FUSED SISTER KINETOCHORES INITIATE THE REDUCTIONAL DIVISION OF MEIOSIS I & PHOSPHOSERINES ON HISTONE H3 AND CENH3 DEMARCATE THE CENTROMERE AND PERICENTROMERE DURING CHROMOSOME SEGREGATION

by

XUEXIAN LI

(Under the Direction of R. Kelly Dawe)

ABSTRACT

The kinetochore, a protein complex that assembles on the centromere, tethers the chromosome to the microtubules and plays fundamental roles in chromosome orientation and faithful segregation during cell division. Here we show that kinetochore structural component MIS12 forms a visible bridge between sister kinetochores that is required for reductional chromosome segregation in meiosis I. MIS12 and microtubule binder NDC80 appear as a bridge between sister kinetochores. In *Mis12* knockdown mutants, the visible MIS12/NDC80 bridge between sister kinetochores is lost, and chromosomes orient randomly. The outcome is severe meiosis II defects and overall meiotic failure. Meiosis-specific Rec8 cohesion and its protector Shugoshin (SGO) have also been implicated in controlling sister chromatid co-orientation in meiosis I. Our analysis shows that the MIS12 function is distinct from the Shugoshin/cohesion system between chromosome arms. The fusion of sister kinetochores by the MIS12/NDC80

bridge provides a unified microtubule binding interface and promotes sister chromatid co-segregation in meiosis I.

Second, we show that phosphorylation of H3 on serine 28 (phH3-Ser28) in maize is a cell cycle dependent and pericentromere-specific posttranslational modification. It is undetectable in interphase, becomes increasingly apparent with cell cycle progression, and disappears in telophase. A unique feature of H3-Ser28 phosphorylation is that it is strictly limited to the pericentromeric domains during cell division. Considering the densely distributed cohesion in this heterochromatic domain, H3-Ser28 phosphorylation may serve as an epigenetic marker to label the cohesive region. Interestingly, CENH3, a histone H3 variant exclusively recruited in the centromere, is phosphorylated on serine 50 (phCENH3-Ser50) following the same temporal pattern as H3Ser28 phosphorylation. Together, we propose that the primary role of the CENH3Ser50 and H3Ser28 phosphorylation is to demarcate the centromere and its flanking pericentromere domains during the cell division.

Lastly, we performed functional analysis of plant aurora kinases in the model plant *Arabidopsis thaliana*. Aurora kinases play pivotal roles in regulating the cell cycle in animals and yeast by phoshorylating versatile substrates including histone H3 and CENH3. Our data shows that plant aurora kinases have distinct functions implicated in cell division and many developmental pathways. Knockdown or overexpression of Arabidopsis aurora kinases leads to pleiotropic developmental defects in plant growth.

INDEX WORDS: Homolog segregation, co-orientation, Rec8 cohesin, Shugoshin, kinetochore, MIS12, NDC80, maize, phosphorylation, histone H3, centromere, CENH3, aurora kinase, *Arabidopsis thaliana*, RNAi, overexpression

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by

XUEXIAN LI

B.S., Shanxi Agricultural University, P. R. China, 1997

M.S., China Agricultural University, P. R. China, 2000

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTER OF PHILOSOPHY

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by

XUEXIAN LI

Major Professors: R. Kelly Dawe

Committee: Michael J. Scanlon
Michelle Momany
Edward Kipreos
Xiaoyu Zhang

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
May, 2009

DEDICATION

To my wife, my daughter, my parents, my sister and my brother for their love and support.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

PART A CELL DIVISION AND THE KINETOCHORE COMPLEX

Mitosis is a fundamental cytological process in which a eukaryotic cell divides its genetic information equally into two daughter cells. The entire process consists of four easily discernable substages called prophase, metaphase, anaphase, and telophase. Prophase is featured by striking chromosome thickening and shortening (chromosome condensation) (Figure 1.1 A); fully condensed chromosomes achieve alignment and bi-polar attachment at metaphase (Figure 1.1 B); anaphase initiates via the release of sister chromatid cohesion and sister chromosome migration towards poles (Figure 1.1 C); at telophase, each set of sister chromosomes nucleate near the spindle pole, and the new cell plate begins to form (Figure 1.1 D) (Nicklas, 1971; Cheeseman and Desai, 2008).

In contrast to direct sister chromatid segregation in mitosis, meiosis undergoes homologous chromosome segregation in meiosis I and sister chromatid separation in meiosis II, resulting in four genetically different gametes. Recombination (crossing-over) between homologous chromosomes is an essential meiosis I-specific event in early prophase when homologous chromosomes pair and synapse forming a bivalent of four chromatids (von Wettstein et al., 1984; Heyting, 1996; Dawe, 1998; Bass et al., 2000).

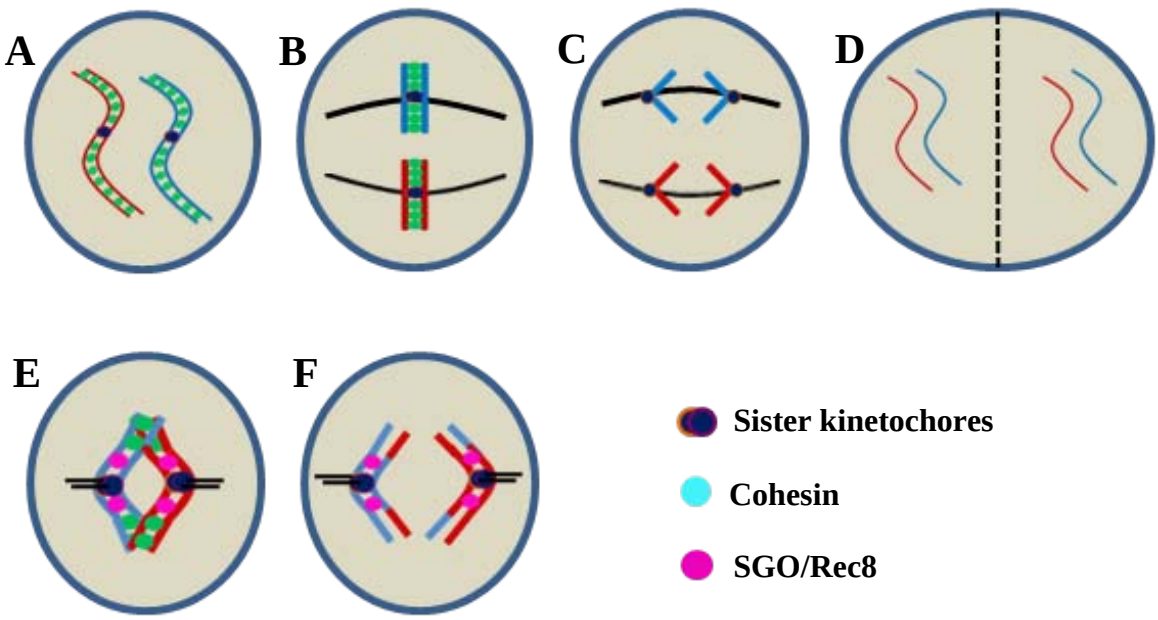

Figure 1.1 Cartoon of different substages in mitosis (A-D) and meiosis (E, F). A: prophase; B: metaphase; C: anaphase; D: telophase; E: metaphase I; F: anaphase I.

It not only provides new interchromosomal genetic combinations and passes them on to next generation; but also results in at least one chiasma between one set of homologues. Chiasmata function as a physical linkage to tether each bivalent together, facilitate sister chromatid co-orientation on metaphase plate (Figure 1.1 E), and remain in place until their resolution upon the removal of chromosomal arm cohesion at the onset of anaphase I (Figure 1.1 F) (Dawe, 1998; Petronczki et al., 2003; Kudo et al., 2006).

Another meiosis I specific cytological event is homologous chromosome segregation with sister chromatids remaining associated throughout the meiosis I. Homolog separation reduces the total number of chromosomes in a daughter cell to half of what it was in the preceding mitosis. Therefore, meiosis I is called the reductional chromosome segregation. Recombination is required for proper homolog segregation. Recombination failure leaves homologs free of chiasmata interlock, and achiasmatic homologs randomly segregate in meiosis I, leading to severe aneuploidy (trisomy or monosomy, the gain or loss of one copy of chromosome) and lethality of progeny (Koehler et al., 1996; Page and Hawley, 2003). In human, aneuploidy has been the major genetic cause of fetal death, mental impairment, and severe developmental defects (Hassold and Hunt, 2001).

In addition to chiasmata, stepwise loss of cohesion plays a critical role in homolog segregation in meiosis I. The cohesion is established by recruitment of the ring-shaped cohesin complex along entire chromosome axis to hold sister chromatids together (Guacci et al., 1997; Michaelis et al., 1997; Milutinovich and Koshland, 2003; Losada and Hirano, 2005). At the onset of anaphase I, cleavage of chromosome arm cohesion by separase releases chiasmata and allows homolog segregation; while sister chromatid association is protected by centromere cohesion until initiation of anaphase II (Figure 1.1 F) (Moore et al., 1998; Buonomo et al., 2000; Siomos

et al., 2001). Cytological and genetic data suggest meiosis-specific cohesin subunit Rec8 is required for maintaining sister chromatid association and promoting reductional segregation in meiosis I (Watanabe and Nurse, 1999; Buonomo et al., 2000; Kitajima et al., 2003; Lee et al., 2003). It localizes to pericentromeric regions and adjacent chromosome arms, and persists in centromere until anaphase II (Figure 1.1 F). Deletion of Rec8 causes precocious sister chromatid separation in meiosis I (Watanabe and Nurse, 1999). Further, Shugoshin (SGO) localizes in centromere region and collaborates with protein phosphatase 2A to protect Rec8 cohesin from phosphorylation and release during meiosis I (Kerrebrock et al., 1995; Lopez et al., 2000; Kitajima et al., 2004; Kitajima et al., 2006; Riedel et al., 2006). Knockout of Shugoshin dissociates Rec8 from centromere region at anaphase I, and leads to premature sister chromatid separation. In addition to SGO, Spo13 is also involved in centromeric cohesion protection (Katis et al., 2004; Lee et al., 2004).

The third, yet not well-addressed, key player in mediating reductional chromosome segregation may be the kinetochore, a large protein complex assembled on the centromere. It primarily serves as the attachment site for spindle microtubules, and generates signals for the checkpoint when improper attachment occurs. Genetic and biochemical analysis revealed around 80 kinetochore proteins required for faithful chromosome segregation (McAinsh et al., 2003; Meraldi et al., 2006; Cheeseman and Desai, 2008). These kinetochore components are further organized into three domains: inner kinetochore, central kinetochore, and outer kinetochore (Westermann et al., 2003). Inner kinetochore contains constitutive components including chromatin proteins and kinetochore foundation proteins. One of the well-known inner kinetochore proteins is CENH3, a histone H3 variant replacing canonical H3 in discontinuous chromatin domains in centromere core region exclusively (Ahmad and Henikoff, 2002). It is

conserved across all species studied, and is required for recruitment of many other inner kinetochore proteins (Palmer et al., 1987; Meluh et al., 1998; Takahashi et al., 2000; Blower and Karpen, 2001; Zhong et al., 2002). Purification of human CENH3 nucleosomes reveals 14 CENH3-dependent proteins including CENPC and CENP-H-I complex (Nishihashi et al., 2002; Foltz et al., 2006; Okada et al., 2006). CENH3 is required for proper cell cycle progression and chromosome segregation (Stoler et al., 1995; Blower and Karpen, 2001; Oegema et al., 2001; Regnier et al., 2005), and over-expression of CENH3 is sufficient to initiate ectopic kinetochores formation in Drosophila (Heun et al., 2006). Another well-studied inner kinetochore protein, CENPC, is a DNA and RNA binding protein (Sugimoto et al., 1994; Trazzi et al., 2002; Wong et al., 2007). Importantly, sequence analysis demonstrates that CENPC underwent adaptive evolution, perhaps in concert with centromere DNA sequence divergence (Talbert et al., 2004). All inner kinetochore proteins form a kinetochore foundation on the centromere for recruitment of more transient proteins in the central domain. Depletion of inner kinetochore proteins, such as CENH3, CENP-C, CENP-H, and CENP-I, leads to disruption of centromeric chromatin, chromosome misalignment, missegregation, or mitotic halt (Stoler et al., 1995; Howman et al., 2000; Fukagawa et al., 2001; Nishihashi et al., 2002; Kwon et al., 2007).

The central kinetochore forms a critical linkage between kinetochore and microtubules. It contains at least 3 biochemically distinct subcomplexes: the MIS12 complex (discussed in MIS12 section below), NDC80 complex, and KNL1 complex (Cheeseman and Desai, 2008). The NDC80 complex, a central component of microtubule-binding interface with four closely associated subunits, is required for reliable chromosome segregation (Wigge and Kilmartin, 2001). The heterotetrameric NDC80 complex forms a rod-like structure with one globular head directly binding to microtubules and another globular head facing the kinetochore (Wei et al.,

2005; Cheeseman et al., 2006; Wei et al., 2007). Recent data demonstrate NDC80's microtubule binding capability is conferred by an 80 aa disordered tail domain at the N-terminus (Guimaraes et al., 2008). The KNL complex is involved in the establishment of microtubule binding interface and chromosome alignment (Desai et al., 2003; Cheeseman et al., 2006; Cheeseman et al., 2008). KNL is also required for targeting of outer kinetochore proteins such as CENP-F and Zwint. Both MIS12 complex and KNL complex are physically associated with NDC80 complex, and are required for NDC80 complex recruitment (Kline et al., 2006; Cheeseman et al., 2008).

The outer kinetochore accommodates three classes of regulatory components: spindle checkpoint proteins such as MAD2 (Sullivan, 2001; Vos et al., 2006), passenger proteins such as Aurora kinase B (Vagnarelli and Earnshaw, 2004), and motor proteins such as CENP-E (Fukagawa, 2004). Therefore, the outer kinetochore functions as a surveillance system during cell division to ensure proper chromosome attachment on one hand, and facilitate chromosome movement by associated motors on the other hand. MAD2 localizes to the unattached kinetochores after chromosome condensation as a spindle checkpoint component to monitor microtubule-kinetochore attachment, and delocalizes from the properly attached kinetochores at metaphase (Chen et al., 1996; Li and Benezra, 1996; Shah and Cleveland, 2000; Logarinho et al., 2004). Occasionally, sister kinetochores are mono-polar attached in mitosis, which retains MAD2's kinetochore localization until the attachment errors are corrected and bi-polar attachment is established (Saitoh et al., 2008). Beyond a checkpoint component, aurora B kinase promotes kinetochore bi-polar attachment and destabilizes incorrect attachment in mitosis (Tanaka et al., 2002; Hauf et al., 2003; Lampson et al., 2004; Morrow et al., 2005; Cimini et al., 2006). In striking contrast, aurora B kinase controls sister chromatid co-orientation by protecting centromeric cohesion from cleavage in meiosis I (Rogers et al., 2002; Hauf et al., 2007; Monje-

Casas et al., 2007; Yu and Koshland, 2007). Similarly, CENP-E also regulates mitotic checkpoint signaling (Abrieu et al., 2000; Yao et al., 2000; Mao et al., 2005), and ensures stable microtubule-kinetochore attachment (Putkey et al., 2002). Importantly, CENP-E is a plus end-directed motor providing a motile kinetochore tether to microtubules, and powers chromosome congression (Kim et al., 2008). Loss-of-function of transitory proteins results in various cytological defects in spindle checkpoint, chromosome segregation, or mitotic arrest (Chen et al., 1996; Wojcik et al., 2001; Putkey et al., 2002).

In plants, maize is a well-established model organism in kinetochore study largely due to its excellent cytology. Maize CENH3 interacts with CentC (a centromere specific satellite repeat) and CRM (maize centromere specific retrotransposable element), and co-localizes with another inner kinetochore protein CENPC (Dawe et al., 1999; Zhong et al., 2002). Interestingly, CENH3 is also tightly associated with CentC and CRM transcripts, suggesting that RNA is an integral part of centromere/kinetochore complex (Topp et al., 2004). NDC80 localizes outside of the CENH3 and CENPC as expected, but is a constitutive component in maize (Du and Dawe, 2007). Outermost is MAD2, which is sensitive to microtubule attachment in mitosis, but sensitive to tension in meiosis (Yu et al., 1999).

Numerous data show that a fully functional kinetochore is required for faithful chromosome segregation, and disruption of kinetochore assembly leads to chromosome congression errors, alignment defects, and chromosome mis-segregation (Amor et al., 2004; Cheeseman and Desai, 2008). A subset of kinetochore proteins may specifically mediate mono-polar attachment of sister chromatids in meiosis I. One such protein is monopolin, a meiosis specific kinetohcore protein required for homolog segregation in S. *cerevisiae* meiosis I (Toth et al., 2000). Monopolin forms a complex with Csm1 and Lrs4 to mediate sister chromatid co-

segregation presumably via clamping two sister's microtubule binding sites together (Rabitsch et al., 2003). The monopolin complex is sufficient to promote sister chromatid co-segregation when artificially expressed during mitosis (Monje-Casas et al., 2007). The identification of monopolin highlights the essential role of kinetochores in controlling meiotic chromosome behavior. Unfortunately, monopolin does not have the same role in fission yeast S. *pombe*, nor is monopolin homolog found outside of fungi. It remains mysterious whether and what kinetochore proteins play key roles in reductional chromosome segregation in meiosis I across species. It's plausible to postulate that other proteins involved in microtubule binding and their immediate associates may function to connect the sister kinetochores in a manner similar to monopolin. MIS12 genetically and physically interacts with microtubule binder NDC80 complex, and has a dual role in promoting inner and outer kinetochore assembly (Kline et al., 2006; Wei et al., 2007); and is one candidate for mediating the association of sisters.

MIS12, first identified in fission yeast, plays a fundamental role in maintaining the kinetochore structure, establishing microtubule attachment, and ensuring proper chromosome segregation (Goshima et al., 1999; Goshima et al., 2003; Westermann et al., 2003; Tanaka et al., 2005). Mis12 depletion leads to disassociation of inner kinetochore proteins CENP-H and CENP-I, and chromosome mis-segregation (Fukagawa et al., 2001; Nishihashi et al., 2002). The budding yeast MIS12 homologue Mtw1p localizes to the kinetochore throughout the cell cycle (Pinsky et al., 2003), and physically associates with the centromeric DNA (Goshima and Yanagida, 2000). It is required for cell viability and determination of metaphase spindle length. Mtw1 mutations result in high frequency of unequal chromosome segregation and 50% longer metaphase spindles (Goshima and Yanagida, 2000).

MIS12 localizes to kinetochore in a CENH3 independent pathway and associates with the centromeric DNA (Goshima and Yanagida, 2000; Goshima et al., 2003). Cytological and biochemical analysis reveals that MIS12/Mtw1p forms a complex with Nnf1p, Dsn1p, and Nsl1p in an interdependent manner (Euskirchen, 2002; Kline et al., 2006). The MIS12 complex contributes to the assembly of the inner kinetochore and outer kinetochore regions and is indispensable for localization of NDC80 complex and KNL complex (Kline et al., 2006; Cheeseman et al., 2008).

Notably, MIS12/Mtw1p is necessary for kinetochore bi-orientation on the mitotic spindle. It plays a role in maintaining physical tension derived from the bipolar attached sister kinetochores and stabilize the proper attachment (Pinsky et al., 2003). Mutation of Mtw1 leads to defects in bi-orientation, unattached chromosomes, and subsequent delayed metaphase due to spindle checkpoint activation. Thus, MIS12 was speculated to play a role in homolog segregation via coordinating sister kinetochore co-orientation in meiosis I. To gain direct evidence of MIS12 function in this intriguing biological process, we choose maize as our experimental system due to its high resolution cytology, beautiful genetics, and a handful of characterized kinetochore markers (Doebley, 1998; Cone et al., 2002; Hamant et al., 2006). Indeed, maize is a long-standing model organism in cytogenetics in addition to its continuous cultivation as one of the major crops (Doebley, 2004; Candela and Hake, 2008). Important historic events in genetics are attributed to pioneering work on maize. In 1931, the genetic recombination was correlated to chromosome crossing-over by close observation on maize meiosis (Creighton and McClintock, 1931). Recently, the power of maize cytology has been envisioned again with the development and improvement of techniques such as immunocytochemistry and fluorescent *in situ* hybridization (FISH) (Kato et al., 2004; Jiang and Gill, 2006; Shi and Dawe, 2006). This is

particularly powerful in centromere and kinetochore studies, where cytogenetics, genetics and genomics have been applied to make important advances. (Dawe et al., 1999; Jin et al., 2004; Jin et al., 2005; Pawlowski et al., 2009).

PART B HISTONE H3 AND CENH3 PHOSPHORYLATION, AND AURORA KINASE B

The nucleosome is the fundamental chromatin unit consisting of a histone octamer (two copies of histone H2A, H2B, H3 and H4) wrapped around by 146 bp DNA. The core histones not only serve as essential structural components to make up chromatin, but also accommodate arrays of covalent modifications that function spatially and temporally to regulate chromatin remodeling, transcription and silencing (Jenuwein and Allis, 2001; Goldberg et al., 2007; Li et al., 2008; Osley, 2008; Smith and Shilatifard, 2009). Histone H3 phosphorylation is an extensively studied covalent modification with a dynamic pattern during cell division. Four histone H3 residues, serine 10 (Ser10), serine 28 (Ser28), threonine 3 (Thr3), and threonine 11 (Thr11), are phosphorylated, and all display a similar cell cycle dependent pattern. Phosphorylation is undetectable at interphase, but becomes apparent in prophase. Maximal phosphorylation correlates to chromosome alignment and a 'ready-to-go' status at metaphase. Phosphorylation declines during anaphase and finally disappears in telophase (Hendzel et al., 1997; Gernand et al., 2003; Preuss et al., 2003; Polioudaki et al., 2004; Houben et al., 2005).

The phosphorylation of histone H3Ser10 and H3Ser28 share very similar spatial pattern in mammals. Both phosphorylation events initiate in pericentromeres, then spread along chromosome arms in prophase and correlate with chromosome condensation (Hendzel et al.,

1997; Goto et al., 1999). Early papers revealed that chromosome condensation defects were caused by mutation of H3Ser10 or peptide competition (Van Hooser et al., 1998; Wei et al., 1998; de la Barre et al., 2000). However, this was disputed by the fact that the same modifications in plants show different temporal and spatial patterns. First, H3Ser10 and H3Ser28 phosphorylation is undetectable until late prophase in plants, which is after the initiation of chromosome condensation during cell division (Kaszas and Cande, 2000; Gernand et al., 2003). Second, mutation of H3Ser10 in S. *cerevisiae* causes no detectable chromosome condensation defects during cell cycle (Hsu et al., 2000). Third, H3Ser10 phosphorylation along the chromosome arms during meiosis I correlates with the distribution of cohesion in plants; H3Ser10 and H3Ser28 phosphorylation is restricted to the pericentromere during mitosis and meiosis II, mirroring the cohesion distribution pattern (Houben et al., 1999; Kaszas and Cande, 2000; Manzanero et al., 2000; Gernand et al., 2003). It now appears, that H3Ser10 and H3Ser28 phosphorylation may be related to sister chromatid cohesion in plants (Kaszas and Cande, 2000; Gernand et al., 2003).

Similarly, H3Thr3 and H3Thr11 phosphorylation has different spatial pattern in plants from that in animals and has been implicated in different functions. H3Thr3 and H3Thr11 phosphorylation is mainly enriched in the centromere region in mammalian cells, and might serve as an epigenetic mark for centromere assembly (Preuss et al., 2003; Dai et al., 2005; Dai et al., 2006). However, in plant cells staining is correlated with chromosome condensation (Houben et al., 2005).

In addition to the close relationship of H3Ser10 phosphorylation with chromosome dynamics, it also plays roles in regulating chromatin state and gene transcription. HP1 (heterochromatin protein 1) is recruited onto chromatin regions with H3 lysine 9 trimethylation,

but displaced when H3Ser10 is phosphorylated (Fischle et al., 2005; Hirota et al., 2005). In ovarian granulose cells, H3Ser10 phosphorylation and H3Lys14 acetylation collaboratively activate the expression of cellular differentiation genes (DeManno et al., 1999; Salvador et al., 2001). H3Ser10 phosphorylation also collaborates with H3Thr3 phosphorylation to activate the downstream stress response in tobacco cells under sucrose and salt deficient conditions (Houben et al., 2007). Taken together, the available data suggest that histone H3 phosphorylation is involved in gene regulation by its own or, sometimes, in collaboration with other covalent modifications. However, all known histone H3 phosphorylation events mostly occur along chromosome arms or the pericentromeres, and are most closely associated with cell cycle progression, chromosome condensation and chromatid cohesion.

The centromere is known as a primary constriction on condensed chromosomes, where the kinetochore builds up for microtubule attachment. With the exception budding yeast (with 125 bp non-repetitive centromeres), centromeres are characterized by repetitive nature and divergence across species (Vafa and Sullivan, 1997; Zhong et al., 2002; Sun et al., 2003; Dawe and Henikoff, 2006). In higher eukaryotes, centromeres contain megabases of tandem repeat arrays interspersed by long retroelement clusters (Schueler et al., 2001; Jin et al., 2004). Retroelements may play fundamental roles in centromere formation and evolution (Chueh et al., 2005). Chromatin immunoprecipitation and fiber FISH analysis suggest that maize centromere specific retroelements (CR) are integral component of maize centromere core (Topp et al., 2004). Nevertheless, centromeric satellites and CR elements are not necessarily required to assemble functional centromeres, suggesting that centromere speciation is primarily a epigenetic process and epigenetics has a primary role in recruiting inner kinetochore proteins (Choo, 2001; Dawe and Henikoff, 2006; Allshire and Karpen, 2008; Nakano et al., 2008).

The common feature of centromeres across species is CENH3 deposition by replacement of canonical histone H3. Centromeric histone H3 (CENH3) is found exclusively in the centromere. CENH3 has a conserved histone-fold domain that interacts with other core histones but has a flexible N-terminal tail that is involved in centromere speciation (Henikoff et al., 2000). CENH3 deposition is independent of replication, but coupled with centromere transcription during interphase, and then serves as a centromeric epigenetic marker and kinetochore assembly platform (Choo, 2001; Ahmad and Henikoff, 2002; Amor et al., 2004; Dawe and Henikoff, 2006). CENH3-containing nucleosomes are organized in blocks that are often interspersed with H3-rich domains (Blower et al., 2002). This may facilitate centromeric chromatin arrangement, perhaps to promote loop or spiral structure that exposes and lines up CENH3 domains for kinetochore buildup (Choo, 2001; Blower et al., 2002; Chueh et al., 2005).

Given the general prevalence of covalent modifications on histones, it is plausible to speculate that CENH3 is also exposed to extensive modifications. Phosphorylation would be particularly likely because it is associated with chromosome dynamics during cell division. Indeed human CENH3 (CENPA) is phosphorylation on the serine 7, a serine that does not exist on H3 (Zeitlin and colleagues, 2001). Following the same temporal pattern as other known phosphorylation events, CENPA phosphorylation initiates and increases in prophase, maximizes in metaphase, then drops in anaphase. Beyond a phospho-mark on centromere, CENPA phosphorylation on Serine 7 is required for proper chromosome alignment (Zeitlin et al., 2001b; Zeitlin et al., 2001a; Kunitoku et al., 2003). Aurora A kinase binds to CENPA in prophase to initiate phosphorylation, then aurora B maintains CENPA phosphorylation (Kunitoku et al., 2003). It has been proposed that CENPA may serve as a docking site for aurora B which functions to correct improper kinetochore-microtubule attachment. To further test whether a

'phospho-code' applies to the centromeres in general and to determine what biological roles may play during cell division, it is important to identify CENH3 phosphorylation events in other species.

Another interesting question beyond H3 and CENH3 phosphorylation is what kinase phosphorylates H3 and CENH3 in plants? Aurora kinase B appears to be the primary histone kinase. In humans there are three Aurora kinases, with aurora B kinase being known to have essential roles in regulating chromosome segregation. Aurora B localizes to the centromeric region from G2 to metaphase, phosphorylates human CENH3 on serine 7, and ensures proper microtubule attachment by correcting improper microtubule attachments, then moves to the central spindle in anaphase (Vagnarelli and Earnshaw, 2004). Aurora B also phosphorylates H3Ser10 and H3Ser28 in other species including *S. cerevisiae*, *C. elegans*, and mammals (Crosio et al., 2002; Goto et al., 2002). Mutation or knock-down of aurora B decreases H3Ser10 and H3Ser28 phosphorylation *in vivo* (Hsu et al., 2000; Giet and Glover, 2001; Goto et al., 2002).

In Arabidopsis, aurora kinases are identified as Ataurora1, Ataurora2, and Ataurora3, which share a conserved catalytic domain similar to animal aurora kinases (Demidov et al., 2005; Kawabe et al., 2005; Kurihara et al., 2006). Arabidopsis aurora kinases are preferentially transcribed in actively dividing tissues such as floral buds and young roots. GFP-tagged Ataurora1 and Ataurora2 have similar dynamic localization patterns: they localize to spindle poles at prophase, move to the spindle midzone, then back to the spindle poles. GFP-Ataurora1 also seems to colocalize with centromeres. GFP-Ataurora1 moves to the mid-phragmoplast by the end of anaphase and then disappears by the end of the cell cycle similar to GFP-Ataurora2. In contrast, GFP-Ataurora3 signals appear as dots around the nucleolus and nuclear periphery in early prophase, concentrate and align at centromeric regions during metaphase, remain there

until the onset of anaphase, and then diffuse into cytoplasm. Functionally, Ataurora1 phosphorylates H3Ser10, but not H3Ser28, as detected by immunostaining and an *in vitro* kinase assays (Demidov et al., 2005; Kawabe et al., 2005). Ataurora3, however, has been shown to mediate the phosphorylation of both H3Ser10 and H3Ser28 *in vitro*, and is inhibited by the aurora kinase inhibitor Hesperadin (Kurihara et al., 2006). It remains unclear whether Ataurora1 or Ataurora3 phosphorylates H3Ser10 and H3Ser28. An absence of *in vivo* analysis of aurora kinase function makes clear functional classification of three plant aurora kinases difficult.

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CHAPTER 2

MIS12, A STRUCTURAL ELEMENT OF THE KINETOCHORE, MEDIATES THE CO-ORIENTATION OF SISTERS IN MEIOSIS I

Li. X and Dawe. R.K. Submitted to Nature Cell Biology, 3/22/2009.

ABSTRACT

Meiosis I is featured by reductional chromosome segregation: sister chromatids co-orient and move to one spindle pole, instead of separating to opposite poles. The kinetochore, a protein complex that assembles on the centromere, tethers the chromosome to the microtubules, and plays fundamental roles in chromosome orientation and faithful segregation during cell division. Here we show that sister chromatids fail to co-orient in plants with reduced quantities of the MIS12 protein. Crosslinked chromatin immunoprecipitation (XChIP) analysis suggests MIS12 is a centromeric protein. It localizes outside of the inner kinetochore protein CENPC, and forms a visible bridge between sister kinetochores. So does microtubule binder NDC80. In *Mis12* knockdown mutants, the visible MIS12/NDC80 bridge between sister kinetochores is lost, and chromosomes orient randomly. The outcome is severe meiosis II defects and overall meiotic failure. Meiosis-specific Rec8 cohesion and its protector Shugoshin (SGO) have also been implicated in controlling sister chromatid co-orientation in meiosis I. Our analysis shows that the MIS12 function is distinct from the Shugoshin/cohesion system between chromosome arms. The fusion of sister kinetochores by the MIS12/NDC80 bridge provides a unified microtubule binding interface and promotes sister chromatid co-segregation in meiosis I.

INTRODUCTION

One of the major differences between meiosis and mitosis is the homologous chromosome segregation in meiosis I that reduces chromosome number in a mother cell by half (Petronczki et al., 2003; Allshire, 2004; Hamant et al., 2006). To make homologous chromosomes segregate properly, at least three cellular functions participate in a coordinative manner. The first function is conferred by the chiasmata derived from the homologous chromosome recombination (von Wettstein et al., 1984; Heyting, 1996; Bascom-Slack et al., 1997). Chiasmata mechanistically link homologous chromosomes together, sustain tension when homologous chromosomes get attached by microtubules, and facilitate establishment of mono-polar attachment of sister chromatids at metaphase I (Figure 1.1). Recombination failure leads to random homolog segregation and severe aneuploidy (Koehler et al., 1996; Hassold and Hunt, 2001; Page and Hawley, 2003; Kouznetsova et al., 2007). The second function is conferred by the cohesin complex which is recruited along the chromosome axis to hold sister chromatids together (Toyoda et al., 2002; Gillespie and Hirano, 2004; Haering et al., 2008). Chromosome arm cohesion is dissolved immediately before anaphase I onset to resolve chiasmata and release homologous chromosomes towards opposite poles; while the meiosis-specific Rec8 cohesin is protected from cleavage by Shugoshin (SGO) throughout the metaphase II (Molnar et al., 1995; Stoop-Myer and Amon, 1999; Watanabe and Nurse, 1999; Buonomo et al., 2000; Kitajima et al., 2004; Hamant et al., 2005). Either Rec8 or SGO deletion leads to sister chromatid separation in meiosis I.

The third but pivotal function is conferred by the kinetochores, protein complexes that tether chromosomes to microtubules (Brar and Amon, 2008; Cheeseman and Desai, 2008). The

primary function of the kinetochore is to establish and maintain proper association of chromosomes with the highly dynamic microtubules. Sister kinetochores are bi-polar attached to microtubules in mitosis and meiosis II, which favors sister chromatid equational segregation. However, in meiosis I, the sister kinetochores must be attached to microtubules from the same spindle pole in agreement with sister chromatid co-segregation (Toth et al., 2000; Tanaka et al., 2002; Dewar et al., 2004; Katis et al., 2004; Lampson et al., 2004). If sister kinetochores are bipolar attached, and this amphitelic attachment escapes from surveillance system, sister chromatids instead of homologous chromosomes separate in meiosis I, which eventually causes aneuploidy (Hassold and Hunt, 2001; Kouznetsova et al., 2007; Monje-Casas et al., 2007). Given the complexity of kinetochore-microtubule interaction, sustainability of this dynamic interaction, and accuracy of the specific interaction mode in meiosis and mitosis, there should be a subset of kinetochore proteins directly modulating kinetochore-microtubule interaction to ensure high-fidelity homolog segregation in meiosis I.

The discovery of monopolin complex, a three units complex required for sister chromatid co-orientation in *S. cerevisiae*, provides solid evidence for kinetochore's critical role in regulating reductional chromosome segregation (Toth et al., 2000; Rabitsch et al., 2003; Monje-Casas et al., 2007). The meiosis specific kinetochore protein Mam1 (monopolin) recruits the other two nuclear proteins Lrs4 and Csm1, and functions as a sister kinetochore clamp to suppress the bi-orientation of sister kinetochores in meiosis I (Rabitsch et al., 2003). Mutation of Mam1 leads to sister kinetochore bipolar attachment, and homologous chromosomes are restrained (lag) at anaphase I due to the remaining pericentromeric cohesion. Knockout of Lrs4 or Csm1 leads to prolonged anaphase I and abnormal nuclear division. Nonetheless, monopolin complex does not confer the same meiosis specific function in more complex fission yeast *S.*

pombe. Pcs1, the Csm1 homologue in *S. pombe*, is only required for centromere integrity maintenance, and Pcs1 null mutants show lagging chromosomes in meiosis II and mitosis (Gregan et al., 2007). Beyond fungi, there are no apparent monopolin homologs (Pidoux and Allshire, 2003; Rabitsch et al., 2003). Failure to identify monopolin orthologs in higher eukaryotes leaves the key question largely unanswered: what kinetochore proteins are modulators in homolog segregation? Of particular interest are the kinetochore structural components that maintain kinetochore integrity and the kinetochore proteins that directly interact with microtubules.

MIS12 and NDC80 are among the best candidates in this regard. The NDC80 complex is conserved across all species studied (Wigge and Kilmartin, 2001; Wei et al., 2005; Cheeseman et al., 2006; Du and Dawe, 2007). The rod-like NDC80 complex directly binds to microtubules with one globular head and interact with other kinetochore complexes with another globular head (Wei et al., 2007). In vitro analysis demonstrates the capability of NDC80 complex to establish persistent dynamic microtubule attachment, generate force by taking advantage of microtubule disassembly, and couple cargo to dynamic microtubules (Powers et al., 2009). MIS12 was first identified in the fission yeast *S. pombe* as a constitutive kinetochore protein necessary for faithful chromosome segregation (Takahashi et al., 1994; Goshima et al., 1999). *Mis12* mutation disrupts inner centromere structure and expands metaphase spindle length. MIS12 is also required for kinetochore assembly and proper chromosome segregation in animals (Goshima et al., 1999; Goshima et al., 2003b; Kline et al., 2006). Further, MIS12 localizes to the kinetochore independent of CENH3. It recruits NDC80 complex and mediates kinetochore-microtubule attachment via NDC80 complex (Cheeseman et al., 2006; Kline et al., 2006; Wei et al., 2007; Ciferri et al., 2008).

S. cerevisiae has been an outstanding model system for studying kinetochore biology due to its tiny 125 bp centromere and simple kinetochore-microtubule interaction (only 1 microtubule per kinetochore). However the simplicity of kinetochore-microtubule interaction in *S. cerevisiae* may potentially undermine the power of this model organism in exploring kinetochore components controlling homolog segregation. A cytological model organism such as maize may be required to address this issue. Maize is an excellent cytogenetic model with high cytological resolution and unique set of genetic markers. Over the past decade, research in maize greatly advanced our understanding of plant kinetochore components, organization, and functions (Dawe et al., 1999; Yu et al., 1999; Zhong et al., 2002a; Dawe et al., 2005; Hamant et al., 2005; Du and Dawe, 2007). The abundance of maize male meiotic cells, high cytological resolution of maize meiosis, and unique set of kernel markers make maize a particularly powerful model to identify kinetochore proteins mediating homolog segregation in meiosis.

RESULTS

There are two *Mis12* genes in the maize genome.

Two maize *Mis12* genes (MAGI4_132977 and MAGI4_143787; AC155386.2) were identified by tBlastx search against the NCBI database. Their cDNAs were then obtained by RT-PCR and shown to have 89% sequence identity (Figure 2.1). *Mis12-1* contains 6 exons and encodes a ~ 24.7 KDa protein of 223 amino acids; while *Mis12-2* contains 7 exons and encodes a ~ 27.2 KDa protein of 244 amino acids. The major difference between two *Mis12* genes is derived from a point mutation of C_{670} to T_{670}, which makes a premature stop codon TAA on exon 6 in *Mis12-1*. We speculate that two Mis12 genes may have tissue specific expression pattern, and RT-quantitative PCR (RT-qPCR) with ubiquitin as internal control was performed to check two gene's relative transcript abundance in four different tissues: leaf, root, tassel (male flower) and ear (female flower). Surprisingly, the two genes have no tissue preference, and are uniformly expressed in all four tissues (data not shown). But *Mis12-2* transcripts are over 30 times more abundant than *Mis12-1* transcripts in the W23 inbred line. Protein sequence alignment of two *Mis12* genes shows 81% amino acid identities between two proteins and both proteins share weak homology with yeast and human MIS12 homologues (Goshima et al., 1999; Kline et al., 2006) (Figure 2.2).

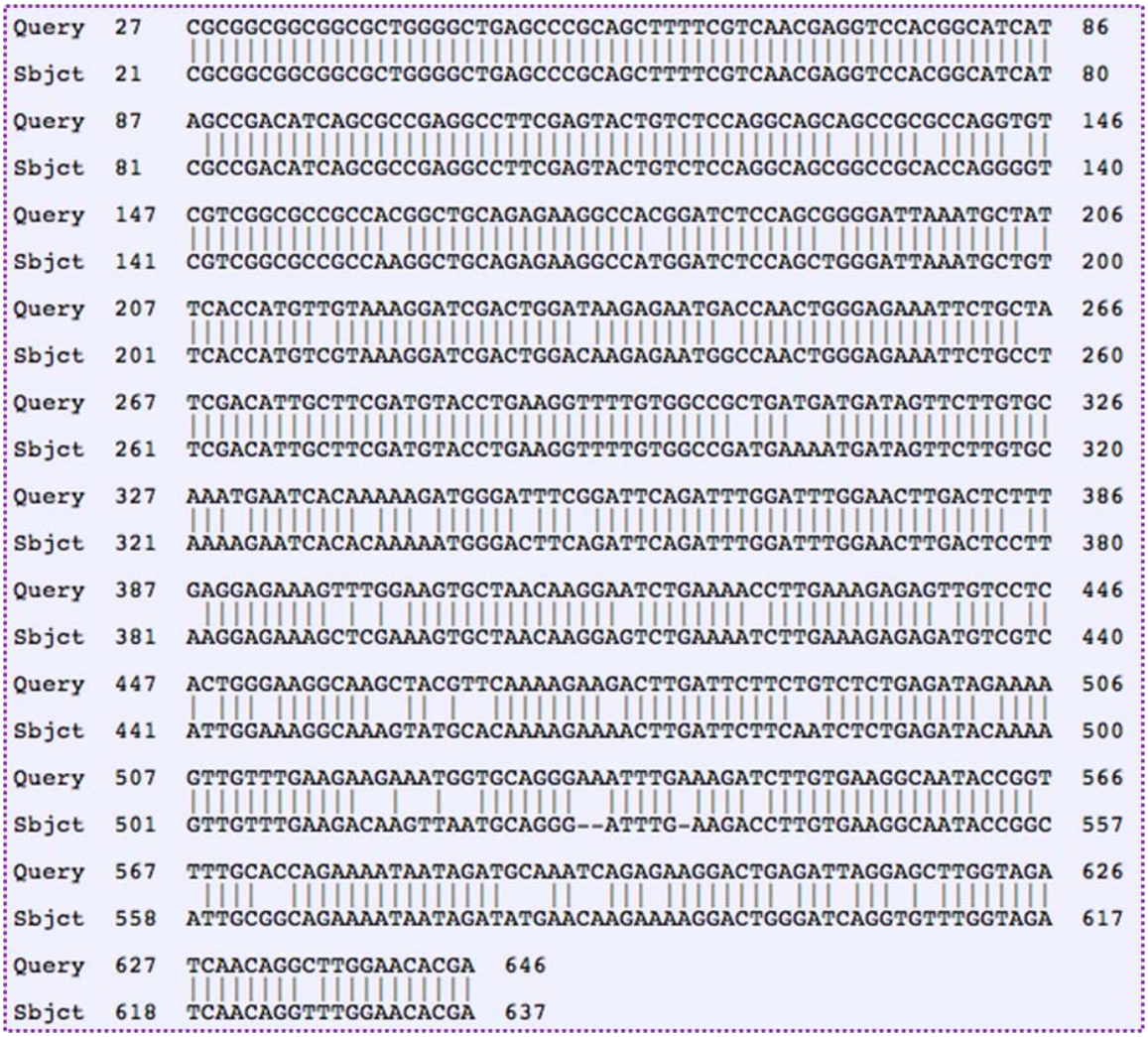

Figure 2.1 High level of sequence identities between maize *Mis12-1* and *Mis12-2* cDNA sequences. The sequence alignment shows 89% identity between *Mis12-1* (query) and *Mis12-2* (sbjct) cDNA coding sequences.

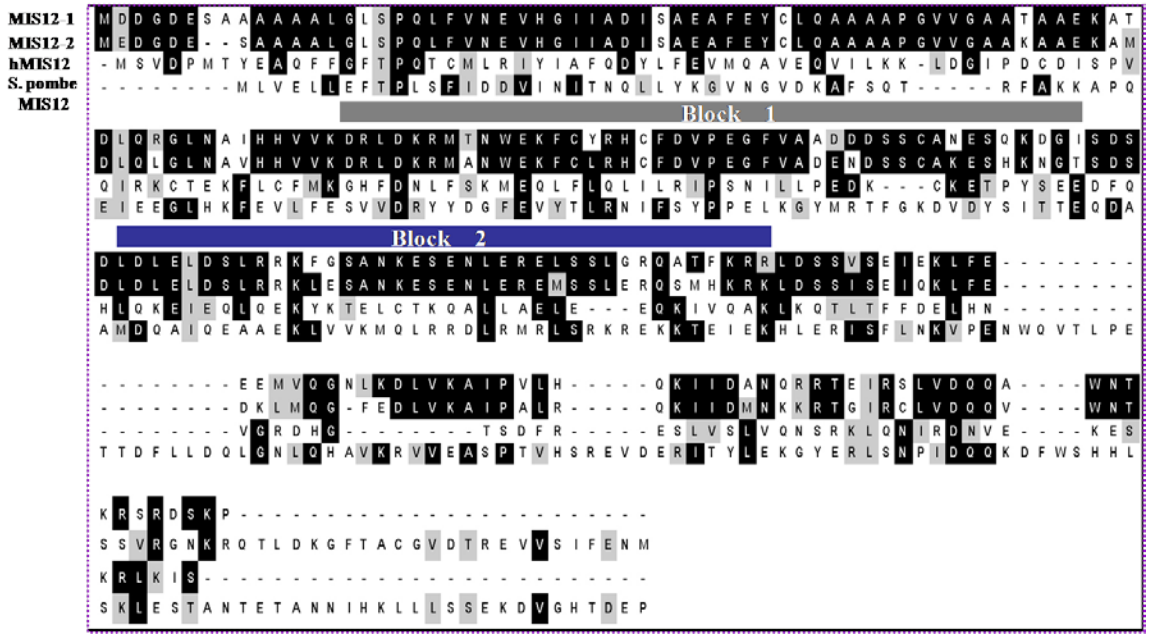

Figure 2.2 Weak homology between maize MIS12s and their counterparts in yeast and humans. Only six amino acids are identical across species, but two conserved blocks (around 50aa per block) were identified based on block maker (Henikoff et al., 1988).

Anti-MIS12 antibodies recognize a 25~27 kDa MIS12 protein in maize nuclear protein.

The complete *Mis12-2* coding sequence was cloned into the pET-28a expression vector and overexpressed in bacteria. The His-tagged MIS12-2 protein was purified by Ni-NTA agarose and used to generate polyclonal antibodies in rabbits. To test whether the resulting MIS12 antibodies recognize both MIS12 proteins, we first did immunoblotting analysis with His-tagged MIS12-1 and MIS12-2, and anti-MIS12-2 antibodies do recognize both His-tagged MIS12 proteins (Figure 2.3 A). The specificity of the antibodies was further tested using maize root and ear nuclear protein extracts. Given the capability of MIS12-2 antibodies to recognize both recombinant MIS12 proteins and obvious size difference between two MIS12 proteins, we expect to see two bands, presumably one strong band (MIS12-2) and one weak band (MIS12-1). However, the MIS12 antibodies recognize native MIS12 specifically as a single band in two different tissues, suggesting that the MIS12 antibodies are specific to MIS12 proteins (Figure 2.3 B). The missing band should correspond to MIS12-1 and it's undetectable in our assay due to its less abundance. Similarly, immunostaining analysis with the same antibodies may just visualize dominant MIS12-2 protein.

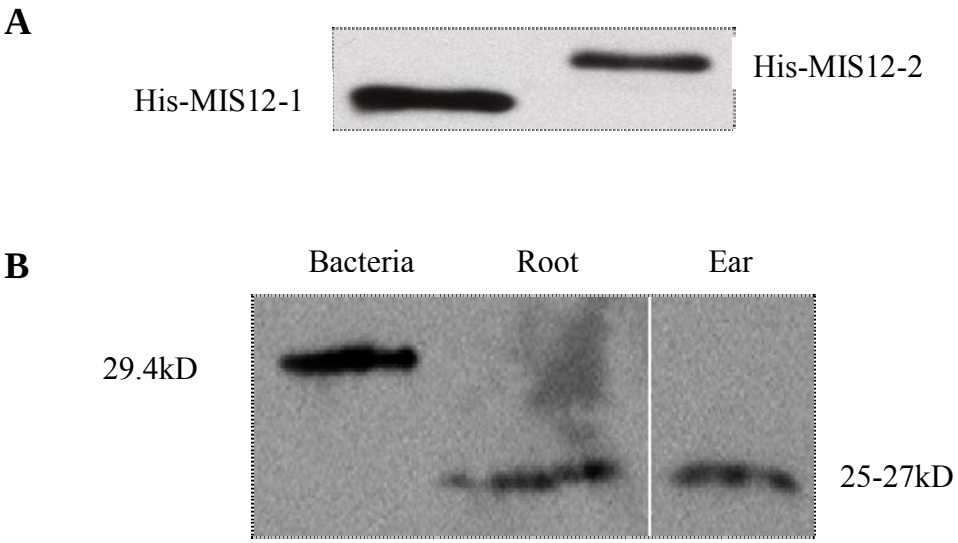

Figure 2.3 Anti-MIS12 antibodies are specific to MIS12 proteins**.** A) MIS12 antibodies recognize both His-tagged MIS12-1 and His-tagged MIS12-2. B) MIS12-2 antibodies recognize a 25-27 kD band in maize root and ear nuclear protein, and recombinant MIS12-2 (HIS tagged) is also detected in bacterial protein extracts.

Maize MIS12 signals are present on chromosomes as unique spots throughout the cell cycle.

The subcellular localization of MIS12 was analyzed in maize male meiotic cells and root tip mitotic cells by immunostaining analysis. MIS12 signals are distributed in the nucleus as distinct spots in interphase (Figure 2.4 A, J). During meiosis and mitosis, MIS12 signals are present on each chromosome as a unique spot throughout the cell cycle (Figure 2.4 B-I; K, L). In somatic C-metaphase (chemical-disrupted metaphase), two MIS12 spots pair nicely on each fully condensed chromosome, indicating bi-oriented sister kinetochores at metaphase (Figure 2.4 L). To confirm that maize MIS12 is a kinetochore protein, we use well-characterized maize kinetochore markers CENH3 and CENPC to do double labeling analysis (Dawe et al., 1999; Zhong et al., 2002a). MIS12 signals (red) almost perfectly overlap with CENH3 (green, Figure 2.5 C) and CENPC (green, Figure 2.5 F) signals on diplotene and pachytene chromosomes. These data suggest that MIS12 is a constitutive component of the kinetochore in maize as in all other studied species.

Maize MIS12 localizes outside of inner kinetochore protein CENPC

The kinetochore structure is highly dynamic and elastic during cell division. At metaphase I, sister kinetochores co-orient and are attached to the microtubules from the same spindle pole. Kinetochores under tension are stretched and generally show a subdomain structure (at metaphase or early anaphase). To further examine MIS12's relative position on the kinetochore, we double label MIS12 with CENPC, a well-known inner kinetochore marker (Dawe et al., 1999; Amor et al., 2004).

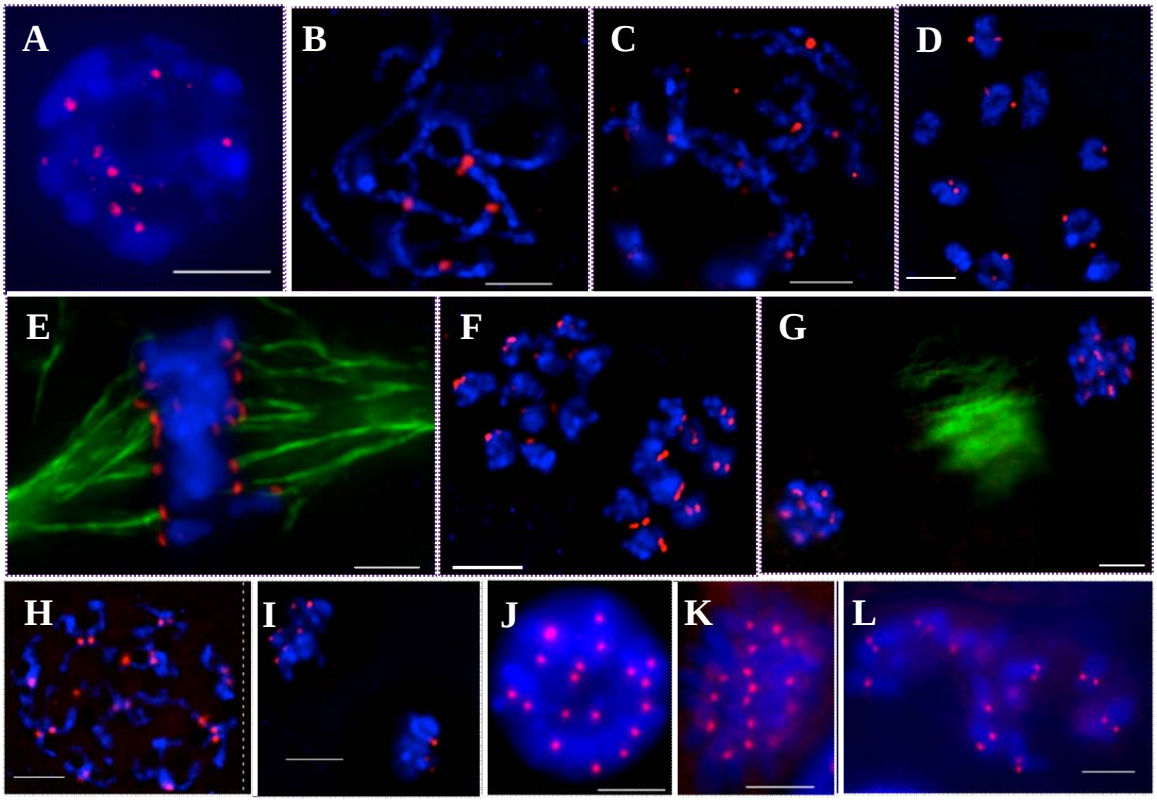

Figure 2.4 Maize MIS12 localizes on the chromosome as unique spots throughout the cell cycle in both meiosis and mitosis. All images are partial projections from 3D data sets. MIS12 signals are shown in red, microtubules in green, and chromosomes in blue. A-G: meiosis I. A: interphase; B: pachytene; C: diplotene; D: diakinesis; E: metaphase I; F: anaphase I; G: telophase I. H-I: meiosis II. H: prophase II; I: anaphase II; J-L: mitosis. J: interphase; K: prophase; L: C-metaphase. Scale bar = 5μm.

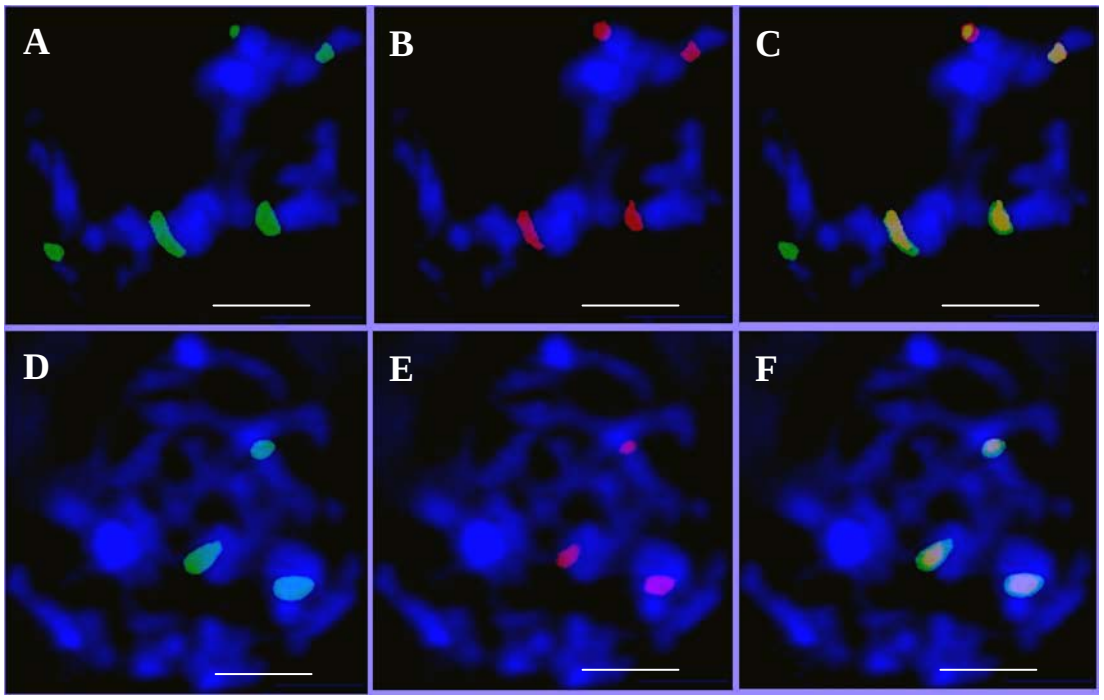

Figure 2.5 Colocalization of MIS12 with CENH3 and CENPC confirms that MIS12 is a kinetochore protein. All images are partial projections from 3D data sets, where CENH3 and CENPC are shown in green, MIS12 in red, and chromosomes in blue. **A-C**) Double labeling of CENH3 (A) and MIS12 (B) at deplotene. MIS12 signals overlap with CENH3 signals shown in yellow (C). **D-F**) Double labeling of CENPC (D) and MIS12 (E) at pachytene. MIS12 signals overlap with CENPC signals shown in yellow (F).

The data show that MIS12 signals are just apart from CENPC signals and mostly outside of CENPC signals along the spindle axis (Figure 2.6 A, D). These data suggest that CENPC and MIS12 occupy two distinct kinetochore domains with CENPC in inner domain and MIS12 in central domain respectively, as illustrated in cartoon (Figure 2.6 E).

Maize MIS12 retains its co-localization with CENH3 and CENPC on chromatin fibers

The extended chromatin fiber technique has been developed into a useful tool to examine histone modifications or chromatin associated proteins at high resolution (Sullivan and Karpen, 2004). In this method, interphase nuclei are gently stretched to the point where kinetochores 'unwind' to reveal their (presumed) substructure. The units observed by this method are presumed to represent subcomplexes at some level, although it is unlikely that we are seeing individual subcomplexes such as the MIS12 complex. To further examine MIS12's association with CENH3 and CENPC, chromatin fibers were prepared following Sullivan and Karpen (2004) method and detected by immunolabeling. Near-perfect co-localization of MIS12 with CENH3 and CENPC suggests that MIS12 remains its association with CENH3 and CENPC on chromatin fibers (Figure 2.7 B, C).

Maize MIS12 interacts with centromeric DNA

Chromatin immunoprecipitation (ChIP) is a powerful technique widely used for characterizing the interaction of chromatin proteins with DNA (Thorne et al., 2004).

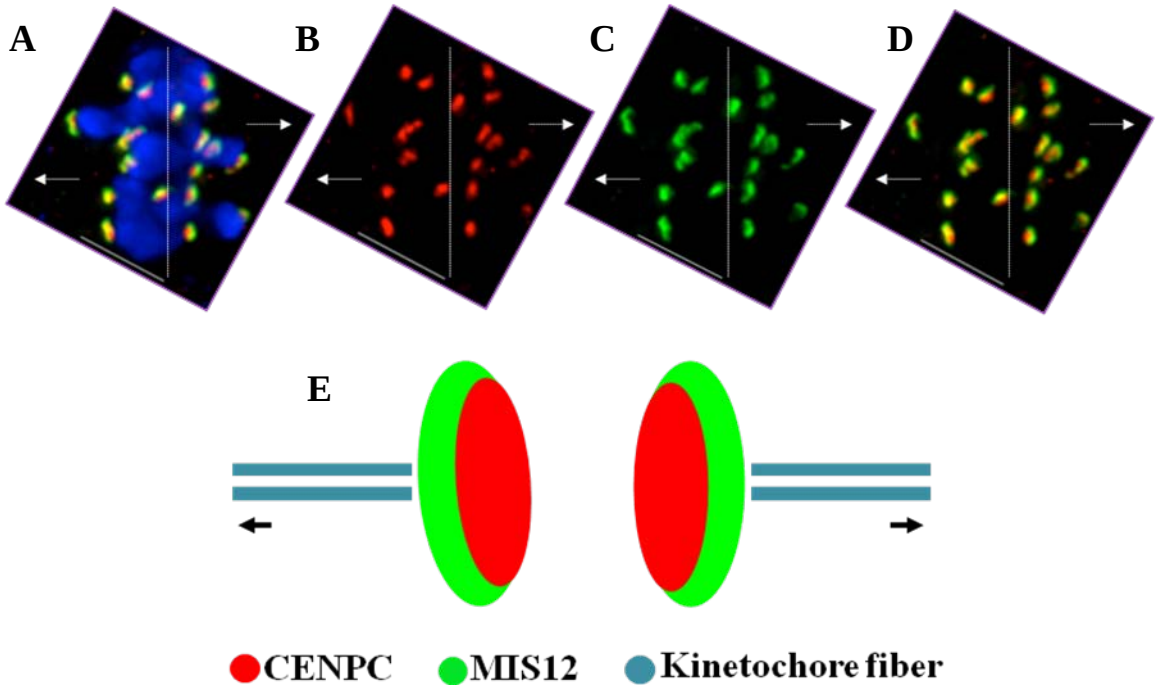

Figure 2.6 Double labeling of MIS12 with CENPC at metaphase shows that MIS12 is a central kinetochore protein. A) a partial projection from 3D data set of a metaphase cell. MIS12 signal is shown in red, CENPC in green, and chromosomes in blue. B) the same image projection with MIS12 only. C) the same image projection with CENPC only. D) the same image projection with both MIS12 and CENPC. E) Cartoon shows that MIS12 localizes outside of CENPC on the metaphase kinetochores.

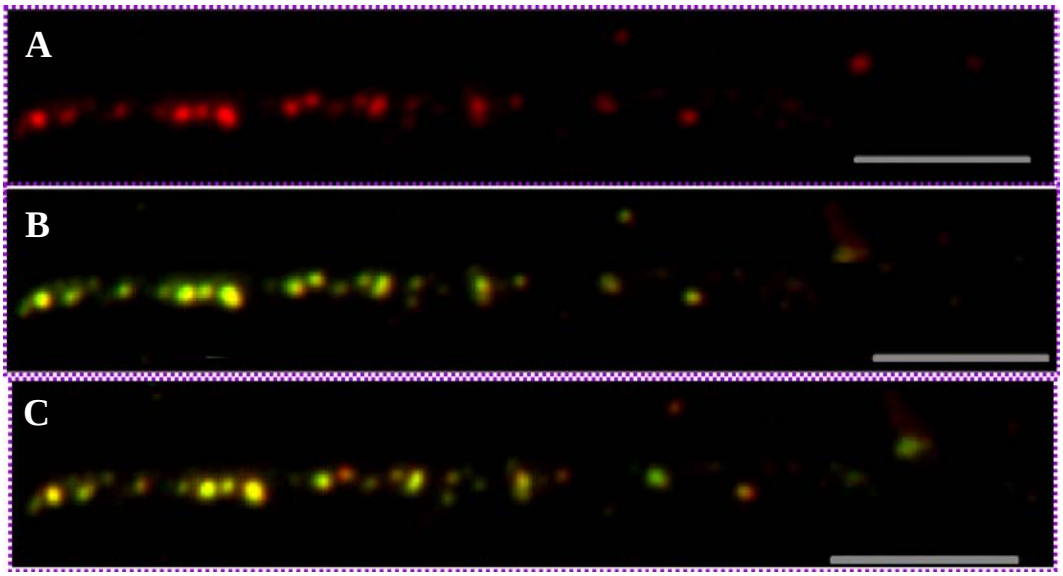

Figure 2.7 MIS12 is associated with CENH3 and CENPC on the extended chromatin fiber. A) MIS12 (red) on the chromatin fiber; B) colocalization of MIS12 (red) and CENH3 (green) on the chromatin fiber, with merged signals in yellow; C) colocalization of MIS12 (red) and CENPC (green) on the chromatin fiber, with merged signals in yellow.

Studying the interaction of nonhistones with DNA requires a technique called XChIP, which involves chemical or physical cross-linking to preserve the protein-DNA association (Kuo and Allis, 1999; Kuras, 2004; Ezhkova and Tansey, 2006). Kinetochore foundation proteins such as CENH3, CENP-C, and MIS12 are presumed to interact with

centromeric DNA, however, only CENH3 has been shown to interact with DNA by ChIP (Zhong et al., 2002a). To test whether MIS12 interacts with centromeric DNA, XChIP using formaldehyde cross-linked B73 (an inbred line) tissue was followed by quantitative PCR (qPCR) analysis (Gendrel et al., 2005). Anti-CENH3 and anti-HTR12 antibodies serve as positive and negative controls respectively (HTR12 is a CENH3 homolog in Arabidopsis; anti-HTR12 antibodies do not recognize maize proteins and show no positive ChIP with maize centromeric DNA).

Primers specific to maize centromeric satellite repeat CentC were used for qPCR with primers specific to 180-bp knob repeat as negative control (the knob repeat doesn't interact with kinetochore proteins in vivo and is similar to CentC repeat in size) (Ananiev et al., 1998a, b; Zhong et al., 2002a). Our analysis shows that CentC is enriched 11-fold in MIS12-precipitated chromatin as compared to a mock (HTR12) control. The data suggest that MIS12 interacts with centromeric DNA and functions as core kinetochore protein.

Maize MIS12 forms a bridge between sister kinetochores in meiosis I

The kinetochore is a key structure in coordinating homologous chromosome segregation in meiosis I (Cheeseman and Desai, 2008). It undergoes a series of structural rearrangements in accordance with the sister chromatid co-orientation in meiosis I and bi-orientation in meiosis II.

Each set of sister kinetochores appear as single spot in interphase, and remain closely associated until late diakinesis. After the transition from prometaphase I to early anaphase I, CENPC-labeled sister kinetochores separate to become two distinct structures in late anaphase I. However, MIS12 shows a different pattern of staining, revealing a clear bridge between sister kinetochores in metaphase. Sister kinetochores indicated by CENPC appear as two distinct spots, but MIS12 and NDC80 signals shows a continuous staining pattern spanning two sister kinetochores (Figure 2.8; Figure 2.15 B, upper right inset). Although MIS12 and NDC80 signals between sister kinetochores are weaker than they are over sister kinetochores, MIS12 and NDC80 links sister kinetochores together as an integral unit for microtubule attachment. The linkage revealed by MIS12 and NDC80 will be referred to as the MIS12/NDC80 bridge.

Mis12 RNAi knocks down *Mis12* gene expression in different transgenic lines

Although cytological functions of MIS12 are well addressed in yeast and animals, it remains unclear how *Mis12* functions in meiosis. Particularly, it appeared that MIS12 may have a role in promoting sister chromatid co-orientation given its novel staining pattern. RNA interference (RNAi) is a well-established reverse genetic approach to study gene families such as maize *Mis12* genes (Boutros and Ahringer, 2008; Mahmood ur et al., 2008). *Mis12* RNAi constructs were made to independently target *Mis12-1* and *Mis12-2* (Figure 2.9).

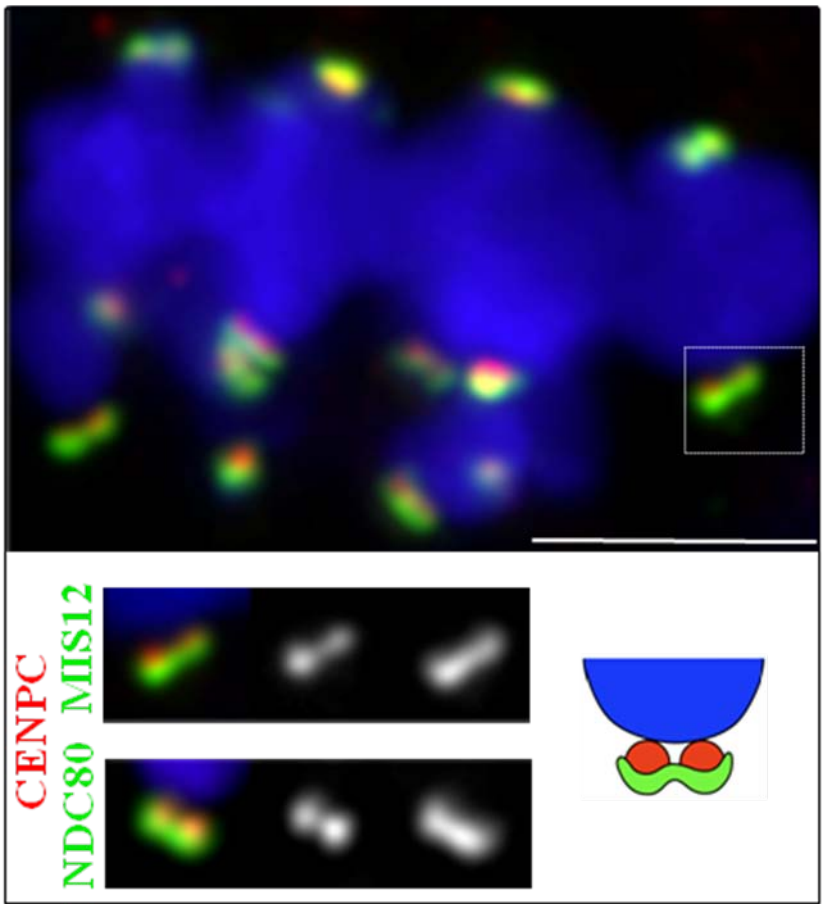

Figure 2.8 MIS12/NDC80 bridge is evident on metaphase I kinetohcores via double labeling of MIS12 (NDC80) and CENPC. An enlarged view of the boxed pair of kinetochores is shown below (labeled MIS12) (left: merged; middle: CENPC; right: MIS12). It is compared to a pair of kinetochores from a different prometaphase cell double stained with NDC80 and CENPC (labeled NDC80) (left: merged; middle: CENPC; right: NDC80). The summary cartoon illustrates MIS12/NDC80 bridge spanning sister kinetochores (CENPC in red, MIS12 and NDC80 in green). Scale bar=5 μm.

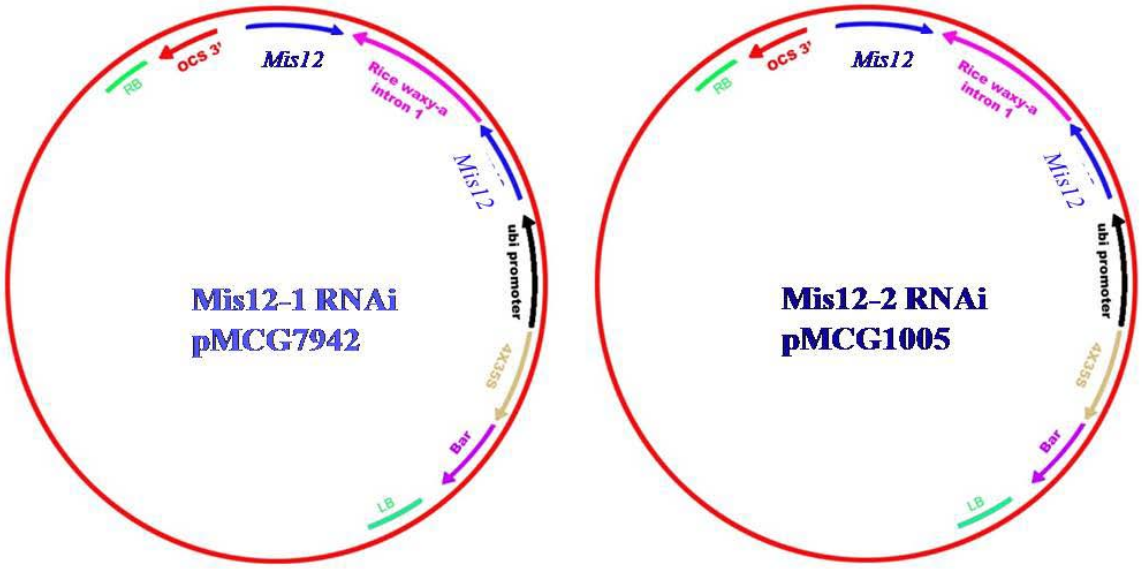

Figure 2.9 Maps of *Mis12-1*(left) and *Mis12-2* (right) RNAi constructs. pMCG7942 and pMCG1005 are the original corresponding vectors, and the inverted *Mis12* cDNA fragments are spaced by Rice *waxy-a* intron 1. Both RNAi construct was expressed under the Ubiquitin promoter, and the Bar gene confers herbicide resistant for mutant screen.

A total of 204 *Mis12-1* RNAi and 200 *Mis12-2* RNAi T0 plants were grown in the greenhouse and many were out crossed to maize inbred lines KYS or B73 for T1 progeny. T1 seeds were planted out and self crossed to bulk seeds as illustrated in pedigree (Figure 2.10). T1 and T2 plants are used for mRNA, protein, and phenotypic analysis.

To assess the effectiveness of RNAi, four-week-old young leaf tissue was used for RNA isolation, and cDNA-qPCR was carried out to examine *Mis12* RNA reduction using primer sets amplifying both *Mis12* genes. The reduction was evaluated by the equation: Relative fold change= $2^{\Delta\Delta C(t)}$, $\Delta\Delta C(t)= (\Delta C(t)_{Mis12} - \Delta C(t)_{ubiquitin})_{wild\ type} - (\Delta C(t)_{Mis12} - \Delta C(t)_{ubiquitin})_{mutant}$ (Livak and Schmittgen, 2001). Different *Mis12* RNAi plants showed different extents of RNA reduction. For a subset of six transgenic events, *Mis12* mRNA was differentially reduced from 20% to 80% (data not shown). The data also revealed that *Mis12-1* RNAi knock down both *Mis12* gene's expression (Figure 2.11). We assume that *Mis12-2* RNAi has the similar effect and interpret the two *Mis12* RNAi experiments as replicates. The six families scored for expression were subsequently studied in detail (Figure 2.10).

Mis12 RNAi reduces MIS12 protein in different transgenic lines

To further examine *Mis12* RNAi efficiency in transgenic plants, we analyzed the MIS12 protein intensity by immunostaining assay. In an effort to reduce experimental variations, wild type cells and mutant cells were prepared on the same slide side by side.

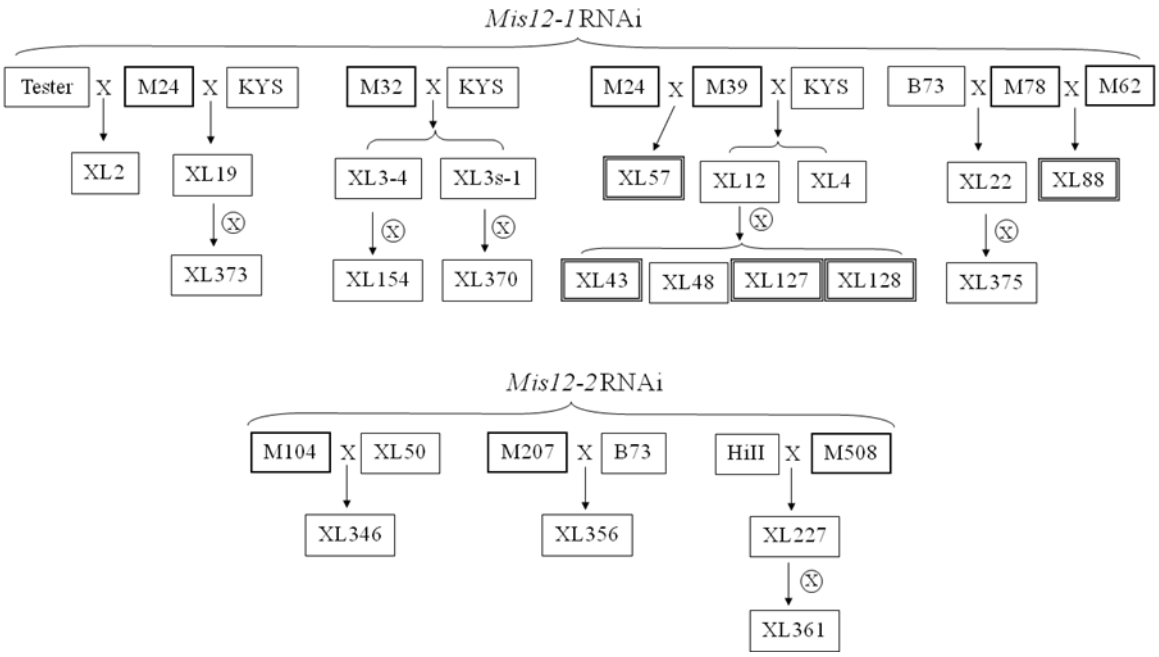

Figure 2.10 Pedigree of the *Mis12* RNAi lines used in this study. Families beginning with 'M' and outlined in bold are primary transformants. Transformants were first crossed to inbred lines (KYS or B73) or hybrid (HiII or lab tester), then self crossed for further analysis. Families marked by double outlines segregated sporadic dwarf plants.

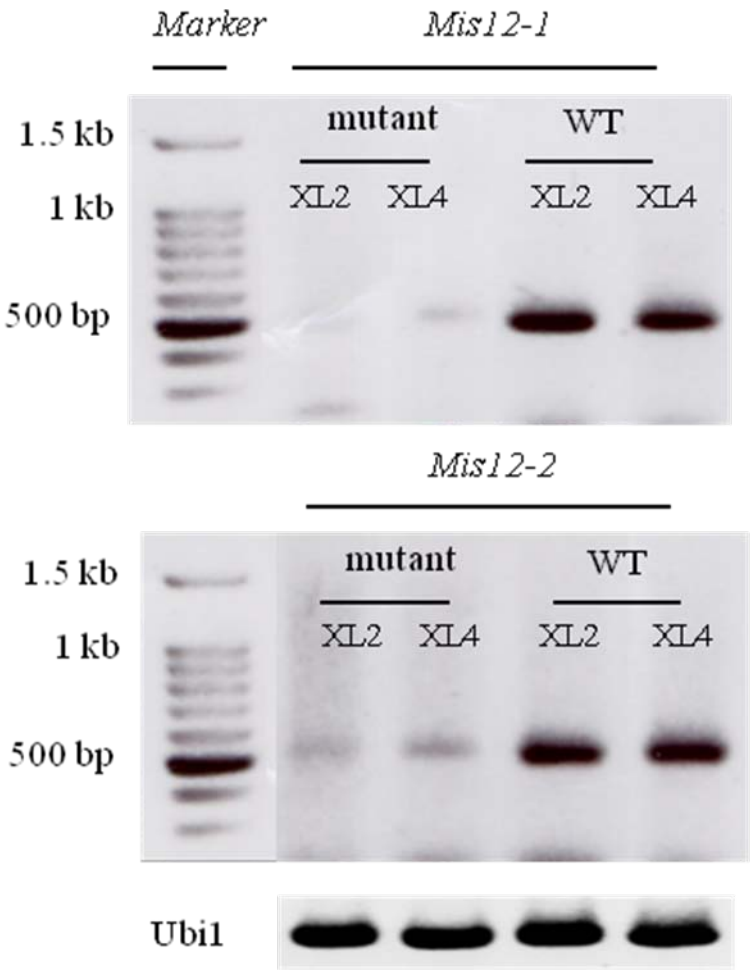

Figure 2.11 *Mis12-1* RNAi reduces the accumulation of both *Mis12-1* and *Mis12-2* mRNA. The gels show the results of a quantitative RTPCR experiment using primers specific to the individual genes. mRNA reduction varied from 40-80% in different experiments. The origin of the RNAi lines used (XL numbers) can be found Figure 2.10.

Given the fact that CENPC is an inner kinetochore protein and MIS12 localizes outside CENPC, we assume CENPC is unaffected in *Mis12* RNAi mutant and take the ratio of MIS12 intensity to CENPC intensity to evaluate MIS12 protein reduction in *Mis12* RNAi lines. The analysis revealed that MIS12 protein is reduced 24~42% in different mutant plants from four transgenic events (two for the *Mis12-1* RNAi and two for the *Mis12-2* RNAi) compared to that in the wild type (Table 2.1).

Dwarf plants segregate in the field

We examined T0 and T1 mutant plants carefully to find potential morphological (mitotic) phenotypes. In some families, there were significantly more leaf splits or cracks on mutant plants than on wild type plants, and the crack/split phenotype is consistent with MIS12's function as an essential kinetochore protein, but this phenotype is not reproducible on T2 plants (Figure 2.12 A). In a further effort to identify mitotic phenotype of *Mis12* RNAi plants, a large-scale screen was conducted in the field. Sporadic and severe dwarf plants segregate in five families (Figure 2.12 B; 2.10). The severity of dwarfing phenotype suggested that these plants were aneuploid progeny caused by errors in meiosis. The inconsistency of the phenotype among different families could be a result of variation in the efficiency of RNAi or environmental effect.

Table 2.1. MIS12 staining, but not SGO staining, is significantly reduced on meiotic kinetochores in *Mis12* RNAi lines, and MIS12 is unaffected in *ameiotic-1* (*am1*) mutants ($p < 0.001$*** for MIS12, $p = 0.82$ for SGO, and $p = 0.1334$ in *am1*, ANOVA). Each value shown is derived from at least ten cells. (for the *am1* data, a 'set' is a randomly chosen pair of cells from a wild type and a mutant plant). The percent reduction was calculated as the ratio of MIS12 (or SGO) intensity to CENPC intensity. The genetic origin of transgenic lines is illustrated in Fig. 2.10.

MIS12 Quantification	**WT**	**Mutant**	**Reduction**
***Mis12 -1* RNAi**			
XL43	2.02 ±0.08	1.55±0.05	23%***
XL375	2.92±0.12	2.20±0.07	25%***
***Mis12 -2* RNAi**			
XL361	3.32±0.21	1.95±0.11	41%***
XL356	2.01±0.05	1.58±0.04	22%***
SGO Quantification	**WT**	**Mutant**	**Reduction**
***Mis12 -1* RNAi**			
XL154	1.19±0.03	1.17±0.06	1%
XL373	0.89±0.04	0.93±0.03	-4%
***Mis12 -2* RNAi**			
XL346	1.12±0.06	1.09±0.05	3%
MIS12 Quantification	**WT**	**am1**	**Reduction**
1st set	2.52±0.07	2.33±0.09	8%
2nd set	2.48±0.05	2.22±0.08	10%
3rd set	1.95±0.06	1.42±0.06	28%
4th set	2.22±0.07	1.59±0.08	29%

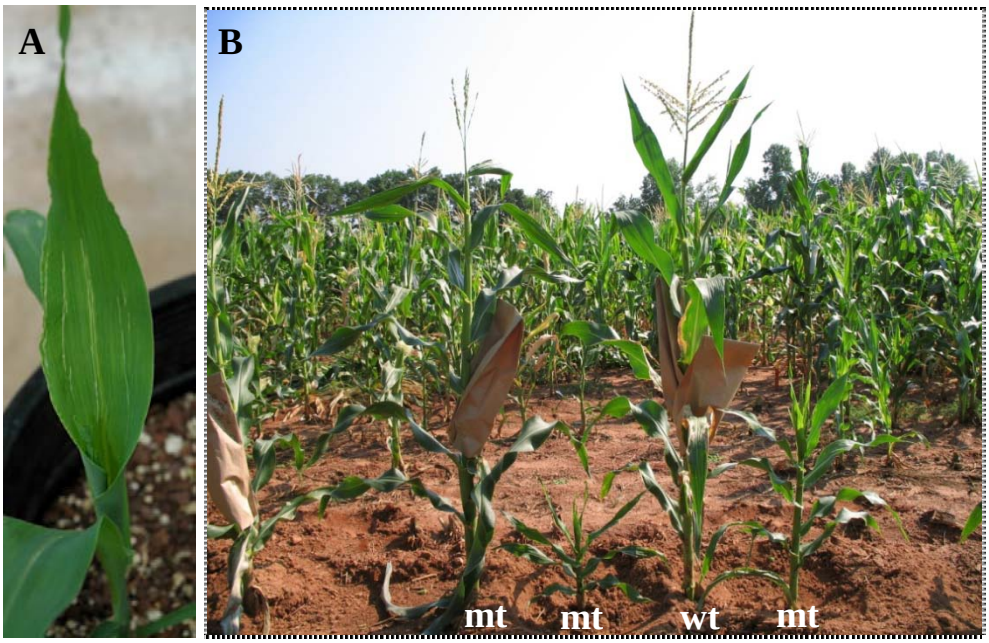

Figure 2.12 Phenotypes of *Mis12* RNAi plants. **A)** Inconsistent leaf split or crack phenotype on mutant plants; **B)** Sporadic dwarf plants segregate in *Mis12* RNAi F2 progeny. Four plants from a family (XL88) (see Figure 2.10) segregating mutant and wild type plants are featured in the forefront. The shortest mutant plant is one ninth as tall as the wild type plant. However, many other mutant plants in the same family appeared normal. Brown bags are used to protect crossed ears.

Maize *Mis12* RNAi cause severe chromosome loss and aneuploidy

If the dwarfing phenotype was caused by aneuploidy progression, we would expect high frequency of chromosome loss events. We chose to score tetrads for initial tests of this hypothesis. Tetrads are direct products of meiosis and more abundant than metaphase and anaphase cells. In these cells, lost chromosomes usually nucleate by themselves as a mininuclei in the cytoplasm (Figure 2.13). Thirteen plants from a family showing dwarfing phenotype are sampled for mininuclei scoring at tetrad stage (each mininuclei represents a chromosome loss event). In wild type plants, the frequency of mininuclei is below 0.06% (SD ± 0.03%; n=2,479 from 3 plants); while the mininuclei frequency is 2.17 % (SD ± 1.35%; n=10,353 from 11 plants) in *Mis12* RNAi plants -- around 30 times higher than that in wild type plants (Figure 2.13). Many tetrads contained multiple mininuclei (Figure 2.13 C); as many as 10 (7 extra mininuclei in Figure 2.13 B). These data suggest that chromosome segregation is severely disrupted and leads to multiple chromosome loss events. Encouraged by the severe phenotype in tetrads, we further examined meiosis I and II in *Mis12* RNAi mutant plants.

Maize *Mis12* RNAi causes sister kinetochore bi-orientation at metaphase I

The key of meiosis I is to reduce chromosome number by segregating homologous chromosomes into different daughter cells. To achieve this, sister kinetochores must co-orient and are attached to the microtubules from the same spindle pole. In wild type, sister kinetochores maintain their association until late anaphase I.

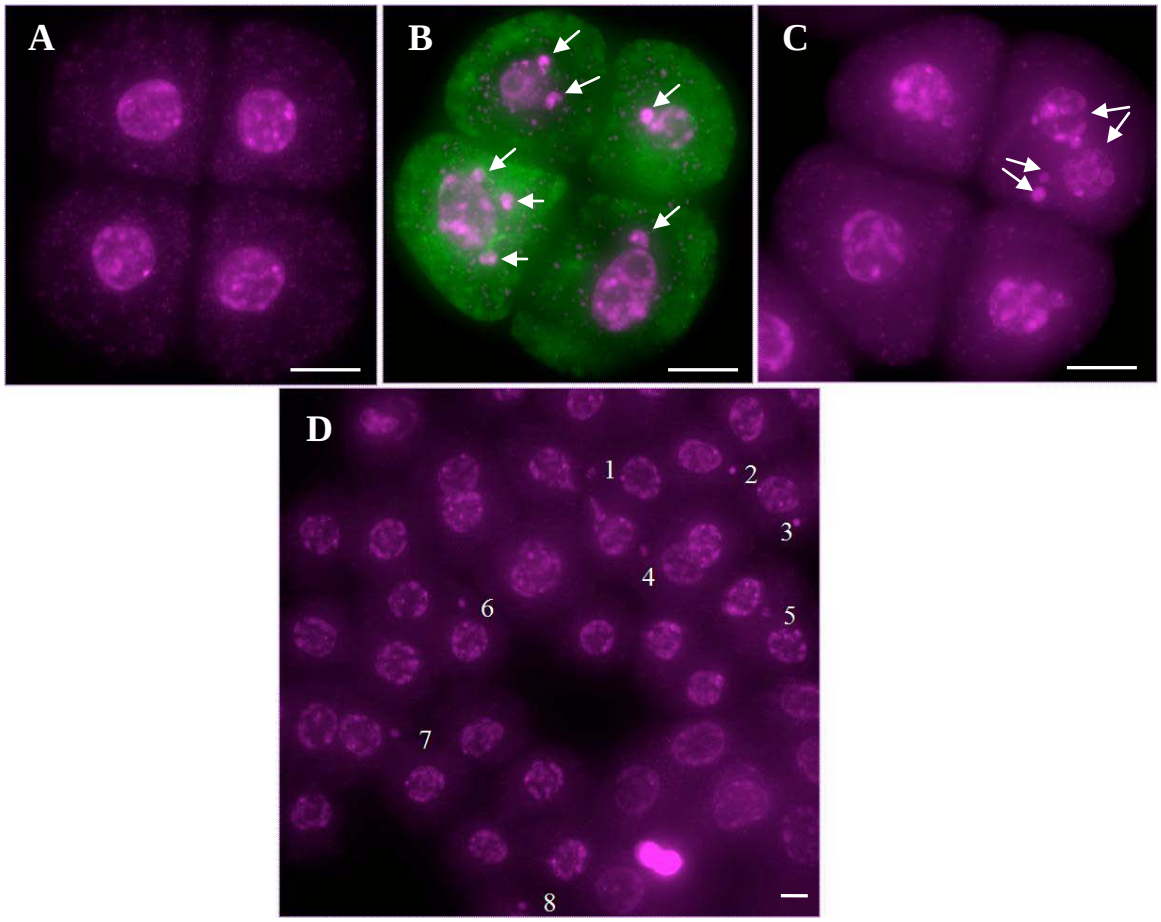

Figure 2.13 Prevalent chromosome loss or aneuploidy in maize *Mis12* RNAi mutant plants. A) A wild type tetrad; B) A mutant tetrad with many mininuclei (arrows); C) A mutant tetrad with twin nuclei (arrows); D) An overview of high frequency of mininuclei in the mutant tetrads (20%, 8 out of 40 nuclei, mininuclei are numbered).

Our data suggest that the MIS12/NDC80 bridge functions as sister kinetochore clamp and directs sister chromatid co-segregation. In *Mis12* RNAi mutant plants, MIS12 protein reduction undermines or breaks the sister kinetochore bridge, leading to sister kinetochore misbehavior at metaphase I. Rather than co-orient, approximately 30% of the sister kinetochores separate and align equationally at meiosis I (Figure 2.14, Table 2.2). On equationally orientated chromosomes the sister kinetochores are wholly separate and distinct as measured by CENPC, MIS12 or NDC80 antisera, align with the spindle axis, and organize distinct microtubule bundles (kinetochore fibers) that emanate to opposite poles. Although CENPC staining usually disjoins cleanly, MIS12 staining often appears to stretch and separate unevenly (Figure 2.14). The number of separated and disjoined sisters varies from two to twenty-four.

In rare cases, single kinetochores form two microtubule binding faces that orient to opposite poles (a process called merotelic attachment; Figure 2.15 B lower right inset). The merotelic phenotype was common in severe MIS12 knockdowns that affect human mitosis (Kline et al., 2006) but was unusual in our analysis. In a sample of 28 anaphase I cells from a Mis12-1 RNAi line (XL373), sister separation followed by equational alignment was observed 145 times, but sister separation followed by merotelic alignment was observed only 13 times. These data suggest that the MIS12/NDC80 bridge that links sister kinetochores is particularly sensitive to reductions in MIS12 abundance. We presume that more severe MIS12 knockdowns would have caused more frequent merotelic alignments in meiosis and pronounced defects at mitosis. However, it is unlikely that such a phenotype would have survived our transformation protocol, which requires full plant regeneration from cultured cells.

Sister kinetochore separation leads to chromosome lagging at anaphase I in *Mis12* RNAi mutant plants

In wild type, homologous chromosomes are released from the chiasmata interlock, and migrate towards opposite poles in anaphase. In *Mis12* RNAi mutants, numerous chromosomes lag or get stuck on the spindle. The kinetochore staining pattern on those lagging or stuck chromosomes suggests that sister kinetochores prematurely separate and are pulled towards different poles (Figure 2.15 A, B). Lagging chromosomes may remain trapped near emerging cell plate (Figure 2.15 C), separate and give rise to single chromatid chromosome (data not shown), or randomly partition into daughter cells in late telophase (data now shown).

***Mis12* RNAi may cause meiotic abortion**

Due to numerous chromosome lagging or segregation errors, some meiotic cells fail to enter into meiosis II. As shown in figure 2.16 A, multiple nuclei interrupt the cell cycle progression, and lead to meiotic abortion. Some cells may undergo a very random meiosis I and II, and give rise to polyploidy (Figure 2.16 B-D). In severe situations, both cell divisions fail and the whole set of chromosomes remain in one nucleus, resulting in reconstitution of the 4n state (Figure 2.16 E).

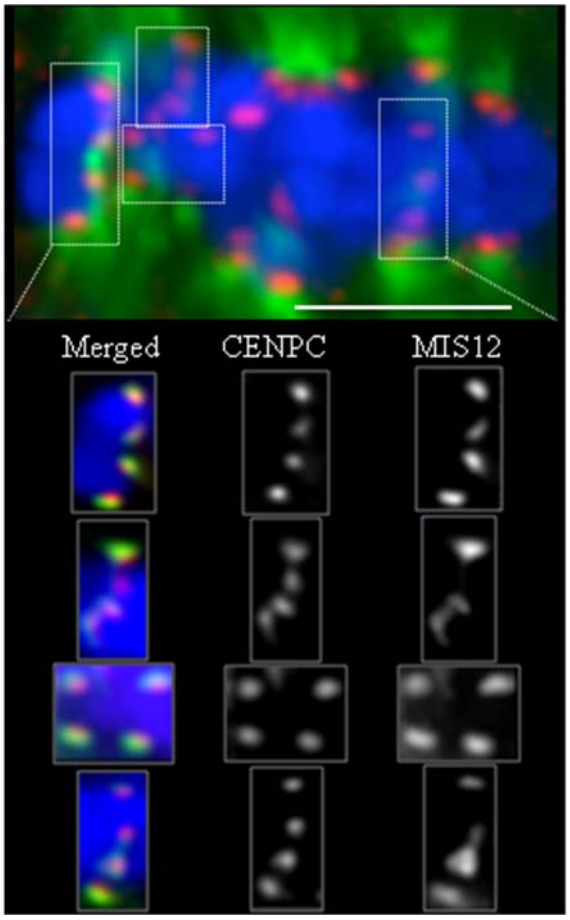

Figure 2.14 Sister kinetochores prematurely separate and bi-orient at metaphase I due to *Mis12* RNAi. At least eight sets of bi-oriented sister kinetochores are shown below, with the CENPC (red) and MIS12 (green) channels shown without color. Image is partial projection from 3D data set. DNA is in red and tubulin in green. Scale bar= 5μm.

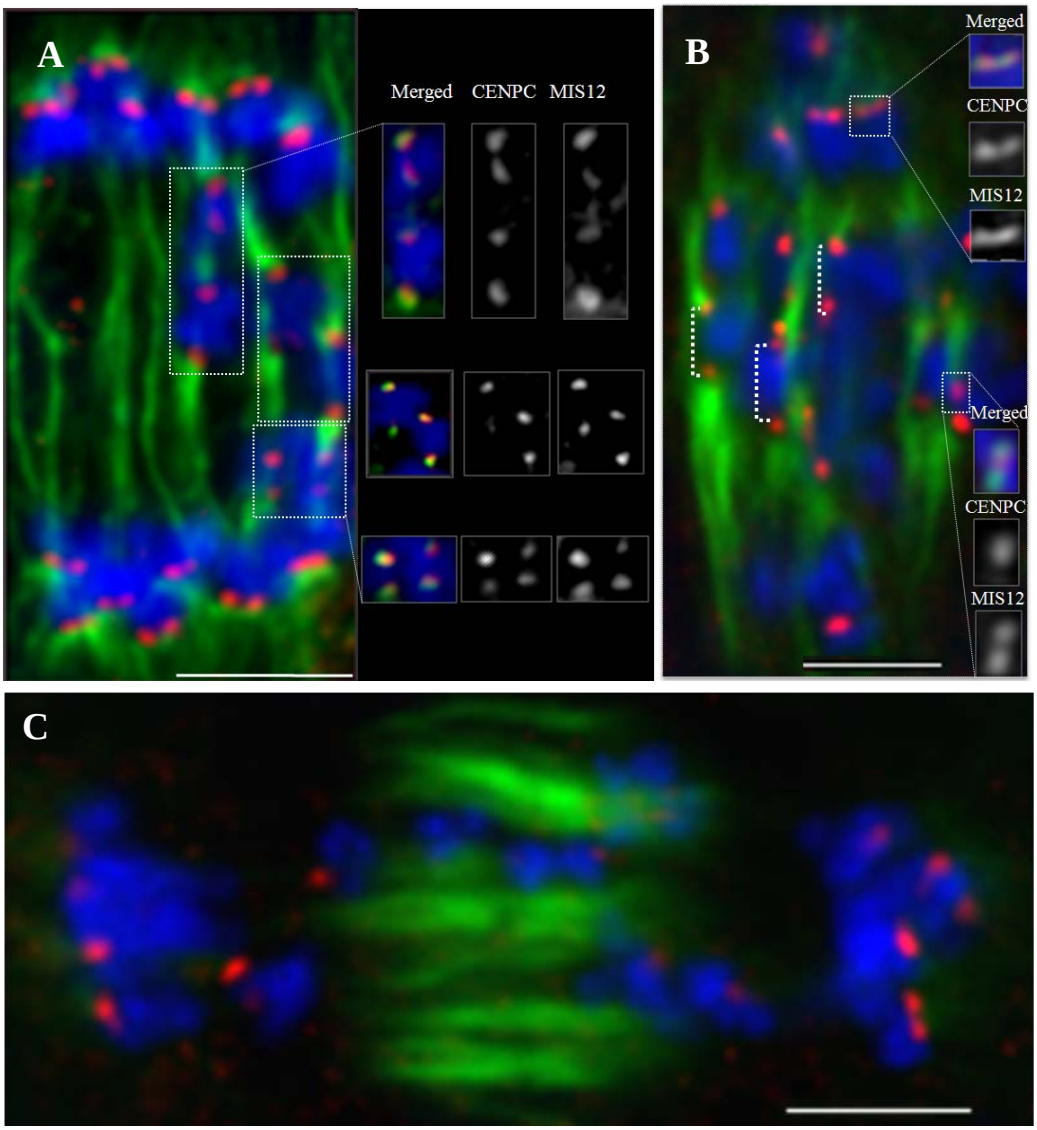

Figure 2.15 Sister kinetochore separation and chromosome lagging at anaphase I and telophase I in the *Mis12* RNAi mutant plants. All images are partial projections from 3D data sets, DNA in blue and tubulin in green. **A)** At anaphase I the chromosomes lag in the spindle midzone. Three homologs showing premature kinetochore separation and equational alignment are shown, with the CENPC (red) and MIS12 (green) channels shown without color. **B)** An anaphase I cell showing a severe phenotype. The sister kinetochores are separated and oriented equationally on

(Figure 2.15 legend continued**)** most chromosomes (three are indicated with brackets). In addition, three single kinetochores aligned merotelically (one is highlighted at lower right). An example of normal co-orientation with MIS12 bridge is highlighted at upper right, an example of merotelic attachment is enlarged at lower right. **C)** Late telophase showing lagging chromosomes trapped in the emerging cell plate (MIS12 in red). Scale bar= 5μm.

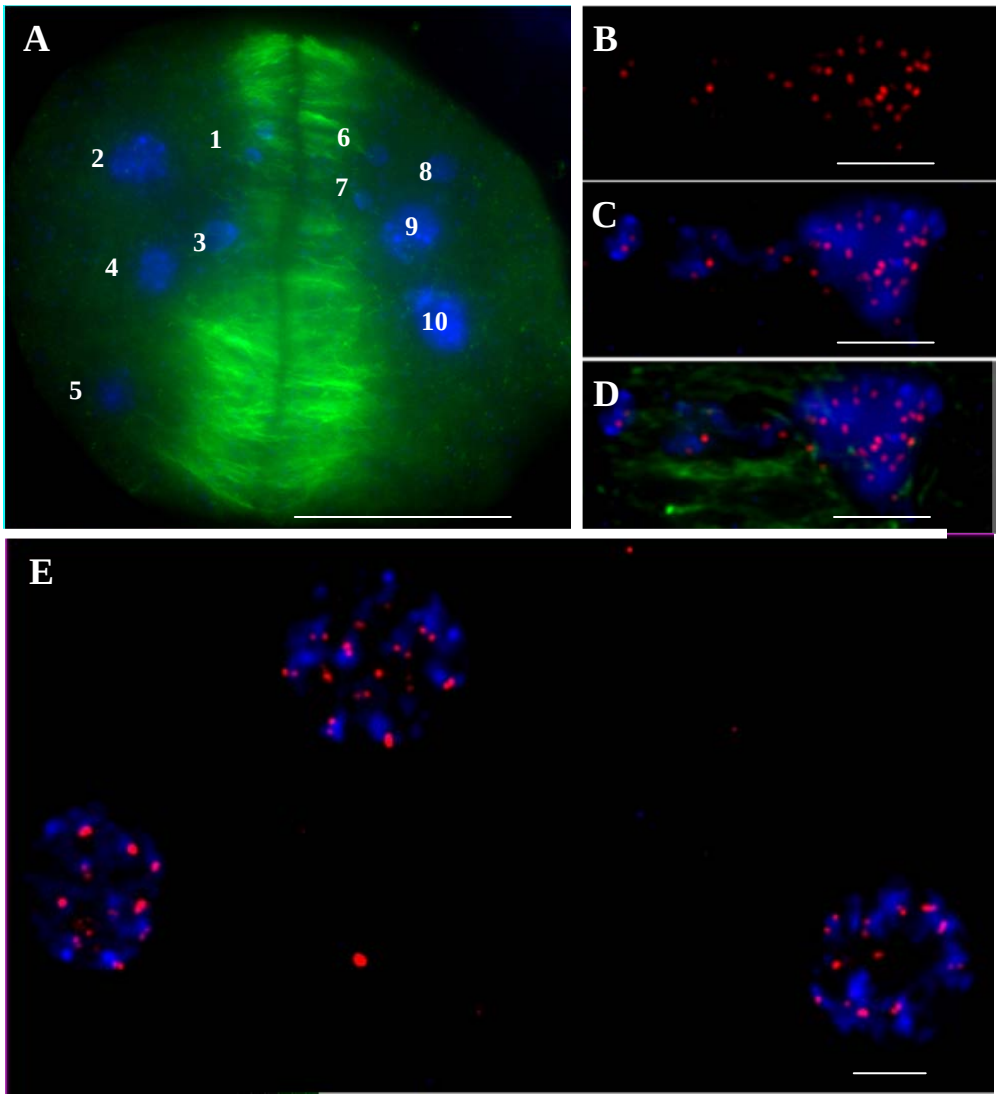

Figure 2.16 Meiotic abortion in *Mis12* RNAi plants. All images are partial projections from 3D data sets. MIS12 in red, microtubules in green, and chromosomes in blue. A) A telophase I mutant cell with 10 mininuclei potentially aborts; B) An anaphase II mutant cell showing around 40 kinetochore spots; C) The same image showing random partition of chromosome mass; D) The same image indicating severe unequal chromosome segregation; E) An overview of mutant cells with 40 kinetochore spots after cell reconstitution.

Mis12 RNAi cause multiple spindle structures, chromosome lagging and aneuploidy in meiosis II

Chromosome misbehavior in meiosis I leads to multiple chromosome masses at metaphase II. Some metaphase II cells have as many as four sets of chromosome masses (Figure 2.17 A inset). Each chromosome mass organize their own spindle, thus form multiple spindles in a single cell (Figure 2.17A). Multiple spindles pass the meiosis I errors on to the anaphase II. Various cytological errors occur, such as over-stretched kinetochore on the single chromatid chromosome (Figure 2.17 B), severe chromosome lagging and unsynchronized cell division (Figure 2.17 C), and random chromosome segregation (Figure 2.17 D). Rather than four nuclei formed at tetrad stage, nuclei twins (Figure 2.13 C; 2.17 E) or multiple mininuclei form in mutant plants (Figure 2.17 E). Sometimes, triads form due to metaphase II arrest (Figure 2.17 F).

To quantify these effects, error frequencies of metaphase I, anaphase I, metaphase II, and anaphase II were scored in five different *Mis*12 RNAi lines and are summarized in Table 2.2. On average, the error frequency in mutant is around 30 times higher than that in wild type plants.

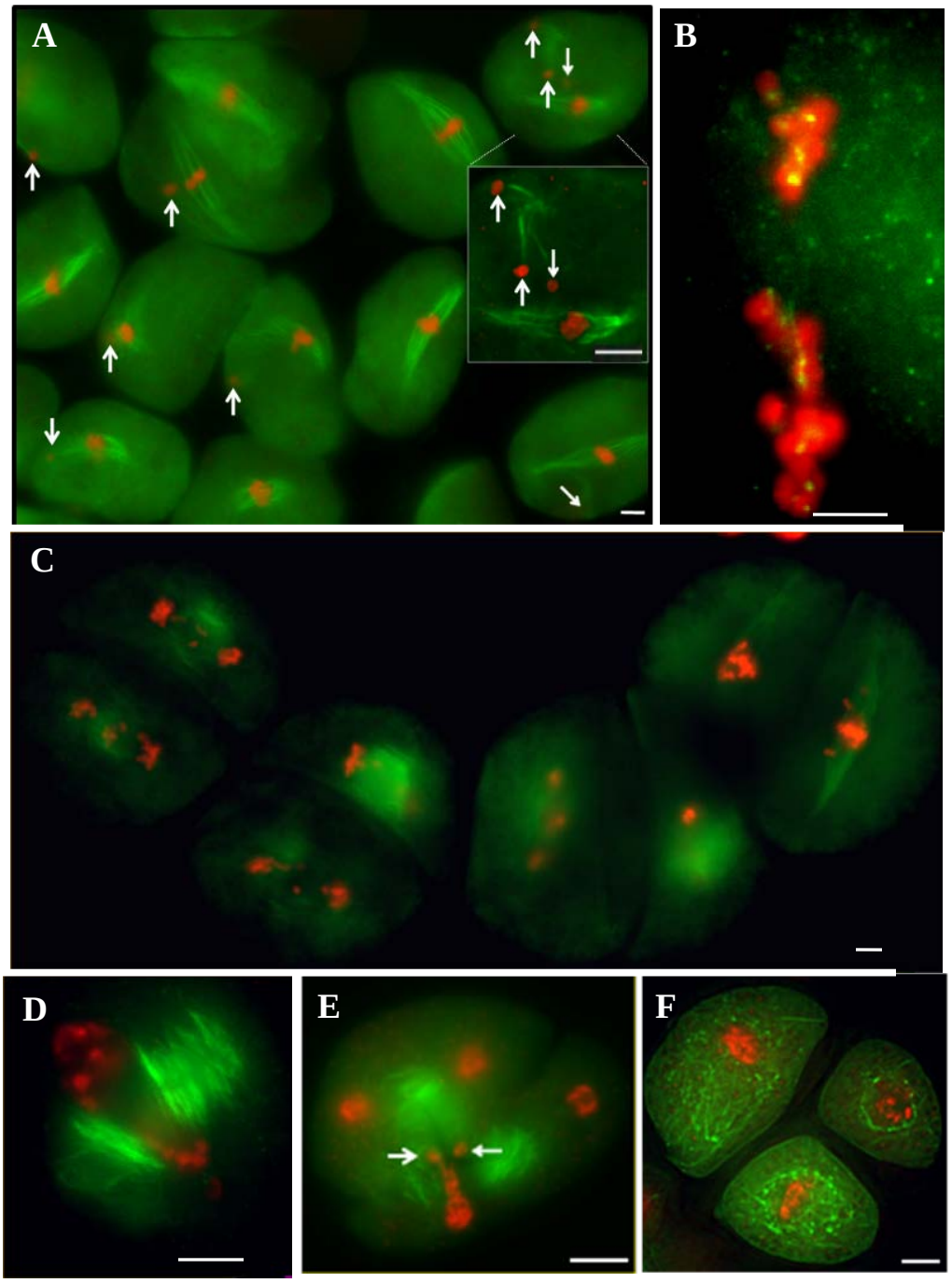

Figure 2.17 Various meiosis II errors in *Mis12* RNAi lines. **A)** A field of metaphase II cells illustrating how lagging chromosomes from meiosis I affect meiosis II spindle morphology and mininuclei (arrows). The inset is an enlarged view of multiple mininuclei and mini-spindles.

(**Figure 2.17** legend continued) **B)** An anaphase II cells with stretched kinetochore fibers (MIS12 in green); **C)** Unsynchronized anaphase II and lagging chromosomes;. **D)** Unequal segregation; **E)** Tetrad defects caused by multiple spindles or lagging chromosomes; **F)** A triad due to metaphase II arrest. Mininuclei are indicated by arrows. DNA is shown in red and tubulin in green. Scale bar= 5μm.

Table 2.2 Quantification of meiotic errors in *Mis12* RNAi mutant plants**.** Data are represented as total cells counted / number of cells with errors (percentage of cells with errors). The errors counted were: diakinesis - unpaired chromosomes; prometaphase/metaphase I - sister kinetochore separation; anaphase I - lagging chromosomes; metaphase II - multiple nuclei; anaphase II - spindle abnormalities. Seventeen wild type (WT) plants were scored to confirm that meiotic errors were limited to transgene-containing lines; two WT are featured to illustrate the natural error rate. Twenty-eight mutant plants were observed and all showed meiotic defects. The five scored in the table were among those with severe phenotypes. Pedigree information for the lines used (XL numbers) is shown in Figure 2.10. *data from sibling line XL43.

	Diakinesis	**Prometaphase-metaphase I**	**Anaphase I**	**Metaphase II**	**Anaphase II**
***Mis12-1* RNAi**					
WT	110/0 (0)	125/0 (0)	142/1 (0.7%)	181/2 (1.1%)	150/0 (0)
XL48	107/0 (0)	216/68 (32%)	139/108 (78%)	193/72 (37%)*	38/12 (32%)*
XL370	102/0 (0)	146/60 (42%)	121/49 (41%)	184/70 (38%)	114/41 (36%)
XL373	115/0 (0)	137/41 (30%)	103/38 (37%)	150/64 (43%)	121/44 (36%)
***Mis12-2* RNAi**					
WT	145/0 (0)	114/0 (0)	121/0 (0)	110/1 (0.9%)	123/0 (0)
XL346	118/0 (0)	98/25 (26%)	126/43 (34%)	221/73 (33%)	121/35 (29%)
XL361	120/0 (0)	106/26 (25%)	101/24 (24%)	110/31 (28%)	118/31 (26%)

SGO remains its pericentromeric localization despite MIS12 Reduction

Cohesion is necessary for sister chromatid co-orientation in meiosis I. Particularly, SGO localizes to pericentromeric regions and protects cohesin from cleavage in meiosis I (Figure 2.18 A, B). Premature depletion of cohesion causes sister chromosome separation at anaphase I. To differentiate *Mis12* RNAi phenotype from the potential SGO/cohesion defects, we quantified SGO intensity by immunoassay. Analysis of three different *Mis12* RNAi lines (two for the *Mis12-1* RNAi and one for the *Mis12-2* RNAi) revealed no SGO intensity variation between *Mis12* RNAi mutant and wild type plants (Table 2.1). SGO localizes to the pericentromere region even when sisters are already separated (Figure 2.18 D), indicating sister kinetochore separation resulting from *Mis12* RNAi is distinct from cohesion's function in mediating sister chromatid co-segregation.

Sister kinetochore separation in *Mis12* RNAi mutants is not caused by defects in recombination

In prophase I, each set of homologous chromosomes crossover, and generate chiasmata between them (forming a bivalent). At diakinesis, all bivalents spread well in the nucleus (Figure 2.19). Recombination failure or no recombination between homologues generates univalents, and sister kinetochores of univalents are likely to be bipolar attached (Kouznetsova et al., 2007). To rule out the possibility that the *Mis12* RNAi phenotypes observed are caused by recombination failures, we measured the frequency of natural recombination failure by scoring univalents at diakinesis stage (extra univalents can be easily recognized and scored).

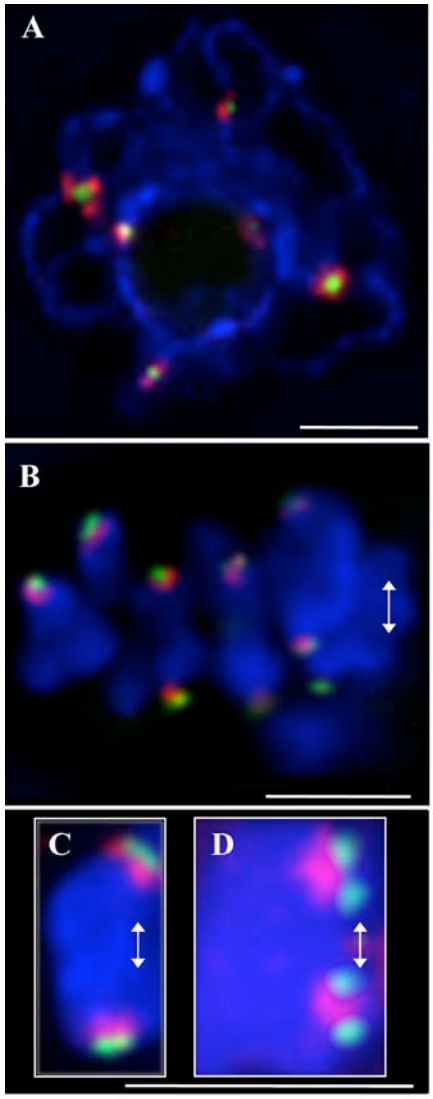

Figure 2.18 Shugoshin (SGO) stains pericentromeric regions and remains in place when sister kinetochores separate in *Mis12* RNAi mutants. **A)** Meiotic prophase (pachytene) showing CENPC (green) and maize SGO (red). **B)** Prometaphase I cell showing that SGO staining trails CENPC (green). **C)** A close-up of one set of homologous chromosomes oriented properly. **D)** A metaphase I chromosome from a *Mis12* RNAi line showing both sets of sisters separated and aligned with the spindle axis. SGO remains between the separated sister kinetochores. Arrows show spindle axis. Scale bar= 5μm.

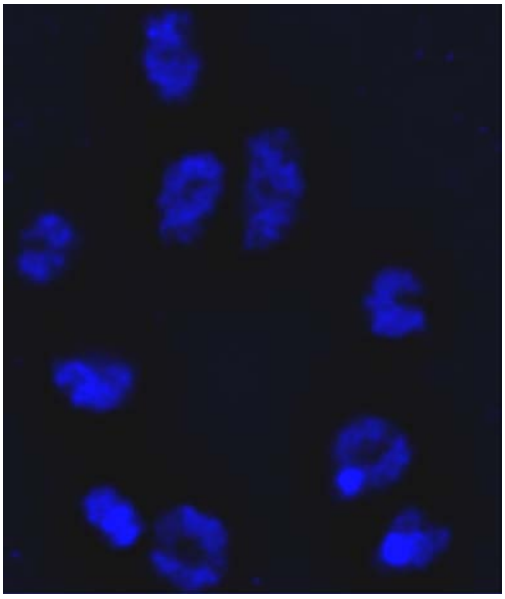

Figure 2.19 A wild type diakinesis cell showing ten well spread bivalents.

We sampled five different *Mis12* RNAi lines, counted over 100 diakinesis cells for each plant (the plants from the same lines for meiotic phenotype characterization; three for *Mis12-1* RNAi and two for *Mis12-2* RNAi), and found no extra univalents (Table 2.2), suggesting that the frequency of natural recombination failure is at least below 1% by our assay.

Sensitivity of meiosis I to *Mis12* RNAi may be related to the unique structure of MIS12 bridge between sisters

Given the differential effect of Mis12 RNA on meiosis and mitosis, we wondered whether meiosis I kinetochores contain more MIS12 protein on a molecular basis. To test this hypothesis, we compared the MIS12 intensity between *ameiotic-1* mutant individuals and their wild type siblings (*ameiotic*-1 is a mutation that has mitosis in place of meiosis but is otherwise wild type; (Staiger and Cande, 1992)). Our analysis shows no MIS12 difference in quantity between wild type and *am1* mutant plants (Table 2.1).

DISCUSSION

One of the key issues in understanding cell division is how the kinetochore assembles and maintains its specific interaction mode with microtubules during cell cycle progression. Meiosis I is different from mitosis in that homologous chromosomes, rather than sister chromatids, segregate at anaphase I (Dawe, 1998; Petronczki et al., 2003). Sister kinetochore co-orientation is one critical feature in this process although two other cytological machineries, cohesion and chiasmata, are also indispensable (Watanabe and Nurse, 1999; Kitajima et al., 2004; Kouznetsova et al., 2007; Kiburz et al., 2008). Efforts in dissecting the kinetochore are making

promising progress, and around 70 kinetochore proteins are identified to date (Cheeseman and Desai, 2008). It remains largely unknown what kinetochore proteins directly contributes to sister chromatid co-orientation in meiosis I. Budding yeast monopolin functions to maintain sister kinetochore co-orientation in budding yeast, but has neither the same function in fission yeast, nor homolog in other species (Toth et al., 2000; Rabitsch et al., 2003). A more general 'sister clamp' has been proposed (Pidoux and Allshire, 2003; Rabitsch et al., 2003) but no one kinetochore protein has yet been identified for this purpose.

MIS12 is an inner kinetochore protein required for kinetochore assembly, kinetochore biorientation, and proper chromosome segregation in yeast and mammals (Goshima et al., 1999; Goshima et al., 2003b; Kline et al., 2006). Further, MIS12 is required for NDC80 deposition and physically interacts with NDC80 complex, a complex that directly associates with dynamic microtubules and is able to power chromosome movement in conjunction with microtubule disassembly (Wei et al., 2007; Powers et al., 2009). Here we take advantage of maize male meiotic cells to identify maize Mis12 homologue, characterize its cellular localization, and address its unique function in sister kinetochore co-orientation and reductional chromosome segregation in meiosis I.

Mis12 is duplicated in maize

Different from yeast and mammalian *Mis12*, we identified two *Mis12* genes in maize. Two *Mis12* genes share 89% sequence identities in coding sequences, and slightly lower level of protein sequence identities (81%) due to small frameshifts. Search results show that there is only one putative Mis12 gene in rice (CI015191), suggesting Mis12 duplication happened after *Zea mays* speciation. The fate of duplicated genes depends on species, biological context and

evolutionary history (Szklarczyk et al., 2008; Zhou and Wang, 2008). One possibility is that duplicated genes adapt to tissue specific expression, and the cDNA-qPCR analysis done here was designed to test this hypothesis. However, both maize Mis12 genes are expressed uniformly in different tissues, with Mis12-2 being more abundant). These data suggest that the duplicate *Mis12* genes show asymmetric expression rather than differential expression (He and Zhang, 2005; Ganko et al., 2007).

Maize MIS12 localizes to the central kinetochore

Yeast is an ideal model system to identify kinetochore proteins by genetic and molecular analysis. But the tiny cells minimize kinetochore cytological resolution. Our chromatin fiber analysis showed co-localization of MIS12 with CENH3 and CENPC on kinetochore subunits, but failed to reveal positional information of MIS12. In large eukaryotes, kinetochore subdomains can be discerned. Human MIS12 was assigned to the inner kinetochore using NDC80 as a marker (Kline et al., 2006). Here we use the well-characterized inner kinetochore marker CENPC to show that MIS12 localizes outside of CENPC on the spindle axis (Figure 2.6). XChIP analysis further confirms our observation indirectly. Chromatin immunoprecipitation experiments suggest that MIS12 is distantly associated with centromeric DNA. MIS12 XChIP studies showed an 11-fold enrichment for CentC, while CENH3 ChIP showed an enrichment of 120 fold.

The MIS12/NDC80 bridge between sister kinetochores mediates sister chromatid co-orientation in meiosis I

Sister kinetochores appear as single spots in early prophase I, and develop into two discernible spots at metaphase I as indicated by inner kinetochore protein CENPC. However the two sisters do not separate completely until late anaphase. Our results show that both MIS12 and NDC80 form a bridge between sisters and direct sister chromatid co-orientation in meiosis I by providing a unified microtubule binding interface (Figure 2.8). This confirmed the earlier postulation that a kinetochore protein may clamp sister kinetochores together for their co-orientation (Pidoux and Allshire, 2003; Rabitsch et al., 2003). During transition stages including prometaphase and metaphase, the inner structures of sister kinetochores separate (e.g. CENPC), while central structures remain intact to maintain sisters as one fused structure for microtubule mono-polar attachment. The co-orientation event may initiate prior to leptotene, presumably in interphase, when both Mis12 and NDC80 are known to be present (Figure 2.20). Maize *Mis12* RNAi weakens or breaks sister kinetochore bridge, and leaves sister kinetochores free for bipolar attachment (Figure 2.14). Pericentromeric cohesion prevents sister chromatids from full disjunction, causing chromosomes to lag on the spindle (Figure 2.15; 2.18)..

Chromosomes caught in the meiosis I midzone often remain through interkinesis and produce multiple nuclei (Figure 2.17). Multiple mininuclei tend to organize their own minispindles at meiosis II, and pass meiotic errors on to gametes (Figure 2.13). In severe cases, failures in disjunction appeared to cause meiotic abortion or polyploidy (Figure 2.16).

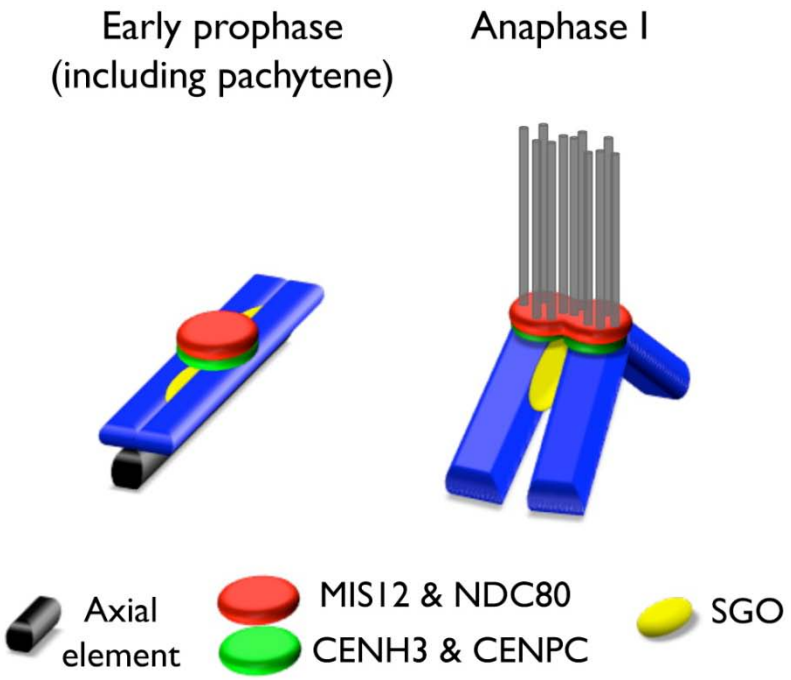

Figure 2.20. A three-dimensional perspective highlighting the role of the MIS12/NDC80 bridge. The left panel shows that meiotic kinetochores are formed in prophase I. The MIS12/NDC80 domain is shown in red and the centromeric regions (marked by CENH3 and CENPC) are shown in green. SGO is visible in pericentromeric regions at this stage (see Fig. 2.18A). Axial elements (Armstrong et al., 2002; Golubovskaya et al., 2006) are removed in late prophase but the MIS12/NDC80 bridge, which provides a unified microtubule binding interface, remains intact during metaphase I and early anaphase I.

While the primary phenotypes are meiotic, we also suspect there are mitotic defects. We observed dead sectors like leaf crack/split on mutant plants, although the mitotic phenotypes were inconsistent over generations or across families. The inconsistency of the phenotype could be due to the mild reduction of Mis12 mRNA and protein.

MIS12's function in sister chromatid co-orientation is distinct from the SGO/cohesion system

Cohesion is loaded to chromatin immediately after replication to keep sisters together, and stepwise cohesion release is required for meiosis progression. At the onset of anaphase I, cohesion along chromosome arms is cleaved and chiasmata are released, but cohesion in pericentromeres persist with the protection of SGO (Watanabe and Nurse, 1999; Kitajima et al., 2004). Mutation of cohesin subunit Rec8 causes sister chromatid premature separation in meiosis I. To differentiate the function of MIS12 from the SGO/cohesion system, we analyzed the SGO intensity in *Mis12* RNAi mutants, and found no SGO reduction (Table 2.1). Convincingly, SGO localizes to pericentromere regions between bipolar attached sister kinetochores at metaphase I (Figure 2.18). Neither Rec8 or SGO bind to kinetochores and are not sufficient to ensure sister chromatid co-orientation in meiosis I (Toth et al., 2000; Yamagishi et al., 2008; Lee et al., 2008). Taken together the data suggest that the MIS12/DNC80 bridge establishes sister kinetochore co-orientation while the SGO/cohesion maintain their orientation (Figure 2.20).

MATERIALS AND METHODS

Identification of maize Mis12 genes and comparison of their mRNA abundance

A presumed Mis12 homolog from Glycine max (sp43a06.y1; (Goshima et al., 2003a)) was used as a query to search maize sequence databases. Another research group followed the same reasoning and identified Arabidopsis MIS12 (Sato et al., 2005). Primers homologous to two maize sequences (MAGI4_132977 and MAGI4_143787; AC155386.2) were used to identify full-length Mis12 cDNAs from inbred B73 ear tissue (sequences to be submitted to GenBank). To compare relative expression levels, specific primers were used in a reverse transcription-quantitative PCR assay with Ubiquitin as an internal control. Primers for Mis12-1 were GAAGAGTCGGAAGAAGAAGCGGGCG (forward) and TAATCTCAGTCCTTCTC TGATTTGCA (reverse), for Mis12-2 were GCCCCCCCACAAATCCACAATCCAA (forward) and ATTTTCTGCCGCAATGCCGGTATTG (reverse), and for maize Ubiquitin-1 (Ubi1) were TAAAGACCCTGACTGGAAAA (forward) and ACGACCCATGACTTACTGAC (reverse).

Protein analysis

The complete Mis12-2 coding sequence was cloned into the pET-28a expression vector (Novagen) and expressed in bacteria. The His-tagged MIS12-2 protein was purified using Ni-NTA agarose (Qiagen). Anti-MIS12 antibodies were prepared in rabbit and affinity-purified by Strategic Biosolutions (Newark, DE). For protein blotting, nuclear protein from fresh root tips (~3 mm in length) and young ears (~7 cm in length) were extracted and blotted as described previously (Zhang et al., 2005).

Indirect immunostaining of male meiotic cells

Male meiocytes were prepared from Mis12 transgenic lines and wild type siblings (Zhang et al., 2005). Cells were incubated with rabbit anti-MIS12-2 (1:100), rabbit anti-NDC80 (1:50; (Du and Dawe, 2007)), chicken anti-CENPC (1:100; (Zhong et al., 2002b)) rabbit anti-Shugoshin (1:50; (Hamant et al., 2005)), or mouse anti-tubulin (1:500).

Indirect immunostaining of chromatin fiber

Young leaf nuclei prep (Zhang et al., 2005) was used to make chromatin fibers following Sullivan and Karpen method (2004). Fibers were blocked 15 minutes at room temperature with 3% BSA in blocking buffer, and incubated with rat anti-MIS12-2 (1:100), rabbit anti-CENH3 (1:50; (Zhong et al., 2002a)), chicken anti-CENPC (1:100; (Zhong et al., 2002b)), or mouse anti-tubulin (1:500).

Image analysis

Data were collected and analyzed using a Zeiss Axioimager and Slidebook software (Intelligent Imaging Innovations, Denver, CO). For quantification of MIS12 and SGO signal intensity, wild type and mutant cells were spotted to the same slide to reduce experimental variation. The total signal intensity from all kinetochores in a cell was subtracted from background signal intensity.

Crosslinked chromatin immunoprecipitation

Young maize ears around 5~10 cm in length were harvested and immediately ground into fine powder and extracted for nuclei prep (Zhang et al., 2005), and crosslinked chromatin immunoprecipitation was done with MIS12, CENH3 (positive control) and HTR12 (negative control) antibodies followed by real time PCR analysis (Zhong et al., 2002a; Nagaki et al., 2003; Bowler et al., 2004; Gendrel et al., 2005). Primers for CentC GATTGGGCATGTTCGTTGTG (forward) and CACTACTTTAGGTCGAAAAC (reverse), for knob repeat (negative control) were ACCAGAAATCCAAAAATGTG (forward) and GAGACCATTTCTTGGTCAAA (reverse). The final relative enrichment (REF) of CentC is defined as (RC_{CentC}/RC_{knob} of MIS12_1) / (RC_{CentC}/RC_{knob} of HTR12) (RC: relative concentration based on standard curve).

Transgenic plant production and propagation

The Mis12-1 cDNA was cloned into pMCG7942 (McGinnis et al., 2007) such that the maize Ubiquitin-1 promoter drives expression over two inverted copies of the same sequence. The Mis12-1 RNAi construct was transformed into hybrid line HiII by biolistic bombardment at Iowa State University. The Mis12-2 RNAi construct was prepared in a similar vector (pMCG1005) and transformed into HiII by Agrobacterium-mediated transformation. Nineteen Mis12-1 RNAi lines and 9 Mis12-2 RNAi lines were crossed and studied. Mis12-1 RNAi lines were screened at the UGA Plant Sciences Farm in the summer of 2007; otherwise, plants were grown in greenhouses.

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CHAPTER 3

PHOSPHOSERINES ON MAIZE CENH3 AND HISTONE H3 DEMARCATE THE CENTROMERE AND PERICENTROMERE DURING CHROMOSOME SEGREGATION[1]

[1] Li, X. *, Zhang, X.*, Marshall, J.B., Zhong, C.X., and Dawe, R.K. (2005). Plant Cell. 17**,** 572-583. *: These authors contributed equally. Reprinted here with permission of publisher.

ABSTRACT

Multiple phosphorylation events on histone H3 N-terminal are coupled to the cell cycle, however they occur on different chromosomal regions and their functions remain controversial. Here we show that phosphorylation of H3 on serine 28 (phH3-Ser28) in maize is a cell cycle dependent and pericentromere specific posttranslational modification. It's undetectable in interphase, becomes increasingly apparent with the cell cycle progression, and disappears in telophase. A unique feature of H3-Ser28 phosphorylation is that it never expands along the chromosome arm or towards the centromere, but is strictly limited to the pericentromeric domains during cell division. Considering the densely distributed cohesion in this heterochromatic domain, H3-Ser28 phosphorylation may serve primarily as an epigenetic marker to label this cohesive region. Interestingly, CENH3, a histone H3 variant exclusively recruited in the centromere, is phosphorylated on serine 50 (phCENH3-Ser50) following the same temporal pattern as H3Ser28 phosphorylation. Together, we propose that the primary role of the CENH3Ser50 and H3Ser28 phosphorylation is to demarcate the centromere and its flanking pericentromere domains during the cell division.

INTRODUCTION

Chromatin undergoes dramatic morphological change during cell division, and the two major complexes to reshape chromatin structure are cohesin and condensin. The cohesin is a four-subunit complex including Smc1 (the structural maintenance of chromosome), Smc3, Scc1, and Scc3 (Guacci et al., 1997; Michaelis et al., 1997; Losada and Hirano, 2005). It serves as a sister chromatid glue to physically hold sisters together until the onset of metaphase II, and may delimit the condensin deposition domains along the chromosomes (Lavoie et al., 2002). The condensin (I and II), a five-subunit complex including Smc2 and Smc4, is required for chromosome condensation and cohesion removal during meiosis (Hirano et al., 1997; Hirano, 2000, 2005; Yu and Koshland, 2007). Condensins are recruited in prophase to compact chromatin into fully condensed individual chromosomes for efficient alignment and subsequent segregation.

Histone H3 phosphorylation is closely related to chromosome cohesion and condensation during cell division, although it is also involved in gene regulation. Four known phosphorylation events on histone H3 all appear in a very similar cell cycle-dependent pattern. H3-Ser10 phosphorylation promotes chromosome condensation in animals. Temporal and spatial staining pattern of H3-Ser10 phosphorylation indicates its correlation with chromosome condensation (Hendzel et al., 1997). Mutation of H3-Ser10 causes abnormal chromosome condensation and severe chromosome loss (Wei et al., 1999). However, plant H3-Ser10 phosphorylation correlates with changes in sister chromatid cohesion rather than condensation. H3-Ser10 phosphorylation distributes along the entire chromosome arms in meiosis I. Disruption of cohesion in meiosis I changes H3-Ser10 distribution, and single-chromatid chromosomes have no H3-Ser10

phosphorylation (Kaszas and Cande, 2000b; Gernand et al., 2003b). Pericentromeric localization of H3-Ser10 phosphorylation in meiosis II and mitosis also correlates it to cohesion.

H3-Ser28 phosphorylation is a poorly characterized posttranslational modification. It was correlated to chromosome condensation in animals (Goto et al., 1999), but to chromosome cohesion in plants (Gernand et al., 2003b). Similar to H3-Ser10 phosphorylation, H3-Ser28 phosphorylation extends through the chromosome arms in meiosis I, but just localizes to pericentromere region in meiosis II and mitosis. Nonetheless, the precise boundary of H3-Ser28 phosphorylation is unclear without a centromere marker, and its biological function is under debate.

CENH3, a histone H3 variant, is an epigenetic marker of the centromere (Dawe and Henikoff, 2006). It contains a conserved histone fold domain and a diverged N-terminus, and colocalizes with another centromeric protein CENPC (Zhong et al., 2002a).

In human, CENH3 (CENP-A) is phosphorylated at serine 7. CENP-A phosphorylation displays a temporal pattern similar to H3-Ser10 phosphorylation during mitosis (Zeitlin et al., 2001b), and is required for chromosome alignment at metaphase (Kunitoku et al., 2003). Similarly, maize CENH3 is phosphorylated at serine 50 in a cell-cycle dependent manner (Zhang et al., 2005). Taking advantage of the excellent maize cytology resolution and our centromere makers, we precisely localize H3-Ser28 phosphorylation onto maize chromosomes, confirm maize CENH3's phosphorylation in mitosis, and discuss the biological implications of H3-Ser28 and CENH3 phosphorylation.

RESULTS

Anti-phH3-Ser28 antibodies stains the pericentromere during cell division

H3Ser28 phosphorylation is reported to mark whole chromosomes in mammalian cells, and is also scattered over the entire condensed chromosomes in *Arabidopsis* and barley during meiosis I (Gernand et al., 2003a). We carried out a detailed analysis of the early prophase stages of maize meiosis I. H3-Ser28 phosphorylation was undetectable in pachytene (Fig. 3.1 A), but was consistently observed in the regions surrounding kinetochores at late diplotene (Fig. 3.1 B). Staining increased in the pericentromeric domains at diakinesis (Fig. 3.1 C) and was visible trailing the kinetochores at prometaphase I (Fig. 3.1 D). In contrast, during prometaphase II, phH3-Ser28 appeared to lie between aligned chromatids at the metaphase plate (Fig. 3.1 E). Following chromatid separation in anaphase II, the staining began to lessen (Fig. 3.1 F), and became undetectable in telophase. Similarly, in mitosis, phH3-Ser28 was undetectable in interphase (Fig. 3.2 C, upper left), but pronounced at metaphase (Fig. 3.2 C, lower down).

Anti-phCENH3 antibodies stain the centromere on condensed chromosomes during mitosis

CENH3 is phosphorylated on serine 50 during meiosis with a similar temporal pattern to H3-Ser28 phosphorylation (Zhang et al., 2005). To test whether it's also phosphorylated on the same residue during mitosis, we carried out immunoassays on root tips and found a similar pattern.

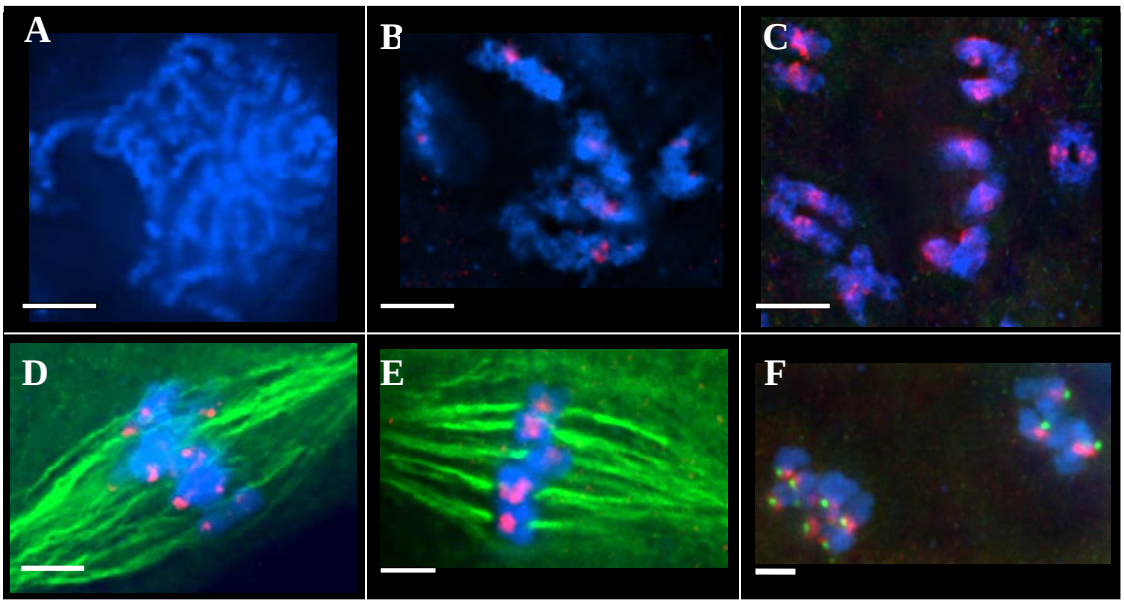

Figure 3.1 phH3-Ser28 localization in maize meiosis. All images are partial projections from 3D data sets. phH3-Ser28 staining is shown in red, microtubules in green, and chromosomes in blue. **A**) phH3-Ser28 staining at pachytene of meiosis I. **B**) phH3-Ser28 at diplotene. **C**) phH3-Ser28 at diakinesis. **D**) phH3-Ser28 at prometaphase I. **E**) phH3-Ser28 at prometaphase II. **F**) phH3-Ser28 at anaphase II. CENPC is shown in green to illustrate that the phH3-Ser28 staining trails kinetochores. Bars =5 µm.

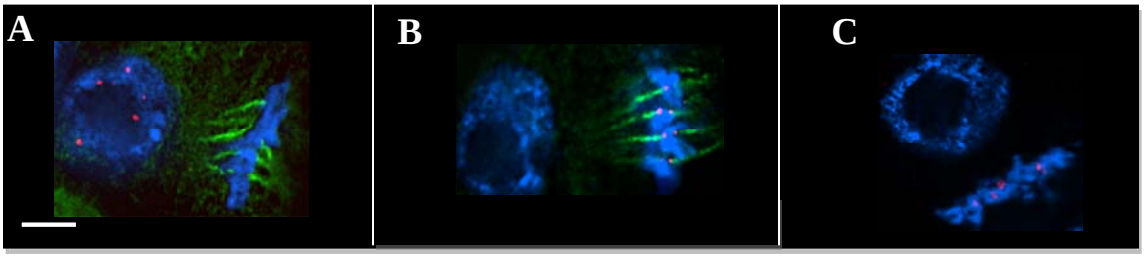

Figure 3.2 CENH3, phCENH3-Ser50, and phH3-Ser28 Localization in Mitosis. CENH3, phCENH3-Ser50, and phH3-Ser28 staining is shown in red, microtubules in green, and chromosomes in blue. **A)** CENH3 antisera stain only those cells in interphase, early prophase, and late anaphase. The cell at left is in interphase and the cell at right is in metaphase. **B**) phCENH3-Ser50 antisera stain only those cells in late prophase through early anaphase. The cell at left is in interphase and the cell at right is in metaphase. **C**) Mitotic phH3-Ser28 staining. The cell at upper left is in interphase and the cell at lower right is in metaphase. Bar (=5 µm) indicates the scale for all images in the row.

phCENH3-Ser50 was undetectable in interphase (Fig 3.2 B, left), while bright staining was observed in metaphase (Fig 3.2 B, right). In an overview of root tip cross section as shown in Figure 3.3A, staining was limited to the mitotic cells in prophase, metaphase, and anaphase. This same patterns was observed in meiosis (Zhong et al., 2002; Zhang et al., 2005); Fig 3.2 A).

Anti-phCENH3-Ser50 antibodies recognize a 17-18 kD protein in oryzalin-treated cells

Given the very limited number of phCENH3-Ser50-positive cells even in the most actively dividing tissue (Fig. 3.3 A), the quantity of phCENH3 is very low in whole protein extracts. One way to increase the amount of phCENH3 for western analysis is to delay cell cycle progression using microtubule-disrupting drug. To this end, root tips were treated with various concentration of the microtubule-depolymerizing drug oryzalin. A 4-8 hour treatment with 10 μM oryzalin was most effective, increasing phCENH3-Ser50-positive cells by 2-4 fold (Figs. 3.3A and 3.3B). Protein extracts from untreated and oryzalin-treated root tips were processed for western analysis side by side (Fig. 3.3C). Although the predicted 17-18 kD band was sometimes observed in untreated roots, the intensity of the band was consistently higher in oryzalin-blocked root tissue. When blots were washed, incubated with phosphatase, and re-probed with anti-CENH3 antisera, a wider band in the same molecular weight range was observed. It is likely that the wide anti-CENH3 band is composed of two bands, since both the phosphorylated and non-phosphorylated forms of the protein should be recognized after phosphatase treatment.

Other bands were also observed to a lesser and variable extent on western blots. Of these, the brightest and most consistently observed band was at ~16 kD (Fig. 3.3C). We cannot rule out the possibility that it represents a second phosphorylated histone or chromatin protein. However, the fact that anti-phCENH3-Ser50 can be detected in cells only at kinetochores, and

that phosphatase removes ~90% of this signal (zhang et al., 2005) demonstrates that the antiserum binds most effectively to phCENH3-Ser50.

Antibodies to phH3-Ser28 and phCENH3-Ser50 stain chromosomes in juxtaposed domains

Given the similar dynamics and neighboring chromosomal location of phH3-Ser28 and phCENH3-Ser50, it is intriguing to test whether phH3-Ser28 domains overlap with or are distinct from phCENH3-Ser50 domains. Because CENPC and phCENH3-Ser50 label the same region of kinetochores (Fig 3.4 A), anti-CENPC antisera were used to mark the presence of the kinetochores in phH3-Ser28-stained cells. Double labeling experiment showed that phH3-Ser28 and kinetochores localize to distinct positions relative to the spindle axis (Fig 3.4 B and 3.4 C). Opposing kinetochores separate early in prometaphase and are never attached by detectable CENPC-positive material. The regions that lie between sister kinetochores are uniformly stained by anti-phH3-Ser28 antisera (Fig 3.4 C). As kinetochores are drawn farther apart in later metaphase II (~1.8-2.1 microns), phH3Ser28 marked precentromeric domains appear to be under tension, strongly indicating phH3-Ser28 localizes within the cohesive region that holds sister chromatids together before anaphase initiation.

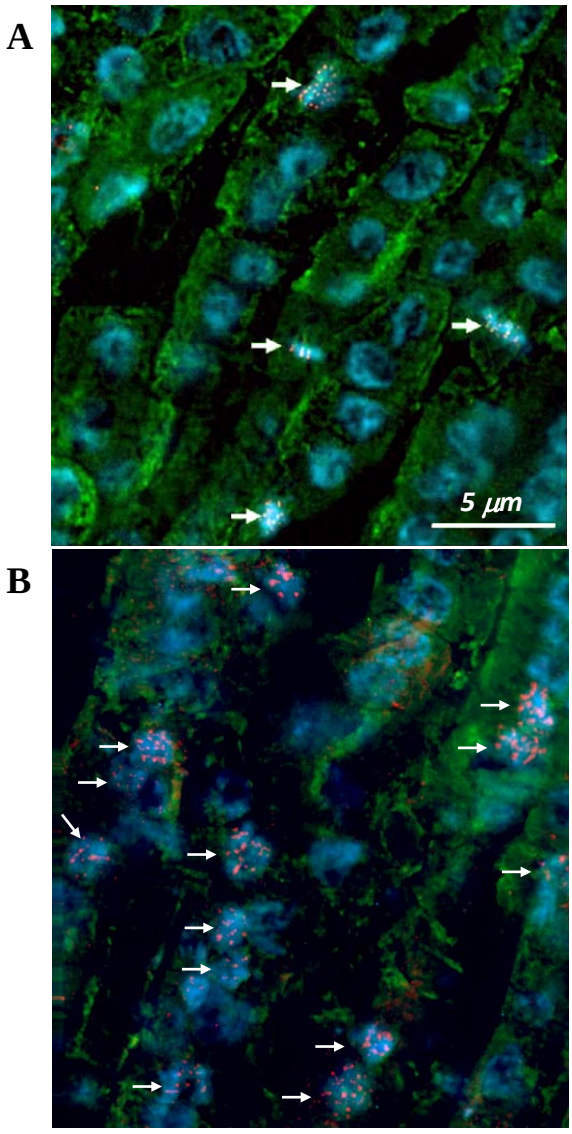
A
5 μm
B
C
kDa marker
1 Control phCENH3
2 Oryzalin phCENH3
3 Phos'tase CENH3
25
20
15
10

Figure 3.3 Effect of the microtubule-destabilizing drug oryzalin on phCENH3-Ser50 staining. **A**) An optical section from the meristematic zone of a (untreated) root tip after incubation with anti-phCENH3-Ser50 antisera (red), anti-α-tubulin antisera (green) and the DNA stain DAPI (blue). Note that phCENH3-Ser50 staining is restricted to cells in prometaphase and metaphase (arrows). **B**) A section from a seedling grown at the same time as the seedling shown in 'A', except in the presence 10 μM oryzalin for 8 hours. The number of phCENH3-Ser50-positive cells increases dramatically. **C**) Protein blot of extracts derived from root tips. Lane 1 shows anti-phCENH3-Ser50 staining in untreated root tip extracts. Lane 2 shows the results after treating root tips with 10μM oryzalin for 8 hours. Oryzalin induces a phCENH3-Ser50-positive band at 17-18 kD. Lane 3 shows the Lane 2 membrane after it was stripped, alkaline phosphatase treated, and re-probed with anti-CENH3 antisera.

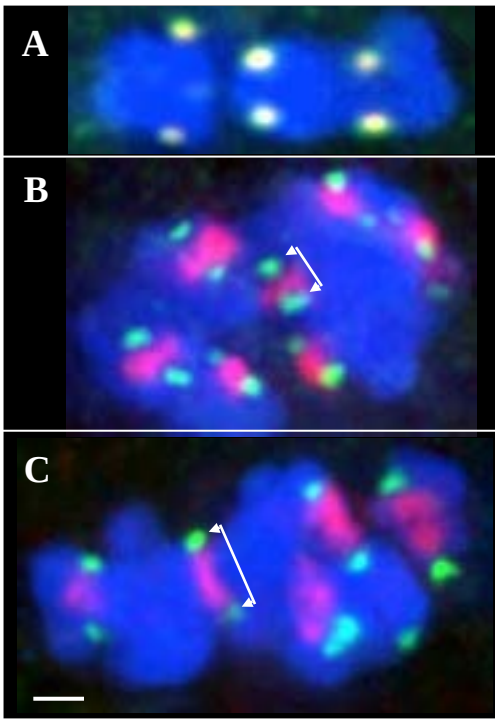

Figure 3.4. Differential localization of phH3-Ser28 and CENPC. **A**) Double labeling of CENPC (green) and phCENH3-Ser50 (red). The two signals overlap to produce a yellow color. **B**) Double labeling of CENPC (green) and phH3-Ser28 (red) at prometaphase (average kinetochore-kinetochore distance in this cell was 1.40 µm, n=7). Note that the phH3-Ser28 lies between sister chromatids and does not overlap with the CENPC staining (arrowheads). **C**) Double labeling of CENPC (green, arrowheads) and phH3-Ser28 (red) at late metaphase (average kinetochore-kinetochore distance in this cell was 1.93µm, n=7). In this case the phH3-Ser28 domain is stretched between the kinetochores as if it were under tension. Bar =1µm.

DISCUSSION

We have shown that H3-Ser28 phosphorylation localizes specifically to pericentromeric regions, and rarely extends along the chromosomal arms or spreads into centromere regions. Although CENH3 phosphorylation follows a similar temporal pattern, the spatial pattern is very different. The data suggest that histone H3 and its CENH3 variant demarcate the pericentromeres and centromeres, respectively.

A centromere-initiated phosphohistone code for the centromere and pericentromere

While the major roles of the centromere/kinetochore complex in chromosome segregation are well known (Nicklas, 1988; Choo, 2001b), pericentromeres have remained relatively vague, often identified only by their characteristic deep staining pattern (e.g. (Dawe, 2003). Only in *Schizosaccharomyces pombe* have pericentromeres been molecularly defined (Bannister et al., 2001), and in this species they are the primary cohesive domains that bind chromatids together during chromosome alignment (Appelgren et al., 2003). In plants, phH3-Ser10 (Kaszas and Cande, 2000a; Shibata and Murata, 2004) and phH3-Ser28 (Gernand et al., 2003b) (Figure. 3.1) are the only known molecular markers for the presumed pericentromeric domain. Our phH3-Ser28 localization data and similar phH3-Ser10 staining from *Arabidopsis* (Shibata and Murata, 2004) appear to confirm the interpretation from *S. pombe* that chromosomes are held together primarily by their pericentromeres (Appelgren et al., 2003).

It is now well established that cohesin preferentially associates with pericentromeres at metaphase (Haering and Nasmyth, 2003). Recent data demonstrate that the centromere/kinetochore complex has a strong influence on cohesin deposition in these

pericentromeric regions (Meluh and Strunnikov, 2002). The most convincing results come from *S. cerevisiae*, where cohesin is poorly recruited unless centromeric DNA is present (Megee and Koshland, 1999), and humans, where mis-targeted CENP-A (but not CENP-C) causes the co-recruitment of cohesin (Van Hooser et al., 2001). Budding yeast centromeres appear to enhance an existing pattern of cohesin distribution, such that the overall quantity of cohesin on either side of a (existing or newly introduced) centromere is elevated 5-6 fold (Weber et al., 2004). Similarly, a cohesin enhancing role for human centromeres would help to explain how 'new' centromeres, such as neocentromeres (Choo, 2001a) or artificial chromosomes (e.g. (Mee et al., 2003) are regularly segregated to progeny. Although the available data are compelling, the molecular basis for centromere-mediated cohesin accumulation remains unclear.

Based on the strict temporal coordination of CENH3 and H3 phosphorylation (Fig. 3.1 and Figure 3.2) we suggest that one signal for centromere-mediated cohesin accumulation is a histone kinase, which binds first at CENH3 and diffuses outwards over histone H3 to define the boundaries of the pericentromeric domains. As shown in Figure 3.5, such CENH3-centered diffusion events would satisfy the need to place the pericentromeres, cell cycle after cell cycle, in discrete domains immediately adjacent to centromeres. Human Aurora B phosphorylates both CENP-A and histone H3, providing a precedent for the idea that a single kinase can regulate the centromere and pericentromere (Zeitlin et al., 2001a; Goto et al., 2002).

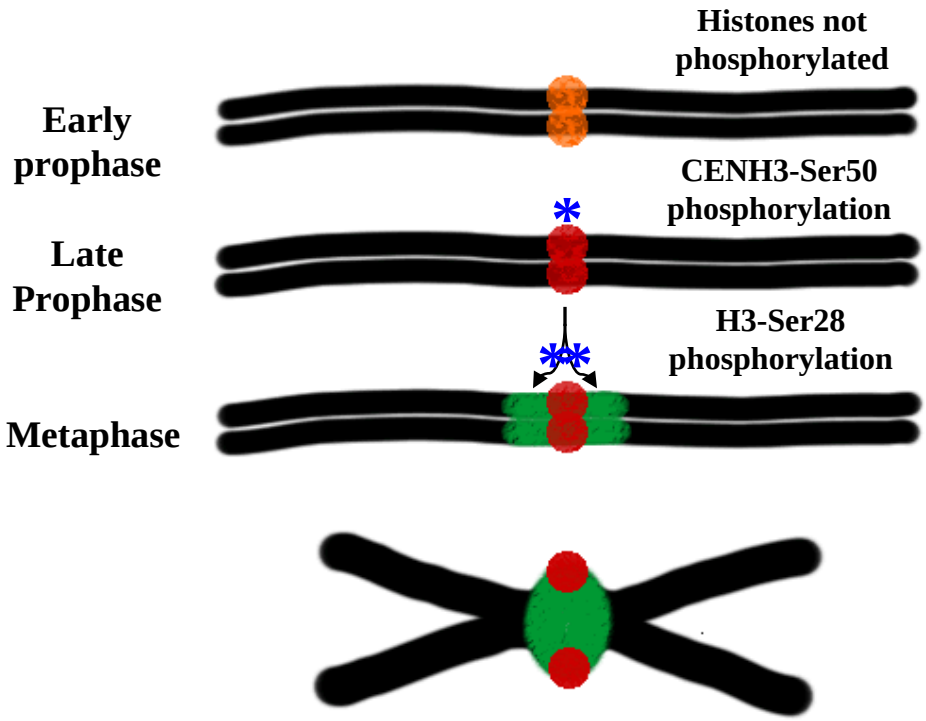

Figure 3.5. A kinase diffusion model for pericentromere determination. At top is a pre-diplotene chromosome and its centromere with unphosphorylated CENH3 (orange). At diplotene a histone kinase phosphorylates CENH3 first (red), then travels outwards over the pericentromere and phosphorylates histone H3 (green) in a diffusion-limited manner. The phosphorylated CENH3 interacts with the spindle, while phosphorylated histone H3 marks the pericentromere and serves to enhance or stabilize cohesion deposition.

By analogy to the affects of centromeres on cohesin distribution in *S. cerevisiae* (Weber et al., 2004), we further suggest that histone H3 phosphorylation stabilizes or enhances cohesin distribution within pericentromeres. Previous data support this view. phH3-Ser10 and phH3-Ser28 are entirely absent on maize and rye chromosomes that lack a sister chromatid at meiosis II, suggesting a causal relationship between histone phosphorylation and cohesin deposition (or vice versa; (Kaszas and Cande, 2000a; Gernand et al., 2003a). In addition, H3-Ser10 phosphorylation is one of the few (if only) phosphorylation events that accompanies the dissolution of sister chromatid linkages in *Xenopus* extracts (Losada et al., 2002).

Whether histone phosphorylation is a cause or consequence (or both) of chromosome alignment and segregation, our data provide compelling correlations between the two events. Foremost among these is the striking temporal coordination between and CENH3-Ser50 and H3-Ser28 phosphorylation (Figure 3.1 and Figure 3.2) and the fact that phH3-Ser28 defines the cohesive pericentromeric domain with apparent precision (Figure 3.4; (Gernand et al., 2003b). Our description of phCENH3-Ser50 also closely parallels the early descriptions of phCENP-A-Ser7 (Zeitlin et al., 2001b), suggesting that CENH3 phosphorylation may have a similar role in regulating anaphase onset (Kunitoku et al., 2003). The availability of well-characterized plant phosphohistone antibodies also has practical implications. The observation that anti-phCENH3-Ser50 antisera identify only segregating chromosomes (Fig. 3.2) opens the door to identifying the DNA in biologically active centromeres (by ChIP), an issue that has yet to be addressed in any organism. In addition, our data and the prior data from Gernand et al (2003b) establish anti-phH3-Ser28 antibodies as an excellent reagent for identifying the DNA sequences of plant pericentromeres.

MATERIALS AND METHODS

Antisera

A peptide was designed to correspond to residues 46-54 of maize CENH3 (Zhong et al., 2002), with a single phosphorylated serine at position 50 (SGGDS[p]VKKT). Anti-phCENH3-Ser50 antibodies were raised against the peptide conjugated to keyhole limpet hemocyanin. The preparation and affinity purification of antisera was performed by BioSource International, Inc. (Camarillo, CA).

Indirect immunolocalization in meiotic cells

Meiocytes were prepared from the W23 inbred line as described by Yu et al. (1999). Fixed samples were incubated with rabbit anti-CENH3 antibodies (1:25), rabbit anti-phCENH3-Ser50 antibodies (1:25), rabbit anti-phH3-Ser28 antibodies (1:25), chicken anti-CENPC antibodies (1:25) (Dawe et al., 1999; Zhong et al., 2002b) and/or mouse anti-tubulin antibodies (1:500) (Asai et al., 1982) as appropriate. Rhodamine-conjugated goat anti-rabbit antibodies (1:25) (Jackson Immunoresearch, West Grove, PA) and/or FITC-conjugated goat anti-mouse or donkey anti-chicken (1:25) (14274020, Boehringer Mannheim) secondary antibodies were then applied for 2 hrs at RT. Procedures for the necessary washing steps, mounting, and 4,6-diamidino-2-phenylindole (DAPI) staining have been described previously (Yu et al., 1997). For alkaline phosphatase treatment, meiocytes were fixed, adhered to cover slips, and incubated with 10 units of calf intestinal phosphatase (p4252, Sigma**,** St. Louis, MO) diluted in AP buffer (100

mM NaCl, 5 mM $MgCl_2$, 100 mM Tris, pH 9.5) at 37°C overnight. Cells were then washed 3X in 1X PBS for 5 min each and processed for immunofluorescence.

Indirect immunolocalization in root tips

Seeds from the maize inbred W23 were germinated in a moist incubator at 26°C for three days. In some experiments (Fig. 5) 3d-old seedlings were treated for 6-8 hours with 10 mM oryzalin (Chem Service) to depolymerize microtubules. Root tips ~3 mm in length were fixed, sectioned, and mounted as described previously (Yu et al. 1999). Slides were washed 3X in 1X PBS and processed for immunofluorescence as above, except secondary antibodies were applied for 3 hours at RT.

Image analysis

Data were acquired and analyzed using a DeltaVision 3D light microscopy workstation and associated software (Applied Precision, Issaquah, WA). Staining intensity measurements were averaged from 4 X4 pixel boxes centered over 10 different kinetochores or spindle fibers (next to kinetochores) as appropriate. Intensity values were divided by background staining (calculated in the same way, from the cytoplasm) to obtain signal to noise (S/N) ratios.

CENH3 extraction and blotting

Root tips ~3 mm in length were ground in liquid nitrogen and protein extracted as described previously (Pilch et al., 2004). Samples were electrophoresed by SDS-PAGE and

blotted to nitrocellulose. Blots were blocked for 1.5 hours with 5% Carnation nonfat milk in 0.1% TBST (20 mM Tris, 137 mM NaCl, 0.05% Tween 20, pH 7.6), and incubated for 4 hours with phCENH3-Ser50 or CENH3 antibodies at dilutions of 1:2000 (0.56 mg/ml) or 1:5000 (0.4 mg/ml) respectively. After washing 3X with TBST, the blots were incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies (1:3000 dilution; Amersham) and detected using the ECL western blotting kit (Amersham).

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CHAPTER 4

FUNCTIONAL ANALYSIS OF PLANT AURORA KINASES

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ABSTRACT

Aurora kinases play pivotal roles in regulating the cell cycle in animals and yeast. Specifically, Aurora B kinase phosphorylates histone H3 and CENP-A (CENH3 homologue in human), which is required for proper chromosome segregation. In plants, three aurora kinases (aurora1-3) have been identified but their functional classification remains unclear. We performed functional analysis of plant aurora kinases by overexpression and RNA interference in the model plant *Arabidopsis thaliana*. Our data shows that overexpression of Ataurora1 leads to bushy plants with either no inflorescence branches or few branches with weak apical dominance, while knockdown of Ataurora1 reduces inflorescence branches dramatically. Overexpression of Ataurora2 causes smaller plants with thicker stems, dark green leaves, and terminal flowers instead of indeterminate inflorescence. Overexpression of Ataurora3 results in dwarf plants or complete developmental suppression within four weeks. The data reveal that plant aurora kinases have important functions in cell division and affect multiple developmental processes.

INTRODUCTION

Cell division is a fundamental cellular process that underlies all growth and development. During cell division, the chromosomes undergo dramatic structural and morphological changes that facilitate cell cycle progression. These include a series of signal cascades that ensure all chromosomes pass through each substage precisely. A key protein in the regulatory cascade is aurora kinase, a broadly conserved serine/threonine kinase that has been implicated in chromosome restructuring, signal transduction, and chromosome partitioning during the cell cycle (Ruchaud et al., 2007). Aurora kinase exists in yeast as a single gene but is generally found in more than one copy in complex eukaryotes. In animals there are three aurora kinases (A, B, and C) which are thought to have partially overlapping but unique roles. Aurora A kinase is thought to function in centrosome separation and spindle assembly (Kufer et al., 2003). Aurora B kinase plays multiple roles in histone H3 phosphorylation (H3 at serine 10 and 28), kinetochore assembly, and spindle attachment (Shannon and Salmon, 2002). Aurora C kinase is unique to mammals and has a relatively minor role (Shannon and Salmon, 2002).

In *Arabidopsis*, the aurora kinases are identified as AtAurora1, AtAurora2, and AtAurora3, which share a conserved characteristic catalytic domain with animal aurora kinases (Demidov et al., 2005). The transcription profile shows *Arabidopsis* aurora kinases are preferentially transcribed in dividing cell-enriched tissues such as floral buds and young roots (Demidov et al., 2005). The authors showed that GFP-tagged AtAurora1 and AtAurora2 have similar dynamic localization patterns. Each are present at spindle poles during prophase, move to the spindle midzone at metaphase, then return to spindle poles from anaphase to telophase (Demidov et al., 2005; Kawabe et al., 2005; Kurihara et al., 2006). In contrast, GFP-tagged

AtAurora3 signals appear as dots around the nuclear periphery in early prophase and then concentrate and align at centromere regions during metaphase, where they remain until anaphase (Kurihara et al., 2006). In vitro assays suggests that AtAurora3 phosphorylates histone H3 on serine 10 and 28 (H3Ser10 and H3Ser28) (Kurihara et al., 2006).

Plant aurora kinases have yet to be functionally classified by *in vivo* analysis. In particular, it is not yet known which of the Arabidopsis Aurora kinases is the functional homolog of human aurora B. Aurora B kinase is particularly important in animals. First, it promotes chromatin condensation (Giet and Glover, 2001; Lipp et al., 2007). Second, aurora B is required for the cohesion of sister chromatids and protects cohesion until the onset of the anaphase. Depletion of aurora B cause precocious separation of sister chromatids in meiosis I (Rogers et al., 2002; Dai et al., 2006; Resnick et al., 2006; Yu and Koshland, 2007). Third, Aurora B is required for spindle assembly and serves in a checkpoint that ensures proper kinetochore orientation and attachment by the spindle (Kallio et al., 2002; Dewar et al., 2004; Ducat and Zheng, 2004; Hauf et al., 2007; Kelly et al., 2007; Monje-Casas et al., 2007). Fourth, Aurora B localizes to centromeres in prometaphase and metaphase where it forms a complex with other proteins to orchestrate a series of key cell division events that culminate in cytokinesis (Giet and Glover, 2001; Zeitlin et al., 2001; Tanaka et al., 2002; Andrews et al., 2004; Jeyaprakash et al., 2007; Ruchaud et al., 2007). Since each of these cell division functions are also required in plants, and the basic localization patterns of Aurora kinases are conserved in plants, identification of plant aurora B homolog is of great interest.

Efforts on the identification of plant aurora B by homology have been inconclusive. Phylogenetic analysis suggests Ataurora1 and AtAurora2 are duplicated genes with similar sequence and subcellular localization patterns. Both auroras are evenly distributed in interphase

nuclei, and primarily associated with spindle microtubules during mitosis (Demidov et al., 2005; Kawabe et al., 2005). Ataurora1 phosphorylates H3Ser10 *in vitro*, although it has no H3Ser28 kinase activity. In contrast, AtAurora3 localizes directly on centromeres similar to animal Aurora B kinase, and could phosphorylate both H3Ser10 and H3Ser28 by *in vitro* assay. Taken together these data suggest that AtAurora3 may be a better candidate than AtAurora1 (Demidov et al., 2005; Kawabe et al., 2005). Nevertheless without *in vivo* analyses, it is not possible to determine which protein(s) is fulfilling the aurora B functions. Therefore we performed *in vivo* functional analysis of plant aurora kinases by RNAi knockdown and constitutive overexpression in the model plant species *Arabidopsis thaliana*.

RESULTS

Ataurora1 is involved in multiple plant developmental pathways

Compared to wild type plants, Ataurora1 knockdown plants have 1-2 times more rosette leaves (Fig 4.1A), and sometimes form rosette leaves where the cauline leaves of the inflorescence stem would normally form (Fig 4.1 B). In addition, axillary branch growth is repressed on the floral stem (3-4 times less than that of wild-type) (Fig 4.1 C).

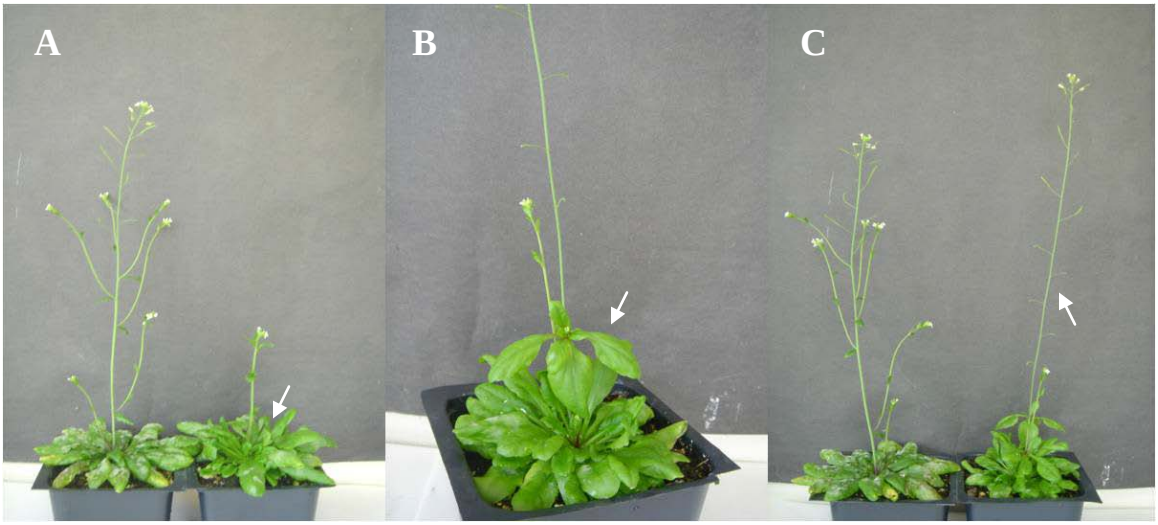

Figure 4.1 Phenotypes of AtAurora1 RNAi plants. A) Knock-down plant with more rosetta leaves (right, arrow) than wild-type plant (left). B) Knock-down plant with rosetta leaves on the primary infloral stem (arrow). C) Knock-down plant with reduced branches (right, arrow) than that of wild-type (left).

Overexpression of Ataurora1, on the other hand, displays a range of phenotypes (Fig 4.2). In the most severe cases the plants appear ball-like with no reproductive development at all. In plants with less severe phenotypes the plants are bushy with few flower shoots; and the least affected plants have many branches, but no one primary shoot probably as a result of loss of apical dominance (Figure 4.2). Due to abnormal reproductive development, overexpression plants generally produce few seed pods (siliques).

We measured the transcription level of Ataurora1 in each group by reverse transcription-PCR (RT-qPCR). As expected, Ataurora1 is overexpressed in all three groups compared to wild-type plants. The more severe developmental phenotypes corresponds to higher levels of Ataurora1 expression (Figure 4.3).

Ataurora2 overexpression results in small plants with terminal flowers

Despite no obvious morphological phenotype observed in the Ataurora2 RNAi lines (data not shown), overexpression of Ataurora2 leads to shorter plants with terminal flowers at the position of indeterminate inflorescent (Figure 4.4). Overexpression plants have sturdy stems and dark green leaves as well (Figure 4.4B, arrows).

Figure 4.2 AtAurora1 overexpression plants with variable phenotypes. Top, wild-type; bottom left, severe phenotype; bottom middle, medium phenotype; bottom right, mild phenotype.

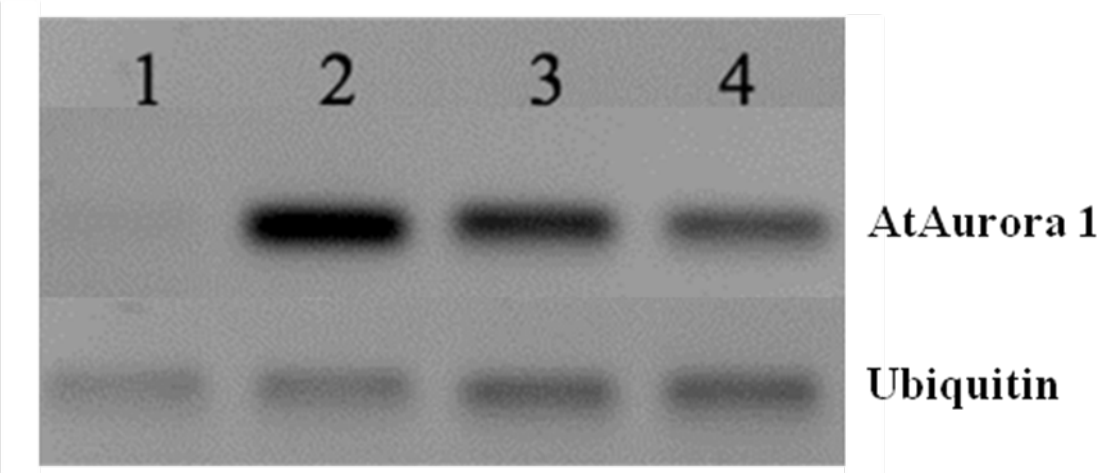

Figure 4.3 Reverse transcription-PCR (RT-qPCR) analysis shows Ataurora1 expression correlates with the plant developmental defects. Lane 1, wild type; lane 2, severe line; lane 3, medium line; line 4, mild line.

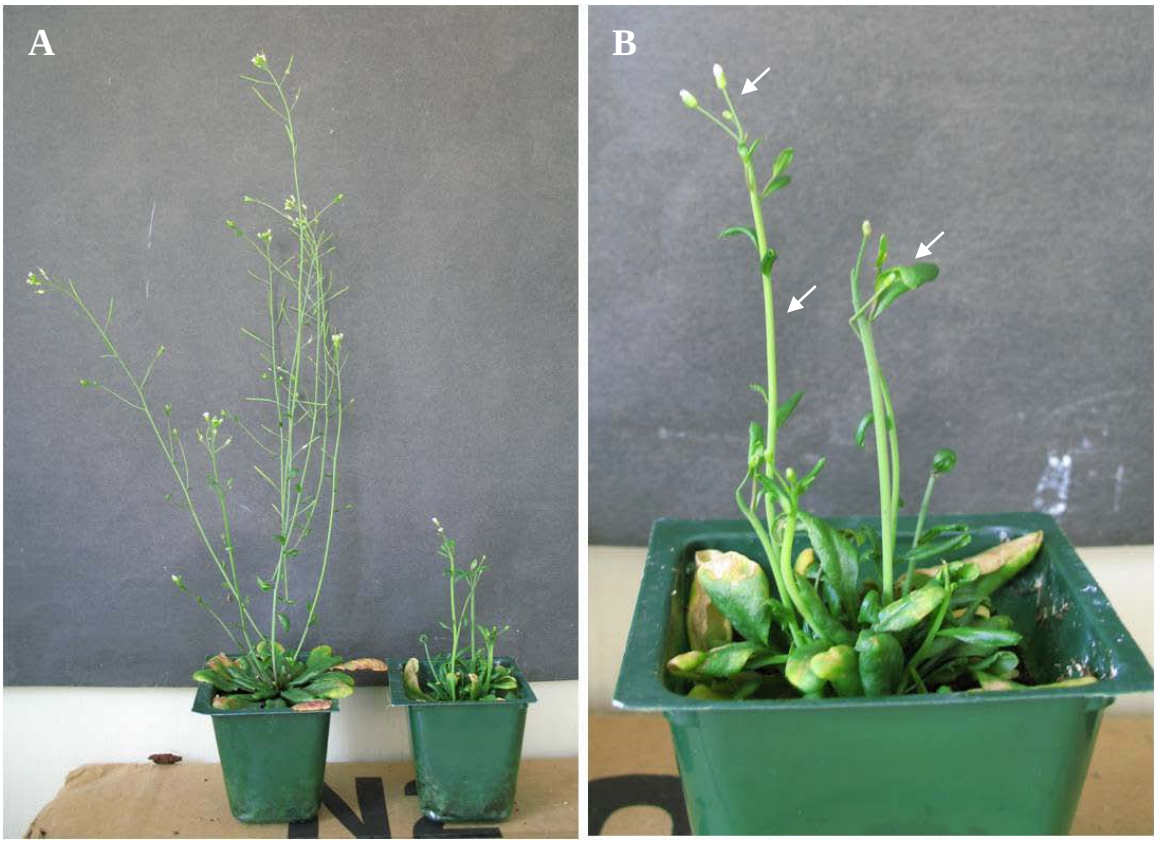

Figure 4.4 Phenotypes of Ataurora2 overexpression lines. A) Overexpression plant (right) is shorter than wild-type (left). B) Close-up view of overexpression plant with terminal flowers, thick stem and dark green leaves (arrows).

Ataurora3 overexpression causes severe growth defects

Similar to Ataurora2, there is no morphological phenotype in Ataurora3 knockdown lines (data not shown), whereas overexpression of Ataurora3 leads to severe growth repression. Over 80% of the plants grow very slowly, and the most severe plants never have fully extended leaves and die with small stature within four weeks (Figure 4.5). Other plants appear closer to normal but with few or no floral stems. In addition, the nodes (leave positions) and internodes are more closely spaced than normal (Figure 4.5). Not surprisingly, there are very few seeds produced in the AtAurora3 overexpression lines.

DISCUSSION

Aurora kinases coordinate cell division by multiple phosphorylation cascades. In human, Aurora B phosphorylates histone H3 and CENH3, ensures proper microtubule-kinetochore attachment, promotes chromosome congression, and provides spatial cues for cell cycle progression in anaphase and during cytokinesis (Fuller et al., 2008; Rosasco-Nitcher et al., 2008); Aurora A is involved in bipolar spindle assembly and chromosome alignment by promoting microtubule polymerization and maintaining microtubule flux (Sasai et al., 2008; Wang et al., 2008), while Aurora C may functionally overlap with aurora B. Plant aurora kinases are identified in *Arabidopsis*, and their localization patterns have been described in detail (Demidov et al., 2005). However, the in vivo functions of aurora kinases have not been reported, and it remains unknown which of the known genes confer the critical aurora B functions in plants.

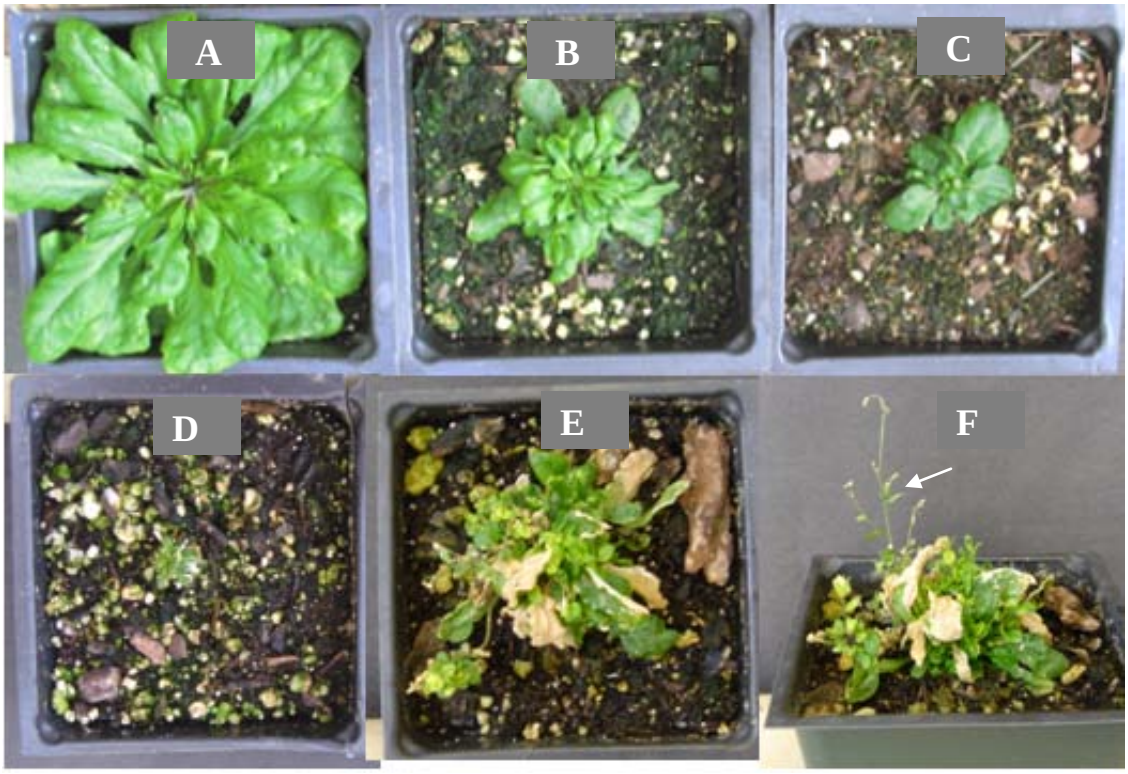

Figure 4.5 Phenotypes of AtAurora3 overexpression plants. A) Wild-type. B) A plant with mild phenotype. C) A plant with medium phenotype. D) A plant with severe phenotype dies within weeks. E) A plant survives with bushy stature. F) The only stem in this plant shows short node and internode (arrow).

Both Ataurora1 and Ataurora3 overexpression repress plant growth and development, and result in severely dwarfed plants. Similarly, knockdown of Ataurora1 leads to obvious morphological defects. Although we have not assayed cell division in this report, these data imply that both genes have essential roles in cell division. Plant vegetative and reproductive development requires specific cell proliferation in a timely manner. Knockdown or overexpression of aurora kinases severely disrupts cell cycle progression, and causes severe chromosome missegregation and extensive aneuploidy, which may hinder or alter developmental program and give rise to pleiotrophic developmental abnormalities. It is also possible that aurora kinases are directly involved in certain specific developmental pathways. Further cytological and genetic analyses are required to confirm these hypotheses.

WORK IN THE FUTURE

Given the multiple functions of aurora kinases in other species, it is not surprising that knockdown and overexpression of aurora kinases cause severe developmental abnormalities. To better understand these phenotypes, cytological analysis will be conducted for all nine RNAi, overexpression, and T-DNA insertion lines (two Aurora1 T-DNA insertion lines are 089167 and 031697c; two Aurora2 T-DNA insertion lines are 143594 and cs354226; and two Aurora3 T-DNA insertion lines are 081949 and 114602; http://www.arabidopsis.org/abrc/index.jsp) with regard to chromosome segregation, spindle structure, and histone H3 phosphorylation defects. We are optimistic that these studies will yield new and valuable data.

MATERIALS AND METHODS

RNAi construct and overexpression construct

RNAi. To generate recessive mutations, an RNAi strategy was utilized to specifically knock down each of the three aurora kinases: AtAurora1, AtAurora2, and AtAurora3. Due to high sequence similarity of the coding sequences, 300 nucleotides in 3' UTR divergent region were cloned into TOPO-PCR4 vector (Invitrogen) with enzymatic linkers at each end and then subcloned into the RNAi vector, FGC5941. Primers for *AtAurora1* RNAi were CCTAGGATTTAAATAAACAAAACGCTTACTGTTC (forward) and TTAATTAAGGCGCGCCGACTGTTGCATTTATCCTTC (reverse), for *AtAurora2* RNAi were CCTAGGATTTAAATAGCGATGAAGCTAGGAAAAG (forward) and TTAATTAAGGCGCGCCAGTAACATCAGTTAACGTTA (reverse), for *AtAurora3* RNAi were CCTAGGATTTAAATTGTATTCTCTCCCAATCTCA (forward) and TTAATTAAGGCGCGCCAGACGACGATGAGGAGTACT (reverse). The resulting RNAi constructs produce hairpin structures when expressed under the control of 35S promoter *in vivo*.

Overxpresssion. The pEarleyGate vector 101 was used to generate overexpression lines. An Arabidopsis floral cDNA library was built, and the coding region of each aurora kinase (without stop codon; Demidov et al., 2005) was cloned into an 'entry' vector (pENTR/D-TOPO; Invitrogen) flanked by attL1 and attL2 sites. Primers for *AtAurora1* overexpression were CACCATGGCGATCCCTACGGAGACACAACA (forward) and AACTCTGTAGATTCCAGAAGGATCAGC (reverse), for *AtAurora2* overexpression were CACCATGTTGTATCAGGCGGCTTCAGAGGCT (forward) and TCCTCTGTAAAGGCCTGATGGGTCTGCGT (reverse), for *AtAurora3* overexpression were

CACCATGAGTAAGAAATCGACAGAATCTGACG (forward) and AATATCAATTGAGGCACACACACCTTT (reverse). The flanking sites attL1 and attL2 enable efficient recombination into a second set of 'destination' vectors known as the pEarleyGate series. The 35S promoter constitutively expresses each of the three proteins with YFP and HA epitope tags at the C-terminus.

Generation of transgenic lines

RNAi and overexpression constructs were transformed into *Agrobacterium* (GV3101 strain), and then introduced into *Arabidopsis* using the floral-dip method. A blank vector was also transformed as a control. On average, around 20 unique genetic events were obtained for each transformation. Both vectors confer resistance to the herbicide BASTA, which allow for a straightforward selection of T1 transformant seedlings by foliar spray.

Reverse transcription-quantitative PCR analysis of AtAurora1 expression level in *AtAurora1* overexpression lines.

Young leaves were harvested from two-week old seedlings for total RNA isolation using Plant RNeasy Mini Kit (Qiagen). Reverse transcription-real time PCR was carried out using AtAurora1 specific primers with Ubiquitin as an internal control. Primers for AtAurora1 were GAGACACAACACCAGGAGAAG (forward) and ATCGCTTAAAGTCCATCTCTT (reverse), for arabidopsis ubiquitin-protein ligase (Skp1) were CTGCTACCTCCGATGACGAT (forward) and GCGAACCTCTTCCTCTTCCT (reverse).

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CHAPTER 5

CONCLUSIONS AND PERSPECTIVES

PART A MIS12 bridge mediates sister kinetochore co-orientation and controls reductional chromosome segregation in meiosis I via organizing an unified microtubule binding face.

The kinetochore tethers the chromosomes to the microtubule and plays a pivotal role in mediating chromosome behavior during cell division. Each set of sister kinetochores bi-orient for sister chromatid segregation during mitosis and meiosis II, and co-orient for homolog segregation during meiosis I (Brar and Amon, 2008; Cheeseman and Desai, 2008). Much is known about kinetochore component, and around 80 proteins are identified to date using genetic and biochemical analysis. However, the fundamental mechanism of how the proper orientation of sister kinetochores is established and maintained remains obscure. MIS12 is a critical kinetochore structural component that contributes to inner and outer kinetochore assembly, and mediates sister kinetochore bi-orientation in mitosis (Goshima and Yanagida, 2000; Kline et al., 2006). Taking advantage of high resolution of maize cytology, we observed a visible MIS12 bridge to link sister kinetochores during meiosis I for the first time, implying a unique molecular mechanism that mediates sister kinetochore co-orientation. Knockdown of *Mis12* reduces MIS12 protein, breaks the sister kinetochore linkage and leads to sister kinetochore bi-orientation instead of co-orientation, suggesting that MIS12 bridge is required for sister kinetochore co-orientation in meiosis I. Interestingly, NDC80 also appears as a bridge between sister kinetochores in a MIS12 dependent manner. Given the broad conservation of MIS12 and NDC80 across species (Chen et al., 1997; Goshima et al., 1999; Zheng et al., 1999; Goshima et al., 2003; Sato et al., 2005; Du and Dawe, 2007), we propose that sister kinetochore co-orientation is mediated, at least partially, by MIS12 bridge and further stabilized by mono-polar attachment of microtubules via the NDC80 bridge.

The second fundamental question is whether and how the kinetochore actively contributes to homolog segregation during meiosis I. Accurate homolog segregation is a prerequisite for a successful gametogenesis. Only two kinetochore proteins are reported to be involved in this process: monopolin and Moa1 (Toth et al., 2000; Yokobayashi and Watanabe, 2005). Monopolin regulates homolog segregation presumably via clamping sister kinetochores together in *S. cerevisiae* (Rabitsch et al., 2003). However, there is no functional homolog of monopolin identified yet outside of this model system. Moa1 physically associates with Rec8 cohesin and regulates monopolar attachment via Rec8 cohesin, rather than an independent kinetochore function (Yokobayashi and Watanabe, 2005). Thus far, no conserved kinetochore protein has been identified to regulate reductional chromosome segregation. Our data showed that each set of sister kinetochores are linked together by the MIS12 bridge. Built upon the MIS12 bridge, microtubule binder NDC80 forms a continuous structure over sister kinetochores for monopolar microtubule attachment. Destruction of MIS12 bridge by *Mis12* RNAi breaks sister kinetochore association, disassociates the NDC80 bridge, and leads to sister kinetochore bi-orientation and missegregation of homologous chromosomes. Thus, MIS12/NDC80 bridge couples sister kinetochore co-orientation to monopolar attachment and controls the homolog segregation, revealing an active role of the kinetochore in mediating reductional chromosome segregation.

Further, we propose that centromeric MIS12/NDC80 bridge and pericentromeric Shugoshin/Rec8 cohesion cooperate to direct reductional segregation. MIS12 and NDC80 are all constitutive kinetochore components (Figure 2.4; (Kline et al., 2006; Du and Dawe, 2007)). They're recruited for kinetochore assembly and microtubule attachment, and may fuse sister kinetochores early in interphase. However, MIS12 bridge is not the only mechanism mediating

homolog segregation. When sister kinetochores are bipolarly attached due to *Mis12* RNAi, the persisting SGO/Rec8 cohesion counteracts the bipolar pulling force and maintains sister chromatids association, causing numerous chromosomes to stall on the spindle mid-zone (Moore et al., 1998; Watanabe and Nurse, 1999; Kitajima et al., 2004; Rabitsch et al., 2004). Also, sister kinetochores naturally separate as anaphase I progresses, during when Rec8 cohesion and Shugoshin would become the primary means of holding the sisters together (Watanabe and Nurse, 1999; Kitajima et al., 2004; Watanabe, 2004). Indeed, Rec8 is required to organize the axial elements and is the binding substrate of Shugoshin (Klein et al., 1999; Stoop-Myer and Amon, 1999; Kitajima et al., 2004; Golubovskaya et al., 2006). Axial elements of the synaptonemal complex hold sisters together and facilitate the establishment of kinetochore co-orientation in prophase when the microtubule binding face is assembled (Counce and Meyer, 1973; Dawe, 1998; Moore and Orr-Weaver, 1998; Cheeseman and Desai, 2008). Loss of Rec8 or Shugoshin causes full disjunction of sister chromatids.

Our findings also have implications in genetics, medicine and the underpinnings of aneuploidy diseases. In human females, most errors in meiosis occur during ovulation as chromosomes align and segregate at meiosis I (Hassold and Hunt, 2001). Our data show that quantitative reductions of a key kinetochore structural protein lead to premature separation of sister chromatids, a major cause of human aneuploidy (Hassold and Hunt, 2001). Age-dependant loss of kinetochore proteins may provide a mechanical basis for many of these meiotic errors.

However, two maize *Mis12* genes are expressed uniformly in different tissues. It remains unclear whether two MIS12 proteins differentially function or closely interact to form a sister kinetochore bridge in meiosis? Two MIS12 proteins might actually form heterodimers to facilitate bridge formation. Future work in single gene knockout, in vitro protein-protein

analysis, and EM scanning may reveal MIS12 bridge in details and uncover the molecular mechanism of MIS12 bridge formation.

Another interesting issue is whether NDC80 has the same functions as MIS12 does in mediating reductional chromosome segregation in meiosis I. NDC80 complex directly binds to dynamic microtubules on the one side, and binds to MIS12 complex on the other side (Wei et al., 2005; Kline et al., 2006; Wei et al., 2007; Powers et al., 2009). Connecting sister kinetochores could be an active role of NDC80 and is required for sister chromatid co-segregation in meiosis I. Alternatively, NDC80 bridge could be a passive structure upon MIS12 bridge. Disruption of NDC80 would not affect MIS12 bridge and therefore has no effect on kinetochore co-orientation and homolog segregation, but causes metaphase arrest or cell cycle progression delay. Construction and analysis of NDC80 knockouts will testify either hyphotheses and further shed light on how the kinetochore controls homolog segregation in meiosis I.

PART B CENH3 phosphorylation initiates phosphorylation wave along the chromosome to demarcate distinct chromosomal domains

Histone H3 phosphorylation is implicated in chromatid cohesion in plants (Kaszas and Cande, 2000; Gernand et al., 2003), however, no consistent H3 phosphorylation in centromere has ever been identified. Our data shows that phH3-Ser28 occurs only in the pericentromere during cell division in maize. Interestingly, CENH3, the centromere specific H3 variant, is phosphorylated with the similar cell cycle dependent pattern (Figure 3.2; (Zhong et al., 2002; Zhang et al., 2005)). Together, phCENH3-Ser50 and phH3-Ser28 demarcate the centromere and pericentromere during chromosome segregation. Given the fact that the centromere enhances

pericentromere cohesion (Megee and Koshland, 1999; Weber et al., 2004), we propose that CENH3 phosphorylation initiates a phosphorylation wave and extends it towards the pericentromere to coordinate chromosome alignment and segregation. One approach to test this model is to replace maize CENH3 serine 50 with a similar but non-phosphorytable amino acid (i.e. alanine). If consistent with our model, mutation of CENH3 may alter distribution pattern and abundance of cohesion in pericentromeric region, and cause chromosome segregation defects. Another interesting question is what is the kinase to phosphorylate maize CENH3 serine 50. Aurora B is a chromosome passenger protein and plays critical roles during cell division by phosphorylating various substrates (Shannon and Salmon, 2002; Tanaka et al., 2002; Lampson et al., 2004; Monje-Casas et al., 2007; Yu and Koshland, 2007; Liu et al., 2009). Particularly, CENP-A (CENH3 homolog) serine 7 is phosphorylated by aurora B (Zeitlin et al., 2001). So we speculate plant aurora B phosphorylates CENH3 in plants. However, the understanding of plant aurora kinases is limited to their subcellular localization and in vitro kinase assay (Demidov et al., 2005; Kurihara et al., 2006). It remains challenging to identify plant aurora B without in vivo analysis. To this end, in vivo functional characterization of plant aurora kinases has been performed and the preliminary data are presented in chapter 4.

Although Ser10 and Ser28 phosphorylation is not conserved on divergent CENH3 N-termini, the similar cell-cycle dependent phosphorylation pattern of CENH3 serine 50 and CENP-A serine 7 implies the conservation of CENH3 phosphorylation in animals and plants, indicating that the 'phospho code' on CENH3 is conserved in principle, but on different serine residues across species. Beyond the phosphorylation, there may be more general 'histone code' on CENH3 given the prevalence of 'histone code' and the epigenetic nature of the centromere (Strahl and Allis, 2000; Dawe and Henikoff, 2006). It's interesting to identify other codes such

as (presumbly) methylation and acetylation and decipher their corresponding functions using genetic, biochemical, and cytological approaches.

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