C (Figure 4-29B).
Figure 4-29. BUBR1(487-700) binds directly to C-MAD2 and inhibits APC/C activity.
(A). GST tagged BUBR1(487-700) was incubated with MAD2L13A at 37 °C for 1 hr followed by GST pull down assay. The samples were subjected to SDS-PAGE and western blot analysis. GST and MAD2 were probed. (B). Mitotic extracts supplemented GST-BUBR1(487-700) or BSA (control) were incubated at 30 °C. Samples were collected at various timepoints and analyzed. ATM, CDC27, cyclin B, GST were probed as indicated.

4.8.3. p31comet does not compete with C-MAD2 for binding to BUBR1(487-700)

Dr. Kexi Wang in our lab found the direct interaction between BUBR1(1-486) and p31comet (Wang, PhD dissertation, 2014). p31comet, which shares similar structural fold to MAD2 (Yang, Li et al. 2007), was first identified as a MAD2-binding protein in a yeast two-hybrid screening (Habu, Kim et al. 2002). As p31comet has been characterized as a mitotic checkpoint silencing protein, the favored hypothesis was that p31comet competes with MAD2 for binding to BUBR1(1-486) thus disrupting the critical interaction between BUBR1 and MAD2, affecting MCC assembly. However, Kexi's results did not support the hypothesis.

As described above, BUBR1(487-700) also directly binds to C-MAD2 and inhibits the APC/C. To test whether BUBR1(487-700) interacts with p31comet directly, we performed in vitro binding assay using purified GST tagged BUBR1(487-700) and His tagged p31comet. Our result indicated that GST-BUBR1(487-700) bound to p31comet directly. We further tested whether p31comet competes with MAD2 for binding to BUBR1(487-700) (Figure 4-30). Addition of p31comet did not reduce the interaction between BUBR1(487-700) and MAD2L13A,
suggesting that $p31^{\text{comet}}$ does not compete with C-MAD2 for binding to BUBR1$^{(487-700)}$.

Our results uncovered additional MAD2 and $p31^{\text{comet}}$ binding sites on BUBR1 and will guide the future study on how MCC assembles and disassembles.

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**Figure 4-30. $p31^{\text{comet}}$ does not compete with C-MAD2 for binding to BUBR1$^{(487-700)}$.** Recombinant BUBR$^{(487-700)}$ was incubated with $p31^{\text{comet}}$ and MAD2$^{L13A}$ either separately or together. The samples after GST pull down were subjected to SDS-PAGE and western blot analysis. GST, $p31^{\text{comet}}$ and MAD2 were probed as indicated.
Chapter 5

Discussion

5.1. Function and regulation of MAD1 during the mitotic checkpoint signaling

During mitosis, when the mitotic checkpoint is activated, O-MAD2 is converted to C-MAD2, which then interacts with BUBR1 and CDC20 to form the MCC---the effector of the mitotic checkpoint (Musacchio 2015, Liu and Zhang 2016). Although O-MAD2 can spontaneously convert to C-MAD2 in vitro, the process is very slow due to the fact that the topological change of MAD2 entails large activation energy (Luo, Tang et al. 2004, Mapelli and Musacchio 2007, Luo and Yu 2008). The conversion of O-MAD2 to C-MAD2 may be the rate-limiting step for accumulating MCC during mitosis.

A single unattached kinetochore is sufficient to activate the mitotic checkpoint (Rieder, Cole et al. 1995), suggesting the existence of a mechanism to catalyze MAD2 O-C conversion. The MAD1:C-MAD2 complex acts as a special catalyst to catalyze MAD2 O-C conversion during mitosis (Mapelli and Musacchio 2007, Luo and Yu 2008). However, the mechanism to catalyze MAD2 O-C conversion by the MAD1:C-MAD2 complex is not fully understood.
MAD1-MIM has been the focus of MAD1 studies for a long time. MAD1-MIM is believed to be important for recruiting MAD2 to kinetochores (Luo, Tang et al. 2002, Izawa and Pines 2012). In contrast to mCherry-Mis12-MAD1WT, mCherry-Mis12-MAD1AA, whose MIM is mutated, cannot maintain mitotic arrest, suggesting that MAD1-MIM domain is important for the catalytic activity of the MAD1:C-MAD2 complex at kinetochores (Maldonado and Kapoor 2011) (Figure 4-1B). However, MIM alone is not sufficient to maintain mitotic arrest, suggesting that other domains of MAD1 are also essential for MAD1 activity at kinetochores (Ballister, Riegman et al. 2014, Heinrich, Sewart et al. 2014, Kruse, Larsen et al. 2014).

MAD1-CTD is required for MAD1 kinetochore localization and the mitotic checkpoint activity (Kim, Sun et al. 2012). Mutating multiple residues in MAD1-CTD caused defective mitotic checkpoint even when MAD2 kinetochore recruitment is not disrupted (Ballister, Riegman et al. 2014, Heinrich, Sewart et al. 2014, Kruse, Larsen et al. 2014). However, how MAD1-CTD is involved in regulating MAD2 O-C conversion remains unclear. In addition, the function of long ignored MAD1-NTD on converting O-MAD2 to C-MAD2 remains largely unknown.

To investigate whether MAD1-NTD and CTD are essential for MAD1 activity at kinetochores, we examined the mitotic durations of cells transfected with mCherry-Mis12-MAD1 lacking either NTD (1-485) or CTD (597-718). Compared with mCherry-Mis12-MAD1WT, deleting MAD1-NTD or CTD led to dramatic decrease in mitotic duration despite the presence of MIM domain and recruitment of MAD2 to kinetochores (Figure 4-1B). Our results suggest that both MAD1-NTD and CTD are required for maintaining a functional mitotic checkpoint.
Although some earlier reports have indicated the importance of CTD for MAD1 (Ballister, Riegman et al. 2014, Heinrich, Sewart et al. 2014), no direct connection has been established between MAD1-NTD and MAD2 O-C conversion. For a long time, it was believed that MAD1-NTD was only involved in MAD1 kinetochore localization (Chen, Shevchenko et al. 1998, Kim, Sun et al. 2012, Rodriguez-Bravo, Maciejowski et al. 2014). MAD1\(^{(485-718)}\) (NTD deletion) can form a complex with C-MAD2 to convert O-MAD2 to C-MAD2 \textit{in vitro} (Luo, Tang et al. 2002, Vink, Simonetta et al. 2006, Yang, Li et al. 2008). In addition, tethering MAD1\(^{(485-718)}\) (MIM+CTD) to kinetochores can maintain cells at mitosis for an average of 100 min, which is longer than the mitotic duration of untransfected cells (Figure 4-1B). However, tethering MAD1\(^{WT}\) to kinetochores cause a mitotic arrest longer than ~12 hours. Clearly, MAD1\(^{(485-718)}\) maintains some activity for catalyzing MAD2 O-C conversion, but the NTD is essential for full activity of MAD1.

We noticed that in IPs shown in Figure 4-1C and Figure 4-14C, MAD1 fragments lacking NTD or CTD seem to bind the same amount of MAD2 as compared to full-length MAD1, despite the \textit{in vitro} binding results showing NTD and CTD also bind to MAD2 (Figure 4-3). It is very likely that \textit{in vivo} the interaction between NTD or CTD and MAD2 is more dynamic and requires proper conformation of the full length MAD1 and regulatory factors such as MPS1 kinase. The IP only detects the stable interaction between C-MAD2 and MAD1-MIM. The interpretation provides an attractive working model to explain the temporal discord between cell cycle independent MAD2:MAD1-MIM interaction and the increased catalytic activity of the MAD1:MAD2 complex.
during mitosis, further emphasizing potential significance of understanding the newly identified interactions between MAD1-NTD or MAD1-CTD and MAD2 molecules.

In current models, the MAD1:C-MAD2 complex is a heterotetramer as MAD1 forms a complex with C-MAD2 through the MIM domain and meanwhile MAD1 dimerizes with another molecule of MAD1 (Sironi, Mapelli et al. 2002). Interestingly, we found that MAD1-CTD is required for dimerization of full-length MAD1 as deleting MAD1-CTD disrupts the association with endogenous MAD1 (Figure 4-1C). This result is different from a previous report, which stated that MAD1-CTD deletion does not influence the dimerization of MAD1 (Kim, Sun et al. 2012). However, in that study, the authors used Myc-MAD1\textsuperscript{ACTD}, whose size is very similar to endogenous MAD1, thus to distinguish the Myc-MAD1\textsuperscript{ACTD} signal from endogenous MAD1 is not easy. To further test whether MAD1-CTD is required for MAD1 dimerization, we can use a different tagged MAD1 construct, such as GFP-MAD1\textsuperscript{ACTD} (CTD deletion) construct. The dimerization of GFP-MAD1\textsuperscript{ACTD} with endogenous MAD1 will be tested. It is still surprising that deleting MAD1-CTD compromised MAD1 dimerization since MAD1-MIM alone adopts coiled coil structure and forms a dimer by itself (Sironi, Mapelli et al. 2002). This hints that dimerization of full-length MAD1 in cells might be regulated. It would also be interesting to test whether MAD1-NTD is required for MAD1 dimerization using the same technique described above.

The MAD1:C-MAD2 complex acts as a special enzyme to catalyze MAD2 O-C conversion. Mutating MAD1-MIM totally abolished MAD1 activity as mCherry-Mis12-MAD1\textsuperscript{AA} transfected cells showed no difference in mitotic duration from untransfected cells (Figure 4-1B), suggesting that MAD1-MIM is critical for MAD1 function. As
mCherry-Mis12-MAD1 ΔNTD or ΔCTD transfected cells showed significantly shorter mitotic arrest as compared with mCherry-Mis12-MAD1 WT, we reasoned that MAD1-NTD and CTD may contribute to the catalytic activity of the MAD1:C-MAD2 complex. To convert O-MAD2 to C-MAD2, the MAD1:C-MAD2 complex works similarly as isomerase, which form transient complexes with both substrates and products. Based on this principle, we reasoned that MAD1-NTD and CTD may interact with either O-MAD2 (substrate) or C-MAD2 (product) during catalyzing MAD2 O-C conversion. Indeed, the in vitro binding assays using recombinant proteins showed that both MAD1-NTD and CTD bind to not only C-MAD2 but also O-MAD2 directly. Structural or at least stoichiometrical information and reliable determination of the Kds using advanced biophysical techniques might be essential to further establish how NTD and CTD bind to MAD2 conformers and drive the MAD2 O-C conversion. Nevertheless, different MAD1 domains may coordinate with each other to catalyze MAD2 O-C conversion.

MAD1 was mostly described as a rigid coiled coil in previous studies. However, the secondary structure prediction clearly indicated that multiple segments of MAD1 can form structures other than a coiled coil (Figure 4-4A). Therefore, full length MAD1 may adopt a structure that allows interactions between different MAD1 domains. We found that MAD1-NTD interacts directly with CTD (Figure 4-6). The result strongly indicates that different MAD1 domains can communicate with each other.

To better characterize the newly discovered protein-protein interactions, we tried to map out MAD2 binding domains on MAD1-NTD and CTD, and the MAD1-CTD binding domains on MAD1-NTD. We started by making MAD1-NTD truncations based on secondary structure prediction. A series of in vitro binding assays indicated that the
integrity of MAD1-NTD is required for the interactions with both MAD2 and CTD (Figure 4-4C and Figure 4-7). Correspondingly, live cell imaging found that MAD1 lacking the first 327 amino acids showed defects in triggering the mitotic checkpoint (Figure 4-8), therefore the integrity of MAD1-NTD is also important to activate mitotic checkpoint.

Similar mapping was conducted on MAD1-CTD in order to find out the binding domain of MAD2 on MAD1-CTD. We found that MAD1-CTD globular domain binds more MAD2 compared with the coiled coil segment (Figure A-2). However, the purified MAD1-CTD truncations were not very stable as high level of degradation happened. To confirm the result that MAD1-CTD globular domain binds more MAD2, we need to improve the protein quality by either adding additional chromatography steps or changing the tag of the protein. The mitotic checkpoint function of full length MAD1 with CTD coiled-coil domain or CTD globular domain deleted can also be tested to better characterize the MAD1-CTD domain.

We also characterized the structural elements of MAD2 required for its binding to either MAD1-NTD or CTD (Figure 4-3B, 4-5). Since both C-MAD2 and O-MAD2 bind to NTD and CTD, the interaction most likely does not depend on MAD2 conformations (Figure 4-3B). Our results further revealed that the interaction does not depend on MAD2 dimerization domain either (Figure 4-5A). Future studies will further characterize which region of MAD2 is required for the interaction with either MAD1-NTD or CTD. For example, MAD2 S195D and V193N were reported to adopt the conformation as O-MAD2 (Mapelli, Massimiliano et al. 2007, Kim, Sun et al. 2010). We can further study whether MAD2 S195D or V193N interacts with either NTD or CTD.
5.2. MPS1 kinase promotes C-MAD2 production

Previously, our lab has demonstrated that BUBR1 directly interacts with C-MAD2 to form the MCC (Tipton, Tipton et al. 2011, Tipton, Wang et al. 2011). The results have been largely confirmed by the crystal structure of the S. pombe MCC and recent human MCC:APC/C structures (Tipton, Wang et al. 2011, Chao, Kulkarni et al. 2012, Alfieri, Chang et al. 2016).

Inhibition of MPS1 results in MAD2 dissociation from the BUBR1-BUB3-CDC20 ternary complex, thus compromising the mitotic checkpoint (Tipton, Ji et al. 2013). This result suggests that MPS1 promotes the production of C-MAD2 as C-MAD2 is selectively incorporated with the BUBR1-BUB3-CDC20 complex. MPS1 may work at a minimum of two discrete steps during C-MAD2 amplification. First, MPS1 targets the catalytic platform, the MAD1:C-MAD2 complex to unattached kinetochores. Second, MPS1 may modify the MAD1:C-MAD2 complex to activate or enhance its catalytic activity. The second mechanism is consistent with the result that MPS1 activity is still required for the mitotic checkpoint even when MAD1 is constitutively tethered at kinetochores by fusion with Mis12 (Maldonado and Kapoor 2011) (Figure 4-16C). Theoretically, MPS1 may work through increasing substrate (O-MAD2) binding, stimulating MAD2 O-C conversion or promoting product (C-MAD2) release.

Formation of the MAD1:C-MAD2 complex is cell cycle independent (Hardwick and Murray 1995, Campbell, Chan et al. 2001). However, only kinetochoore-localized MAD1:C-MAD2 complex can efficiently catalyze MAD2 O-C conversion, despite a recent report that in interphase cells nuclear envelope localized MAD1:C-MAD2 retains some catalytic activity (Rodriguez-Bravo, Maciejowski et al. 2014). We reasoned that
posttranslational modifications may activate the MAD1:C-MAD2 complex. Inhibition of MPS1 or Aurora B abolished the mitotic arrest caused by mCherry-Mis12-MAD1, suggesting that both MPS1 and Aurora B are required for the catalytic activity of the MAD1:C-MAD2 complex at kinetochores (Maldonado and Kapoor 2011). Using IF, we found that a fraction of O-MAD2 remains at unattached kinetochores in mCherry-Mis12-MAD1 expressing cells even when MPS1 is inhibited (Tipton, Ji et al. 2013) (Figure 4-10E). This result suggests that O-MAD2 recruitment to kinetochore is not enough to convert MAD2 from O to C. Instead, MPS1 kinase may directly affect the MAD2 O-C conversion.

To further dissect how MPS1 facilitates the catalysis of MAD2 O-C conversion by the MAD1:C-MAD2 complex, we tested whether MPS1 kinase can directly phosphorylate MAD1 or MAD2. The *in vitro* kinase assay indicated that MPS1 phosphorylates both MAD1-NTD and CTD (Figure 4-12A) and MPS1 does not phosphorylate MAD2 *in vitro* (Figure 4-12A). Mass spectrometry was used to identify eight putative phosphorylation sites on MAD1 by MPS1. Four sites are on MAD1-NTD (T8, S22, S62 and T323) while the other four are on MAD1-CTD (S598, S610, T624 and T716) (Figure 4-12 B).

To analyze whether these sites are important for the MAD1:C-MAD2 activity during mitosis, we mutated all eight sites to alanine on mCherry-Mis12-MAD1 to create a phospho-resistant MAD1 mutant. We found that MAD1<sup>8A</sup> cannot maintain mitotic arrest as efficiently as MAD1<sup>WT</sup>, suggesting that at least one of the putative sites is essential for MAD1 activity (Figure 4-13A). Further characterization indicated that the potential important sites lie among the four sites on MAD1-CTD as live cell imaging...
showed that mCherry-Mis12-MAD1_{CTD4A} cannot maintain mitotic arrest (Figure 4-13A). Consistently, MAD1-CTD_{4A} is defective in binding to either O-MAD2 or C-MAD2 \textit{in vitro} (Figure 4-14A). More interestingly, the dimerization of MAD1-CTD is also abolished between CTD_{4A} molecules (Figure 4-14B). These results confirmed the importance of the newly discovered interaction between CTD and MAD2 on the mitotic checkpoint.

However, it is also surprising to see that CTD_{4A} binds to less MAD2 compared with CTD_{WT} \textit{in vitro}. Supposedly, CTD_{4A} and CTD_{WT} should be considered as unphosphorylated \textit{in vitro} and both should perform similarly in terms of interacting with MAD2 or dimerization. We reasoned that at least one site among the four is critical for the interaction between CTD and MAD2 regardless of MPS1 phosphorylation. To test that, we mutated the 4 sites to glutamic acids to create phospho-mimic mutants. If our reasoning was correct, it would be expected that CTD_{4E} cannot interact with either O-MAD2 or C-MAD2 efficiently as one or more critical sites required for interacting with MAD2 were mutated. That turned out to be the case (Figure 4-15B). Interestingly, like mCherry-Mis12-MAD1_{CTD4A}, mCherry-Mis12-MAD1_{CTD4E} cannot maintain mitotic arrest as efficiently as WT. All these results consolidated the correlation of the capability of MAD1-CTD mutants to interact with MAD2 and their capability to support the mitotic checkpoint.

MPS1 phosphorylation on MAD1-CTD_{4A} was dramatically diminished in the \textit{in vitro} kinase assay, suggesting that our mass spectrometry covered almost all the sites on MAD1-CTD phosphorylated by MPS1 \textit{in vitro} (Figure 4-13B). However, whether those sites were all phosphorylated \textit{in vivo} or there are any additional phosphorylation sites by
MPS1 *in vivo* remain to be elucidated. It is possible that certain *in vivo* phosphorylation events by MPS1 kinase require priming phosphorylation, which is missing in the *in vitro* kinase assays. It is also possible that certain phosphorylation events on MAD1-CTD fragment by MPS1 kinase *in vitro* might not happen on full-length MAD1 *in vivo*. To find out *in vivo* phosphorylation sites on MAD1 potentially by MPS1 kinase, we can carry out MAD1 IPs using mitotic cell lysates treated with or without reversine and then subject the MAD1 IP samples to mass spectrometry analysis.

To further investigate the four putative MPS1 phosphorylation sites on MAD1-CTD, we created four groups of single-site mutations: CTD S598A/E, S610A/E, T624A/E and T716A/E. Even though our CTD$^{4E}$ as well as CTD$^{4A}$ showed mitotic checkpoint defects (Figure 4-13A, 4-15A), it remains possible that one or more sites among the four are phosphorylated by MPS1 during mitosis and the phosphorylation is essential for activating the MAD1:C-MAD2 complex, if another crucial site was left untouched. The mitotic durations of HeLa cells transfected with the mutants were recorded using live cell imaging. We found that S598 and T624 are not required for the MAD1:C-MAD2 activity during mitosis (Figure 4-16A). MAD1 S610A, S610E and T716A all showed defects in maintaining mitotic arrest compared with WT, suggesting that S610 and T716 are essential for the mitotic checkpoint (Figure 4-16A). Interestingly, MAD1$^{T716E}$ stayed in mitosis significantly longer than the T716A mutant (Figure 4-16A). The results suggested that phosphorylation at T716 by MPS1 may be required for MAD1 activity. On the other hand, MAD1 S610 may be the critical site that is required for the interaction between CTD and MAD2 or CTD dimerization. We noticed that the site is in proximity to Y634, another critical residue for MAD1 activity (Figure 4-1B). Further
characterization of MAD1 S610 is still needed. We noticed that the mitotic durations of MAD1 S610A/E and MAD1 T716A/E are distributed over a wide range. We have not decided whether this was caused by different protein expression levels or endogenous MAD1 but preliminary tests argued against these possibilities. We noticed a recent publication suggesting a positive feedback mechanism to control the duration of mitosis (Araujo, Gelens et al. 2016). It is unclear whether the positive feedback mechanism is perturbed when MAD1 is mutated.

Cells transfected with mCherry-Mis12-MAD1<sup>WT</sup> are arrested in mitosis, most likely due to "constitutive" generation of C-MAD2 by the MAD1:C-MAD2 complex at kinetochores (Maldonado and Kapoor 2011) (Figure 4-1B). However, MPS1 activity is required for the mitotic arrest caused by mCherry-Mis12-MAD1 as inhibiting MPS1 by reversine drives cells out of mitosis even when MAD1 is constitutively localized at kinetochores. If MAD1 phosphorylation of T716 is sufficient for maintaining active mitotic checkpoint, the phospho-mimic mutant MAD1<sup>T716E</sup> should bypass the inhibition of MPS1 by reversine. However, our results showed that reversine still drove cells transfected with mCherry-Mis12-MAD1<sup>T716E</sup> out of mitosis (Figure 4-16C). Besides the function of phosphorylating MAD1 during mitosis, MPS1 is required for activating the mitotic checkpoint through regulating many other important events such as the recruitment of BUB1 (Faesen and Musacchio 2015). It is possible that mimicking T716 phosphorylation alone is necessary but insufficient to bypass the inhibition of MPS1. It is also possible that the T716E mutant cannot faithfully mimic T716 phosphorylation.

As discussed above, different MAD1 domains may coordinate to catalyze MAD2 O-C conversion through facilitating O-MAD2 recruitment to and C-MAD2 release from
the catalyst. MPS1 phosphorylation may directly influence the interactions between MAD2 and MAD1-NTD or CTD or between MAD1-NTD and CTD. *In vitro* binding assay revealed that MPS1 kinase activity reduces the interaction between MAD1-NTD and CTD. Since MPS1 functions positively to promote C-MAD2 production, we reasoned that the interaction of MAD1-NTD and CTD may inhibit the catalytic activity of the MAD1:C-MAD2 complex by blocking O-MAD2 recruitment or C-MAD2 release (Figure 5-1). It would be interesting to test whether the interaction between NTD and CTD blocks MAD2 binding to CTD or NTD.

**Figure 5-1. Model on the MAD2 O-C conversion catalyzed by the MAD1:C-MAD2 complex.** The MAD1:C-MAD2 complex is inactive during interphase, when the MAD1-NTD binds to MAD1-CTD. During mitosis, active MPS1 kinase reduces the interaction between MAD1-NTD and CTD to activate the MAD1:C-MAD2 complex. Different MAD1 domains coordinate to catalyze the MAD2 O-C conversion.
While characterizing the effects of MPS1 on the novel protein-protein interactions, we found that MAD1-NTD and CTD directly interact with MPS1. The function of the interaction between MAD1 and MPS1 needs to be further characterized. To map the MAD1 binding region on MPS1 in vitro can be a good start. Following that, a MPS1 construct with MAD1 binding domain deleted can be created to study the function of the interaction in cells.

5.3. Kinases other than MPS1 regulate mitosis

Many kinases such as Aurora B and BUB1 are also essential for mitosis regulation (Musacchio 2015). In particular, Aurora B kinase activity seems also required for the MAD1:C-MAD2 activity at kinetochores (Maldonado and Kapoor 2011). However, the in vitro kinase assay showed that Aurora B does not directly phosphorylate MAD1 nor MAD2 (Figure 4-18). Aurora B may regulate the MAD1:C-MAD2 activity through activating MPS1 or recruiting other essential elements for mitotic checkpoint. How Aurora B regulates C-MAD2 production needs to be further investigated.

The other important kinase is BUB1. It remains controversial whether BUB1 directly regulates C-MAD2 production during mitosis. BUB1 directly phosphorylates human MAD1 in vitro (Seeley, Wang et al. 1999). BUB1 also interacts with MAD1 in yeast and C. elegans (London and Biggins 2014, Moyle, Kim et al. 2014). Knocking down BUB1 abolished the mitotic arrest caused by mCherry-Mis12-MAD1 (Figure 4-19), suggesting that BUB1 either directly regulates MAD1:C-MAD2 catalytic activity or regulates some events downstream of C-MAD2 production. Whether BUB1 regulates MAD1 directly also needs further investigation.
Small molecule inhibitors are widely used when studying protein kinases. They have also been extensively explored as targeted therapeutic agents for disease treatment. OTSSP 167 was developed as a potent inhibitor of MELK and is currently in clinical trials (Chung, Suzuki et al. 2012). We found that OTSSP167 abolished mitotic arrest caused by microtubule-targeted drugs (Figure 4-21). This prompted us to investigate a potential role of MELK in the mitotic checkpoint. Unfortunately, inhibiting MELK using OTSSP 167 and shRNA showed inconsistent results. Further studies revealed that like many other kinase inhibitors that are ATP analogs, OTSSP167 may exhibit unintended "off-target" effects against other kinases that are essential for mitosis regulation. We found that OTSSP167 is a relatively potent inhibitor of Aurora B kinase but its dramatic impact on mitosis progression cannot be fully explained by Aurora B inhibition alone (Figure 4-24 A, C). Our data showed that OTSSP167 also inhibits BUB1 and Haspin (Figure 4-24 D, E). A comprehensive kinase profiling of OTSSP167 was not available during the course of our work but off-target effects of MELK was indicated in a web source (http://www.kinase-screen.mrc.ac.uk/kinase-inhibitors) although inhibition of BUB1 and Haspin kinases were not tested there. Compromising the mitotic checkpoint could be an alternative mechanism for the cytotoxic effects of OTSSP167, distinct from its proposed mechanism by inhibiting MELK.

MELK has become an attractive target for novel anti-cancer therapy due to its characteristic overexpression in cancer cells and cancer stem cells (Carter, Eklund et al. 2006, Hebbard, Maurer et al. 2010, Du, Qu et al. 2014, Ganguly, Mohyeldin et al. 2015). Although the molecular functions of MELK remain to be fully characterized, several lines of evidence suggested it might play a role in mitosis that include G2/M transition or
cytokinesis (Davezac, Baldin et al. 2002, Gray, Jubb et al. 2005, Badouel, Korner et al. 2006, Badouel, Chartrain et al. 2010, Tipton, Wang et al. 2012). MELK knockdown showed that MELK does not have a major role in the mitotic checkpoint signaling. However, MELK kinase activity and protein level peak during prometaphase, implicating some function in this stage of mitosis. One possibility is that MELK is required to coordinate cell cortex changes with chromosome segregation. As reported before (Chartrain, Couturier et al. 2006, Tipton, Wang et al. 2012), in unperturbed mitosis a fraction of MELK is re-located to cell cortex after the metaphase-to-anaphase transition, concurring with lower activity of MELK. We observed premature cortex association of GFP-MELK in prometaphase cells when OTSSP167 was applied to cells and presumably inhibited MELK kinase activity. Therefore, it is possible that subcellular distribution of MELK activity is regulated by its kinase activity, and MELK activity might be important in maintaining the rounded shape of prometaphase cells. Future work will address this possibility with the help of several other recently characterized MELK small molecule inhibitors together with OTSSP167 (Mahasenan and Li 2012, Canevari, Re Depaolini et al. 2013, Chung and Nakamura 2013, Beke, Kig et al. 2015).

As previously described, the MAD1:C-MAD2 complex is cell cycle independent, however, it is only activated during mitosis, suggesting that the MAD1:C-MAD2 activity is cell cycle regulated. Phosphorylation is one of the most common post-translational modifications in the cell cycle. In addition to the putative MPS1 phosphorylation sites on MAD1, a public database (phosphosite.org) listed more MAD1 in vivo phosphorylation sites. To systematically study the function of MAD1 phosphorylation on the MAD1:C-MAD2 activity, we screened the mitotic checkpoint effects of the reported
MAD1 phosphorylation sites. We found that MAD1 Y634 is essential for MAD1 activity during mitosis. Mutating Y634 to E (an imperfect phospho-mimic mutant) abolished the mitotic checkpoint (Figure 4-1B). In constrast, mutating Y634 to F (phospho-resistant mutant) has no substantial mitotic checkpoint defects. The checkpoint defect caused by MAD1<sup>Y634E</sup> can be at least partially explained by the fact that MAD1<sup>Y634E</sup> showed compromised interaction with both O-MAD2 and C-MAD2 (Figure 4-26C). The results suggest that potentially a tyrosine kinase may phosphorylate MAD1 to facilitate mitotic checkpoint silencing. Although MAD1 Y634 is phosphorylated <i>in vivo</i>, whether the phosphorylation happens during the metaphase to anaphase transition needs to be further confirmed. Along the line, we noticed that multiple Abl kinase inhibitors caused mitotic arrest (Figure 4-27). Abl shRNA can be used to further confirm the result. To find out the tyrosine kinases that silence mitotic checkpoint, live cell imaging will be used to check mitotic duration of cells transfected with shRNAs. Whether Abl kinases directly phosphorylates MAD1 on Y634 should also be tested.

5.4. The second CDC20 binding domain of BUBR1 directly interacts with C-MAD2 and inhibits APC/C

BUBR1 acts as a scaffold for assembling MCC. The crystal structure of MCC from <i>S. pombe</i> shows that C-MAD2 directly interacts with BUBR1 and CDC20 (Chao, Kulkarni et al. 2012), which is consistent with our previous discovery for human MCC (Tipton, Wang et al. 2011). Although conserved, human MCC shows some difference from the yeast MCC. Human MCC contains BUB3 and human BUBR1 contains the BUB3 binding domain, second CDC20 binding domain and a pseudo kinase domain. The recently solved structures on human MCC:APC/C complexes revealed how different
BUBR1 segments interact with two CDC20 molecules (Alfieri, Chang et al. 2016, Yamaguchi, VanderLinden et al. 2016) (Figure 1-4), however, in the proposed model, BUB3 and a large part of BUBR1 cannot be detected.

We started to pay attention to the second CDC20 binding domain of BUBR1 more than four years ago. Using BUBR1 truncations, we confirmed that BUBR1\textsuperscript{(487-700)} associates with CDC20 but not APC/C subunits, which is consistent with the recent structural information (Figure 4-28B). Moreover, we found that BUBR1\textsuperscript{(487-700)} also directly interacts with C-MAD2 and p31\textsuperscript{comet} \textit{in vitro} (Figure 4-29A, 4-30). Adding recombinant BUBR1\textsuperscript{(487-700)} to the cell-free mitotic extract delays the degradation of Cyclin B, suggesting that BUBR1\textsuperscript{(487-700)} can inhibit APC/C (Figure 4-29B). Our discovery suggested there is still knowledge gap on the function of the second CDC20 binding domain in BUBR1 and better understanding of the domain would help future study on MCC assembly and MCC inhibition of the APC/C.


leucine zipper kinase is a key regulator of the proliferation of malignant brain tumors, including brain tumor stem cells." J Neurosci Res 86(1): 48-60.


Appendix A

Supplementary Data

Figure A-1. MAD1-NTD and CTD bind to both O-MAD2 and C-MAD2.

(A). GST tagged MAD1-NTD, or (B) GST tagged MAD1-MIM, or (C) GST tagged MAD1-CTD, or (D) GST tagged BUBR1\textsuperscript{(1-371)} was incubated with His-TEV-tagged MAD2\textsubscript{L13A} or MAD2\textsubscript{ΔC10} at 37 °C for 1 hr followed by GST pull down assay. In all four experiments, in the Control 1 lane, recombinant GST was added instead of MAD1 fragments. In the Control 2 lane, only GSH beads were incubated with MAD2.
Figure A-2. MAD1-CTD globular domain binds to MAD2.
GST tagged MAD1-CTD, MAD1-CTD$^{597-638}$ or MAD1-CTD$^{638-718}$ was incubated with either MAD2$^{L13A}$ or MAD2$^{ΔC10}$ at 37 °C followed by GST pull down assay. The samples were subjected to western blot analysis. GST and MAD2 were probed as indicated.

Figure A-3. MPS1 kinase activity does not influence the interactions between MAD2 and MAD1-CTD or MAD1-NTD.
(A). Recombinant GST-Mad1-CTD or GST purified from E. coli were incubated with MAD2$^{L13A}$ or MAD2$^{ΔC10}$, in the absence or presence of MPS1 and reversine (MPS1 inhibitor) and then GST pull down was performed. (B). Similar to (A) except recombinant GST-Mad1-NTD was used.
Figure A-4. Aurora B is inhibited by OTSSP167.
HeLa cells were arrested in mitosis with nocodazole (Noc) or taxol. The arrested cells were further treated with DMSO ("-"") or OTSSP167("+") and the lysates were subjected for western blot. The BUBR1, CDC20, BUB3, MAD2, MELK and pT232 on Aurora B were probed.
**Appendix B**

**Supplementary Tables**

**Supplemental Table B.1. Antibodies used in this study**

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Supplemental Table B.2. Results on the mitotic checkpoint responses of MAD1 phosphorylation site mutants

All mutants were made on the mCherry-Mis12-MAD1 backbone and tested for their capability in inducing mitotic arrest in HeLa cells after transfection. The phosphorylation sites were from Phosphosite Plus (Hornbeck, Zhang et al. 2015) or based on our mass spectrometry results.

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Appendix C

Publications, Abstracts and Posters

Publications


Abstracts and Posters

2016/12  56th Annual Meeting of American Society for Cell Biology, San Francisco
Title: "Different MAD1 domains coordinate to catalyze MAD2 O-C conversion"

2016/05  13th Biennial Meeting on The Cell Cycle, Cold Spring Harbor
Title: "Different MAD1 domains coordinate to catalyze MAD2 O-C conversion"

**Ji, W.**, Liu, S.
Title: "Regulation of the mitotic checkpoint by p31comet-BUBR1 and p31comet-TRIP13 interactions"

Wang, K., Ji, W., Tipton, A.R., Zhang, H., Sturt-Gillespie, B., Liu, S.