이학박사학위논문

세포분열 과정에서의 Mis18α 인산화에 대한 기능 연구

Functional studies on the phosphorylation of Mis18α in the regulation of cell division

2018년 2월

서울대학교 대학원

생명과학부

이민경

세포분열 과정에서의 Mis18α 인산화에 대한 기능 연구

Functional studies on the phosphorylation of Mis18α in the regulation of cell division

지도교수 백 성 희

이 논문을 이학 박사 학위논문으로 제출함

2017년 12월

서울대학교 대학원
생명과학부

이 민 경

이민경의 이학박사 학위논문을 인준함.
2017년 12월

위 원 장 강찬희 (인)
부위원장 백 성희 (인)
위 원 김 근일 (인)
위 원 김 건동 (인)
위 원 황 성순 (인)

Functional studies on the phosphorylation of Mis18α in the regulation of cell division

A dissertation submitted in partial fulfillment of the requirement for the degree of

DOCTOR OF PHILOSOPHY

TO THE FACULTY of
THE SCHOOL of BIOLOGICAL SCIENCES
at
SEOUL NATIONAL UNIVERSITY
by

Minkyoung Lee

Date approved

2017. 12. 26

Sung Key Baek

Functional studies on the phosphorylation of Mis18α in the regulation of cell division

by

Minkyoung Lee

Advisor

Professor Sung Hee Baek, Ph.D.

A Thesis for the Degree of Doctor of Philosophy

February, 2018

School of Biological Sciences

Seoul National University

ABSTRACT

Minkyoung Lee
School of biological sciences
The Graduate school
Seoul National University

Mis18α, a component of Mis18 complex comprising of Mis18α, Mis18β, and M18BP1, is known to localize at the centromere from late telophase to early G1 phase and plays a priming role in CENP-A deposition. Although its role in CENP-A deposition is well established, the other function of Mis18α remains unknown. Here, I elucidate a new function of Mis18α that is critical for the proper progression of cell cycle independent of its role in CENP-A deposition. I find that Aurora B kinase phosphorylates Mis18α during mitosis not affecting neither centromere localization of Mis18 complex nor centromere loading of CENP-A. However, the replacement of endogenous Mis18α by phosphorylation-defective mutant causes mitotic defects including micronuclei formation, chromosome misalignment, and the chromatin bridges or lagging chromatids. Interestingly, PLK1, another mitotic kinase, shows decreased recruitment in Mis18α phosphorylation-defective cell lines. PBD of PLK1 recognizes Mis18α phosphorylation so that its kinetochore recruitment can be enhanced. Together, my data demonstrate that Aurora B kinase-mediated mitotic phosphorylation of

Mis18α is a crucial event for faithful cell cycle progression through the enhanced recruitment of PLK1 to the kinetochore.

Key words:

Mis18α, Aurora B kinase, PLK1, Mitosis-specific phosphorylation, Polo Box Domain, kinetochore

Student number: 2009-22939

CONTENTS

LIST OF FIGURES AND TABLES

CHAPTER I

Introduction

I-1 Cell cycle

1.1 Introduction of cell cycle

Development from a single cell to multi-cellular organism needs many rounds of cell division and cells complete the division with an ordered series of events, called 'Cell cycle' (Figure I-1). Cell cycle is composed largely with DNA synthesis phase (S phase) and chromosome segregation phase (M phase). The somatic cells also have a gap phase before each phase, G1 phase before S phase and G2 phase before M phase. Dependent on environmental and developmental signals, cells in G1 may temporarily or permanently leave the cell cycle and enter a quiescent or arrested phase known as G0 (Otto and Sicinski, 2017).

1.2 Regulation of cell cycle

The cell cycle is a highly organized and regulated process that ensures duplication of genetic material and cell division. This regulation governed by growth-regulatory signals as well as signals from proteins that monitor any genetic damage (Fernández-Miranda et al., 2010). Cyclin-dependent kinases (CDKs) are master regulators of the cell cycle. CDKs are controlled by cyclins and the different CDKs are activated by different cyclins. As cyclin's expression is oscillated during cell cycle, each CDK shows cell cycle dependent activation. Besides CDKs, many kinases are involved in checkpoint which controls the cell cycle depending on external or internal signal of cell damage. These

kinases are primarily Polo-Like Kinases (PLKs) and Aurora Kinases. Unlike CDKs, which promote cell cycle progression, these kinases are responsible for avoiding errors during cell cycle progression. (Archambault and Glover, 2009; Barr et al., 2004; Carmena and Earnshaw, 2003; Fu et al., 2007)

For this reason, the phosphorylation is the most important protein modification during the cell cycle (Figure I-2). The kinases phosphorylates substrates on serine/ threonine/ tyrosine and the phosphorylated residue is recognized by "phospho-peptide binding domain" of other proteins. Therefore, the phosphorylation status triggers the assembly between proteins and the de-phosphorylation turns back to basal status. This signaling cascade by kinase and phosphatase is precise for cell cycle regulation. The blockade of this regulation induces cell cycle arrest or apoptosis in normal cell to protect cells from increasing genomic instability that is a cancer hallmark. In cancer, cells bypass this error and shows increased genomic instability such as aneuploidy in many cases.

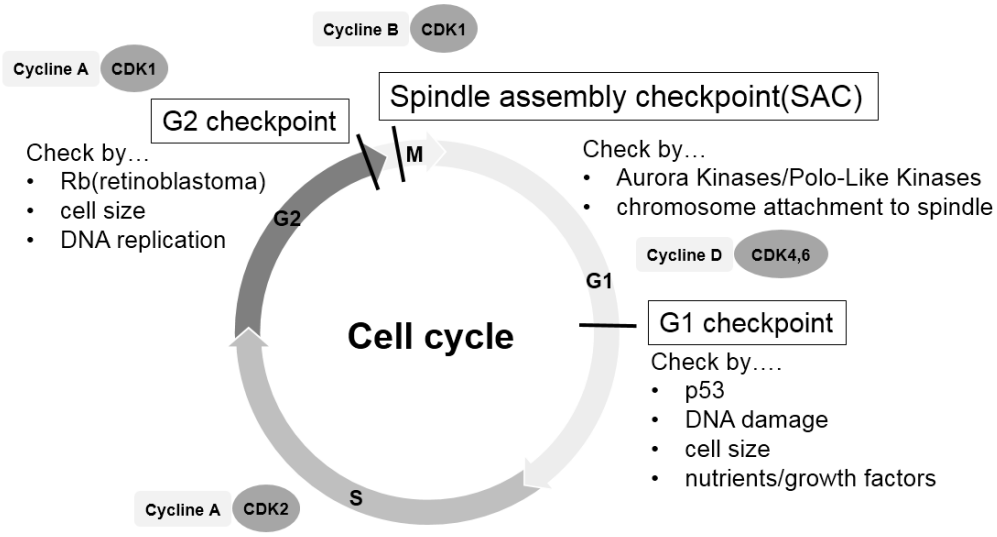


Figure I-1. Regulation of cell cycle

Cell cycle is largely divided into 2 phases including S phase and M phase. It is controlled by checkpoints that are activated at the interphase between two major phases. And spindle assembly checkpoint (SAC) is in M phase to control kinetochore-microtubule attachment. All these processes are regulated by several proteins which can detect physiological changes in cells.

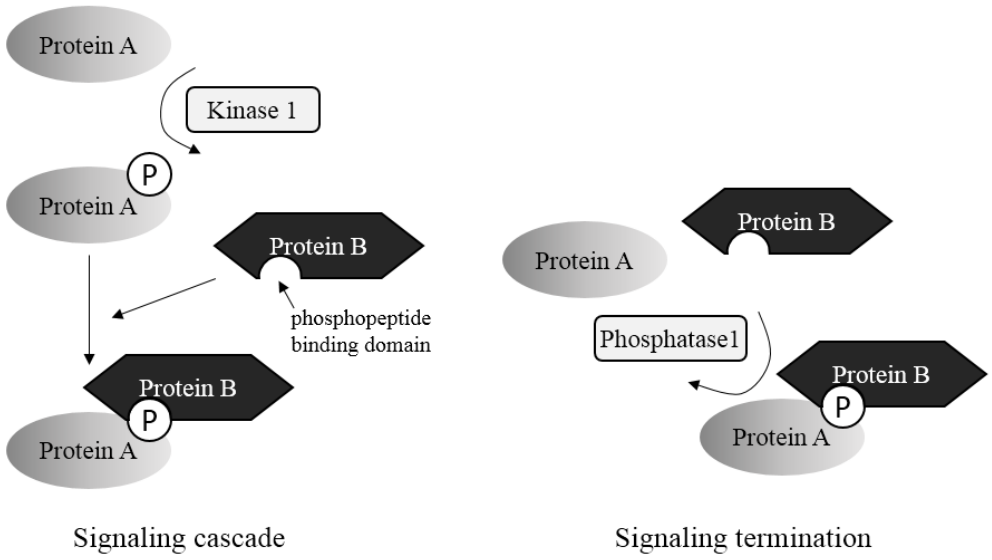


Figure I-2. Signaling cascade by kinase and phosphatase

During cell cycle, phosphorylation has a major role in controlling the signaling cascade. Phosphorylated protein is recognized by another protein by 'phospho-peptide binding domain' for activation and this cascade is terminated by phosphatase mediated de-phosphorylation.

I-2 Aurora B kinase

2.1 Functions of Aurora B kinase in cell cycle

Among mitotic kinases, Aurora serine/threonine kinases work crucially during mitosis. Aurora A kinase locates pericentrosome and regulates mitotic spindle assembly, centrosome separation and G2/M transition at the beginning of mitosis (Berdnik and Knoblich, 2002; Hirota et al., 2003). Aurora B kinase locates innercentromere from prometaphase to metaphase regulating chromatin modification and chromatid separation, and relocates to midzone for cytokinesis (Fu et al., 2007). During prometaphase, the mitotic spindle generates forces to align the sister chromatids at the metaphase plate pulling the sister chromatids in opposite directions for the same daughter cells in two. Kinetochores initially bind to spindles in any configuration, but each pair of sister kinetochores needs to ultimately attach to spindles in bi-orientation states for an accurate chromosome segregation (Figure I-3). Phosphorylation of Aurora B kinase targets in the innercentromere participates in this control by regulating the kinetochore-microtubule interaction. Bi-oriented spindles ensure the accurate segregation of chromosome and induces dephosphorylation of the Aurora B kinase targets giving strong tension between microtubule and kinetochore allowing the cells to go to anaphase (Lesage et al., 2011). During the cytokinesis, Aurora B kinase localizes to the cleavage furrow that possesses the intermediate filament proteins. In general, phosphorylation by Aurora B kinase destabilizes

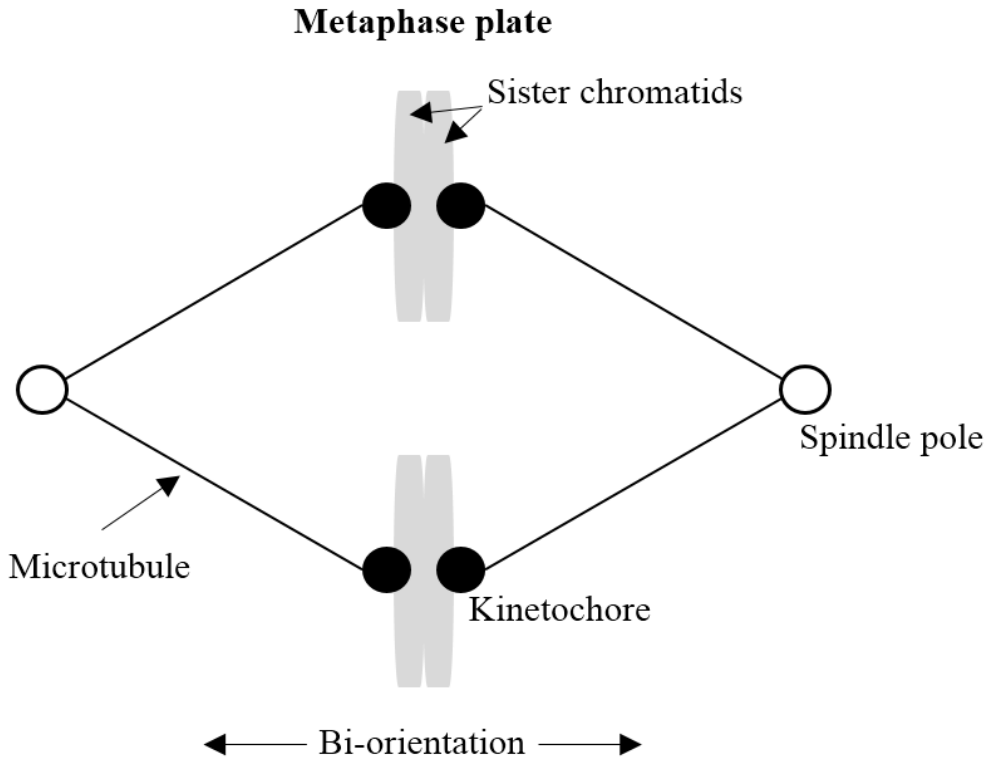


Figure I-3. Bi-oriented microtubules

During mitosis, chromosomes need to be aligned on a metaphase plate by kinetochore-microtubule interactions. Adequate interaction between kinetochore-microtubule achieves bi-orientated tension for an accurate chromosome segregation.

Intermediate filaments in preparation for cytokinesis. Failure in phosphorylation of targets at cleavage furrow leads to defects in filament deformation and prevents the final stage of cytokinesis. Aurora B kinase also phosphorylates myosin II regulatory light chain at the cleavage furrow. Improper phosphorylation of myosin II prevents its accurate localization to the cleavage furrow and disrupts spindle midzone organization (Goto et al., 2003; Kawajiri et al., 2003; Mackay et al., 1998; Terada et al., 1998).

2.2 Substrates of Aurora B kinase

It initially has been found that Aurora-B phosphorylates histone H3 at Ser10 and Ser 28 (Goto et al., 2002; Hsu et al., 2000) as well as H3 variant CENP-A at Ser 7 (Kunitoku et al., 2003; Zeitlin et al., 2001) in mitosis. H3S10 phosphorylation is now used as a positive marker in Aurora-B kinase assay; yet, the function remains largely unknown. H3S10 phosphorylation seems to be required for chromosome condensation (Sawicka and Seiser, 2014; Wei et al., 1998; Wei et al., 1999; Wilkins et al., 2014) but there is a controversial in mammalian cells (Equilibrina et al., 2015; Wang et al., 2013). A recent study showed that H3S10 phosphorylation by Aurora-B allows the disassociation of HP1 from heterochromatin (Fischle et al., 2005; Hirota et al., 2005), by facilitating chromosome condensation, or by serving as the indicator for the mitotic checkpoint to control proper cell division (Li et al., 2006). However, the exact reason of HP1 release in mitosis is still unclear. Interestingly, Aurora-A also phosphorylates CENP-A (Kunitoku et al., 2003). Phosphorylation of CENP-A by Aurora-A at Ser^{7} in prophase is required for Aurora-B concentration to inner centromeres in prometaphase. Maintenance

of later CENP-A phosphorylation state depends on Aurora-B activity and is required for proper kinetochore-microtubule attachment (Kunitoku et al., 2003). It would be meaningful to explore how Aurora-A and Aurora-B cooperate in understanding the subtle regulation of mitosis.

Recently, the studies showed that Aurora-B phosphorylates centromeric MCAK to inhibit its microtubule depolymerization activity during the mitotic spindle assembly (Andrews et al., 2004; Lan et al., 2004). When the kinetochore-microtubule attachment is properly bi-oriented, Aurora-B phosphorylates MCAK for a stable attachment of microtubules onto kinetochore. Aurora B kinase-PLK1-MCAK (mitotic centromere-associated kinesin) axis has also been shown to be required for accurate chromosome segregation (Shao et al., 2015). At the kinetochore, Aurora B kinase activates PLK1 by phosphorylation and the activated PLK1 in turn phosphorylates MCAK, which is essential for accrurate chromosome segregation with its increased microtubule depolymerase activity. Inhibition of either Aurora B kinase or PLK1 reduces MCAK phosphorylation on PLK1 target sites and induces formation of impolar mitotic spindle and the chromatin bridges.

I-3 PLK1

3.1 Functions of PLK1 in cell cycle

Polo kinases from different organisms have common but precise functions in cell division for the conserved cellular roles. The main kinase among all Polo kinases is PLK1. The Polo kinases regulate the set of conserved, oscillating enzymatic activities that drive cell division (Barr et al., 2004; Petronczki et al., 2008; van de Weerdt and Medema, 2006; van Vugt and Medema, 2005). Entry into M phase is controlled through the activation of cyclin-dependent kinase 1 (CDK1)–cyclin B, the protein kinase that regulates the mitosis. CDK1 is inactivated by Wee1/MYT1 medicated phosphorylation during interphase and CDC25 dephosphorylates this phosphorylation to activate CDK1 during mitosis. In this regulation, PLK1 has a function in the positive-feedback loop as phosphorylating CDC25 for translocation to nucleus at prophase and thereby dephosphorylating of inactivated CDK1(Toyoshima-Morimoto et al., 2002). At the same time, PLK1 also phosphorylates Wee1/MYT1 for blocking inactive phosphorylation of CDK1 (Figure I-4).

Approximately 5 h before mitosis, PLK1 activity starts to rise and activation of PLK1 at this time in G2 phase requires Aurora A to phosphorylate Thr210 in the activation loop of its kinase domain depending on the binding of PLK1 cofactor Bora (Jang et al., 2002; Macurek et al., 2008; Seki et al., 2008). The precise regulation of the Bora–PLK1 interaction is unclear, but seems to be initiated by CDK1-dependent

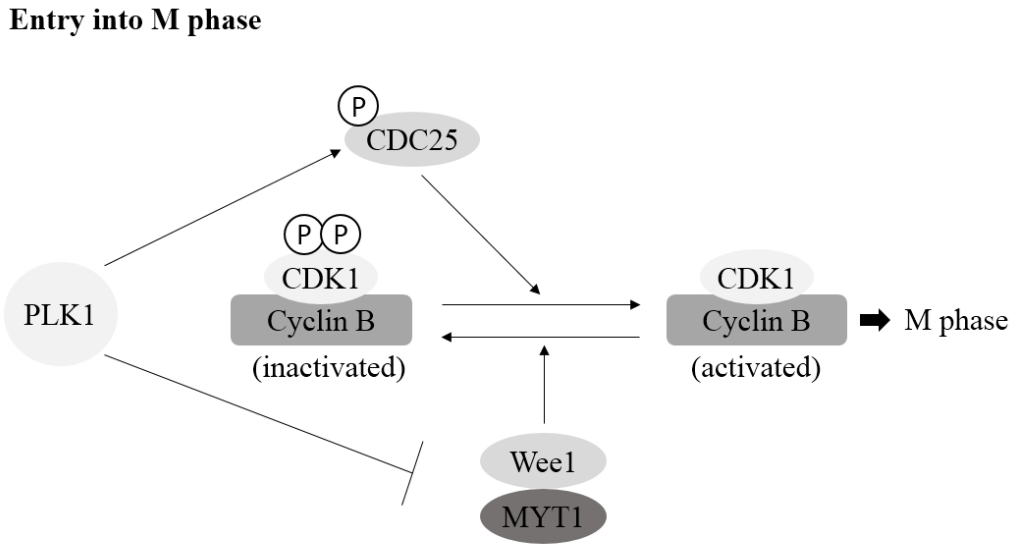


Figure I-4. Functions of PLK1 at mitosis entry

CDK1 is phosphorylated by Wee1/MYT1 during interphase for inactivation and it is de-phosphorylated at mitotic entry by CDC25. In this regulation, PLK1 has a crucial role by activating CDC25 and inhibiting Wee1/MYT1 at mitotic entry.

phosphorylation of Bora (Chan et al., 2008). This suggests that CDK1 activity is required for the activation of PLK1 in G2 phase, whereas Plk1 activation in turn is required for the activation of CDK1-Cyclin B. This type of regulation is often seen in cell cycle transitions, which typically depend on such interlinked feedback and feed-forward loops to establish bistable systems that render cell cycle transitions irreversible.

At the beginning of mitosis, the nuclear envelope disappears and thereby the possibility of distinct compartmental regulation of PLK1 also dissolves. In addition, Bora is degraded just prior to mitotic entry in a PLK1- and SCF-bTrCP-dependentmanner. Regulation of Aurora A is taken over by another cofactor, targeting protein for Xklp2 (TPX2), which targets Aurora A to the mitotic spindle (Kufer et al., 2002). Around the same time, regulation of PLK1 activity seems to switch from being Aurora A dependent to Aurora A independent. Interestingly, recent study has found that Drosophila Aurora B kinase is able to phosphorylate Polo at residue Thr182, the equivalent of human Thr210, and that this Aurora-B-dependent regulation is potentially conserved in mammalian cells (Carmena et al., 2012).

Once centrosomes are separated and a cell enters prometaphase it has to break down its nuclear envelope (NEB) to allow the association of the chromosomes with the mitotic spindle. After NEB, PLK1 remains at the centrosomes and kinetochores where it functions to regulate kinetochore–microtubule attachment (Lenart et al., 2007). Early association of PLK1 with the kinetochore depends on polo-box-interacting protein (PBIP)1 (Kang et al., 2006b). However, PBIP1 is degraded early in mitosis in a PLK1-dependent manner. After degradation of PBIP1, PLK1 is retained at the kinetochores and

several kinetochore/centromere-associated proteins have been shown to contribute to its localization (Goto et al., 2006; Qi et al., 2006). Although this phosphorylation coincides with a lack of tension at the kinetochores, the exact functional implication remains to be determined. Besides regulating kinetochore-microtubule attachments, PLK1 functions also early in mitosis to activate the anaphase promoting complex/cyclosome (APC/C). PLK1 phosphorylates the APC/C inhibitor early mitotic inhibitor 1 (Emi1), for facilitating the recognition by its ubiquitin ligase SCF-bTrCP (Hansen et al., 2004; Moshe et al., 2004). Emi1 degradation by this interaction allows the APC/C to become active. However, non-degradable mutant of Emi 1 did not show any defect in cyclin A, cyclin B1 and securin degradation suggesting PLK1-mediated destruction of Emi1 is not a prerequisite for APC/C activation at mitotic onset (Di Fiore and Pines, 2007).

Furthermore, PLK1 is inactivated in response to DNA damage to resume the cell cycle (Smits et al., 2000) and PLK1 is activated by the Aurora A kinase and its activator BorA27 to promote mitotic entry after the DNA damage response as recovery (Macurek et al., 2008; van Vugt et al., 2004). Coordinating the time of entry into M phase in response to cellular physiology seems to be its ancient function of PLK1 as a central role.

3.2 Physical interaction with the substrates

PLK1 consists of a kinase domain and two polo box regions. The two polo box regions fold together to form a polo box domain (PBD), a functional domain that can bind phosphorylated peptides. This priming phosphorylation is mainly provided by CDK1 but also Plk1 self-priming phosphorylation (Elia et al., 2003a; Elia et al., 2003b; Neef et al.,

2007) (Figure I-5). This mechanism ensures targeted substrate recognition and recruitment of PLK1 to selected sites within the cell. It provides a very important aspect of spatiotemporal control for PLK1, because it allows sequential recruitment to distinct substrates through timely phosphorylation of the respective peptides that create a PBD-binding site. Many of its interactions and substrates have been studied extensively (Archambault and Glover, 2009), and analysis of interaction partners of the PBD of Plk1 shows that it interacts with proteins acting in a wide variety of processes, many of which are still unexplored (Lowery et al., 2007). Indeed, several recent papers have been published that implicate Plk1 in novel processes, providing more insight into its functions and the regulation of its activity.

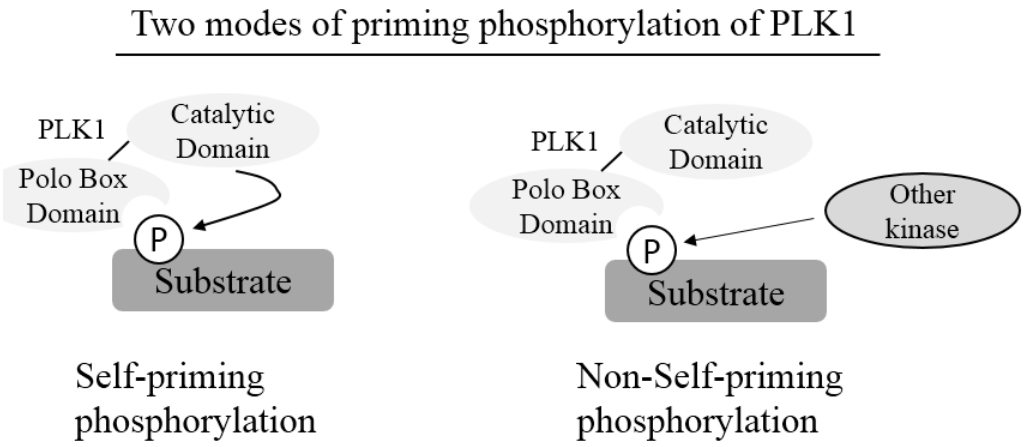


Figure I-5. PLK1 activation by priming phosphorylation

PLK1 is activated by recognizing the phosphorylated peptide and this phosphorylation is achieved by two modes. In self-priming phosphorylation, PLK1 phosphorylates substrate and itself recognizes this phosphorylation by PBD. In non-self-priming phosphorylation, substrate is phosphorylated by other kinase, such as CDK1, and PLK1 recognizes this phosphorylation by PBD.

I-4 Mis18α

4.1 Previous studies on Mis18α

A BLAST search revealed that all of the vertebrate genomes examined encode one or two similar proteins to S. pombe spMis18. In human, two Mis18s are designated Mis18α and Mis18β, respectively. Similar proteins have not yet been found in other fungi, fly, or nematode,as the sequence similarity may be rather low (Fujita et al., 2007). Fujita et al. observed Mis18α localization by GFP tag expression and found that it concentrates on centromeres during mitotic exit beginning in anaphase/telophase. They applied RNAi mediated knockdown of Mis18α to clarify the function of Mis18α in HeLa cells and found the increase in metaphase misalignment of chromosome. And 72hrs after RNAi, CENP-A signal was barely seen at centromere. CENP-A is a histone H3 variant that determines centromere location in eukaryotes (Earnshaw and Rothfield, 1985; Palmer et al., 1987) and every cell cycle recruits newly synthesized CENP-A by several proteins to maintain the centromere identity (Dunleavy et al., 2009; Foltz et al., 2006). Therefore, CENP-A mis-regulation is frequently related to cancer (Tomonaga et al., 2003). Interestingly, this disappearance was recovered by HDAC inhibitor, TSA treatment suggesting the possibility of epigenetic regulation of CENP-A centromeric localization.

Although CENP-A centromeric localization is regulated by Mis18α, they do not bind each other. Therefore, it strengthened the possibility of epigenetic regulation by Mis18α. With this hypothesis, Kim et al. generated *Mis18α* conditional knockout mice

and found mis-localization of CENP-A in blastocysts leading to early embryonic lethality of the mice. Histone modification patterns were also altered, concomitant with increased noncoding transcripts at centromere region. They identified DNMT3A/3B as interacting partners of Mis18α (Figure I-6), and their interaction turned out to be crucial for maintaining methylation status of centromeric DNA and licensing CENP-A loading (Kim et al., 2012).

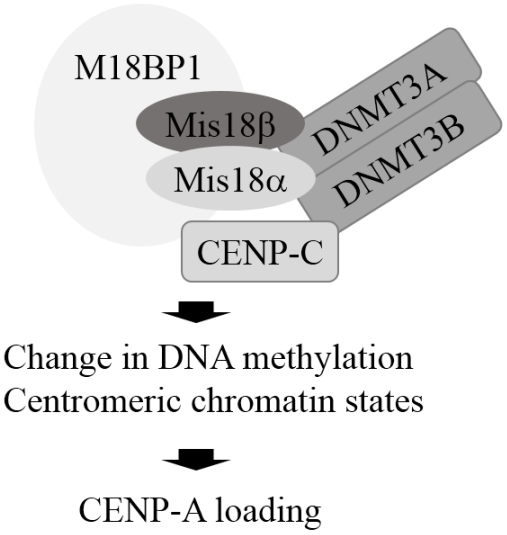


Figure I-6. Epigenetic regulation of CENP-A loading by Mis18α

Mis18α/Mis18β complex interacts with DNMT3A/DNMT3B by LRR sequence in Mis18α. This interaction leads to change in DNA methylation and centromeric chromatin states. Mis-regulation of epigenetic states by Mis18α knockdown or DNMT3A/3B loss causes critically decreased CENP-A loading.

CHAPTER II.

Mis18α is phosphorylated by Aurora B kinase during mitosis

II-1. Summary

Recent studies revealed that Mis18α functions in newly synthesized CENP-A deposition during telophase to G1 phase to assure the accurate chromosome segregation. However, none of studies revealed the upstream mechanisms of Mis18α regulation during cell cycle.

In the present study, I focus on the upstream signaling of Mis18α function especially by phosphorylation. In cell cycle, many proteins are regulated by several kinases and phosphorylation status is usually oscillated for a timely regulated cell cycle function. For this reason, I synchronize cells by double thymidine block, which arrests cells at S phase, and released for hours to observe the phosphorylation pattern of Mis18α. Interestingly, Mis18α is phosphorylated at mitotic phase and phosphorylation is disappeared as the cells exit mitosis. With this result, I hypothesize that Mis18α would have a role in mitosis by phosphorylation besides the regulation of CENP-A deposition.

To discrete the function, I screen the kinases, which are responsible for the mitotic phosphorylation of Mis18α and find that Aurora B kinase interacts with Mis18α and directly phosphorylates Mis18α on Ser36 in human/Ser13 in mice. The stable cell lines of Mis18α SA mutants show the increased mitotic defects including micronuclei, misalignment of chromosome and the chromatin bridges or lagging chromatids. However, newly synthesized CENP-A deposition is not changed in this stable cell lines of Mis18α SA mutants suggesting the possibility of other targets of Mis18α phosphorylation at mitosis. Here, I confirm the Mis18α phosphorylation during

mitosis with various methods and prove that mitotic defects shown in stable cell lines of Mis18α SA mutants are not caused by the failure of newly synthesized CENP-A deposition.

II-2. Introduction

Accurate segregation of duplicated chromosomes is crucial for daughter cells to have one copy of each chromosome during cell division. To complete accurate segregation, the chromosome should be condensed properly and mitotic spindle should bind to kinetochore bi-oriented (Lesage et al., 2011). The kinetochore is formed on a chromosomal locus called centromere that is composed of DNA segments and histone proteins containing centromere-specific H3 variant, CENP-A (Cleveland et al., 2003). Mis18 complex (Mis18α, Mis18β and M18BP1) in higher eukaryote is a critical factor for the recruitment of newly synthesized CENP-A to the centromere at early G1 phase (Fujita et al., 2007; Kim et al., 2012).

Previously, it has been reported that deletion of *Mis18α* in mice causes embryonic lethality as well as defect in epidermal stratification, which are accompanied with CENP-A loss at the centromere and defects in chromosome segregation (Kim et al., 2012; Park et al., 2017). Mis18 complex localizes to the centromere from telophase to early G1 phase of cell cycle prior to the CENP-A deposition to centromere (Fujita et al., 2007; Silva and Jansen, 2009). Phosphorylation of M18BP1 is involved in the regulation of the timing of centromere localization and licensing function of Mis18 complex. CDK1/2-mediated phosphorylation of M18BP1 on multiple sites blocks its interaction with Mis18α/Mis18β and hence centromere localization during S/G2/M phases, whereas phosphorylation of M18BP1 by PLK1 at early G1 phase facilitates centromere localization of Mis18 complex and its licensing function (McKinley and Cheeseman,

2014; Silva et al., 2012).

Among mitotic kinases, Aurora serine/threonine kinases work crucially during mitosis. Aurora A kinase locates pericentrosome and regulates mitotic spindle assembly, centrosome separation and G2/M transition at the beginning of mitosis (Berdnik and Knoblich, 2002; Hirota et al., 2003). Aurora B kinase locates innercentromere from prometaphase to metaphase regulating chromatin modification and chromatid separation, and relocates to midzone for cytokinesis (Fu et al., 2007). Phosphorylation of Aurora B kinase targets in the innercentromere participates in spindle checkpoint and regulates the kinetochore-microtubule interaction (Cheeseman et al., 2002; Lampson and Cheeseman, 2011). Dephosphorylation of the Aurora B kinase targets gives strong tension between microtubule and kinetochore allowing the cells to go to anaphase (Lesage et al., 2011).

Here, I presented the new mechanism of Mis18α regulation during mitosis and revealed it has a distinct role during mitosis besides its ancient role in CENP-A deposition. Mis18α was phosphorylated by Aurora B kinase depending on its activity during mitosis. Mis18α has a consensus sequence of Aurora B kinase and LC-MS/MS analysis revealed that Ser36 is the phosphorylation site. Mutation of Ser36 to Alanine did not change the Mis18 complex formation and newly synthesized CENP-A deposition; however, dramatically increased in mitotic defects. I confirmed this phenotype also in *Mis18α*$^{f/f}$*/ESR-Cre* SA MEFs suggesting the role of Mis18α phosphorylation is conserved among organisms.

II-3. Result

Mis18α is phosphorylated during mitosis by Aurora B kinase

Although Mis18α has been shown to function as a licensing factor for the recruitment of newly synthesized CENP-A to centromere at G1 phase, whether Mis18α is involved in the processes of cell division cycle has not been investigated. As Mis18α protein level is not changed through the cell cycle, I anticipated that post-translational modification of Mis18α might act as a signal for the regulating Mis18α function. Therefore, I analyzed whether Mis18α is phosphorylated during cell cycle progression by the mitotic kinases that actively regulate mitosis. HeLa cells stably expressing Flag-Mis18α were mitotically synchronized by nocodazole treatment and the phosphorylation level of Mis18α was analyzed. Interestingly, I detected increased phosphorylation level of Mis18α from the mitotic cell extracts comparable to the H3S10 phosphorylation, a mitotic marker (Figure II-1A). Consistently, Mis18α phosphorylation increased at mitotic phase after release from G1/S cell cycle synchronization by double thymidine block (Figure II-1B), confirming mitosis-specific phosphorylation of Mis18α. I next screened for potential kinases that are responsible for Mis18α phosphorylation during mitosis. Among several mitotic kinases tested, only Aurora B kinase was able to phosphorylate Mis18α (Figure II-1C). I also found the increased binding between Mis18α and Aurora B kinase during mitosis (Figure II-1D and II-1E), which matches

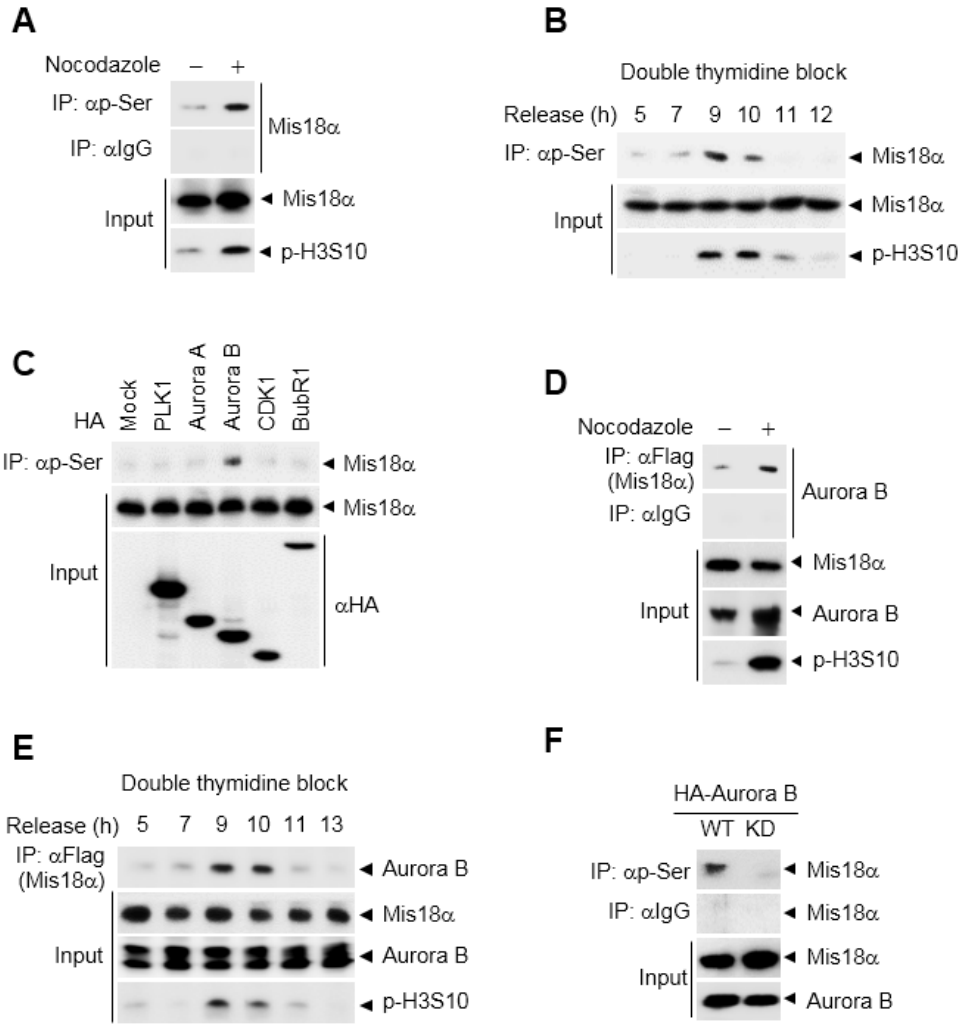

A
Nocodazole – +
IP: αp-Ser
IP: αIgG
Mis18α
Input
Mis18α
p-H3S10
B
Double thymidine block
Release (h) 5 7 9 10 11 12
IP: αp-Ser
Mis18α
Input
Mis18α
p-H3S10
C
HA Mock PLK1 Aurora A Aurora B CDK1 BubR1
IP: αp-Ser
Mis18α
Mis18α
Input
αHA
D
Nocodazole – +
IP: αFlag (Mis18α)
IP: αIgG
Aurora B
Input
Mis18α
Aurora B
p-H3S10
E
Double thymidine block
Release (h) 5 7 9 10 11 13
IP: αFlag (Mis18α)
Aurora B
Input
Mis18α
Aurora B
p-H3S10
F
HA-Aurora B
WT KD
IP: αp-Ser
Mis18α
IP: αIgG
Mis18α
Input
Mis18α
Aurora B

Figure II-1. Mis18α is phosphorylated during mitosis

A. HeLa cells stably expressing Flag-Mis18α (HeLa/Flag-Mis18α) were synchronized by nocodazole treatment. Cell extracts were subjected to immunoprecipitation (IP) with an antibody against phosphorylated-serine (p-Ser) followed by immunoblotting with anti-Flag antibody. Phosphorylation of ser10 of histone H3 (p-H3S10) was used as a mitosis indicator. **B.** HeLa/Flag-Mis18α cells were synchronized at G1/S by double thymidine block and released into indicated time points and were analyzed as in **A**. **C.** Mitotic kinases were transfected into HeLa/Flag-Mis18α cells and cell extracts were applied to IP with the anti-p-Ser antibody followed by immunoblotting with anti-Flag antibody. **D.** Mitotically arrested HeLa/Flag-Mis18α cells with nocodazole treatment were applied for IP with anti-Flag antibody and detected with anti-Aurora B antibody. **E.** HeLa/Flag-Mis18α cells prepared as in **B** were used for IP assay with anti-Flag antibody and detected with anti-Aurora B antibody. **F.** HeLa/Flag-Mis18α cells transfected with Aurora B wild-type (Aurora B WT) or K160A kinase dead mutant (Aurora B KD) were used for IP with anti-p-Ser antibody.

well with the phosphorylation pattern of Mis18α. Furthermore, the kinase dead (KD) mutant of Aurora B kinase failed to phosphorylate Mis18α (Figure II-1F), indicating that Aurora B kinase activity is crucial for Mis18α phosphorylation.

Next, I searched for the phosphorylation site by Aurora B kinase in Mis18α. LC-MS/MS analysis of Mis18α from mitotically synchronized 293T cells confirmed Ser36 as the phosphorylation site during mitosis (Figure II-2). And it was in a consensus sequence of Aurora B kinase, [R/K]-X-[S/T], and found that Mis18α contains only one serine residue that matches with the consensus sequence in both mouse (Ser13) and human (Ser36) (Figure II-3A). Thus, I generated phosphorylation-specific antibody against the peptide of Mis18α and the resulting antibody detected phosphorylated form of peptide much stronger than non-phospho peptide control (Figure II-3B). I then evaluated the specificity of our phospho-specific Mis18α antibody. The antibody detected a specific band corresponding to Mis18α only in nocodazole-treated cell extracts and λ-phosphatase (a Ser/Thr/Tyr phosphatase) treatment abolished the signal (Figure II-4A). In addition, the antibody efficiently detected the increased phosphorylation of Mis18α WT in nocodazole-arrested cells. However, the expression of Mis18α SA, a mutant Mis18α containing alanine substitution of Ser36, did not give rise to any significant signal (Figure II-4B). In an attempt to clarify the time of Mis18α Ser36 phosphorylation with this new phospho-specific antibody, the antibody generated the strongest signal during mitosis in consistency with H3S10 phosphorylation (Figure II-5A). Furthermore, synchronization of cells by mitotic drugs other than nocodazole,

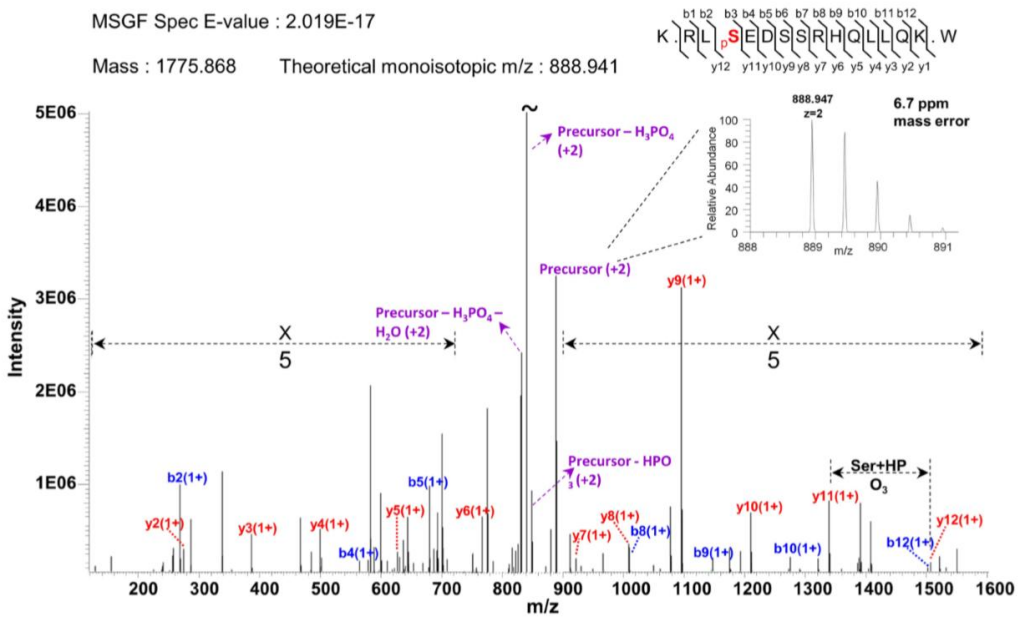


Figure II-2. LC-MS/MS analysis of Mis18α phosphorylation in mitosis

Flag-Mis18α was overexpressed in 293T cells and the cells were treated with nocodazole for 15 h. The lysates were subjected to overnight incubation with anti-Flag M2 beads. The beads were washed with lysis buffer and eluted with 3xFlag peptide. The elute was subjected to SDS-PAGE and the gel slice corresponding to Flag-Mis18α was used for subsequent LC-MS/MS analysis.

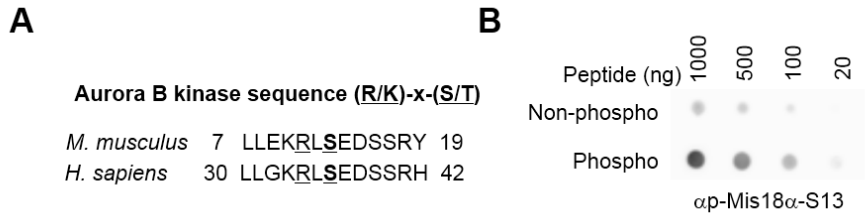


Figure II-3. Generation of phosphor-specific antibody of Mis18α

A. Aurora B kinase consensus sequences in mouse and human Mis18α. **B.** Dot blot analysis for a phosphorylation-specific antibody of Mis18α on Ser36 residue (p-Mis18α) by comparing non-phospho peptide with phospho-peptide at indicated concentrations.

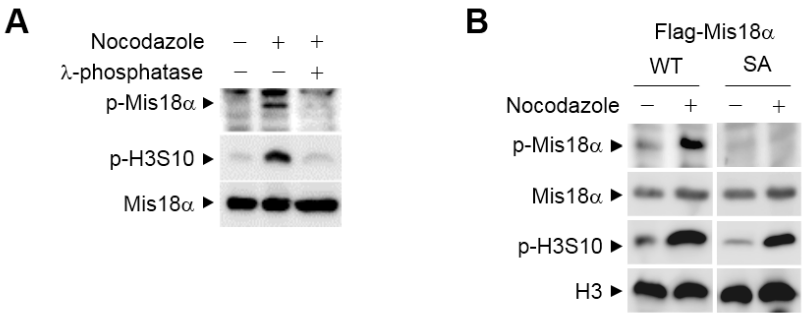


Figure II-4. A. Confirmation of phosphor-specific antibody of Mis18α

A. Extracts from 293T cells transfected with Flag-Mis18α were treated with λ-phosphatase and used for immunoblotting with anti-p-Mis18α antibody. **B.** 293T cells were transfected with Flag-Mis18α WT, Flag-Mis18αSA and synchronized by nocodazole treatment. Cell extracts were used for immunoblotting with anti-p-Mis18α antibody.

such as monastrol and taxol, increased phosphorylation of Mis18α (Figure II-5B). In an *in vitro* kinase assay with bacterially expressed Mis18α, purified active Aurora B kinase phosphorylated wild-type Mis18α but not Mis18α SA (Figure II-6A). Mis18α phosphorylation increased in mitotic cells, but decreased as the cells exited mitosis (Figure II-7A). MG132, which induces metaphase arrest by inhibiting APC-mediated proteolysis (Iimori et al., 2016), maintained Mis18α phosphorylation in parallel with H3S10 phosphorylation, although the cells were released from nocodazole-mediated arrest (Figure II-7B). Concurrently, the binding between Aurora B kinase and Mis18α increased during mitosis and decreased as the cells exited mitosis, but not under APC block, in parallel with Mis18α phosphorylation pattern (Figure II-7C). Moreover, the treatment of cells with Aurora B kinase inhibitor, Hesperadin (Sessa et al., 2005) diminished the phosphorylation of Mis18α induced by mitotic arrest (Figure II-7D), indicating that Aurora B kinase is responsible for the phosphorylation of Mis18α. Taken together, these results indicate that the phosphorylation of Mis18α is a mitosis-specific event mediated by Aurora B kinase.

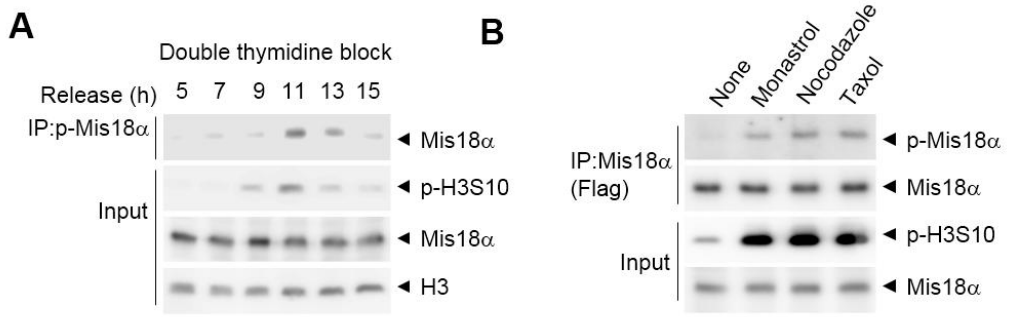


Figure II-5. Mitosis specific phosphorylation of Mis18α was confirmed by cell synchronization

A. HeLa cells stably expressing Flag-Mis18α (HeLa/Flag-Mis18α) were synchronized at G1/S by double thymidine block and released into indicated time points. Cell lysates were subjected to immunoprecipitation with anti-p-Mis18α antibody. **B.** HeLa/Flag-Mis18α cells were synchronized by monastrol, nocodazole, or taxol and the cells were collected by shake-off. Cell lysates were subjected to immunoprecipitation with anti-Flag (Mis18α) antibody and analyzed by immunoblotting with anti-p-Mis18α antibody.

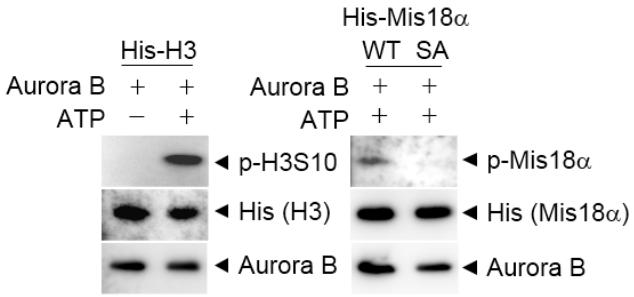


Figure II-6. *In vitro* kinase assay using purified Aurora B kinase

Recombinant His-H3 or His-Mis18α were incubated with purified Aurora B kinase in the presence of ATP for 30 min at 30°C for i*n vitro* kinase assay. Phosphorylation of Mis18α was detected using anti-p-Mis18α.

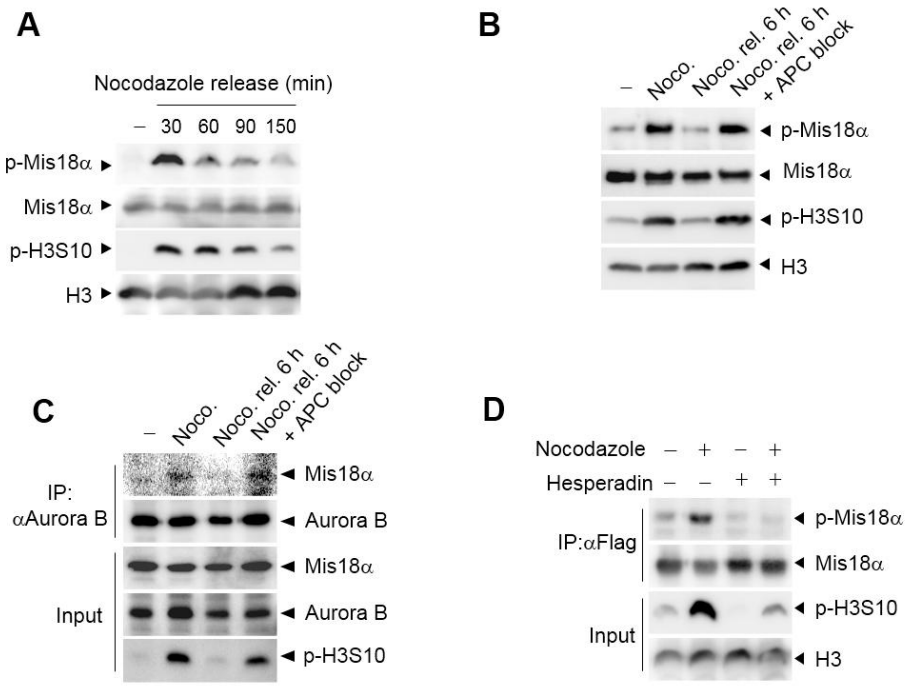


Figure II-7. Mis18α phosphorylation decreased as the cells exit mitosis

A. 293T cells expressing Flag-Mis18α WT were synchronized by nocodazole treatment. After releasing, cells were harvested at indicated time points and applied for immunoblotting. **B.** 293T cells expressing Flag-Mis18α WT were released for 6 h from nocodazole-mediated synchronization with or without MG132 treatment to block APC activity. **C.** HeLa/Flag-Mis18α cells were released for 6 h from nocodazole-mediated synchronization as in **B** and subject to IP analysis with anti-Aurora B antibody. **D.** HeLa/Flag-Mis18α cells expressing Flag-Mis18α were treated with Aurora B inhibitor, Hesperadin and the phosphorylation of Mis18α was evaluated by using anti-p-Mis18α antibody under nocodazole treatment.

Mis18α phosphorylation is necessary for faithful mitotic division

To find out the role of Mis18α phosphorylation, I generated HeLa cells stably expressing shRNA-resistant form of Mis18α WT (WT^R) or Mis18α SA (SA^R). The knockdown of endogenous Mis18α was achieved by lentiviral infection of pLKO-shMis18α just before experiment. The infection of cells with lentivirus reduced the level of endogenous Mis18α efficiently (Figure II-8A). The specific phosphorylation of reconstituted Mis18α proteins was validated by immunoblot analysis (Figure II-8B). With these reconstituted cell lines, I then checked the cell division and interestingly, the number of cells showing misaligned chromosomes (white arrow) at metaphase increased 2.5-fold in Mis18α SA-reconstituted cells compared with Mis18α WT-reconstituted cells (Figure II-9A). In addition, either the chromatin bridges or lagging chromatids and micronuclei (white arrow) increased approximately two folds in Mis18α SA-reconstituted cells than Mis18α WT-reconstituted cells (Figure II-9B).

To verify whether Mis18α phosphorylation is necessary for mitosis, I next examined the mitotic defects in *Mis18α$^{f/f}$/ESR-Cre* MEFs in which endogenous Mis18α can be depleted by the treatment with tamoxifen (Figure II-10A). *Mis18α$^{f/f}$/ESR-Cre* MEFs were reconstituted with either Mis18α WT or Mis18α SA, and I detected mitotic phosphorylation of Mis18α only in Mis18α WT-reconstituted MEFs, but not in Mis18α SA-reconstituted MEFs (Figure II-10B). Interestingly, the depletion of endogenous Mis18α induced an increase of aneuploidy in Mis18α SA-reconstituted MEFs compared

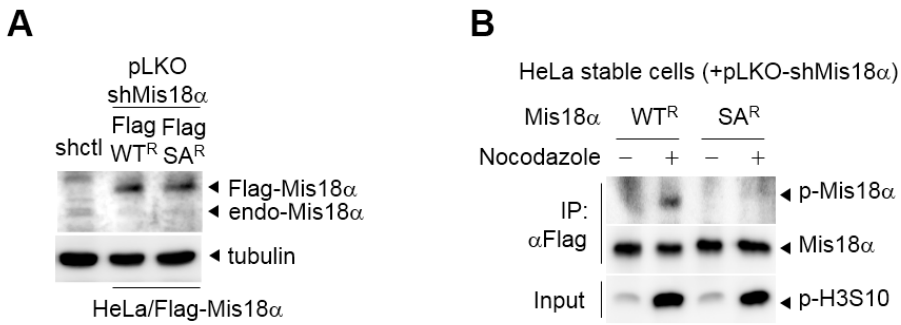


Figure II-8. Generation of HeLa/Flag-Mis18α stable cell lines

A. HeLa cells stably expressing shRNA resistant form of Flag-Mis18α (WT^R or SA^R) were infected with lentivirus generated from pLKO-shMis18α. Regular HeLa cells infected with lentivirus generated from shRNA pLKO-shControl (shctl) were used as a control. The knockdown efficiency was evaluated by immunoblot analysis with anti-Mis18α antibody. **B**. Immunoblotting for Mis18α phosphorylation in nocodazole-synchronized HeLa/Flag-Mis18α stable cells. WT^R or SA^R represents shRNA-resistant form of Mis18α proteins.

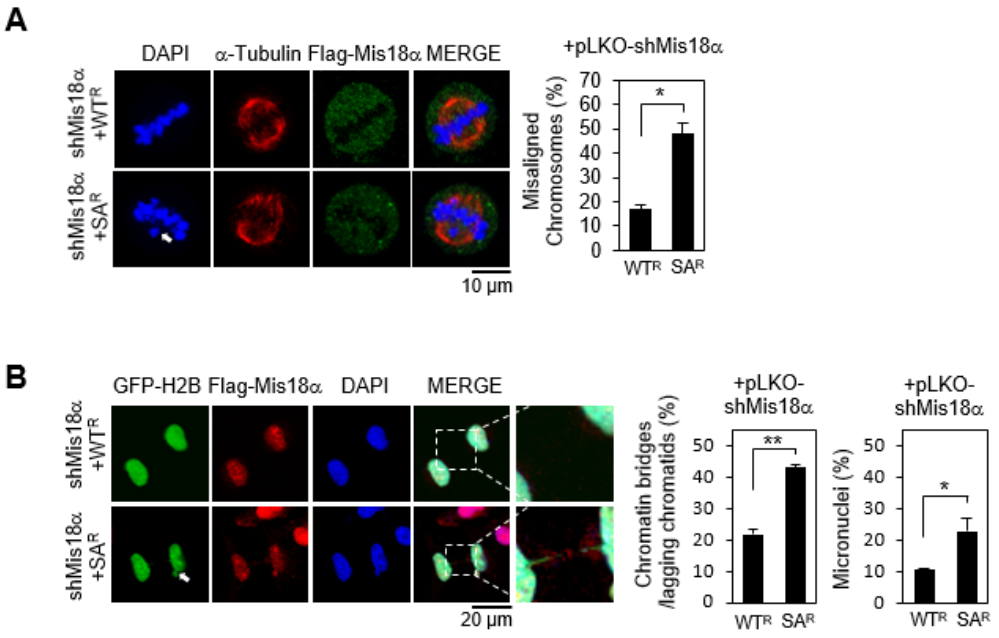


Figure II-9. Mitotic defects increased in HeLa/Flag-Mis18α SA stable cell lines

A. Images of misaligned chromosomes in HeLa/Flag-Mis18α stable cells (left) and the number of cells showing misaligned chromosomes presented in percentage (right). P value is calculated by t-test (*$p < 0.05$). **B.** Images of the chromatin bridges or lagging chromatids and micronuclei in HeLa/Flag-Mis18α stable cells (left) and the number of cells showing the chromatin bridges or lagging chromatids presented in percentage (right). P value is calculated by t-test (*$p < 0.05$).

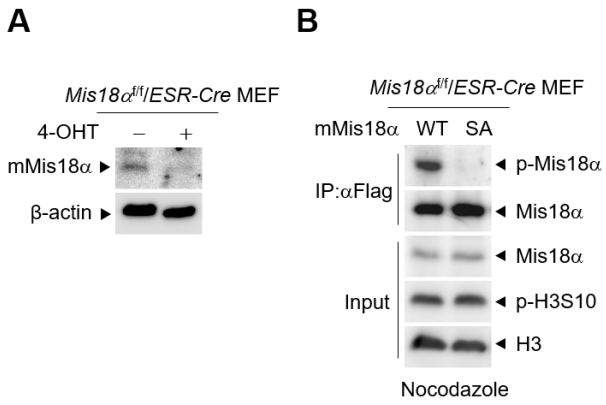


Figure II-10. Generation of *Mis18α*$^{f/f}$*/ESR-Cre* MEFs stable cell lines

A. *Mis18α*$^{f/f}$*/ESR-Cre* MEFs were treated with 4-hydroxy-tamoxifen (4-OHT) for four days and the depletion of endogenous mMis18α was validated by immunoblot using anti-mMis18α antibody. **B.** The phosphorylation of mMis18α proteins in *Mis18α*$^{f/f}$*/ESR-Cre* MEFs reconstituted with Flag-mMis18α WT or Flag-mMis18α SA were validated by immunoblotting with anti-p-Mis18α antibody.

with Mis18α WT-reconstituted MEFs; especially the population of cells containing the number of chromosome over 4N were increased in Mis18α SA-reconstituted MEFs compared with Mis18α WT-reconstituted MEFs (Figure II-11). This is consistent with the previous studies that showed Aurora B kinase depletion or overexpression increasing aneuploidy (Fernández-Miranda et al., 2010; González-Loyola et al., 2015). Furthermore, the depletion of endogenous Mis18α increased the number of the chromatin bridges or lagging chromatids by four times when compared with untreated control cells (Figure II-12A). While Mis18α WT-reconstitution significantly reduced the chromatin bridges or lagging chromatids formation in *Mis18α*-deficient MEFs, Mis18α SA-reconstitution failed to do so (Figure II-12A and II-12B). In addition, the micronuclei formation and chromosome misalignment also increased in *Mis18α*-deficient MEFs and these were not recovered by Mis18α SA-reconstitution similarly to the chromatin bridges or lagging chromatids formation (Figure II-12A, II-12C and II-12D). However, CENP-A dots were intact indicating that these mitotic defects in Mis18α SA-reconstituted MEFs are independent of CENP-A deposition. Taken together, mitotic phosphorylation of Mis18α by Aurora B kinase is necessary both for the faithful segregation of chromosome.

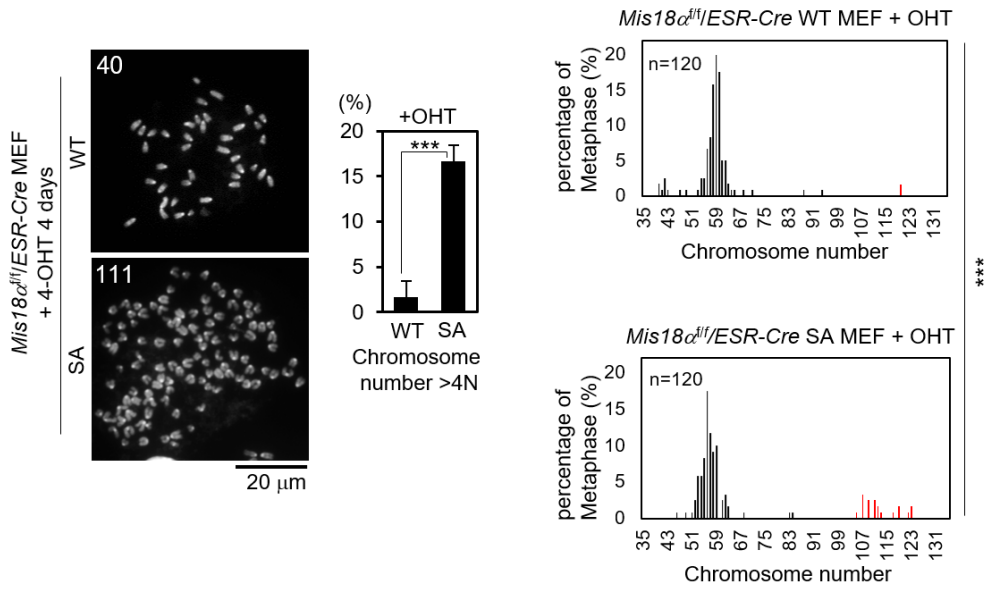
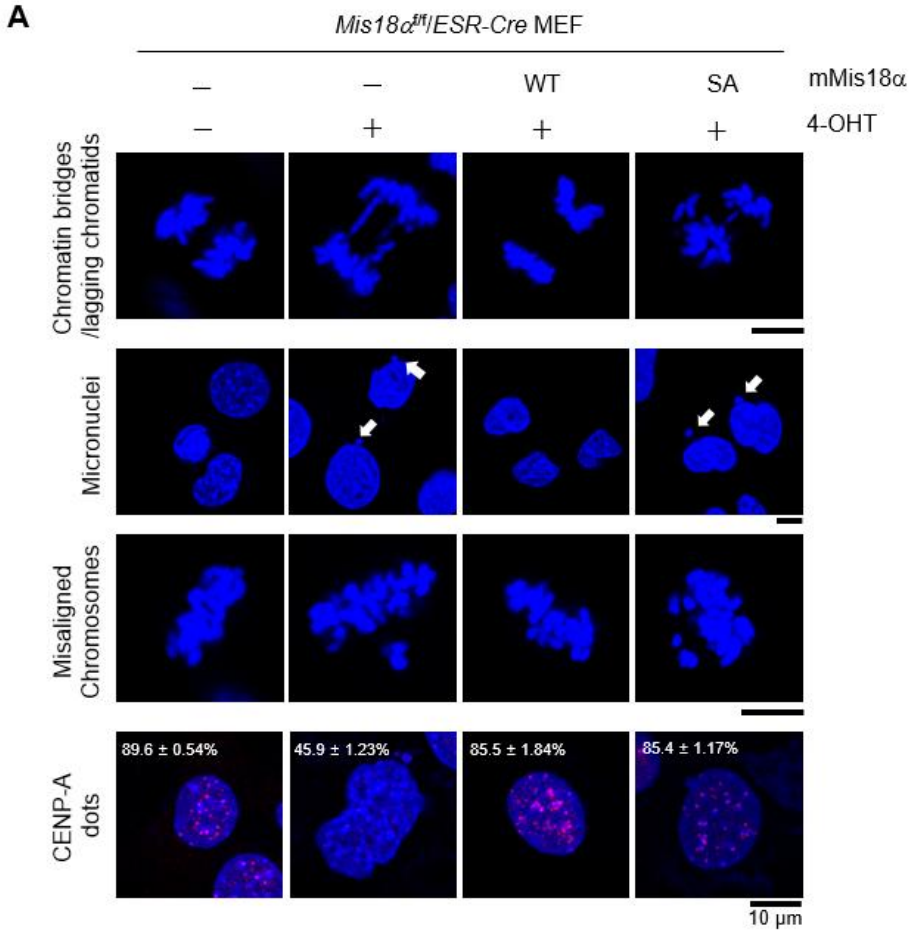


Figure II-11. Aneuploidy increased in *Mis18α*$^{f/f}$*/ESR-Cre* SA MEFs

Reconstituted *Mis18α*$^{f/f}$*/ESR-Cre* MEFs were analyzed by chromosome spreading assay. Histogram in right side shows the percentage of cells containing chromosome number over 4N for each genotype. The number of chromosome spreads is 120 each and chromosomes were counted using Image J software by identifying the centromeres, the brighter part than the rest of the chromosome. P value is calculated by t-test (***p<0.001).


A
Mis18α^f/f/ESR-Cre MEF
–
–
WT
SA
mMis18α
–
+
+
+
4-OHT
Chromatin bridges /lagging chromatids
Micronuclei
Misaligned Chromosomes
CENP-A dots
89.6 ± 0.54%
45.9 ± 1.23%
85.5 ± 1.84%
85.4 ± 1.17%
10 μm

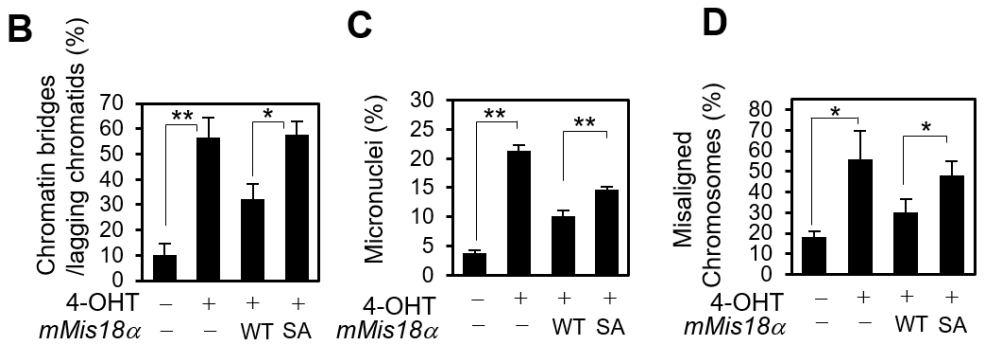


Figure II-12. Mitotic defects increased in *Mis18α*$^{f/f}$*/ESR-Cre* SA MEFs

A. The chromatin bridges or lagging chromatids, micronuclei and misaligned chromosomes were analyzed in reconstituted *Mis18α*$^{f/f}$*/ESR-Cre* MEFs in parallel with CENP-A dots. **B.-D.** The percentage of cells showing the chromatin bridges or lagging chromatids (**B**), micronuclei (**C**), and misaligned chromosomes (**D**) was calculated from the images in **G**. *P* value is calculated by t-test (*$p < 0.05$, **$p < 0.01$).

Mis18α phosphorylation is not required for CENP-A loading

Since Mis18α plays a role in CENP-A loading process as a licensing or priming factor (Fujita et al., 2007; Silva et al., 2012), I questioned whether the phosphorylation of Mis18α by Aurora B kinase is important for this function. Therefore, we first checked the Mis18 complex formation which is essential for CENP-A loading process and found that Mis18α SA has little or no defect in the binding with either Mis18β or M18BP1 (Figure II-13A and II-13B). Furthermore, Mis18α WT began to show as dots at centromere from anaphase and stayed there until G1 phase (Figure II-14) as reported previously (Fujita et al., 2007). Mis18α SA showed the similar pattern with WT, indicating that Mis18α phosphorylation at Ser36 by Aurora B kinase is not crucial for its centromere localization.

Next, I examined whether the phosphorylation of Mis18α is required for the CENP-A loading process by adopting and modifying the experimental scheme that was used to show the prerequisite function of Mis18α for the centromere loading of newly synthesized CENP-A (Fujita et al., 2007). HeLa cells stably expressing siRNA-resistant form of Mis18α WT or Mis18α SA were transfected with siRNA against Mis18α to get rid of endogenous Mis18α and then transfected with GFP-CENP-A as shown in the scheme of Figure 3D. After 24 and 9 hours of siRNA and GFP-CENP-A transfection, respectively, GFP-CENP-A dots were clearly observed at late telophase of both WT and SA-expressing cells (Figure II-15).

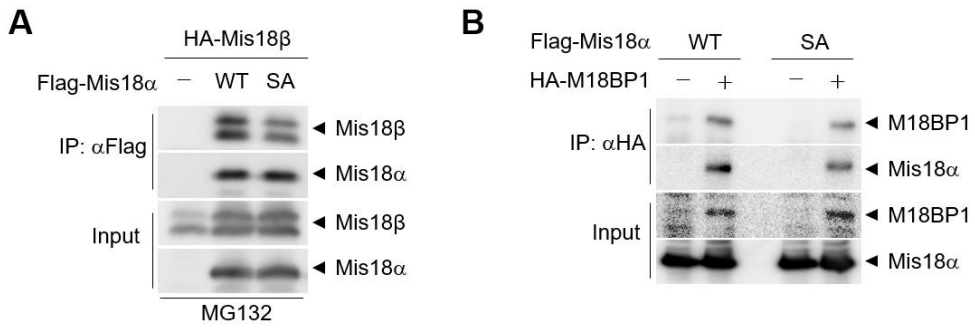


Figure II-13. Phosphorylation of Mis18α did not affect Mis18 complex formation

A. HA-Mis18β with either Flag-Mis18α WT or Flag-Mis18α SA were transfected into 293T cells and the extracts were applied for co-IP assay. **B.** The binding between Flag-Mis18α and HA-M18BP1 was analyzed as in **A**.

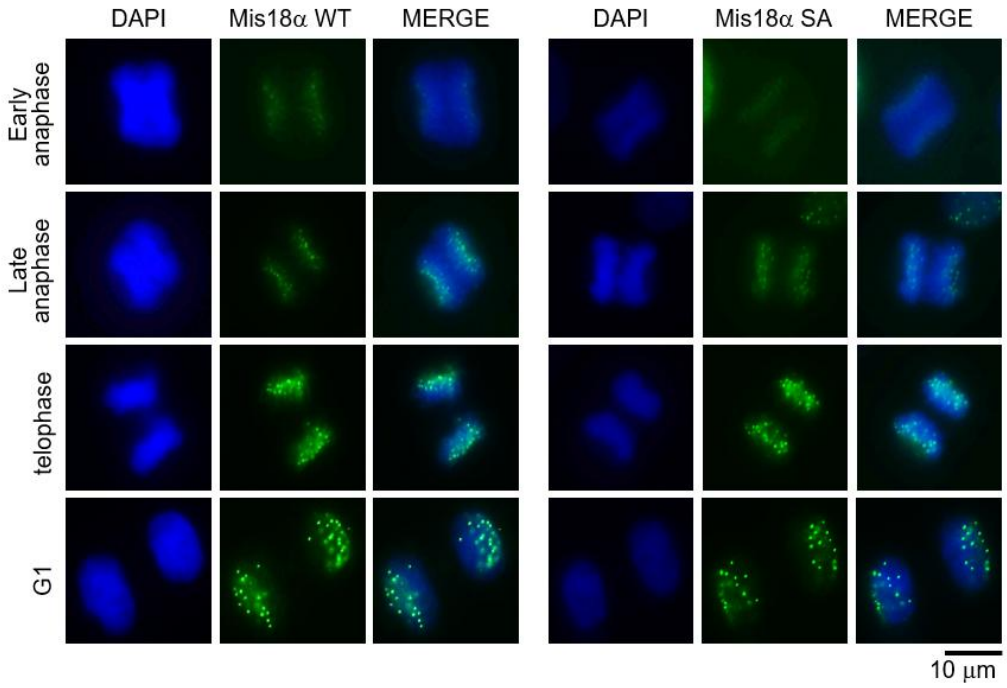


Figure II-14. Phosphorylation of Mis18α did not affect Mis18α centromere localization

HeLa/Flag-Mis18α stable cells were synchronized by double-thymidine block and released into indicated phase. The cells were stained with anti-Flag antibody. The green dots indicate centromeric localization of Mis18α. Confocal image with 1,000x magnification.

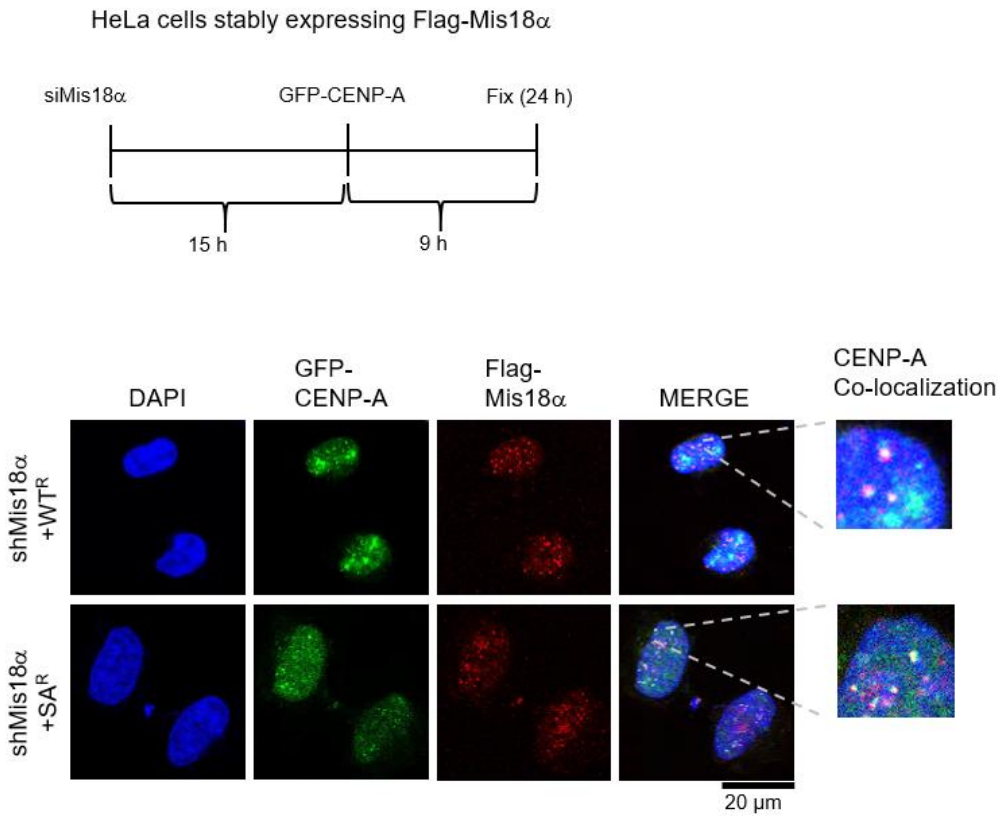
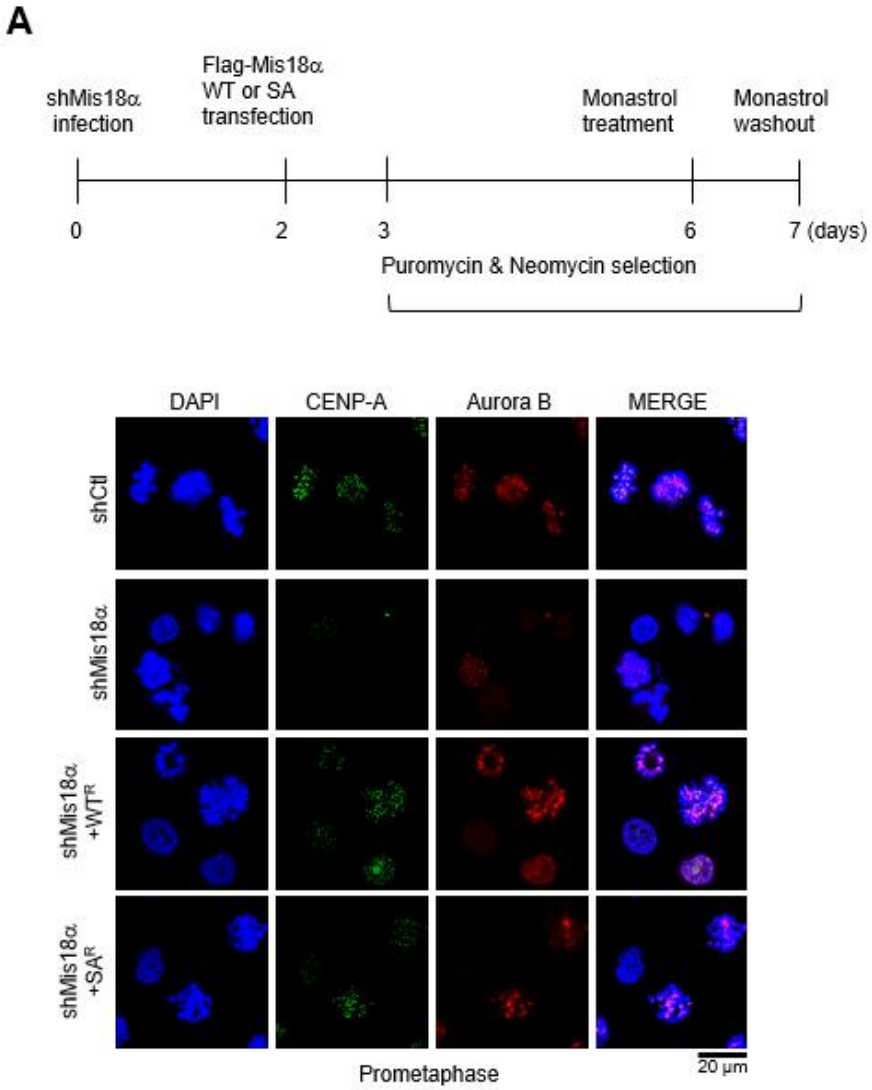


Figure II-15. Phosphorylation of Mis18α did not affect CENP loading

Analysis scheme for the centromere recruitment of newly synthesized CENP-A (left). HeLa cells stably expressing siRNA-resistant form of Mis18α WT (WT^R) or SA (SA^R) were transfected sequentially with siRNA against Mis18α and with GFP-CENP-A (mimic newly synthesized CENP-A) as indicated in the scheme. Immunocytochemistry for Mis18α with anti-Flag antibody and GFP-CENP-A (right).

I applied an alternative scheme to confirm that Mis18α phosphorylation is unrelated to CENP-A loading process. In this experiment, I could check the effect of prolonged Mis18α knockdown and the reconstitution Mis18α WT or Mis18α SA on CENP-A loading process. HeLa cells were infected with lentivirus expressing shRNA against Mis18α and reconstituted with either Mis18α WT or Mis18α SA as shown in the scheme of Figure II-16A. Cells were synchronized by treating with monastrol and then released for 30 min. With the representative data for prometaphase cells (Figure II-16A) and G1 phase cells (Figure II-16B), knockdown of Mis18α in HeLa cells diminished CENP-A dots dramatically on day 7; only 13 % of cells showed positive signal for CENP-A dots compared with the control shRNA-infected cells (Figure II-16B). However, reconstitution of Mis18α WT and Mis18α SA recovered CENP-A dots approximately up to 80 % in centromere. Taken together, we could exclude the effect of Mis18α phosphorylation on newly synthesized CENP-A loading into centromere.


A
shMis18α infection
Flag-Mis18α WT or SA transfection
Monastrol treatment
Monastrol washout
0
2
3
6
7 (days)
Puromycin & Neomycin selection
DAPI
CENP-A
Aurora B
MERGE
shCtl
shMis18α
shMis18α +WTR
shMis18α +SAR
20 μm
Prometaphase

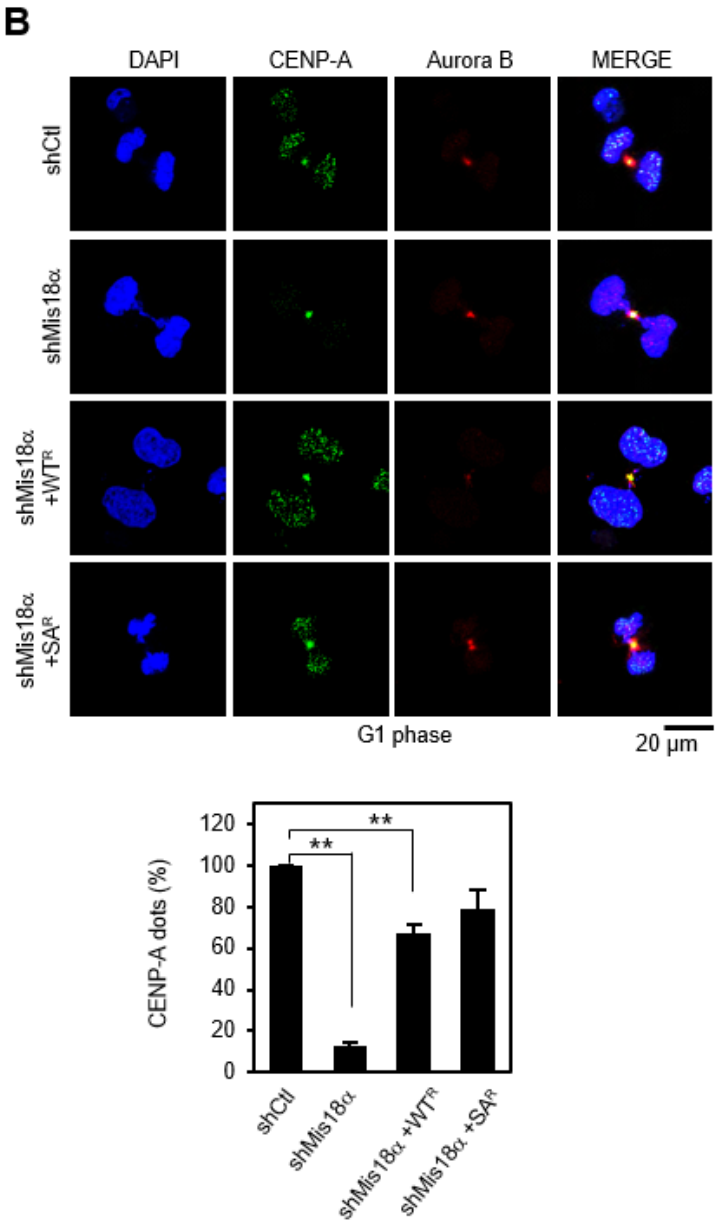

B
DAPI
CENP-A
Aurora B
MERGE
shCtl
shMis18α
shMis18α +WTR
shMis18α +SAR
G1 phase
20 μm
CENP-A dots (%)
120
100
80
60
40
20
0
**
**
shCtl
shMis18α
shMis18α +WTR
shMis18α +SAR

Figure II-16. Phosphorylation of Mis18α did not affect CENP loading

A. Scheme for the centromeric recruitment of CENP-A under prolonged Mis18α knockdown (upper). Knockdown of endogenous Mis18α was achieved by infecting lentivirus that is expressing shRNA against Mis18α. Lower left panel shows CENP-A dots in prometaphase cells and lower right panel represents G1 phase cells. **B.** The number of CENP-A dot positive cells from **A** were calculated and expressed as a percentage of total cells. *P* value is calculated by t-test (**$p < 0.01$).

II-4. discussion

For a long time, Mis18α has been known to function only for newly synthesized CENP-A deposition. A member of Mis18 complex, M18BP1 was phosphorylated by CDK1/2-mediated phosphorylation and this phosphorylation inhibits the Mis18 complex formation before precise time of CENP-A licensing. However, PLK1-medicated phosphorylation of M18BP1 enhances Mis18 complex recruitment onto centromere. Unlike M18BP1 phosphorylation, Mis18α phosphorylation did not affect neither Mis18 complex formation nor CENP-A licensing.

Indeed, Aurora B kinase localizes to midzone at a time of CENP-A licensing and there is no chance to participate in regulation of CENP-A deposition at centromere (Fuller et al., 2008). Furthermore, Aurora B kinase is mostly activated from prometaphase to metaphase as proven by H3S10 phosphorylation (Emanuele et al., 2008), which is well known substrate. Mis18α phosphorylation was also in consistent with H3S10 phosphorylation. Therefore, I hypothesized that Mis18α has a new function during mitosis and it is related to the precise regulation during prometaphase to metaphase by Aurora B kinase.

Considering that Mis18α is de-phosphorylated as cells exit mitosis, the phosphatase would have a crucial role for the next stage of cell cycle. Indeed, de-phosphorylation of Aurora B kinase targets is the fastest way for an abrupt activation of APC/C (Huang et al., 2008). For an example, the phospho-mimetic mutants of some KMN or spindle checkpoint proteins or the inhibition of their de-phosphorylation failed

to step-forward from the metaphase (Kemmler et al., 2009; Kim et al., 2010; Vanoosthuyse and Hardwick, 2009). Hence, Mis18α phosphorylation would be reversed by phosphatase for the precise regulation. This remained to be studied for the concrete axis of Aurora B kinase dependent phosphorylation of Mis18α.

II-5. Materials and Method

Cell culture, generation of stable cells and transfection

HeLa, 293T and *Mis18α$^{f/f}$/ESR-Cre* MEFs were cultured in 37℃ humidified CO_2 incubator with DMEM containing 10% FBS and antibiotics. All cell lines were regularly tested for mycoplasma contamination. For the generation of Mis18α-stably reconstituted HeLa cell lines, cells transfected with shRNA-resistant Flag-Mis18α were selected with neomycin for two weeks. The cells were then infected with shMis18α expressing lentivirus (pLKO-shMis18α) followed by selection with puromycin. Lentivirus was generated by transfecting lentiviral shRNA and packaging plasmids (psPAX2 and pMD2.G) into 293T cells. The culture supernatant were collected two days later and concentrated by Lenti-concentrator (Takara Bio, USA). The targeting sequences of shRNA are as follows; human Mis18α, 5'-CAGAAGCTATCCAAACGTG-3'; human M18BP1, 5'-GGATATCCAAATTATCTCA-3'. The targeting sequence of siRNA for human Mis18α is as follows; 5'-CAGAAGCUAUCCAAACGUGUU-3'. For the generation of Mis18α-reconstituted *Mis18α$^{f/f}$/ESR-Cre* MEF cell lines, cells were infected with Flag-mMis18α expressing lentivirus (pLJM1-Flag-Mis18α) and selected with puromycin for two weeks. For the depletion of endogenous mMis18α, 4-hydroxy-tamoxifen (200 nM) was added for 4 days.

Cell synchronization

To arrest cells at G1/S, the cells were incubated with DMEM media containing 4 mM thymidine (Sigma, St Louis, MO) for 16 h and were washed with PBS twice. After 9 h of release, cells were incubated with thymidine containing media again for 15 h, and were released and harvested at indicated time points. To arrest cells at metaphase, the cells were incubated with DMEM media containing 0.4 μg nocodazole (Sigma, St Louis, MO) for 15 h. For metaphase-aligned chromosomes, MG132 (Sigma, St Louis, MO) was added while cells were released from 100 nM monastrol incubation for 15 h.

Immunoprecipitation

The whole cell lysates were prepared using lysis buffer (50 mM Tris-HCl pH 8.0 containing 200 mM NaCl, 0.5% NP-40, and freshly added protease and phosphatase inhibitors). Cell lysates were briefly sonicated to shear the chromatin structure. For immunoprecipitation, 1 mg of lysates were incubated sequentially with primary antibody for 4 h followed by protein A/G coated beads for 1 h at 4℃.

Immunoblot

For immunoblot, normalized cell lysates or immunoprecipitation samples were separated on SDS-PAGE gels and transferred on nitrocellulose membrane (GE Healthcare, Little Chalfont, UK). The blots were probed with the following primary antibodies; anti-Flag (Sigma, St Louis, MO), anti-HA (Covance, Princeton, NJ), anti-H3 (Cell signaling, Danvers, MA), anti-p-H3S10 (Abcam, Cambridge, UK), anti-Aurora B (Abcam,

Cambridge, UK), anti-ACA (Antibodies Incorporated, Davis, CA), anti-CENP-A (Cell Signaling, Danvers, MA), anti-phospho serine (Sigma, St Louis, MO). Phosphorylation-specific antibody for Mis18α was generated by injecting synthetic phospho-peptide to rabbits and purified using phospho-peptide affinity chromatography (AbClone, Seoul, South Korea). Peptide sequence used for injection is as follows; 5'-CESPLLEKRL(pS)EDSSR-3'.

Immunocytochemistry

For immunocytochemistry, the cells were cultured on poly-L-Lysine coated coverslip or chamber slide and were fixed with 2% formaldehyde for 15 min at 25℃. Cells were then permeabilized with PBS containing 0.5% Triton X-100 for 5 min followed by incubation with primary antibodies for overnight. After washing, secondary antibodies conjugated with Alexa Fluor 488 or 594 (Invitrogen, Carlsbad, CA**)** were applied in the dark for 1 h at 25ºC. DAPI was incubated for short time just before mounting and the slides were observed with confocal microscope (Carl Zeiss, Germany) at 1,000x magnification with immersion oil. The images were analyzed by Image J software.

Chromosome spreading assay

Mis18α$^{f/f}$*/ESR-Cre* WT MEFs and *Mis18α*$^{f/f}$*/ESR-Cre* SA MEFs were incubated with colcemid (Sigma, USA) to a final concentration of 1 μg/ml for 4 h at 37℃. After incubation, cells were trypsinized and harvested by centrifugation at 1,000 rpm for 4 min.

The pellet was resuspended with 75 mM KCl solution and incubated for 6 min. After harvesting by centrifugation at 1,000 rpm for 4 min, cells were fixed with methanol/glacial acetic acid (3:1) by dropping and mixing slowly. The fixed chromosomes were released as a single drop at a time onto the slide and were allowed to air-dry. The air-dried slide was covered by coverslip with DAPI-containing mounting solution. The image was observed by confocal microscope (Carl Zeiss, Germany) at 1,000x magnification, and centromeres that are brighter than the rest of the chromosome were counted by Image J software.

***In vitro* kinase assay**

Recombinant His-H3 and His-Mis18α was incubated with purified Aurora B kinase (Eurofins, UK) for 30 min at 30°C in the kinase buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM EGTA, 2 mM DTT, 0.01% Tween-20, 1 mM ATP, protease inhibitors and phosphatase inhibitors). The reaction was stopped by adding SDS sampling buffer and subjected to SDS-PAGE followed by immunoblotting with anti-p-H3S10 antibody and anti-p-Mis18α antibody.

Statistical analysis

All experiments were performed independently at least three times. More than 100 cells were counted to evaluate mitotic defects in each experiment. Values are expressed as mean ± s.e.m. Significance was analyzed using two-tailed, unpaired *t*-test. $P<0.05$ was

considered statistically significant.

CHAPTER III.

Phosphorylated Mis18α enhances PLK1 recruitment on kinetochore

III-1. Summary

Mis18α is phosphorylated during mitosis by Aurora B kinase on Ser36 in human and Ser13 in mice. However, this phosphorylation did not affect neither Mis18 complex formation nor newly synthesized CENP-A deposition. Nevertheless, failure of Mis18α phosphorylation still showed critical defects in chromosome segregation leading to increased micronuclei, misaligned chromosome and the chromatid bridges or lagging chromatids.

In the present study, I extensively analyzed mutant stable cell lines to find the downstream targets of Mis18α phosphorylation. Among many mitotic proteins, PLK1 signal on kinetochore was dramatically decreased under Mis18α knockdown or Mis18α SA mutant reconstitution. I hypothesized that Mis18α phosphorylation is crucial for PLK1 interaction through PBD and this interaction enhances PLK1 localization on kinetochore. Indeed, either Mis18α SA mutant or PLK1 PBD mutant had a deleterious effect on Mis18α and PLK1 interaction. Furthermore, the interaction between Mis18α and PLK1 increased during mitosis or by Aurora B kinase overexpression proving phosphorylation dependent binding in a cell cycle dependent manner.

During prometaphase, Aurora B kinase and PLK1 cooperate to regulate the kinetochore-microtubule attachment. The misregulation of Aurora B kinase or PLK1 often causes mis-segregation of chromosome. I conclude that Mis18α phosphorylation

mediates this regulation by providing PLK1 docking site and suggest that Mis18α has a distinct role in the cell cycle besides newly synthesized CENP-A deposition, which has long been known for the only function of Mis18α.

III-2. Introduction

Recently, Aurora B kinase-PLK1-MCAK (mitotic centromere-associated kinesin) axis has been shown to be required for accurate chromosome segregation (Shao et al., 2015). At the kinetochore, Aurora B kinase activates PLK1 by phosphorylation and the activated PLK1 in turn phosphorylates MCAK, which is essential for accrurate chromosome segregation with its increased microtubule depolymerase activity. Inhibition of either Aurora B kinase or PLK1 reduces MCAK phosphorylation on PLK1 target sites and induces formation of impolar mitotic spindle and the chromatin bridges. Interestingly, PLK1 is also needed for the full activation Aurora B kinase at the beginning of prometaphase. Aurora B kinase, Survivin, INCENP, and borealin are members of chromosomal passenger complex (CPC) and Survivin phosphorylation by PLK1 elicits Aurora B kianse acitivity around kinetochore (Chu et al., 2011). Thus, the cooperation between Aurora B kinase and PLK1 is a very important biological process for accurate chromosome segregation.

Here, I report that Aurora B kinase phosphorylates Mis18α during mitosis, specifically at prometaphase which is critical for the faithful chromosome segregation. During prometaphase, microtubule dynamically interacts with kinetochore for the proper attachment and the process is regulated by Aurora B kinase and PLK1. Notably, I found Mis18α phosphorylation by Aurora B kinase is important for the recruitment of PLK1 to the kinetochore and for preventing the mitotic defects.

III-3. Result

Mis18α phosphorylation enhances PLK1 kinetochore recruitment

Aurora B kinase functions to regulate kinetochore-microtubule attachment during prometaphase and PLK1 is another key regulator for this function with Aurora B kinase (Liu et al., 2012; Shao et al., 2015). To achieve accurate microtubule binding to kinetochore, Aurora B kinase and PLK1 phosphorylate each substrate at a balanced level for microtubule dynamics. If either kinase is abnormally activated, the cells divide with abnormal microtubule binding inducing misaligned chromosomes (Liu et al., 2012; Shao et al., 2015). I questioned whether the mitotic defects shown in Mis18α SA-reconstituted cells are caused by dysregulation of PLK1 at the kinetochore during prometaphase. Based on this hypothesis, I examined PLK1 recruitment to the kinetochore during prometaphase compared to *Mis18α*-depleted HeLa cells. Mis18α knockdown was validated by the disappearance of CENP-A dots, while anti-centromere antibody (ACA) recognized centromere regions in the chromosomes (Figure III-1A). In control cells, PLK1 dots were clearly stained consistently with centromere markers, whereas the intensity of PLK1 dots was significantly reduced at the kinetochore in *Mis18α*-knockdown cells (Figure III-1B), indicating that Mis18α is necessary for the proper recruitment of PLK1 to the kinetochore. Interestingly, introduction of Mis18α WT recovered the intensity of PLK1 dots; however, introduction of Mis18α SA was not sufficient to substitute for Mis18α WT (Figure III-2A). The evaluation of the ratio of

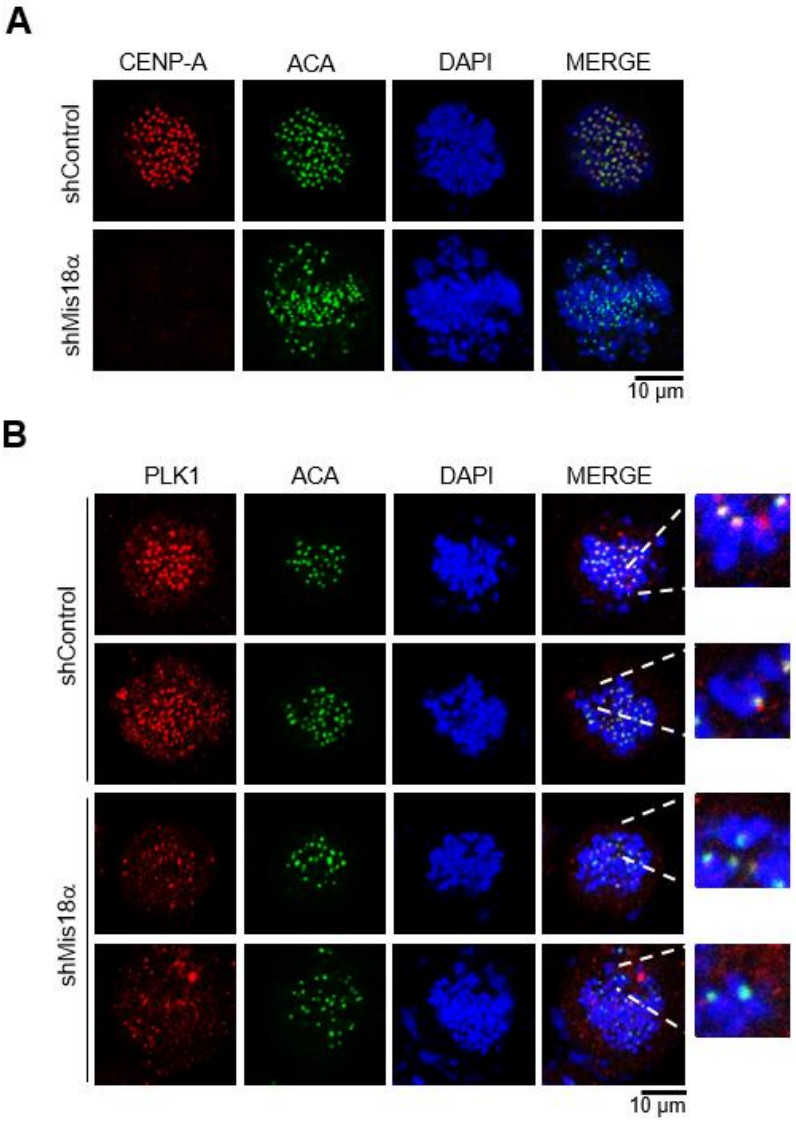

A
CENP-A
ACA
DAPI
MERGE
shControl
shMis18α
10 μm
B
PLK1
ACA
DAPI
MERGE
shControl
shMis18α
10 μm

Figure III-1. Knockdown of Mis18α did affect PLK1 kinetochore localization

A. HeLa cells were infected with lentivirus expressing either control shRNA (shControl) or shRNA against Mis18α (shMis18α). Cells were fixed at prometaphase by releasing for 30 min after monastrol treatment and stained with anti-ACA (centromere marker) or anti-CENP-A antibody. Confocal image with 1,000x magnification. **B.** Cells prepared as in **A** were co-stained with anti-PLK1 and anti-ACA antibodies.

PLK1 dots to ACA centromere maker confirmed that Mis18α phosphorylation is necessary for the proper recruitment of PLK1 to kinetochore (Figure III-2B). Since Aurora B kinase is also responsible for PLK1 activation at kinetochore through Thr210 phosphorylation (Shao et al., 2015), I next checked whether Mis18α phosphorylation is also involved in it. Interestingly, Thr210 phosphorylation of PLK1 was also decreased in Mis18α SA-reconstituted cells, indicating that Mis18α phosphorylation is essential for the function of PLK1 at kinetochore (Figure III-3). Moreover, Mis18α SD, a phospho-mimic form, recovered and further maintained PLK1 recruitment even during the metaphase when PLK1 starts to leave kinetochore (Liu et al., 2012) (Figure III-4). The level of PLK1 in Mis18α WT- or Mis18α SA-reconstituted cells was comparable, indicating that reduced kinetochore recruitment of PLK1 in Mis18α SA-reconstituted cells is not related to its protein level (Figure III-5). Taken together, these results indicate that Mis18α phosphorylation by Aurora B kinase is necessary for the PLK1 kinetochore localization at early mitosis.

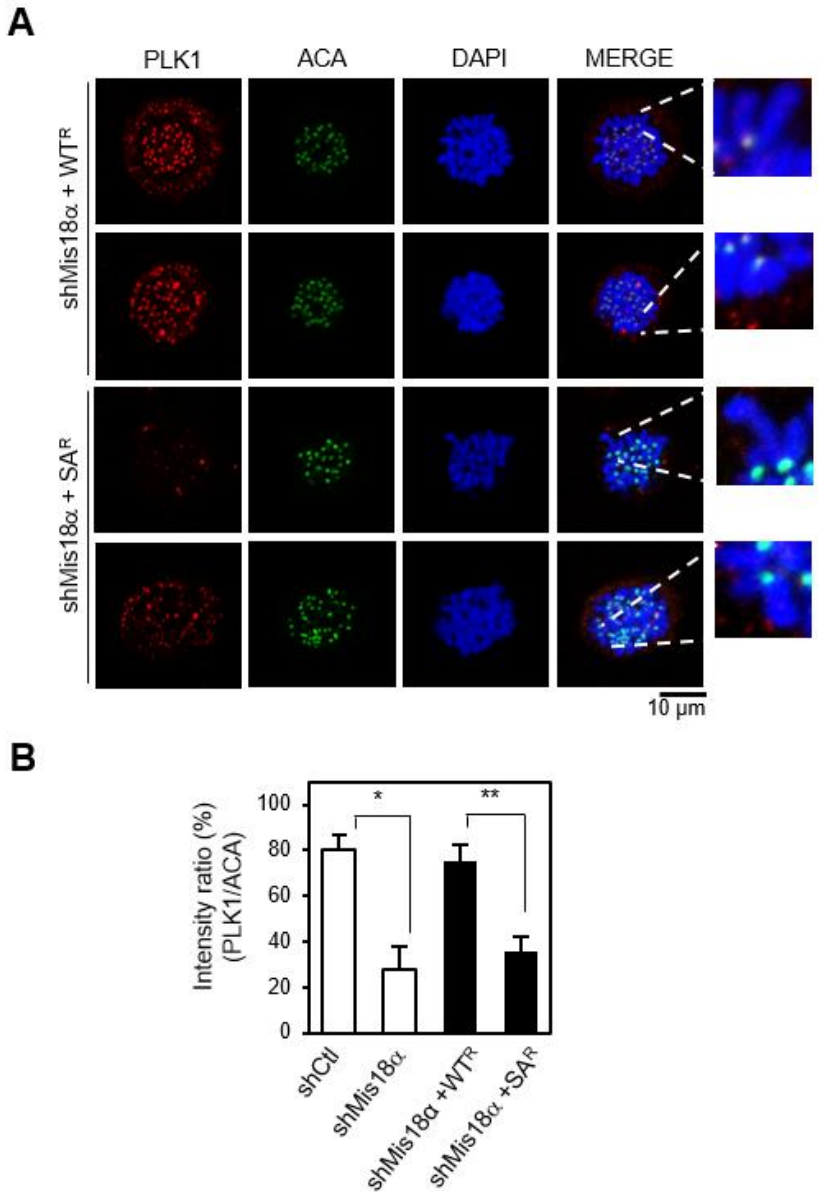

A
PLK1
ACA
DAPI
MERGE
shMis18α + WTR
shMis18α + SAR
10 μm
B
Intensity ratio (%)
(PLK1/ACA)
100
80
60
40
20
0
*
**
shCtl
shMis18α
shMis18α +WTR
shMis18α +SAR

Figure III-2. Reconstitution of Mis18α SA mutants impairs PLK1 kinetochore localization

A. HeLa cells stably expressing shRNA-resistant form of Mis18α (WT^R and SA^R) were infected with lentivirus expressing shMis18α. Cells were co-stained with anti-PLK1 and anti-ACA antibodies at prometaphase. **B.** The number of cells showing high intensity of PLK1 staining, ACA signal as a control (PLK1/ACA) was presented in percentage. P value is calculated by t-test (*$p < 0.05$, **$p < 0.01$).

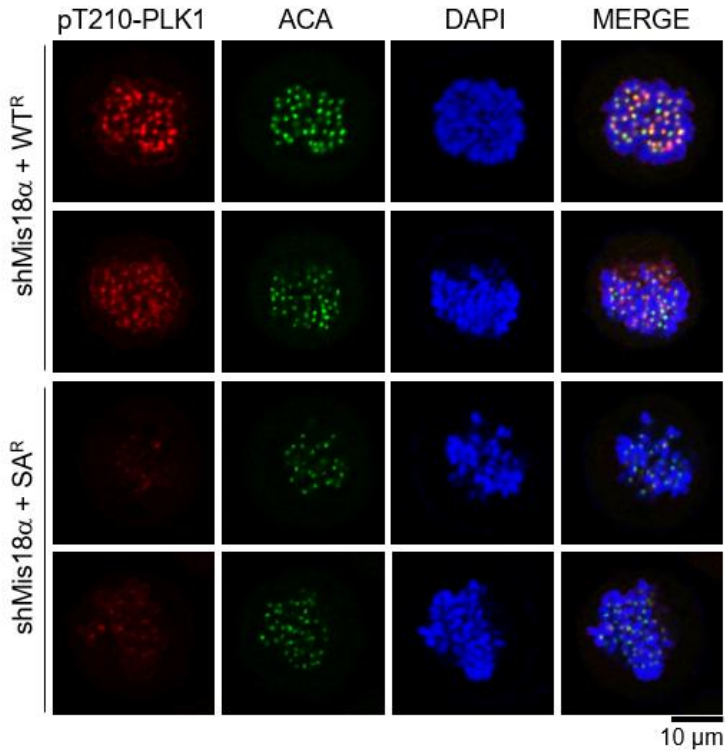


Figure III-3. Reconstitution of Mis18α SA mutants impairs PLK1 activation on kinetochore

HeLa cells stably expressing shRNA-resistant form of Mis18α (WT^R and SA^R) were infected with lentivirus expressing shMis18α. Cells were co-stained with anti-pT210-PLK1 and anti-ACA antibodies at prometaphase.

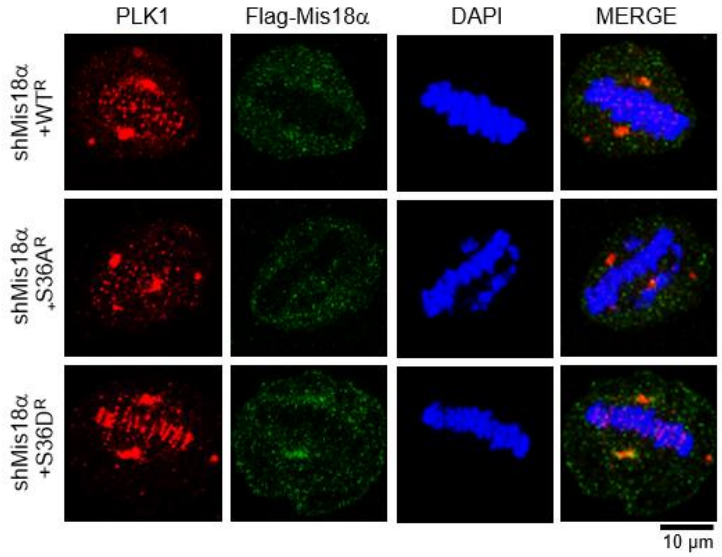


Figure III-4. Reconstitution of Mis18α SA mutants showed decreased PLK1 kinetochore staining at metaphase

HeLa cells stably expressing shRNA-resistant form of Mis18α (WT^R, SA^R and SD^R) were co-stained with anti-PLK1 and anti-Flag Mis18α antibody at metaphase.

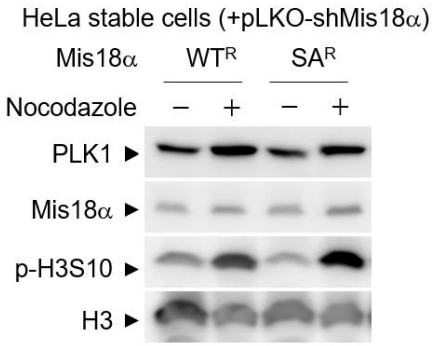


Figure III-5. HeLa/Mis18α SA stable cell lines did not affect PLK1 expression

Immunoblot for PLK1 level in reconstituted HeLa stable cell lines.

PLK1 recognizes Mis18α phosphorylation through its Polo Box Domain (PBD)

To examine whether Mis18α-PLK1 binding is important for PLK1 kinetochore recruitment and whether Mis18α phosphorylation mediates the binding, I performed co-immunoprecipitation assay. Interestingly, PLK1 bound with Mis18α WT, but the binding with Mis18α SA decreased significantly (Figure III-6A). In addition, the binding between PLK1 and Mis18α SD phospho-mimic mutant increased more than Mis18α WT (Figure III-6B). *In vitro* binding assay was also performed to confirm if Mis18α phosphorylation is necessary for its interaction with PLK1. Mis18α interacted with PLK1 only in the presence of ATP and Aurora B kinase, whereas Mis18α SA did not bind with PLK1 even in the presence of ATP and Aurora B kinase (Figure III-7A). In a separate assay, phosphorylation mimic mutant form of Mis18α (Mis18α SD) interacted with PLK1 without addition of Aurora B kinase (Figure III-7B). Furthermore, knockdown of Aurora B kinase diminished the binding between PLK1 and Mis18α (Figure III-8A), and overexpression of Aurora B kinase increased their binding (Figure III-8B), revealing Aurora B kinase dependent binding of PLK1. Nocodazole treatment increased the binding between PLK1 and Mis18α (Figure III-8C). Thus, phosphorylation of Mis18α enhances its binding with PLK1.

PLK1 localization is enhanced by phospho-binding domain, called Polo Box Domain (PBD) (Jang et al., 2002; Lee et al., 1998). PLK1 recognizes substrates through its PBD, and substrate binding through PBD leads to further activation of PLK1. Therefore, I checked whether the PBD of PLK1 is involved in the binding with the

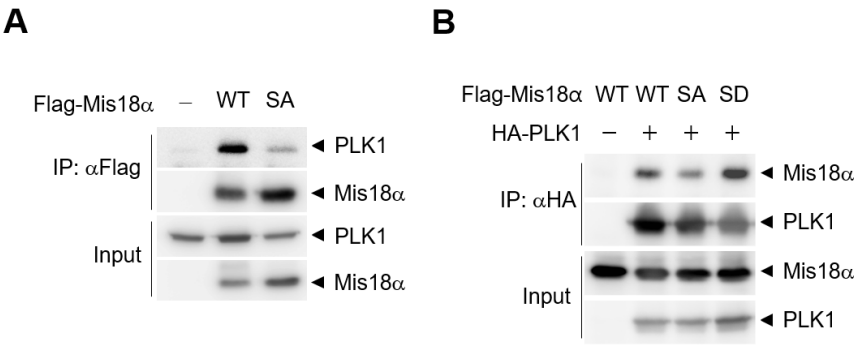


Figure III-6. PLK1 binding with Mis18α is enhanced by Mis18α phosphorylation

A. Flag-Mis18α and HA-PLK1 constructs were transfected in 293T cells and cell extracts were applied for IP analysis by using anti-Flag antibody. **B.** Flag-Mis18α and HA-PLK1 constructs were transfected in 293T cells and cell extracts were applied for IP analysis by using anti-HA antibody.

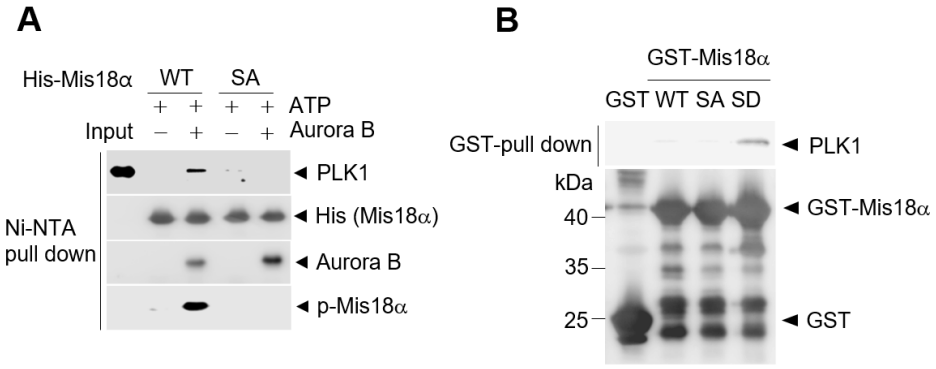


Figure III-7. *In vitro* kinase-binding assay confirming phosphorylation dependent binding of PLK1 with Mis18α

A. HA-PLK1 was synthesized *in vitro* by using a coupled Transcription/Translation system and incubated with recombinant His-Mis18α in the presence of Aurora B kinase for in vitro binding assay. The sample was subjected to immunoblotting with anti-HA antibody. **B.** HA-PLK1 was synthesized *in vitro* by using a coupled Transcription/Translation system and incubated with bacterially expressed recombinant GST-Mis18α. After GST-pull-down, samples were separated on SDS-PAGE and analyzed by immunoblotting with anti-HA antibody.

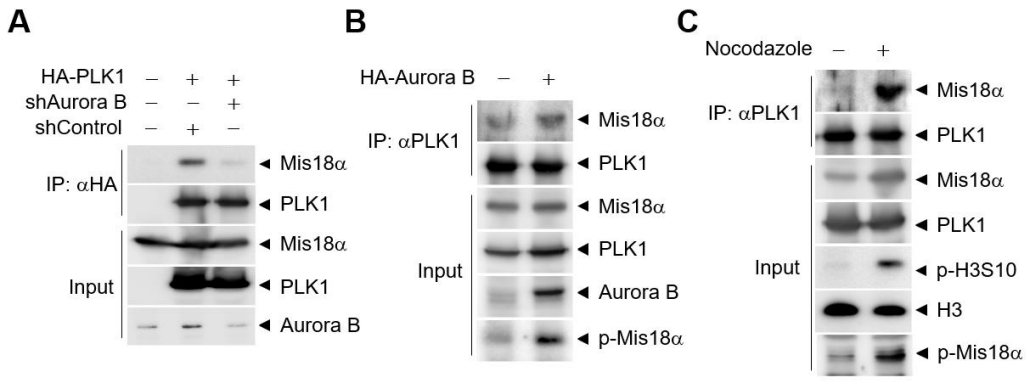


Figure III-8. Binding of PLK1 with Mis18α increased by Aurora B kinase activation

A. 293T cells were transfected with Flag-Mis18α and HA-PLK1 constructs in the presence or absence of shRNA against Aurora B kinase. IP was performed by using anti-HA antibody. **B.-C.** Flag-Mis18α constructs were transfected in 293T cells in the presence of Aurora B kinase overexpression (**B**) or nocodazole treatment (**C**), and IP was performed by using anti-PLK1 antibody.

phosphorylated Mis18α. Since two sites (His538 and Lys540) in PBD are important for the binding of PLK1 to other substrates (Hanisch et al., 2006), I generated PLK1 AA mutant by substituting each amino acid to alanine. Interestingly, the binding of PLK1 AA with Mis18α decreased considerably compared to PLK1 WT (Figure III-9A). PLK1 AA mutant also exhibited decreased binding to Aurora B kinase (Figure III-9B). To gain more insight into PLK1 and Mis18α interaction, I performed bimolecular fluorescence complementation assay (Kerppola, 2006). The N-terminal half and the C-terminal half of VENUS were fused with Mis18α and PLK1, respectively. When the two proteins were co-expressed, Mis18α/PLK1 interaction-mediated complementation of VENUS showed green fluorescence signals. However, Mis18α SA or PLK1 AA mutant failed to show any fluorescence signal (Figure III-10). Taken together, PLK1 binds to phosphorylated Mis18α through its PBD and the binding enhances PLK1 recruitment to the kinetochore at prometaphase.

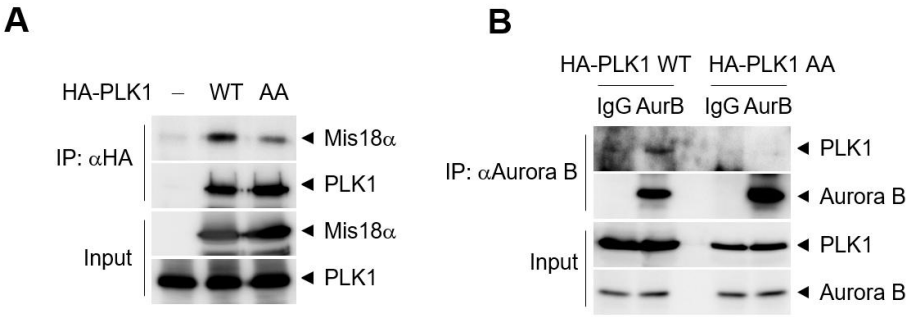


Figure III-9. Polo-box domain of PLK1 is needed for PLK1 binding with Mis18α

A. Flag-Mis18α was transfected into 293T cells together with either wild-type PLK1 (HA-PLK1 WT) or PBD-mutant form of PLK1 (HA-PLK1 AA). IP was performed using anti-HA antibody. **B.** 293T cell extracts expressing either HA-PLK1 WT or HA-PLK1 AA were applied for IP analysis using anti-Aurora B kinase antibody.

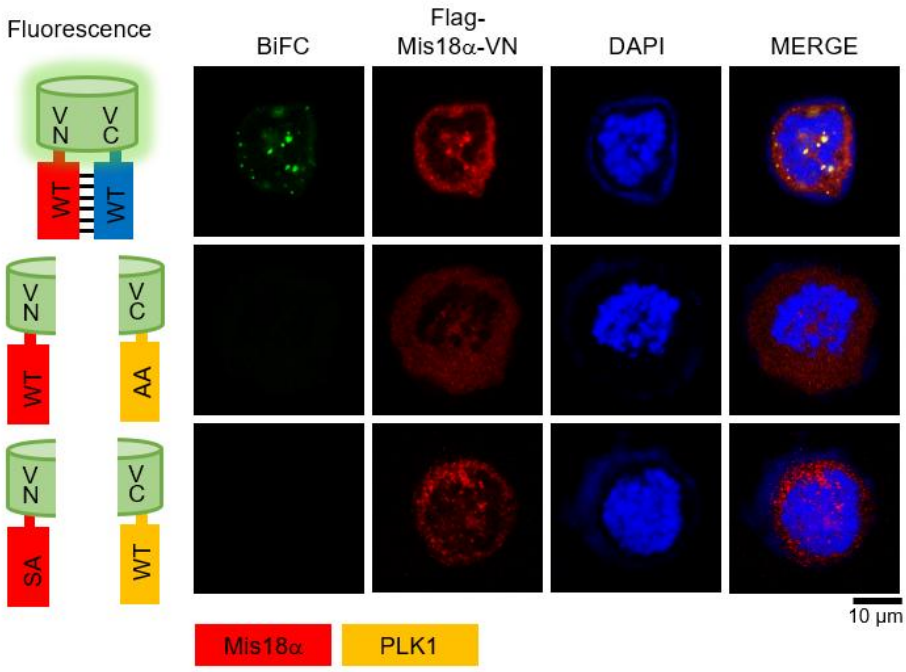


Figure III-10. Confirmation of PLK1 binding depending on Mis18α phosphorylation

Bimolecular fluorescence complementation assay. Flag-Mis18α-VN constructs and HA-PLK1-VC constructs were transfected into HeLa cells and the fluorescence images were detected under confocal microscope (green fluorescence for BiFC and red fluorescence for Mis18α). Confocal image with 1,000x magnification.

III-4. discussion

The present approach to elucidate the physiological role of Mis18α phosphorylation first revealed its pivotal role in KT-MT attachment regulation during mitosis. At first, I speculated that Mis18α phosphorylation would affect Mis18α centromeric localization and thereby affect newly synthesized CENP-A deposition. However, Mis18α phosphorylation mutant still could localize to centromere and functions as a 'priming factor' of CENP-A deposition. Rather, stable cell lines of Mis18α phosphorylation mutant under endogeneous Mis18α knockdown showed dramatically increased mitotic defects. Furthermore, aneuploidy, chromosome number more than 4N, increased in *Mis18α*$^{f/f}$*/ESR-Cre* SA MEFs as shown in the chromosome spreading assay.

To determine the direct targets of Mis18α phosphorylation, I had looked into the Mis18α interacting partners from the datasets (BioGRID), first. Many centrosome proteins and microtubule interacting proteins were provided by affinity purification-mass spectrometry methodology. On account of this aspect, I had checked some proteins in these sets after selecting by high possibility of interaction. However, as the purification in this analysis was not performed using mitotically arrested cells, none of interacting partners did show distinct difference in binding with Mis18α phosphorylation mutant. Even in the analysis of cells by immunostaining, their localization was not changed in the stable cell lines of Mis18α phosphorylation mutant. For the next step for

determining the direct targets of Mis18α phosphorylation, I had looked into the Aurora B kinase interacting partners that has a similar phenotype when they are loss. Recently, Lera and Burkard group demonstrated that Plk1 has a function in chromosome segregation in anaphase. Moreover, this impaired chromosome segregation was observed only when Plk1 was inhibited specifically upon anaphase onset (Brennan et al., 2007; Burkard et al., 2007; Petronczki et al., 2007). They suggested that the observed anaphase phenotype is related to an earlier mitotic event that requires a high level of Plk1 activity. This was not related to the mitotic checkpoint or impaired resolution of sister chromatid cohesion or DNA topology. However, merotelic attachment shown by the stretched CREST signals at kinetochores suggested Aurora B kinase is a key mediator of PLK1 regulation. (Cimini et al., 2002; Cimini et al., 2001). Intriguingly, Aurora B kinase, a known mediator of resolving merotelic attachment, has recently been described as a direct activator of Plk1 at the centromere/inner kinetochore (Carmena et al., 2012). Moreover, improper syntelic attachment due to kinetochore dysfunction have been reported with Polo depletion in Drosophila S2 cells (Moutinho-Santos et al., 2012). These observations raised the questions how PLK1 might mediate resolution of the merotelic microtubule attachment (Lera and Burkard, 2012).

In the present study, I solved the questions how PLK1 interacts with Aurora B kinase to achieve proper microtubule attachment. Mis18α was phosphorylated by Aurora B kinase during prometaphase and this phosphorylation provides a docking site for PLK1 recruitment at kinetochore. I believe that the precise regulation by Mis18α

phosphorylation between Aurora B kinase and PLK1 would contribute the accurate chromosome segregation by fully bi-oriented microtubule attachment.

III-5. Materials and Method

Cell culture

HeLa and 293T was cultured in 37℃ humidified CO_2 incubator with DMEM containing 10% FBS and antibiotics. All cell lines were regularly tested for mycoplasma contamination. For the generation of Mis18α-stably reconstituted HeLa cell lines, cells transfected with shRNA-resistant Flag-Mis18α were selected with neomycin for two weeks. The cells were then infected with shMis18α expressing lentivirus (pLKO-shMis18α) followed by selection with puromycin. Lentivirus was generated by transfecting lentiviral shRNA and packaging plasmids (psPAX2 and pMD2.G) into 293T cells. The culture supernatant were collected two days later and concentrated by Lenti-concentrator (Takara Bio, USA). The targeting sequences of shRNA are as follows; human Mis18α, 5'-CAGAAGCTATCCAAACGTG-3'; human M18BP1, 5'-GGATATCCAAATTATCTCA-3'.

Cell synchronization

To arrest cells at metaphase, the cells were incubated with DMEM media containing 0.4 μg nocodazole (Sigma, St Louis, MO) for 15 h.

Immunoblot

For immunoblot, normalized cell lysates or immunoprecipitation samples were separated on SDS-PAGE gels and transferred on nitrocellulose membrane (GE Healthcare, Little Chalfont, UK). The blots were probed with the following primary antibodies; anti-Flag (Sigma, St Louis, MO), anti-HA (Covance, Princeton, NJ), anti-H3 (Cell signaling, Danvers, MA), anti-p-H3S10 (Abcam, Cambridge, UK), anti-Aurora B (Abcam, Cambridge, UK), anti-PLK1 (Santa Cruz, Dallas, TX), anti-ACA (Antibodies Incorporated, Davis, CA), anti-CENP-A (Cell Signaling, Danvers, MA), anti-phospho serine (Sigma, St Louis, MO). Phosphorylation-specific antibody for Mis18α was generated by injecting synthetic phospho-peptide to rabbits and purified using phospho-peptide affinity chromatography (AbClone, Seoul, South Korea). Peptide sequence used for injection is as follows; 5'-CESPLLEKRL(pS)EDSSR-3'.

Immunoprecipitation

The whole cell lysates were prepared using lysis buffer (50 mM Tris-HCl pH 8.0 containing 200 mM NaCl, 0.5% NP-40, and freshly added protease and phosphatase inhibitors). Cell lysates were briefly sonicated to shear the chromatin structure. For immunoprecipitation, 1 mg of lysates were incubated sequentially with primary antibody for 4 h followed by protein A/G coated beads for 1 h at 4℃.

Immunocytochemistry

For immunocytochemistry, the cells were cultured on poly-L-Lysine coated coverslip or chamber slide and were fixed with 2% formaldehyde for 15 min at 25℃. Cells were then

permeabilized with PBS containing 0.5% Triton X-100 for 5 min followed by incubation with primary antibodies for overnight. After washing, secondary antibodies conjugated with Alexa Fluor 488 or 594 (Invitrogen, Carlsbad, CA**)** were applied in the dark for 1 h at 25ºC. DAPI was incubated for short time just before mounting and the slides were observed with confocal microscope (Carl Zeiss, Germany) at 1,000x magnification with immersion oil. The images were analyzed by Image J software.

Bimolecular fluorescence complementation assay

The N-terminal half of Venus fluorescent protein with Flag-tag was fused with Mis18α and the C-terminal half of Venus with HA-tag was fused with PLK1. Phosphorylation-defective mutant of Mis18α and PBD mutant of PLK1 were fused with Venus in the same way, respectively. These constructs were transfected into HeLa cells in combination. The cells were synchronized by monastrol treatment and then released to obtain prometaphase population. The expression of each construct was validated by immunostaining with anti-Flag or anti-HA antibodies. The complemented Venus protein was detected by green fluorescence signal using a confocal microscope (Carl Zeiss, Germany).

***In vitro* binding assay**

HA-PLK1 was synthesized *in vitro* by using a coupled transcription and translation systems (Promega, USA). HA-PLK1 was incubated with recombinant GST-Mis18α for

GST-pull down in binding buffer (125 mM NaCl, 20 mM Tris-HCl pH 8.0, 10% Glycerol, 0.1% NP-40, 0.5 mM DTT and protease inhibitors). The reaction was stopped by adding SDS sampling buffer and was subjected to SDS-PAGE. PLK1 was analyzed by immunoblotting with anti-HA antibody. Amount of GST-Mis18α was analyzed by anti-GST antibody.

In vitro kinase-binding assay

Recombinant His-Mis18α that is bound on Ni-NTA-agarose was incubated with purified Aurora B kinase (Eurofins, UK) for 30 min at 30°C in the kinase buffer. The sample was washed with binding buffer and then incubated with *in vitro* synthesized HA-PLK1 in binding buffer for 2 h with rotating. After collecting the beads and washing with binding buffer, samples were boiled for 5 min with SDS sampling buffer and subjected to SDS-PAGE. PLK1 was analyzed by immunoblotting with anti-HA antibody.

Statistical analysis

All experiments were performed independently at least three times. More than 100 cells were counted to evaluate mitotic defects in each experiment. For PLK1/ACA intensity ratio, an average number of 150 kinetochores was examined for each group. Values are expressed as mean ± s.e.m. Significance was analyzed using two-tailed, unpaired *t*-test. $P<0.05$ was considered statistically significant.

CHAPTER IV.

Conclusion

Mis18α is a component of Mis18 complex that is crucial for centromere deposition of newly synthesized CENP-A at early G1 phase of cell cycle (Fujita et al., 2007; Silva and Jansen, 2009). In this study, I identified a distinct role of Mis18α at mitosis, independent of its known function in CENP-A deposition at early G1 phase. The mitotic function of Mis18α is accompanied by Aurora B kinase-mediated phosphorylation on a conserved serine residue (Ser36 in human and Ser13 in mouse) during mitosis and this phosphorylation enhances the interaction between Mis18α and PLK1 resulting in the increased kinetochore recruitment of PLK1. The Mis18α phosphorylation I have shown here is distinct from previously identified phosphorylations of Mis18 proteins by CDK1/2 and PLK1, which mainly regulate the function for CENP-A deposition. First, it occurs during mitosis specifically by Aurora B kinase, and the association with Mis18BP1 is not necessary for the phosphorylation. Second and most importantly, the phosphorylation is independent of CENP-A loading. Phosphorylation-defective mutation of Mis18α does not affect either timely scheduled centromere localization of Mis18 complex during cell cycle progression or its function as a priming factor for CENP-A deposition. In detail, the replacement of endogenous Mis18α with phosphorylation-defective Mis18α in two cell types, HeLa and MEFs, did not induce CENP-A loss, whereas Mis18α depletion without Mis18α reconstitution clearly caused CENP-A loss.

It has been previously shown that the depletion of Mis18α causes mitotic defects such as misaligned chromosomes and chromosomal bridges (Kim et al., 2012), which

finally leads to cell death resulting in developmental failure of embryos and skin stratification in mice (Kim et al., 2012; Park et al., 2017). Interestingly, the expression of phosphorylation-defective mutant form of Mis18α in *Mis18α*-depleted cells can rescue severe lethal phenotype of *Mis18α* depletion; however, still exhibits mitotic defects, indicating that although the phosphorylation is not critical for CENP-A deposition, it contributes to the faithful chromosome alignment and segregation. Indeed, Aurora B kinase and PLK1 are essential kinases for accurate KT-MT attachment by interdependent regulation and the loss of Aurora B kinase activity is known to cause abnormally stable KT-MT attachment, as the cooperation with PLK1 to balance KT and MT tension is collapsed (Herman et al., 2015; Krenn and Musacchio, 2015; Maresca and Salmon, 2009; Suijkerbuijk et al., 2012). This results in abnormally increased inter-kinetochore distance (Tauchman et al., 2015). Aurora B kinase destabilizes KT-MT attachment, whereas PLK1 stabilizes it by phosphorylating BubR1 to disturb Aurora B kinase activity (Suijkerbuijk et al., 2012). However, PLK1 activates Aurora B kinase by phosphorylating Survivin, which is a member of chromosomal passenger complex and Aurora B kinase activates PLK1 by direct phosphorylation (Chu et al., 2011; Shao et al., 2015). Due to this complicated cooperation, cells may have multilayers of self-checking system for accurate KT-MT attachment and Mis18α phosphorylation by Aurora B kinase would have a role of enhancing PLK1 recruitment.

The most striking feature of PLK1 regulation is PBD-dependent interaction with its substrates resulting in the kinetochore localization. There are many studies on the

proteins that recruit PLK1 to the kinetochore by investigating their interaction with PBD. The PBIP1 recruits PLK1 by PBD-mediated binding at early mitosis and PLK1 phosphorylates PBIP1 for self-primed enrichment (Kang et al., 2006). Interestingly, PLK1-mediated phosphorylation of PBIP1 on another site induces its degradation and releases PLK1 for the interaction with other recruiting factors. Indeed, PLK1 kinetochore signals remain even when the PBIP1 is depleted (Petronczki et al., 2008), indicating multilayered regulation of PLK1 kinetochore recruitment. In addition, INCENP and RSF1 are known to function in PLK1 kinetochore recruitment. Knockdown of INCENP, which is a member of passenger complex with Aurora B kinase, resulted in the loss of PLK1 kinetochore recruitment (Goto et al., 2006) providing the evidence of Aurora B kinase complex's involvement in PLK1 recruitment. RSF1 partially regulates PLK1 kinetochore recruitment and dual knockdown of RSF1 and INCENP further reduces PLK1 kinetochore recruitment (Lee et al., 2015). In our study, Mis18α SA reconstitution did not completely inhibit PLK1 recruitment to the kinetochore. Moreover, there was no obvious mitotic delay. Therefore, I speculate that the mitotic defects observed from Mis18α SA-reconstituted cells would be the accumulated defects from prolonged dysregulation of PLK1.

In summary, I have identified mitosis-specific phosphorylation of Mis18α by Aurora B kinase, which is not essential for the previously known function of Mis18 complex in CENP-A deposition to centromere. Instead, the phosphorylation of Mis18α contributes the recruitment of PLK1 to the kinetochore, which requires PBD-mediated

binding of phosphorylated Mis18α at prometaphase (Figure IV-1). Since the phosphorylation-defective mutation on Mis18α causes mitotic problems, Mis18α plays a critical role in mitosis in addition to its well-known function in CENP-A deposition at G1 phase. Our finding opens up a possibility that Mis18α may play a diverse role in a wide range of cell cycle regulation.

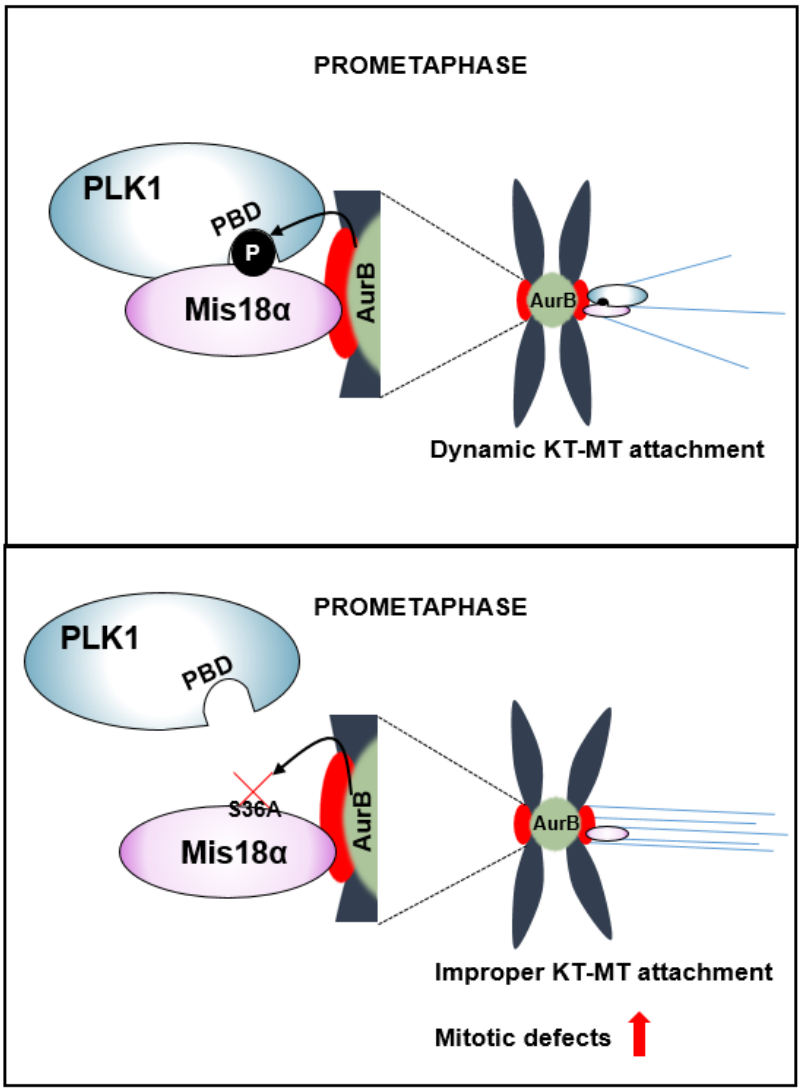


Figure IV-1. A schematic model showing how Mis18α phosphorylation enhances PLK1 recruitment at the kinetochore

REFERENCES

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

Archambault, V., and Glover, D.M. (2009). Polo-like kinases: conservation and divergence in their functions and regulation. Nat Rev Mol Cell Biol *10*, 265-275.

Barr, F.A., Sillje, H.H., and Nigg, E.A. (2004). Polo-like kinases and the orchestration of cell division. Nat Rev Mol Cell Biol *5*, 429-440.

Berdnik, D., and Knoblich, J.A. (2002). Drosophila Aurora-A is required for centrosome maturation and actin-dependent asymmetric protein localization during mitosis. Curr Biol *12*, 640-647.

Brennan, I.M., Peters, U., Kapoor, T.M., and Straight, A.F. (2007). Polo-Like Kinase controls vertebrate spindle elongation and cytokinesis. PLoS One *2*, e409.

Burkard, M.E., Randall, C.L., Larochelle, S., Zhang, C., Shokat, K.M., Fisher, R.P., and Jallepalli, P.V. (2007). Chemical genetics reveals the requirement for Polo-like kinase 1 activity in positioning RhoA and triggering cytokinesis in human cells. Proc Natl Acad Sci U S A *104*, 4383-4388.

Carmena, M., and Earnshaw, W.C. (2003). The cellular geography of Aurora kinases. Nature Reviews Molecular Cell Biology *4*, 842.

Carmena, M., Pinson, X., Platani, M., Salloum, Z., Xu, Z., Clark, A., Macisaac, F., Ogawa, H., Eggert, U., Glover, D.M., et al. (2012). The chromosomal passenger

complex activates Polo kinase at centromeres. PLoS Biol *10*, e1001250.

Chan, E.H., Santamaria, A., Sillje, H.H., and Nigg, E.A. (2008). Plk1 regulates mitotic Aurora A function through betaTrCP-dependent degradation of hBora. Chromosoma *117*, 457-469.

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

Chu, Y., Yao, P.Y., Wang, W., Wang, D., Wang, Z., Zhang, L., Huang, Y., Ke, Y., Ding, X., and Yao, X. (2011). Aurora B kinase activation requires survivin priming phosphorylation by PLK1. J Mol Cell Biol *3*, 260-267.

Cimini, D., Fioravanti, D., Salmon, E.D., and Degrassi, F. (2002). Merotelic kinetochore orientation versus chromosome mono-orientation in the origin of lagging chromosomes in human primary cells. J Cell Sci *115*, 507-515.

Cimini, D., Howell, B., Maddox, P., Khodjakov, A., Degrassi, F., and Salmon, E.D. (2001). Merotelic Kinetochore Orientation Is a Major Mechanism of Aneuploidy in Mitotic Mammalian Tissue Cells. The Journal of Cell Biology *153*, 517-528.

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

Di Fiore, B., and Pines, J. (2007). Emi1 is needed to couple DNA replication with mitosis but does not regulate activation of the mitotic APC/C. J Cell Biol *177*, 425-437.

Dunleavy, E.M., Roche, D., Tagami, H., Lacoste, N., Ray-Gallet, D., Nakamura, Y., Daigo, Y., Nakatani, Y., and Almouzni-Pettinotti, G. (2009). HJURP is a cell-cycle-

dependent maintenance and deposition factor of CENP-A at centromeres. Cell *137*, 485-497.

Earnshaw, W.C., and Rothfield, N. (1985). Identification of a family of human centromere proteins using autoimmune sera from patients with scleroderma. Chromosoma *91*, 313-321.

Elia, A.E., Cantley, L.C., and Yaffe, M.B. (2003a). Proteomic screen finds pSer/pThr-binding domain localizing Plk1 to mitotic substrates. Science *299*, 1228-1231.

Elia, A.E., Rellos, P., Haire, L.F., Chao, J.W., Ivins, F.J., Hoepker, K., Mohammad, D., Cantley, L.C., Smerdon, S.J., and Yaffe, M.B. (2003b). The molecular basis for phosphodependent substrate targeting and regulation of Plks by the Polo-box domain. Cell *115*, 83-95.

Emanuele, M.J., Lan, W., Jwa, M., Miller, S.A., Chan, C.S.M., and Stukenberg, P.T. (2008). Aurora B kinase and protein phosphatase 1 have opposing roles in modulating kinetochore assembly. The Journal of Cell Biology *181*, 241-254.

Equilibrina, I., Krayukhina, E., Inoue, K., Ishikawa, Y., Kawamoto, A., Kato, T., Suetake, I., Tajima, S., Takata, H., Uchiyama, S., et al. (2015). The role of phosphorylation of histone H3 at serine 10 in chromatin condensation in *vitro*. Chromosome Science *18*, 9-14.

Fernández-Miranda, G., de Castro, I.P., Carmena, M., Aguirre-Portolés, C., Ruchaud, S., Fant, X., Montoya, G., Earnshaw, W.C., and Malumbres, M. (2010). SUMOylation modulates the function of Aurora-B kinase. J Cell Sci *123*, 2823-2833.

Fischle, W., Tseng, B.S., Dormann, H.L., Ueberheide, B.M., Garcia, B.A., Shabanowitz,

J., Hunt, D.F., Funabiki, H., and Allis, C.D. (2005). Regulation of HP1-chromatin binding by histone H3 methylation and phosphorylation. Nature *438*, 1116-1122.

Foltz, D.R., Jansen, L.E., Black, B.E., Bailey, A.O., Yates, J.R., 3rd, and Cleveland, D.W. (2006). The human CENP-A centromeric nucleosome-associated complex. Nat Cell Biol *8*, 458-469.

Fu, J., Bian, M., Jiang, Q., and Zhang, C. (2007). Roles of Aurora kinases in mitosis and tumorigenesis. Mol Cancer Res *5*, 1-10.

Fujita, Y., Hayashi, T., Kiyomitsu, T., Toyoda, Y., Kokubu, A., Obuse, C., and Yanagida, M. (2007). Priming of centromere for CENP-A recruitment by human hMis18α, hMis18β, and M18BP1. Dev Cell *12*, 17-30.

Fuller, B.G., Lampson, M.A., Foley, E.A., Rosasco-Nitcher, S., Le, K.V., Tobelmann, P., Brautigan, D.L., Stukenberg, P.T., and Kapoor, T.M. (2008). Midzone activation of aurora B in anaphase produces an intracellular phosphorylation gradient. Nature *453*, 1132.

González-Loyola, A., Fernández-Miranda, G., Trakala, M., Partida, D., Samejima, K., Ogawa, H., Cañamero, M., de Martino, A., Martínez-Ramírez, Á., de Cárcer, G., et al. (2015). Aurora B overexpression causes aneuploidy and p21Cip1 repression during tumor development. Mol Cell Biol *35*, 3566-3578.

Goto, H., Kiyono, T., Tomono, Y., Kawajiri, A., Urano, T., Furukawa, K., Nigg, E.A., and Inagaki, M. (2006). Complex formation of Plk1 and INCENP required for metaphase-anaphase transition. Nat Cell Biol *8*, 180-187.

Goto, H., Yasui, Y., Kawajiri, A., Nigg, E.A., Terada, Y., Tatsuka, M., Nagata, K.-i., and Inagaki, M. (2003). Aurora-B regulates the cleavage furrow-specific vimentin phosphorylation in the cytokinetic process. J Biol Chem *278*, 8526-8530.

Goto, H., Yasui, Y., Nigg, E.A., and Inagaki, M. (2002). Aurora-B phosphorylates Histone H3 at serine28 with regard to the mitotic chromosome condensation. Genes Cells *7*, 11-17.

Hanisch, A., Wehner, A., Nigg, E.A., and Silljé, H.H.W. (2006). Different Plk1 functions show distinct dependencies on Polo-Box Domain-mediated targeting. Mol Biol Cell *17*, 448-459.

Hansen, D.V., Loktev, A.V., Ban, K.H., and Jackson, P.K. (2004). Plk1 regulates activation of the anaphase promoting complex by phosphorylating and triggering SCFbetaTrCP-dependent destruction of the APC Inhibitor Emi1. Mol Biol Cell *15*, 5623-5634.

Herman, J.A., Toledo, C.M., Olson, J.M., DeLuca, J.G., and Paddison, P.J. (2015). Molecular pathways: regulation and targeting of kinetochore-microtubule attachment in cancer. Clin Cancer Res *21*, 233-239.

Hirota, T., Kunitoku, N., Sasayama, T., Marumoto, T., Zhang, D., Nitta, M., Hatakeyama, K., and Saya, H. (2003). Aurora-A and an interacting activator, the LIM protein ajuba, are required for mitotic commitment in human cells. Cell *114*, 585-598.

Hirota, T., Lipp, J.J., Toh, B.H., and Peters, J.M. (2005). Histone H3 serine 10 phosphorylation by Aurora B causes HP1 dissociation from heterochromatin. Nature *438*, 1176-1180.

Hsu, J.Y., Sun, Z.W., Li, X., Reuben, M., Tatchell, K., Bishop, D.K., Grushcow, J.M., Brame, C.J., Caldwell, J.A., Hunt, D.F., et al. (2000). Mitotic phosphorylation of histone H3 is governed by Ipl1/aurora kinase and Glc7/PP1 phosphatase in budding yeast and nematodes. Cell *102*, 279-291.

Huang, H., Hittle, J., Zappacosta, F., Annan, R.S., Hershko, A., and Yen, T.J. (2008). Phosphorylation sites in BubR1 that regulate kinetochore attachment, tension, and mitotic exit. J Cell Biol *183*, 667-680.

Iimori, M., Watanabe, S., Kiyonari, S., Matsuoka, K., Sakasai, R., Saeki, H., Oki, E., Kitao, H., and Maehara, Y. (2016). Phosphorylation of EB2 by Aurora B and CDK1 ensures mitotic progression and genome stability. Nat Commun *7*, 11117.

Jang, Y.J., Lin, C.Y., Ma, S., and Erikson, R.L. (2002). Functional studies on the role of the C-terminal domain of mammalian polo-like kinase. Proc Natl Acad Sci U S A *99*, 1984-1989.

Jang, Y.J., Ma, S., Terada, Y., and Erikson, R.L. (2002). Phosphorylation of threonine 210 and the role of serine 137 in the regulation of mammalian polo-like kinase. J Biol Chem *277*, 44115-44120.

Kang, Y.H., Park, J.E., Yu, L.R., Soung, N.K., Yun, S.M., Bang, J.K., Seong, Y.S., Yu, H., Garfield, S., Veenstra, T.D., et al. (2006). Self-regulated Plk1 recruitment to kinetochores by the Plk1-PBIP1 interaction is critical for proper chromosome segregation. Mol Cell *24*, 409-422.

Kawajiri, A., Yasui, Y., Goto, H., Tatsuka, M., Takahashi, M., Nagata, K.-i., and Inagaki, M. (2003). Functional significance of the specific sites phosphorylated in desmin

at cleavage furrow: Aurora-B may phosphorylate and regulate Type III intermediate filaments during cytokinesis coordinatedly with Rho-kinase. Mol Biol Cell *14*, 1489-1500.

Kemmler, S., Stach, M., Knapp, M., Ortiz, J., Pfannstiel, J., Ruppert, T., and Lechner, J. (2009). Mimicking Ndc80 phosphorylation triggers spindle assembly checkpoint signalling. EMBO J *28*, 1099-1110.

Kerppola, T.K. (2006). Design and implementation of bimolecular fluorescence complementation (BiFC) assays for the visualization of protein interactions in living cells. Nat Protoc *1*, 1278-1286.

Kim, I.S., Lee, M., Park, K.C., Jeon, Y., Park, J.H., Hwang, E.J., Jeon, T.I., Ko, S., Lee, H., Baek, S.H., et al. (2012). Roles of Mis18α in epigenetic regulation of centromeric chromatin and CENP-A loading. Mol Cell *46*, 260-273.

Kim, Y., Holland, A.J., Lan, W., and Cleveland, D.W. (2010). Aurora kinases and protein phosphatase 1 mediate chromosome congression through regulation of CENP-E. Cell *142*, 444-455.

Krenn, V., and Musacchio, A. (2015). The Aurora B kinase in chromosome bi-orientation and spindle checkpoint signaling. Front Oncol *5*, 225.

Kufer, T.A., Sillje, H.H., Korner, R., Gruss, O.J., Meraldi, P., and Nigg, E.A. (2002). Human TPX2 is required for targeting Aurora-A kinase to the spindle. J Cell Biol *158*, 617-623.

Kunitoku, N., Sasayama, T., Marumoto, T., Zhang, D., Honda, S., Kobayashi, O.,

Hatakeyama, K., Ushio, Y., Saya, H., and Hirota, T. (2003). CENP-A phosphorylation by Aurora-A in prophase is required for enrichment of Aurora-B at inner centromeres and for kinetochore function. Dev Cell *5*, 853-864.

Lampson, M.A., and Cheeseman, I.M. (2011). Sensing centromere tension: Aurora B and the regulation of kinetochore function. Trends Cell Biol *21*, 133-140.

Lan, W., Zhang, X., Kline-Smith, S.L., Rosasco, S.E., Barrett-Wilt, G.A., Shabanowitz, J., Hunt, D.F., Walczak, C.E., and Stukenberg, P.T. (2004). Aurora B phosphorylates centromeric MCAK and regulates its localization and microtubule depolymerization activity. Curr Biol *14*, 273-286.

Lee, H.-S., Park, Y.-Y., Cho, M.-Y., Chae, S., Yoo, Y.-S., Kwon, M.-H., Lee, C.-W., and Cho, H. (2015). The chromatin remodeller RSF1 is essential for PLK1 deposition and function at mitotic kinetochores. Nat Commun *6*, 7904.

Lee, K.S., Grenfell, T.Z., Yarm, F.R., and Erikson, R.L. (1998). Mutation of the polo-box disrupts localization and mitotic functions of the mammalian polo kinase Plk. Proc Natl Acad Sci U S A *95*, 9301-9306.

Lenart, P., Petronczki, M., Steegmaier, M., Di Fiore, B., Lipp, J.J., Hoffmann, M., Rettig, W.J., Kraut, N., and Peters, J.M. (2007). The small-molecule inhibitor BI2536 reveals novel insights into mitotic roles of polo-like kinase 1. Curr Biol *17*, 304-315.

Lera, R.F., and Burkard, M.E. (2012). High mitotic activity of Polo-like Kinase 1 is required for chromosome segregation and genomic integrity in human epithelial cells. J Biol Chem *287*, 42812-42825.

Lesage, B., Qian, J., and Bollen, M. (2011). Spindle checkpoint silencing: PP1 tips the balance. Curr Biol *21*, R898-903.

Li, Y., Kao, G.D., Garcia, B.A., Shabanowitz, J., Hunt, D.F., Qin, J., Phelan, C., and Lazar, M.A. (2006). A novel histone deacetylase pathway regulates mitosis by modulating Aurora B kinase activity. Genes Dev *20*, 2566-2579.

Liu, D., Davydenko, O., and Lampson, M.A. (2012). Polo-like kinase-1 regulates kinetochore-microtubule dynamics and spindle checkpoint silencing. J Cell Biol *198*, 491-499.

Lowery, D.M., Clauser, K.R., Hjerrild, M., Lim, D., Alexander, J., Kishi, K., Ong, S.E., Gammeltoft, S., Carr, S.A., and Yaffe, M.B. (2007). Proteomic screen defines the Polo-box domain interactome and identifies Rock2 as a Plk1 substrate. EMBO J *26*, 2262-2273.

Mackay, A.M., Ainsztein, A.M., Eckley, D.M., and Earnshaw, W.C. (1998). A dominant mutant of inner centromere protein (INCENP), a chromosomal protein, disrupts prometaphase congression and cytokinesis. The Journal of Cell Biology *140*, 991-1002.

Macurek, L., Lindqvist, A., Lim, D., Lampson, M.A., Klompmaker, R., Freire, R., Clouin, C., Taylor, S.S., Yaffe, M.B., and Medema, R.H. (2008). Polo-like kinase-1 is activated by aurora A to promote checkpoint recovery. Nature *455*, 119-123.

Maresca, T.J., and Salmon, E.D. (2009). Intrakinetochore stretch is associated with changes in kinetochore phosphorylation and spindle assembly checkpoint activity. J Cell Biol *184*, 373-381.

McKinley, K.L., and Cheeseman, I.M. (2014). Polo-like kinase 1 licenses CENP-A deposition at centromeres. Cell *158*, 397-411.

Moshe, Y., Boulaire, J., Pagano, M., and Hershko, A. (2004). Role of Polo-like kinase in the degradation of early mitotic inhibitor 1, a regulator of the anaphase promoting complex/cyclosome. Proc Natl Acad Sci U S A *101*, 7937-7942.

Moutinho-Santos, T., Conde, C., and Sunkel, C.E. (2012). POLO ensures chromosome bi-orientation by preventing and correcting erroneous chromosome–spindle attachments. J Cell Sci *125*, 576-583.

Neef, R., Gruneberg, U., Kopajtich, R., Li, X., Nigg, E.A., Sillje, H., and Barr, F.A. (2007). Choice of Plk1 docking partners during mitosis and cytokinesis is controlled by the activation state of Cdk1. Nat Cell Biol *9*, 436-444.

Otto, T., and Sicinski, P. (2017). Cell cycle proteins as promising targets in cancer therapy. Nat Rev Cancer *17*, 93-115.

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

Park, K.C., Lee, M., Jeon, Y., Jeon, R., Baek, S.H., Lee, H., and Kim, K.I. (2017). Skin-specific deletion of Mis18a impedes proliferation and stratification of epidermal keratinocytes. J Invest Dermatol *137*, 414-421.

Petronczki, M., Glotzer, M., Kraut, N., and Peters, J.-M. (2007). Polo-like Kinase 1 triggers the initiation of cytokinesis in human cells by promoting recruitment of the RhoGEF Ect2 to the central spindle. Dev Cell *12*, 713-725.

Petronczki, M., Lenart, P., and Peters, J.M. (2008). Polo on the rise-from mitotic entry to cytokinesis with Plk1. Dev Cell *14*, 646-659.

Qi, W., Tang, Z., and Yu, H. (2006). Phosphorylation- and polo-box-dependent binding of Plk1 to Bub1 is required for the kinetochore localization of Plk1. Mol Biol Cell *17*, 3705-3716.

Sawicka, A., and Seiser, C. (2014). Sensing core histone phosphorylation — A matter of perfect timing. Biochim Biophys Acta *1839*, 711-718.

Seki, A., Coppinger, J.A., Jang, C.Y., Yates, J.R., and Fang, G. (2008). Bora and the kinase Aurora a cooperatively activate the kinase Plk1 and control mitotic entry. Science *320*, 1655-1658.

Sessa, F., Mapelli, M., Ciferri, C., Tarricone, C., Areces, L.B., Schneider, T.R., Stukenberg, P.T., and Musacchio, A. (2005). Mechanism of Aurora B activation by INCENP and inhibition by Hesperadin. Mol Cell *18*, 379-391.

Shao, H., Huang, Y., Zhang, L., Yuan, K., Chu, Y., Dou, Z., Jin, C., Garcia-Barrio, M., Liu, X., and Yao, X. (2015). Spatiotemporal dynamics of Aurora B-PLK1-MCAK signaling axis orchestrates kinetochore bi-orientation and faithful chromosome segregation. Sci Rep *5*, 12204.

Silva, M.C., and Jansen, L.E. (2009). At the right place at the right time: novel CENP-A binding proteins shed light on centromere assembly. Chromosoma *118*, 567-574.

Silva, M.C., Bodor, D.L., Stellfox, M.E., Martins, N.M., Hochegger, H., Foltz, D.R., and Jansen, L.E. (2012). Cdk activity couples epigenetic centromere inheritance to cell cycle progression. Dev Cell *22*, 52-63.

Smits, V.A., Klompmaker, R., Arnaud, L., Rijksen, G., Nigg, E.A., and Medema, R.H. (2000). Polo-like kinase-1 is a target of the DNA damage checkpoint. Nat Cell Biol *2*, 672-676.

Suijkerbuijk, S.J., Vleugel, M., Teixeira, A., and Kops, G.J. (2012). Integration of kinase and phosphatase activities by BUBR1 ensures formation of stable kinetochore-microtubule attachments. Dev Cell *23*, 745-755.

Tauchman, E.C., Boehm, F.J., and DeLuca, J.G. (2015). Stable kinetochore-microtubule attachment is sufficient to silence the spindle assembly checkpoint in human cells. Nat Commun *6*, 10036.

Terada, Y., Tatsuka, M., Suzuki, F., Yasuda, Y., Fujita, S., and Otsu, M. (1998). AIM-1: a mammalian midbody-associated protein required for cytokinesis. The EMBO Journal *17*, 667-676.

Tomonaga, T., Matsushita, K., Yamaguchi, S., Oohashi, T., Shimada, H., Ochiai, T., Yoda, K., and Nomura, F. (2003). Overexpression and mistargeting of centromere protein-A in human primary colorectal cancer. Cancer Res *63*, 3511-3516.

Toyoshima-Morimoto, F., Taniguchi, E., and Nishida, E. (2002). Plk1 promotes nuclear translocation of human Cdc25C during prophase. EMBO Rep *3*, 341-348.

van de Weerdt, B.C., and Medema, R.H. (2006). Polo-like kinases: a team in control of the division. Cell Cycle *5*, 853-864.

van Vugt, M.A., and Medema, R.H. (2005). Getting in and out of mitosis with Polo-like kinase-1. Oncogene *24*, 2844-2859.

van Vugt, M.A., Bras, A., and Medema, R.H. (2004). Polo-like kinase-1 controls recovery from a G2 DNA damage-induced arrest in mammalian cells. Mol Cell *15*, 799-811.

Vanoosthuyse, V., and Hardwick, K.G. (2009). Overcoming inhibition in the spindle checkpoint. Genes Dev *23*, 2799-2805.

Wang, B., Ma, W., Xu, X., Wang, C., Zhu, Y., An, N., An, L., Wu, Z., and Tian, J. (2013). Phosphorylation of histone H3 on Ser10 by auto-phosphorylated PAK1 is not essential for chromatin condensation and meiotic progression in porcine oocytes. Journal of Animal Science and Biotechnology *4*, 13.

Wei, Y., Mizzen, C.A., Cook, R.G., Gorovsky, M.A., and Allis, C.D. (1998). Phosphorylation of histone H3 at serine 10 is correlated with chromosome condensation during mitosis and meiosis in Tetrahymena. Proc Natl Acad Sci U S A *95*, 7480-7484.

Wei, Y., Yu, L., Bowen, J., Gorovsky, M.A., and Allis, C.D. (1999). Phosphorylation of histone H3 is required for proper chromosome condensation and segregation. Cell *97*, 99-109.

Wilkins, B.J., Rall, N.A., Ostwal, Y., Kruitwagen, T., Hiragami-Hamada, K., Winkler, M., Barral, Y., Fischle, W., and Neumann, H. (2014). A cascade of histone modifications induces chromatin condensation in mitosis. Science *343*, 77-80.

Zeitlin, S.G., Shelby, R.D., and Sullivan, K.F. (2001). CENP-A is phosphorylated by Aurora B kinase and plays an unexpected role in completion of cytokinesis. The Journal of Cell Biology *155*, 1147-1158.

국문초록/Abstract in Korean

Mis18α는 Mis18β/M18BP1과 함께 Mis18 복합체를 이루는 것으로 늦은 세포분열 시기에서부터 이른 G1 시기에 동원체 부위에 위치하는 것으로 알려져 있다. 이는 히스톤 H3의 변이체 중 하나인 CENP-A가 동원체에 들어오도록 유도하는 과정으로 그 기능이 잘 알려져 있지만, Mis18α의 다른 기능은 알려진 바가 없다. 이 연구에서는 세포 주기가 정확하게 이루어 질 수 있도록 Mis18α가 담당하는 기능을 밝히고 그 기능이 기존에 밝혀진 CENP-A가 동원체에 위치하는 과정과는 무관함을 밝혔다. Aurora B 인산화 효소에 의해 Mis18α가 세포분열 시기에 인산화가 되는 것을 보았으며 이는 Mis18α가 Mis18 복합체를 이루거나 CENP-A의 동원체 위치지정을 돕는 것에는 전혀 영향이 없음을 확인하였다. 하지만 인산화가 되지 않는 세포주를 제작하여 표현형을 관찰하였을 때 소핵 형성이 늘어나거나 염색체가 제대로 중앙에 배열되지 않고 염색사가 늘어지는 현상 또한 많이 관찰이 되었다. 흥미롭게도, 세포분열 시기에 Mis18α가 인산화가 되지 못할 때에는 PLK1이 동원체 부위로 제대로 들어오지 못한다는 것을 확인하였다. 따라서 본 연구에서는 세포주기가 정확하게 이루어지기 위해서는 Aurora B에 의한 Mis18α 인산화가 정확하게 일어나는 것이 중요하며 이는 PLK1이 동원체 부위로 들어오는 것에 결정적인 역할을 한다는 것을 보이고자 했다.

주요어

Mis18α, Aurora B 인산화효소, PLK1, 세포분열 시기 인산화, Polo Box Domain, 동원체