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Interaction Between Centromeric Histone H3 Variant And Shugoshin

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INTERACTION BETWEEN CENTROMERIC HISTONE H3 VARIANT AND
SHUGOSHIN

Thesis

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of

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Visarut Buranasudja

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" วิทยานิพนธ์ฉบับนี้เปรียบเสมือนเครื่องเตือนใจในการดำเนินชีวิตของข้าพเจ้า

คนเราเมื่อล้มแล้วต้องลุกขึ้นให้ได้ อย่ามัวจมปลักกับความผิดหวัง

คนเราหากโดนเหยียบอยู่ในดิน ก็ต้องหาหนทางที่จะใช้ชีวิตอยู่ให้ได้ในโคลนตม

คนเราต้องอย่ายึดติดกับความสำเร็จในอดีต จนลืมตัวว่าปัจจุบันเรายืนอยู่ในจุดไหน

คำว่า แพ้ นั้นไม่มีอยู่จริง หากเราไม่ยอมแพ้

คำว่า ชนะ จะไม่เกิดขึ้น หากเราไม่ทุ่มเทที่จะได้มันมา

ขออุทิศวิทยานิพนธ์ฉบับนี้ให้กับคุณพ่อ คุณแม่

สำหรับทุกสิ่งทุกอย่างที่ได้ให้ทุ่มเทกับลูกคนนี้ตลอดเวลาเกือบ 30 ปีที่ผ่านมา

ขออุทิศวิทยานิพนธ์ฉบับนี้ให้กับครูบาอาจารย์ทุกท่าน

สำหรับวิชาความรู้ต่างๆที่นำมาซึ่งความสำเร็จในวันนี้

ขออุทิศวิทยานิพนธ์ฉบับนี้ให้กับประชาชนชาวไทย

สำหรับโอกาสอันแสนล้ำค่าที่ทำให้ผมได้มาศึกษาเล่าเรียน ณ. ที่แห่งนี้

ผมหวังว่าการเดินทางของผมจะถึงจุดหมายในไม่ช้า

ผมหวังว่าจะได้กลับไปตอบแทนบุญคุณทุกท่านในอนาคตอันใกล้ "

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Buranasudja, Visarut. M.S., Purdue University, May 2013. The interaction between centromeric histone H3 and shugoshin. Major Professor: Tony Hazbun Ph.D.

Precise and faithful segregation of chromosome segregation during mitosis depends on the ability of the cell to regulate chromosome bi-orientation on the mitotic spindle. Shugoshin (Sgo1), the protector of meiotic centromeric cohesin, is required for proper establishment of chromosome bi-orientation. Sgo1 plays a crucial role as part of a mitotic tension sensor between sister chromatids. Recently, Sgo1 has been reported to interact with histone H3 at the pericentromere region, as an important factor for tension sensing and chromosome segregation. However, the role of Sgo1 in tension sensing at centromere is still elusive. The centromere is the region of attachment of chromatin fiber to mitotic spindle via the kinetochore and these structures assist in segregation of chromosomes to opposite spindle poles during mitosis. Cse4, budding yeast centromere specific histone variant, is thought to substitute histone H3 when assembling into a centromeric nucleosome. Cse4 plays key roles in kinetochore formation and proper chromosome segregation. Cse4 contains conserved C-terminal histone fold domain and unique 135-amino-acid N-terminal tail that extends from the nucleosome core making it accessible to interacting proteins and modification. To date, there is no evidence of

 direct physical interaction reported between the Cse4 tail and the kinetochore or cell cycle related-proteins.

In our study, we first established a direct interaction between the Cse4 N-terminal tail and Sgo1 by using an *in vitro* pull down assay. Sgo1 has a strong ability to associate with Cse4 tail, while it is not able to bind with another kinetochore protein tail, Cnn1, indicating the specificity of Sgo1-Cse4 interaction. From our kinetic binding study, interaction between Sgo1 and N-terminal tail of Cse4 has an equilibrium dissociation constant (KD) of approximately 33 nM. Moreover, we identified the minimal region on Cse4 tail (residue 49-65) that is sufficient for associating with Sgo1. Interestingly, part of this binding motif (residue 49-56) is conserved from present throughout eukaryotes. Furthermore, our pull down analysis and multiple sequence alignment analysis of Cse4 tail homologues suggest that there is an additional conserved motif, located within residues 95-102 of Cse4-tail, that is responsible for the Sgo1 interaction. In addition, an N-terminal proteolytic fragment of Sgo1 can interact with Cse4. The finding of the Sgo1 binding motifs, present in Cse4, suggests an attractive model in which the orthologous interaction is conserved in higher eukaryotes and this interaction could have an important role in tension sensing throughout the eukaryotic kingdom .

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CHAPTER 1. LITERATURE REVIEW

1.1 <u>Centromeric Nucleosome</u>

1.1.1 Organization of the Centromeric Nucleosome

A nucleosome is the fundamental unit of DNA packaging in eukaryotes, consisting of 147 bp of DNA wrapped in 1.7 turns around an evolutionarily conserved protein core, called the histone octamer (Luger et al., 1997). The histone octamer is composed of eight histone proteins (two copies each of histone H2A, H2B, H3 and H4). The eight core histones are organized into a $(H3)_2(H4)_2$ tetramer and a pair of H2A-H2B dimers (Arents et al., 1991). In addition, each core histone has a histone fold domain and amino-terminal tail protruding from the core nucleosome. These flexible amino tails are subjected to post-translational modifications, such as phosphorylation, acetylation, methylation and ubiquitination (Morales and Richard-Foy, 2000; Nowak and Corces, 2004; Gatti et al., 2012; Villar-Garea et al., 2012). However, not all nucleosomes are comprised of the conventional histone octamer. In some regions of chromosome, specific histones are substituted by variant histones. For example, histone variant H2AZ is an evolutionary conserved histone variants from yeast to human, comprising around 5-10% of total H2A in the cell (West and Bonner, 1980; Talbert and Henikoff, 2010). In budding

yeast, histone H2AZ is enriched at non-coding region and important for transcriptional activation and genome integrity (Zhang et al., 2005; Morillo-Huesca et al., 2010).

The centromere is a specialized chromosomal locus that mediates chromosomal segregation. The centromere acts as the site for kinetochore formation. The proteinaceous kinetochore complex ensures fidelity of chromosome segregation by linking chromatin fibers to microtubule spindles (Cleveland et al., 2003). Eukaryotic centromeres are typically categorized as either a point centromere or regional centromere. Some simple eukaryotes, such as the budding yeast, *Saccharomyces cerevisiae*, have point centromeres, specified by unique DNA sequence found on all chromosomes, while higher eukaryotic organisms and other yeast species (e.g., *Schizosaccharomyces pombe*, fruit fly and mammals) have larger regional centromeres, defined by hierarchical arrays of satellite DNA repeats. The point centromere of budding yeast is occupied by a single centromeric nucleosome and attaches to single spindle tubules, whereas regional centromeres of higher eukaryotes consist of arrays of centromeric nucleosomes that regulate attachment to multiple spindle tubules (Cleveland et al., 2003; Verdaasdonk and Bloom, 2011). Centromeric DNA sequences are not conserved across eukaryotic organisms. Centromeres vary in size from 125 bp found in budding yeast to several megabases in human (Cleveland et al., 2003). In budding yeast, each of the 16 centromeres contains a conserved, single copy 125 bp CEN sequence that is crucial for chromosome segregation. The CEN consists of 3 conserved DNA elements, termed CDEI, CDEII and CDEIII. CDEI, a partially conserved 8 bp sequence, is important for proper chromosome segregation (Niedenthal et al., 1991); CDEII is 78-86 bp A/T rich spacer, which is required for chromosome segregation (Espelin et al., 2003); and CDEIII is a highly

conserved 25 bp fragment, which is required for kinetochore assembly (Kaplan et al., 1997; Espelin et al., 2003; Bellizzi et al., 2007).

1.1.2 Centromere-Specific Histone Variants

Despite variation in organization and length of centromeres, all eukaryotic centromeres share one reserved property: they are universally marked by centromere-specific histone variant (CenH3), which localizes exclusively to the centromere (Palmer et al., 1991; Yoda et al., 2000). These histone variants have been identified in human (CENP-A), *Drosophila melanogaster* (CID), *Caenorhabditis elegans* (HCP-3), *S. pombe* (Cnp1) and *S. cerevisiae* (Cse4). At point centromeres of budding yeast, a single CenH3 nucleosome is nucleated base kinetochore assembly and microtubule attachment. Larger regional centromeres, such as those of fission yeast, Drosophila and mammals, contain blocks of CenH3 nucleosomes interspersed between canonical histone H3 nucleosomes (Verdaasdonk and Bloom, 2011).

CenH3 replaces the conventional histone H3 at centromere and together with centromere-specific-DNA binding factors provides the basis for kinetochore assembly. In vertebrates, once CENP-A is incorporated into nucleosomes, it directly recruits the nucleosome-associated complex (NAC), comprising CENP-C, CENP-H, CENP-M, CENP-N, CENP-T and CENP-U, together with CENP-I, which contribute to the inner kinetochore region of the centromere (Foltz et al., 2006). The CENP-A-NAC complex then serves to load other kinetochore components that located distal to CENP-A (CENP-K, CENP-L, CENP-O, CENP-Q, CENP-R and CENP-S) (McClelland et al., 2007). RNAi experiments have shown the crucial role of CENP-A in kinetochore assembly. Chicken

DT40 CENP-A depleted cells showed mislocalization defects of inner kinetochore CENP-I, CENP-H and CENP-C, as well as outer kinetochore proteins Nuf2/HEC1, CENP-E and Mad2 (Regnier et al., 2005). Localization of CENP-I and CENP-C at centromere is abolished in CENP-A-RNAi in HeLa cells (Goshima et al., 2003). Then, CenH3 nucleosome constitutes a nucleation site for kinetochore assembly.

In addition to roles in kinetochore formation, CenH3 also plays an important role in mitosis. Disruption of CenH3 function in eukaryotes results in severe chromosome missegregation rates. Mutation of budding yeast CenH3, Cse4, abolished kinetochore assembly at centromere, which caused a defect in separation of sister chromatids (Samel et al., 2012). Inhibiting Drosophila CenH3, CID, with antibody against CID demonstrated various defects in anaphase chromosome segregation, including failure to move toward the poles at anaphase onset, unequal chromosome segregation and failure to maintain spindle contact (Blower and Karpen, 2001). Moreover, disruption of the mouse *CENPA* gene demonstrated severe chromosomal missegregation phenotypes, such as micronuclei and macronuclei formation, nuclear bridging and blebbing, and chromatin fragmentation and hypercondensation (Howman et al., 2000). However, the molecular details of CenH3 participation throughout the chromosome segregation process needs further investigation to determine its role in the process.

1.1.3   Centromere-Specific Histone Variant in Budding Yeast

Budding yeast is an ideal system to investigate the molecular genetics of centromere structure and function because of its simple and small genetic size, which is easily manipulated genetically and biochemically (Clarke, 1990). The structure of the

budding yeast centromere is less complex than those in higher eukaryotes. In *S. cerevisiae*, the centromere is occupied by a single centromeric nucleosome that attaches to a single spindle microtubule (Cleveland et al., 2003). The generally accepted model is that a histone variant, Cse4, replaces histone H3 and assembles into nucleosome at the budding yeast centromere; however, the overall centromeric nucleosome structure is still in debate. Several models have been proposed for budding yeast centromeric nucleosome, including hemisomes (Dalal et al., 2007; Dalal et al., 2007), hexasomes (Mizuguchi et al., 2007) and octasomes (Camahort et al., 2009). The most conventional model is the octameric nucleosome, containing two copies each of histone H2A, histone H2B, histone H4 and Cse4, and DNA wrapping in a left-handed supercoil (Figure 1-1) (Camahort et al., 2009; Kingston et al., 2011).
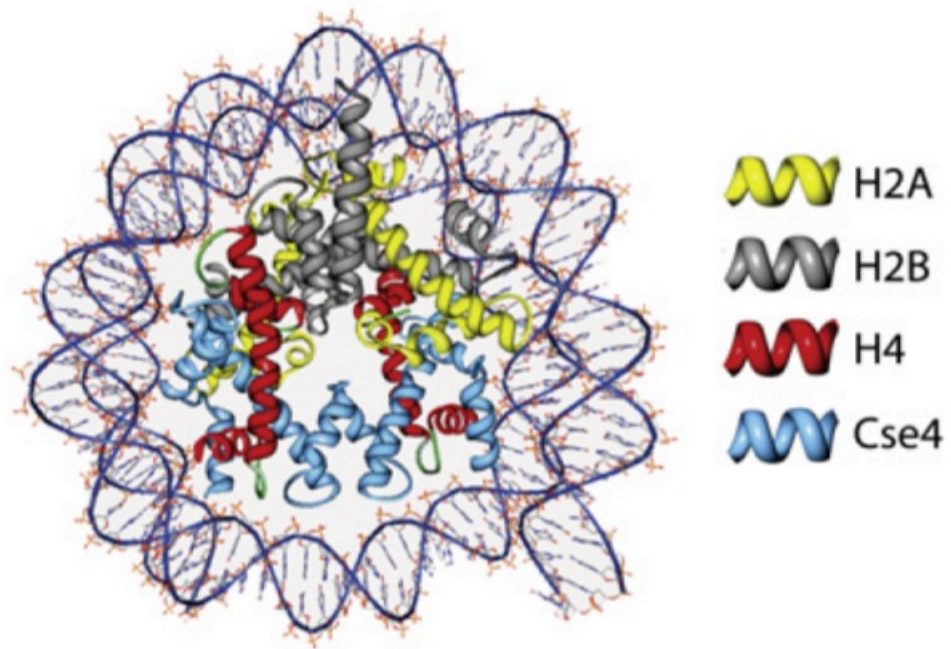
Figure 1- 1 Budding yeast centromeric nucleosome. Cse4 substitutes for histone H3 at the centromere and assembles into budding yeast centromeric nucleosome. The octamer model proposes that centromeric nucleosome consists of two dimers of histone H2A/H2B and one tetramer of histone H4/Cse4, and DNA wrapping in a left-handed supercoil. This figure was modified from (Camahort et al., 2009).

Cse4 has 135-amino-acid-long N-terminal amino acid tail extending from a conserved histone fold domain. The homology between Cse4 and canonical histone H3 is situated at the C-terminal histone fold domain of the protein (more than 60% identity) (Luger et al., 1997). On the other hand, the N-terminal tails of histone H3, Cse4 and its homologs are highly diverged among species. Eukaryotic CenH3 have no sequence similarity to H3 in their N-terminal amino acid tails. These tails can vary from 20 to ~200 amino acids (Malik and Henikoff, 2003). N-terminus of Cse4 extends from the nucleosome core, making it accessible for interaction with other kinetochore proteins involved in mitotic function. Synthetic lethality and yeast two-hybrid assays demonstrated the interaction between Cse4 tail and central kinetochore complex, COMA (Chen et al., 2000). Mutation of Cse4 tail also resulted in reduction of kinetochore component levels at centromere (Samel et al., 2012). Cse4 N-terminus tail may also be a target for posttranslational modification, as observed in N-terminus tail of standard core histone (Bannister and Kouzarides, 2011). However, there is no direct evidence thus far for Cse4 being interacted with other kinetochore proteins.

The N-terminal domain of yeast histone H3 can be deleted without loss of cell viability (Mann and Grunstein, 1992); however, the N-terminal tail of Cse4 is essential (Keith et al., 1999; Chen et al., 2000) and deletion of first 50 amino acids of Cse4 N-terminal tail is lethal to cell (Keith et al., 1999). Chen et al. delineated the essential N-terminal domain, which is essential for Cse4 function, by using systematic deletion analysis strategy. A specific region of the Cse4 was identified called END which was a 33 amino-acid domain between residues 28 and 60 in Cse4 N-terminus. Strains carrying either END mutations or END deletions exhibited an increase in chromosome loss rate

(Chen et al., 2000). In addition, END is involved in interaction between Cse4 and other kinetochore proteins. Using synthetic dosage lethality, it was revealed that a genetic interaction between *cse4* END mutations and *MCM21*, which encodes components of the COMA complex. Moreover, Cse4 N-terminal tails with deletion of END abolished the interaction with kinetochore component Ctf19, as determined by yeast two-hybrid assay. The yeast cells co-expressing AD-Cse4 wild type and BD-Ctf19 were able to grow on selection media. However, cell growth of yeast cells carrying AD-Cse4 with END deletion and BD-Ctf19 was barely to detect. (Chen et al., 2000).

1.1.4   Organization of Kinetochore

The kinetochore is a proteinaceous complex containing at least 80 different proteins assembled at the centromere of each sister chromatid. The kinetochore physically links centromeric chromatin to the plus end of microtubule. Proper assembly of the kinetochore on the centromere and attachment of kinetochore components to microtubules are crucial for accurate and efficient transmission of genetic information. Structural and regulatory components of the kinetochore in eukaryotes are conserved throughout evolution with some specific differences. For example, budding yeast has a point centromere, which contains only a single Cse4 nucleosome but the regional centromere of human contains multiple copies of CENP-A (Cleveland et al., 2003). Notably, each budding yeast kinetochore is attached to only single spindle microtubule, while the kinetochore in human provides a platform for connecting multiple microtubules (Sullivan et al., 2001). The kinetochore is generally built of 3 layers of protein complexes, most of which are conserved among eukaryotic organisms (Joglekar et al., 2009). First,

the inner kinetocore plate provides a structurally distinct interface between centromeric nucleosomes and kinotochores. The inner kinetochore plate contains conserved DNA-binding protein CENP-C (Mif2 in budding yeast), which associates with CenH3 containing nucleosome (Santaguida and Musacchio, 2009). Second, the central kinetochore plate connects between inner and outer kinetochore. Ctf19 complex (COMA subcomplex (Ctf19, Okp1, Mcm21, Ame1), Ctf3, Mcm16, Mcm19, Mcm22, Chl4, Iml3, Nkp1, Nkp2 and Cnn1), which is functionally equivalent to human constitutive centromere-associated network (CCAN), is important for loading of KMN network: KNL-1 complex (Spc105 complex in budding yeast), Mis12 complex (Mtw1 complex in budding yeast) and Ndc80 complex (Hori and Fukagawa, 2012). KMN network generate a bridge connecting centromere-associated protein and plus ends of microtubules (Lampert and Westermann, 2011; DeLuca and Musacchio, 2012). In addition, Mtw1 complex and Spc105 complex create a linker between Ndc80 and CENP-C (Santaguida and Musacchio, 2009; DeLuca and Musacchio, 2012). Third, the outer kinetochore plate consists of Ndc80 complex (Ndc80, Nuf2, Spc24 and Spc25) that interact with the plus ends of microtubules. Ndc80 complex is crucial for load-bearing attachments to spindle microtubules (DeLuca and Musacchio, 2012). However, there are 2 major components that made budding yeast kinetochores differ from general organization of kinetochore. First, centromere DNA binding protein, Cbf3, is important for deposition of Cse4 and initiation of kinetochore formation (Shivaraju et al., 2011). Second, the Dam1 complex, which cooperates with Ndc80 complex, interacts directly with the microtubule (Lampert et al., 2010; Tanaka, 2010).

1.2   Mitotic Spindle Checkpoint and Tension-Sensing Mechanism

1.2.1   The Chromosome Passenger Complex

Equal chromosome segregation during mitosis is required for stability of chromosome transmission. For accurate chromosome segregation, duplicated chromosome must be attached to mitotic spindle in a bi-polar fashion (bi-orientation) and separation of chromosome does not begin until all chromosomes have bi-oriented. This means sister kinetochores have to be captured by mitotic spindles emanating from opposite spindle poles (amphitelic or bipolar attachment) before an onset of segregation (Tanaka, 2010). Nevertheless, error of attachment, including syntelic attachment (both sister kinetochore are attached to mitotic spindles derived from single spindle pole) and merotelic attachment (single kinetochore is captured by microtubules from both spindle poles), frequently happen during mitosis, which lead to chromosome missegregation in anaphase (Figure 1-2) (Watanabe, 2012). Defects in chromosome segregation during mitosis such as these results in aneuploidy, which is a major cause of tumorigenesis (Gordon et al., 2012).
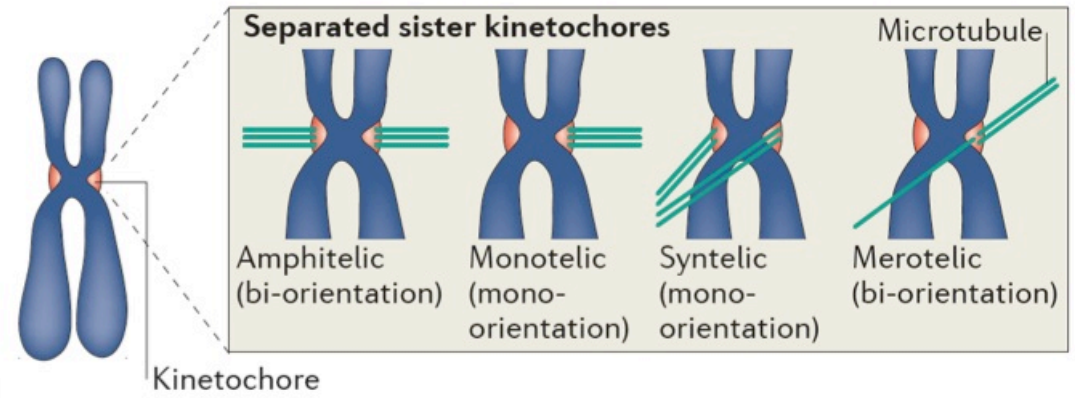
Figure 1- 2 Types of kinetochore-microtubule attachment. This figure was modified from (Watanabe, 2012).

The chromosome passenger complex (CPC) is an important regulator of chromosome segregation during mitosis by correcting non-bipolar attachment. To serve in this role, CPC promotes re-orientation of improperly attached kinetochore, which subsequently is sensed by spindle assembly checkpoint, until proper amphitelic attachments are made (Carmena et al., 2012). CPC is composed of Aurora B kinase (Ipl1 in yeast), with non-enzymatic components INCENP (Sli15 in yeast), Survivin (Bir1 in yeast) and Borealin. The functions of this complex are conserved among eukaryotes. Mutants of Ipl1 lead to impairment of spindle assembly checkpoint activation, and chromosome missegregation in budding yeast (Biggins et al., 1999; Kim et al., 1999; Biggins and Murray, 2001). Disruptions of Aurora B kinase expression with RNA-mediated interference in *C. elegans* and *Drosophila* results in incomplete anaphase chromosome separation and abortive cytokinesis (Schumacher et al., 1998; Speliotes et al., 2000; Adams et al., 2001; Giet and Glover, 2001). Moreover, Aurora B kinase is important for correcting improper kinetochore-microtubule attachment in human (Hauf et al., 2003; Lampson et al., 2004; Cimini, 2007).

Aurora B kinase is a serine/threonine kinase, which is crucial for re-orientation of improper kinetochore-microtubule attachment. However, it is still unclear how the molecular mechanism of this process occurs. Since bi-orientation of the chromosome ensures equal separation of genetic material, it has been hypothesized that the cell would detect the accuracy of kinetochore-microtubule orientation by controlling the elevated tension state of centromeric chromatin and kinetochores (McIntosh, 1991). Since the Aurora B-INCENP complex localizes at the centromere from G1 until onset of anaphase,

then it is reasonable to assume that this complex facilitates correction of improper kinetochore-microtubule attachment by phosphorylating kinetochore components (He et al., 2001; Buvelot et al., 2003). Moreover, it was found that Ipl1 kinase phosphorylates components of outer kinetochore plate and this phosphorylation is important to ensure bi-orientation attachment (Cheeseman et al., 2002; Cheeseman et al., 2006). The phosphorylation possibly diminishes the association of outer kinetochore complexes, then promotes the re-orientation of kinetochore to pole connection (Cheeseman et al., 2006). Aurora B kinase has been revealed that it promotes error correction in a tension-dependent manner. As syntelic attachments (mono-orientation) are tensionless, Aurora B kinase facilitates re-orientation by phosphorylating kinetochore components, such as the Dam1 complex and the Ndc80 complex. When bi-orientation attachment is established, sister chromatids are under tension. The distance between centromere and kinetochore is increased. Then, the ability of Aurora B kinase to reach substrate at kinetochore is eliminated, and as a result Aurora B kinase ceases its re-orientation (Tanaka et al., 2002; Liu et al., 2009).

1.2.2   The Spindle Assembly Checkpoint

Spindle assembly checkpoint (SAC) is a quality control mechanism that prevents onset of anaphase until bi-orientation is achieved (Musacchio and Salmon, 2007; Foley and Kapoor, 2013). SAC is conserved across eukaryotic organisms. SAC contains MPS1, BUB1, MAD1, MAD2, BUB3, BUBR1 (Mad3 in yeast) (Hoyt et al., 1991; Li and Murray, 1991; Weiss and Winey, 1996). To prevent the precocious separation of sister

chromatids, SAC is activated in the presence of syntelic attachment or merotelic attachments (Musacchio and Salmon, 2007). The target of SAC is an activator of anaphase promoting complex/cyclosome (APC/C), CDC20 (Hwang et al., 1998; Kim et al., 1998). APC/C triggers anaphase progression by ubiquitylation and subsequent proteasome-dependent degradation of cyclin B and securin (Peters, 2006). Cyclin B is a mitotic kinase that mediates mitotic progression. Proteolysis of cyclin B triggers exit from mitosis by inactivation of CDK1 (Glotzer et al., 1991; Sudakin et al., 1995). Securin (Pds1 in budding yeast) is an inhibitor of separase. Separase is a proteolytic enzyme that cleaves the cohesin complex, which hold sister chromatids together (Yamamoto et al., 1996; Shindo et al., 2012). SAC inhibits APC/C activity by forming a CDC20 inhibitory complex, called MCC (Mitotic Checkpoint Complex), thus protecting degradation of cyclin B and securin. MCC is composed of CDC20, MAD2, BUBR1 and BUB3 (Sudakin et al., 2001). SAC delays an onset of anaphase by inhibiting CDC20 until all sister chromosomes have amphitelic attachment to mitotic spindles. SAC is inactivated when chromosome bi-orientation is finally achieved (Musacchio and Salmon, 2007). This phenomenon releases Cdc20 from MCC, which can now stimulate APC/C (Mansfeld et al., 2011; Varetti et al., 2011). Activation of APC/C results in polyubiquination of cyclin B and securin, which are subsequently degraded by 26S proteasome (Clute and Pines, 1999; Hagting et al., 2002). The destruction of securin releases active separase which then cleaves the cohesin complex that holds sister chromatids together, leading to loss of cohesion and chromosome segregation (Waizenegger et al., 2002). Concomitantly, the degradation of cyclin B triggers the mitotic-exit program by suppressing CDK1 (Yu, 2007).

1.2.3 Cohesin Complex

Sister chromatids must be held together from the time of replication until segregation in anaphase during both mitosis and meiosis. The cohesion between sister chromatids is carried out by multi-subunit complex, called cohesin complex. Cohesin is a four-subunit complex which comprises two SMC family proteins, Smc1 and Smc2, together with two accessory subunits, Scc1 (Mcd1 in budding yeast and Rad21 in fission yeast) and Scc3. Meiotic cohesin complex contains the same protein subunit, with the exception that the Scc1 subunit is replaced by its meiosis-specific homologue, Rec8. Cohesin complex acts as a glue that connects sister chromatids together by forming a ring and trapping two sister DNA molecules inside complex. Cohesin complex counteracts the splitting force exerted by microtubules to generate tension at kinetochores. Once proper bipolar attachment is achieved, cohesin complex is degraded due to cleavage of Scc1 subunit by separase, thereby triggering chromosome segregation to opposite pole during anaphase (Haering and Nasmyth, 2003).

In vertebrate mitosis, a majority of cohesin complex is removed from chromosome arms before metaphase in a non-proteolytic pathway (Figure 1-2). This removal of cohesin complex is separase-independent which requires phosphorylation by Polo-like kinase (Plk1) and Aurora B kinase. This process is known as the prophase pathway. The residual cohesin complex that persists around the centromere is sufficient to connect sister chromatids together until it is cleaved by separase at metaphase-anaphase transition, enabling chromosome segregation (Waizenegger et al., 2000; Schockel et al., 2011). This is in contrast in to budding and fission yeast mitosis where all cohesin complex is

removed along the length of chromosome by separase at the onset of anaphase (Uhlmann et al., 2000; Haering and Nasmyth, 2003).

In meiosis, Scc1 is replaced by meiosis-specific cohesin subunits, Rec8. During the first round of meiosis (meiosis I), homolog chromosomes, not sister chromatids, separate away from each other. In order to segregate, cohesin complexes along the chromosome arms have to be cleaved by separase for resolving chiasmata, the linkage between homologous chromosomes. However, centromeric cohesion is preserved throughout anaphase I until meiosis II, when sister chromatids segregate as they do in mitosis. At the onset of anaphase II, centromeric Rec8 is cleaved by separase, triggering chromosome segregation into each gametes. Hence, centromeric cohesin complex and cohesin complex along chromosome are released in stepwise manner in meiosis (Watanabe and Kitajima, 2005; Watanabe, 2012).
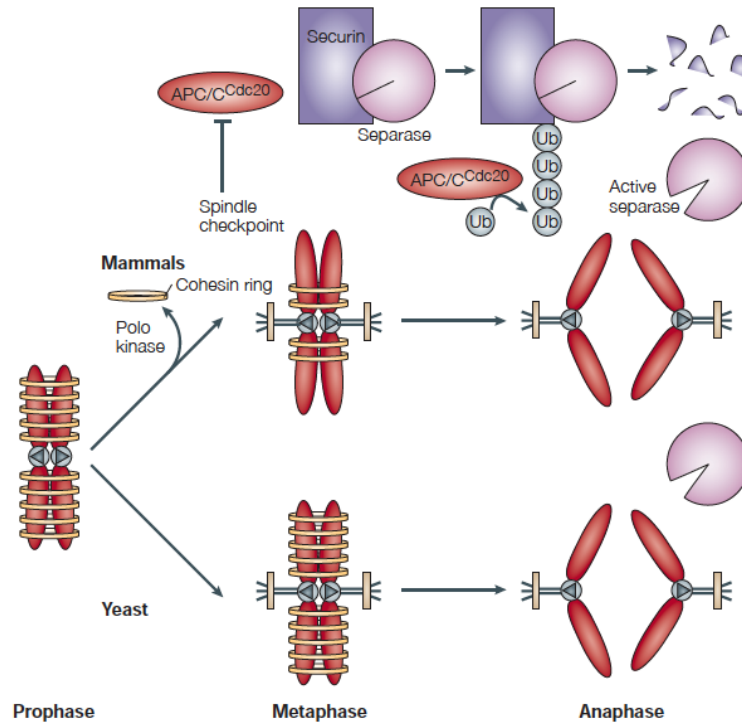
Figure 1- 3 Chromosome segreagation during eukaryotic mitosis. The spindle assembly checkpoint inhibits activation of APC/C until chromosome bi-orientation is established. Activation of APC/C triggers anaphase progression by targeting securin for proteasome destruction. The degradation of securin releases active separase which then cleaves the cohesin complex that holds sister chromatids together, leading to loss of cohesion and chromosome segregation. In mammals, most of cohesin is cleaved from chromosome arms during prophase pathway in Aurora-B- and Polo-kinase dependent manner. A pool of cohesin complex that persists around the centromere is sufficient to hold sister chromatids together until it is cleaved by separase at the onset of anaphase, enabling chromosome segregation. In yeast, all cohesin complexes are removed along the length of chromosome by separase at the metaphase-anaphase transition. This figure is originally from (Marston and Amon, 2004).

1.2.4   Shugoshin

Shugoshin, which means "guardian spirit" in Japanese, is a protector of cohesin complex. Shugoshin was initially discovered in *Drosophila melanogaster* mutant MEI-S332, which showed random segregation of sister chromatids in meiosis. Moreover, MEI-S322 specifically localizes around centromere region of meiotic chromosome and disappears from this region during anaphase II, concomitantly with loss of centromeric cohesion (Kerrebrock et al., 1992; Kerrebrock et al., 1995). These data revealed an important role of MEI-S322 in protection of centromeric cohesion. Shugoshin proteins are conserved across eukaryote from yeasts to mammals (Katis et al., 2004; McGuinness et al., 2005). In budding yeast and fruit fly, there is only a single Shugoshin, called Sgo1, and MEI-S332 respectively. In contrast, there are 2 paralogs, Sgo1 and Sgo2, in fission yeast, plants and mammals. In addition to maintenance of cohesin complex during cell division, Shugoshin also acts as a tension sensor for correcting improper chromosome attachment. Both of functions are found in budding yeast Sgo1 (Katis et al., 2004; Indjeian et al., 2005; Kiburz et al., 2008; Liu et al., 2013). In fission yeast, Sgo1 contributes to maintenance of cohesion during meiosis, while Sgo2 is required for checkpoint sensing loss of tension (Kitajima et al., 2004; Kawashima et al., 2007; Ishiguro et al., 2010). Mammalian Sgo1, as well as Sgo2, also functions in cohesin complex protection in mitosis; however, role of mammalian Sgo2 is still largely elusive (McGuinness et al., 2005; Kitajima et al., 2006; Gomez et al., 2007; Tanno et al., 2010).

In meiosis, the Rec8 subunit is removed by separase only along chromosome arms during meiosis I. However, centromeric Rec8 must be protected from removal since

cohesin around this area is important for bi-orientation on the meiosis II spindle (Watanabe, 2012). This protection is regulated by Shugoshin. In both budding and fission yeast, Sgo1 forms a complex with a specific form of serine/threonine protein phosphatase 2A (PP2A) (Kitajima et al., 2006; Riedel et al., 2006). PP2A is recruited to the centromere and required for maintenance of centromeric Rec8 during meiosis I. Protection of centromeric cohesin requires the catalytic subunit of PP2A. Inactivation of PP2A catalytic subunit results in loss of centromeric cohesin at anaphase I, and random chromosome segregation at meiosis II (Riedel et al., 2006). Purified shugoshin complex from HeLa cells, containing human Sgo1 and PP2A, has an ability to remove phosphates from cohesin *in vitro* (Kitajima et al., 2006). Moreover, phosphorylation of Rec8 in yeast is required for removal of Rec8 by separase (Ishiguro et al., 2010; Katis et al., 2010). Together, theses findings suggested that collaboration of Sgo1 and PP2A protects cohesin removal by counteracting Rec8 phosphorylation.

In vertebrate mitosis, the cohesin complex is lost in 2 steps. Most of vertebrate Scc1 subunit dissociates from chromosome arms during prophase and prometaphase (prophase pathway). In the prophase pathway, removal of cohesin complex is mediated by phosphorylation of Plk1 and Aurora B kinase. At the onset of anaphase, the residual centromeric Scc1 subunit is cleaved by separase, triggering chromosome segregation. The protection of centromeric Scc1 subunit during mitotic prophase is contributed by centromeric shugoshin. Depletion of human shugoshin by RNAi results in mitotic arrest and loss of sister kinetochore cohesion (Salic et al., 2004; McGuinness et al., 2005). Interestingly, expression of Scc3-SA2, a nonphosphorytable Scc3 mutant, alleviated precocious loss of sister chromatid cohesion and mitotic arrest in Sgo1 depleted HeLa

cells (McGuinness et al., 2005). LC-MS/MS and immunoprecipitation experiments showed that human Sgo1 associates with PP2A in mitotic cells (Kitajima et al., 2006). These lines of evidence suggest that shugoshin forms a complex with PP2A to oppose phosphorylation of cohesin mediated by Plk1, then protects dissociation of centromeric cohesin.

In fission yeast, functions of shugoshin were divided into Sgo1 and Sgo2. Sgo1 is meiosis specific and required for protecting centromeric cohesin (Ishiguro et al., 2010). Sgo2 is dispensable for centromeric cohesin protection in mitosis but instead exclusively responsible for tension sensing. Sgo2 promotes correction of tensionless attachment by loading Aurora kinase complex to kinetochore (Kawashima et al., 2007). On the other hand, Sgo1 plays a major role in tension sensing in budding yeast mitosis, instead of cohesin protection. The *sgo1* mutants show a major defect in arresting cells that have tensionless kinetochores, not a defect in mitotic cohesion. Also, *sgo1* mutants are unable to detect lack of tension at kinetochores and failed to activate SAC and delay APC/C activation. However, *sgo1* mutants still detect unattached kinetochore (Indjeian et al., 2005). Hence, role of Sgo1 in budding yeast is specific for sensing the tensionless kinetochore. This property makes Sgo1 a good candidate as a mitotic tension sensor in budding yeast.

1.3 <u>References</u>

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CHAPTER 2. INTERACTION BETWEEN CENTROMERIC HISTONE VARIANT H3 AND SHUGOSHIN

2.1 <u>Introduction</u>

Centromeres are specialized chromosomal loci that regulate chromosome segregation. Centromeres are the chromosomal region where proteinaceous kinetochore assemblies are built on (Cleveland et al., 2003). The main function of the kinetochore is to regulate the attachment of centromeric chromatid to spindle microtubule. Eukaryotic centromeres are classified into 2 groups. First, simple eukaryotes, such as budding yeasts, have point centromeres, which are only a few hundred base pairs in size. Budding yeast point centromeres are packaged into a single nucleosome, which attaches to a single spindle microtubule. Second, higher eukaryotes (e.g., fission yeast, fruit fly, mammals) have larger regional centromeres, which range from several kilobases to hundred kilobases. Regional centromeres consist of arrays of centromeric nucleosomes that regulate attachment to multiple spindle tubules (Cleveland et al., 2003; Verdaasdonk and Bloom, 2011). Despite variation in organization and length of centromeres, all eukaryotic centromeres are generally marked by centromeric-specific histone variant (CenH3). These histone variants have been discovered in human (CENP-A), *Drosophila melanogaster* (CID), *Caenorhabditis elegans* (HCP-3), *Schizosaccharomyces pombe* (Cnp1) and *Saccharomyces cerevisiae* (Cse4).

In budding yeast, it is universally accepted that Cse4 substitutes for histone H3 when assembled into the centomeric nucleosome (Camahort et al., 2009; Kingston et al., 2011). Cse4 contains a conserved C-terminal histone fold domain and unique 135-amino-acid N-terminal tail domain. The C-terminal histone fold domain is more than 60% identical to canonical histone H3 (Luger et al., 1997). The histone fold domain of Cse4, as well as histone fold domain of human CENP-A, is important for centromere localization (Sullivan et al., 1994; Morey et al., 2004). In contrast to the histone fold domain, eukaryotic CenH3s have highly divergent N-terminal tail. These tails can vary from 20 to ~200 amino acids in length (Malik and Henikoff, 2003). The architecture of the budding yeast centromeric nucleosome is such that the conserved histone fold domain of Cse4 is embedded into the octameric nucleosome core with the N-terminal amino acid tail projecting from the core (Camahort et al., 2009). The protrusion of the N-terminus Cse4 from the nucleosome core make it possibly accessible for protein interactions and enzymatic modifications from other proteins, including kinetochore or cell cycle components involved in mitotic function. Mutations of the *cse4* N-terminus showed synthetic lethal interactions in combinations with defects in the COMA subcomplex (Chen et al., 2000; Samel et al., 2012). COMA subcomplex, a component of budding yeast central kinetochore, functions as a bridge between inner kinetochore subunits contacted with centromeric DNA and outer kinetochore subunits bound to microtubule (Lampert and Westermann, 2011). Moreover, Cse4 tail mutants abolished localization of Mtw1 and COMA complex to the centromeric region (Samel et al., 2012).

Cse4 is different from histone H3 in the length of N-terminal tail. Cse4 contains a protruding 135 amino acid N-terminal domain, while N-terminal tail of canonical histone

H3 is only 36 amino acids in length. Histone H3 N-terminus is dispensable for cell viability (Mann and Grunstein, 1992); however, the N-terminal domain of Cse4 is essential (Keith et al., 1999; Chen et al., 2000). Deletion of first 50 residues from Cse4 N-terminal tail is lethal to the cell (Keith et al., 1999). Systematic deletion studies revealed a region that is vital for mitotic function of the Cse4 N-terminal tail. This specific region of Cse4, called as essential N-domain (END), contains 33 amino acid-domain between residue 28 and 60 in Cse4 N-terminus. Deletion of END is lethal, whereas mutation of END or partial deletion of END leads to chromosome missegregation. Additionally, END is involved in genetic interactions between Cse4 and other kinetochore proteins. Mutations of *cse4* N-terminus at END demonstrated specific synthetic lethal interactions in combinations with defects in genes responsible for encoding central kinetochore complex, *MCM21*, *CTF19* and *OKP1*. Furthermore, deletion of END alleviated interaction between Cse4 and kinetochore component Ctf19, determined by yeast two-hybrid assay (Chen et al., 2000). Nevertheless, the interaction between Cse4 tail and kinetochore protein detected by yeast two-hybrid could be an indirect protein-protein interaction (e.g. endogenous yeast protein serves as a bridge between bait and pray protein). To date, there is no evidence establishing direct physical interactions between N-terminal tail of Cse4 and kinetochore components.

The fidelity of chromosome segregation is crucial for maintaining chromosome stability. For accurate chromosome segregation during mitosis, it is important that sister kinetochores must capture spindle microtubule from opposite poles (bi-orientation), and that segregation does not begin until all chromosomes have bi-oriented (Tanaka, 2010). The opposing pulling force exerted by microtubules generates tension between sister

kinetochores (McIntosh, 1991). Any errors in chromosome segregation lead to aneuploidy, which ultimately cause disease and death (Gordon et al., 2012). However, attachment errors, including synthelic attachment and merotelic attachment, frequently happen during mitosis, which lead to chromosome missegregation in anaphase (Watanabe, 2012). Spindle assembly checkpoint (SAC) is a cell cycle control mechanism that detects unattached kinetochore and tensionless spindle-kinetochore attachment. To correct an erroneous of kinetochore-microtubule attachment, the metaphase-anaphase transition must be delayed by SAC to give time for sister kinetochores to attach properly to spindle microtubules (Musacchio and Salmon, 2007; Foley and Kapoor, 2013). Once all sister chromatids have established biorientation, the activation of APC/C liberates separase from inhibition of securin, resulting in degradation of cohesin complex and separation of sister chromatids (Musacchio and Salmon, 2007).

Sgo1 is the single budding yeast member of Shugoshin family. Members of this family play a crucial role in protection of the centromeric cohesin complex, particularly in meiosis. In vertebrates, Sgo1 is required for protection centromeric cohesin complex from prophase dissociation during mitosis. However, budding yeast Sgo1 is dispensable for maintenance of cohesion of sister chromatids in mitosis, but instead appears to be exclusively crucial for tension sensing at kinetochores (Indjeian et al., 2005; Kiburz et al., 2005). Using genome-wide location analysis and chromatin immunoprecipitation assay, Sgo1 was found enriched at pericentric and centromeric regions in budding yeast mitosis (Kiburz et al., 2005; Luo et al., 2010), which are both regions in which tension is generated and controlled (Maresca and Salmon, 2010; Watanabe, 2012). The localization of Sgo1 to these regions depends on a member of SAC, the Bub1 kinase. Bub1 without a

kinase domain (*bub1ΔK* strain), resulted in chromosome missegregation and mislocalization of Sgo1. The *bub1ΔK* strain was used because this strain maintained a stable truncated Bub1 protein, which may not be the case compared to catalytically inactivating point mutation that was used in other studies. In addition to segregation defects, depletion of Sgo1, as well as depletion of the Bub1 without a kinase domain, results in a defect in the delay of anaphase onset in cells with lack of tension at kinetochore. (Fernius and Hardwick, 2007). Sgo1 appears to detect only tensionless kinetochores and does not appear to have a role in unattached kinetochores. Sgo1 mutants failed to sense lack of tension of kinetochore, as indicated by their inability to stabilize Pds1 under genetically induced tensionless conditions. However, a delay of anaphase onset in response to unattached kinetochores was still detected (Indjeian et al., 2005). Fission yeast Sgo2, which is not required for protection of both mitotic and meiotic centromeric cohesin, has an important role in checkpoint-mediated sensing of the tensionless situation in mitosis. Deletion of Sgo2, as well as deletion of checkpoint component Mad2, abolished the mitotic delay in the absence of tension at the kinetochore (Kawashima et al., 2007). These lines of evidence establish the importance of Sgo1 in tension sensing in mitosis.

Recently, histone H3 was also revealed to have crucial role in sensing the lack of tension at kinetochore. A yeast strain carrying a mutation at the junction between the N-terminal tail and histone fold domain of histone H3 (G44S) was not able to activate the spindle checkpoint in response to tensionless kinetochores, resulting in chromosome instability and chromosome missegregation. Moreover, the histone H3 mutant specifically abolished recruitment of Sgo1 at pericentric region during mitosis.

Interestingly, this study found that Sgo1 is a specific suppressor of the histone H3 mutant. Restoring Sgo1 to pericentromere alleviated mitotic defects, including benomyl hypersensitivity, loss of viability and inability to activate checkpoint in response to tensionless kinetochore. Together, these results suggest that association between Sgo1 and the histone H3 N-terminal tail at the pericentromere is crucial for mitotic tension sensing (Luo et al., 2010). However, the mitotic role of centromeric Sgo1 is still elusive. **According to function and localization of both Sgo1 and Cse4, we hypothesize that Sgo1 localizes to the centromere and associates with Cse4 at its N-terminal tail. To support our hypothesis, we sought to establish Sgo1-Cse4 interaction and investigate the Sgo1 binding site in the Cse4 N-terminus. We expected that the END sequence was probably an important domain for the Sgo1-Cse4 interaction.** Establishment of this interaction and investigating its role in mitotic regulation may provide insights the mechanisms involved in maintaining centeromeric cohesion and tension sensing.

2.2 <u>Materials and Methods</u>

Strains and Plasmids

All plasmids and primers used in this study are listed in Supplementary Materials Table 2-4, and Table 2-5, respectively. Bacterial plasmids for *Cse4* N-terminal tail, *Cse4* truncations, *H3* N-terminal tail, *Cnn1* N-terminal tail and *SGO1* were made by PCR amplification of yeast genomic DNA. The *Cse4_11, Cse4_12, Cse4_13* and *Cse4_14* truncation constructs were generated by annealing complementary pairs of oligonucleotides. All *Cse4* mutants were generated by PCR-driven overlap extension (Heckman and Pease, 2007), using plasmid containing N-terminus of *CSE4* as template. PCR amplified *Cse4, H3, Cnn1* were subcloned into BamHI and XhoI sites of pGEX-6P-1 vector, whereas PCR amplified *Sgo1* was subcloned into EcoRI and NdeI sites of pET-28b vector. All plasmids were then transformed into bacterial DH5α competent cells and growing on antibiotic selection marker. To determine the accuracy of cloning, all plasmids were sequenced by using Sanger sequencing. After verifying DNA sequence, all plasmid were transformed into bacterial BL21-DE3 strain to express the protein of interest. The pET-28b Sgo1 Y317X and pGEX-6P-1 Cse4 4SA/4SD were kindly provided by Dr. Min-Hao Kuo and Dr. Munira A. Barsai, respectively.

Purification of Recombinant Proteins from Bacteria

To express and purify recombinant proteins from bacteria, BL21-DE3 cells (optical density at 600 nm of 0.4 to 0.6) were induced with 1mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) at 37°C for 3 hours. Cells were harvested at 4°C and sonicated in ice-cold IPP150 buffer (10 mM Tris-Cl, pH 8.0, 150 mM Nacl and 0.1% NP-

40) six cycles (sonicate 20 seconds/cycle with 1 minute rest between cycle). Samples were kept on ice all the time during sonication. The soluble fraction was collected by centrifugation at 12,000 RPM for 30 minutes at 4°C. GST-tagged proteins were purified by incubating with 200 μL of glutathione agarose (Pierce) at 4°C for 2 hours. Glutathione agarose beads bound to GST fusion proteins were washed with ice-cold 500 μL of IPP150 3 times for 5 minutes each times. The bound proteins were eluted by gently rotating beads on rotator with 10 mM reduced glutathione in IPP150 for 2 hours at 4°C.

Pull Down Assay

To investigate protein-protein interactions, we performed a pull down assay between purified GST-fusion proteins and cell lysate containing Sgo1 (His6-Sgo1, His6-SUMO-Sgo1 or His6-SUMO-Sgo1 Y317X). GST-fusion proteins were incubated with 50 μL glutathione agarose for 2 hour at 4°C. After incubation with bait protein, beads were washed with ice-cold 500 μL IPP150 3 times for 5 minutes each times, followed by incubation with cell lysate containing His6-Sgo1 or His6-SUMO-Sgo1 Y317X for another hour at 4°C. The beads were washed with ice-cold 500 μL IPP150 3 times for 5 minutes each times, and bound-protein were eluted by boiling in 100 μL of SDS-PAGE loading buffer. Samples were resolved by 10% SDS-PAGE and analyzed by western blot analysis with 1:1,000 dilution of primary mouse monoclonal anti-His6 (GeneCopoeia), 1:1,000 dilution of primary mouse monoclonal anti-GST (GeneCopoeia) and 1:10,000 dilution of secondary anti-mouse-HRP conjugated antibody (GE Healthcare). Eluents were also examined by Commassie staining assay.

Kinetic Study

The binding kinetic studies were performed in kinetic buffer (1XPBS with 1%BSA and 0.02% tween20) using the BLItz instrument (ForteBio). Briefly, the anti-GST biosensors were hydrated in 0.2 mL kinetic buffer for 10 minutes, followed by equilibration for 5 minutes. Subsequently, 4 μL of 25 μg/mL GST-fusion proteins were loaded to the anti-GST biosensor (ForteBio) for 1-5 minutes, followed by an additional equilibration for 3 minutes. Biosensors were then incubated with various concentrations of cell lysates containing Sgo1 Y317X for 1-5 minutes, and dissociation was performed for 10 minutes. The shaking speed of each step was 2200 rpm. The experiments were performed at room temperature. Rate and affinity constants of binding interaction were obtained using BLItz Pro software.

Conserved Sequence Motif Identification

Protein sequences for CenH3 were obtained from public databases: Saccharomyces Genome Database (http://www.yeastgenome.org) and National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). Motif searching was performed using the online implementation of MEME at http://meme.nbcr.net. All searches were analyzed with these parameters; one occurrence per sequence, minimum motif width of 8, maximum motif width of 25 and number of different motifs was 10.

2.3   Results

Sgo1 specifically associates with the Cse4 N-terminal.

During budding yeast mitosis, Sgo1 is localized at pericentric and centromeric region, where tension is created and regulated (Maresca and Salmon, 2010; Watanabe, 2012). Interaction between Sgo1 and N-terminal tail of Histone H3 at pericentromere is required for sensing tension in a tensionless crisis (Luo et al., 2010). However, roles of Sgo1 during mitosis at the centromere are still elusive. We hypothesized that Sgo1 is associated with Cse4 N-terminus at centromere for mitotic tension signaling. To investigate the interaction between Cse4 tail and Sgo1, we expressed His6-tagged Sgo1 in *E. coli* and subjected the crude lysate from this strain to an *in vitro* pull down study using GST-tagged Cse4 N-terminal tail purified from bacterial cells. Crude lysate was used rather than purified protein because the Sgo1 protein was highly susceptible to proteolysis during purification. For the pull down assay, GST-Cse4 tail or GST alone was immobilized on glutathione agarose. Beads were then incubated with whole cell lysate containing His6-Sgo1. Western blot analysis revealed a strong interaction between Sgo1 and GST-Cse4 N-terminal tail (Figure 2-1). We also confirmed the presence of Sgo1 in the pull down sample by peptide mass mapping using MALDI TOF/TOF mass spectrometry. Briefly, the pull down samples were separated by SDS PAGE and stained with Coomassie Brilliant Blue. The expected Sgo1 band was excised and further identified protein fingerprint with mass spectrometry. The result from mass spectrometry confirmed that Sgo1 was co-purified with Cse4 N-terminal (Supplementary material Table 2-6). In addition to Sgo1, Sgo1 proteolytic products were also pulled down with Cse4 N-terminal tail (Figure 2-1). One of Sgo1 proteolytic products was also subjected to identify its protein fingerprint with mass spectrometry (Figure 2-1, Supplementary material Table 2-7).

Consistent with the tension sensing role at pericentromeric region (Luo et al., 2010), Sgo1 is bound to the Histone H3 N-terminal tail. Importantly, we also examined if Sgo1 has an interaction with other N-terminal tail of a kinetochore protein that has similar structural organization to histone H3 and Cse4. To answer this question, we used Cnn1, which has a histone fold domain and a putative unstructured N-terminal tail (Bock et al., 2012; Malvezzi et al., 2013). We used GST-Cnn1 N-terminal tail as a bait for our pull down study with His6-Sgo1. We found that Sgo1 did not associate to N-terminal tail of Cnn1 (Figure 1). Our findings strongly suggested that Sgo1 has a specific interaction with N-terminal tail of Cse4 and Histone H3 (Figure 2-1).
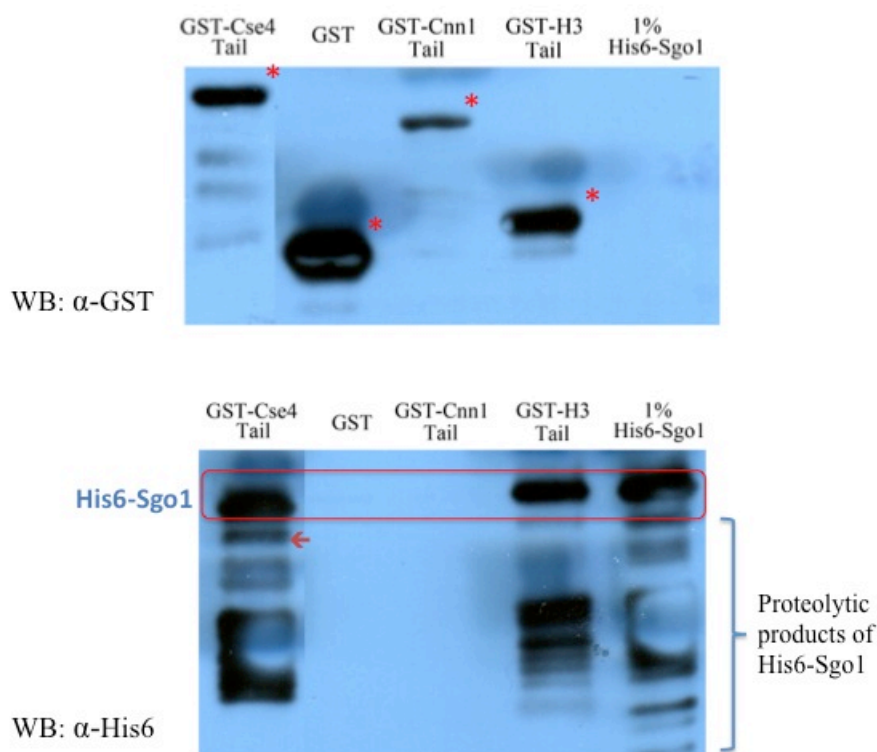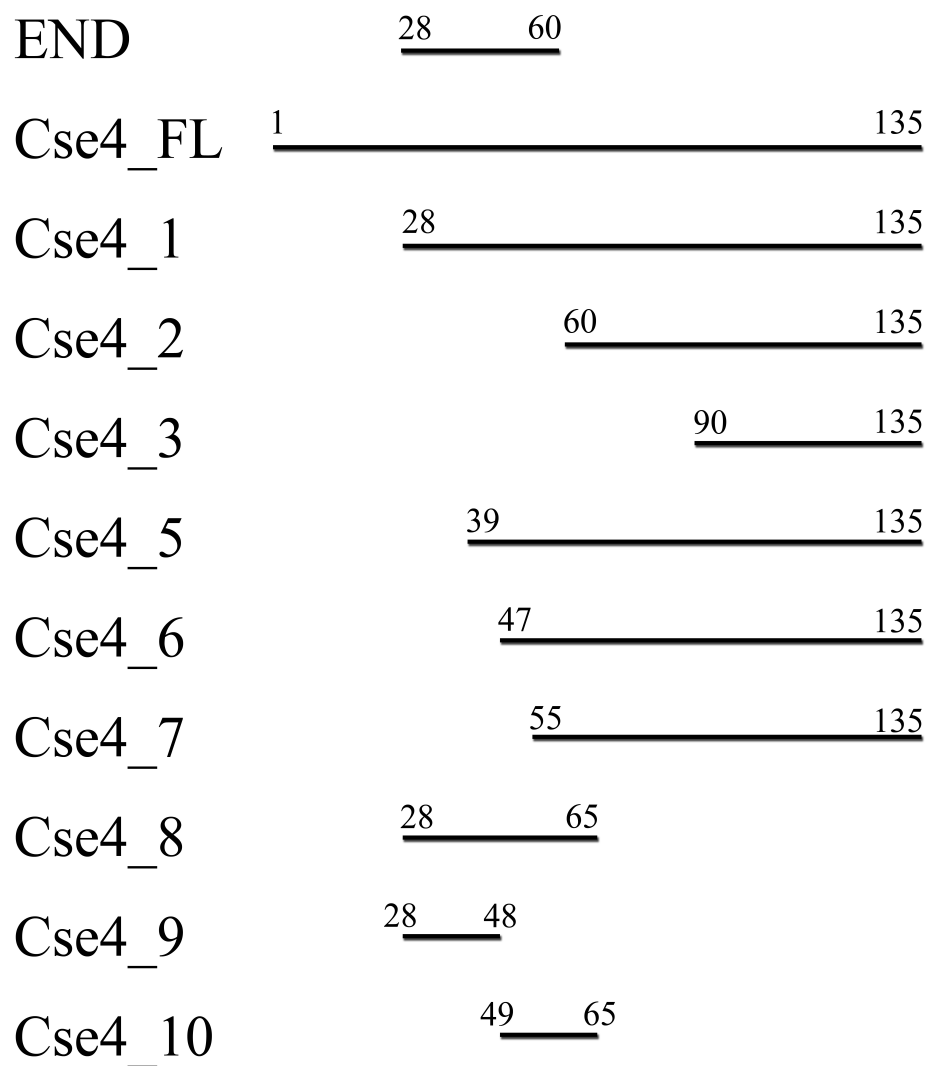
Figure 2- 1 Sgo1 has a specific interaction with Cse4 N-terminal tail. *In vitro* pull

down assay were performed to determine the interaction between Sgo1 and N-terminal

tail of Cse4, Histone H3 and Cnn1. Purified GST-Cse4 tail, GST-Histone H3 tail or GST-

Cnn1 tail were used to pull down bacterial cell lysate containing His6-Sgo1. GST was

used as control for non-specific binding. The 1% of total cell lysate (1% His6-Sgo1) and

bound proteins on agarose beads were analyzed by western blot using α-GST monoclonal

antibody and α-His6 monoclonal antibody. The asterisks mark the position of GST-

tagging proteins. The arrow marks the proteolytic product of Sgo1 subjected to identify

its protein fingerprint with mass spectrometry.

END of Cse4 is important for association with Sgo1.

The Essential N-terminal Domain of Cse4 (residue 28 to 60) is crucial for Cse4 mitotic function because deletion of this domain abolished interaction between Cse4 and other kinetochore components determining by yeast two-hybrid analysis (Chen et al., 2000). To examine the importance of END for the Sgo1-Cse4 interaction, Cse4 tail deletion mutants lacking END (Cse4_2, Cse4_3) and Cse4 tail truncation mutant containing END (Cse4_1) were created and investigated for an interaction with Sgo1. The schematic map of Cse4 N-terminal deletion is presented in Figure 2-2. Cse4 tail mutant containing END (Cse4_1) could associate with Sgo1, similar to full length of Cse4 N-terminal (Cse4_FL) (Figure 2-3 A, B). Cse4_2 mutant was relatively deficient in binding with Sgo1, while Sgo1 association was mostly abolished when pulling down with Cse4_3 mutant (Figure 2-3 A, B). These results clearly indicated that END is important for association with Sgo1.

Since our His6-Sgo1 construct was observed to have very low expression, solubility and stability we used various methods to improve protein expression and solubility, including reducing rate of protein synthesis (lowering growth temperature, lowering IPTG concentration), changing growth media (addition of NaCl, addition of polyols, addition of ethanol and addition of glucose), and using other fusion protein system, like maltose binding protein (MBP). Unfortunately, we did not successfully enhance solubility, expression and stability of Sgo1 with all the aforementioned strategies. SUMO fusion technology has been reported to enhance protein expression and improve solubility in difficult-to-express proteins (Marblestone et al., 2006). Hence, we attempted to use the SUMO fusion expression system, kindly provided by Dr. Min-Hao Kuo

(Michigan State University), to promote expression and solubility of Sgo1. We discovered that using the SUMO tagging system dramatically enhanced both solubility and expression of Sgo1 (data not shown). The SUMO-Sgo1 also contains an N-terminal His6 tag, hence we are able to use α-His6 and α-GST for immunoblotting to detect Sgo1-Cse4 interaction. To confirm the involvement of END in Sgo1 association, full length of Cse4 N-terminal, its truncation mutants containing END (Cse4_1) and its deletion mutant lacking END (Cse4_2, Cse4_3) were examined for association with His6-SUMO-Sgo1. Consistent with our previous finding, both full-length Cse4 N-terminal tail and Cse_1 had the ability to pull down His6-SUMO-Sgo1 from whole cell extract, while ability to associate with Sgo1 was partially abolished in Cse4_2 mutant (Figure 2-3 C, D). Also, Sgo1 binding was mostly eliminated when using Cse4_3 (Figure 2-3 C, D) although the amount of Cse4_3 was relatively less then the other constructs used for the pull down. These evidences confirmed an importance of END in Sgo1 binding.

END   28   60

Cse4_FL   1   135

Cse4_1   28   135

Cse4_2   60   135

Cse4_3   90   135

Cse4_5   39   135

Cse4_6   47   135

Cse4_7   55   135

Cse4_8   28   65

Cse4_9   28   48

Cse4_10   49   65

Figure 2- 2 Schematic representation of Cse4 N-terminal mutation. The N-terminal of Cse4 is depicted as a line. Number above the lines inform the amino acid residue position relative to the N-terminal methionine.

Figure 2- 3 END of Cse4 is important for Sgo1 association. *In vitro* pull down assay was performed to determine the necessity of END for Sgo1-Cse4 tail interaction. Purified full length GST-Cse4, GST-Cse4 mutants were used to pull down bacterially expressed His6-Sgo1 (A,B) or His6-SUMO-Sgo1 (C,D) cell lysates. GST was used as the control for non-specific binding (A,B). GST-Cnn1 tail was used as negative control as previously described. The 1% of total cell lysate (1% His6-Sgo1) and bound proteins on agarose beads were analyzed by Coomassie Brilliant Blue Staining (A,C), and western blot using α-GST monoclonal antibody and α-His6 monoclonal antibody (B,D). The asterisks mark the position of GST-tagging proteins.

Figure2-3
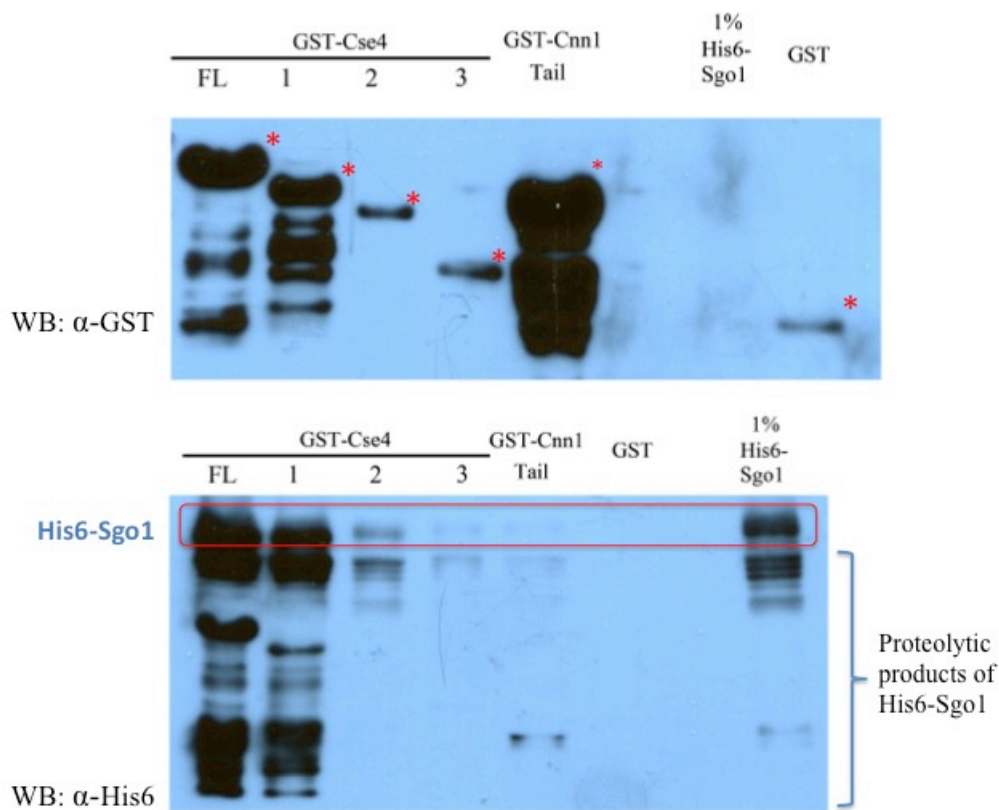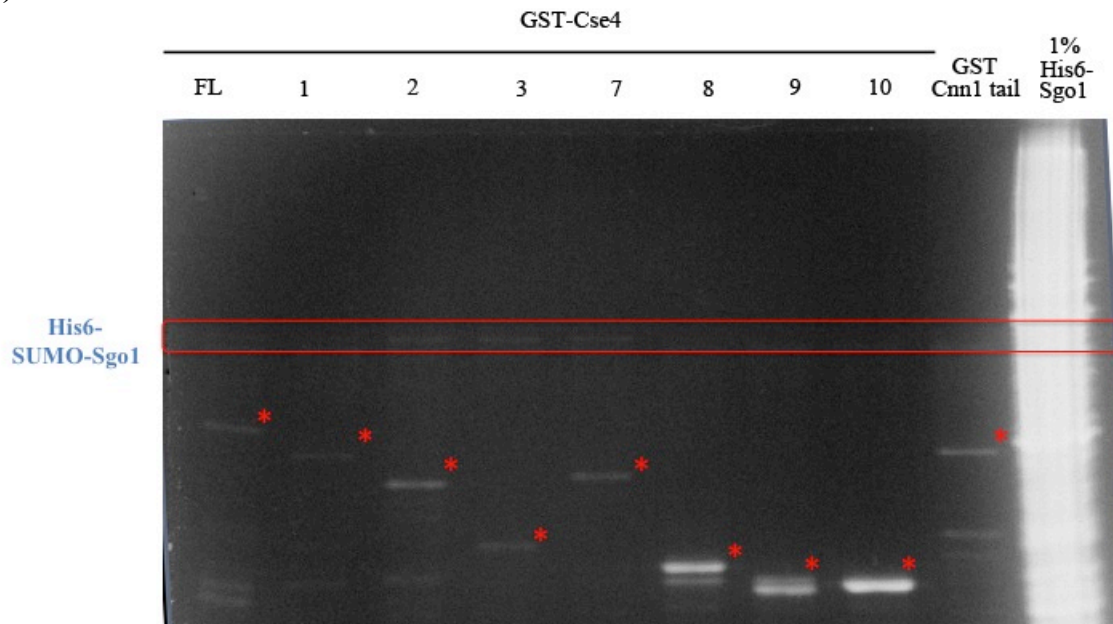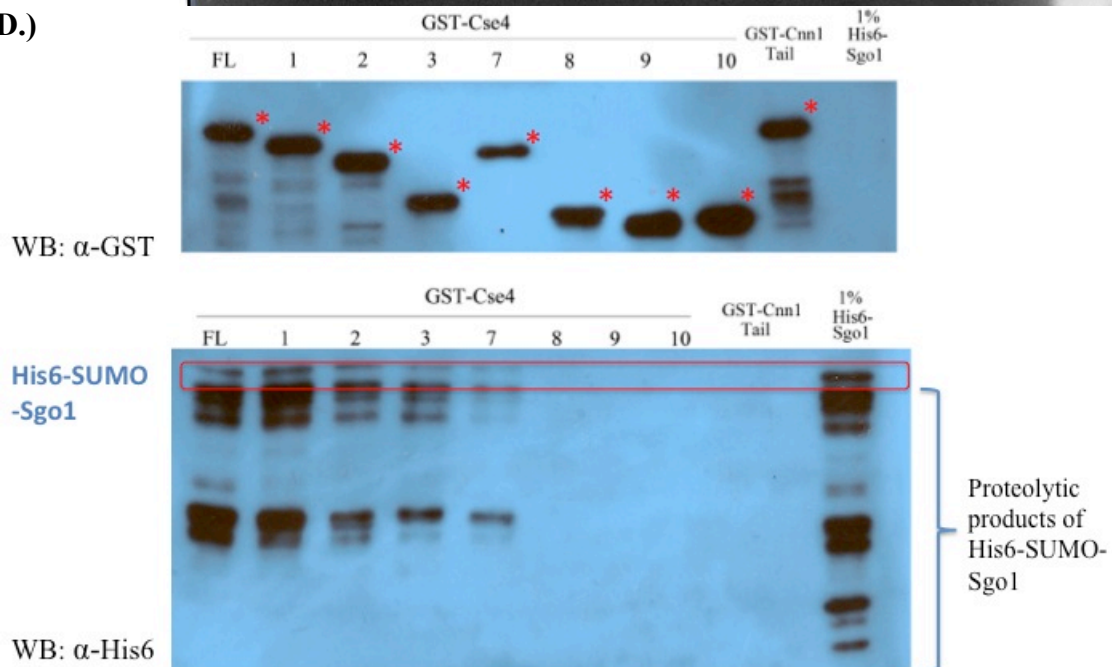
A.)
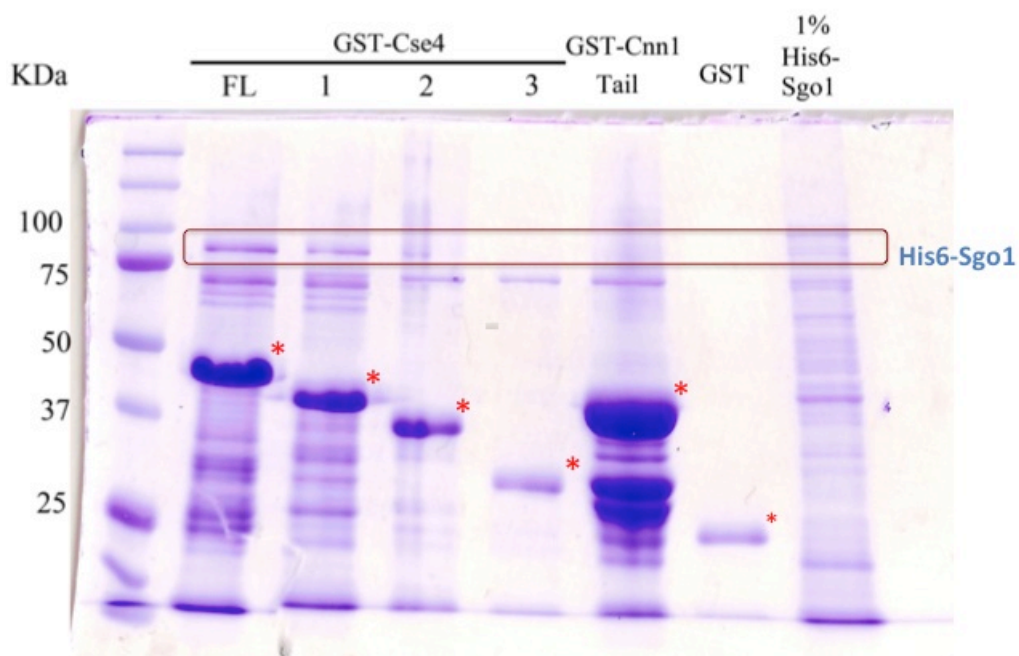
B.)

Figure 2-3 Continued

C.)

D.)

Residue 49 to 65 of Cse4 END is sufficient to interact with Sgo1.

Since we demonstrated that END of Cse4 is important for Sgo1 interaction, we further investigated the region of Sgo1 that is responsible for Cse4 binding. To examine if C-terminal amino acids of Sgo1 are required for binding with the Cse4 tail, Sgo1 mutants lacking C-terminal 270 amino acid (His6-SUMO-Sgo1 Y317X) was tested for association with Cse4 N-terminal tail. Interestingly, Sgo1 deletion mutant could still bind to Cse4 tail, suggesting that C terminal of Sgo1 is dispensable for association (Figure 2-4 A, B). An additional advantage of the Sgo1 Y317X construct is that it generated smaller proteolytic products resulting in only two prominent products of similar size. These proteolytic products could still be pulled down suggesting that further deletion analysis of the Sgo1 could further minimize the Cse4-binding domain. Based on size it appears that the N-terminal 100 amino acids of Sgo1 are sufficient to interact with Cse4 (Figure 2-4 B).

To further examine the minimal region of Cse4 N-terminal tail that is required for Sgo1 binding, we generated another series of Cse4 mutants containing partial deletion of END (Cse4_5, Cse4_6 and Cse4_7) and examined binding with Sgo1 Y317X. Surprisingly, Sgo1 Y317X was able to interact with all Cse4 deletion mutants, even mutants lacking END (Cse4_2, Cse4_3) (Figure 2-4 A-D). This phenomenon also occurred when we performed a pull down experiment with cell lysate containing high level of His6-Sgo1 or His6-SUMO-Sgo1 (Supplementary materials Figure 2-12). These evidences clearly revealed that END of Cse4 is important, but not essential for interaction with Sgo1 and suggested the possibility that another unknown region, located around residue 90 to 135, is responsible for association with Sgo1.

Our findings demonstrated that END is important for Sgo1 binding (Figure 2-3). To investigate whether this region is sufficient to associate with Sgo1, END construct (Cse4_8) was generated and performed pull down with Sgo1 Y317X. Interestingly, END alone was capable to bind to Sgo1, although its binding ability to Sgo1 is less than full length of Cse4 tail (Figure 2-4). Therefore, END of Cse4 tail is not only important but also sufficient to interact with Sgo1. To further examine the minimal amino acid sequence in END sufficient for binding to Sgo1, two truncation mutants of END (Cse4_9, Cse4_10) were generated and investigated for their ability to pull down Sgo1 Y317X from cell lysates. Our experiments showed that Sgo1 could still bind to Cse4_10 mutant, but was unable to interact with Cse4_9 (Figure 2-4 A-D). We also created another series of Cse4 truncations (Cse4_11, Cse4_12, Cse4_13, Cse4_14) to further identify precisely the domain between residues 49 to 65 responsible for Sgo1 interaction. The schematic map of this truncation series are shown in Figure 2-5. However, we failed to narrow down the exact Sgo1 binding site in this region because all of truncations were able to bind Sgo1 at the same level (Figure 2-4 E, F). From these evidences, we concluded that residue 49 to 65 of Cse4 N-terminal tail is enough for Sgo1 association. The ability of Cse4 tail and its mutants to His6-Sgo1, His6-SUMO-Sgo1 and His6-SUMO-Sgo1-Y317X were summarized on Table 2-1.

In summary, our pull down analysis first revealed the direct interaction between Cse4 N-terminal tail and Sgo1. Moreover, we have defined the region of END that is sufficient for associating with Sgo1. However, we suspected that there is another Sgo1-binding motif located within residue 90 to 135 thats needs further investigation.

Futhermore, we also have evidence suggesting the N-terminal region of Sgo1 is important for binding to Cse4.

Figure 2- 4 Residue 49 to 65 of Cse4 END is sufficient for association with Sgo1. *In vitro* pull down assay was performed to further identify minimal sequence that sufficient to interact with Sgo1. Purified full length GST-Cse4, GST-Cse4 deletion mutants were used to pull down bacterial His6-SUMO-Sgo1 Y317X from cell lysate. GST was used as control for non-specific binding (A, B). GST-Cnn1 tail was used as negative control as previously described. The 1% of total cell lysate (1% His6-Sgo1) and bound proteins on agarose beads were analyzed by Coomassie Brilliant Blue Staining (A, C, E), and western blot using α-GST monoclonal antibody and α-His6 monoclonal antibody (B, D, F). The asterisks mark the position of GST-tagging proteins.
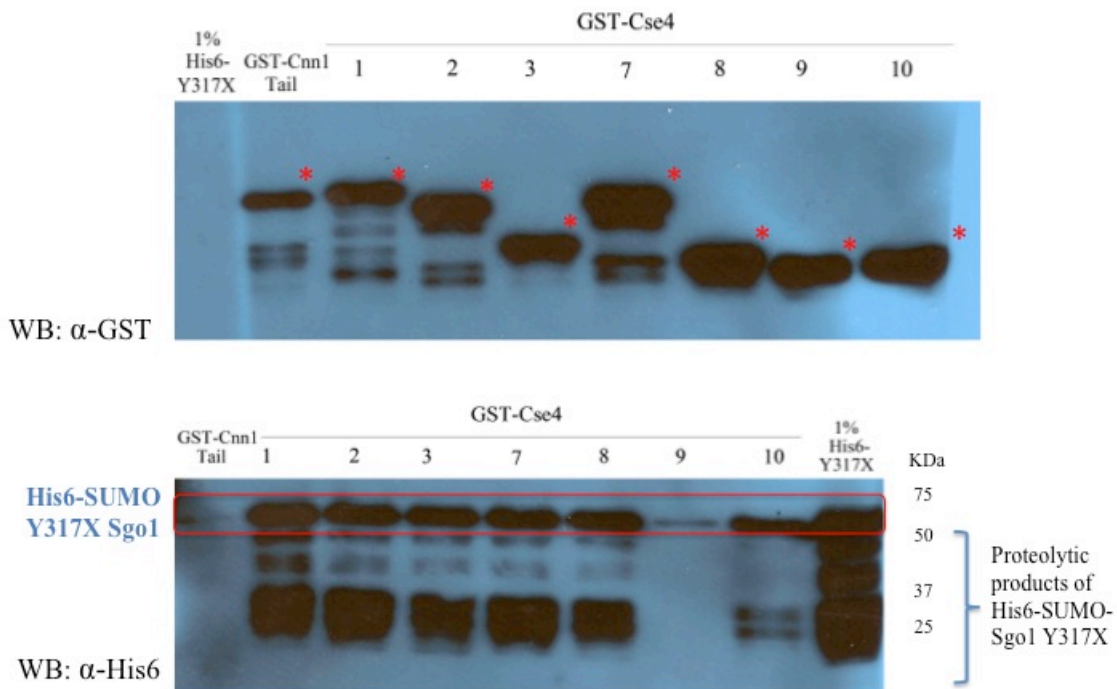
Figure 2-4

A.)
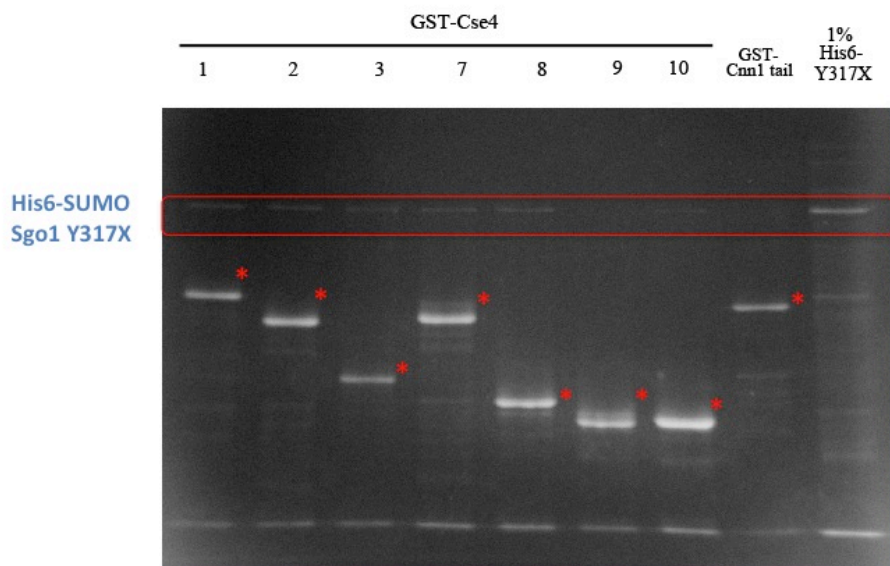
B.)

Figure 2-4 Continued

C.)

D.)

Figure 2-4 Continued

E.)

F.)

Cse4_11   55_____65

Cse4_12   49_____60

Cse4_13   55_____70

Cse4_14   61_____75
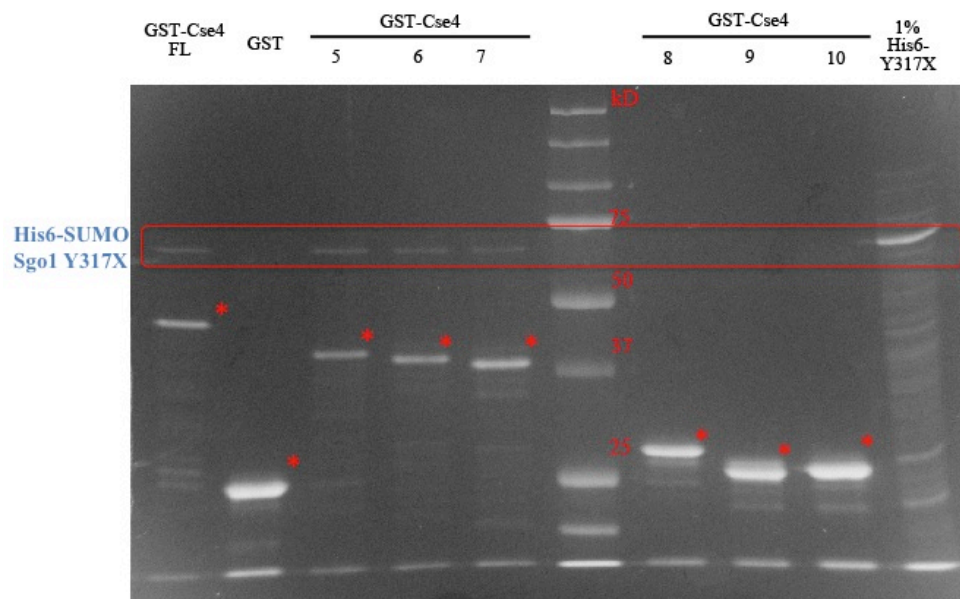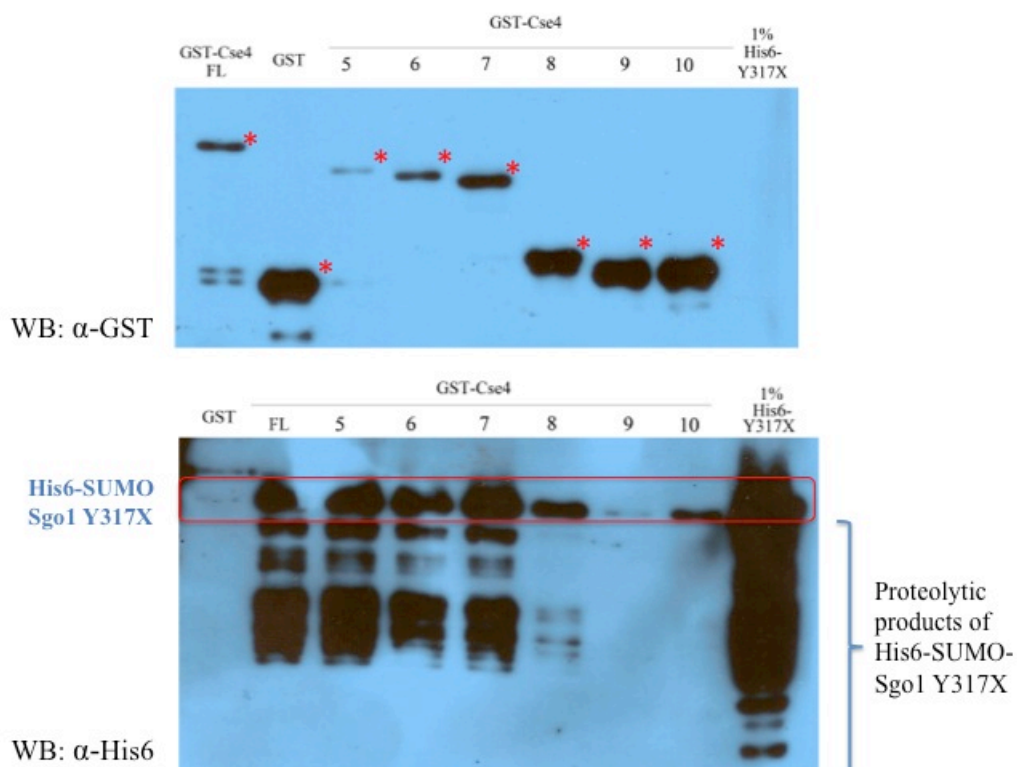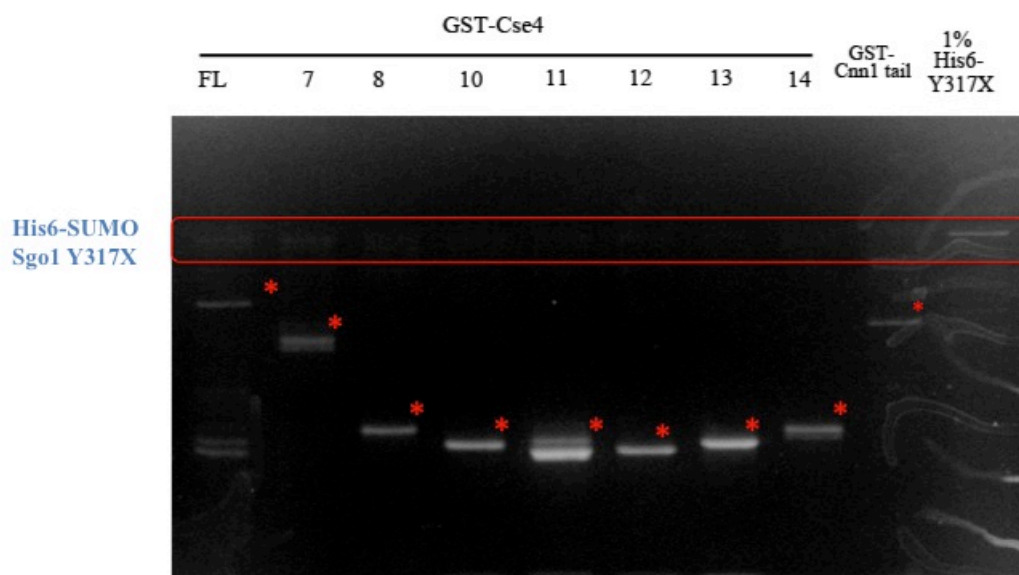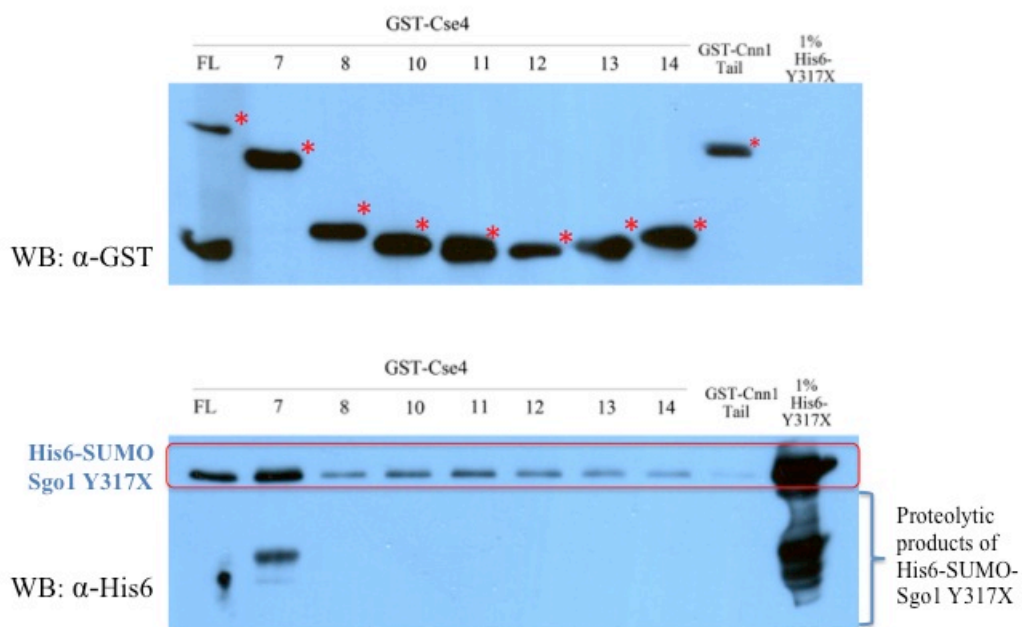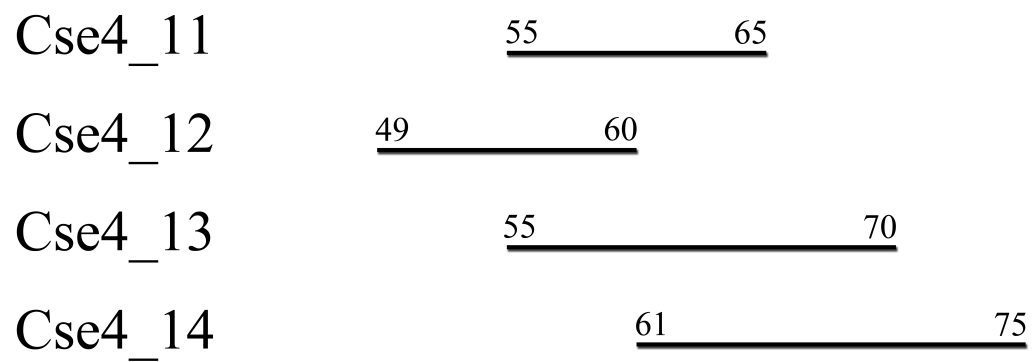
Figure 2- 5 Schematic map representation of END truncation mutations

Table 2- 1 Ability of Cse4 tail and its mutants to associate with Sgo1. To classify the association of Cse4 tail and its mutants to His6-Sgo1, His6-SUMO-Sgo1 and His6-SUMO-Sgo1 Y317X, we estimated the binding affinity from pull down result and graded each constructs with *.   ***** = highest, * = lowest, - = no binding, N/A = information not available

Cse4 constructs	His6-Sgo1 / His6-SUMO-Sgo1	His6-SUMO-Sgo1 Y317X
Cse4_FL	*****	*****
Cse4_1	*****	*****
Cse4_2	**	****
Cse4_3	**	****
Cse4_5	N/A	****
Cse4_6	N/A	****
Cse4_7	*	****
Cse4_8	-	***
Cse4_9	-	*
Cse4_10	-	**
Cse4_11	N/A	**
Cse4_12	N/A	**
Cse4_13	N/A	**
Cse4_14	N/A	**

Kinetic study of Sgo1-Cse4 interaction

Our pull down data was instructive in identifying binding regions generated qualitative data on their binding abilities. We sought to determine quantitative binding data for these proteins to further inform on their binding abilities. Bio-Layer Interferometry (BLI) technology is a label-free method that can be used for monitoring binding kinetics of protein-protein interactions in real time. For our kinetic studies using the BLI approach, ligand is loaded to a surface of pre-coated antibody biosensor, followed by incubation with the interacting protein. The binding between ligand and its binding partner alters the interference pattern of light reflected from the biosensor surface, allowing molecular association and dissociation events to be measured in real-time.

To investigate Sgo1-Cse4 binding kinetic, we immobilized GST-fusion proteins (GST-Cse4_FL, GST-Cse4_8 or GST-Cnn1 tail) to the surface of an anti-GST biosensor, followed by incubating the loaded biosensor with cell lysate containing various concentrations of Sgo1 Y317X. We first performed kinetic study with the condition listed for Condition A Table 2-2. The result from these kinetic studies showed that there was non-specific binding since Cnn1-tail could bind to Sgo1 Y317X (Figure 2-6 B). Moreover, the dissociation step did not perform normally compared to other well-behaved protein-protein interactions performed by our lab or provided by the manufacturer (Figure 2-6 A). To solve this problem, we modified some critical conditions, including reducing association time, increasing concentration of BSA in the kinetic buffer and increasing the shaking speed. Reducing association time would prevent evaporation of sample in drop position. Increasing BSA concentration in the kinetic buffer was recommended by the manufacturer to reduce non-specific binding, while increasing

shaking speed should help to mitigate mass transport effect, especially in the drop position. The modified conditions are listed on Condition B Table 2-2. With these modified conditions, the association and dissociation curves performed properly (Figure 2-7 A). Moreover, Cnn1 tail was not able to associate with Sgo1, indicating that non-specific binding was eliminated (Figure 2-7 B). However, there were bumps for the beginning of dissociation curves. These bumps are mostly caused by buffer mismatch. The bumps could affect KD calculation since they resulted in inaccurate curve fitting. To solve the bump issue, we performed a study by preparing all protein samples in similar buffer to the dissociation buffer (kinetic buffer) by buffer exchange through a gel filtration spin column. From figure 2-8, the bumps at the beginning of the dissociation curve disappeared after matching buffers. Then, we could use this condition for studying binding kinetics between Sgo1 and Cse4 tail constructs. Our Sgo1 binding kinetic analysis showed that full length of Cse4 N-terminal has equilibrium dissociation constant (KD) around 32.6 nM, while END alone (Cse4_8) has KD around 461.7 nM. These kinetic values are consistent with our pull down experiment, showing that full length of Cse4 tail has increased ability to interact with Sgo1 relative to END alone (Table 2-1, Figure 2-4 C, D). The kinetic values of Cse4_FL and Cse4_8 are summarized in Table 2-3.

Table 2- 2 Parameters and conditions used for kinetic study

Step Name / Parameters	Condition A	Condition B
Equilibration	20 sec	20 sec
Condition (10 mM Glycine)	20 sec	20 sec
Equilibration	20 sec	20 sec
Condition (10 mM Glycine)	20 sec	20 sec
Equilibration	20 sec	20 sec
Condition (10 mM Glycine)	20 sec	20 sec
Equilibration	300 sec	300 sec
Loading	300 sec	300 sec
Baseline	180 sec	180 sec
Association	600 sec	300 sec
Dissociation	600 sec	600 sec
Shake Speed	1,100 RPM	2,200 RPM
% BSA	0.1%	1%

Figure 2- 6 Improper dissociation and non-specific binding. To perform binding kinetic studies, GST-fusion proteins (GST-Cse4_FL (A) and GST-Cnn1 tail (B)) were loaded to anti-GST biosensors, followed by associating with cell lysate containing Sgo1 Y317X in a concentration range between 437.5 nM to 1,750 nM. The parameters and conditions used for these experiments are shown in Condition A Table 2-2.

Figure 2-6

A.)
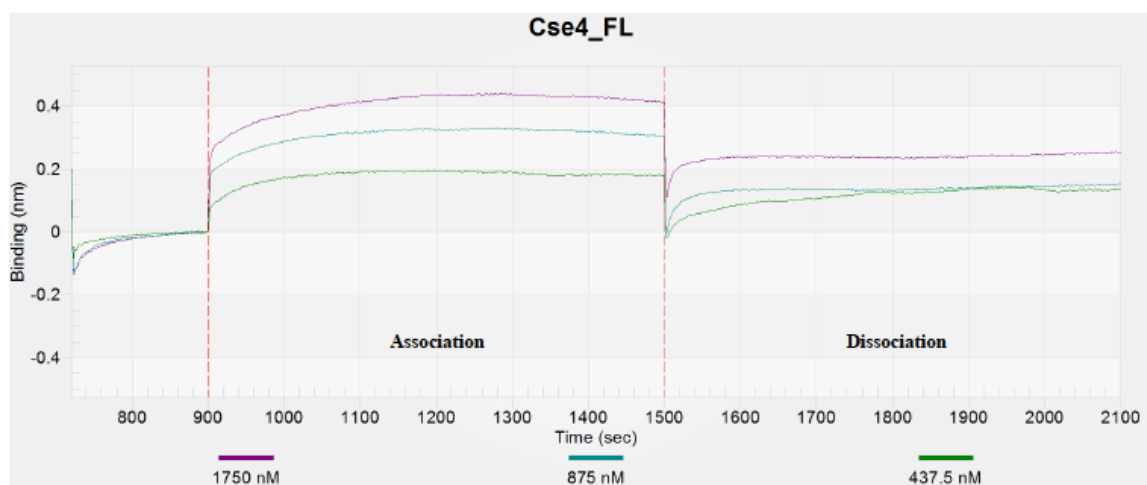
B.)
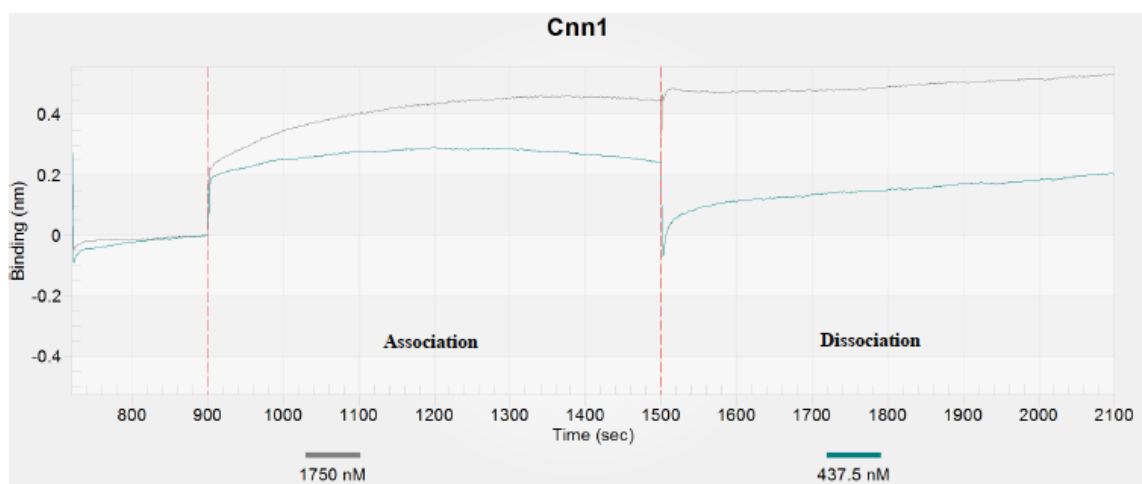
Figure 2- 7 Improvement of kinetic binding curves after changing critical experimental parameters. To improve the binding kinetic studies, we used parameters and conditions in Condition B Table 2-2 to perform kinetic study between Sgo1-GST-Cse4 tail (A) / Sgo1-GST-Cnn1-tail (B) with anti-GST biosensor. The cell lysate used in this study contained Sgo1-Y317X in concentration range 218.8 nM to 1,750 nM.

Figure 2-7

A.)
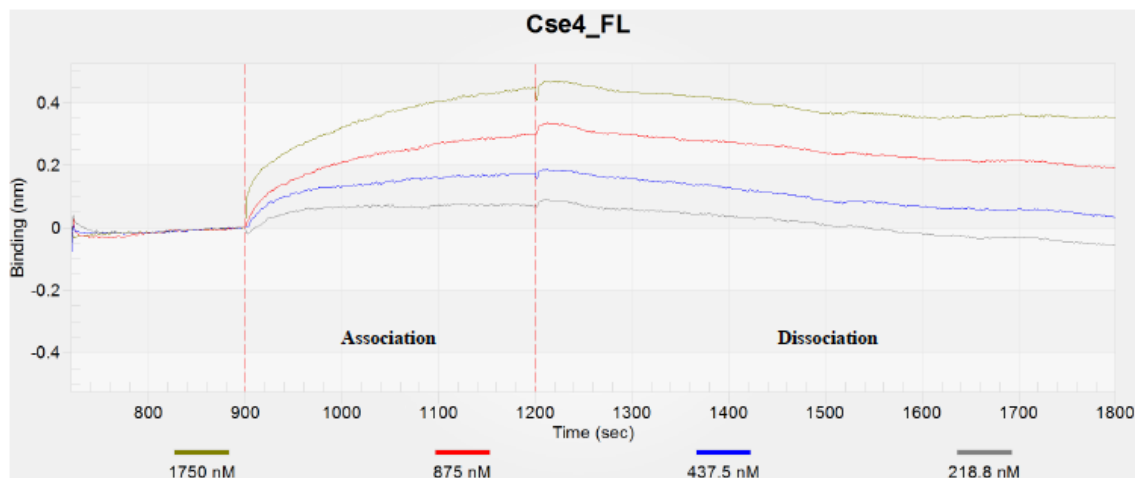
B.)
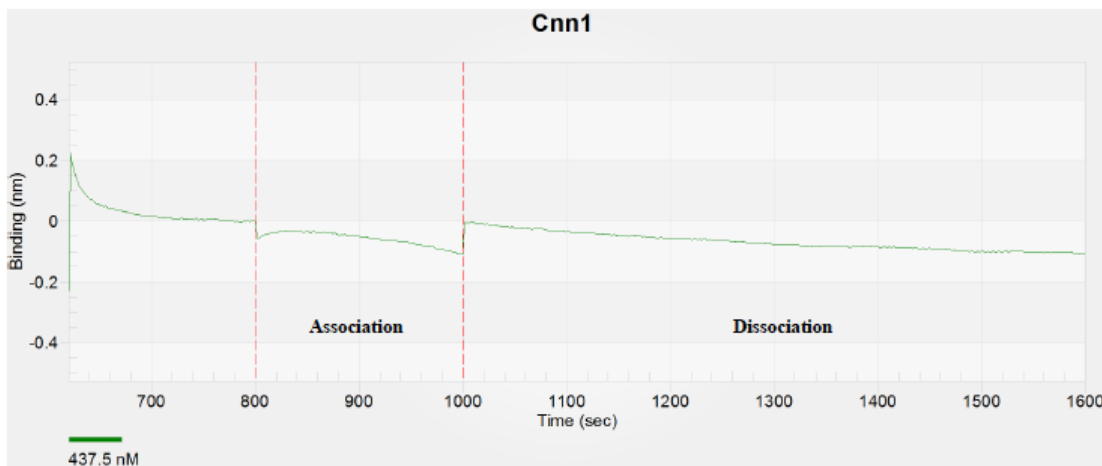
Figure 2- 8 Kinetic analysis of Sgo1-Cse4 tail and Sgo1-END association. To perform binding kinetic studies, GST-Cse4_FL (A) and GST-Cse4_8 (B) were loaded to anti-GST biosensors, followed by incubating with cell lysate containing Sgo1 Y317X in a concentration range between 500 nM to 1,000 nM. The parameters and conditions used for these experiments are shown in Condition A Table 2-2, except 200 sec for association step.

Figure 2-8

A.)

Cse4 FL

B.)
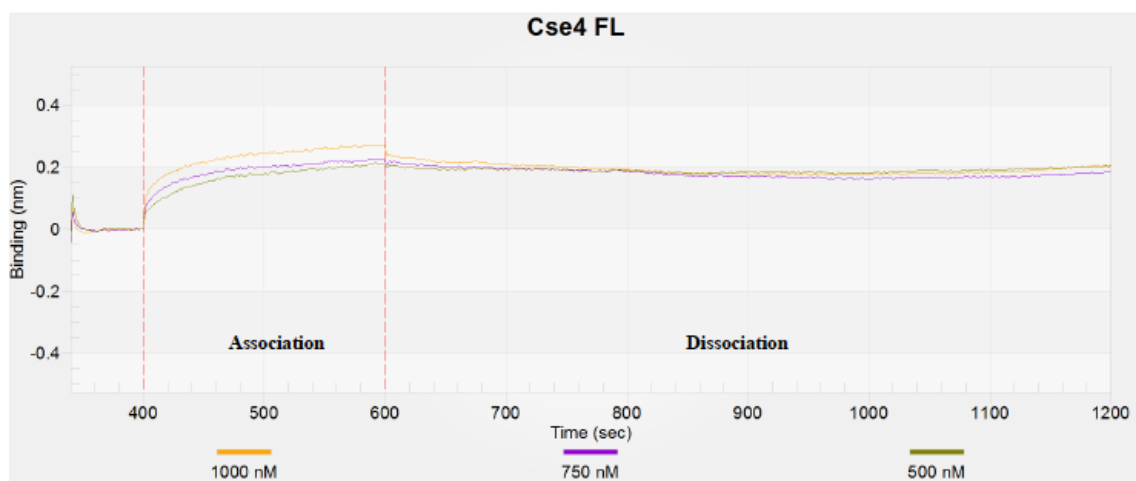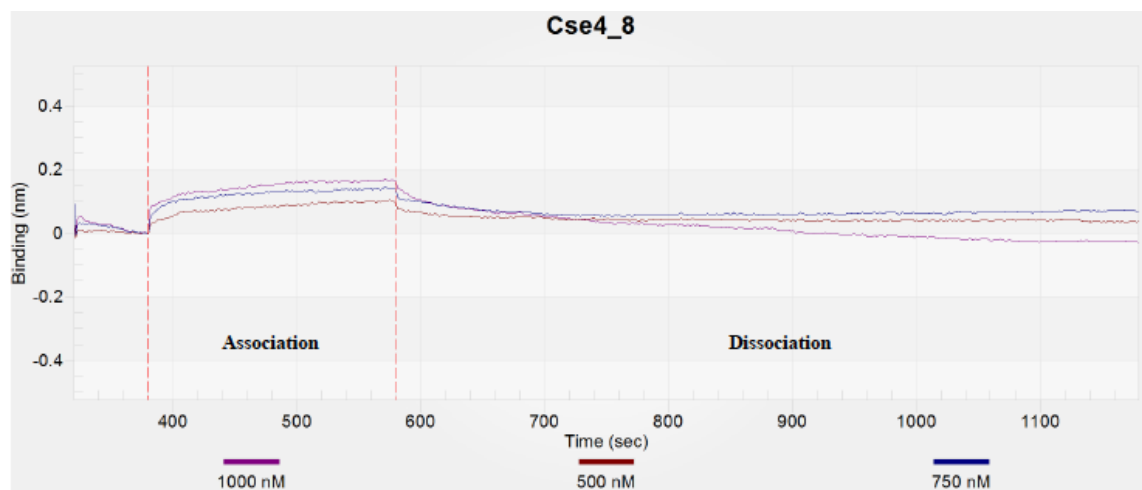
Cse4_8

Table 2- 3 Kinetic values for Sgo1-Cse4 tail and Sgo1-END interaction

Ligand	Ka $(M^{-1}s^{-1})$	Kd (s^{-1})	KD (M)
Cse4_FL	$6.59X10^4$	$2.151X10^{-3}$	$3.26X10^{-8}$
Cse4_8	$3.423 X 10^4$	$1.58X10^{-2}$	$4.617x10^{-7}$

Identification of Sgo1 binding motif in Cse4 N-terminal tail

To identify a conserved motif correlated with Sgo1 binding ability in the Cse4 N-terminal tail in eukaryotic organisms, we used MEME (http://meme.nbcr.net/meme/) to perform multiple sequence alignment of Cse4 tail homologues. We performed multiple sequence alignment of Cse4 tail from various eukaryotic organisms, including yeast (*S. cerevisiae*, *S. pombe*), plant (*Arabidopsis thaliana*, *Zea mays*), fly (*Drosophila melanogaster*), worm (*Caenorhabditis elegans*) and mammal (*Homo sapiens*, *Mus musculus*). The protein sequences of Cse4 tail homologues were obtained from public databases. Based on motif searching result from MEME, we revealed two motifs of Cse4 tail that are highly conserved from yeast to human, including residue 49 to 56 (Figure 2-9 A, B) and residue 95 to 102 (Figure 2-9 C, D) of budding yeast Cse4 tail. The first domain (residue 49-56) is rich in positively charged amino acid, while the second motif (residue 95-102) contains conserved serine/threonine, a possible proline and hydrophobic amino acids. Further bioinformatic analysis is needed because the conservation of the second motif a weaker and it appears difficult to detect in some species but appears to be well conserved in others. Interestingly, the first motif is part of END of Cse4 that is sufficient to pull down Sgo1 from cell lysate. Moreover, the second motif is located on the region we suspected for another Sgo1 binding site. Hence, based on our pull down study and multiple sequence alignment analysis, we suggest that these two conserved motifs are putative Sgo1 binding sites. However, the exact binding boundaries and interaction kinetics for the second motif in Sgo1 association needs to be further investigated.

Figure 2- 9 Multiple sequence alignment of a conserved motif present in Cse4 tail.
To search conserved motifs in Cse4 N-terminal tail, we performed a multiple alignment of Cse4 homologues by using motif search program MEME. (A, C) The conserved motifs are depicted in sequence LOGOS format. The total height of a stack reflects the information content of that position in the motif. The height of the letter in a stack represents their frequency at that position. (B, D) The occurrences of eukaryotic Cse4 tail motif were aligned and colored in MEME format. The sites are listed in order of increasing statistical significance. (A, B) represents the first putative motif, while (C, D) represents the second putative motif of the Cse4 tail.

Figure 2-9

A.)
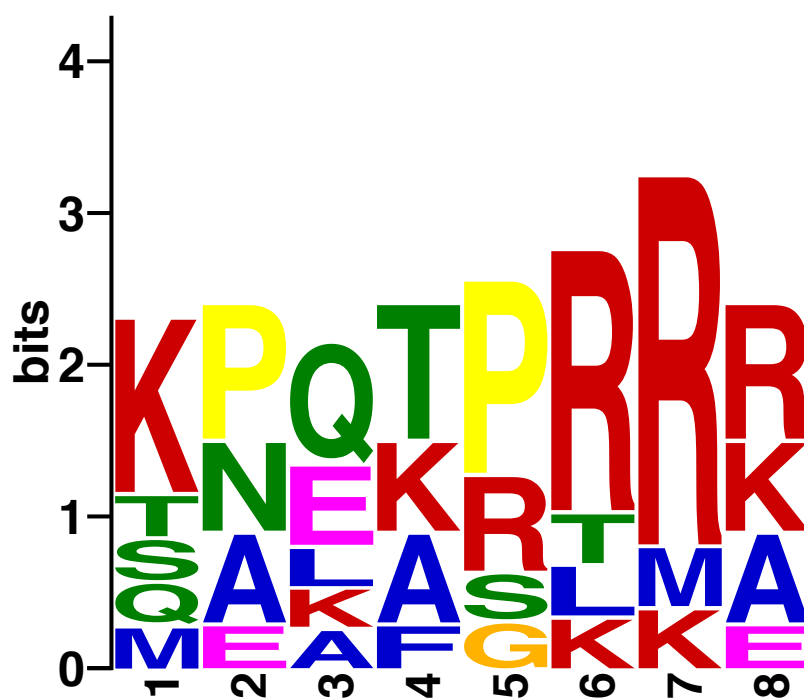
B.)

Name	Start	p-value	Sites ?
Mmusculus	6	1.41e-08	MGPRR KPQTPRRR PSSPAPGPSR
Hsapien	9	1.13e-07	MGPRRRSR KPEAPRRR SPSPTPTPGP
Scerevisiae	49	4.36e-07	LSLLQRTRAT KNLFPRRE ERRRYESSKS
Dmelenogaster	115	4.48e-06	KTRAAGPVTS QNQTRRRK AAN
Celegans	100	1.29e-05	KQAEDEYHAR KEQARRRA SSMDFTVGRN
Zmays	12	1.46e-04	ARTKHQAVRK TAEKPKKK LQFERSGGAS
Athaliana	53	5.02e-04	DNTQQTNPTT SPATGTRR GAKRSRQAMP
Spombe	1	2.40e-03	MAKKSLMA EPGDPIPRPR

Figure 2-9 Continued

C.)
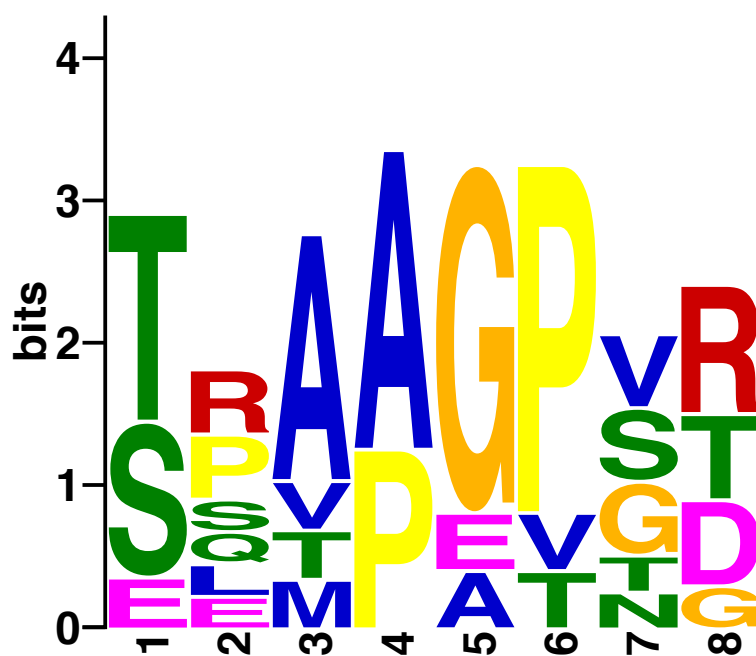
D.)

Name	Start	*p*-value	Sites ?
Dmelenogaster	106	1.46e-08	RRMTVQQESK TRAAGPVT SQNQTRRRKA
Mmusculus	16	2.77e-07	KPQTPRRRPS SPAPGPSR QSSSVGSQTL
Athaliana	25	3.05e-07	NQTDAAGASS SQAAGPTT TPTRRGGEGG
Celegans	40	8.95e-07	QRVTSVPGFN TSAAGVND LIDILNQYKK
Hsapien	21	2.93e-06	EAPRRRSPSP TPTPGPSR RGPSLGASSH
Scerevisiae	95	1.25e-05	TENEEEAEME TEVPAPVR THSYALDRYV
Spombe	5	2.83e-05	MAKK SLMAEPGD PIPRPRKKRY
Zmays	35	3.60e-05	SGGASTSATP ERAAGTGG RAASGGDSVK

Roles of phosphorylation in Sgo1-Cse4 interaction

Ipl1, mitotic serine/threonine kinase, plays a crucial role in chromosome segregation. In serving this role, Ipl1 promotes re-orientation of improperly attached kinetochores, which is subsequently sensed by the spindle assembly checkpoint, until proper amphitelic attachment are made (Carmena et al., 2012). Ipl1 is known to phosphorylate serine 10 of histone H3 and detection of S10 phosphorylation is commonly used as a mitotic marker in yeast and higher eukaryotic systems (Hans and Dimitrov, 2001; Prigent and Dimitrov, 2003; Fu et al., 2007). Based on Ipl1 consensus sites (Cheeseman et al., 2002), Cse4 could probably be an Ipl1 substrate since it has a putative Ipl1 phosphorylation site at serine 124. To investigate whether phosphorylation at serine 124 is important for Sgo1-Cse4 interaction, we created Cse4 tail mutants, which replaced serine 124 with alanine (S124A) or aspartic acid (S124D), and examined interaction with Sgo1 by using pull down analysis. Both Cse4 S124A and S124D has ability to pull down His6-Sgo1 from cell lysate in the same level as wild type, suggesting that phosphorylation at 123 is not necessary for Sgo1-Cse4 association (Figure 2-10). Recently, serine 22, 33, 40 and 105 of Cse4 tail have been identified as phosphorylation sites for Ipl1 *in vitro* and possibly *in vivo* (Munira Basrai, personal communication). These sites are atypical to the consensus site and their significance is not clear. To examine whether phosphorylation at these 4 sites is important for Sgo1-Cse interaction, we performed pull down study by using Cse4 tail mutants, Cse4 4SA and Cse4 4SD. Cse4 4SA and Cse4 4 SD have alanine mutation and aspartic acid mutation, respectively, at all serine 22, 33, 40 and 105. Our results showed that both mutants were able to bind to

Sgo1 in the same level as wild type, suggesting that phosphorylation at these sites is dispensable for Sgo1-Cse4 association (Figure 2-10).

Mps1 is an essential mitotic kinase that is implicated in spindle checkpoint system. Mps1 activation is required for mitotic arrest in response to attachment defect and tensionless crisis at kinetochore (Liu and Winey, 2012). Based on recognition feature of Mps1 (Liu and Winey, 2012), Cse4 tail has Mps1 putative a phosphorylation site at serine 33. To examine if phosphorylation at this site is involved in Sgo1-Cse4 tail binding, we created Cse4 S33A, Cse4 S33D mutants and investigated association with Sgo1. Sgo1 could still associate to both Cse4 S33A, S33D. This result suggests that phosphorylation at serine 33 does not modulate the Sgo1-Cse4 interaction (Figure 2-10).
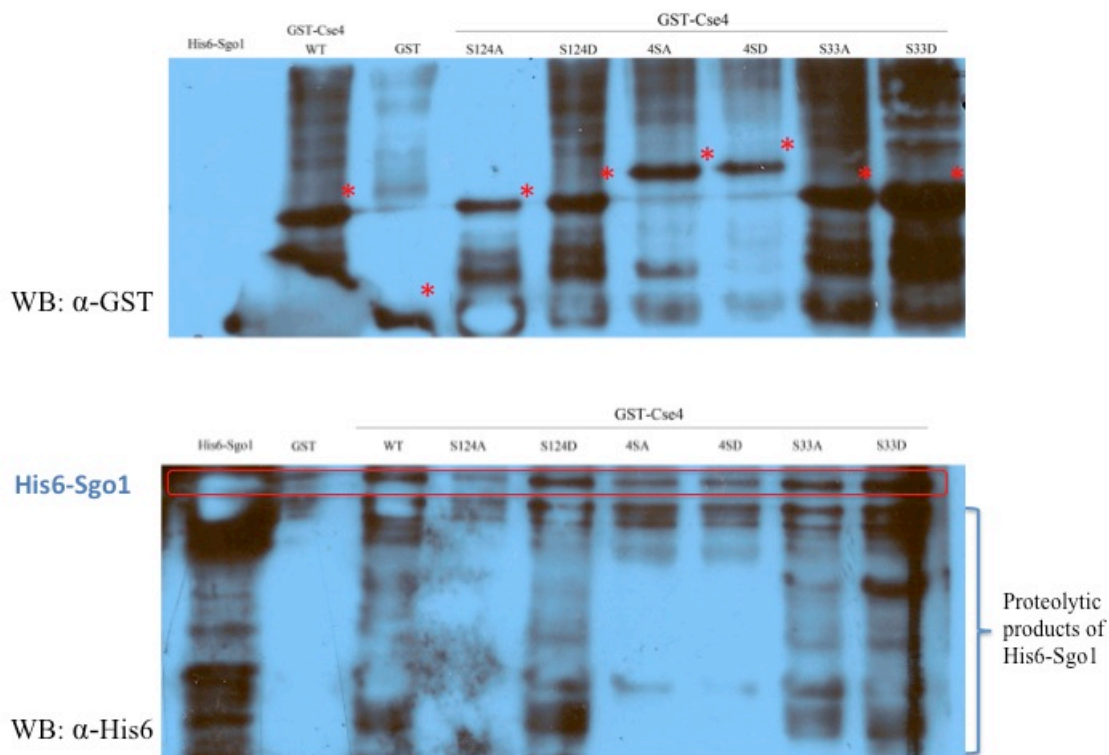
Figure 2- 10 Roles of phosphorylation in Sgo1-Cse4 tail interaction. An *in vitro* pull down assay was performed to investigate if phosphorylation on Cse4 tail mediates association with Sgo1. Purified full length GST-Cse4 and GST-Cse4 deletion mutants were used to pull down bacterial His6-Sgo1from cell lysate. GST was used as control for non-specific binding. GST-Cnn1 tail was used as negative control as previously described. The 1% of total cell lysate (1% His6-Sgo1) and bound proteins on agarose beads were analyzed by western blot using α-GST monoclonal antibody and α-His6 monoclonal antibody. The asterisks mark the position of GST-tagging proteins.

2.4   **Discussion**

Budding yeast Cse4 is thought to substitute for canonical histone H3 when assembling into a centomeric nucleosome (Camahort et al., 2009; Kingston et al., 2011). Cse4 contains a conserved C-terminal histone fold domain and unique 135-amino-acid N-terminal tail domain. The architecture of budding yeast centromeric nucleosome is such that the conserved histone fold domain of Cse4 is embedded into the octameric nucleosome core and the N-terminal tail protrudes from the core (Camahort et al., 2009). Extension of Cse4 tail from centromeric nucleosome core make it accessible for association with other kinetochore components involved in mitotic function (Chen et al., 2000; Samel et al., 2012). Prior to this study, there is no evidence of a direct physical interaction reported between the Cse4 tail and kinetochore or cell cycle related-proteins. A possible interacting protein is Sgo1, a protein that is a protector of the meiotic cohesin complex in budding yeast (Watanabe and Kitajima, 2005). The mitotic roles of Sgo1 in budding yeast are still not fully understood but several pieces of evidence suggest that Sgo1 plays an important role in sensing tension at the kinetochore during mitosis (Indjeian et al., 2005; Luo et al., 2010). This led to our hypothesis that Sgo1 is recruited to the centromere and interacts with the Cse4 N-terminal tail for mediating microtubule attachment as a tension sensor at the kinetochore. Our study demonstrates for the first time that Cse4 tail has a specific direct interaction with Sgo1 (Figure 2-1). From our pull down analysis, Sgo1 has a strong ability to interact with the Cse4 N-terminal tail, while it is not able to associate with another kinetochore protein tail, Cnn1, indicating the specificity of Sgo1-Cse4 interaction (Figure 2-1). Future studies investigating mitotic role of Sgo1-Cse4 tail interaction would be useful to clarify tension-sensing role of Sgo1 at

the centromere. In addition to the Sgo1-Cse4 interaction, we also revealed direct interaction between histone H3 tail and Sgo1 (Figure 2-1). This result is consistent with previous finding that demonstrated roles of histone H3 tail-Sgo1 interaction at pericentromeric region in mitotic tension sensing (Luo et al., 2010).

The N-terminal tail of Cse4 is essential for viability. Deletion of the first 50 amino acid of the extended Cse4 tail causes cell lethality (Keith et al., 1999). Comprehensive and systematic mutagenesis analysis of Cse4 N-terminal tail revealed the domain that is vital for its mitotic function. This specific region of Cse4 tail, called as essential N-domain (END), contains 33 amino acid-domain between residue 28 and 60 in Cse4 N-terminus. END is essential for cell viability since deletion of END causes cell death. Mutation of END or partial deletion of END leads to chromosome missegregation. Additionally, yeast two hybrid demonstrated that END is an important motif for interaction between Cse4 and other kinetochore components. This led to our hypothesis that END of Cse4 N-terminal tail is important for Sgo1-Cse4 association and for mitotic tension sensing role of Sgo1 at centromere. Our pull down analysis demonstrate that the interaction between Sgo1 and Cse4 tail were mostly abolished in Cse4 tail mutant lacking END, Cse4_2 and Cse4_3 (Figure 2-3). Moreover, END alone has ability to pull down Sgo1 from cell lysate (Figure 2-4). These results clearly suggested that END of Cse4 N-terminus is responsible for interaction between Cse4 and Sgo1. Using truncation mutants of END, we discovered the minimal sequence of END that is sufficient for association with Sgo1. From pull down analysis, we revealed that residue 49 to 65 of END is sufficient for binding with Sgo1, while the first 21 amino acid of END is dispensable for Sgo1 interaction (Figure 2-4 A-D).

Surprisingly, not only was Sgo1 able to associate with the Cse4 tail, but also with Cse4 mutant lacking END (Cse4_2, Cse4_3) when we performed a study with cell extract containing Sgo1 Y317X (Figure 2-4 A, B, Table 2-1). The Sgo1-Cse4 association in the absence of END could be explained in 2 ways. One explanation is that the amount of His6-SUMO-Sgo1 Y317X in cell lysate (Figure 2-4 A, B) was much higher than His6-SUMO-Sgo1 (Figure 2-3 A, B) and His6-Sgo1 (Figure 2-3 C,D). The other explanation is that there is probably another Sgo1 binding motif around 90-135 of Cse4-tail. To test this hypothesis, we used higher amount of either His6-Sgo1 or His6-SUMO-Sgo1 to associate with Cse4 tail and its mutants in the pull down experiment. Our results demonstrated that either His6-Sgo1 (Supplementary Materials Figure 2-12 A) or His6-SUMO-Sgo1 (Supplementary Materials Figure 2-12 B) was able to bind with Cse4 truncation mutants lacking END (Cse4_2 and Cse4_3). These results clearly support our hypothesis that there is another association domain located around residue 90 to 135 of Cse4 tail. Interestingly, multiple sequence alignment analysis of Cse4 tail homologues revealed two conserved motif from yeast to human, including residue 49 to 56 (Figure 2-9 A, B) and residue 95 to 102 (Figure 2-9 C, D) of budding yeast Cse4 tail. The finding of these two motifs is strongly consistent with our pull down analysis and suggests an attractive model in which the orthologous interactions are conserved in higher eukaryotes where tension sensing is also important and conserved during the chromosome segregation process. The first conserved motif (residues 49 to 56) is part of Cse4 truncation mutant (Cse4_10; residue 49 to 65) that is sufficient to interact with Sgo1 (Figure 2-4), while the second conserved motif (residue 95 to 102) is located within the region we hypothesized as an additional Sgo1 binding motif. The pull down analysis and location of two conserved

motifs and in each Cse4 mutants are summarized in figure 2-11. Hence, this led to our hypothesis that these two conserved motifs could possibly form binding sites for association with Sgo1. Further future studies with an X-ray crystallography approach would be useful to better understand the importance of these two motifs in the Sgo1-Cse4 interaction. In addition to binding analysis, both of these two conserved motifs need further investigation into their roles in Sgo1 mediated tension sensing during mitosis. There are several recommendations for examining mitotic roles of conserved binding motifs on Cse4 N-terminal tail. First, the two dimensional-density mapping analysis of Sgo1 localization raises the possibility that Sgo1 co-localize with Cse4 at centromere (Haase et al., 2012). Together this possibility with our findings, two conserved motifs of Cse4 could be responsible for localization of Sgo1 at centromere. Future microscopy studies with GFP-Sgo1 in the presence of mutation at conserved motifs of Cse4 tail would be attractive for studying Sgo1 localization. Moreover, future studies with chromatin immunoprecipitation assay of Sgo1 in the presence of mutations at conserved motifs of Cse4 tail would be useful in clarifying the importance these two motifs in Sgo1 localization at the centromere and pericentromere. Second, spindle assembly checkpoint is activated in the response to unattached and tensionless kinetochore. Activation of SAC results in inhibition of APC/C, stabilization of Pds1 and inhibition of separase. Thus, future biochemical studies with measuring the Pds1 level in response to tensionless crisis of yeast strains carrying mutations at the two conserved motifs of Cse4 tail will provide insight into roles of Cse4 and Sgo1 in mediating tension-sensing at centromere.

In this study, we also developed methods for studying binding kinetics of Sgo1 and Cse4 N-terminal tail. Our preliminary experiment found that full length of Cse4 has

equilibrium dissociation constant (KD) around 32.6 nM, while KD of END is around 461.7 nM. These kinetic analyses are consistent with our pull down experiments, demonstrating that full length Cse4 tail was able to associate with Sgo1 with higher affinity then the END alone (Table 2-1, Figure 2-11). However, this kinetic analysis is a preliminary study and more studies are needed to confirm the kinetic values we measured, especially since we used cell lysate containing Sgo1 Y3179X. We measured concentration of Sgo1 Y317X by comparing intensity of the Sgo1 Y317X band to standards of known concentration on Coomassie Briliant Blue stained SDS-PAGE. To obtain more precise kinetic values of Sgo1-Cse4 interaction, using purified Sgo1 is recommended. Furthermore, future kinetic studies with synthesized peptides corresponding to the two putative conserved binding motifs, residue 49-56 and residue 95-102, will provide insight into the relative importance these two domains in associating with Sgo1.
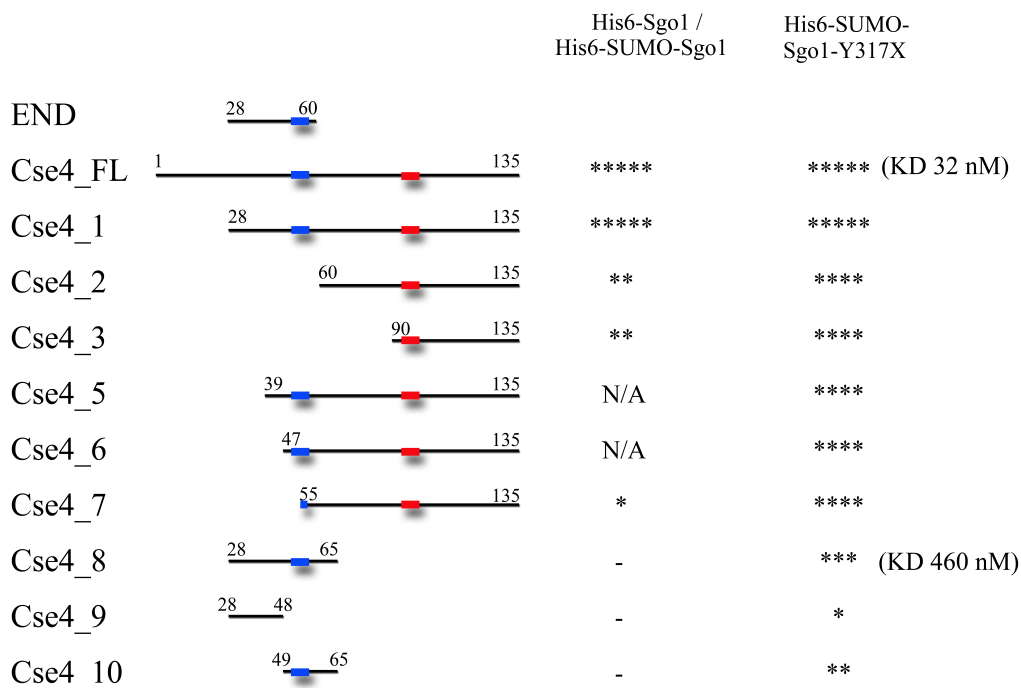
Figure 2- 11 Schematic map representation of two conserved motifs in Cse4 N-terminal tail and its mutants. The blue block represents the first conserved motif (residue 49-56), while red block represents the second conserved motif (residue 95-102) of Cse4 tails. The binding affinity between Sgo1 and Cse4 constructs was estimated from pull down result and graded each constructs with *.   ***** = highest, * = lowest, - = no binding, N/A = information not available   The number in parentheses indicate equilibrium dissociation constants (KD).

Based on an *in vitro* kinase assay (Munira, Basrai (personal communication)) and Ipl1, Mps1 recognition sites, and the role these kinases have in modulating other kinetochore protein interactions, we predicted that Ipl1 and Mps1 mitotic kinase could modulate the Sgo1-Cse4 N-terminal by phosphorylation of the Cse4-tail. To investigate roles of phosphorylation on Sgo1-Cse4 tail, we performed an experiment by using phosphomimetic mutation approach together with pull down analysis. For phosphomimetic approach, we substituted putative serine to aspartic acid (phosphorylated form) or alanine (unphosphorylatable form). Our results demonstrated that phosphorylation at serine 22, 33, 40, 105 and 124 on Cse4 tail do not affect the Sgo1-Cse4 interaction in an *in vitro* pull down assay. However, we cannot exclude the possibility that phosphorylation at Sgo1 is involved in regulation of Sgo1-Cse4 interaction *in vivo* because we may be missing other phosphorylation sites. Future studies with additional mutants are needed to determine if phosphorylation can modulate this interaction in any way.

In conclusion, we first established a direct interaction between Sgo1 and the Cse4-tail. We discovered that residue 49 to 56 of Cse4 tail is sufficient to associate with Sgo1. However, we suspected that there is another binding motif of Sgo1 around residue 95-102. These two binding motifs are an excellent starting point to investigate the biological role of the Sgo1-Cse4 interaction, and especially its possible role in Sgo1-mediated mitotic tension sensing. The tension-sensing mechanism is an essential and evolutionarily conserved mechanism in mitosis and we hypothesize that the conserved motifs we have identified in Cse4 play an important role in this process.

2.5   <u>Supplementary Materials</u>

Table 2- 4 Plasmids constructs used in this study

Plasmid	Inserted Gene	Promoter	Source
pGEX-6p-1	*Cse4_FL*	Tac	This study
pGEX-6p-1	*Cse4_1*	Tac	This study
pGEX-6p-1	*Cse4_2*	Tac	This study
pGEX-6p-1	*Cse4_3*	Tac	This study
pGEX-6p-1	*Cse4_5*	Tac	This study
pGEX-6p-1	*Cse4_6*	Tac	This study
pGEX-6p-1	*Cse4_7*	Tac	This study
pGEX-6p-1	*Cse4_8*	Tac	This study
pGEX-6p-1	*Cse4_9*	Tac	This study
pGEX-6p-1	*Cse4_10*	Tac	This study
pGEX-6p-1	*Cse4_11*	Tac	This study
pGEX-6p-1	*Cse4_12*	Tac	This study
pGEX-6p-1	*Cse4_13*	Tac	This study
pGEX-6p-1	*Cse4_14*	Tac	This study
pGEX-6p-1	*Cse4 S33A*	Tac	This study
pGEX-6p-1	*Cse4 S33D*	Tac	This study
pGEX-6p-1	*Cse4 4SA*	Tac	Munira Basrai

Table 2-4 Continued

pGEX-6p-1	*Cse4 4SD*	Tac	Munira Basrai
pGEX-6p-1	*Cse4 S124A*	Tac	This study
pGEX-6p-1	*Cse4 S124D*	Tac	This study
pGEX-6p-1	*Cnn1 1-150*	Tac	This study
pGEX-6p-1	*H3 1-38*	Tac	This study
pET-28b	*Sgo1*	T7	This study
pET-28b	*SUMO-Sgo1 Y317X*	T7	Min-Hao Kuo
pET-28b	*SUMO-Sgo1*	T7	Min-Hao Kuo
pGEX-4T-2	None	Tac	This study

Table 2- 5 Primers for cloning

Constructs	5' Primers	3' Primers
Cse4_FL	GCCCCTGGGATCCATGTCAAGTAAACAACAATGGGTTAGTTCTG	GCCCCTGGGATCCATGTCAAGTAAACAACAATGGGTTAGTTCTG
Cse4_1	TGGGATCCGCAGGAGACCAACAATCTATTAACG	CGCTCGAGCTAACTAGGAGTATATTTCTTTTCGACGC
Cse4_2	TGGGATCCCGTTATGAAAGCTCAAAAAGTGACC	CGCTCGAGCTAACTAGGAGTATATTTCTTTTCGACGC
Cse4_3	TGGGATCCGAAGCTGAAATGGAAACTGAAGTAC	CGCTCGAGCTAACTAGGAGTATATTTCTTTTCGACGC
Cse4_5	GGCCCCTGGGATCCTTATCGTTATTGCAGAGAACAAGAG	CGCTCGAGCTAACTAGGAGTATATTTCTTTTCGACGC
Cse4_6	GGCCCCTGGGATCCGCGACAAAGAACCTGTTTCC	CGCTCGAGCTAACTAGGAGTATATTTCTTTTCGACGC
Cse4_7	GGCCCCTGGGATCCAGAGAGGAAAGAAGACGTTATGA	CGCTCGAGCTAACTAGGAGTATATTTCTTTTCGACGC
Cse4_8	GGCCCCTGGGATCCGCAGGAGACCAACAATCTATTAAC	TGCGGCCGCTCGAGCTATTTTGAGCTTTCATAACGTCTTCT
Cse4_9	GGCCCCTGGGATCCGCAGGAGACCAACAATCTATTAAC	TGCGGCCGCTCGAGCTATGTCGCTCTTGTTCTCTGC

Table 2-5 Continued

Cse4_10	GGCCCCTGGGATCCAAGAACCTGTT TCCAAGAAGAGA	TGCGGCCGCTCGAGCTATTTTGAGCTTT CATAACGTCTTCT
Cse4_11	GATCCAGAGAGGAAAGAAGACGTT ATGAAAGCTCAAAATAAC	TCGAGTTATTTTGAGCTTTCATAACGTC TTCTTTCCTCTCTG
Cse4_12	GATCCAAGAACCTGTTTCCAAGAAG AGAGGAAAGAAGACGTTAAC	TCGAGTTAACGTCTTCTTTCCTCTCTTC TTGGAAACAGGTTCTTG
Cse4_13	GATCCAGAGAGGAAAGAAGACGTT ATGAAAGCTCAAAAAGTGACCTAG ATATCTAAC	TCGAGTTAGATATCTAGGTCACTTTTTG AGCTTTCATAACGTCTTCTTTCCTCTCT G
Cse4_14	GATCCTATGAAAGCTCAAAAAGTG ACCTAGATATCGAAACAGACTACG AATAAC	TCGAGTTATTCGTAGTCTGTTTCGATAT CTAGGTCACTTTTTGAGCTTTCATAG
Cse4 S33A	CGTCAACAGGCTTGCAGGAGACCA ACAAGCAATTAACGATCGTGCG	CGCACGATCGTTAATTGCTTGTTGGTCT CCTGCAAGCCTGTTGACG
Cse4 S33D	CGTCAACAGGCTTGCAGGAGACCA ACAAGACATTAACGATCGTGCG	CGCACGATCGTTAATGTCTTGTTGGTCT CCTGCAAGCCTGTTGACG

Table 2-5 Continued

Cse4 S124A	GGAGGGAAAAACAAAGAAAGCAG GCATTAAAGCGCGTCGAAAAG	CTTTTCGACGCGCTTTAATGCCTGCTTT CTTTGTTTTTCCCTCC
Cse4 S124D	GGAGGGAAAAACAAAGAAAGCAG GACTTAAAGCGCGTCGAAAAG	CTTTTCGACGCGCTTTAAGTCCTGCTTT CTTTGTTTTTCCCTCC
Cnn1 1-150	TGGGATCCATGAGCACTCCCAGGA AGG	CGCTCGAGCTAGCCTTTGTCTCTTTCCT GC
H3 1-38	TGGGATCCATGGCCAGAACAAAGC AAACA	CGCTCGAGTCACTTCTTAACACCACCG GT
Sgo1	TTATTGTTTGCATATGCCGAAGAGA AAAATTGCTC	AAAGACTAGAATTCATTTTTTGGTGCG ATATGTT

Table 2- 6 Mass spectrometry results (Sgo1)

| %Cov(95)	Contrib	Conf	Sequence	dMass	Prec MW	Prec m/z	Theor MW	Theor m/z
16	2	99	AVDYTLPSLR	-0.00231308	1133.605713	1134.613	1133.608032	1134.615356
16	2	99	FDEIFYMFENVR	-0.075920902	1560.648682	1561.656	1560.724854	1561.732178
16	2	99	ISQLVQENVTLR	-0.00751754	1398.775635	1399.783	1398.783081	1399.790405
16	2	99	KISQLVQENVTLR	-0.00909677	1526.868652	1527.876	1526.878052	1527.885376
16	2	99	LSNHENNLSHESSFNKDDGPDLEPK	-0.0394326	2822.234619	2823.242	2822.274414	2823.281738
16	2	99	LSNQLQVIENGIIQR	-0.0141868	1723.943726	1724.951	1723.95813	1724.965332
16	2	99	SLSQDSIPDEPQLR	-0.0136862	1583.765625	1584.773	1583.779175	1584.786377
16	2	99	TSISEAIYR	0.00429169	1038.538696	1039.546	1038.534546	1039.54187

Table 2- 7 Mass spectrometry results (Sgo1 proteolytic product)

%Cov(95)	Contrib	Conf	Sequence	dMass	Prec MW	Prec m/z	Theor MW	Theor m/z
16	2	99	AVDYTLPSLR	-0.00231308	1133.605713	1134.613	1133.608032	1134.615356
16	2	99	FDEIFYMFENVR	-0.075920902	1560.648682	1561.656	1560.724854	1561.732178
16	2	99	ISQLVQENVTLR	-0.00751754	1398.775635	1399.783	1398.783081	1399.790405
16	2	99	KISQLVQENVTLR	-0.00909677	1526.868652	1527.876	1526.878052	1527.885376
16	2	99	LSNHENNLSHESSFNKDDGPDLEPK	-0.0394326	2822.234619	2823.242	2822.274414	2823.281738
16	2	99	LSNQLQVIENGIIQR	-0.0141868	1723.943726	1724.951	1723.95813	1724.965332
16	2	99	SLSQDSIPDEPQLR	-0.0136862	1583.765625	1584.773	1583.779175	1584.786377
16	2	99	TSISEAIYR	0.00429169	1038.538696	1039.546	1038.534546	1039.54187

Figure 2- 12 Pull down experiment with high level of His6-Sgo1 and His6-SUMO-Sgo1. *In vitro* pull down assay were performed with cell lysate containing high level of His6-Sgo1 (A) or His6-SUMO-Sgo1. Purified full length GST-Cse4, GST-Cse4 deletion mutants were used to pull down bacterial His6-Sgo1 (A) or His6-SUMO-Sgo1 (B) from cell lysate. GST-Cnn1 tail was used as negative control as previously described. The 1% of total cell lysate (1% His6-Sgo1) and bound proteins on agarose beads were analyzed by western blot using α-GST monoclonal antibody and α-His6 monoclonal antibody. The asterisks mark the position of GST-tagging proteins.

Figure 2-12

A.)

Figure 2-12 Continued

B.)

WB: α-GST

His6-SUMO-Sgo1

WB: α-His6

Proteolytic products of His6-SUMO-Sgo1
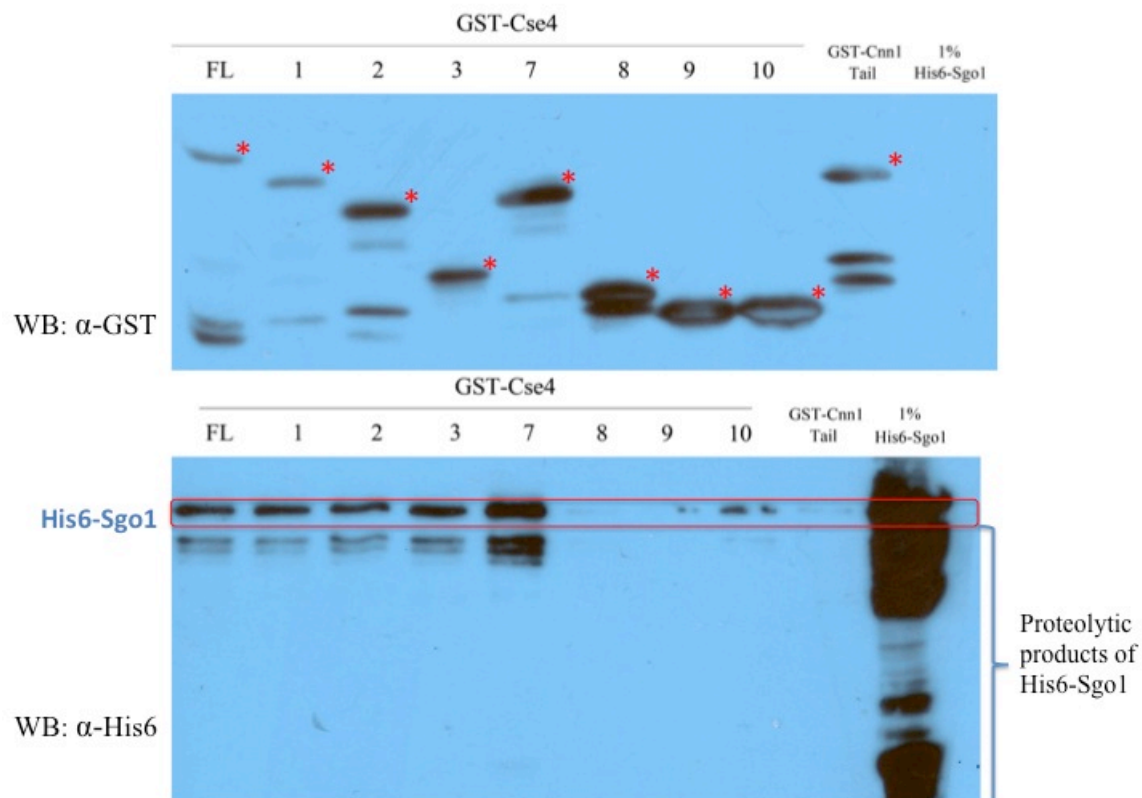
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