THE MECHANISMS OF HOW SISTER CHROMATID SEPARATION IS
REGULATED IN MITOSIS

AN ABSTRACT
SUBMITTED ON APRIL 16TH, 2021
TO THE DEPARTMENT OF BIOCHEMISTRY AND MOLECULAR BIOLOGY
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
OF THE GRADUATE SCHOOL
OF TULANE UNIVERSITY
FOR THE DEGREE OF DOCTOR
OF
PHILOSOPHY BY

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Abstract

Faithful chromosome segregation in mitosis is required for maintaining chromosome stability, thus ensuring the accurate propagation of genetic information. Multiple mitotic pathways have evolved to regulate this process including kinetochore-microtubule (KT-MT) attachment and centromeric cohesion. My studies focus on the regulation of KT-MT attachment and centromeric cohesion. The kinetochore is a multi-protein complex that serves as spindle-microtubule binding sites for chromosome segregation. The spindle kinetochore assembly complex (Ska) is required for proper KT-MT attachment, but the underlying mechanisms are unknown. I demonstrate that multisite phosphorylation of Ska by Cdk1 enables Ska binding to Ndc80C and recruits Ska to the outer kinetochore to promote chromosome segregation. Duplicated sister chromatids are linked by the cohesin complex which is termed cohesion. Proper regulation of centromeric cohesion is essential for chromosome segregation. Cyclin-dependent-kinase (Cdk) 11 has been shown to promote centromeric cohesion. My results demonstrate that Cdk11 localizes to centromeres and binds to RNA polymerase (RNAP) II. Cdk11 depletion significantly decreases RNAPII levels at centromeres and centromeric transcription. As centromeric transcription is essential for centromeric cohesion, I propose that Cdk11 binds to and phosphorylates RNAPII to enhance centromeric transcription, thus promoting centromeric cohesion. All the above results reveal the critical mechanisms underlying chromosome segregation. As chromosome missegregation drives aneuploidy that promotes tumorigenesis, these mechanisms may provide potential therapeutic targets for cancer.
THE MECHANISMS OF HOW SISTER CHROMATID SEPARATION IS REGULATED IN MITOSIS

A DISSERTATION
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ACKNOWLEDGEMENT

“Where there is a will, there is a way” ---- Pauline Kael. I am always motivated and inspired by this quotation. However, I could not have come this far without standing on the shoulders of giants. It is their help and support that encourage me to explore the way to success, to find the light of dim tunnel, and to dig out the treasure of my long-term journey. For my PhD, I need to thank many people.

Firstly, I would like to thank my mentor, Dr. Hong Liu, for providing unconditional support during my time at Tulane. I am so grateful for his patience, for teaching me from zero, and for guiding me towards the right path. I have learned from him the value of tolerance, the respect to science and the insistence on the truth. The knowledge he has imparted to me has been a great asset throughout my career. I will always be inspired by his energy, curiosity, enthusiasm, and compassion. One day, I hope to pass on what he has taught me to others.

I would also like to thank my dissertation committee members Dr. Zachary Pursell, Dr. Heather Machado, Dr. Jeffrey Han, and my previous committee member Dr. Zubaida Saifudeen for their invaluable insights, guidance and encouragement throughout my pre-doctoral training. They are pushing me to become a better graduate student and scientist. I also want to thank the graduate students and postdocs in Biochemistry and Molecular Biology department for their kindness, helps and support I will always cherish.
I would also like to thank the chairman of Biochemistry and Molecular Biology department Dr. Hua Lu, for encouraging me and sticking up for me over the years. I would also like to thank the stuff in our department and Biomedical Sciences program Teyana Antoshchenko, Gilbert Estrada, Marlene Jones, Miriam Ruiz, John H. (Jake) Korn, and Zylkia Lozano for their support and all of the work they put into facilitating my success.

I would also like to thank our collaborators and colleagues that have assisted us. It’s their contributions that made this work possible. I am so grateful for all they have done and deeply value our collaborations.

I would also like to thank my friends for always being there and backing me up. I could not achieve this without their continuous support. The laughs, dinners, movies, and all the memories we share will always cheer me up along the journey. Meeting them was one of the best things that ever happened in my life.

Last but not least, I want to thank my family. I would like to thank my Mom and Dad for their love, unconditional support and all the sacrifices they have made to make who I am. They always stand by my side and encourage me to pursue my dreams. I would also like to thank my Grandpa for igniting my passion for biology when I was young and teaching me to see the bright side of each situation. Though I can no longer be with him, he will be living in my heart forever. I would also like to thank my Grandma for spending time with me during summer break. I appreciate that more than anything. I would also like to thank my uncle and my aunt for being wonderful listeners and sharing their interesting stories. I would also like to thank
my high school classmate, who happens to be my boyfriend, for being there for me no matter how challenging and difficult things may be and bringing cares and laughs to my parents when I am 7,791 miles away in these years.

To everyone I mentioned, this work is for you.
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CHAPTER 1: BACKGROUND FOR CHROMOSOME SEGREGATION IN MITOSIS

1.1 Chromosome segregation in mitosis and genome integrity

Somatic cell cycle in humans is generally divided into interphase and mitosis. Interphase consists of four phases (G0, G1, S and G2) during which cells prepare their genetic materials necessary for cell cycle progression and duplicate their chromosomes for subsequent cell division. Mitosis proceeds in five phases: prophase, prometaphase, metaphase, anaphase and telophase. In mitosis, sister chromatids separate from each other into two daughter cells [1]. Proper chromosome segregation is the prerequisite for maintaining genome stability [2-4]. Error in chromosome segregation usually leads to aneuploidy, which is a very common feature of many cancers [5-7]. As aneuploidy is known to drive tumorigenesis, a better understanding of chromosome segregation would help provide better therapeutics for cancer.

To prevent the occurrence of aneuploidy, faithful chromosome segregation must be guaranteed. It requires coordination of chromosome movement and cell cycle regulation during mitosis [8, 9]. At least four major pathways function essentially in this coordination, including centrosome number and integrity, KT-MT attachment and dynamics, spindle assembly checkpoint, and regulation of sister-chromatid cohesion [10-14]. Centrosome serves as the main microtubule organizing center (MTOC) and contributes to the assembly of the bipolar spindle apparatus during mitosis. Aberrant centrosome numbers and functions generate the formation of mono- or multipolar spindles, which
compromises the fidelity of chromosome segregation [15]. Spindle microtubules emanating from centrosomes can interact with chromosomes via kinetochores and establish KT-MT attachment at early mitosis. KT-MT attachment serves as the major driving force for chromosome movement and aligns chromosomes onto metaphase plate. Defects in mitotic spindle assembly and chromosome alignment activate the spindle assembly checkpoint (SAC) to halt the cell cycle progression, thus allowing cells to repair the defects [16, 17]. Defective SAC causes mitotic slippage and results in the formation of aneuploid progeny [18]. Not until the SAC is silenced can the sister-chromatid cohesion at centromeres be removed to initiate chromosome segregation. Timely dissolution of centromeric cohesion is crucial for proper mitotic progression [14, 19]. Loss of centromeric cohesion ahead of anaphase leads to premature sister-chromatid separation and mitotic arrest. Conversely, cells with delayed removal of centromeric cohesion may exhibit lagging chromosomes and produce progeny with abnormal chromosome content. Collectively, all these pathways are essential to ensure the fidelity of chromosome segregation and the integrity of the whole genome. Dysfunction of mitotic pathways can drive chromosome instability (CIN), which is a hallmark of human cancer [20, 21].

Considering the key role of CIN in promoting aneuploidy and tumorigenesis, a better understanding of the mechanisms controlling faithful chromosome segregation would help to decipher the origin of tumor malignancy and shed light on cancer therapies in future. Thus, in this thesis, I investigate the general mechanisms underlying the regulation of sister chromatid separation in
mitosis and demonstrate the importance of two mitotic regulators, Ska3 and Cdk11, in promoting proper mitotic progression and ensuring faithful chromosome segregation.

1.2 Regulation of KT-MT attachments and dynamics

Accurate segregation of chromosomes during cell division requires proper KT-MT interactions [22, 23]. The kinetochore is a multi-protein complex assembled on the centromere region of each sister chromatid in mitosis [24]. Spindle microtubules are dynamic and undergo cycles of growing (polymerizing) and shrinking (depolymerizing) [25, 26]. During their growth and shrinkage, microtubules adopt important conformational changes: “straight” during polymerization and “curved” during depolymerization. To couple depolymerizing microtubule ends to chromosomes robustly, the kinetochore adopts so-called “force-bearing” binding to microtubules, which is also dynamic [27, 28]. These properties allow the kinetochore to track with both straight and curved protofilaments of microtubules and harness the energy released by depolymerizing microtubules to pull paired sister chromatids to opposite spindle poles at anaphase [29, 30].

At prometaphase, the kinetochore is initially captured by the lattice of microtubules (lateral-attachments) and subsequently binds to the plus ends (end-on attachments). The end-on attachments are stable and force-bearing, whereas the initial KT-MT attachments are transient, unstable and error-prone [31].

Erroneous attachments include: 1) monotelic attachments (only one of the
kinetochore pairs is captured by spindle microtubules; 2) syntelic attachments (both kinetochore pairs are attached by spindle microtubules emanating from the same spindle pole); and 3) merotelic attachments (single kinetochore is attached by spindle microtubules emanating from both sides of spindle poles) [32]. These erroneous attachments activate the SAC. It has been shown that even a single unattached kinetochore is sufficient to activate the SAC, thus preventing mitotic progression. In order to satisfy the SAC and ensure faithful chromosome segregation, erroneous attachments must be corrected to establish bioriented attachments (the kinetochore pairs are attached by spindle microtubules emanating from the opposite spindle poles) [27, 33, 34]. The repair of erroneous attachments requires dynamic interactions between kinetochores and microtubules [35].

1.3 The KMN network and Ndc80 complex

The “force-bearing” capability of kinetochores in tracking with shortening microtubules is determined by various outer kinetochore proteins, which play a crucial part in regulating KT-MT attachments at KT-MT interface [36, 37]. The nomenclature “outer kinetochore” was named according to its relative proximity to the centromere, which means “centromere-distal”. Correspondingly, “inner kinetochore” means “centromere-proximal”. The outer kinetochore provides the major binding sites for spindle microtubules, within which the 10-subunit Knl1-Mis12-Ndc80 (KMN) network is the key component [38, 39]. In vertebrates, the KMN network includes the Knl1 complex (Knl1C) consisting of Knl1 and ZwinT; the
Mis12 complex (Mis12C) comprising of Mis12, Nsl1, Nnf1 and Dsn1 (or Mis13 in humans); and the Ndc80 complex (Ndc80C), which consists of Ndc80 (or Hec1 in humans), Nuf2, Spc24 and Spc25. The KMN network assembles onto the kinetochore in prophase and dissociate from the kinetochore in telophase. Knl1C and Ndc80C are the major MT-binding components at kinetochores [40, 41]. Both complexes associate with dynamic plus ends of microtubules through their very outer ends, whereas their inner ends are anchored to either the inner kinetochore proteins, like CENP-T of the CCAN network (constitutive centromere-associated network), or to Mis12C. Although Mis12C does not directly bind spindle microtubules, it serves as a scaffold for outer kinetochore assembly [42]. Mis12C is also responsible for recruiting Knl1C and Ndc80 to kinetochores at early mitosis and synergizes with other components to enhance the MT-binding activities [43]. In addition to directly binding spindle microtubules, the KMN network recruits spindle checkpoint proteins to kinetochores during mitosis [42, 44]. KNL1 is the key receptor for the Bub1-Bub3 and BubR1-Bub3 checkpoint complexes at outer kinetochores. Ndc80 is required for the kinetochore targeting of MPS1 as well as Mad1-Mad2 [45, 46]. Because of its essential role in both KT-MT interactions and SAC, removal of any of the components of the KMN network disrupts outer kinetochore structure, abolishes end-on attachment, causes chromosome misalignment and compromises SAC signaling [38, 39].

The Ndc80 complex adopts a 57-nm-long rod-shaped structure containing two globular domains at each end [47, 48]. It is composed of two heterodimers, Ndc80-Nuf2 and Spc24-Spc25 complexes, which are connected together by a
central α-helical coiled coil. The Spc24-Spc25 complex anchors the whole complex into the kinetochore while the Ndc80-Nuf2 complex directly bind microtubules. Ndc80C is very flexible and adopts various conformations. This flexibility is conferred by the loop in the coiled coil and determines Ndc80C capacity to switch between straightened and bent conformations, which is important for its tracking with microtubules [49, 50]. Loss of this ability causes aberrant KT-MT attachments and failure in proper mitotic progression. In addition, the Ndc80 loop functions as a platform for recruiting multiple mitotic factors crucial for chromosome segregation and deletion of the loop results in delocalization of these factors from the kinetochore [51-53].

Extensive studies have identified two distinct MT-binding elements in the globular head of Ndc80 that are important for the formation of stable KT-MT attachments and alignment: the disordered N-terminal tail and the adjacent calponin homology (CH) domain [54-56]. The N-terminal tail of Ndc80 is very basic so that it is capable of interacting with the negatively charged C-terminal tails of tubulin monomers. This interaction is mainly regulated by mitotic kinase Aurora B [57, 58]. Aurora B-dependent phosphorylation of Ndc80 is critical for destabilizing KT-MT attachments and correcting attachment errors during early mitosis. The CH domain of Ndc80 binds to spindle microtubules independently of the N-terminal tail and directly interfaces microtubule lattice [55]. Cells with removal or disruption in charges of the Ndc80 CH domain failed to establish stable KT-MT attachments and were arrested in mitosis. Additionally, the Ndc80 CH domain is able to sensitize the conformational changes of microtubules and accordingly adjust its
MT-binding affinities. Consequently, Ndc80C binds to straight microtubule protofilaments with stronger affinity and to curved protofilaments with weaker affinity. The CH domain of Nuf2 is also important for the binding to microtubules but to a lesser extent of Ndc80 [59].

Despite that Ndc80C provides the key KT-MT binding sites, the stable end-on attachments cannot be finalized without the presence of other outer-kinetochore proteins like the spindle kinetochore assembly (Ska) complex [60-62]. The absence of Ska impaired the binding activity of Ndc80 to microtubule plus ends in vitro. How these factors collaborate with Ndc80C to achieve proper end-on attachments is an important question in the field.

1.4 The role of the Ska complex in end-on attachments

The human Ska complex (Ska) is a “W” shaped dimer composed of triple helical bundles (Ska1, Ska2 and Ska3) [63]. Ska localizes at kinetochores, microtubules and centrosomes, and serves as a crucial factor in promoting chromosome segregation in mitosis [62, 64, 65]. In yeast, Ska has a functional ortholog named the Dam/Dash1 complex though they share no substantial similarities in amino acid sequences [65, 66]. Both complexes clamp onto kinetochores and track the tip and lattice of depolymerizing microtubules. The major functions of Ska in promoting chromosome segregation is to establish and/or maintain stable KT-MT attachments and to silence the SAC when sister-kinetochore pairs are bioriented [64, 67]. At KT-MT interface, Ska interacts with other outer kinetochore proteins like Ndc80C and Cdt1 to regulate proper KT-MT
interactions [60, 68]. Ska also recruits PP1 (protein Ser/Thr phosphatase 1) to the kinetochore for inactivation of the SAC and enriches APC/C (the anaphase promoting complex) on chromosomes for transition to anaphase [69, 70]. Depletion of any member of Ska causes chromosome misalignment, prolonged anaphase onset or permanent mitotic arrest, and mitotic cell death. Furthermore, Ska is an essential regulator for centrosome integrity [71]. Cells with depletion of Ska exhibited fragmented centrosomes and multiple spindle poles. Ska is also important for the maintenance of centromeric cohesion [67].

Ska directly binds to dynamic spindle microtubules in mitosis [63, 72, 73]. The MT-binding domain of Ska is located within its C-termini within Ska1 and Ska3 extruding from both ends. Truncation of either of the C-termini impaired the formation of robust KT-MT attachments and reduced sister chromatid oscillations in cells. The C-terminal domain of Ska1 provides the primary MT-binding site [74]. It forms into a winged-helix-like structure, which contains multiple Aurora B phospho-sites responsible for suppressing Ska interaction with microtubules in early mitosis [75]. Ska also promotes Aurora B functions [76]. It remains unclear how Ska3 C-terminus binds to microtubules. Biochemical studies showed that the C-terminus of Ska3 directly interacts with tubulin monomers and indirectly interacts with tubulin contacting regions of Ska1 [77], providing a likely mechanism whereby it associates with microtubules. It is noteworthy that cells expressing Ska1 mutant lacking the MT-binding domain aligned chromosome at equator normally, indicating that the MT-binding activity of Ska is dispensable for chromosome alignment.
Ska begins to enrich at kinetochores at prometaphase, peaks at metaphase and dislodges at middle anaphase. Progressive recruitment of Ska to kinetochores is essential since premature kinetochore localization of Ska compromises the chromosome segregation fidelity [75]. Once onto kinetochores, Ska tracks dynamic microtubules in a load-bearing manner and promotes the conversion from lateral to end-on attachments [73]. Depletion of Ska largely diminished the number of kinetochores with stable microtubule attachments without affecting chromosome congression in cells. The establishment of stable KT-MT attachments by Ska is through its collaboration with Ndc80C [60, 61, 68]. Although Ndc80C alone is unable to form stable end-on attachments, accumulative evidence suggest that Ska imparts the tip-tracking capacity to it and hence strengthens the KT-MT interactions. Additionally, Ska and Ndc80C bind to microtubules synergistically. This synergy may grant further enhancement to the KT-MT associations. Ska functions in assisting end-on attachments are suppressed by Mps1 in early mitosis [78]. Mps1 phosphorylation on Ska disrupts its interaction with microtubules and thus destabilizes KT-MT attachments. This regulation allows for the correction of erroneous attachments.

Nevertheless, the proper function of Ska at KT-MT interface must be based on the fact that Ska is recruited and binds to kinetochores. However, how this is achieved at the molecular levels remains unknown. Lack of this knowledge prevents us from further investigating how the KT-MT attachments are properly regulated to ensure faithful chromosome segregation.
1.5 the recruitment of Ska to the kinetochore

Extensive studies have focused on the mechanism whereby Ska is recruited to kinetochores. Multiple mitotic factors have been identified to function essentially in this process including Aurora B, PP1, PP2A (protein phosphatase 2A), as well as Ndc80 [75, 79]. Aurora B kinase activity has been implicated to counteract the recruitment of Ska to kinetochores [75]. At early mitosis, Aurora B with high activity phosphorylates Ska to restrain Ska from localizing to kinetochores. At metaphase, Aurora B with low activity relieves the restraining and Ska localizes to kinetochores at its maximum capacity. The kinetochore recruitment of Ska relies on PP1 and PP2A [79]. Knockdown of PP1 or PP2A remarkably reduced Ska loading onto kinetochores, resulting in chromosome alignment defects and delayed chromosome segregation. *In vitro* cross-linking assay showed that Ndc80 provides the major binding site for Ska [60]. In cells, Ndc80 knockdown prominently decreased Ska levels at kinetochores. The Ndc80 loop may serve as a platform for Ska binding [52]. Thus, Ndc80C may be the direct kinetochore receptor for Ska, but the compelling evidence is still lacking.

In this thesis, I attack this important question by showing that Cdk1 phosphorylation on Ska3 promotes its binding to Ndc80 both *in vitro* and *vivo*. I further demonstrate that Cdk1 phosphorylation-mediated Ska-Ndc80 binding is required for Ska localization to kinetochores and proper chromosome segregation. Detailed results are included in Chapter 3.
1.6 Protection of sister-chromatid cohesion at centromeres by Sgo1

Sister-chromatid cohesion is required to entrap DNA topologically until anaphase. Cohesion is usually established at S phase when DNA replication occurs and removed at mitosis when sister-chromatids separate from each other [14, 80, 81]. It is mainly mediated by the ring-shaped cohesin complex and its regulator Sororin [81, 82]. The cohesin complex is composed of four core subunits, Smc1, Smc3, SA1/2 and Scc1, which are highly conserved from yeast to human. Proper sister-chromatid cohesion is important for maintaining the chromosome segregation fidelity and protecting the genome stability [83]. During vertebrate mitosis, cohesion dissolution occurs in a stepwise manner. In prophase, most of the cohesin on chromosome arms is phosphorylated and removed by mitotic kinases (Cdk1, Plk1 and Aurora B) in Wapl-dependent manner, thus forming iconic X-shaped chromosomes with a small pool of cohesin preserved at centromeres [84-86]. At meta-to-anaphase transition, this centromeric pool of cohesin is cleaved by Seperase to initiate chromosome segregation. The Shielding of centromeric cohesin from Wapl is dependent on Shugoshin/MEI-S332 family proteins that are conserved across species [87, 88]. In vertebrates, Shugoshin depletion results in severe cohesion defects, premature chromosome segregation, mitotic arrest, and chromosome missegregation. In yeast and plant, while Shugoshin is dispensable for the protection of centromeric cohesion in mitosis, it is essential in centromeric cohesion maintenance in meiosis. In addition to function critically in protecting centromeric cohesion, Shugoshin also plays a role in regulating chromosome attachment and spindle checkpoint pathway [89-92].
*Drosophila melanogaster* Mei-S332 was the first member identified in the Shugoshin family, but it is a major guardian of centromeric cohesion in meiosis not mitosis [88, 93]. Vertebrates possess two shugoshin-like proteins Sgo1 and Sgo2. Sgo1 is believed as the major protector for centromeric cohesin in mitosis, whereas Sgo2 is the major one in meiosis [94-97]. Both shugoshins associate with PP2A, the chromosome passage complex (CPC), and PP2A inhibitor SET at centromeres to regulate centromeric cohesion [89, 98-102]. How is Sgo1 modulated to protect centromeric cohesion in mitosis? Firstly, Cdk1 phosphorylation on Sgo1 directs its binding to cohesin at inner centromeres [103]. Then, cohesin-bound Sgo1 escorts PP2A to the vicinity of cohesin to counteract the phosphorylation of mitotic kinases on cohesin and Sororin [104, 105]. Sgo2 may also play a role in this process because the recruitment of PP2A is mainly through Sgo2, though Sgo2 knockdown barely induced cohesion defects in mitotic cells [97, 106, 107]. Finally, hypo-phosphorylated cohesin and Sororin alleviate the cohesin-releasing effects imposed by Wapl and maintain cohesion at centromeres [108]. In addition to recruit PP2A, Sgo1 competitively binds to cohesin with Wapl, thus serving as a second barrier between Wapl and centromeric cohesin [103].

1.7 The recruitment of Sgo1 to the inner centromere

The recruitment of Sgo1 to inner centromeres is a two-step process: Sgo1 is firstly recruited to kinetochores and then translocated to inner centromeres for cohesin binding. Two essential mitotic kinases functions importantly in this process: Bub1 and Cdk1[103, 109, 110]. Bub1 phosphorylates histone H2A on T120 at
kinetochore-proximal region, a marker that can be recognized by Sgo1 and recruits it to kinetochores initially [111]. In yeast and human, Bub1 depletion delocalized Sgo1 from inner centromeres to chromosome arms [109-111]. Either inactivation of Bub1 kinase activity or mutation of H2A-T120 to alanine largely reduced centromeric Sgo1 levels. Once at kinetochores, Sgo1 directly binds to histone through its C-terminal basic region (also known as “Shugoshin” domain). Sgo1 C-terminus is important for its association with mitotic chromosomes. In cells, truncation of the C-terminus (ΔC) or mutation of the key residue from lysine to glutamate (K492E) resulted in the delocalization of Sgo1 from centromeres and chromosome arms. Biochemical data also proved that ΔC or K492E severely disrupted the binding of Sgo1 to histone. After initial enrichment at kinetochores, Cdk1-mediated phosphorylation of Sgo1 promotes its binding to centromeric cohesin. The cohesin binding domain of Sgo1 is critical for its inner centromere localizations. Either mutation of the key phospho-site to alanine (T346A) or complete ablation of the cohesin binding domain delocalized Sgo1 from centromeres and relocated Sgo1 at kinetochore-proximal region. Interestingly, Sgo1 T346A failed to rescue the cohesion defects in Sgo1-depleted cells while Sgo1 K492A could, suggesting that the predominant factor determines Sgo1 function in protecting centromeric cohesion is Cdk1-mediated cohesin binding instead of Bub1-mediated kinetochore enrichment.

How is Sgo1 translocated from kinetochores to inner centromeres? Our recent studies suggested that RNAPII-mediated mitotic transcription enables the kinetochore-bound Sgo1 to penetrate into the inner centromere for binding to
cohesion [112, 113]. We found that Sgo1 directly binds to RNAPII. Mitotic-specific degradation of RNAPII subunit Rpb2 or inhibiting transcription by α-amanitin, a drug that suppresses RNAPII elongation, led to Sgo1 redistribution from inner centromeres to kinetochores. As a result, centromeric cohesion defects were observed in these cells. We also found that Sgo1 binds to centromeric RNAs, but the biological functions remain unknown. Given that transcription is globally suppressed in mitosis but centromeric DNAs are still actively transcribed, it is possible that centromeric transcription plays a key role in protecting centromeric cohesion. However, the evidence supporting this notion is still lacking.

1.8 Centromeric transcription and its functions in cells

The centromere is the region of chromosome that forms the assembly for the kinetochore and dictates sister-chromatid separation in mitosis and meiosis [114, 115]. This core function of centromere is highly conserved, but the DNA sequences vary dramatically across evolution and species. Human centromeres are built on a series of tandem repeats of 171 bp DNA named α-satellite, which further forms into higher-order repeat (HOR) units and gives rise to mb-sized human centromeres accounting for 3% of the genome. More than one distinct α-satellite array can be found in 70% of human chromosomes within their centromeric region, while only one can form into active centromere [116, 117]. With few exceptions like the point centromere in budding yeast, centromeric DNA sequences are not required nor sufficient to maintain the centromere identity. The functional centromere is defined epigenetically by the specific incorporation of a
histone H3 variant CENP-A, which is interspersed with the canonical H3 nucleosomes to constitute centromeres. All the centromeres of human chromosomes except Y chromosome, contain a 17 bp α-satellite monomer called CENP-B box that can be bound by CCAN protein CENP-B [118]. Centromeric chromatin is surrounded by constitutive heterochromatin ---- the pericentromere. The pericentromeric DNA is also composed of tandem and short repeats mainly containing Satellite I, II and III, but less ordered [119].

The centromere and pericentromere had been previously considered highly condensed and transcriptionally inert, but mounting evidence proved that these regions are being actively transcribed and producing non-coding RNA transcripts [112, 120, 121]. The delicate balance between centromeric- and pericentromeric- transcription is important for maintaining the genome stability [114, 122]. In human and mouse cells, long noncoding RNAs produced from pericentromeres promotes the formation of heterochromatin [123]. Defects in silencing the pericentromere led to chromosome missegregation. Centromeres from different chromosomes produce their own-specific RNA transcripts, which remain stable throughout the cell cycle [117, 124]. Centromeric RNAs are indispensable for proper cell cycle progression. In Drosophila, depletion of centromeric RNAs induced mitotic defects [125]. In human, targeting array-specific RNA transcripts for depletion arrested cells prior to mitosis [117]. Centromeric transcription is finely tuned at low levels. Enhancing or weakening centromeric transcription results in centromere dysfunction, chromosome mis-segregation and aneuploidy in progeny cells.
RNAPII mainly dictates centromeric transcription. Even in mitosis, when most transcription factors are evicted from chromosomes, elongating RNAPII (RNAPII-Ser2) is still presented at kinetochores [120]. Inhibition of RNAPII activity led to centromeric cohesion defects and chromosome missegregation in human cells [113]. Histone modifications also play a critical part in regulating centromeric transcription [126-129]. The centromere contains transcriptionally active marks such as dimethylated H3K4 (H3K4me2) and H3K36 (H3K36me2), which are essential for promoting RNAPII activity. These marks also promote the recruitment of CENP-A and its chaperon HJURP to centromeres [126]. In comparison, the pericentromere contains transcriptionally silencing marks such as hyper-methylated H3K9 (H3K9me2 and H3K9me3) and H3K27(H3K27me2 and H3K27me3), which prevent the pericentromere from expanding into the centromere region [121].

The key functions of centromeric transcription are to maintain centromere features and direct cell cycle division. This is achieved through its interaction with several centromeric proteins. In eukaryotes, centromeric transcription aids the recruitment of CENP-A at G1 [130, 131]. Inhibiting RNAPII-mediated transcription or targeted depletion of centromeric RNAs reduced CENP-A and HJURP levels [132, 133]. Centromeric transcription also promotes the integration of CENP-C [117, 134, 135]. Inhibition of RNAPII elongation by α-amanitin in mitotic cells destabilized CENP-C at kinetochores and induced lagging chromosomes. In animals and plants, the binding of CENP-C to centromeric RNA transcripts is important for its association with centromeric chromatin. Furthermore, centromeric
transcription directs the recruitment of the CPC during prometaphase. In mouse and Xenopus, centromeric RNAs are required for Aurora B inner centromere localization as well as its kinase activation [136, 137].

While there is a growing body of work demonstrating the biological significance of centromeric transcription, several important questions remain to be addressed. Firstly, it is unclear whether promoter exists in centromere region to fuel transcription machinery; Secondly, it is important to discern the effect of RNAPII-mediated transcription activity from that of centromeric RNA transcripts for better characterizing centromeric transcription; Thirdly, it is unknown whether there are any transcription factors specifically regulating this process; Lastly, the mechanisms whereby centromeric transcription regulates chromosome segregation remain elusive. In this thesis, I mainly focus on the last two questions by demonstrating how Cdk11 specifically regulates centromeric transcription and how important centromeric transcription is to preserve centromeric cohesion in mitosis. Detailed results are included in Chapter 4.

1.9 Transcriptional regulator Cdk11 and its role in mitosis

Cyclin-dependent kinase (Cdk) 11 belongs to the transcriptional Cdk subfamily and plays essential roles in multiple cellular pathways [138, 139]. Depletion of Cdk11 in mice led to early embryonic lethality [140]. In cells, its knockdown provoked cell proliferation defects and mitotic arrest [141, 142]. Cdk11 has various isoforms with distinct functions including Cdk11p110, Cdk11p58 and Cdk11p46. The major kinase isoform Cdk11p110 is constantly expressed throughout
the cell cycle and is a key regulator for mRNA processing and splicing. It is also believed to function critically in RNAPII-directed transcription. *In vitro* studies proved that Cdk11<sup>p110</sup> directly binds and phosphorylates the C-terminal domain of RNAPII at Ser2 and Ser5, marks for transcription elongation and initiation [143, 144]. In yeast, Cdk11-dependent phosphorylation of mediator subunits Med27 and Med4 promotes the assembly of the RNAPII mediator complex [145]. The shorter isoform Cdk11<sup>p58</sup> is specifically synthesized through an internal ribosome entry site (IRES) during mitosis. It is required for centrosome maturation, spindle assembly, centromeric cohesion protection and cytokinesis. Cdk11<sup>p46</sup> is generated through the cleavage of the larger or mitosis-specific isoform of Cdk11 by caspases 1 and 3 in response to apoptosis. All three types of Cdk11 proteins are partnered with L-type cyclins. Cdk11<sup>p58</sup> also binds cyclin D3.

The role of Cdk11 in protecting centromeric cohesion in mitosis was firstly reported by Lahti et al in 2007. By ectopic targeting cohesin complex subunit Scc1 to centromeres, they found that Cdk11 depletion caused premature rejection of Scc1 from mitotic chromosomes. Further scrutiny demonstrated that Cdk11 knockdown abrogated Bub1 localizations at kinetochores and relocated Sgo1 from inner centromeres to kinetochores, suggesting that Cdk11-dependent centromeric cohesion protection is partially dependent on Bub1. Consistently, a follow-up study in 2014 by another research group also confirmed the role of Cdk11 in the maintenance of centromeric cohesion and additionally proved that the kinase activity of Cdk11<sup>p58</sup> is essential for this process [141]. Different from the previous finding, however, they found that both Bub1 and Sgo1 were completely removed
from chromosomes upon Cdk11 depletion, suggesting the dominant role of Bub1 in this regulation. Therefore, how Cdk11 promotes centromeric cohesion is poorly understood. In this thesis, I attempt to determine the underlying mechanism(s) by which Cdk11 promotes centromeric cohesion. Detailed results are included in Chapter 4.
CHAPTER 2: MATERIALS AND METHODS

2.1 Mammalian cell culture, siRNAs, and transfection

HeLa Tet-On cells (Clontech) were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum and 10mM L-glutamine. RPE-1 cells were cultured in DMEM: F-12 medium (Invitrogen) supplemented with 10% FBS and 10 mM L-glutamine. To arrest cells at G1/S, cells were incubated in medium containing 2mM thymidine (Sigma) for 16 h. Nocodazole, MG132 and RO3306 were purchased from Sigma Aldrich. ZM447439 was bought from Tocris Bioscience. Reversine was purchased from Cayman Chemical. BI2536 was purchased from Selleck Chemicals.

Plasmid transfection was done using the Effectene reagent (Qiagen) according to the manufacturer’s protocols. For H2B-mCherry stable cells, HeLa Tet-On cells were transfected with pIRES vectors encoding H2B-mCherry and selected with 0.4 μg ml⁻¹ puromycin (Invitrogen). For Myc-Ska3/CENP-B/Cdk11 stable cells, HeLa Tet-On cells were transfected with pTRE2 vectors encoding RNAi-resistant Myc-Ska3/CENP-B/Cdk11 and selected with 350 μg ml⁻¹ hygromycin (Invitrogen). The surviving clones were screened for expression of the desired proteins in the presence of 1μg ml⁻¹ doxycycline (Invitrogen). Expression of Myc-Ska3/CENP-B/Cdk11 was also induced with 1μg ml⁻¹ doxycycline in the subsequent experiments.
For RNAi experiments, the siRNA oligonucleotides were purchased from Dharmaco or Thermo Scientific. HeLa cells were transfected using Lipofectamine RNAiMax (Invitrogen) according to the manufacturer’s protocols. Ska3-depleted cells were analyzed at 32-42 h after transfection, Cdk11-depleted and Wapl-depleted cells were analyzed at 48h after transfection, and CENP-B-depleted cells were analyzed at 72h after transfection. The sequences of the siRNAs used in this study are: siSka3, GGAUAUAGUCCACGUGUCA; siNdc80, GAGUAGAACUAGAAUGGA; siCENP-B #6, GCACGAUCCUGAAGAACAA; siCENP-B #7, GGAGGAGGGUGAUGUUGAU; siWapl, CGGACTACCCTTAGCACAA; siCdk11 #1361, AGCGGCUGAAGAGAGAA; siCdk11 #Design, GAGCGAGCAGCAGCGUGUGUU.

The following transcriptional inhibitors were used in this study. Alpha-amanitin (MilliporeSigma, A2263), triptolide (MilliporeSigma, T3652), and THZ1 (Selleckchem, S7549). These inhibitors were dissolved in DMSO and working concentrations were specified in each experiment.

2.2 Antibodies, Immunoblotting, and Immunoprecipitation

Antibodies used in this study were listed in the following: anti-centromere antibody (ACA or CREST-ImmunoVision, HCT-0100), anti-Tubulin (Thermo Scientific, 62204), anti-Actin (Invitrogen, MA5-11869), anti-Ska3 (Bethyl, A304-215A), anti-GFP (Abcam, ab1218; Aves, GFP-1010), anti-Myc (Roche, 11667203001), anti-Smc1 (Bethyl, A300-055A), anti-pRpb1 (Abcam, 4H8), anti-Rpb2-pSer2 (H5, Biolegend), anti-CENP-B (abcam, ab25734), anti-CENP-A (EMD
Millipore, 07-574), anti-Cdk11 (Bethyl, A300-310A). Anti-pSka3, anti-APC2, anti-Sgo1, anti-Wapl and anti-Bub1 antibodies were made in-house as described previously [101, 103]. Anti-Sororin antibodies were a gift from Dr. Susannah Rankin.

For immunoblotting, the secondary antibodies were purchased from LI-COR: IRDye® 680RD Goat anti-Mouse IgG Secondary Antibody (926-68070) and Goat anti-Rabbit IgG Secondary Antibody (926-32211).

For immunostaining, the secondary antibodies were purchased from Invitrogen: Goat anti-Rabbit IgG Secondary Antibody (A11008), Goat anti-Human IgG Secondary Antibody (A21090), and Donkey anti-Mouse IgG Secondary Antibody (A31571).

Antibody dilution was often 1:1000 unless specified.

Immunoprecipitation was performed as follows. Collected cells were lysed with lysis buffer (25 mM Tris–HCl at pH 7.5, 50 mM NaCl, 5 mM MgCl₂, 0.1% NP-40, 1 mM DTT, 0.5 μM okadaic acid, 5 mM NaF, 0.3 mM Na₃VO₄ and 100 units ml⁻¹ Turbo-nuclease (Accelagen). After a 1-hr incubation on ice and then a 10-min incubation at 37 °C, the lysate was cleared by centrifugation for 15 min at 4 °C at 20,817g. The supernatant was incubated with the antibody beads for 2 hr at 4 °C. The beads were washed four times with wash buffer (25 mM Tris–HCl at pH 7.5, 50 mM NaCl, 5 mM MgCl₂, 0.1% NP-40, 1 mM DTT, 0.5 μM okadaic acid, 5 mM NaF, 0.3 mM Na₃VO₄). The proteins bound to the beads were dissolved in SDS
sample buffer, separated by SDS–PAGE and subjected to mass-spectrometric analysis or blotted with the appropriate antibodies.

2.3 Immunofluorescence and chromosome spread

For regular immunostaining, mitotic cells were collected and then spun onto with a Shandon Cytospin centrifuge. Cells were immediately fixed with 4% ice-cold paraformaldehyde for 4 min, and then extracted with ice-cold PBS containing 0.2% Triton X-100 for 2 min. Cells were washed with PBS containing 0.1% Triton X-100 and then incubated with primary antibodies (1:1000 dilution) overnight at 4 °C. After washed with PBS containing 0.1% Triton X-100, cells were incubated at room temperature for 1 hr with the appropriate secondary antibodies conjugated to fluorophores (Molecular Probes, 1:1000 dilution). After incubation, cells were washed again with PBS containing 0.1% Triton X-100, stained with 1 μg ml⁻¹ DAPI and mounted with Vectashield.

For Chromosome spreads and immunostaining, mitotic cells were swelled in a pre-warmed hypotonic solution containing 50 or 75 mM KCl for 15 min at 37 °C and then spun onto slides with a Shandon Cytospin centrifuge. Cells were first extracted with ice-cold PBS containing 0.2% Triton X-100 for 2 min and then fixed in 4% ice-cold paraformaldehyde for 4 min. Cells were washed with PBS and then incubated with primary antibodies (1:1000 dilution) overnight at 4 °C. Cells were washed with PBS containing 0.1% Triton X-100 and incubated at room temperature for 1 hr with the appropriate secondary antibodies conjugated to fluorophores (Molecular Probes, 1:1000 dilution). After incubation, cells were
washed again with PBS containing 0.1% Triton X-100, stained with 1 μg ml⁻¹ DAPI and mounted with Vectashield.

The images were taken by a Nikon inverted confocal microscope (Eclipse Ti2, NIS-Elements software) with a ×60 objective. Image processing was carried out with ImageJ and Adobe Photoshop. Quantification was carried out with ImageJ. Statistical analysis was performed with GraphPad Prism

2.4 Microtubule cold-sensitivity assay

For microtubule cold-sensitivity assay, mitotic cells were collected and then spun onto with a Shandon Cytospin centrifuge. Slides with cells on were treated with ice for 5min and then cells were immediately fixed with methanol precooled at -20°C for 5min followed by extraction with ice-cold PBS containing 0.2% Triton X-100 for 2 min. Cells were washed with PBS containing 0.1% Triton X-100 and then incubated with primary antibodies (1:1000 dilution) overnight at 4 °C. After washed with PBS containing 0.1% Triton X-100, cells were incubated at room temperature for 1hr with the appropriate secondary antibodies conjugated to fluorophores (Molecular Probes, 1:1000 dilution). After incubation, cells were washed again with PBS containing 0.1% Triton X-100, stained with 1 μg ml⁻¹ DAPI and mounted with Vectashield.

The images were taken by a Nikon confocal microscope (Eclipse Ti2, NIS-Elements software) with a ×60 objective. Image processing was carried out with
ImageJ and Adobe Photoshop. Quantification was carried out with ImageJ. Statistical analysis was performed with GraphPad Prism.

**2.5 Time-lapse microscopy**

HeLa Tet-on cells stably expressing H2B-mCherry were treated as indicated, and then long-term imaging was performed. Images were collected every 6 min for 12-15 hr using a Nikon confocal microscope (Eclipse Ti2, NIS-Elements software) equipped with a 20X objective and an environment chamber that controls temperature and CO₂. Image panels displaying the elapsed time between consecutive frames were assembled using the software designed for Nikon confocal microscope. The time taken for each cell to progress from nuclear envelope breakdown (NEB) to anaphase onset (chromatid separation) was calculated in minutes and plotted in GraphPad Prism. The experiments were repeated for at least two times and the results were highly reproducible. Quantification was performed based on the results from a single experiment. Average and standard deviation were calculated using GraphPad Prism.

For checking Ska3 localization in live cells, GFP-Ska3 WT, 2A, 4A-3, 6A, and 6D were expressed in HeLa Tet-On cells. After treated with MG132 for 1 h, cells were immediately subjected to imaging using a Nikon confocal microscope (Eclipse Ti2, NIS-Elements software) with a ×60 objective. Images were processed in ImageJ and Adobe Photoshop. Statistical analysis was performed with GraphPad Prism.
2.6 Protein purification

The full-length Ska3 was subcloned into pGEX-6p-1 (GE Healthcare) vectors with an N-terminal GST tag with a 3C-cleavage site. The full-length Ska1-Ska2 were subcloned into the modified pET vector (Novagen) with or without 6x His tag. All Ska3 mutants were generated with standard two-step methods using PCR and Dpn1 and confirmed by DNA sequencing. For full-length Ska complex, GST-Ska3 was expressed in *E.coli* BL21 (DE3) cells, cultured in Luria-Bertani medium, Untagged or 6×His-tagged Ska1-Ska2 was cultured in Terrific Broth medium. All the cells were induced by 0.2 mM IPTG at 16°C overnight. Two sorts of cells were harvested and mixed for co-purification, and then disrupted by high-pressure homogenizer (ATX Engineering) in the PBS buffer. The cell lysates were clarified by centrifuged at 35000 g for 30 min at 4 °C. GST agarose beads (GE Healthcare) was added into the supernatant followed by rotation at 8 °C for 1.5 hr. The protein-bound beads were then transferred into gravity columns, washed with PBS extensively, and treated with 3C protease overnight at 4 °C to remove the GST tags. After being dialyzed against 25 mM Tris pH 8.0, 50 mM NaCl and 1 mM DTT, the harvested Ska complex was further purified by anion exchange chromatography (HiTrap Q FF, GE Healthcare) and gel filtration chromatography (Superdex 200 10/300 GL, GE Healthcare). The purified Ska complex was finally concentrated to 15~20 mg/ml in buffer containing 20 mM Tris pH 8.0, 150 mM NaCl and 1 mM DTT, and stored at -80 °C for later use.
GST-Nuf2-Hec1 complexes, GST-Spc24-Spc25 complexes and Ndc80 Bosai were purified as previously described [146]. To remove GST tags, cell lysates of these complexes were firstly treated with GST agarose beads (GE Healthcare), and then the protein-bound beads were transferred into gravity columns and treated with 3C protease overnight at 4 °C. The eluted complexes were subjected to gel filtration chromatography (Superdex 200 10/300 GL, GE Healthcare) for further purification.

2.7 In vitro binding and kinases assays

5 ug-10 ug GST fusion protein (GST-Nuf2:Hec1/GST-Spc24:Spc25), in buffer containing 20 mM Tris pH 8.0, 150 mM NaCl and 1 mM DTT, was bound to previously-equilibrated GST beads at 8 °C for 1 hr, incubated with Ska complex or mutants proteins at 8 °C for 1.5 hr, and washed three times with buffer containing 20 mM Tris pH 8.0, 300 mM NaCl, 1 mM DTT and 0.02% TritonX-100. The proteins retained on the beads were resolved with SDS-PAGE and stained with Coomassie Blue. GST proteins were also included as control.

Purified Ska complexes (WT and 6A) were treated with Cdk1-CyclinB1 plus 10 mM ATP with or without 100 uM RO3306 in kinase buffer containing 20 mM Tris pH 8.0, 50mM NaCl, 10 mM MgCl2 and 1 mM DTT at room temperature for 1 hr. Then Ska complexes were incubated with GST beads pre-bound with the GST-Nuf2-Hec1 complex for 1 hr at 8 °C. After washed with kinase buffer containing 0.02%Triton, the proteins bound with beads were resolved by SDS-PAGE and stained with Coomassie Brilliant Blue.
2.8 EU chasing and purification of EU-RNAs

Purification of EU-RNAs was performed according to the protocol from Click-iT™ Nascent RNA Capture Kit (C10365, ThermoFisher). Cells with a confluency of 60–80% in 10 cm petri dishes were treated with EU in a final concentration of 0.5 mM for 1 hr. EU-chased cells were then collected and dissolved in TRIzol solution (Invitrogen, 15596026). Total RNAs were extracted, dissolved in nuclease-free water and treated with TURBO DNase (Invitrogen, AM2238) in the presence of RNase inhibitor (NEB, M3014) at 37°C for 1 hr. Total RNAs were then extracted with Phenol/Chloroform/Isoamyl alcohol (Invitrogen, 15593-031), precipitated with ice-cold ethanol solution containing glycogen (Roche, 34990920) and sodium acetate (Invitrogen, AM9740), and finally dissolved in nuclease-free water (Invitrogen, 10977-015). Purified total RNA was then further incubated with streptavidin dynabeads (Invitrogen, 65602) pretreated with Salmon sperm DNA (Invitrogen, 12321D) in binding buffer for 45 min. With the help of DynaMag™-2 Magnet (Invitrogen, 12321D), dynabeads were washed with wash buffer I and II.

2.9 Reverse transcription and real-time PCR analysis

EU-RNA bound dynabeads were mixed with iScript Reverse Transcription Supermix (Bio-Rad, 1708841) and reverse transcription was performed according to the manufacturer’s protocols. After being mixed with the SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad, 1725274), the synthesized cDNA
was subjected to real-time PCR analysis using QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems).

The primers for human cells were used in this study: GAPDH-F, TGATGACATCAAGAAGGTGGTGAAG; GAPDH-R, TCCTTGGAGGCCATGTGGGCCAT; Rpl30-F, CAAGGCAAAGCGAATTGGT; Rpl30-R, GCCCGTTCAGTCTCTTCGATT; SAT-1-F: AAGGTCAATGGCAGAAAAGAA; SAT-1-R, CAACGAAGGCCACAAGATGTC; SAT-4-F: CATTCTCAGAAACTTCTTGATGTG; SAT-4-R: CTTCTGTCTAGTTTTTAGTGAATATA; SAT13/21-F: TAGACAGAAGCATTCTCAGAAACT; SAT13/21-R: TCCCGCTTCCAACGAAATCCTCCAAAC; pTRS-63-F: ATTGAAAACCTGCTCGATTGG; pTRS-63-R: TCGGTTTGATTCCATTCCAT; Satellite III-F: CTGGACTTTGTTGGAAGGA; Satellite III-R: ACAATCTCAGCCCACATTCC; pTRS-47-F: GGATCAGAAGGAACAGAGC; pTRS-47-R: AGTCCACTGCTCCATTCC.

2.10 Quantification and Statistical analysis

Image J was used to obtain numeric intensities of experimental subjects under investigation. In the experiments of Figures 3.3A, 3.3C, 3.4A, 3.4C, 3.6A, 3.7A, 3.8A, 3.8D, 3.9A, 3.12A, 4.1A, 4.1E, 4.2A, 4.4A, 4.4D, 4.5D, 4.6B, 4.7A, 4.9A, 4.10C, 4.10E, 4.12A, 4.12C, 4.14A, 4.14D, 4.15A, 4.15D, and 4.16C, six kinetochores were randomly selected from each cell. A mask was generated to mark centromeres on the basis of ACA staining in the projected image. After
background subtraction, the intensities of GFP-Ska1/2/3, Ska3, Myc-Ndc80, Sgo1, RNAPII, RNAPII-Ser2, Bub1, GFP-Mis12-Bub1 and ACA fluorescence signals within the mask were obtained in number. Relative intensity was derived from the intensity of GFP-Ska1/2/3, Ska3, Myc-Ndc80, Sgo1, RNAPII, RNAPII-Ser2, Bub1, or GFP-Mis12-Bub1 signals normalized to the one of ACA signals and plotted with the GraphPad Prizm software. All the experiments were repeated at least twice or three times.

For quantification of H3pS10 intensity on kinetochores in Figures 3.8A, a mask was generated to mark the whole DNA mass. After background subtraction, the intensities of H3pS10 and DAPI fluorescence signals within the mask were obtained in number. Relative intensity was derived from the intensity of H3pS10 signals normalized to the one of DAPI signals and plotted with the GraphPad Prizm software.

For quantification of GFP-Ska3 intensity on kinetochores and microtubules in Figures 3.5A and 3.5C, masks were generated to mark the cytoplasm, kinetochores and microtubules. After background subtraction, the intensities of GFP-Ska3 fluorescence signals within the masks were obtained in number. Relative intensity was derived from the intensity of GFP-Ska3 signals on kinetochores or microtubules normalized to the one of GFP-Ska3 signals in cytoplasm and plotted with the GraphPad Prism software.

For quantification of microtubule intensity in Figure 3.11B, a mask was generated to mark the nucleus within a cell. After background subtraction, the
intensities of microtubules and DAPI fluorescence signals within the mask were obtained in number. Relative intensity was derived from the intensity of microtubule signals normalized to the one of DAPI signals and plotted with the GraphPad Prism software.

For quantification of CENP-A levels in Figures 4.3A and 4.8A, a mask was generated to mark centromeres on the basis of ACA staining in the projected image. After background subtraction, the intensities of CENP-A and DNA fluorescence signals within the mask were obtained in number. Relative intensity was derived from the intensity of CENP-A signals normalized to the one of DNA signals and plotted with the GraphPad Prizm software.

Quantification was usually performed based on the results from a single experiment unless specified. Differences were assessed using ANOVA followed by pairwise comparisons using Tukey’s test. All the samples analyzed were included in quantification. Sample size was recorded in figures and their corresponding legends. No specific statistical methods were used to estimate sample size. No methods were used to determine whether the data met assumptions of the statistical approach.
CHAPTER 3: MULTISITE PHOSPHORYLATION DETERMINES THE FORMATION OF SKA-NDC80 MACRO-COMPLEXES AND RECRUITS SKA TO KINETOCHORES TO PROMOTE MITOTIC PROGRESSION

3.1 Introduction

In this chapter, I aim to investigate: 1) how Ska is recruited to kinetochores and 2) how Ska binds to Ndc80C in mitosis. Gaining such knowledge would provide insights for the mechanisms controlling proper KT-MT attachments for faithful chromosome segregation. By analyzing Cdk1 phosphorylation on Ska3 using mass spectrometry, I identify multiple phospho-sites within its C-terminus, which are highly conserved across species. Immunostaining and live-imaging analysis demonstrate that these sites are critical for Ska recruitment to kinetochores and its functions. Mutation of these sites to alanine largely reduces Ska3 kinetochore levels, impairs the KT-MT attachments, and delays chromosome segregation. Among them, Thr358 and Thr360 are particularly important and is very likely prime Ska loading at kinetochores. Consistent with previous findings, I find that Ndc80 loop is required for Ska recruitment to kinetochores. By reconstituting Ska and Ndc80C, I further prove that Cdk1 phosphorylation on Ska promotes its binding to Ndc80-Nuf2 and enables the formation of stable Ska-Ndc80 macro-complexes. Thus, I propose that Cdk1-mediated phosphorylation is the decisive factor that docks Ska onto kinetochores to enable its functions in mitosis.
3.2 Results

The Ska3 C-terminal region is heavily phosphorylated at multiple conserved Cdk1 sites

The Ska3 proteins undergoes a gel mobility shift on SDS-PAGE during mitosis that can be reversed by λ phosphatase treatment, indicating that Ska3 is hyperphosphorylated during mitosis (Figure 3.1A). To determine which mitotic kinase is responsible for this hyperphosphorylation, I treated Hela Tet-on cells with chemical inhibitors to inactivate various mitotic kinases, including Cdk1 (RO3306), Aurora B (ZM447439), Mps1 (Reversine), or Plk1 (BI2536). Only Cdk1 inhibition prevented the hyperphosphorylation of Ska3 (Figure 3.1B), suggesting that Cdk1 is required for Ska3 hyperphosphorylation in cells. The Ska3 protein has fourteen Ser/Thr phospho-sites that conform to the minimal consensus sequence of Cdk1 substrates (Figure 3.1E). Mutation of all these sites to alanine (Ska3 14A) abolished Ska3 recruitment to the kinetochore (Figure 3.1C), suggesting that Ska3 might play a key role in its kinetochore localizations. I firstly analyzed the conservation of these Cdk1 sites across species, including human (hs), mouse (mm), chicken (gg) and xenopus laevis (xl). I found that six sites (Thr203, Thr217, Ser283, Thr291, Thr358 and Thr360) distribute within three conserved regions. Four of them (Thr203, Ser283, Thr291, and Thr358) are highly conserved across the species we tested and the other two (Thr217 and Thr360) are less conserved (Figure 3.1D). Secondly, I performed mass-spectrometric analyses on immunoprecipitated Myc-Ska3 isolated from nocodazole-arrested HeLa Tet-On
cells. Five out of six sites (Ser265, Ser267, Ser283, Thr291, Ser346, Thr358 and Thr360) were found to be subjected to Cdk1 phosphorylation (Figure 3.1E). I also included Thr203 for further analysis because of its high conservation, though it was not retrieved from our mass-spectrometric data. All the six sites were able to be phosphorylated by Cdk1 in vitro (Figure 3.1F).

To specify the functions of these Cdk1 phosphorylation sites, I constructed various mutants containing different sets of mutations on these sites (Figure 3.2A). Because Ska3 is hyperphosphorylated in mitosis and exhibits significantly reduced gel mobility in SDS-PAGE, I then examined how mutations of these sites affected its migration behavior. Nocodazole-arrested HeLa Tet-On cells were transfected with GFP-Ska3 WT, 2A, 4A1/2/3, 6A, and 6D, and cell lysates were analyzed with SDS-PAGE followed by Western blots. Consistently, the slower-migration species of Ska3 WT appeared on SDS-PAGE (Figure 3.2B). Double mutations to non-phosphorylatable alanine (2A) accelerated the gel mobility of the slower species, and quadruple mutations to alanine (4A1/2) that included Thr358 and Thr360 further accelerated the gel mobility. Strikingly, mutations of all the six sites to alanine (6A) almost completely abolished the slower-migration species. Conversely, mutations of them to phospho-mimetic aspartic acid (6D) largely restored them, suggesting that these six sites are the major phosphorylation sites controlling gel mobility behavior of Ska3. Surprisingly, another quadruple-mutation mutant (4A3) that excluded Thr358 and Thr360 exhibited similar gel mobility to WT, suggesting that Thr358 and Thr360 play a more important role in controlling gel
mobility behavior of Ska3. Taken together, Ska3 is phosphorylated at multiple sites during mitosis.

A phosho-specific antibody was then generated that recognized the phosphorylated Thr358 and Thr360 residues. In lysates of mitotic Hela cells stably expressing GFP-Ska3 WT, T358A, T360A, 2A, or 2D, the phospho-antibody specifically recognized the slower-migrating hyperphosphorylated WT-Ska3 proteins, but not the T360A, 2A, and 2D mutant proteins (Figure 3.2C), attesting to its specificity. Furthermore, the phospho-antibody recognized only the hyperphosphorylated endogenous Ska3 in nocodazole-arrested mitotic cells, but not Ska3 in cells arrested at G1/S by thymidine (Figure 3.2D). Histone H3 serine 10 phosphorylation (H3-pS10) showed that the nocodazole lysates were indeed mitotic. Thus, Ska3 phosphorylation on Thr358 and Thr360 occurs specifically during mitosis and can be detected by our phospho-antibody.

To test whether Cdk1 phosphorylate Ska3 in vitro, recombinant His$_6$-Ska3 (residues 141-412) WT and 2A were purified and treated with recombinant Cdk1 in kinase reactions containing cold ATP. A slower-migrating band was detected in the WT sample on SDS-PAGE gel stained with Coomassie (Figure 3.2E). This band was recognized by the phospho-Ska3 antibody. Thus, Ska3 can be phosphorylated by Cdk11 in vitro.

**Multisite Cdk1 phosphorylation determines Ska3 localization to the kinetochore**
To determine how the mutations of these sites affect its localization to kinetochores, I firstly examined Ska3 localization in unperturbed mitotic cells. Thymidine-arrested HeLa Tet-On cells expressing GFP-Ska3 WT, 2A, 6A, and 6D, were released to fresh medium and mitotic cells were collected for immunostaining analysis. As shown in Figures 3.3A and 3.3B, GFP-Ska3 WT robustly localized at kinetochores in prometaphase and metaphase cells, whereas the signals of GFP-Ska3 2A were largely reduced but still detectible. In contrast, GFP-Ska3 6A with all the six sites mutated to alanine completely lost its kinetochore signals, suggesting that in addition to Thr358 and Thr360, the other four sites (Thr203, Thr217, Ser283 and Thr291) also play a key role in kinetochore localization of Ska3. Surprisingly, while the signals of the phospho-mimetic GFP-Ska3 6D were detectable at kinetochores, they were about two-fold weaker than the ones of WT, implying that the phosphomimic mutant only partially mimics WT in cells. Nevertheless, these data strongly support the notion that Cdk1 phosphorylation predominantly determines Ska localization to kinetochores. The observed localization defects for these mutants are unlikely due to distinct protein expression levels as they were all comparable to WT (Figure 3.2B). Similar localization patterns for these mutants were also observed in MG132-arrested metaphase cells (Figures 3.4A and 3.4B). I next tested whether Ska3 was required for Ska1 or Ska2 kinetochore localization. In Hela cells depleted of endogenous Ska3, neither GFP-Ska1 nor GFP-Ska2 localized to kinetochores (Figures 3.4C and 3.4D), suggesting that Ska3 is required for Ska1 and Ska2 localization to the kinetochore.
To rule out the possible artifacts caused by formaldehyde fixation in the immunostaining, I performed live-cell imaging. Consistently, GFP-Ska3 WT was robustly localized to kinetochores and the three mutants, the 2A, 6A and 6D, all exhibited reduced kinetochore localization (Figures 3.5A-3.5D). Interestingly, the tested mutants were all normally localized to spindle microtubules (Figures 3.5A-3.5D). Thus, Cdk1 phosphorylation of Ska3 is the decisive factor for Ska localization to kinetochores, but dispensable for its localization on microtubules.

Differential contributions of Ska3 phosphorylation sites to its kinetochore localization

Mutations of the six phosphorylation sites to alanine abolished Ska3 localization to kinetochores, whereas mutations of Thr358 and Thr360 to alanine only reduced it, suggesting that the other four sites also contribute to Ska3 localization to kinetochores. I then tested this hypothesis by examining the kinetochore localization of Ska3 4A3 that excludes the mutations of Thr358 and Thr360. Unexpectedly, GFP-Ska3 4A3 was still localized to kinetochores as robustly as WT in unperturbed mitotic HeLa Tet-On cells (Figures 3.3C and 3.3D). This observation was further confirmed by the results from live-cell imaging (Figures 3.5C and 3.5D). Thus, the six phosphorylation sites contribute differentially to Ska3 localization to kinetochores and Thr358/Thr360 play a dominant role in Ska kinetochore localization. To further confirm this, I examined the effects of other phospho-defective quadruple mutations (4A1/2) that include Thr358 and Thr360 on the kinetochore localization of Ska3. As expected, the
signals of GFP-Ska3 4A1 and 4A2 were also significantly reduced compared to WT, but still stronger than the ones of GFP-Ska3 6A (Figures 3.3A and 3.3B). To rule out the possibility that the remaining signals of 2A and 4A mutants on kinetochores are due to their dimerization with endogenous Ska complexes, I re-examined their kinetochore localization in cells depleted of endogenous Ska3. Their localization patterns were similar to the ones in cells without depletion of endogenous Ska3 (Figures 3.6A and 3.6B).

I then sought to examine the behavior of their corresponding phospho-mimetic quadruple mutants (4D1/2) in Ska3 kinetochore recruitment. Again, the localizations of GFP-Ska3 4D1 and 4D2 were mitigated but could still be detected at the comparable levels to 2D (Figures 3.7A-3.7C), suggesting that these phospho-mimetic Ska3 mutants, including 6D, can only partially mimic WT in cells. Based on these observations, we conclude that Ska3 localization to kinetochores is predominantly determined by Thr358 Thr360-centered multisite phosphorylation. However, it remains unknown how the additional four mutations have a significant effect on Ska3 localization to kinetochores on the base of mutations at Thr358 and Thr360.

**Spindle microtubules may directly promote Ska3 recruitment to kinetochores**

It was previously shown that nocodazole treatment significantly reduced Ska localization to kinetochores [147]. I repeated the experiment in HeLa Tet-On and observed a similar phenomenon. Treatment of the Aurora B inhibitor ZM did
not restore the kinetochore localization of Ska3 at all while it completely abrogated the fluorescence signals of histone H3pS10 (Figures 3.8A-3.8C). These results suggest that spindle microtubules other than Aurora B plays a role in Ska recruitment to kinetochores. Similarly, the Dam1 complex, the functional Ska analog in budding yeast, was previously shown to be loaded to kinetochores by spindle microtubules [148]. I hypothesized that spindle microtubules may facilitate Ska loading to kinetochores by enriching Ska to the proximity of kinetochores. If this was the case, Ska overexpression could override the nocodazole-induced Ska decrease at kinetochores. To test this, I compared the kinetochore localization of GFP-Ska3 in between the absence and presence of nocodazole. Strikingly, GFP-Ska3 intensity on kinetochores was barely altered by addition of nocodazole (Figures 3.8D and 3.8E), suggesting that increasing Ska protein concentration is able to bypass the requirement of spindle microtubules for Ska recruitment to kinetochores. Thus, spindle microtubules per se may be important for Ska localization to kinetochores.

I then examined how spindle microtubules affected the kinetochore localization of these Ska3 mutants. GFP-Ska3 WT, 2A, 4A1/2/3, 6A, and 6D, were expressed in nocodazole-treated HeLa Tet-On cells and kinetochore localization was examined. In the presence of nocodazole, GFP-Ska3 WT was detected at kinetochores. Surprisingly, all the tested mutants except for 4A-3 completely lost the kinetochore signals (Figures 3.9A and 3.9B), seemingly in contrast to the results from cells with intact microtubules (Figure 3.3). Considering that all the mutants but 4A-3 had more or less defects in kinetochore localization in the
unperturbed mitosis (Figures 3.3A and 3.3B), the above observations suggest that spindle microtubules may help stabilize the weak Ska-Ndc80 interactions in these mutants. Taken together, my results here suggest that although Cdk1-mediated multisite phosphorylation plays a decisive role in Ska localization to kinetochores, spindle microtubules may regulate this process. In addition, the contrasting behavior of 4A3 further confirmed that our previously identified Thr358 and Thr360 are major sites contributing to Ska localization to kinetochores [146].

**Ska functions in chromosome segregation are predominantly determined by multisite Cdk1 phosphorylation**

As the Ska complex is required for chromosome segregation, I next determined how Cdk1 phosphorylation on Ska3 affected chromosome segregation. GFP-Ska3 WT, 6A and 6D were expressed in Ska3-depleted HeLa Tet-On cells stably expressing mCherry-H2B, and then chromosome dynamics was monitored by time-lapse microscopy. As shown in Figures 3.10A and 3.10B, mock cells spent an average of 48 min from nuclear envelop breakdown (NEB) to anaphase onset. Depletion of Ska3 gave rise to two major groups of cells — the one with prolonged metaphase followed by anaphase onset and the other with prolonged metaphase followed by cohesion fatigue and cell death. I quantified mitotic duration (from NEB to anaphase onset or from NEB throughout cohesion fatigue to cell death) that these two groups of cells spent and found an average of 307 min. Expression of GFP-Ska3 WT largely rescued the defects caused by Ska3 depletion and significantly reduced the mitotic duration with an average of 55 min,
which exhibited no significant difference from mock cells. Strikingly, expression of GFP-Ska3 6A almost failed to rescue the delay caused by Ska3 depletion with an average mitotic duration of 234 min. As Ska3 6A completely lost its kinetochore localization (Figure 3.3), the above results strongly support the notion that Ska localization to kinetochore is crucial for its functions in chromosome segregation. These results also reveal a decisive role of Cdk1 phosphorylation in activating Ska functions. In addition, I also found that while phospho-mimetic GFP-Ska3 6D exhibited a significantly shorter mitotic duration (150 min) compared to 6A (234 min), its performance was much worse than WT (55 min) (Figures 3.10A and 3.10B), suggesting that Ska3 6D is not a good phospho-mimicry in cells. The underlying reason will be discussed in the section of Discussion.

I also examined chromosome segregation in siSka3-treated cells expressing GFP-Ska3, 2A, 4A2, and 4A3 (Figures 3.10C and 3.10D). Expression of GFP-Ska3 2A showed a mild delay with an average mitotic duration of 82 min. Cells expressing GFP-Ska3 4A2 that includes the mutations of Thr358A and Thr360A were also delayed in chromosome segregation with an average mitotic duration of 154 min. Strikingly, expression of GFP-Ska3 4A3 that excludes the mutations of Thr358A and Thr360A did not delay chromosome segregation with an average mitotic duration of 70 min, compared with GFP-Ska3 WT (64 min). Again, these results support that Thr358 and Thr360 are the major sites contributing to Ska functions. Of note, cells with endogenous Ska3 depletion or expressing GFP-Ska3 6A only exhibited 5 min delay from NEB to metaphase compared with mock cells (Figure 3.11A), suggesting that the significant delay in
our experiments is mainly derived from metaphase-to-anaphase transition (Figures 3.10A-D) and thus the Ska complex may be dispensable for chromosome congression to metaphase plates from initial attachments, which is consistent with the previous findings.

**Stability of KT-MT attachments is determined by multisite Cdk1 phosphorylation on Ska3**

Ska depletion has been shown to significantly decrease the stability of KT-MT attachments [146, 149, 150]. As Ska localizes on both microtubules and kinetochores, we wanted to know which pool of Ska could be more important for the stability of KT-MT attachments. I examined the microtubule cold sensitivity for cells expressing the Ska3 6A mutant that completely lost its kinetochore localization. As a comparison, the 2A and 4A2 mutants that retained partial kinetochore localization were also included. As shown in Figures 3.11B and 3.11C, Ska3 depletion significantly increased the microtubule cold sensitivity. Expression of GFP-Ska3 WT and 2A largely rescued the sensitivity and no significant difference was observed between WT and 2A. Expression of GFP-Ska3 4A2 also rescued the cold sensitivity but at a reduced level compared with WT. Strikingly, expression of GFP-Ska3 6A completely failed to rescue the cold sensitivity. Considering that the 6A mutant completely lost its kinetochore localization but retained normally on microtubules, the above observations strongly indicate that the Cdk1 phosphorylation-dependent kinetochore pool of Ska is the major determinant for the KT-MT stability. The fully rescue of microtubule cold sensitivity
by Ska3 2A might suggest that there is a threshold in the Ska levels on kinetochores, above which, KT-MT attachments become much more stable. Alternatively, the implemented assay here may not be sensitive enough to detect the moderate defects caused by Ska3 2A.

**Multisite Cdk1 phosphorylation in Ska3 promotes Ska binding to the Ndc80-Nuf2 complex**

Previous studies have implicated Ndc80 to be the receptor of the Ska complex at the kinetochore [64, 73, 151]. One study has proposed that the Ndc80 loop region may play a role in binding to the Ska complex [52]. To test this hypothesis, Myc-tagged full-length (FL) Ndc80 or Ndc80 ΔLoop was expressed in Hela cells depleted of endogenous Ndc80 and the localization of Ska3 was determined. The cells were treated with 5 μM nocodazole to avoid any contribution from microtubules on Ska localization. Ska3 localized to the kinetochores in mock-treated cells. As shown before, depletion of Ndc80 largely decreased the localization of Ska3 at the kinetochore. Expressing the siRNA-resistant Myc-Ndc80 FL fully rescued the kinetochore localization of Ska3, whereas expressing the Myc-Ndc80 ΔLoop construct did not (Figures 3.12A and 3.12B). Both Myc-Ndc80 FL and ΔLoop localized to kinetochores and were expressed at similar levels based on both immunostaining and western blotting (Figures 3.12B and 3.12C). Total endogenous Ska3 protein levels were also similar when Ndc80 WT or ΔLoop was expressed (Figure 3.12C). These results indicate that the kinetochore localization of Ska3 depends on the loop region of Ndc80. This finding strongly implicates
Ndc80C as the kinetochore receptor of Ska3. To further confirm this, I examined the Ndc80-Ska3 interaction in cells by performing co-immunoprecipitation assays. Myc-Nuf2 and Myc-Ndc80 WT or ΔLoop were co-expressed in MG132-arrested Hela cells and precipitated by anti-Myc antibodies. Myc-Ndc80 WT interacted with Ska3, and depletion of the loop reduced this interaction (Figure 3.12D), suggesting that the Ndc80 loop is important for the Ndc80-Ska3 interaction.

I next wanted to know whether Cdk1 phosphorylation was important for the binding of the full-length Ska complex to Ndc80C. To test this, the full-length Ska complex and the GST-Nuf2-Ndc80 complex were reconstructed and purified and in vitro Cdk1 phosphorylation followed by GST-pull down assays was performed. As shown in Figure 3.13A, an interaction was detected between the Ska complex and GST-Nuf2-Ndc80 even without Cdk1 treatment. This basal interaction does not require Cdk1 phosphorylation, which was also demonstrated in an in vitro cross-linking experiment, and its nature is not quite understood. Cdk1 treatment significantly enhanced the interaction between Ska WT and GST-Nuf2-Ndc80. Treatment of RO3306, a potent Cdk1 inhibitor, completely abolished the increased binding between these two complexes, suggesting that the increased binding is Cdk1 phosphorylation dependent. As a comparison, the interaction between the Ska 6A and GST-Nuf2-Ndc80 complexes was also examined under the same conditions. Again, the basal interaction was detected as well between these two complexes (Figure 3.13A). Neither Cdk1 treatment nor Cdk1 together with RO3306 treatment altered the interaction between Ska 6A and GST-Nfu2-Ndc80. Western blots using our home-made phospho-specific antibody against
T358 and T360 showed that Cdk1 kinase was effective in our experiments. Taken the above results together, I conclude that Cdk1 phosphorylation on the six sites in Ska3 promotes Ska binding to Ndc80C.

As Ska3 6D partially mimicked the behavior of Ska3 WT in kinetochore localization (Figure 3.3), I wanted to know if the Ska complex containing this phospho-mimetic Ska3 mutant (Ska 6D) could as well exhibit enhanced binding to Ndc80C. To test this, the interaction between the Ska 6D and GST-Nuf2-Ndc80 complexes was examined using GST-pull down assays. Consistent with the previous results, the similar basal interaction was again detected between the Ska WT complex and GST-Nuf2-Ndc80 (Figure 3.13B). Interestingly, an enhanced interaction (about 2.5-fold increase) between the Ska 6D and GST-Nuf2-Ndc80 complexes was also observed, further confirming the phospho-dependent interaction between Ska and Ndc80C. As Ndc80C contains the other two subunits Spc24 and Spc25, I then tested if these two subunits could also interact with the Ska 6D complex. As a result, the GST-Spc24-Spc25 complex did not bind the Ska 6D complex at all (Figure 3.13C), suggesting that the subunits Ndc80 and Nuf2 in Ndc80C are mainly responsible for the interaction with Ska.

**The Ska 6D complex forms a stable macro-complex with the Ndc80-Nuf2 complex in vitro**

Although Cdk1 phosphorylation has been shown to significantly enhance the interaction between the Ska and Ndc80 complexes, it is tempting to know whether they could form a stable macro-complex *in vitro*, and if so, could Cdk1
phosphorylation be the decisive factor for the formation of this macro-complex? To overcome the potential heterogeneity of Ska3 phosphorylation by Cdk1, the phospho-mimetic Ska3 6D mutant was utilized as a substitute for phosphorylated Ska3. Because Spc24 and Spc25 are dispensable for Ska binding (Figure 3.13C), only Nuf2-Ndc80 complexes were applied in the in vitro reconstitution experiments. As shown Figure 3.14A, the Nuf2-Ndc80 complex was eluted at a high molecular weight, which was in the middle of 669 and 440 kDa. Both Ska WT and 6D complexes were also eluted at high molecular weights, slightly more than 440 kDa (Figures 3.14B and 3.14C). The mixture of the Ska 6D and Nuf2-Ndc80 complexes was eluted as a major peak appearing at a molecular weight slightly less than 440 kDa (Figure 3.15A). Strikingly, SDS-PAGE analysis on the major peak demonstrated that the Ska 6D and Nuf2-Ndc80 complexes were perfectly co-eluted, revealing the formation of a stable macro-complex. These findings are consistent with a very recent study showing that Cdk1 treatment is able to promote the formation of a stable Ska-Ndc80 macro-complex in vitro [152]. In contrast, the mixture of the Ska WT and Nuf2-Ndc80 complexes was eluted as two major peaks appearing at the molecular weights that were slightly higher and lower than 440 kDa (Figure 3.15B). SDS-PAGE analysis on these two major peaks demonstrated that the Ska WT and Nuf2-Ndc80 complexes were not co-eluted well with each other. All these interesting observations strongly suggest that Cdk1 phosphorylation is the decisive factor for the formation of the stable Ska-Ndc80 macro-complex in vitro. Thus, it is the first time to successfully reconstitute this macro-complex using phospho-mimetic mutants. In addition, the Ska 6D complex
was not co-eluted with the Ndc80 Bosai that lacks the Ndc80 internal loop and the flanking regions (Figure 3.15C), although it did so with the full-length Nuf2-Ndc80 complex, further confirming the previous findings that the Ndc80 internal loop together with the flanking regions contains main binding sites for the Ska complex [146, 153]. Interestingly, the Ndc80 loop is well conserved across species and also responsible for recruiting other important factors [154-157]. Notably, a very recent study further demonstrated that the flanking regions of the Ndc80 internal loop other than the loop itself plays a more important role in Ska binding in vitro [152].

3.3 Discussion

The Ska complex localizes to both kinetochores and microtubules. Our previous and current studies have provided strong evidence to support that Ska recruitment to kinetochores is essential for its functions in chromosome segregation [146]. Especially, in this study, I isolated a Ska mutant (Ska3 6A) that completely loses kinetochore localization and does not support Ska functions at all, suggesting that loading Ska onto kinetochores is the only way to enable Ska functions in chromosome segregation. As these sites are phosphorylated by Cdk1, my findings also suggest that Cdk1 phosphorylation is the decisive factor for activating Ska functions.

How is the Ska complex recruited to kinetochores?

Our previous and current findings demonstrate that Cdk1 phosphorylation-enabled Ska-Ndc80 binding is required for docking Ska to kinetochores. Recent
studies indicated that the Ndc80 tail and/or the calponin-homology domain facilitate Ska recruitment to kinetochores [158, 159]. Thus, multiple mechanisms may co-exist to recruit Ska to kinetochores. As the binding between Cdk1-phosphorylated Ska3 and the Ndc80 loop with its flanking regions is sufficient for forming a stable Ska-Ndc80 macro-complex in vitro, and it is so predominant for Ska localization at kinetochores in cells, I believe that spindle microtubules and the Ndc80 N-terminal tail and/or the calponin-homology domain play regulatory roles in Ska recruitment to kinetochores. I therefore propose a model to explain how the Ska complex is recruited to kinetochores, in which spindle microtubules facilitate the delivery of Ska to the KT-MT interface, and then Ska binds to Ndc80 in a multisite Cdk1 phosphorylation-dependent manner. Based on this model, one predicts that perturbations on Ska-microtubules interactions could affect Ska localization to kinetochores. Actually, mutations on Ska1 and depletion of other microtubule-interacting proteins, which both impair Ska-microtubule interactions, have been shown to moderately decrease Ska at kinetochores.

**Why is multisite phosphorylation needed?**

Why is multisite phosphorylation needed? The Ska complex starts to accumulate at kinetochores in prophase, peaks at metaphase and disappears at middle anaphase [160, 161], suggesting that the binding affinity of Ska to kinetochores may increase with mitotic progression. Multisite phosphorylation may provide such regulation. In support of this idea, we found that the state of Cdk1 phosphorylation on Ska3 is positively correlated with the robustness of Ska3
localization to kinetochores. Although mutations on all the six Cdk1 sites abolished Ska localization to kinetochores and its functions in chromosome segregation, their contributions seem to be different. Mutations (2A and 4A1/2) that include the two sites T358 and T360 are always associated with the phenotypes of decreased kinetochore localization and prolonged mitotic progression; whereas mutations (4A3) that exclude T358 and T360 resulted in no detectable defects in kinetochore localization and normal mitotic progression. Thus, phosphorylation on the two sites Thr358 and Thr360 likely lays a foundation for Ska localization to kinetochores; and the additional phosphorylation on the other sites could further enhance its kinetochore recruitment. Of note, although the Ska 6D complex bound to Ndc80C stronger than WT, it exhibited partial defects in kinetochore localization and chromosome segregation, suggesting that Ska3 6D is not a perfect mimicry to phosphorylated Ska3 in cells. Alternatively, as a recent study showed that dynamic phosphorylation-dephosphorylation cycles on Ska3 is important for proper Ska functions [162], it is possible that similar phosphorylation-dephosphorylation cycles is also critical for proper Ska functions in chromosome segregation [163, 164]. It is worth mentioning here that at early anaphase when Cdk1 activity has significantly declined, the Ska complex is still robustly retained at kinetochores, suggesting that either the Cdk1 phosphorylation-dependent Ska-Ndc80 binding is preserved or other unknown mechanisms tether Ska onto kinetochores. It would be interesting to test these possibilities in future.

What is the significance of Ska binding to the Ndc80 complex?
Our *in vitro* reconstitution results showed the phospho-mimetic Ska 6D complex is able to form a stable macro-complex with the Ndc80-Nuf2 complex, suggesting that a tight Ska-Ndc80 interaction can be formed in cells albeit its existence might be transient. As the Ndc80 internal loop together with its flanking regions is mainly responsible for Ska binding and the loop confers structural flexibility to Ndc80C [146, 153, 157, 165], such a tight Ska-Ndc80 interaction might be able to alter the conformation of Ndc80C, thus promoting proper KT-MT interactions. In this sense, the Ska complex not only functions as a scaffold to bring PP1 or other factors in proximity to the KT-MT interface [166], but also might serve as a structural modifier [167]. In future, it is tempting to test if this could be the case; and if so, how does it affect the behavior of Ndc80C on dynamic microtubules [168]?

In budding yeast, the DASH/Dam1 complex, the functional Ska homolog, contains 10 subunits and shares no structural and sequence similarity to Ska. The Dam1 complex was reconstituted *in vitro* and its structure was solved using cryo-EM [169]. It was also shown that the Ndc80 complex bridges two Dam1 complex rings [170]. Although integrating the structure of the Ndc80 complex and published interaction data into that of the Dam1 complex yielded an interesting molecular view of KT-MT interactions [169], how these complexes indeed interact with each other still remains unclear. In future, by solving the structure of the Ska (6D)-Ndc80 macro-complex using cryo-EM, people would gain profound insights into how Cdk1 phosphorylation on Ska3 promotes the Ska-Ndc80 interaction at the atomic level.

In summary, our findings unveil the essential role of multisite Cdk1 phosphorylation-enabled Ska-Ndc80 macro-complexes in controlling Ska
localization and functions during mitosis, and also reveal that coordinated actions of microtubules and kinases load the Ska complex to kinetochores.
Figure 3.1 The Ska3 C-terminus contains multiple Cdk1-phospho sites [146, 171].

Ska3 is phosphorylated during mitosis. Lysates of log-phase or mitotic Hela Tet-on cells were incubated with antibody against Ska3. Immunoprecipitated proteins were treated with mock or λ phosphatase and then resolved with SDS-PAGE and blotted with the indicated antibodies. B. Cdk1 kinase phosphorylates Ska3. Nocodazole-arrested Hela Tet-on cells were treated with the inhibitor, RO3306 (10 μM); Aurora B inhibitor, ZM447439 (25 μM); MPS1 inhibitor, Reversine (1 μM); and Plk1 inhibitor, BI2356 (500 nM). MG132 was added to prevent mitotic exit. The cell lysates were resolved on SDS-PAGE and blotted with anti-Ska3 antibodies. C. GFP-Ska3 14A fails to localize at kinetochores. Hela Tet-on cells depleted of endogenous Ska3 were transfected with GFP-Ska3 WT or 14A. Mitotic cells were collected for immunostaining using the indicated antibodies. Representative images were shown here. Scale bars, 5 μm. D. Analysis on the conservation of Cdk1 sites in Ska3. Six Cdk1 sites (*) in human Ska3 are distributed in three conserved domains identified from a sequence alignment across four species, human (hs), mouse (mm), chicken (gg), and *xenopus laevis* (xl). Among the six sites, four are highly conserved across the tested species. The other two are conserved at least among human and mouse. In the conserved regions, identical amino-acids were marked in red and similar amino-acids in orange. E. Phosphorylated Cdk1 sites identified by mass-spectrometric analyses on immunoprecipitated Myc-Ska3 isolated from nocodazole-arrested HeLa Tet-On cells. The sites firstly identified were marked in orange and the newly identified
ones in the second time were marked in red. Totally eleven Cdk1 sites were identified being phosphorylated. F. Phosphorylation sites identified by Mass spectrometry on recombinant Ska3 fragments (141-412) treated with recombinant Cdk1/Cyclin B1.
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Figure 3.2 Ska3 is highly phosphorylated by Cdk1 in vivo and in vitro [146, 171]

A. Summary of Ska3 mutants applied in this study.  B. Lysates of nocodazole-arrested HeLa Tet-On cells expressing GFP-Ska3 WT, 2A, 4A1/2/3, 6A, or 6D were resolved with SDS-PAGE and blotted with the indicated antibodies.  C. GFP-Ska3 is phosphorylated at Thr358 and Thr360 residues. Lysates of GFP-Ska3 WT, Thr358A, Thr360, 2A, or 2D were resolved on SDS-PAGE and blotted with the indicated antibodies.  D. Ska3 is specifically phosphorylated during mitosis. Lysates of log-phase (thymidine) or mitotic (nocodazole) Hela cells were resolved on SDS-PAGE and blotted with the indicated antibodies.  E. Ska3 is phosphorylated by Cdk1 in vitro. Recombinant 6xHis-Ska3 (141-412) WT or 2A fragments were treated with Cdk1 in the presence or absence of ATP. Proteins were then resolved on SDS-PAGE and subjected to Coomassie Brilliant Blue (CBB) staining and blotted with the indicated antibodies.
Figure 3.3 Cdk1 phosphorylation on Ska3 is essential for Ska localization at kinetochores in unperturbed mitotic cells [171]

A. Thymidine-arrested HeLa Tet-On cells expressing GFP-Ska3 WT, 2A, 4A1/2, 6A, or 6D were released into fresh medium. Mitotic cells were collected for immunostaining using the indicated antibodies. Representative images were shown here. Scale bars, 5 µm. B. Quantification of GFP-Ska3 intensity on kinetochores in (A). Detailed description about quantification was recorded in the section of Methods. At least 60 kinetochores (6 kinetochores per cell) were analyzed for each condition. Average and standard deviation were shown in lines. C. Cells expressing GFP-Ska3 WT, 4A3 or 6D were treated similarly to the ones in (A). Scale bars, 5 µm. D. Quantification of GFP-Ska3 intensity on kinetochores in (C). At least 60 kinetochores (6 kinetochores per cell) were analyzed for each condition. Average and standard deviation were shown in lines. P<0.0001 (****). n.s. denotes no significance.
WT Mock

GFP - Ska3

GFP - Ska3 (MG132)

Relative GFP-Ska3 intensity on kinetochores

n=50-52 kinetochores each condition

DNA/GFP/ACA

DNA/GFP/ACA
Figure 3.4 Cdk1 phosphorylation on Ska3 is essential for Ska localization at kinetochores in MG-132 treated mitotic cells [146, 171]

**A.** Thymidine-arrested HeLa Tet-On cells expressing GFP-Ska3 WT, 2A, 4A1/2, 6A, or 6D were released into fresh medium. Mitotic cells were harvested after 2-hour MG132 treatment and then subjected to immunostaining with the indicated antibodies. Representative images are shown here. **B.** Quantification of GFP-Ska3 intensity on kinetochores in (A). Detailed description about quantification was recorded in the section of Methods. At least 60 kinetochores (6 kinetochores per cell) were analyzed for each condition. Average and standard deviation were shown in lines. P<0.0001 (****). n.s. denotes no significance. **C.** Ska3 is required for the kinetochore localizations of GFP-Ska1 and GFP-Ska2 at the kinetochore. Thymidine-arrested Hela Tet-on cells expressing GFP-Ska1, Ska2, or Ska3 with or without Ska3 depletion were released and incubated with nocodazole for 2 hr before harvest. Mitotic cells were then harvested and subjected immunostaining with the indicated antibodies. Representative images are shown here. **D.** Quantification of GFP-Ska1/2/3 intensities on kinetochores in (C). GFP-Ska1, n=52 kinetochores (5 kinetochores per cells); GFP-Ska2, n=50 kinetochores; GFP-Ska3, n=50 kinetochores; GFP-Ska1 and siSka3, n=50 kinetochores; GFP-Ska2 and siSka3, n=50 kinetochores; GFP-Ska3 and siSka3, n=51 kinetochores.
**Figure 3.5 Cdk1 phosphorylation on Ska3 is required for its kinetochore localization but not for its microtubule localization [171]**

**A** and **C**. Live-cell imaging of HeLa Tet-On cells expressing GFP-Ska3 WT, 2A (A), 6A (A), 4A3 (C) or 6D (C) treated with MG132 for 1h. Scale bars, 5 µm. **B** and **D**. Quantification of GFP-Ska3 intensity on kinetochores (in the left panel) and microtubules (in the right panel) in (**A** and **C**) normalized to that of cytoplasm. Detailed description about quantification was recorded in the section of Methods. For both **B** and **D**, at least 10 cells (10 kinetochores and 10 microtubules per cell) were analyzed for each condition. Average and standard deviation were shown in lines. P<0.05 (*); P<0.0001(****). n.s. denotes no significance.
Figure 3.6. The kinetochore localization of Ska3 mutants in cells depleted of endogenous Ska3 [171]

A. siSka3-treated HeLa Tet-On cells expressing GFP-Ska3 WT, 2A, 4A1/2/3, or 6A were firstly arrested with thymidine and released into fresh medium. Mitotic cells were subjected to immunostaining with the indicated antibodies. Representative images are shown here. B. Quantification of GFP-Ska3 intensity on kinetochores in (A). Detailed description about quantification was recorded in the section of Methods. At least 60 kinetochores (6 kinetochores per cell) were analyzed for each condition. Average and standard deviation were shown in lines. P<0.0001 (***) n.s. denotes no significance.
Figure 3.7. Localization of phospho-mimetic Ska3 mutants on kinetochores [171]

A. Thymidine-arrested HeLa Tet-On cells expressing GFP-Ska3 WT, 2D, 4D1 or 4D2 were released into fresh medium. Mitotic cells were collected for immunostaining using the indicated antibodies. Representative images were shown here. Scale bars, 5 μm. B. Quantification of GFP-Ska3 intensity on kinetochores in (A). Detailed description about quantification was recorded in the section of Methods. At least 60 kinetochores (6 kinetochores per cell) were analyzed for each condition. Average and standard deviation were shown in lines. P<0.0001 (****). n.s. denotes no significance. C. Lysates of HeLa Tet-On cells expressing GFP-Ska3 WT, 2D, 4D-1 and 4D-2 were resolved with SDS-PAGE and blotted with the indicated antibody.
Figure 3.8. Aurora B is not important for Ska recruitment to kinetochores [171].

A. Aurora B kinase activity is dispensable for Ska3 localization at kinetochores. Thymidine-arrested HeLa Tet-On cells were released into fresh medium. At 7 hours after release, nocodazole with a final concentration of 5 µM was added. Aurora B inhibitor ZM447439 (ZM) was then added 2 hours later. Mitotic cells with 1-hour ZM treatment were collected for staining with DAPI and the indicated antibodies. B. Quantifications of Ska3 intensity (left) and H3pS10 intensity (right) on kinetochores in (A). Detailed description about quantification was recorded in the section of Methods. At least 60 kinetochores (6 kinetochores per cell) were analyzed for each condition. Average and standard deviation were shown in lines. P<0.0001 (**); P<0.001 (**). n.s. denotes no significance. C. Cell lysates in (A) were separated with SDS-PAGE and then blotted with the indicated antibodies. D. Ska3 overexpression overrides the effects of nocodazole on its kinetochore localization. HeLa Tet-On cells transfected with plasmids containing GFP-Ska3 were treated with DMSO or nocodazole. Mitotic cells were collected for staining with DAPI and the indicated antibodies. E. Quantification of GFP-Ska3 intensity on kinetochores in (D). Only prometaphase cells were selected for analysis. Detailed description about quantification was recorded in the section of Methods. At least 60 kinetochores (6 kinetochores per cell) were analyzed for each condition.
Average and standard deviation were shown in lines. \( P<0.0001 \) (****). n.s. denotes no significance.
Figure 3.9. Spindle microtubules facilitate Ska recruitment to kinetochores [171].

A. HeLa Tet-On cells were treated under the same conditions described in (Figure 3.3A) except that nocodazole was added at 7 hours after thymidine release. Scale bars, 5 μm. B. Quantification of GFP-Ska3 intensity on kinetochores in (A). Detailed description about quantification was recorded in the section of Methods. At least 60 kinetochores (6 kinetochores per cell) were analyzed for each condition. Average and standard deviation were shown in lines. P<0.0001 (**). n.s. denotes no significance.
Figure 3.10. Cdk1 phosphorylation in Ska3 is essential for chromosome segregation [171].

A and C. HeLa Tet-On cells stably expressing H2B-mCherry were co-transfected with siSka3 and vectors (V) or plasmids containing GFP-Ska3 WT, 2A (C), 4A2 (C), 4A3 (C), 6A (A), or 6D (A). Time-lapse microscopic analysis was performed. Scale bars, 5 µm. B and D. Quantification of the duration from nuclear envelop breakdown (NEB) to anaphase onset in (A) and (C). At least 100 mitotic cells in (A) and 80 mitotic cells in (C) were analyzed for each condition. Average and standard deviation were shown in lines. P<0.05 (*); P<0.01 (**); P< 0.0001 (****). n.s. denotes no significance. Scale bars, 5 µm.
Figure 3.11. Cdk1 phosphorylation in Ska3 is dispensable for chromosome alignment but essential for the stabilization of end-on attachment [171].

A. Duration from NEB to metaphase for cells in Figures 3.10A and 3.10C. P<0.001 (**); P<0.0001 (****). n.s. denotes no significance. B. Cdk1 phosphorylation in Ska3 is required for stability of KT-MT attachments. siSka3-treated HeLa Tet-On cells transfected with vectors or plasmids containing GFP-Ska3 WT, 2A, 4A2 or 6A were treated with MG132 for 1h after 9h release from thymidine and incubated on ice for 5 min and then subjected to staining with DAPI and the indicated antibodies. Representative images are shown here. C. Quantification of microtubule intensity in (B) normalized to that of DNA. At least 15 mitotic cells (10 microtubules per cell) were analyzed for each condition. Average and standard deviation were shown in lines. P<0.01 (**); P< 0.0001 (****). n.s. denotes no significance.
Figure 3.12 Ndc80 loop is required for Ska3 localization to the kinetochore and promotes Ndc80-Ska3 interaction \textit{in vivo} [171].

A. Nocodazole-arrested Hela cells transfected with RNAi-resistant plasmids containing Myc-Ndc80 WT or ΔLoop were treated with Ndc80 siRNAs. Representative images of chromosome spread were shown in here. The outlined regions were amplified and shown on the right panels. B. Quantification of the kinetochore intensities of Ska3 (upper) and Myc-Ndc80 (lower) in (A) normalized to that of ACA. Average and standard deviation were shown in lines. Mock, n=15 cells; siNdc80 and vector, n=15 cells; siNdc80 and Ndc80-FL, n=16 cells; siNdc80 and Ndc80 ΔLoop, n=15 cells. C. Cell lysates from the experiment in (A) were resolved on SDS-PAGE and blotted with the indicated antibodies. D. Ndc80 loop promotes the Ndc80-Ska3 interaction. Nocodazole-arrested Hela Tet-on cells expressing Myc-Ndc80 WT or ΔLoop and Myc-Nuf2 were released into MG132. Lysates of these cells were incubated with anti-Myc antibodies. Immunoprecipitated proteins were resolved on SDS-PAGE and blotted with anti-Ska3 and anti-Myc antibodies.
Figure 3.13 Cdk1 phosphorylation on Ska3 promotes the formation of a sable Ska-Ndc80 macro-complex [171]

A. Cdk1 phosphorylation promotes Ska binding to Ndc80C. The Ska1-Ska2-Ska3 WT or 6A complex treated with Cdk1/Cyclin B1 with or without RO3306 were mixed with the GST-Nuf2-Ndc80 complex and GST beads. The bead-bound proteins were resolved with SDS-PAGE and then stained with Coomassie Blue or blotted with the indicated antibody. 

B. The Ska 6D complex binds to Ndc80C stronger than the WT complex. The Ska1-Ska2-Ska3 WT or 6D complex was mixed with the GST-Nuf2-Ndc80 complex and GST beads. The bead-bound proteins were resolved with SDS-PAGE and then stained with Coomassie Blue. The binding activity in fold change was derived from the amount of pelleted Ska2 by GST-Nuf2-Ndc80 sequentially normalized to the ones of input Ska2 and pelleted GST-Nuf2-Ndc80. The average and standard deviation were calculated from two independent experiments and shown in the bottom panel. 

C. Spc24 and Spc25 are dispensable for Ska binding. The Ska1-Ska2-Ska3 6D complex was mixed with the GST-Nuf2-Ndc80 or GST-Spc24-Spc25 complex and GST beads. The bead-bound proteins were resolved with SDS-PAGE and then stained with Coomassie Blue.
Figure 3.14. Purification of the Ska and Ndc80 complexes [171].

Size-exclusion chromatographic analysis on purified Nuf2-Ndc80 complexes (A), Ska1-Ska2-Ska3 6D complexes (B), Ska1-Ska2-Ska3 WT complexes (C), and Ndc80 Bosai (D). The thicker lines marked the eluted fractions that were analyzed by SDS-PAGE.
Figure 3.15. A stable macro-complex is formed by the Ska 6D and Ndc80-Nuf2 complexes in vitro [171]

A, B and C. The Ska 6D, not WT complex, forms a stable macro-complex with the full-length Ndc80C, not Bonsai. Size-exclusion chromatographic analysis on the mixer of the Ska1-Ska2-Ska3 6D and Nuf2-Ndc80 complexes (A), or the one of the Ska1-Ska2-Ska3 WT and Nuf2-Ndc80 complexes (B), or the one of the Ska1-Ska2-Ska3 6D and the Ndc80 Bosai (C). The thicker lines marked the eluted fractions that were analyzed by SDS-PAGE. Arrows indicated the peaks of the analyzed complexes.
CHAPTER 4: CENTROMERIC COHESION PROTECTION REQUIRES CENTROMERIC TRANSCRIPTION AND ITS KEY REGULATOR CDK11

4.1 Introduction

In this chapter, I aim to investigate: 1) the requirement of centromeric transcription in centromeric cohesion protection; and 2) identify key factors responsible for centromeric transcription. The determination of the role of centromeric transcription in cohesion regulation at centromeres would reveal the crucial mechanisms controlling chromosome segregation and provide promising molecular targets for cancer therapeutics in future. Here, by analyzing centromeric cohesion using immunostaining, I show that inhibiting centromeric transcription by α-amanitin abrogates elongating RNAPII (RNAPII-pSer2) localization at centromeres and induces centromeric cohesion defects. Reversely, enhancing centromeric transcription by THZ1 increases centromeric RNAPII-pSer2 levels and strengthens centromeric cohesion. Further analysis demonstrate that CENP-B serves as a suppressor for centromeric transcription. Either expression of the CENP-B DB (DNA-binding domain) or depletion of endogenous CENP-B that boosts centromeric transcription reinforces centromeric cohesion. Besides, I identify that Cdk11 is a key factor in promoting centromeric transcription. It localizes at centromeres and binds RNAPII. Its knockdown decreases RNAPII-pSer2 levels on centromeres, reduces centromeric transcription and leads to severe centromeric cohesion defects. Based on these results, I propose that
centromeric transcription together with its regulator Cdk11 is indispensable for preserving cohesion at centromeres during mitosis.

4.2 Results

α-amanitin treatment induces severe centromeric cohesion defects

By labeling newly transcribed α-satellite RNAs at centromeres, we recently showed that α-amanitin is a competent inhibitor that can suppress on-going transcription both on genes and at centromeres, whereas triptolide is an effective inhibitor for gene transcription but not for centromeric transcription (data unpublished). To determine how important on-going centromeric transcription is for the maintenance of centromeric cohesion, I applied these two drugs in mitotic-arrested Hela Tet-on cells and examined centromeric cohesions by performing immunostaining following chromosome spread. The results showed that 5-hour treatment of α-amanitin induced severe cohesion defects in ~70% of mitotic cells in sharp comparison with DMSO treatment, which induced cohesion defects in ~30% of cells. Intriguingly, only ~32% of mitotic cells treated with triptolide exhibited cohesion defects. Cells with cohesion defects (Type II) were featured by reduced Sgo1 and RNAPII loadings at centromeres (Figures 4.1A and 4.1B). Further analysis of Sgo1 and RNAPII localizations in drug-treated cells proved that α-amanitin significantly compromised centromeric Sgo1 and RNAPII signals compared with DMSO. (Figures 4.1 C and 4.1 D). In contrast, the Sgo1 localization was slightly reduced, while the RNAPII loadings at centromeres were marginally increased in triptolide-treated cells. It has been shown that the
centromere-bound RNAPII is phosphorylated at Ser2 (RNAPII-pSer2), a mark for elongating RNAPII. Thus, I also included H5 antibody that can specifically recognize this active form of RNAPII in our analysis. I found that the RNAPII-pSer2 signals were almost abolished upon α-amantin treatment while moderately increased in triptolide-treated cells (Figures 4.1E and 4.1F). These results raised an interesting perspective that on-going centromeric transcription is crucial for maintaining centromeric cohesion in mitosis. Similar results were also observed in non-transformed RPE-1 cells (Figures 4.2A-4.2D), suggesting that the phenotype observed is not specific for cancer cells. To determine whether the centromeric cohesion defects caused by α-amantin are due to reduced protein levels of cohesin and/or its regulators, I examined the protein expressions of Scc1, Sororin, Sgo1 and Bub1 upon different drug treatments and found that no discernable changes could be detected (Figure 4.2E).

Inhibition of centromeric transcription can impair CENP-A incorporation, which may potentially cause centromeric cohesion defects [117, 131, 172]. I then examined the centromeric localizations of CENP-A by staining it with both CENP-A and CREST (ACA) antibodies. The latter can recognize both CENP-A and CENP-B. As a result, the levels of CENP-A were comparable among different treatments (Figure 4.3A-4.3C), excluding the role CENP-A in this process. I then examined the centromeric levels of cohesin subunit Scc1 in drug-treated cells. I found that ~ 75% of cells treated with α-amantin showed no discernable Myc-Scc1 signals, whereas more than 85% of cells treated with triptolide displayed strong Myc-Scc1 localizations at centromeres comparable to DMSO-treated cells (Figure
4.3D), supporting the notion that inhibiting centromeric transcription impairs centromeric cohesion. Together, my data suggest that on-going centromeric transcription functions to maintain centromeric cohesion in mitosis.

**THZ1 treatment causes ectopic RNAPII on chromosome arms and strengthens cohesion-chromosome association**

We recently showed that THZ1 is an enhancer for on-going centromeric transcription (data unpublished). Since suppression of on-going centromeric transcription by α-amanitin weakened centromeric cohesion, I reasoned that boosting on-going centromeric transcription by THZ1 could strengthen centromeric cohesion. To test my hypothesis, thymidine-arrested Hela Tet-on cells were released into THZ1-containing medium and incubated for 12 hr followed by nocodazole treatment in the last 2 hr. Mitotic cells were then collected and subjected to chromosome spread followed by immunostaining analysis. I found remarkable increase of RNAPII at centromeres in THZ1-treated cells compared to DMSO-treated cells (**Figures 4.4A and 4.4B**). The RNAPII-Ser2 signals were also significantly augmented (**Figures 4.4D and 4.4E**), supporting our previous finding that THZ1 enhances centromeric transcription. Strikingly, ~55% of THZ1-treated cells also displayed increased ectopic RNAPII signals on chromosome arms, which were rarely observed in DMSO-treated cells (**Figures 4.4A, 4.4D and 4.4F**). In addition, THZ1 treatment significantly reduced sister-kinetochore distance (**Figure 4.4C**), suggesting that the centromeric cohesion is reinforced. These cells mostly were featured by diffused Sgo1 along chromosomes and closed arms,
presumably due to the gain of arm cohesin. Further analysis confirmed that ~ 67% of THZ1-treated cells displayed ectopic Myc-Scc1 localizations on arms, while only ~24% of DMSO-treated cells showed Myc-Scc1 arm retention (Figure 4.5A), indicating that increased centromeric transcription retains arm cohesin. To further confirm that enhancing centromeric transcription strengthens centromeric cohesion, I examined centromeric cohesion in the presence of proteasome inhibitor MG132, which can induce cohesion defects. As shown in Figure 4.5B, ~37% of DMSO-treated cells showed centromeric cohesion defects after 2-hour treatment of MG132. In comparison, cohesion defects were largely suppressed in THZ1-treated cells (~11%). The resistance to MG132 in THZ1-treated cells might due to the gain of arm cohesin not strengthened centromeric cohesion, so I measured the sister-kinetochore distance in these cells. Compared to DMSO-treated cells, the sister-kinetochore distance was significantly reduced upon THZ1 treatment (Figure 4.5C), suggesting that increased centromeric transcription does reinforce centromeric cohesion.

I then sought to determine whether the arm localization of RNAPII in THZ1-treated cells is dependent on transcription. Thymidine-arrested Hela Tet-on cells were released into THZ1-containing medium and incubated for 12 hr with nocodazole treatment in the last 2 hr. 4 hr ahead of collection, cells were treated with α-amanitin or triptolide. 4-hour treatment has been proved to effectively suppress on-going gene transcription but barely affect the total amount of RNAs of centromeric transcription. The centromeric RNAPII levels were not reduced upon treatment of both drugs in THZ1-treated cells (Figures 4.5D and 4.5E),
supporting our previous results that 4-hour treatment of α-amanitin or triptolide does not trigger reduction in centromeric transcription. In contrast, ectopic RNAPII and Sgo1 along chromosome arms were entirely removed upon treatment of both drugs (Figure 4.5D and 4.5F). Thus, ectopic RNAPII on chromosome arm induced by THZ1 is dependent on transcription. Surprisingly, THZ1-induced closed-arm morphology was also completely resolved (Figure 4.5G), suggesting that ectopic cohesion on chromosome arms is also dependent on transcription.

Ectopic cohesion and RNAPII along the entire length of chromosomes have been observed in Wapl-depleted cells [173, 174]. It is possible that THZ1-induced phenotype is though Wapl not centromeric transcription, thus I examined the protein levels of Wapl in THZ1-treated cells. Wapl depletion remarkably reduced Wapl protein levels (Figure 4.6A). In comparison, the proteins levels of Wapl in THZ1-treated cells were comparable to the ones in mock cells, suggesting that THZ1 treatment does not affect Wapl proteins. I also examined RNAPII levels in Wapl-depleted cells. Consistent with previous findings, ectopic RNAPII on chromosomes and closed-arm morphology were observed upon Wapl depletion (Figure 4.6B). However, neither of the phenotypes could be reversed by α-amanitin or triptolide (Figures 4.6B-4.6E), suggesting that ectopic RNAPII and cohesion in these cells are not dependent on transcription. Thus, it is unlikely that THZ1-induced phenotype is though Wapl.

Expression of CENP-B DNA-binding domain recapitulates the phenotype of THZ1 treatment
Although my data strongly support the notion that centromeric transcription promotes centromeric cohesion, I could not exclude the possibility that all I found by using general transcription inhibitors were not directly driven by centromeric transcription. The DNA-binding domain of CENP-B (DB, 1-163) can specifically recognize a 17-bp DNA element within α-satellite repetitive sequences called CENP-B box. Fusion with CENP-B DB, proteins can be ectopically targeted to the vicinity of the centromere region [175]. Recently, we have demonstrated that expression of CENP-B DB per se prominently augmented centromeric transcription without affecting gene transcription (data unpublished). Additionally, cells stably expressing Myc-CENP-B DB showed increased H3K4 di-methylation, an epigenetic marker for active transcription, and decreased H3K9 tri-methylation, an indicator for suppressive transcription, specifically at centromeres (data unpublished). Therefore, CENP-B DB expression provides an alternative strategy for me to confirm the role of centromeric transcription in regulating centromeric cohesion. Thymidine-arrested Myc-CENP-B stable cells were incubated with doxycycline for 48 hr and nocodazole was added in the last 2 hr. Mitotic cells were then collected and subjected to chromosome spread followed by immunostaining. I found that expression of CENP-B DB increased the centromeric signals of RNAPII and RNAPII-S2 (Figures 4.7A, 4.7B and 4.9A), supporting our previous finding that CENP-B DB boosts centromeric transcription. To determine whether centromeric cohesion is reinforced upon CEN-B DB overexpression, I examined centromeric cohesion in Myc-CENP-B DB stable cells after 2 hr-treatment of MG132. As shown in Figure 4.7D, MG132 treatment induced cohesion defects in
~ 60% of mock cells, whereas less than 32% of Myc-CENP-B DB stable cells exhibited cohesion defects. I also noted that expression of CENP-B DB induced ectopic RNAPII along the entire chromosomes and arm close similar to THZ1 treatment (Figures 4.7A and 4.7C). To exclude the possibility that the resistance of CENP-B DB stable cells to MG132 is due to arm cohesin retention instead of strengthened centromeric cohesion, I measured the sister-kinetochore distance of these cells. Compared to mock cells, CENP-B DB stable cells displayed significantly reduced sister-kinetochore distance (Figure 4.7E), indicating that the centromeric cohesion is strengthened in these cells. Additionally, no discernable changes of the CENP-A integration were detected in cells expressing CENP-B DB (Figures 4.8A-C), excluding the role of CENP-A in this regulation. Altogether, these data strongly support that enhancing centromeric transcription strengthens centromeric cohesion.

To determine whether the ectopic RNAPII on chromosome arms is induced by augmented transcription, thymidine-arrested Myc-CENP-B DB stable cells were released into fresh medium and treated with nocodazole for 2 hr before harvest. In the last 4 hr, cells were treated with α-amanitin and triptolide. The centromeric RNAPII levels were either slightly decreased or barely unaffected upon drug treatments (Figures 4.9A and 4.9C). In comparison, treatment of both drugs completely removed ectopic RNAPII and Sgo1 and resolved closed-arm morphology of CENP-B stable cells (Figures 4.9A, 4.9D and 4.9E), suggesting that ectopic RNAPII and cohesion is dependent on transcription. Myc-CENP-B DB protein levels were comparable among different treatments (Figure 4.9B),
excluding the possibility that the observed phenotype is due to decreased CENP-B DB expression. Further analysis of sister-kinetochore distance showed that the centromeric cohesion of CENP-B stable cells were loosened upon drug treatments (Figure 4.9F), but no cohesion defects were observed. Again, these data underscore the importance of transcription in promoting ectopic RNAPII and sister chromatid cohesion in Myc-CENP-B DB expressing cells.

**CENP-B depletion increases centromeric transcription and reinforces centromeric cohesion**

CENP-B DB-induced centromeric transcription suggest that CENP-B might function as a suppressor to centromeric transcription. To test it, Hela Tet-on cells were transfected with siRNA oligos for CENP-B depletion and total RNAs were extracted for real-time PCR analysis using two gene primers (GAPDH and RPL30) and three centromere primers (α-1, α-4, and α-13/21). Overall, CENP-B knockdown increases centromeric transcription while barely affected gene transcription (Figure 4.10A). CENP-B depletion enhanced centromeric transcription on α-1 DNAs by more than 20-fold, similar to what I previously observed in CENP-B DB expression. These results are consistent with a recent report showing that CENP-B knockout increased centromeric RNA-FISH signals [176]. Cells transfected with siRNA oligos showed remarkable decrease of CENP-B proteins (Figure 4.10B), confirming the efficiencies of the CENP-B depletion.

I next examined if CENP-B depletion could increase RNAPII localization at centromeres. CENP-B depleted Hela Tet-on cells were arrested by thymidine at
G1 and released into fresh medium followed by 2 hr-treatment of nocodazole. Mitotic cells were then harvested for chromosome spread and immunostaining. Similar to CENP-B DB expression, CENP-B depletion significantly increased centromeric levels of RNAPII and RNAPII-Ser2 (Figures 4.10C-F). I then examined if CENP-B knockdown could strengthen centromeric cohesion in MG132-treated cells. About 50% of mock cells displayed centromeric cohesion defects (Figure 4.10G). In comparison, CENP-B knockdown suppressed cohesion defects to a great extent. Taken these results together, I conclude that centromeric transcription promotes centromeric cohesion.

**CDK11 is an important transcription factor in centromeric transcription**

I next sought to identify key regulators involved in this process. I focused on Cdk11 because it falls into two important categories: 1) it is important for centromeric cohesion maintenance; 2) it regulates transcription. I reasoned that Cdk11 might promotes centromeric cohesion by regulating centromeric transcription. To test this, Hela Tet-on cells were transfected with Cdk11 siRNAs and total RNA were extracted for real-time PCR analysis using two gene primers (GAPDH and RPL30), three centromere primers (α-1, α-4, and α-13/21), and three pericentromere primers (SatIII-pR1, -pTRS47, and -pTRS63). Cdk11 knockdown imposed minimal or no effects on the amount of gene RNAs (Figure 4.11A). Except for RNA transcripts on α-1, the total amounts of centromeric RNAs were moderately but significantly reduced upon Cdk11 depletion. In comparison, the amounts of pericentromeric RNAs in all the tested primers were increased (Figure
4.11A, lower panel). These data suggest that Cdk11 promotes centromeric transcription.

To further determine whether Cdk11 is important for centromeric transcriptional activity, newly transcribed RNAs were labeled with EU (5'-Ethyly Uridine, an analog of uridine) in Cdk11-depleted cells. After click-reaction with biotin, nascent RNAs were purified and subjected to real-time PCR analysis using two gene primers (GAPDH and RPL30) and two centromere primers (α-4, and α-13/21). The EU-labeled RNA levels were much higher than the ones without EU labeling, confirming the efficiencies of our method to examine nascent RNA transcripts (Figure 4.11B). For genes, depletion of Cdk11 decreased the amount of GAPDH EU-RNAs but exerted no effects on that of Rpl30. Similar to our analysis of total RNAs, centromeric EU-RNAs on α-4 and α-13/21 were significantly reduced upon Cdk11 depletion. Taken together, these results suggest that Cdk11 promotes centromeric transcriptional activity.

Previous studies showed that Cdk11 directly phosphorylates the C-terminal domain of RNAPII at Ser2 and Ser5 [143]. To determine whether the Cdk11-mediated centromeric transcription requires its kinase activity, I examined the total amount of RNAs in Cdk11-depleted cells that stably express Myc-Cdk11 WT or KD (kinase dead). Expression of Cdk11 WT or KD in Cdk11-depleted cells barely affected the amount of total RNAs on genes (Figure 4.11C). In contrast, expression of Cdk11 WT but not KD increased centromeric RNAs on α-4 and α-13/21. I note that despite of the large variations in my data, the results were highly
reproducible in multiple repeats. Collectively, these results suggest that the kinase activity of Cdk11 is important for regulating centromeric transcription.

**CDK11 depletion delocalizes RNAPII from centromeres and weakens centromeric cohesion in mitosis**

I next examined the RNAPII levels at centromeres in Cdk11-depleted cells. Hela Tet-on cell were transfected with Cdk11 siRNAs and treated with nocodazole for 2 hr. Mitotic cells were then collected for chromosome spread followed by immunostaining. Consistent with previous studies, Cdk11 depletion caused prominent cohesion defects. Strikingly, these cells exhibited significantly reduced signals of RNAPII and RNAPII-Ser2 at centromeres (**Figure 4.12A-4.12C**), suggesting that Cdk11 is important for RNAPII centromeric localizations. I then examined whether expression of Myc-Cdk11 WT could rescue centromeric RNAPII levels and centromeric cohesion defects in Cdk11-depleted cells. As shown in **Figures 4.12C, 4.12E, and 4.12F**, expression of Cdk11 WT restored the levels of RNAPII and largely rescued the cohesion defects caused by Cdk11 knockdown, whereas KD failed to do so. The protein levels of Myc-CDK11 WT and KD were comparable to each other (**Figure 4.12D**), excluding the possibility that the defects in Cdk11 KD expressing cells were due to decreased protein expression. Thus, centromeric transcription may be required for Cdk11-dependent cohesion maintenance at centromeres.

Cdk11 is important for mRNA splicing and processing [139]. It has been shown that defective mRNA splicing decreased the protein levels of Sororin, thus
leading to cohesion defects [177]. I then examined the protein levels of cohesin and its regulators in Cdk11-depleted cells. The protein levels of Cdk11 were diminished, demonstrating the siRNA efficiency (Figure 4.13A). I found that Cdk11 depletion barely altered the protein levels of Smc1, Sororin, RNAPII, Bub1 or Sgo1, excluding the possibility that the cohesion defects observed in Cdk11-depleted cells are an outcome of decreased protein levels. The chromatin-immunoprecipitation (ChIP) assay confirmed that Cdk11 is localized at centromeric chromatin (Figure 4.13B). To determine whether Cdk11 interacts with RNAPII in cells, cross-linking immunoprecipitation assay was performed using Myc-Cdk11 WT and KD stable cells. As shown in Figure 4.13C, Cdk11 WT physically bound to RNAPII though the binding was weak, supporting the previous in vitro results that Cdk11 directly interacts with RNAPII. Notably, RNAPII bound Cdk11 KD at comparable levels to Cdk11 WT, indicating that the kinase activity of Cdk11 is not required for this interaction. Mitosis-specific isoform Cdk11-P58 was also found to weakly interact with RNAPII (Figure 4.13D). The weak binding between Cdk11 and RNAPII suggest that it might be dynamic in cells. Based on these results and previous findings, centromeric localized Cdk11 directly binds and phosphorylates RNAPII, through which it may promote centromeric transcription.

**CDK11 depletion only partially affects Bub1-Sgo1 pathway**

Previous studies found that the kinetochore localization of Bub1 was compromised upon Cdk11 depletion, raising a possibility that the cohesion defects in Cdk11-depleted cells is through Bub1 [141, 142]. Furthermore, our recent
studies also demonstrated the necessity of Bub1 in the recruitment of RNAPII to centromeres [113]. Thus, it is possible that Cdk11-depletion defects are, at least partially, attributed to Bub1 disfunction. To test this, Hela Tet-on cells were transfected with Cdk11 siRNAs and treated with nocodazole for 2 hr. Shake-off mitotic cells were then subjected to chromosome spread and subsequent immunostaining to examine Bub1 localization at centromeres. Bub1 knockdown completely abolished Bub1 localizations at kinetochores (Figure 4.14A). In contrast, although the overall kinetochore signals of Bub1 mildly decreased, they largely remained detectable in Cdk11-depleted cells (Figures 4.14A and 4.14B). Under this condition, Cdk11 knockdown induced much severe cohesion defects than Bub1 knockdown, which only slightly increased the sister-kinetochore distance (Figure 4.14C). I also examined the amount of EU-RNAs upon Bub1 ablation on genes and at centromeres in log-phase cells. Different from what we observed upon Cdk11 depletion, Bub1 knockdown barely affected centromeric transcription (Figure 4.11B). Thus, Cdk11 very likely promotes centromeric cohesion directly through RNAPII not Bub1. Notably, although overall the signals of Sgo1 at centromeres were moderately reduced (Figure 4.12G), they were retained in most of the Cdk11-depleted cells (Figures 4.14D and 4.14E). In addition, Sgo1 tended to localize at kinetochore-proximal regions instead of inner centromeres in these cells (Figure 4.14E, III).

Ectopic targeting of Bub1 to kinetochores fails to fully rescue the cohesion defects caused by Cdk11 depletion
To further determine the contribution of Bub1 to Cdk11 knockdown-induced phenotypes, I fused Bub1 with outer-kinetochore protein Mis12 to ectopically target Bub1 to kinetochores. I firstly examined the functionality of this fusion protein in Bub1-depleted cells. Hela Tet-on cells depleted of Bub1 were transfected with plasmids containing GFP-Mis12-Bub1 WT or KD lacking Bub1 kinase activity and were treated with nocodazole for 2 hr. Mitotic cells were then harvested for chromosome spread and immunostaining analysis. As expected, Bub1 depletion dramatically reduced centromeric Sgo1 localizations, while ectopically targeting Bub1 WT, not KD, to kinetochores completely restored Sgo1 levels (Figures 4.15A and 4.15B). Both Mis12-Bub1 WT and KD signals were comparable to each other (Figure 4.15C), excluding the possibility that the failure of Mis12-Bub1 KD in restoring Sgo1 levels is due to its defective kinetochore localization. Therefore, Mis12-Bub1 fusion proteins are able to target functional Bub1 to kinetochores.

I then examined whether ectopic targeting of functional Bub1 could rescue the cohesion defects provoked by Cdk11 knockdown. Hela Tet-on cells depleted of Cdk11 were transfected with GFP-Mis12-Bub1 WT or KD and treated with nocodazole for 2 hr. Mitotic cells were collected and subjected to chromosome spread followed by immunostaining. Although expression of Mis12-Bub1 WT in Cdk11-depleted cells prominently restored the Sgo1 signals, it was unable to fully rescue the cohesion defects (Figure 4.15D-4.15F). Further analysis revealed that ~40% of these cells exhibited kinetochore-proximal localizations of Sgo1, similar to Cdk11-depleted cells (Figure 4.15G, III). Therefore, the cohesion defects
caused by Cdk11 depletion were due to Sgo1 relocations at kinetochores rather than decreased localizations of Bub1. Considering the essential role of active RNAPII in Sgo1 translocation from kinetochores to inner centromeres [113], these results support the notion that centromeric transcription plays a decisive role in centromeric cohesion protection by Cdk11.

THZ1 enhances centromeric transcription and rescues cohesion defects in Cdk11-depleted cells

We next sought to determine whether enhanced centromeric transcription could rescue Cdk11-depletion defects. Firstly, we examined the total RNA transcripts in Cdk11-depleted cells upon treatment of THZ1, an enhancer for centromeric transcription. Hela Tet-on cells were incubated with medium containing THZ1 for 12 hr and total RNAs were extracted for real-time PCR analysis using two gene primers (GAPDH and RPL30) and three centromere primers (α-1, α-4, and α-13/21). Consistently, depletion of Cdk11 decreased the amount of total RNAs on GAPDH but barely affected that on RPL30. For centromeres, Cdk11 depletion reduced centromeric RNAs on all the tested primers (Figure 4.16A). In comparison, the amount of total centromeric RNAs were increased upon THZ1 treatment in Cdk11-depleted cells. Similar results were also obtained in CENP-B DB stable cells depleted of Cdk11 (Figure 4.16B). Collectively, these results demonstrate that enhanced centromeric transcription can rescue Cdk11-depletion defects in centromeric transcription.
We then examined whether enhanced centromeric transcription could strengthen centromeric cohesion after Cdk11 depletion. Thymidine arrested Hela Tet-on cells depleted of Cdk11 were released into fresh medium containing THZ1 and incubated for 12 hr followed by nocodazole treatment in the last 2 hr. Mitotic cells were then collected for chromosome spread and immunostaining. Consistently, Cdk11 knockdown decreased the levels of RNAPII at centromeres and induced pronounced cohesion defects (Figures 4.16C and 4.16D). In contrast, THZ1 treatment not only largely rescued the centromeric RNAPII signals (Figure 4.16E), but also completely rescued the cohesion defects (Figure 4.16D), strongly supporting that Cdk11 promotes centromeric cohesion through centromeric transcription.

4.3 Discussion

Centromeric transcription is important for centromere functions and the genome stability. Based on our previous and current studies, I propose that a major function of centromeric transcription is to promote centromeric cohesion in mitosis. In this thesis, using transcriptional inhibitors and manipulating CENP-B levels, I demonstrate that inhibiting centromeric transcription dislodges elongating RNAPII from centromeres and induces severe centromeric cohesion defects, whereas enhancing it increases RNAPII levels and strengthens centromeric cohesion. Because the short-term treatment of transcriptional inhibitors dramatically changed the ongoing centromeric transcription without significantly affecting total RNAs, I believe that transcriptional activity instead of RNA transcripts plays a more
important function in this regulation. I also identify Cdk11 as a key factor for centromeric transcription, which may directly bind RNAPII at centromeres to phosphorylate its CTD, thus promoting centromeric transcription.

**How does centromeric transcription maintain cohesions at the centromere in mitosis**

Our previous and current studies demonstrate that centromeric transcription facilitates Sgo1 translocation from kinetochores to inner centromeres, where it binds to cohesin and protects centromeric cohesion. Inhibition of RNAPII by α-amanitin or mitosis-specific degradation of Rpb2 has been shown to trap Sgo1 at kinetochores and compromise centromeric cohesion [113]. In this study, I also found that centromeric transcription inhibition decreases Sgo1 centromeric signals and relocates Sgo1 at kinetochore-proximal regions, leaving centromeric cohesion unprotected. Thus, centromeric transcription may promote centromeric cohesion through Sgo1. Cohesin is a direct receptor for RNAPII [173]. A recent study showed that cohesin *per se* can serve as a promoting factor for mitotic transcription. Consistently, I found ectopic RNAPII localizations on mitotic chromosomes in Wapl-depleted cells (Figure 4.6A). Using THZ1 to enhance centromeric transcription, I demonstrated that transcription can also promote cohesin (Figure 4.5A). Taken all these findings together, cohesin, Sgo1, and transcription may form a positive feedback loop to regulate cohesion in mitosis. Notably, despite that the centromeric localization of Sgo1 was decreased, the signals of Scc1 could still be detected in most of the triptolide-treated cells (Figure...
4.3D), raising a possibility that only a small amount of Sgo1 at inner centromeres is sufficient to protect centromeric cohesion.

**Key factors for regulating centromeric transcription**

Ablation of CENP-B has been shown to increase RNA-FISH signals [176], suggesting that CENP-B might be a suppressing factor for centromeric transcription. In support of this idea, I found that either expression of CENP-B DB or depletion of CENP-B enhances centromeric transcription, increases centromeric RNAPII levels, and strengthens centromeric cohesion (Figures 4.7 and 4.10). How CENP-B suppresses centromeric transcription remains elusive. We have recently showed that CENP-B DB expression increased active transcription mark H3K4 di-methylation and decreased suppressive transcription mark H3K9 tri-methylation (data not shown). Thus, it may epigenetically regulate centromeric transcription. Notably, CENP-B is required for heterochromatin maintenance [175], raising a possibility that CENP-B-mediated transcription may be important for maintaining the centromere identity. It also forms a complex with RNAs produced from inactive α-satellite arrays [117], but the function of this interactions is unknown.

In addition, I identified Cdk11 as a promoting factor for centromeric transcription. This regulation is likely through RNAPII. Cdk11 aids the assembly of the RNAPII mediator complex [145]. Previous *in vitro* studies proved that Cdk11 binds and phosphorylates the RNAPII C-terminus [178-180]. Using cross-linking immunoprecipitation assay, I demonstrate that Cdk11 also physically interacts with
RNAPII in cells, although the binding is weak. The weak binding may be due to its dynamics.

**What determines Cdk11 specificity at centromeres**

Analysis of RNA transcripts using real-time PCR revealed that Cdk11 depletion prominently reduced the amount of RNAs on α-satellite DNAs, suggesting that a major function of Cdk11 is to promote centromeric transcription. Then what determines its specificity at centromeres? Mounting evidence suggest that transcription initiation might be dispensable for transcription at the centromere [120, 173]. Firstly, only RNAPII-Ser2 but not RNAPII-Ser5 was found to localize at centromeres on mitotic chromosomes; Secondly, THZ1 or triptolide that inhibits transcriptional initiation failed to suppress centromeric transcription albeit it did so on genes. Thus, unlike the transcription on genes where Cdks play redundant functions, Cdk11 may plays more specific role in centromeric transcription. Alternatively, the centromere is a specialized region with non-B type DNA structures [181]. The special structures and/or specific centromeric proteins might recruit Cdk11 rather than other Cdks to regulate centromeric transcription. In future, it would be of interest to test these possibilities. In addition, identifying more Cdk11 substrates functioning at centromeres would be another important question to be addressed.

In summary, my findings unveil that a major function of centromeric transcription is to maintain centromeric cohesion during mitosis, thus ensuring
faithful chromosome segregation. I also identify Cdk11 as a key factor which directly binds RNAPII and promotes centromeric transcription.
Figure 4.1 α-amanitin, not triptolide, that suppresses ongoing centromeric transcription impairs centromeric cohesion (DOI: 10.1083/jcb.202008146)

A. Nocodazole-arrested HeLa Tet-On cells were treated with DMSO, α-amanitin (Aman), or triptolide (Trip) for 5 hr and then subjected to chromosome spread and immunostaining with the indicated antibodies. Two major types of chromosome morphology were observed: type 1, chromosomes with cohesed sister centromeres and robust localization of Sgo1 and RNAPII at centromeres; and type 2, chromosomes with separated centromeres and decreased localization Sgo1 and RNAPII at centromeres. B. Quantification of chromosome morphology with unseparated (type I) and separated sister chromatids (type II) in (A). The average and standard error calculated from at least three independent experiments were shown here. At least 30 mitotic cells were analyzed for each condition in one single experiment. C and D. Relative intensity of RNAPII (RNAPII/ACA) and Sgo1 (Sgo1/ACA) at centromeres in (A). Quantification details were recorded in the section of Methods and Materials. At least 90 centromeres (6 per cell) were scored for each condition. E and F. Nocodazole-arrested HeLa Tet-On cells were treated under the same conditions as in (A). Relative intensity of RNAPII-S2 at centromeres (F) was shown here. Quantification details were recorded in the section of Methods and Materials. At least 90 centromeres (6 per cell) were scored for each condition. n.s. denotes not significant; **, P<0.01; ***, P<0.001; ****, P<0.0001.
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Figure 4.2 Cohesion defects induced by α-amanitin is not specific for cancer cells and not due to decreased protein levels of cohesin and its regulators (DOI: 10.1083/jcb.202008146)

A, B, C and D. Non-transformed RPE-1 cells were treated with DMSO, α-amanitin (Aman), or triptolide (Trip) for 5 hr and then subjected to chromosome spread and immunostaining with the indicated antibodies. Quantification of chromosome morphology with unseparated and separated sister chromatids (B) and relative intensity of Sgo1 (C) and RNAPII (D) at centromeres were shown here. Quantification details were recorded in the section of Methods and Materials. The experiment was repeated at least three times. The average and standard error were shown in (B). The average and standard deviation were shown in (C and D). At least 30 mitotic cells were analyzed for each condition in one single experiment (B). At least 90 centromeres (6 per cells) were scored for each condition (C and D). E. Lysates of HeLa Tet-On cells treated with DMSO, α-amanitin (Aman), triptolide (Trip), flavopiridol (Flav) or THZ1 were subjected to Western blots with the indicated antibodies. L, light exposure; H. heavy exposure. n.s. denotes not significant; *, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001.
**Figure 4.3** The integration of CENP-A is not impaired upon α-amanitin treatment (DOI: 10.1083/jcb.202008146)

A. Nocodazole-arrested HeLa Tet-On cells were treated with DMSO, α-amanitin (Aman), or triptolide (Trip) for 5 hr and then subjected to chromosome spread and immunostaining with the indicated antibodies. B and C. Relative intensity of CENP-A (CENP-A/DAPI) and ACA (ACA/DAPI) at centromeres in (A). D. Thymidine-arrested HeLa Tet-On cells transiently expressing Myc-Scc1 were released into fresh medium and treated with nocodazole in the last 2 hr before harvest. Mitotic cells were collected and subjected for chromosome spread and immunostaining with the indicated antibodies. 88.2% of DMSO-treated cells (n=34) and 87.1% of triptolide-treated cells (n=31) showed centromeric Myc-Scc1 signals. Centromeric Myc-Scc1 was only present in 25% of cells (n=52) treated with α-amanitin, featured by a bar-shaped structure. n.s. denotes not significant.
Figure 4.4 THZ1 that increases ongoing centromeric transcription induces ectopic RNAPII localizations and arm close (DOI: 10.1083/jcb.202008146)

A. Thymidine-arrested HeLa Tet-On cells were released into fresh medium containing DMSO or THZ1 and incubated for 12 hr with the treatment of nocodazole in the last 2 hr. Mitotic cells were collected and subjected for chromosome spread and immunostaining with the indicated antibodies.  

B. Relative RNAPII intensity at centromeres in (A). Quantification details were recorded in the section of Methods and Metatrails. The average and standard deviation were shown here. At least 90 centromeres (6 per cell) were scored for each condition.

C. Quantification of sister-kinetochore distance in (A). The average and standard deviation were shown here. At least 150 kinetochore pairs (10 per cell) were scored for each condition.

D. Thymidine-arrested HeLa Tet-On cells were treated under the same conditions as in (A).

E and F. Relative RNAPII intensity at centromeres (E) or on chromosomes (F) of mitotic cells in (A). Quantification details were recorded in the section of Methods and Metatrails. The average and standard deviation were shown here. At least 90 centromeres (6 per cell, E) and 90 chromosome arms (6 per cell, F) were scored for each condition.

****, P<0.0001.
**Figure 4.5** THZ1 that increases ongoing centromeric transcription strengthens centromeric cohesion (DOI: 10.1083/jcb.202008146)

**A.** Thymidine-arrested HeLa Tet-On cells transiently expressing Myc-Scc1 were released into fresh medium containing DMSO or THZ1 and incubated for 12 hr with the treatment of nocodazole in the last 2 hr. Mitotic cells were collected and subjected for chromosome spread and immunostaining with the indicated antibodies. Both DMSO-treated and THZ1-treated cells showed strong centromeric Myc-Scc1 localizations. 24.0% of DMSO-treated cells (n=25) and 66.7% of THZ1-treated cells (n=31) showed ectopic Myc-Scc1 on the entire length of chromosomes. **B.** Thymidine-arrested HeLa Tet-On cells were released into fresh medium containing DMSO or THZ1 and incubated for 12 hr with the treatment of MG132 in the last 2 hr. Mitotic cells were subjected to chromosome spread. Quantification of chromosome morphology with unseparated and separated sister chromatids was shown here. The average and standard error were calculated based on three independent experiments. At least 30 mitotic cells were scored for each condition in one single experiment. **C.** Quantification of sister-kinetochore distance in (B). The average and standard deviation were shown here. At least 150 kinetochore pairs (10 per cell) were scored for each condition. **D.** Thymidine-arrested HeLa Tet-On cells were released into fresh medium containing DMSO or THZ1 and incubated for 12 hr with the treatment of nocodazole in the last 2 hr. Cells were further treated with DMSO, α-amanitin (Aman), or triptolide (Trip) for 4 hr before harvest. Collected mitotic cells were subjected for chromosome spread and immunostaining with the indicated antibodies. **E** and **F.** Relative pRpb1
intensity at centromeres (E) or on chromosome arms (F) of mitotic cells in (D). Quantification details were recorded in the section of Methods and Materials. The average and standard deviation were shown here. At least 90 centromeres (6 per cell, E) and 90 chromosome arms (6 per cell, F) were scored for each condition.

G. Quantification for cells in (D) with open and closed chromosome arms. The average and standard error were calculated based on three independent experiments. At least 30 mitotic cells were scored for each condition in one single experiment. n.s. denotes not significant; **, P<0.01; ***, P<0.001; ****, P<0.0001.
Figure 4.6 Wapl depletion does not recapitulate the phenotype induced by THZ1 (DOI: 10.1083/jcb.202008146)

A. Lysates of HeLa Tet-On cells (Ctl), THZ1-treated Hela cells, and Wapl-depleted Hela cells were resolved with SDS-PAGE and blotted with the indicated antibodies.

B. Thymidine-arrested HeLa Tet-On cells depleted with Wapl were released into fresh medium and treated with DMSO, α-amanitin (Aman), or triptolide (Trip) for 4 hr before harvest. In the last 2 hr, cells were further treated with nocodazole. Collected mitotic cells were subjected for chromosome spread and immunostaining with the indicated antibodies. C and D. Relative RNAPII intensity at centromeres (C) or on chromosome arms (D) of mitotic cells in (A). Quantification details were recorded in the section of Methods and Materials. The average and standard deviation were shown here. At least 90 centromeres (6 per cell, C) and 90 chromosome arms (6 per cell, D) were scored for each condition.

E. Quantification for cells in (B) with open and closed chromosome arms. The average and standard error were calculated based on three independent experiments. At least 30 mitotic cells were scored for each condition in one single experiment. n.s. denotes not significant; **, P<0.01; ***, P<0.001; ****, P<0.0001.
Figure 4.7 Expression of CENP-B DB (DNA-binding domain) induces ectopic RNAPII on mitotic chromosomes and strengthens centromeric cohesion (DOI: 10.1083/jcb.202008146)

A. Nocodazole-arrested HeLa Tet-On cells and HeLa Myc-CENP-B DB stable cells treated with doxycycline were subjected to chromosome spread and stained with the indicated antibodies. B and C. Relative RNAPII intensity at centromeres (B) or on chromosome arms (C) of mitotic cells in (A). Quantification details were recorded in the section of Methods and Materials. The average and standard deviation were shown here. At least 90 centromeres (6 per cell, B) and 90 chromosome arms (6 per cell, C) were scored for each condition. D. HeLa Tet-On cells and HeLa Myc-CENP-B DB stable cells treated with doxycycline were incubated with MG132 for 2 hr. Mitotic cells were subjected to chromosome spread. Quantification of chromosome morphology with unseparated and separated sister chromatids was shown here. The average and standard error were calculated based on three independent experiments. At least 30 mitotic cells were scored for each condition in one single experiment. E. Quantification of sister-kinetochore distance in (D). The average and standard error were shown here. At least 150 kinetochore pairs (10 per cell) were scored for each condition. E. Quantification of sister-kinetochore distance in (D). The average and standard deviation were shown here. At least 150 kinetochore pairs (10 per cell) were scored for each condition. n.s. denotes not significant; *, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001.
Figure 4.8 Expression of CENP-B DB (DNA-binding domain) does not affect the integration of CENP-A (DOI: 10.1083/jcb.202008146)

**A.** Nocodazole-arrested HeLa Tet-On cells and HeLa Myc-CENP-B DB stable cells treated with doxycycline were subjected to chromosome spread and stained with the indicated antibodies. **B** and **C.** Relative intensity of CENP-A (CENP-A/DAPI) and ACA (ACA/DAPI) at centromeres in (A). Quantification details were recorded in the section of Methods and Materials. The average and standard deviation were shown here. At least 90 centromeres (6 per cell) were scored for each condition. n.s. denotes not significant.
Figure 4.9 Ectopic RNAPII and arm close induced by CENP-DB expression are dependent on transcription (DOI: 10.1083/jcb.202008146)

A. Thymidine-arrested HeLa Tet-On cells (Ctl) and HeLa Myc-CENP-B DB stable cells treated with doxycycline were released into fresh medium and further incubated for 12 hr. At 8 hr after release, DMSO, α-amanitin (Aman), and triptolide (Trip) were added. 2 hr before harvest, cells were treated with nocodazole. Collected mitotic cells were subjected to chromosome spread and staining with the indicated antibodies. B. Lysates of HeLa Tet-On cells in (A) were resolved with SDS-PAGE and blotted with the indicated antibodies. C and D. Relative RNAPII intensity at centromeres (C) and on chromosome arms (D) was shown here. The quantification details were recorded in the section of Methods and Materials. The average and standard deviation were shown. At least 90 centromeres (6 per cell, C) and 90 chromosome arms (6 per cell, D) were scored for each condition in one single experiment. E. Quantification of chromosome morphology with closed and open chromosome arms was shown here. The average and standard error were calculated based on three independent experiments. At least 30 mitotic cells were scored for each condition in one single experiment. F. Quantification of sister-kinetochore distance in (E). The average and standard deviation were shown here. At least 150 kinetochore pairs (10 per cell) were scored for each condition. n.s. denotes not significant; *, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001.
Figure 4.10 CENP-B depletion enhances centromeric transcription and strengthens centromeric cohesion (DOI: 10.1083/jcb.202008146)

A. HeLa Tet-On cells were transfected with distinct CENP-B siRNA oligos and RNA was extracted and purified for real-time PCR analysis with the indicated primers. The average and standard error were calculated based on three independent experiments. B. Lysates of HeLa Tet-On cells and CENP-B depleted Hela cells were resolved with SDS-PAGE and blotted with the indicated antibodies. C. Thymidine-arrested HeLa Tet-On cells and CENP-B-depleted Hela cells were released into fresh medium and treated with nocodazole in the last 2 hr before harvest. Collected mitotic cells were subjected to chromosome spread and staining with the indicated antibodies. D. Relative RNAPII-S2 intensity at centromeres (C) was shown here. The quantification details were recorded in the section of Methods and Materials. The average and standard deviation were shown. At least 90 centromeres (6 per cell) were scored for each condition in one single experiment. E. Thymidine-arrested HeLa Tet-On cells were treated under the same conditions as in (C). Collected mitotic cells were subjected to chromosome spread and staining with the indicated antibodies. F Relative RNAPII-S2 intensity at centromeres (E) was shown here. The quantification details were recorded in the section of Methods and Materials. The average and standard deviation were shown. At least 90 centromeres (6 per cell) were scored for each condition in one single experiment. G. HeLa Tet-On cells and CENP-B-depleted Hela cells were incubated with MG132 for 2 hr. Mitotic cells were subjected to chromosome spread. Quantification of chromosome morphology with unseparated and separated sister
chromatids was shown here. The average and standard error were calculated based on three independent experiments. At least 30 mitotic cells were scored for each condition in one single experiment. n.s. denotes not significant; *, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001.
A

Total GAPDH  Total Rp30  Total Sat-1  Total Sat-4  Total Sat-13/21

Fold change

1: Ctrl  4: si-CDK11-3#  
2: si-CDK11-design  5: si-CDK11-4#  
3: si-CDK11-1361

B

Nascent GAPDH  Nascent Rp30  Nascent Sat-4  Nascent Sat-13/21

Fold change

1: Ctrl  2: Ctrl with EU  3: si-CDK11-1361 with EU  4: si-CDK11-design with EU  5: si-Bub1 with EU

C

Total GAPDH  Total Rp30  Total Sat-1  Total Sat-4  Total Sat-13/21

Fold change

WT  KD  si-CDK11-design

in-CDK11-design
Figure 4.11 Cdk11 is important for regulating centromeric transcription and the kinase activity is required in this process

A. HeLa Tet-On cells were transfected with various types of si-Cdk11 RNAs including design, 1361, 3 and 4. Total RNAs were extracted and purified for real-time PCR analysis with the indicated primers. The details were recorded in the section of Methods and Materials. The average and standard deviation calculated from at least three independent experiments were shown here. B. HeLa Tet-On cells were transfected with distinct siRNA oligos for depletion of Cdk11 or Bub1 and EU was added in the last 1 hour before collection. EU-RNAs were prepared and analyzed by real-time PCR with the indicated primers. The details were recorded in the section of Methods and Materials. The average and standard deviation calculated from at least three independent experiments were shown here. C. Hela Tet-on cells stably expressing Myc-CENP-B DB were deleted of Cdk11 and total RNAs were extracted and purified for real-time PCR analysis with the indicated primers. The details were recorded in the section of Methods and Materials. The average and standard deviation calculated from at least three independent experiments were shown here. n.s. denotes not significant; *, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001.
**Figure 4.12 Cdk11 and its kinase activity is required for the centromeric localization of RNAPII and maintenance of centromeric cohesion**

A. HeLa Tet-On cells were transfected with distinct Cdk11 siRNA oligos and nocodazole was added in the last 2 hr before harvest. Collected mitotic cells were subjected to chromosome spread and staining with the indicated antibodies. B. Relative RNAPII-S2 intensity at centromeres (A) was shown here. The quantification details were recorded in the section of Methods and Materials. The average and standard deviation were shown. At least 90 centromeres (6 per cell) were scored for each condition in one single experiment. C. HeLa Tet-On cells stably expressing Myc-Cdk11 WT or KD were depleted of Cdk11 and nocodazole was added in the last 2 hr before harvest. Collected mitotic cells were subjected to chromosome spread and staining with the indicated antibodies. D. Lysates of HeLa Tet-On cells in (C) were resolved with SDS-PAGE and blotted with the indicated antibodies. E. Quantification of sister-kinetochore distance in (C). The average and standard deviation were shown here. At lease 100 kinetochore pairs (10 per cell) were scored for each condition. F and G. Relative intensities of RNAPII (F) and Sgo1 (F) at centromeres were shown here. The quantification details were recorded in the section of Methods and Materials. The average and standard deviation were shown. At least 60 centromeres (6 per cell) were scored for each condition in one single experiment. n.s. denotes not significant; *, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001.
Figure 4.13 Cdk11 depletion does not affect protein levels of cohesin or its regulators and RNPII directly interacts with Cdk11

A. Lysates of HeLa Tet-On cells transfected with various siCdk11 RNAs were resolved with SDS-PAGE and blotted with the indicated antibodies. B. Hela Tet-on cells and Hela Myc-Cdk11 WT stable cells treated with doxycycline were cross-linked with formaldehyde, sonicated, and then subjected to immunoprecipitation with anti-Myc antibodies. DNA extracted from the immunoprecipitates was subjected to real-time PCR analysis with the indicated primers. The average and standard error were calculated based on three independent experiments. C. RNPII weakly interacts with Cdk11 in cells. Lysates of Hela Tet-on cells stably expressing Myc-Cdk11 WT or KD were cross-linked with formaldehyde and incubated with antibody against RNAPII. The immunoprecipitated proteins were resolved with SDS-PAGE and blotted with the indicated antibodies. WT, Cdk11-P110 full length; KD, Cdk11-P110 lacking kinase activity. D. RNAPII weakly interacts with P58 in cells. Lysates of Hela Tet-on cells stably expressing Myc-P58 were cross-linked with formaldehyde and incubated with antibody against RNAPII. The immunoprecipitated proteins were resolved with SDS-PAGE and blotted with the indicated antibodies. P58, mitosis-specific isoform Cdk11-P58 full length.
Figure 4.14 Cdk11 depletion only partially affects Bub1 and Sgo1 localization

A. HeLa Tet-On cells were transfected with Bub1 siRNA or distinct Cdk11 siRNA oligos and nocodazole was added in the last 2 hr before harvest. Collected mitotic cells were subjected to chromosome spread and staining with the indicated antibodies. B. Relative Bub1 intensity at kinetochores (A) was shown here. The quantification details were recorded in the section of Methods and Materials. The average and standard deviation were shown. At least 90 centromeres (6 per cell) were scored for each condition in one single experiment. C. Quantification of sister-kinetochore distance in (A). The average and standard deviation were shown here. At least 100 kinetochore pairs (10 per cell) were scored for each condition. D. HeLa Tet-On cells were depleted of Cdk11 with distinct siRNA oligos and nocodazole was added in the last 2 hr before harvest. Collected mitotic cells were subjected to chromosome spread and staining with the indicated antibodies. D. Lysates of HeLa Tet-On cells in (C) were resolved with SDS-PAGE and blotted with the indicated antibodies. E. HeLa Tet-On cells were transfected with Cdk11 siRNAs (design) and nocodazole was added in the last 2 hr. Mitotic cells were subjected to chromosome spread. The major types of chromosome morphology observed after depletion of Cdk11 were shown in upper panel. Quantification of chromosome morphology was shown in the bottom panel. The average and standard error were calculated based on three independent experiments. At least 30 mitotic cells were scored for each condition in one single experiment. n.s. denotes not significant; *, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001.
Figure 4.15 Ectopic targeting of Bub1 at kinetochores fails to completely rescue cohesion defects in Cdk11-depleted cells.

A. HeLa Tet-On cells transiently expressing GFP-Mis12-Bub1 were transfected with Bub1 siRNA and nocodazole was added in the last 2 hr before harvest. Collected mitotic cells were subjected to chromosome spread and staining with the indicated antibodies. B and C. Relative Bub1 (B) and Sgo1 (C) intensities at kinetochores (A) were shown here. The quantification details were recorded in the section of Methods and Materials. The average and standard deviation were shown. At least 60 centromeres (6 per cell) were scored for each condition in one single experiment. D. HeLa Tet-On cells transiently expressing GFP-Mis12-Bub1 were depleted of Cdk11 (design) and nocodazole was added in the last 2 hr before harvest. Collected mitotic cells were subjected to chromosome spread and staining with the indicated antibodies. E. Relative Sgo1 intensities at kinetochores (D) were shown here. The quantification details were recorded in the section of Methods and Materials. The average and standard deviation were shown. At least 60 centromeres (6 per cell) were scored for each condition in one single experiment. F. Quantification of sister-kinetochore distance in (D). The average and standard deviation were shown here. At lease 100 kinetochore pairs (10 per cell) were scored for each condition. G. The major types of chromosome morphology were observed in (D). Quantification of chromosome morphology was shown in the bottom panel.
Figure 4.16 THZ1 enhances centromeric transcription and rescues cohesion defects in Cdk11-depleted cells

A. HeLa Tet-On cells depleted of Cdk11 were incubated with medium containing THZ1 for 12 hr and EU was added in the last 1 hour before collection. EU-RNAs were prepared and analyzed by real-time PCR with the indicated primers. The details were recorded in the section of Methods and Materials. The average and standard deviation calculated from at least three independent experiments were shown here. B. Hela Tet-on cells stably expressing Myc-CENP-B DB were deleted of Cdk11 and total RNAs were extracted and purified for real-time PCR analysis with the indicated primers. The details were recorded in the section of Methods and Materials. The average and standard deviation calculated from at least three independent experiments were shown here. C. HeLa Tet-On depleted of Cdk11 (design) were incubated with medium containing THZ1 and nocodazole was added in the last 2 hr before harvest. Collected mitotic cells were subjected to chromosome spread and staining with the indicated antibodies. D. Quantification of sister-kinetochore distance in (C). The average and standard deviation were shown here. At lease 100 kinetochore pairs (10 per cell) were scored for each condition. E. Relative RNAPII intensities at centromeres (C) were shown here. The quantification details were recorded in the section of Methods and Materials. The average and standard deviation were shown. At least 60 centromeres (6 per cell) were scored for each condition in one single experiment. n.s. denotes not significant; *, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001.
CHAPTER 5: CONCLUSION

Proper regulations of KT-MT attachment and centromeric cohesion are required for faithful chromosome segregation during mitosis. Many endeavors have been devoted to illustrating the mechanisms of how they are regulated. In my study, I have two major findings: firstly, I demonstrate that Cdk1 phosphorylation is important for Ska functions in mitosis and determines its binding to the Ndc80 complex; Secondly, I prove that centromeric transcription and its key regulator Cdk11 promotes centromeric cohesion. These findings are critical in advancing our understandings of the mechanism of mitotic regulation. In addition, given that Ska3 and Cdk11 are overexpressed in a wide variety of cancer cells, gaining such knowledge provides the insights of the occurrence of chromosome instability and its contribution to tumorigenesis, which are important for developing novel therapeutic strategies for human malignancies. Future studies will be needed to illustrate the structures of the Ska-Ndc80 macro-complexes and determine the underlying mechanisms whereby centromeric cohesion is promoted by centromeric transcription.
Reference


