

MECHANISM OF KINETOCHORE ASSEMBLY IN MAIZE

by

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(Under the Direction of R. KELLY DAWE)

ABSTRACT

The centromere is an essential chromatin structure for cell division and involves centromeric DNAs and their associated kinetochore. The kinetochore is a large complex of proteins that links centromere to spindle microtubules. Although studies of plant centromere/kinetochore have been especially active in recent years, few kinetochore proteins have been well characterized and the mechanism of kinetochore assembly remains unclear. Here I present cytological, biochemical, and transgenic data that contribute to our understanding of plant kinetochores.

NDC80 is important for formation of the kinetochore-microtubule interface in yeast and animals. Although NDC80 sequences were found in plants, their function as a kinetochore protein had not been established. Using antibodies specific to maize NDC80, the protein was localized at centromeres and outside of the inner kinetochore protein CENP-C (CENPC), supporting the general view of NDC80 as a central kinetochore protein. NDC80 is constitutively present on maize kinetochores, which is different from non-plants where the association of NDC80 varies with the cell cycle. The continuous presence of NDC80 at centromeres suggests it has an important role in the kinetochore.

With the complexity and variety of centromeric DNA, it is a major challenge to illustrate how kinetochores recognize centromeres. CENPC is a good candidate to investigate the interaction among the foundation proteins that are constitutively and closely associated with centromeres. By gel mobility shift assays, maize CENPC was shown to have DNA and RNA binding capacity. The major binding domain maps to a duplicated exon region. *In vivo* transgenics demonstrated that the binding domain is required for the centromere localization of CENPC. Based on a novel observation that RNA facilitates the CENPC-DNA interaction, we propose a mechanism for the involvement of centromere RNA in kinetochore assembly.

INDEX WORDS: kinetochore, centromere, NDC80, mitosis, meiosis, Maize, CENPC, centromere DNA, centromere RNA

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DEDICATION

To my husband and my parents for their love.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Overview

Kinetochores are a term derived from Greek, with 'kineto' meaning 'move' and 'chore' meaning 'means for distribution' (Cheeseman and Desai, 2008). It is used to name a multiprotein complex that forms at the centromere, the primary constricted region of the chromosome. During cell division this organelle serves as an attachment site for the spindle, drives chromosome movement, and ensures accurate chromosome transmission. Though underlying centromeric DNAs change quickly, kinetochores as cellular structures have been found well conserved among all eukaryotes (Meraldi et al., 2006).

High-fidelity chromosome segregation requires that kinetochores correctly capture microtubules, promote bi-orientation, and trigger anaphase onset (Yu et al., 2000, Vagnarelli et al., 2008). A dysfunctional kinetochore causes chromosome missegregation and generates aneuploidy (Mikami et al., 2005, Gurzov and Izquierdo, 2006, Lin et al., 2006b). Kinetochore defects have been shown to be connected with human cancers (Tomonaga and Nomura, 2007, Diaz-Rodriguez et al., 2008). The kinetochore is composed of many proteins/subcomplexes, and each of them contributes very specific functions to the whole (Yu et al., 2000, Houben and Schubert, 2003, McAinsh et al., 2003, Cheeseman and Desai, 2008). Some components assemble on centromeres earlier during kinetochore formation and build a foundation for other kinetochore proteins, including the subcomplexes that form the kinetochore-microtubule interface. When

kinetochores and microtubules are attached, forces can be applied, including those provided by molecular motors and the mechanics of microtubule polymerization/depolymerization. Some kinetochore proteins play a checkpoint role and inhibit anaphase initiation until sister kinetochores are attached by microtubules from opposite poles. To understand these events, it is important to identify the components and their functions and to discover how they are recruited to perform their respective roles.

Kinetochore Ultrastructure

Kinetochore morphology as observed by electron microscopy gave rise to the idea that kinetochores can be divided into different layers. At the inside is the layer interacting with centromeric chromatin and on the outside is a layer forming the interface for spindle microtubules. It is commonly accepted that the different functions of kinetochore can be partially dependent on their positions/subdomains (Campbell and Gorbsky, 1995, Cooke et al., 1997, Warburton et al., 1997). The ultrastructural studies in higher eukaryotes have been more satisfying than the ones in fungi (Westermann et al., 2007).

Both traditional chemical fixation and modern high-pressure freezing methods have been used to prepare samples for electron microscopy of the mammalian kinetochore and provide inconsistent descriptions of kinetochore ultrastructure (Brinkley and Stubblefield, 1966, McEwen et al., 1998). Trilaminar morphology is the conventional description of the mammalian kinetochore. Observed under electron microscopy, the kinetochore is composed of three disk-like plates: the inner one is next to the centromere DNA; the outer one is the surface for microtubule termination; and the middle plate between them is electron translucent. When microtubule polymerization is prevented by drug and the attachment with the kinetochore is

absent, a dense array of fibers project from the outer plate and is called the fibrous corona. However, such layered structure appears less distinct when samples were treated with high-pressure freezing (McEwen et al., 1998). Instead, only one dense domain and the corona-like fibers were observed, with the translucent interstitial space missing. Because high-pressure freezing is believed to be a better way of preserving ultrastructure than chemical fixation, the trilaminar plates may represent an artifact.

Observations of plant kinetochores suggest a less structured organization. It has been thought for many years that the plant kinetochore is a “ball” embedded within the “cup” of chromatin and that there are no substructural domains (Braselton and Bowen, 1971, Bajer and Mole-Bajer, 1972). Recent studies of the maize kinetochore using high pressure freezing confirmed the previous descriptions (Dawe et al., 2005).

Though plant ultrastructural studies do not provide support for a subdomain organization, immunocytological microscopic observations do. Maize meiotic chromosomes have unusually large and well-resolved kinetochores when viewed under the light microscope. When co-stained for two different kinetochore proteins, two distinct domains were observed (Yu et al., 1999, Dawe et al., 2005). Therefore, it was proposed that maize kinetochores have an inner domain, containing chromatin-binding proteins and appearing as part of the electron-dense chromatin, and an outer domain, the diffuse domain where microtubules terminate (Dawe et al., 2005).

Kinetochore Composition

Kinetochores in the **budding yeast** *Saccharomyces cerevisiae* have been best characterized. Compared to other eukaryotes, the budding yeast has a simple centromere (Cheeseman et al., 2002, Westermann et al., 2007). As a single-cell organism, the budding yeast

is easily manipulated for genetic and biochemical studies. A large number of mutant strains that show defects in cell division and chromosome segregation is available for yeast (for example, *ctf* and *mcm* mutants) (SGD, 2003). Genetic markers and maps of all yeast chromosomes are well developed (Carbon, 1984). Proteins that interact with centromere DNA or kinetochore proteins have been identified using the powerful one-hybrid (for DNA-protein interaction) and two-hybrid (for protein-protein interaction) systems. Recently, additional kinetochore proteins have been identified via proteomics and microscopy (Westermann et al., 2007). At least 65 proteins are known to comprise the budding yeast kinetochore in a hierarchical manner (De Wulf et al., 2003). Based on their relationship with centromere DNA and microtubules, kinetochore proteins can be classified as inner kinetochore proteins, central kinetochore proteins, and outer kinetochore proteins (McAinsh et al., 2003, Westermann et al., 2007).

Identification of **mammalian** kinetochore components was initially hampered by the fact that they are low-abundance and essential for the host. The first key contribution was the discovery that autoantibodies produced from patients with CREST autoimmune scleroderma can recognize kinetochore proteins (Earnshaw and Rothfield, 1985). Many kinetochore proteins were found to be antigens in CREST patients and the human sera were used to study those proteins in detail (Saitoh et al., 1992, Choo, 1997). In addition, several different approaches such as genetics, RNA interference, and biochemistry have also been utilized to characterize components within the kinetochore. Though sequence similarity between species is very low, searches based on sequence homology have proved to be useful tools to identify kinetochore proteins in many species (Goshima et al., 2003, Cheeseman et al., 2004, Hayashi et al., 2004). In recent years, purification followed by mass-spectrometry-based analysis have greatly facilitated the illumination of kinetochore composition (Cheeseman et al., 2004, Obuse et al., 2004, Foltz et

al., 2006). To date, there are about 80 kinetochore proteins identified in humans and these can be used to search for counterparts in other organisms, for example kinetochore proteins in plants (Cheeseman and Desai, 2008).

Plant kinetochore research at molecular level was not attempted until two decades ago when antibodies against mammalian kinetochore were found to recognize proteins in plant kinetochores (Yu et al., 2000). The antibodies were from patients with an autoimmune disease (CREST) and have been used in *Haemaphysalis*, *Tradescantia*, maize, and field bean (Mole-Bajer et al., 1990, Palevitz, 1990, Houben et al., 1995, Dawe, 1998). Monoclonal antibodies against mammalian kinetochores provided another source for recognition of presumed plant kinetochores (Binarova et al., 1993, Schmit et al., 1994, Yu et al., 1999). With the advent of expressed sequence tags (ESTs), proteins of known identity were found and confirmed (Dawe et al., 1999, Yu et al., 2000, Meraldi et al., 2006).

Currently, only a handful of kinetochore proteins in plants have been well characterized and all show similar functions as their counterparts in yeast and human (Houben and Schubert, 2003, Sato et al., 2005), reinforcing the idea that kinetochores are structurally and functionally conserved. Recent sequence analysis of kinetochore genes from fungi, metazoans, and plants has identified a core set of conserved components (Meraldi et al., 2006). It is reasonable to speculate that the most conserved parts of kinetochores are the major contributors to kinetochore formation and function. In this section, I will focus on the conserved components including those that have been well studied and those that have not been investigated in plants (Figure 1.1).

CENH3

CENH3, CENtrome re Histone 3, is a variant of histone H3 that identifies centromere cores (Figure 1.1). It was first recognized as CENP-A in human (Earnshaw and Rothfield, 1985, Palmer et al., 1991, Sullivan et al., 1994) and is constitutively present at all active centromeres (Warburton et al., 1997, Amor et al., 2004). CENP-A substitutes for histone H3 in centromeric nucleosomes and associates with α -satellite repeats (Shelby et al., 1997, Vafa and Sullivan, 1997, Black et al., 2004, Orthaus et al., 2008). It is required for recruitment of other kinetochore proteins (Howman et al., 2000, Van Hooser et al., 2001, Regnier et al., 2005, Foltz et al., 2006, Liu et al., 2006). CENH3 is widely conserved and the homologs are: Cse4 (Chromosome SEgregation 4) in budding yeast (Stoler et al., 1995), Cid in *D. melanogaster* (Henikoff et al., 2000), SpCENPA or Cnp1 in *S. pombe* (Takahashi et al., 2000), and HCP-3 from *Caenorhabditis elegans* (Buchwitz et al., 1999). CENH3 and its budding yeast homolog Cse4 can function for each other (McAinsh et al., 2003, Wieland et al., 2004). In plants, homologs were sequentially reported in *Arabidopsis* as HTR12 and maize as CENH3 (Talbert et al., 2002, Zhong et al., 2002). The whole protein can be separated into two domains: the amino-terminal tail that diverges dramatically from H3 and a larger histone core domain that is relatively well conserved (Henikoff et al., 2001, Talbert et al., 2002). The N-terminal region of CENP-A seems dispensable for centromere targeting and there is no evidence to show specific interaction of this region with other kinetochore proteins (Black et al., 2007). In contrast, the histone core domain, with strong similarity to H3, is sufficient to load at the centromere (Lermontova et al., 2006, Black et al., 2007). Specifically, it's the small Loop1 region, variable in the histone core domain, that confers centromere specification (Furuyama and Henikoff, 2006). The loading time for CENH3 varies among organisms, from late mitosis and early G1 in animals to G2 in plants

(Lermontova et al., 2006, Schuh et al., 2007, Maddox et al., 2007, Jansen et al., 2007, Fujita et al., 2007).

CENPC (CENP-C)

CENPC, CENTromere Protein C, is a kinetochore protein that changes rapidly in plants and animals (Figure 1.1) (Talbert et al., 2004). It was identified in human as an inner kinetochore protein that constitutively localizes at active centromeres (Saitoh et al., 1992, Sullivan and Schwartz, 1995, Amor et al., 2004). Its yeast homolog, designated as Mif2 (MITotic Fidelity of chromosome transmission), diverges significantly with only two small blocks of similarity to human CENPC, and the mutations in these conserved regions are lethal (Meeks-Wagner et al., 1986, Brown et al., 1993, Brown, 1995, Meluh and Koshland, 1995). Based on this limited homology, plant CENPC was first identified in maize and later shown to co-localize with CENH3 throughout meiosis (Dawe et al., 1999, Zhong et al., 2002). CENPC is essential for kinetochore assembly in animals (Tomkiel et al., 1994, Oegema et al., 2001, Cheeseman et al., 2004, Liu et al., 2006, Kwon et al., 2007, Przewloka et al., 2007). Suppression of protein function in animals and yeast leads to mitotic delay and chromosome mis-segregation (Brown et al., 1993, Fukagawa and Brown, 1997, Kalitsis et al., 1998, Kwon et al., 2007). However, CENPC alone is not sufficient for the establishment of a new centromere (Fukagawa et al., 1999). CENPC is the only kinetochore protein other than CENH3 that shows DNA binding properties (Talbert et al., 2004). No sequence-specific binding activities have been found for CENPC in human or yeast (Sugimoto et al., 1994, Yang et al., 1996, Meluh and Koshland, 1997, Sugimoto et al., 1997, Cohen et al., 2008).

MIS12/MTW1

MIS12, Minichromosome InStability 12, is a constitutive kinetochore protein (Amor et al., 2004). Mis12 was first reported in fission yeast (Goshima et al., 1999) and the homolog in budding yeast is named as MTW1 (Mis TWelve-like) (McAinsh et al., 2003). Its human homolog, designated as hMis12, localizes at the inner kinetochore plate and shows weak sequence similarity from yeast to humans (Goshima et al., 2003). *Mis12* is an essential gene and dysfunction of the gene causes errors in chromosome segregation without mitotic delay (Goshima et al., 1999, Goshima et al., 2003). In both fission yeast and human, hMis12 and CENH3 do not depend on each other to accurately assemble at centromeres (Takahashi et al., 2000, Goshima et al., 2003). Recently, a homolog of MIS12 was characterized immunocytologically in *Arabidopsis* (Sato et al., 2005). MIS12 belongs to the MTW1 complex (MTW1, NNF1, NSL1, DSN1) (Figure 1.1) and the Mtw1 complex shows interaction with the inner kinetochore using biochemical approaches (Westermann et al., 2003). CENP-H, the homolog of NNF1 in vertebrates, is required for CENPC recruitment onto the kinetochore (Fukagawa et al., 2001, Westermann et al., 2003, Kwon et al., 2007).

NDC80

NDC80, Nuclear Division Cycle 80, has been identified from yeast to human (Chen et al., 1997, Wigge et al., 1998, Wigge and Kilmartin, 2001, Desai et al., 2003, Hori et al., 2003, McClelland et al., 2003, Meraldi et al., 2006). Its human homolog HEC1 (Highly Expressed in Cancer) can replace NDC80 in budding yeast (Zheng et al., 1999). NDC80 possesses microtubule binding ability, recruits outer kinetochore proteins, and may be involved in spindle checkpoint signaling (He et al., 2001, Martin-Lluesma et al., 2002, De Wulf et al., 2003, Hori et

al., 2003, McClelland et al., 2003, DeLuca et al., 2005, DeLuca et al., 2006, Lin et al., 2006a, Wei et al., 2007). In general, NDC80 is not constitutively present at centromeres and functions primarily at prometaphase-anaphase when microtubules are present (Chen et al., 1997, Wigge and Kilmartin, 2001, Hori et al., 2003, McClelland et al., 2003, Asakawa et al., 2005). Although NDC80 homologues were suggested to exist in Arabidopsis, their identification and characterization had remained undetermined (Meraldi et al., 2006). NDC80 is a member of the NDC80 complex (NDC80, NUF2, SPC24, SPC25) (Figure 1.1) (Wigge and Kilmartin, 2001, Westermann et al., 2003, Mikami et al., 2005). The components of this subcomplex are broadly conserved (Kline-Smith et al., 2005, Mikami et al., 2005, Meraldi et al., 2006) and have been characterized biochemically in yeast and animals (Janke et al., 2001a, Wigge and Kilmartin, 2001, McClelland et al., 2003, Bharadwaj et al., 2004). Structural studies with electron microscopy have shown that Ndc80 complexes bind directly to the microtubule lattice, providing a coupling mechanism between the kinetochore and microtubules (Davis and Wordeman, 2007).

CENP-E

CENP-E, CENTromere Protein E, is a kinesin motor protein (Figure 1.1) (Yu et al., 2000, Houben and Schubert, 2003). It is involved in chromosome movement in some species by attaching kinetochores to the plus ends of microtubules (Wood et al., 1997). CENP-E can sense microtubule attachment and is also involved in regulating the spindle checkpoint (Abrieu et al., 2000, Yu et al., 2000, Mao et al., 2005). Deletion of CENP-E protein in mammalian cells leads to chromosome misalignment and mitotic arrest (McEwen et al., 2001). Anti-human CENP-E antibodies were initially used to recognize plant kinetochores in *Vicia faba* and/or *Hordeum vulgare* (Yen et al., 1991, ten Hoopen et al., 2000). Genomic analysis has identified CENP-E-

like sequences in Arabidopsis (Lawrence et al., 2002). Two presumed plant CENP-E homologs were cloned and characterized as Cpel1 and Cpel2 (centromere protein E-like 1 and 2) in barley (ten Hoopen et al., 2002). Their localization was detected at centromeres by antibodies specific for Cpel1 and Cpel2, suggesting their role as kinetochore proteins. Cpel1 lacks a kinesin motor domain and Cpel2 was suggested to be a functional homolog of human CENP-E.

MAD2

MAD2, Mitotic Arrest-Deficient 2, is a spindle checkpoint protein. MAD2 was originally identified in the budding yeast (Figure 1.1) (Hardwick, 1998). Though it is not an essential gene, deletion of MAD2 causes reduced fidelity of chromosome disjunction (Li and Murray, 1991). When the function of MAD2 was blocked in mammalian cells, anaphase began early (Gorbsky et al., 1998). In human and frog cells, MAD2 labels free kinetochores without microtubule attachment and disappears when correct attachment has been established (Chen et al., 1996, Li and Benezra, 1996, Waters et al., 1998). Since unaligned chromosomes activate the spindle checkpoint, the observation that MAD2 is only present at unaligned chromosome suggests that MAD2 is a key checkpoint signaling molecule (Chen et al., 1996, Nicklas, 1997, Waters et al., 1998). Additional evidence suggests MAD2 activates the spindle checkpoint by inhibiting the function of APC, anaphase-promoting complex (Gorbsky et al., 1998, Kallio et al., 1998). Studies during mitosis show that microtubule attachment instead of tension seems more likely to cause delocalization of MAD2 from the kinetochore (Waters et al., 1998). The role of MAD2 in the spindle checkpoint has also been characterized in maize (Yu et al., 1999). Consistent with observations in animals, MAD2 staining indicates that mitotic spindle

checkpoint depends more on microtubule attachment. This is different from maize meiosis where tension plays a key role.

Kinetochores Assembly

The kinetochore is a multi-protein complex and plays multiple roles. Each component occupies a different space and has an individual function. With the key kinetochore proteins identified and functions interpreted, the question arises as to how they assemble so they can work together as the whole kinetochore.

There are two possible mechanisms for kinetochore assembly: pre-assembly of all kinetochore proteins into a complex in solution followed by recruitment of the intact complex to centromeric DNA; or pre-assembly of discrete subcomplexes followed by sequential recruitment of subcomplexes to the centromere. The latter assembly mechanism seems the more likely, since the resulting structure is multilayered (Ando et al., 2002, McAinsh et al., 2003). In support of this mechanism, while constitutive components are always present at the centromere, many proteins/subcomplexes associate or disassociate from the kinetochore as a function of the cell cycle (Cheeseman and Desai, 2008).

Elucidation of assembly order has been very successful in yeast and animals (Fukagawa et al., 1999, Fukagawa et al., 2001, Nishihashi et al., 2002, Goshima et al., 2003, Liu et al., 2003, McAinsh et al., 2003, Liu et al., 2006, Foltz et al., 2006, Izuta et al., 2006, Okada et al., 2006, Orthaus et al., 2006, Westermann et al., 2007). This success can be attributed to the variety of kinetochore mutant strains available, and creative ways of combining the mutants with chromatin immunoprecipitation (ChIP) and immunolocalization (Meluh and Koshland, 1997, Ortiz et al., 1999, He et al., 2001, Janke et al., 2001b, Measday et al., 2002, Pot et al., 2003, Westermann et

al., 2003, Capiaghi et al., 2004). The principle of the ChIP assay (Lo et al., 2001) is that antibodies can collect the molecules such as DNA and proteins that are associated with antigens after a cross-linking treatment. If association with centromeric DNA of one protein is broken in the absence of the other protein, the latter is very likely to assemble earlier. The recruitment order of known proteins/subcomplexes is similar from organism to organism (Blower et al., 2002, Zinkowski et al., 1991, Wieland et al., 2004) and will be summarized here using budding yeast as a model. In general, kinetochore components assemble in a step-wise manner beginning with centromeric chromatin.

Cse4, the CENH3 variant in yeast, replaces histone H3 in centromeric nucleosomes and hallmarks the kinetochore (Westermann et al., 2007). Cse4 is a primary foundation protein for the assembly of other kinetochore proteins. Depletion of the protein perturbs recruitment of all kinetochore proteins, including the central and outer ones (Collins et al., 2005). Mif2, the yeast CENPC, is another inner kinetochore protein and co-purifies with Cse4 by tandem affinity (Westermann et al., 2003). Without Cse4, ChIP of Mif2 failed to bring down centromeric DNA (Westermann et al., 2003). It was speculated that Mif2 can bind directly to centromere DNA and support has been provided by recent biochemical studies (Meluh and Koshland, 1997, Cohen et al., 2008).

Mif2 plays an important role in connecting with the central and outer kinetochore. The central kinetochore MTW1 complex (the yeast MIS12 complex) co-purifies with Mif2 (Westermann et al., 2003). Interestingly, ChIP assays revealed that Mif2 and the MTW1 complex depend on each other for centromere binding, raising the possibility that the MTW1 complex is the linker between Mif2 and other essential kinetochore components (Meluh and Koshland, 1997). Reciprocally, recruitment of Mif2 stabilizes the MTW1 complex. It is not

clear whether Mif2 or the MTW1 complex targets the centromere first. These data support a circular one-requires-another-requires-the-first pathway, analogous to the chicken and egg dilemma.

Combined genetic and biochemical studies have revealed the interactions between the MTW1 complex and the Ndc80 complex, another central kinetochore component (Nekrasov et al., 2003). The MTW1 complex may be closer to centromeric chromatin and some components of the complex are required for the association of NDC80 with the centromere (Scharfenberger et al., 2003, Westermann et al., 2003). Consistently, *NDC80* mutation has no effect on the assembly of Mif2 and the MTW1 complex.

The outer kinetochore proteins require the NDC80 complex for their assembly, such as the microtubule-binding components and the motor proteins (He et al., 2001, Tytell and Sorger, 2006). Because the mutants of the NDC80 complex show checkpoint deficiency, it was suggested that the NDC80 complex may recruit the checkpoint protein MAD2 directly or indirectly (McClelland et al., 2003, Gillett et al., 2004).

Centromere DNA for Kinetochore Recognition

Both centromere and kinetochore were once referred to only as the primary constriction. With advances in our knowledge of this complex at the molecular level, the term centromere now is often used to describe the underlying DNA sequence, and the term ‘kinetochore’ represents the proteinaceous structure that forms with the DNA. It is always a major topic to elucidate how centromeric DNA contributes to kinetochore assembly.

Like the kinetochore, centromeric DNA in eukaryotes is best understood in the budding yeast (McAinsh et al., 2003, Westermann et al., 2007). Segments as short as ~125 bp of DNA

(*CEN*) can fulfill centromere activity (Choo, 1997). Each of yeast's 16 chromosomes contains a *CEN* sequence with three conserved domains: the imperfect palindromes CDEI (centromere DNA element I) and CDEIII and the highly AT-rich CDEII. The key kinetochore proteins of the CBF3 complex bind to CDEIII and initiate overlying kinetochore formation. Loss of Cbf3 function results in the "kinetochore null" phenotype (Ortiz et al., 1999, He et al., 2001, Cheeseman et al., 2002, McAinsh et al., 2003).

Such a straightforward situation does not apply to higher eukaryotes, where DNA varies remarkably (Table 1.1) (Houben and Schubert, 2003, Meraldi et al., 2006, Cheeseman and Desai, 2008). The range of centromere size is different and can be from 3,000 ~ 4,000 kb in human (Schueler et al., 2001), ~300 kb in *Drosophila* (Sun et al., 1997), to 40 kb in *Schizosaccharomyces pombe* (Baum et al., 1994). Although the human primary repeat is ~171 bp, in *Drosophila* the centromeres contain much smaller repeats (Sun et al., 1997, Schueler et al., 2001). In case of *S. pombe*, long tandem repeats are in the flanking regions instead of the central cores of the centromere (Baum et al., 1994). No universal motif has been found among these DNA sequences (Baum et al., 1994, Sun et al., 1997, Henikoff et al., 2001).

DNA variation was also observed in higher plants with sizes ranging from 3,000 to 9,000 kb and tandem repeats of ~150 to 180 bp (Table 1.1) (Kaszas and Birchler, 1996, Ananiev et al., 1998, Copenhaver et al., 1999, Kumekawa et al., 2000, Cheng et al., 2002, Jin et al., 2004, Lee et al., 2005). In addition, retrotransposons of the Ty3/gypsy family (CR elements) are abundant in cereal centromeres (Miller et al., 1998b, Presting et al., 1998, Langdon et al., 2000, Jin et al., 2004). The primary sequence of CR is relatively well conserved in cereals such as maize, rice, sorghum, barley, wheat, and rye (Aragon-Alcaide et al., 1996, Jiang et al., 1996, Miller et al., 1998b), but there is limited similarity between tandem repeats in the same species (Ananiev et al.,

1998, Miller et al., 1998a, Nagaki et al., 1998, Zwick et al., 2000, Cheng et al., 2002, Dawe, 2005, Lee et al., 2005).

In plants and many non-mammalian animals, centromere repeats are neither necessary nor sufficient for centromere function (Jin et al., 2004, Dawe, 2005, Cheeseman and Desai, 2008). Underpinning this view is the fact that centromeres with no satellite repeats have been shown to appear spontaneously (Karpen and Allshire, 1997, Choo, 2001, Lo et al., 2001, Amor and Choo, 2002). They recruit key kinetochore proteins and function exactly same as normal centromeres. Composition analysis of new centromeric DNA reveals gene sequences but no centromere-specific repeats. In addition, a spontaneous centromere on a maize chromosome arm was recently described (Topp and Dawe, submitted).

In all complex eukaryotes, the presence of centromere repeats does not guarantee centromere function. This was observed on dicentric human and maize chromosomes where two regions containing centromeric DNA are present but only one recruits a kinetochore (Page et al., 1995, Han et al., 2006). This idea has been extended to all species where detailed information is available. Although centromere-specific repeats exist, they are not specific to centromere cores – generally dispersing into pericentromeric areas where no kinetochore exists (Vafa and Sullivan, 1997, Warburton et al., 1997, Blower et al., 2002, Zhong et al., 2002, Nagaki et al., 2003, Jin et al., 2004). The widely held view is that centromeres are established by epigenetic means.

However, these observations present a genetic quandary: tandem repeats occupy the majority of centromere sequences in most of plants and animals even though an epigenetic mode of inheritance explains most of the data. To explain the complexity and variety of centromere repeats, the centromere drive hypothesis has been proposed (Henikoff et al., 2001, Dawe and Henikoff, 2006). In the model, centromere repeats selfishly amplify themselves and variants that

attract kinetochore proteins more effectively arrive in reproductive cells more often. A genetic mode of inheritance can then evolve, which can allow some kinetochores to be preferentially accumulated (Talbert et al., 2004). This could be selected against by minor changes in inner kinetochore proteins, destroying the emerging genetic relationship and pushing the determination mechanism into an epigenetic mode. Epigenetics would presumably be the dominant mode of assembly but the process should result in a cycle of epigenetic and genetic inheritance that is observable over evolutionary time (Dawe and Henikoff, 2006). It would be exciting to capture the evolutionary ‘moment’ that a species is witnessing genetic control.

Centromere RNA for Kinetochore Recognition

Centromeres are transcriptionally active (Dawe, 2003, Saffery et al., 2003, Nagaki et al., 2004) and RNA transcribed from centromere repeats has been detected in newt (Baldwin and Macgregor, 1985), maize (Topp et al., 2004), human (Fukagawa et al., 2004), Arabidopsis (May et al., 2005), rice (Zhang et al., 2005), mouse (Kanellopoulou et al., 2005, Bouzinba-Segard et al., 2006), and beetles (Pezer and Ugarkovic, 2008). The centromeric RNA varies in size from ~20nt to 5000nt. It was suggested that the transcripts could come from abnormal read-through transcription initiated by centromeric retroelements (Topp et al., 2004).

The process of centromeric RNA generation may be required for kinetochore formation since packing of centromeric nucleosome is replication-independent (Shelby et al., 2000, Ahmad and Henikoff, 2002). Transcription, the alternative way to unfold nucleosomes (Boeger et al., 2003), seems to be a good candidate to facilitate incorporation of centromeric histone variant (Jiang et al., 2003). Supporting this model are the facts that CENH3 recruitment in humans appears to occur where transcription is active and delocalization from the fission yeast

centromere happens when transcription is hampered by the absence of a transcription factor (Chen et al., 2003, Nakano et al., 2003).

From a mechanistic perspective, centromeric RNA has been suggested to serve as a stabilizer to tether the kinetochore to the centromere (Topp et al., 2004, Wong et al., 2007). In maize, ChIP experiments indicate that centromeric repeat RNA is an integral part of the centromere core and tightly associates with CENH3 (Topp et al., 2004). Most recently, human CENPC was observed to delocalize from centromeres after RNA transcripts of centromeric satellite repeats were digested (Wong et al., 2007). Several mechanisms for how RNA may support kinetochore assembly have been proposed (Maison et al., 2002, Dawe, 2003, Jiang et al., 2003, Zappulla and Cech, 2004, Dawe and Henikoff, 2006). However, no empirical data are yet available.

Purpose of the Study

Studies of plant kinetochores are in demand for better understanding of centromere function and the development of plant artificial centromeres. To this end, maize is an ideal model organism for several reasons. Number one, maize kinetochores are large and easily seen, especially during meiosis. Combined immunocytochemistry and high-resolution 3D light microscopy have been well developed to observe kinetochore proteins in maize. Number two, genome sequencing in maize had been under way (nearly complete to date). Number three, several kinetochore proteins have been well characterized in maize (e.g., CENH3, CENPC, MAD2) and there is a good collection of reagents for protein detection. Number four, cDNA clones of kinetochore genes and known sequences of centromere DNA make *in vitro*

preparations and tests feasible. Number Five, maize transformation techniques are mature and many vectors that express fluorescent tags allow us to track kinetochore proteins in vivo.

Chapter 2 of this dissertation, published in *Chromosome Research*, reports the characterization of a new kinetochore protein (NDC80) in maize. In terms of position and function, NDC80 is different from previously described maize kinetochore proteins (CENPC at the inner kinetochore, and MAD2 at the outer kinetochore). This protein was classified in many species as a linker between inner and outer proteins. Recent evidence indicates its involvement in microtubule attachment. Consistent with its role as a connector, this protein shows transient centromere localization in other eukaryotes. It is conserved broadly from yeast to human and plant homologs were detected by sequence similarity. Our studies show that maize NDC80 is a kinetochore protein and localizes at the center of maize kinetochores like its counterparts in animals and fungi. Furthermore, it is constitutively present at the centromere, probably indicating that maize centromeres have a continuous ability for microtubule attachment.

Maize CENPC, the first well characterized plant kinetochore protein, was chosen for our investigation of the mechanisms underlying centromere recognition and kinetochore recruitment. Although the process is currently interpreted as epigenetically controlled, details of the mechanisms are unavailable yet. It was suggested that more than one kinetochore protein (i.e., CENH3, centromeric histone 3) is involved. CENPC is a good candidate because it is a conserved kinetochore protein that shows the ability to bind directly to centromere DNA. In addition, human CENPC binds to centromeric RNA and RNA is required for protein assembly at centromeres. By exploring the DNA/RNA binding properties of maize CENPC, we extend our understanding to the plant kingdom. An exon duplication event in the grasses has been shown to be correlated with the interaction of CENPC with centromeric DNA/RNA. Furthermore, we

provide evidence to support the proposal that RNA is a stabilizer for the targeting of CENPC to centromere DNA. The details are described in **Chapter 3**.

Conclusions of the work from Chapter 2 (NDC80) and Chapter 3 (CENPC) are summarized and discussed in **Chapter 4**.

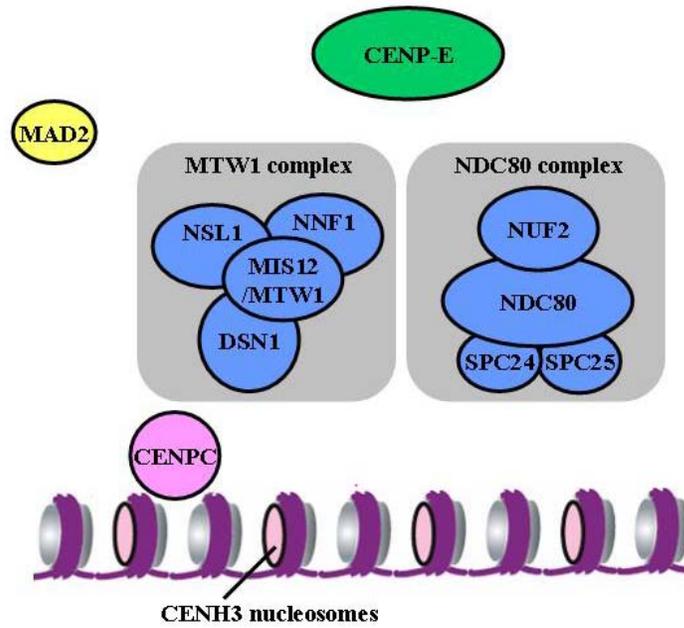


Figure 1.1 A simplified model of the kinetochore. (Redrawn and modified from Meraldi et al., 2006).

Table 1.1 Rapidly Evolving Centromeric DNA. Data are collected and organized from the reference listed in the text. Note the lack of universal sequence motifs in centromeric satellite repeats.

Species	Size of Centromere	Composition of Centromere
Budding yeast	125bp	Three single copy DNA elements
Drosophila	~300kb	Small repeats (5, 7 or 10bp)
Human	3000~4000kb	Alpha satellites(~171bp)
Maize	~300 to >2800 kb	CentC(~157bp) CRM
Rice	750kb (chromosome8)	CentO(155bp) CRR
Arabidopsis	4100kb~9000kb	Satellite repeat (180bp)

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CHAPTER 2
MAIZE NDC80 IS A CONSTITUTIVE FEATURE OF THE CENTRAL
KINETOCHORE¹

¹Du, Y and Dawe, R.K. (2007). Maize NDC80 is a constitutive feature of the central kinetochore. *Chromosome Research* 15 (6), 767-775. The material is copyrighted by Springer Netherlands, reprinted here with permission of the publisher (See APPENDICES A).

Abstract

In yeast and animals, Nuclear Division Cycle 80 (NDC80) is an important kinetochore protein that binds to microtubules and mediates chromosome movement. Its localization pattern is unusual, since it is generally not viewed as either an inner (centromeric chromatin) or outer (regulatory) component of the kinetochore. Here we report the characterization of NDC80 in a higher plant. By taking advantage of the large meiotic kinetochores of maize, we were able to show that NDC80 localizes outside of the constitutive kinetochore protein CENP-C. Further, a detailed analysis of mitosis indicates that NDC80 is stably present on kinetochores throughout the cell cycle. The quantity of NDC80 positively correlates with measured quantities of DNA and CENP-C, suggesting that NDC80 rapidly associates with DNA following replication and is stably maintained at centromeres during cell division. The data suggest that in plants NDC80 is on par with 'foundation' kinetochore proteins such as CENH3 and CENP-C.

Key words kinetochore - centromere - NDC80 - mitosis - meiosis - *Zea mays*

Introduction

Kinetochores are large proteinaceous structures that bind to DNA on one face and to microtubules on the other. In species with complex genomes, centromeres may be megabase-sized and the associated kinetochores nearly a micrometer in diameter. Over 50 different proteins have been characterized as kinetochore-associated in yeast, and at least 30 of these are conserved in humans (Meraldi *et al.* 2006). The functions of most are poorly understood, but have been inferred from their biochemical properties (e.g., DNA or microtubule binding) and the phenotypes of mutants. Depending on the type of kinetochore protein disrupted, kinetochore-defect phenotypes can range from subtle changes in the timing of anaphase onset to gross missegregation of chromosomes and cell death (McAinsh *et al.* 2003, Fukagawa 2004, Pidoux & Allshire 2004).

Kinetochore proteins can also be categorized with respect to how and when they associate with centromeres. The innermost ‘foundation proteins’ are characterized by their constitutive association with centromeres and broad evolutionary conservation (Amor *et al.* 2004). The plant homologs for three of the most important foundation proteins have been described (Houben & Schubert 2003, Sato *et al.* 2005). These are Centromeric Histone H3 (CENH3), a key variant of histone H3 that is thought to nucleate centromeric chromatin (Talbert *et al.* 2002, Zhong *et al.* 2002), Centromere Protein C (CENP-C; Dawe *et al.* 1999), a large rapidly evolving chromatin protein (Talbert *et al.* 2004), and Minichromosome Instability 12 (MIS12), which in yeast is part of a four-protein MIND complex that is required for accurate chromosome segregation (McAinsh *et al.* 2003, Sato *et al.* 2005). The outer kinetochore contains a host of proteins that are involved in positioning chromosomes at metaphase and ensuring that

anaphase occurs on cue. Well-studied examples are MAD2 and CENP-E, which are involved in sensing microtubule attachment (Yu *et al.* [2000](#), Mao *et al.* [2005](#)). Yet a third type of kinetochore protein appears to lie in the middle of the structure and connect the inner and outer domains. Chief among these is NDC80 (Nuclear Division Cycle 80) and its associated proteins (NUF2, SPC24, SPC25) that make up the NDC80 complex (Wigge & Kilmartin [2001](#), Westermann *et al.* [2003](#), Mikami *et al.* [2005](#)).

Recent experiments suggest that NDC80 is an essential protein that is directly involved in microtubule attachment (He *et al.* [2001](#), DeLuca *et al.* [2005](#), [2006](#), Cheeseman *et al.* [2006](#), Wei *et al.* [2007](#)). It is also broadly conserved throughout the eukaryotes, suggesting that it may mediate microtubule attachment in many if not most species (Wigge & Kilmartin [2001](#), Desai *et al.* [2003](#), Hori *et al.* [2003](#), McClelland *et al.* [2003](#), Meraldi *et al.* [2006](#)). The human homolog Highly Expressed in Cancer (HEC1) can substitute for NDC80 in *Saccharomyces cerevisiae* (Zheng *et al.* [1999](#)). The entire NDC80-NUF2-SPC24-SPC25 subcomplex has been identified biochemically in budding yeast, *Xenopus*, and humans (Janke *et al.* [2001](#), Wigge & Kilmartin [2001](#), McClelland *et al.* [2003](#), Bharadwaj *et al.* [2004](#)) and detected by sequence homology in numerous other animals, fungi, and plants (Kline-Smith *et al.* [2005](#), Mikami *et al.* [2005](#), Meraldi *et al.* [2006](#)).

Given its key role in chromosome segregation, it is particularly interesting that the localization pattern of NDC80 differs among species. In humans and *Xenopus*, NDC80 is not detectable during G₁ but appears as centromeric dots from prophase to anaphase (Chen *et al.* [1997](#), Wigge & Kilmartin [2001](#), McClelland *et al.* [2003](#)). In chicken, NDC80 is a component of both kinetochores and centrosomes/spindle pole bodies (Hori *et al.* [2003](#)). NDC80 is a relatively stable feature of mitosis in fungi; however, in fission yeast NDC80 (and NUF2) detaches from

kinetochores in early meiotic prophase (Wigge & Kilmartin 2001, Asakawa *et al.* 2005). These data suggest that NDC80 is generally assembled onto kinetochores late in the cell cycle, consistent with its proposed role as a connector protein that bridges the inner and outer kinetochores (McAinsh *et al.* 2003).

In this study we sought to determine whether maize NDC80 is a kinetochore protein, and if so whether its localization patterns are consistent with what has been observed in other species. Our data suggest that NDC80 has the same central kinetochore localization in maize as it does in animals and fungi, and further, that it is fully constitutive. These data suggest that maize centromeres are rarely if ever kinetochore-free, and may be continuously competent to interact with microtubules.

Materials and Methods

Identification of the maize *Ndc80* gene

Using the protein sequence of mouse HEC1 as a query, we identified a putative full-length cDNA as an expressed sequence tag (EST) clone (CD439119). The cDNA was obtained from Jinsheng Lai (J. Messing laboratory, Rutgers University, NJ, USA) and fully sequenced. The sequence of the C-terminal section was confirmed by RT-PCR using RNA extracted from young ear tissue of the inbred W23 (using primers F-TACAAGGTCACCCGCTCCGCACTC and R-AACATACCAACTACTACCTCACCAG).

Generation of peptide antibodies

The N-terminal 20 amino acids of the predicted ZmNDC80 protein (VIRNLDSAFSRRDSDANSLC) were used to prepare peptide antibodies (by Quality Controlled Biochemicals, Hopkinton, MA, USA). The affinity-purified antisera were used in immunolocalization and western analysis.

Western blot analysis

Maize root tips were ground in a mortar with liquid nitrogen and suspended in protein extraction buffer (10% w/v trichloroacetic acid and 0.07% v/v 2-mercaptoethanol in cold acetone). After being washed with cold acetone, proteins were separated by SDS-PAGE and transferred to nitrocellulose as described previously (Zhang *et al.* 2005). Supersignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA) was used for chemiluminescent immunodetection.

The entire ZmNDC80 coding sequence was inserted into the pET28a vector (Novagen) and expressed as a 6X histidine-ZmNDC80 fusion protein. The bacterially expressed ZmNDC80 was further purified with Ni-NTA agarose (Qiagen, Valencia, CA, USA) and used on western blots as a positive control.

Immunolocalization

Anthers from the inbred W23 were removed from maize tassels and fixed for 3 h in buffer A with 0.1% Triton X-100 (Hiatt *et al.* 2002). The fixed meiocytes were extruded and spun down at 100 g for 1 min onto coverslips precoated with polylysine. Coverslips were incubated with a combination of rabbit anti-ZmNDC80 antibodies (1:50) and chicken anti-

CENP-C antibodies (1:100) (Zhong *et al.* 2002) overnight. After blocking with goat serum (1:10) for 1.5 h, rhodamine-conjugated goat anti-rabbit (1:100; Jackson ImmunoResearch, West Grove, PA, USA) and fluorescein isothiocyanate (FITC)-conjugated donkey anti-chicken antibodies (1:100; Boehringer Mannheim, Mannheim, Germany) were applied as secondary antibodies for 3 h (Zhang *et al.* 2005). The DNA was stained with 0.1 µg/ml 4,6-diamidino-2-phenylindole (DAPI).

Root tips ~3 mm long were fixed in PHEMS buffer as described by Zhang *et al.* (Zhang *et al.* 2005). Sections 10 µm in thickness were prepared on a cryostat (−20°C) and transferred to polylysine slides. Tissue sections were processed as for meiocytes except that microtubules were detected using a mouse α-tubulin antibody (1:500; generously provided by David Asai; Asai *et al.* 1982) and FITC-conjugated goat anti-mouse antibodies (1:100; Boehringer Mannheim).

Localization data were captured as 3D data sets using an Intelligent Imaging Innovations (Denver, CO, USA) Everest Digital Microscope Workstation. SlideBook 4.0 (Intelligent Imaging Innovations) and SoftWoRx (Applied Precision, Issaquah, WA, USA) software packages were used for further image analysis.

Staining intensity analyses

The staining intensities of DNA and ZmNDC80 were measured for 69 mitotic cells from the same root tip section. Background was calculated for each wavelength (from the average of five cytoplasmic areas), and the resulting number was uniformly subtracted from all pixels in the data set. After background removal, the sum intensities for both DNA and ZmNDC80 were measured using SlideBook 4.0 software. As a control, the staining intensities for maize CENP-C

were measured for 45 cells. Total intensity (voxel) values were normalized to a three-point scale to facilitate comparisons.

Results

Identification of maize NDC80

Homology searches suggest that plants have clear NDC80 homologs (Meraldi *et al.* 2006); however, it is not known whether plant NDC80-like proteins are localized to kinetochores. A candidate cDNA (as an EST) homologous to NDC80/HEC1 was identified in a full-length maize cDNA library described by Lai *et al.* (2004). We will refer to this protein as NDC80 (Nuclear Division Cycle 80), which is the accepted generic term for this family of proteins (Zheng *et al.* 1999, Wigge & Kilmartin 2001, McClelland *et al.* 2003). As judged by BLAST analysis and Southern blotting (not shown), *Ndc80* is a single-copy gene in maize. Complete sequencing revealed that the cDNA is full-length and encodes a 576-amino-acid protein with a predicted molecular mass of 64 kDa. Sequence comparisons revealed strong similarity (greater than 40% in all comparisons) to the yeast (NP_012122), human (AAB80726), *Xenopus* (AAN87031), and chicken (BAC81642) homologs of NDC80.

Rabbit polyclonal antibodies against maize NDC80 (ZmNDC80) were produced using a 20-amino-acid peptide from the N-terminal region of the protein. As shown in Figure 2.1, affinity-purified antibodies recognized a single 75 kDa protein in maize root tissue. The size of the identified protein is greater than the predicted molecular mass of ZmNDC80 (64 kDa), which

may be due to posttranslational modification in root tips (the 68 kDa mass in *E. coli* is expected because of the additional 6X His tag).

ZmNDC80 localizes outside of CENP-C at meiotic metaphase

Maize is especially well suited to analyzing kinetochores by immunolocalization. This is particularly true at meiosis where the chromosomes are large and the substages of meiotic prophase are easily distinguished (Dawe *et al.* 1994). Several well-characterized anti-kinetochore antisera are available, making it possible to assess the localization of a new kinetochore component such as NDC80 within an already established substructure (Yu *et al.* 1999).

Analysis of male meiocytes revealed that ZmNDC80 is readily detectable on meiotic chromosomes of all stages. In leptotene and early zygotene, when chromosomes first condense, NDC80 was visible as distinct single (unpaired) and double (paired) spots that co-localized almost perfectly with the inner kinetochore protein CENP-C (Figure 2.2A,B). Later in pachytene, all kinetochores are paired and the centromere/kinetochore complexes are at their largest (Figure 2.2C,D; containing the kinetochores from four chromatids). NDC80 stained brightly in all subsequent stages, including metaphase (Figure 2.2E), late anaphase, and early telophase I (Figure 2.2F). Clear kinetochore localization was also observed throughout meiosis II, as well as in cells that had completed meiosis (i.e., tetrads and young spores; data not shown).

NDC80 is thought to mediate the connection of centromeric chromatin with the outer kinetochore and microtubules (DeLuca *et al.* 2006). A connector protein would be expected to lie outside of the inner kinetochore, at least when kinetochores are under tension at metaphase and anaphase. Our analysis revealed that maize NDC80 localizes slightly outside of CENP-C on metaphase II kinetochores (Figure 2.3), consistent with a role as a connector protein. These

interpretations are limited by the resolution of the light microscope. However, we can say with confidence that unlike CENH3 (Zhong *et al.* [2002](#)), NDC80 does not perfectly co-localize with CENPC at metaphase II.

ZmNDC80 is a constitutive component of the mitotic kinetochore

To determine the localization of ZmNDC80 in mitotic cells, maize root tips were sectioned, treated with ZmNDC80 antisera, and inspected by 3D light microscopy. This strategy makes it possible to assess cells in their native context. Stages can be identified by cell size, diagnostic microtubule arrays, and the proximity of nuclei to each other (for example, newly separated nuclei are identifiable as pairs). Collecting 3D data also allowed us to focus only on complete, undisturbed cells. Analysis of 600 cells from six root tips demonstrated that ZmNDC80 is present at all stages of the cell cycle, including interphase (Figure 2.4A,B), prophase (Figure 2.4C), metaphase (Figure 2.4D), and late telophase (Figure 2.4E). NDC80 was also detected in fully differentiated root cells, such as those from root caps and the non-dividing lower elongation zone (not shown).

Previous data had shown that, in some species, NDC80 does not assemble on kinetochores until G₂ of interphase (e.g., Hori *et al.* [2003](#)). Since root tips are actively growing structures, they contain cells in all stages of interphase, i.e., G₁, S, and G₂ stages. To calibrate the expected staining intensity at G₁ we used telophase nuclei (which, having just separated their chromosomes, have unreplicated DNA). To calibrate the expected staining for G₂, we used cells with a visible preprophase band (a ring of microtubules that forms at the cell cortex just prior to nuclear envelope breakdown in plants; Figure 2.4C; Granger & Cyr [2001](#)). There was an excellent correlation between nuclear diameter and DNA staining intensity, with small nuclei (G₁;

similar in size to telophase) having about half the DNA staining as the large nuclei (G_2 ; often with variously-staged preprophase bands).

If NDC80 levels are tied to DNA content, we would expect a linear relationship between DNA and NDC80 staining intensity. We would also expect that a known constitutive kinetochore protein such as CENP-C (Dawe *et al.* 1999) would show the same or similar relationship. For both NDC80 and CENP-C, appropriate antisera were incubated with sectioned root tips and the cells were scored for G_1 , G_2 , and other visible stages such as prophase or metaphase (Figure 2.4F,G). As shown in Figure 2.5, DAPI staining intensity was then plotted against CENP-C (45 cells) or NDC80 (69 cells) staining intensities. The data reveal a clear linear relationship between DNA, CENP-C, and NDC80 levels (Figure 2.5). Cells with unreplicated G_1 genomes have roughly half the NDC80 staining of cells in G_2 . The fact that cells with intermediate DAPI staining levels (presumed to be in S) have intermediate levels of NDC80 suggests that NDC80 is rapidly assembled onto newly replicated chromatin. These data, and the fact all mitotic cells have detectable NDC80 staining, strongly suggest that NDC80 is a fully constitutive feature of maize centromeres.

Discussion

Kinetochores are generally thought to have two functional faces, an inner chromatin-like domain and an outer microtubule binding and sensing domain. However, there is a distinct third class of proteins that appear to lie in the middle, bridging centromeric chromatin with the large outer regulatory domain (De Wulf *et al.* 2003). It is not yet clear what the primary 'linker' roles

may be. However, recent experiments with NDC80 have provided important clues, showing that NDC80 is required for microtubule attachment (He *et al.* [2001](#), DeLuca *et al.* [2005](#), [2006](#)), has microtubule binding properties (Cheeseman *et al.* [2006](#), Wei *et al.* [2007](#)), and requires phosphorylation for proper kinetochore orientation (Cheeseman *et al.* [2006](#), DeLuca *et al.* [2006](#)). The current view is that microtubules extend well into the central domain, where they are anchored in part by the NDC80 complex.

NDC80 has been identified by sequence homology in fungi, animals, and plants, suggesting that it is among a small group of (about 12) core kinetochore proteins that can be traced to the origins of eukaryotes (Meraldi *et al.* [2006](#)). Although the sequence is well conserved, the concept of an ‘ancestral’ kinetochore rests in large measure on whether proteins such as NDC80 have similar functions in a variety of species. By taking advantage of maize cytology and 3D light microscopy, we provide empirical evidence that maize NDC80 is indeed a kinetochore protein and that it is closely associated with CENP-C on the poleward side (Figure 2.3). These data are in line with biochemical studies (De Wulf *et al.* [2003](#)), ultrastructural interpretations (DeLuca *et al.* [2005](#)), and sequence comparisons (Meraldi *et al.* [2006](#)), which suggest that NDC80 is a highly conserved component of the kinetochore central domain.

Although NDC80 is generally considered to be an indispensable feature of active kinetochores, it is also known for its propensity to detach and return to kinetochores as a function of the cell cycle. For instance, human NDC80 (HEC1) is present in interphase nuclei but not interphase kinetochores (Chen *et al.* [1997](#)). In human, *Xenopus*, and chicken cells, NDC80 is a kinetochore protein only in M phase (Chen *et al.* [1997](#), Wigge & Kilmartin [2001](#), Hori *et al.* [2003](#), McClelland *et al.* [2003](#)). In *S. pombe*, the kinetochores detach from spindle poles during

karyogamy and coincidentally lose NDC80; after the nuclei fuse and meiosis begins, NDC80 reassociates with kinetochores and mediates microtubule attachment. This on-and-off characteristic of NDC80 has undoubtedly contributed to its interpretation as a central kinetochore component that is separate in function from the foundation protein class.

In contrast, we find that maize NDC80 is a constitutive kinetochore protein. Although we cannot rule out the possibility that NDC80 and CENP-C are preferentially assembled at G₂ (as is the case for *Arabidopsis* CENH3; Lermontova *et al.* 2006), we can say with confidence that both proteins are present in abundance at G₁ and presumably at S. An association with chromatin at all stages suggests that NDC80 has an affinity either for DNA or for the kinetochore foundation proteins that constitutively associate with DNA. Our unpublished data indicate that maize NDC80 does not bind DNA. Therefore, the most likely scenario is that NDC80 interacts with proteins of the foundation complex. Among the known foundation proteins is MIS12, a NDC80 binding partner (De Wulf *et al.* 2003, Westermann *et al.* 2003). In addition to Mis12, *C. elegans* NDC80 interacts with the protein KNI-1 in a loose association referred to as the KMN network (Cheeseman *et al.* 2006). The authors proposed that the KMN network functions as the primary microtubule-binding site on kinetochores. If a similar situation exists in plants, then most of the functional kinetochore, including the capacity to bind microtubules, may be an inherent property of plant centromeric chromatin. Further studies with other conserved linker proteins such as NUF2, SPC25, and NNF1 (Meraldi *et al.* 2006), will help to resolve this issue.

Acknowledgements

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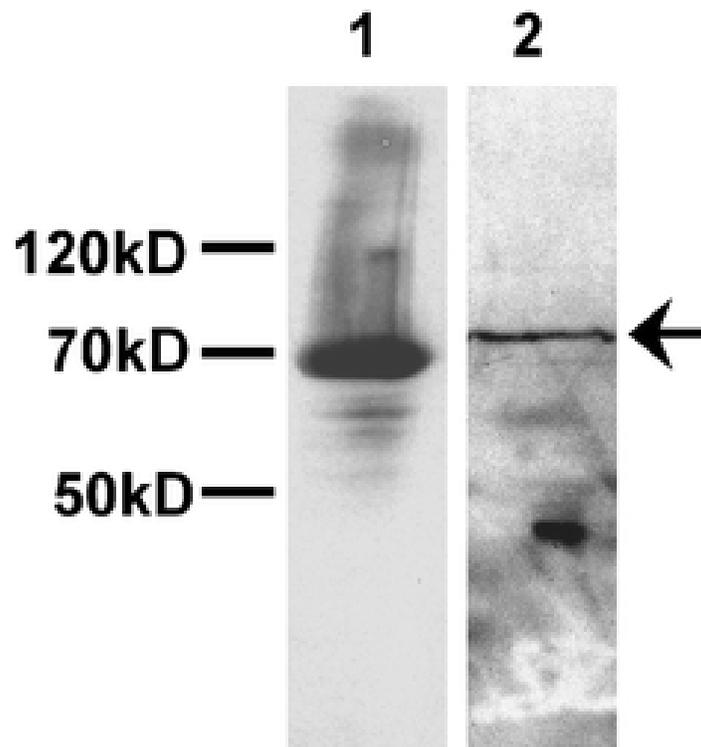
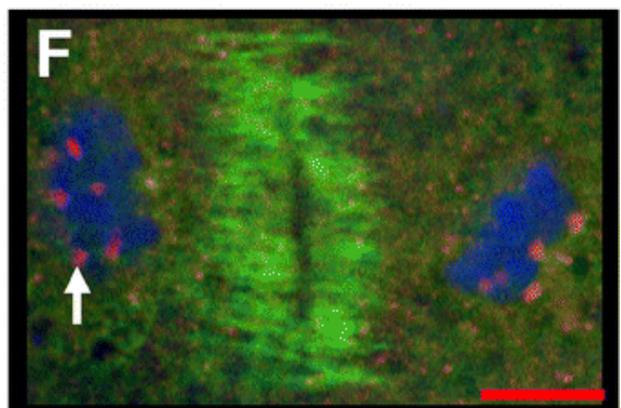
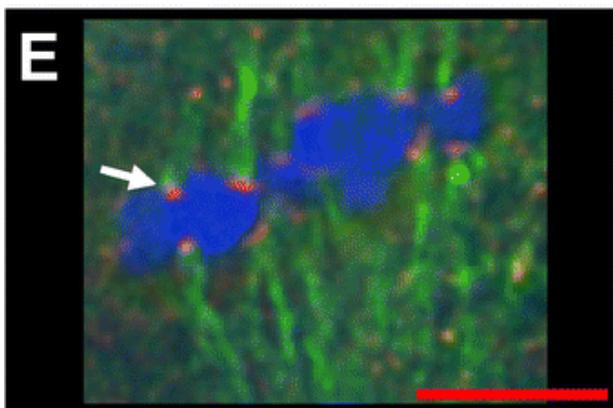
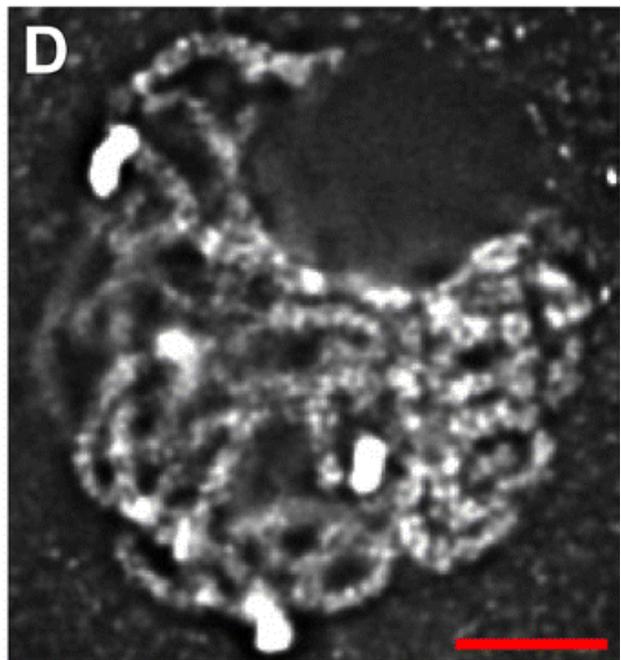
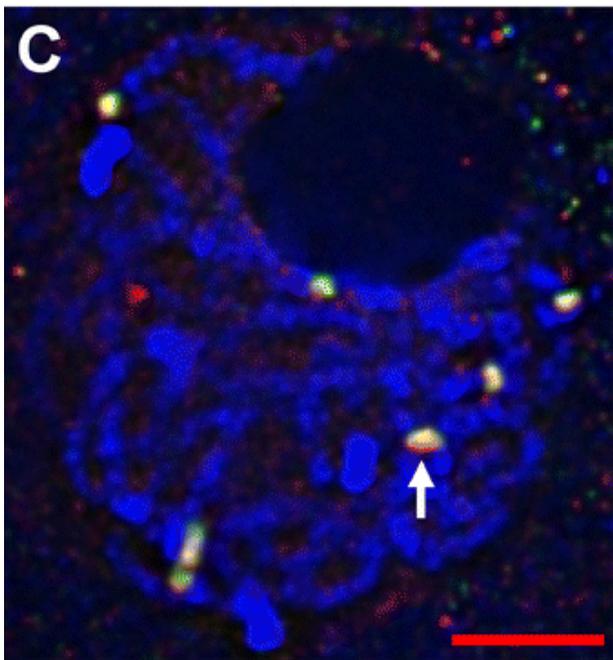
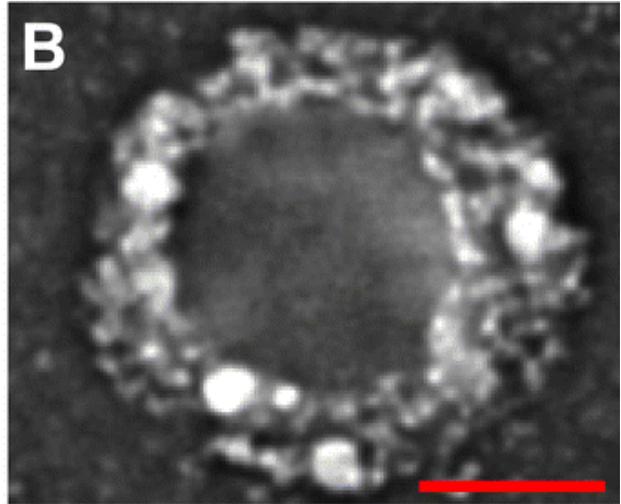
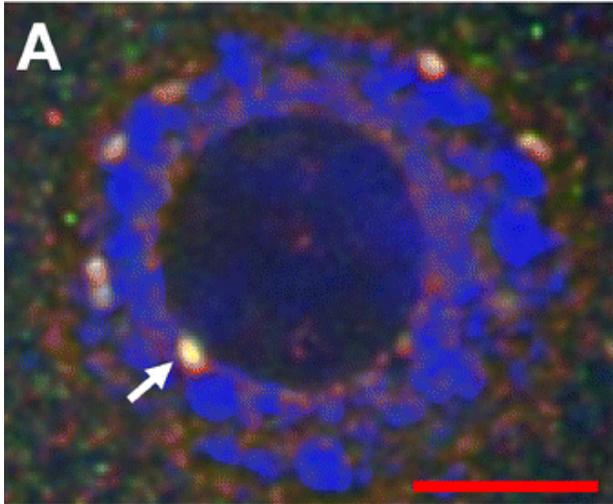


Figure 2.1 ZmNDC80 protein blot. Lane 1: a 6X histidine-ZmNDC80 fusion protein expressed in bacteria. Lane 2: protein extract from root tips. A single protein with an apparent mass of 75 kDa (arrow) was identified in maize root tissue.

Figure 2.2 Immunolocalization of ZmNDC80 in meiotic cells. Images are single optical sections; only a subset of the kinetochores is visible. Arrows highlight kinetochore locations. (A) Leptotene, showing overlay of CENP-C and NDC80. DNA is in blue, CENP-C in green, and ZmNDC80 in red. (B) Chromosomes only from (A). (C) Pachytene. DNA is in blue, CENP-C in green, and ZmNDC80 in red. (D) Chromosomes only from (C). (E) Metaphase I. (F) Telophase I. In (E) and (F), DNA is blue, tubulin is green, and ZmNDC80 is red. Bar = 5 μ m.



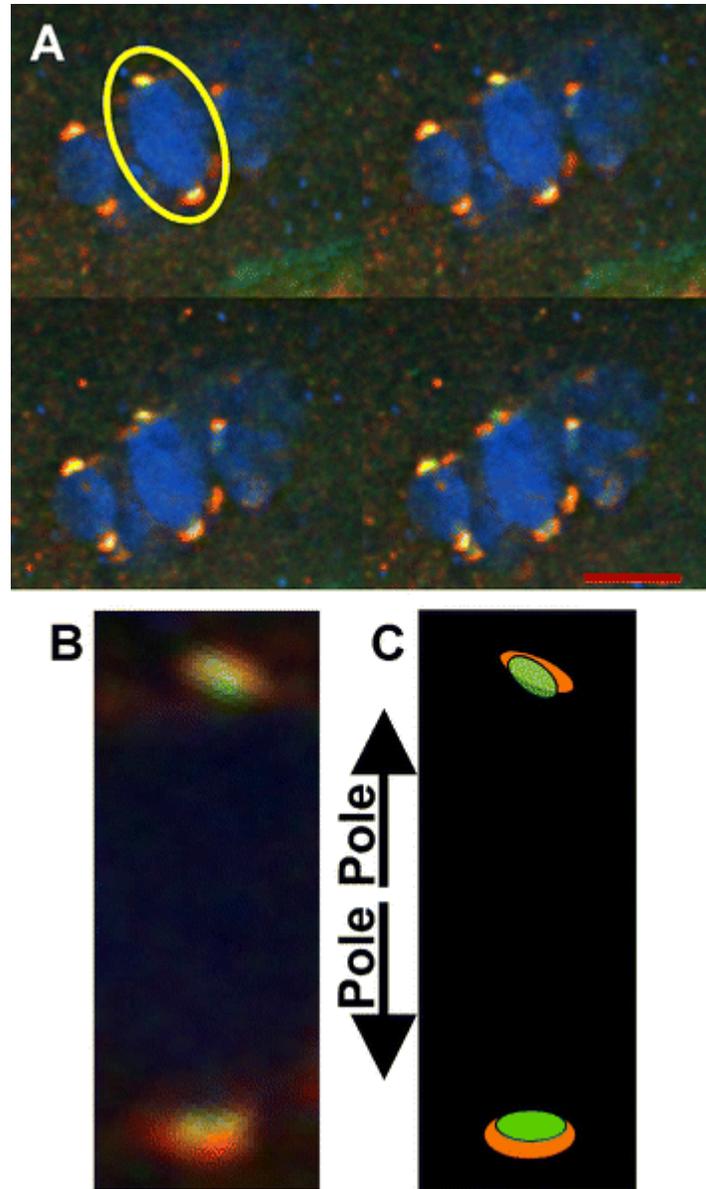


Figure 2.3 ZmNDC80 localizes outside of CENP-C at metaphase II. (A) Four consecutive sections of a prometaphase II cell co-stained for CENP-C (green) and ZmNDC80 (red). (B) An enlarged region (circled in A), showing two kinetochores with red signal (ZmNDC80) on the poleward side of the green signal (CENP-C). (C) A cartoon of (B). Bar = 5 μm .

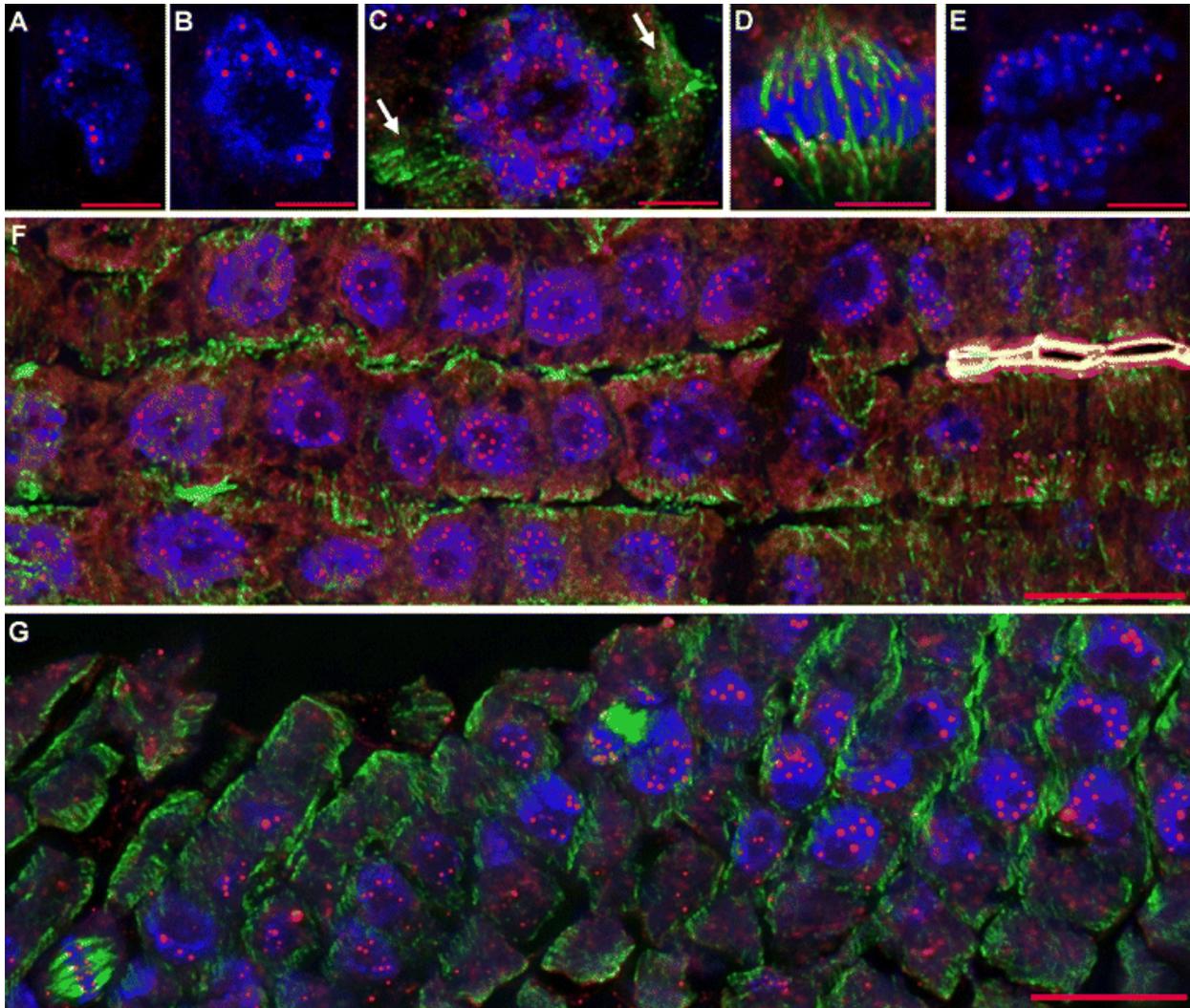
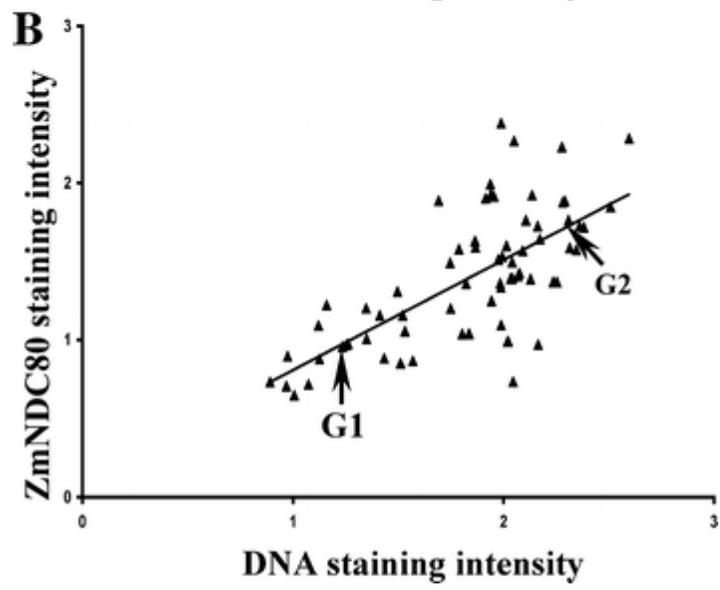
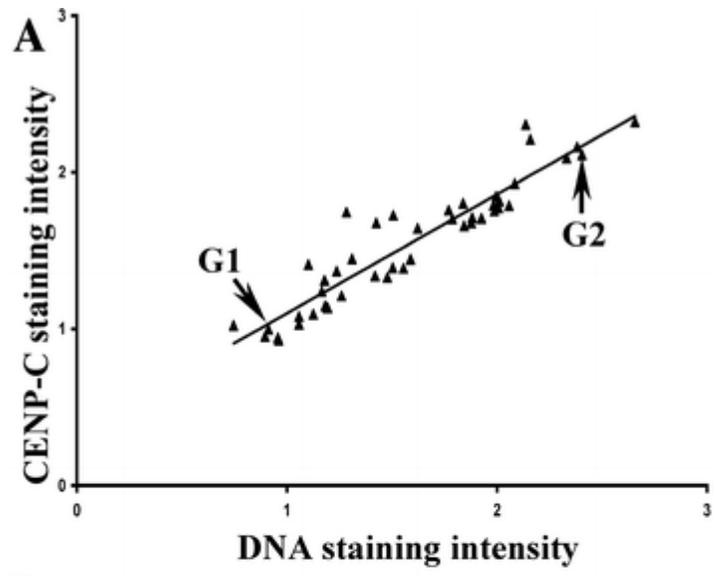


Figure 2.4 Immunolocalization of ZmNDC80 and CENP-C in mitotic cells. DNA is shown in blue, tubulin is shown in green, and ZmNDC80 (A–F) or CENP-C (G) is shown in red. The images in (A–E) are partial projections. (A) A G₁ cell. (B) A G₂ cell. (C) A prophase cell with the remnant of a preprophase band (arrows). (D) A metaphase cell. (E) A telophase cell. (F) Multiple root tip cells stained for ZmNDC80. (G) Multiple root tip cells stained for maize CENP-C. Bar = 5 μm for (A–E); bar = 20 μm for (F) and (G).

Figure 2.5 Linear relationship between DNA, CENP-C, and ZmNDC80 staining intensity. G_1 and G_2 cells are expected to differ in DNA content by a factor of 2. Known G_1 and G_2 cells are indicated: G_1 was scored by the presence of a remnant phragmoplast between two neighboring cells (indicating very recent cell division), and G_2 was scored by the presence of a preprophase band. (A) Maize CENP-C staining intensity is plotted with respect to DNA staining intensity. All 45 data points are from the same root tip. There is a strong linear correlation ($p < 0.01$). (B) Linear relationship between ZmNDC80 and DNA staining intensity ($p < 0.01$). All 69 data points are from the same root tip.



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CHAPTER 3
CENTROMERE PROTEIN C (CENPC) TARGETS CENTROMERES BY A NOVEL
RNA-STABILIZED INTERACTION¹

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Abstract

Centromeric DNAs are repetitive and non-conserved, making it difficult to understand how centromeres maintain their interactions with kinetochore proteins. An excellent candidate for mediating this interaction is Centromere Protein C (CENPC). Our data from gel mobility shift assays show that maize CENPC is a DNA binding protein and that DNA binding activity is localized to a 122 aa exon (9-12) duplication that shows evidence of adaptive evolution. The exon duplication region also binds to RNA. Removal of the DNA/RNA binding region from YFP-tagged CENPC causes the protein to be delocalized from the centromere. A similar disruption of targeting was also observed after replacement of exon 9-12 by the HIV integrase DNA binding domain. Further, our biochemical assays show that single-stranded RNA facilitates the binding of CENPC to DNA. RNA-facilitated DNA binding occurs by stabilizing or folding the exon 9-12 polypeptide. Together, our results demonstrate that coordinated DNA and RNA binding are required for accurate targeting of CENPC.

Introduction

Centromeres are important features of the genome that connect chromosomes to spindles. The connection occurs through a large multifunctional kinetochore complex that binds DNA, binds microtubules, and regulates the timing of anaphase (Yu et al., 2000, Kline-Smith et al., 2005, Kotwaliwale and Biggins, 2006, Fukagawa, 2008). The fact that many of the proteins can be identified by homology suggests that the basic architecture is conserved from yeast to animals and plants (Meraldi et al., 2006). Nevertheless yeast centromeres differ markedly from those in complex eukaryotes, and in plants the biology of the DNA interface remains obscure (Henikoff et al., 2001, McAinsh et al., 2003). The classical concept of a conserved motif interacting with a specific binding protein does not appear to apply to centromeres outside of fungi. Most plants contain diagnostic repeats that can be used to identify centromere boundaries, but the sequences evolve rapidly and do not appear to be necessary or sufficient to organize the kinetochore (Jin et al., 2004, Dawe, 2005).

Current interpretations emphasize a mixed epigenetic-genetic process where particular repeats are preferred (or at least common) binding substrates within centromeres (Dawe and Henikoff, 2006). However, centromeres are neither heterochromatic nor euchromatic in the classic sense making it difficult to interpret what form of epigenetics may be in play. They are generally undercondensed, not tightly packed and deeply staining (Dawe, 2003); centromere repeats are transcribed (Saffery et al., 2003, Topp et al., 2004); embedded genes can be expressed even though canonical histone H3 is rare (Nagaki et al., 2004, Lam et al., 2006, Shi and Dawe, 2006); and the DNA is undermethylated (Zhang et al., 2008). Thus, while the epigenetic framework may help to explain the long-term evolution of centromere repeats, it

provides little guidance on the question of how kinetochores are maintained from one generation to the next.

Centromeric chromatin is associated with a histone H3 variant known as CENH3. CENH3 is widely viewed as the ‘first’ protein to be laid down when a centromere is established. Most of the evidence underlying this view is based on the chemical consideration that CENH3 is a part of the nucleosome and as such is deeply embedded in centromeric DNA. Indeed in the absence of CENH3, all other proteins fail to localize and the chromosomes cannot move on the spindle (Howman et al., 2000, Collins et al., 2005, Regnier et al., 2005, Blower et al., 2006). However, it is not clear how the histone variant composition of a nucleosome might be copied from one generation to the next, especially since the proteins that assemble centromeric nucleosome are similar to those that assemble canonical nucleosomes (Furuyama et al., 2006). Recent data showing that CENH3 is assembled onto chromatin well after S phase (Lermontova et al., 2006, Schuh et al., 2007) point to a complex replication process that requires CENH3 to anchor the kinetochore foundation, but also an intact foundation to accurately target CENH3 (Kline et al., 2006, Okada et al., 2006). This scenario can be likened to the chicken and egg dilemma.

Supporting the self-reinforcing mechanism is the fact that other proteins are installed at the same early stage as CENH3 and have the same essential role (Kline et al., 2006, Okada et al., 2006). One such protein is CENPC, a universally present if poorly conserved inner kinetochore protein (Dawe et al., 1999, Talbert et al., 2004). CENPC is the only known DNA binding protein within kinetochores (aside from CENH3). It constitutively associates with centromeres (Amor et al., 2004) and is essential for kinetochore assembly (Tomkiel et al., 1994, Oegema et al., 2001, Kwon et al., 2007, Przewloka et al., 2007). Although human CENPC selectively binds

to centromeric satellite repeats *in vivo* (Politi et al., 2002, Trazzi et al., 2002), the CENPC protein *in vitro* shows sequence-independent DNA binding (Sugimoto et al., 1994, Yang et al., 1996, Sugimoto et al., 1997, Cohen et al., 2008). Thus, CENPC may be the best candidate for maintaining the epigenetic interaction of kinetochores with non-conserved centromeric DNA (Dawe and Henikoff, 2006). Exactly how CENPC or any other protein might carry out such a role is unclear.

Centromere-associated RNA may mediate a stable interaction between kinetochore proteins and centromere repeats. In maize, centromere repeats are transcribed and maintained in tight association with kinetochore proteins (Topp et al., 2004). Maize centromeric RNA is medium sized (40-200 nt), transcribed from both strands, and present in the single stranded state, suggesting that it is protected from the RNAi machinery. It was proposed that RNA may serve as a structural template to help recruit kinetochore proteins (Jiang et al., 2003). Recent studies in human provide support for this view and further demonstrate that CENPC is involved in the process (Wong et al., 2007). Removal of RNA by RNase displaced human CENPC in human cells (Wong et al., 2007) but it is not known whether CENPC is directly involved in mediating the RNA-centromere interaction. Further work with human CENPC is confounded by the fact that the DNA (and presumably RNA) binding properties are localized in several regions of the protein, and as such are difficult to correlate or separate from other functions.

Our understanding of CENPC binding capacity has been restricted to animals and fungi (Sugimoto et al., 1994, Yang et al., 1996, Sugimoto et al., 1997, Politi et al., 2002, Trazzi et al., 2002, Wong et al., 2007, Cohen et al., 2008). In an effort to broaden and clarify the role of CENPC, we analyzed maize CENPC to discover how it interacts with centromeric DNA and/or RNA. We were encouraged by the sequence analysis of Talbert et al. (2004), who found a small

basic region of the CENPC protein that has been repeatedly duplicated in the grasses. Here we use a combination of *in vitro* and *in vivo* studies to show that maize CENPC has DNA-binding and RNA-binding capacities, that the DNA/RNA-binding domain maps to the exon duplication region, and that the binding domain is required for efficient centromere localization. We further show that RNA directly facilitates the binding of CENPC to DNA *in vitro*, providing a biochemical mechanism for the involvement of RNA in centromere specification. We argue that CENPC and RNA are a part of the structural template that directs CENH3 to the newly replicated DNA.

Results

CENPC binds to DNA

As a first step towards understanding the biochemical properties of CENPC, we used a standard gel shift assay to test whether the full-length protein binds DNA. The maize centromere repeats (CentC) have been isolated (Ananiev et al., 1998) and the known sequences were used here for probe preparation (details in Methods). Bacterially expressed CENPC was mixed with 33P-labeled CentC DNA and the products were resolved by non-denaturing PAGE. Consistent with expectations, the data show that the mobility of CentC is shifted upwards in the presence of CENPC, indicating that DNA and protein are associated in a complex (slower) on gels (Fig. 3.1). As controls we used bovine serum albumin and maize NDC80, another kinetochore protein (Du and Dawe, 2007). There was no gel shift with either control protein (data not shown).

CentC fragments of different lengths were incubated with CENPC to identify the optimal binding substrate. As shown in Fig. 3.1A, CENPC complexes form with increasing efficiency as DNA length increases: 23.1% (shifted) with a 24 bp fragment, 73.7% with a 44 bp fragment,

85.3% with a 67 bp fragment, and 95.9% with a 156 bp fragment. Since the 67 bp and 156 bp fragments produced complexes that were too large to enter the polyacrylamide matrix (Fig. 3.1A, lanes 2 and 4), we opted for a 44 bp probe in subsequent assays.

Titration experiments of CENPC were performed with both 44 bp and 24 bp DNA. When the amount of protein is increased, a second, supershifted band is observed on the 44 bp probe (Fig. 3.2A). This phenomenon was not observed with 24bp DNA (Fig. 3.2B). These data suggest that larger DNA strands (44bp) can accommodate two forms of CENPC-DNA complex. The stoichiometry could be skewed in either of two ways. Either there is more than one CENPC protein/per DNA molecule on the supershifted band (i.e., two CENPC/one DNA), or each CENPC binds to more than one DNA (i.e., one CENPC/two DNA). The first option seems more likely, since the shift occurs as more CENPC is added. Further, if CENPC could bind to more DNA in high concentrations, we would expect the same type of supershift with a 24 bp DNA fragment.

DNA binding is not sequence-specific

Binding specificity can be determined by competition experiments in which unlabeled DNA ('challenger') is added as a competitor to a mix of protein and labeled ('defender') DNA. If the DNA-protein binding is sequence-specific, the defender DNA will not be competed by the challenger DNA (Marian et al., 2003). Here, three sequences were used as competitors. The data show that 180 bp knob repeat (Peacock et al., 1981), 134 bp centromere repeat from sorghum (Zwick et al., 2000), or a 44 bp fragment of Ndc80 (Du and Dawe, 2007) were all efficient competitors for CENPC binding (Fig. 3.1B). In no case did CENPC appear to bind more tightly to CentC than other molecules. While it remains possible that CENPC has minor

binding preferences *in vitro*, our results suggest that the differences (if any) cannot be reliably detected by the gel shift assay.

These results suggest that CENPC is a non sequence-specific DNA binding protein that can bind to fragments as small as 24 bp. The data further suggest that the protein can occupy sites side by side to create a supershifted product.

The DNA binding domain maps to duplicated exons

Since CENPC shows poor conservation among species (similarity over a 23-amino acid sequence) (Dawe et al., 1999, Heeger et al., 2005), it is not possible to identify functional domains by sequence analysis. To determine the DNA binding sites on maize CENPC empirically, fourteen subdomains of CENPC were tested for their capacity to shift DNA on gels (Fig. 3.3A and 3.3B). The amount of protein required to confer a quantifiable shift was used as a measure of binding affinity.

The data reveal that full-length CENPC has the highest DNA binding and that partial proteins bind DNA much less efficiently (Fig. 3.3A and 3.3B). By comparing the location of the proteins we can conclude that the major DNA binding region maps to an area between exons 9 and 12 (Fig. 3.3A). A 9-12 deletion construct that is otherwise identical to full length CENPC had no detectable binding activity (lane 3 in Fig. 3.3B).

In prior work, Talbert et al (2004) showed that the exon 9-12 domain represents a duplication of an ancient exon pair (a progenitor of exons 9-10 and 11-12). The region has been repeatedly subjected to duplication over the course of grass evolution. In some species like maize and sorghum there are four exons, while in others like rice there are six exons,

representing a triplication. Exons 10 and 12 are rich in arginine and lysine, as are many other DNA binding regions in nature (Lambert and Thomas, 1986, Twining et al., 2001).

An RNA binding domain maps to the same duplicated exons as DNA binding

Since maize centromere/kinetochores also contain RNA homologous to the centromeric repeats (Topp et al., 2004), CentC RNA was used in gel shift assays. As shown in Fig. 3.3A and 3.3C, different CENPC subdomains show a similar pattern of affinity to RNA as to DNA, suggesting CENPC is a RNA binding protein and that the RNA binding domain overlaps the DNA binding domain.

Competition experiments were performed to test the sequence specificity of RNA binding. The data revealed that RNAs transcribed from Sorghum centromeric repeat sequence (Zwick et al., 2000), 180 bp knob repeat, and CentC were roughly equivalent in their binding reaction with CENPC (data not shown). We also tested whether CENPC can bind RNAs as small as 24 nt RNA. The results show that CENPC does bind to small RNA, though with much lower efficiency than to long RNA, or to DNA of the same length (Fig. 3.4B, also Fig. 3.1A and 3.3C).

RNA promotes DNA binding by CENPC

The synthesized small RNA was used in competition assays with CentC DNA. Unlike other competitors such as DNA, small RNA does not challenge (compete with) CentC DNA. Instead, the small RNA promotes the formation of a larger supershifted product (Fig. 3.4A). The position of the RNA-supershifted band is similar or identical to the band observed when more CENPC is added (Fig. 3.2A), which we presume represents two or more CENPC proteins on a single DNA substrate.

We further discovered that RNA is required for the purified (122 aa) exon 9-12 to bind DNA efficiently (Fig. 3.5). Even with high concentrations of the exon 9-12 polypeptide, the shift incurred is minor and non-discrete. However, when small amounts of single-stranded RNA are added concurrently, a clear and strongly shifted band appears. The RNA does not compete with DNA binding even at very high concentrations: when a 4,000-fold molar excess of 24 nt RNA (relative to DNA) was added to the reaction, the effect was indistinguishable from the effect when small amounts were added. We note that since the DNA is labeled in this reaction the RNA must be facilitating the binding of CENPC to DNA. To further limit the binding domain, we prepared the exon 9-10 peptide and showed that it also requires RNA to bind effectively to DNA (Fig. 3.6A). In a final test, a single (36 aa) exon 12 was synthesized biochemically, and it too was shown to require RNA to bind DNA (Fig. 3.6B).

It has been suggested that RNA may incorporate within the complex and act as a structural component for its function (Topp et al., 2004). If this were the case, we might expect RNA to change the affinity of the complex for DNA (under increased ionic strength or temperature). However, these studies revealed that RNA does not change the affinity of CENPC for DNA (Supplemental Figure). These data, and the facts that RNA alone binds weakly to CENPC, that small RNA does not compete in gel shift assays, and that RNA promotes the binding of a single 36 aa binding module to DNA, suggest that RNA associates transiently with exon 9-12 to stabilize or facilitate the folding of the CENPC-binding domain.

The structure and size of nucleic acids that stabilize the CENPC-DNA interaction

The majority of centromere-associated CentC and CRM transcripts are single-stranded and larger than ~50 nt (Topp et al., 2004). To test whether RNAs of this type were effective as

stabilizers in our assay, we tested a variety of nucleic acids in single- and double-stranded forms. As a first test the same 24 nt RNA sequence used in the original tests (Fig. 3.4, 3.5, and 3.6) was tested as a DNA molecule. We found that both ssRNA and ssDNA of this size function as facilitators (Fig. 3.7), suggesting that the 2' OH is not involved in the reaction. RNA of 10 nt has no effect on the CENPC-DNA interaction (Fig. 3.7B), further suggesting that the stabilization event requires relatively long oligomers.

Additional length tests are confounded by the fact that long single-stranded nucleic acids tend to form hairpin secondary structures based on partial homology. Indeed, full 167 bp transcripts of the CentC repeat do not stabilize the complex, and instead compete in the binding reaction (Fig. 3.7). Therefore, we used long DNA polydinucleotides with a repeating GTGT motif that cannot form a stable-double stranded state. The GTGT polynucleotides stabilize the CENPC-DNA binding reaction efficiently, as does small RNA (Fig. 3.7B). Thus, the long single-stranded RNA most common *in vivo* is also effective as stabilizers *in vitro*.

We performed the same tests on the well-studied HIV Integrase DNA-binding domain. The HIV Integrase whole protein and purified binding domain are non-specific DNA-binding proteins (Esposito and Craigie, 1999) (simplified as IntBD here). IntBD is also similar in size to exon 9-12 from maize CENPC (51 amino acids as compared to 61 amino acids for exon 9-10). The IntBD region was synthesized *in vitro* and used in DNA gel shift assays. As shown in Fig. 3.8A, IntBD binds strongly to DNA without the need for RNA. Further, when RNA is added to the IntBD-DNA mixture, there is no observable effect (Fig. 3.8B). These data support the view that stabilization by RNA is a unique feature of the CENPC DNA-binding domain.

Exon 9-12 are required *in vivo* for accurate CENPC targeting

We tested the function of the CENPC binding domain *in vivo* using transient assays and stable transgenic lines. Transient assays were conducted on the surface of embryogenic callus. Three constructs were tested: the full length CENPC gene, a gene with exon 9-12 deleted (delCENPC), and a construct with the natural exon 9-12 replaced with HIV IntBD (IntCENPC). The genes were constitutively expressed under control of the 35S promoter and CENPC was tagged by YFP at the N-terminus. In transient assays using the full-length construct as a control, we found (39 cells) that deletion of the DNA binding domain (delCENPC) reduces centromere localization to 56% (39 cells), and substitution of exon 9-12 with IntBD (32 cells) decreases it to 72% (Fig. 3.9).

The same constructs were then introduced into whole plants by *Agrobacterium*-mediated transformation (Frame et al., 2002). During these studies we discovered that the 35S promoter is not expressed in maize meiosis. Therefore, we focused our assays on root tips where expression was strong and uniform. In most transgenic events of three CENPC variants YFP signals were detected at the centromeres during mitosis, including prophase, metaphase, anaphase, and telophase (Fig. 3.10). However, the loading efficiency at interphase differed. Compared to full CENPC (the control, scaled to 100%), the recruitment efficiency of delCENPC was reduced to 80% ($P < 0.05$). Substitution of exon 9-12 with IntBD caused severe displacement of the protein in the young dividing cells of the root tip. Only a few sporadic cells showed specific IntCENPC-YFP labeling in root tip zones (Fig. 3.10C). However, later in root development (the zone of elongation) the IntCENPC protein does target centromeres, but at reduced levels, similar to those of delCENPC (83% for CENPC IntBD and 76% for CENPC deletion in Fig. 3.11).

Discussion

Exon 9-12 is required for centromere localization of maize CENPC

CENPC is one of a few foundation proteins within the kinetochore (Amor et al., 2004). In apparent conflict to its importance, it has poorly conserved protein sequence (Talbert et al., 2004). Maize CENPC was initially found based on homology of a 23 amino-acid sequence (Dawe et al., 1999). The divergence of CENPC makes it uniquely difficult to make functional comparisons across species. CENPC has been heavily studied in humans but the information can not be applied to fungi or plants because of the complete lack of homology in binding regions (Sugimoto et al., 1994, Yang et al., 1996, Sugimoto et al., 1997, Politi et al., 2002, Trazzi et al., 2002). Recently, CENPC was proposed to play a key role in kinetochore recognition and centromere evolution because of its DNA binding ability and rapid sequence change (Talbert et al., 2004, Dawe and Henikoff, 2006). By biochemical and transgenic approaches, we extend the investigation of the CENPC function to plants.

Our subdomain analysis using gel shift experiments revealed that the major DNA binding domain of maize CENPC localizes to a duplicated region encompassing exon 9-12 (Fig. 3.3). Sequence BLAST of the NCBI database found no homology with any known conserved domain. Basic residues are distributed in the region and Talbert et al. predicted that it may bind to DNA (Talbert et al., 2004). As featured in many DNA-binding regions, an enrichment of arginine and lysine residues is found in exons 10 and 12 (Lambert and Thomas, 1986, Twining et al., 2001). Our work identifies exon 9-12 as a strong DNA-binding domain and further reveals that the same region is responsible for RNA binding. Human CENPC also binds DNA and RNA. These data reinforce the view that CENPC has strongly conserved features, if not sequence.

We have demonstrated that removal of exon 9-12 causes delocalization of CENPC from the centromere (Fig. 3.9, 3.10, and 3.11). Therefore, CENPC recruitment requires exon 9-12 and very likely the DNA-binding region within it. A similar correspondence between DNA binding and centromere targeting were found for human CENPC (Talbert et al., 2004). The presence of a DNA-binding domain, and prior observations that CENPC is among a core set of kinetochore proteins recruited earliest (Oegema et al., 2001, Cheeseman et al., 2004, Liu et al., 2006, Kwon et al., 2007), suggests that CENPC plays an adaptor role at the kinetochore/DNA interface (Talbert et al., 2004). Of particular interest is the observation that exon 9-12 have repeatedly duplicated in grass species by exon duplication, suggesting an adaptive role (Sunita et al., 2007).

Although exon 9-12 is important for the recruitment of maize CENPC, centromere localization can still be detected in the wild-type background of transgenic lines that express wild-type CENPC as well. A likely factor contributing to this observation is CENPC dimerization. A dimerization domain has been found at the C-terminus of human and yeast CENPC, and crystal structures have revealed dimers that include this domain (Sugimoto et al., 1997, Cohen et al., 2008). Additionally, the interaction with other kinetochore proteins may be involved in centromeric assembly of CENPC. In human, CENP-B was suggested to be involved in recruiting CENPC (Suzuki et al., 2004). Reduced CENPC accumulation at centromeres was seen with deletion of MIS12 (Kline et al., 2006).

Single-stranded RNA may facilitate CENPC assembly at centromeres

We show that RNA can cause a supershift of the whole protein/DNA complex and that the purified binding domain alone is incapable of binding DNA efficiently unless single-stranded nucleic acids are present (Fig. 3.4A and 3.5). The data reveal that the RNA must be single-

stranded and larger than 10nt (Fig. 3.7), corresponding well with the previous observation that maize centromeric chromatin contains abundant 40 to 200 nt single-stranded RNAs (Topp et al., 2004). Although centromeric RNA is required for centromere assembly of CENPC in human, how RNA may confer this function is not clear. Our data suggest that one role for centromere-associated RNA could be as a direct facilitator of CENPC recruitment.

This appears to be a unique feature of CENPC, since our assays show that another similar DNA-binding domain (HIV IntDB) does not require an RNA stabilization step (Fig. 3.8). Replacement of exon 9-12 with HIV IntBD *in vivo* does not restore the efficiency of centromere targeting (Fig. 3.9, 3.10, and 3.11); in fact, it promotes ectopic localization in root tips (Fig. 3.10C). These data suggest that the DNA-binding domain in CENPC is uniquely adapted to the unusual requirements of the centromere. A non-specific DNA-binding protein such as HIV integrase, in principle, should bind non-discriminately to regions all over the genome and this appears to be true. In contrast, CENPC DNA binding, with its novel RNA stabilization feature, corrects the mis-localization defect observed with the IntBD substitution construct.

A model for exon duplication binding and RNA facilitation in centromeric assembly of CENPC

Given the results described here, we propose a model to summarize our conclusions (Fig. 3.12). In this model, CENPC is proposed to be an inherently unstable protein, and inefficiently binds DNA unless single-stranded RNA (or ssDNA) is locally abundant. We know that maize centromeric chromatin is rich in this form of RNA (Topp et al., 2004). Therefore, within centromeres the CENPC-DNA complex is particularly stable (Fig. 3.12B).

In support of our model, the sequence of exon 9-12 was analyzed in detail and shown to possess characteristics of an intrinsically unstructured protein (Tompa, 2002). RNA, and perhaps other molecules that are abundant in kinetochores, stabilize the structure of exon 9-12 and promote its interaction with DNA. Although we cannot rule out the possibility that RNA remains with the complex following DNA binding, this seems unlikely. RNA binds weakly to CENPC, excessive small RNA does not compete with a DNA-CENPC complex, and RNA stabilizes a single 36 amino acid-binding module that would appear to be too small to accommodate the binding of both DNA and RNA.

In maize, ChIP experiments revealed that centromeric repeat RNA is tightly associated with CENH3, indicating that RNA is a consistent feature of the centromere core (Topp et al., 2004). When RNA transcripts of centromeric satellite repeats were digested away in human, CENPC delocalized from centromeres (Wong et al., 2007). In addition, replicative assembly of kinetochores appears to require reinforcement from existing kinetochore proteins from the prior cell cycle (Kline et al., 2006, Okada et al., 2006). We propose that this occurs through an epigenetic templating cycle. Weak DNA binding of the CENPC protein, in combination with stabilizing features of the chromatin environment (single-stranded RNA and existing CENPC) helps to recruit new CENPC. Incorporation of new CENPC in turn promotes the assembly of a mature kinetochore, presumably including the most basal features (i.e., CENH3), which can then proceed to initiate the replication cycle again.

Methods

Preparation of recombinant CENPC protein and its variants

The clone CIMMH01 was a gift from Michael Muszynski and Pioneer Hi-Bred International and contains a full-length maize CENPC cDNA (GenBank accession numbers AF129857) (Dawe et al., 1999). The corresponding fragments of different CENPC subdomains were amplified from CIMMH01 plasmid by PCR with Accu Taq polymerase (Sigma):

Forward primers,

5'-**ATGCTAGCATGGACGCCGCCGACCCCCTC**-3' for pET28-CENPC, exon1-5.7, exon1-8, and exon1-11.3,

5'-**ATGCTAGCGATAAGTCAAGCCAGTTGTTGGAG**-3' for pET28-exon 9-12,

and the reverse primers,

5'-**TAAAGCTTTCAGTACCTTGCAGATTTAGCAAC**-3' for pET28-CENPC,

5'-**TAAAGCTTTCACCTTTCATATGCATGATCAGG**-3' for pET28-exon1-5.7,

5'-**TAAAGCTTTCATTGTAGGACACCACCTCTTT**-3' for pET28-exon1-8,

5'-**TAAAGCTTTC AACCATTTGGTGAGAGTGTGCT**-3' for pET28-exon1-11.3,

5'-**TAAAGCTTTCATCCAAGGATTTTTCTTTGATTCCT**-3' for pET28- exon 9-12,

(restriction endonucleases *Nhe* I and *Hind* III recognition sites in bold).

To generate CENPC variant without exon 9-12, a 1~1506 bp fragment at the 5'-end and an 1873~2106 bp fragment at the 3'-end of the maize *CenpC* gene were amplified from CIMMH01 plasmid using PCR. The amplified 5'-end was joined to the 3'-end by a secondary PCR with the primers for a full-length CENPC. The CENPC deletion variant was ligated into the pCR4 vector (Invitrogen). The integrity of cDNA was confirmed by sequencing. The same

strategy is applied for generation of CENPC integrase variant except that amplified DNA-binding domain of HIV integrase (GenBank accession numbers AAC83550) (Esposito and Craigie, 1999) was added as a third template for the secondary PCR.

PCR products were inserted into pET28a vector (Novagen) and the ligation products were used to transform Rosette Blue (DE3) competent cells (Novagen). Single-colony PCR was used to detect colonies that contained the insert. The plasmids were purified and sequenced. Recombinant CENPC subdomains were expressed as histidine-fused proteins respectively, and purified according to the Qiagen's protocols (QIAGEN, 2003). The expression and purification of recombinant proteins were verified by His-tag staining (Invitrogen) and Western blotting (Pierce). Protein concentration was determined using the Bradford assay (Bio-rad). The purified proteins were stored at -80°C in aliquots until use.

The peptide for exon 12 of maize CENPC was synthesized by the Sigma-Genosys Peptide Synthesis Facility. The peptides for exon 9&10 of maize CENPC and the DNA-binding domain of HIV integrase were synthesized by the Abgent Peptide Synthesis Facility. Peptide stocks were prepared by dissolving lyophilized peptide in Elution Buffer for protein purification (QIAGEN) and then diluted with water.

Preparation of DNA probes and competitors

156 bp centromeric repeat monomer was synthesized by annealing two long primers (5'-GGTTCCGGTGGCAAAAACCTCGTGCTTTGTATGCACCCCGACACCCGTTTTTCGGAATG GGTGACGTGCGGCAACGAAATTGCGCGAAACCACCCCAAACATGAGTTTTGGACCT AAAGTAGTGGATTGGGCATGTTCGTTGCGAAAAACGAAGAAAT- 3') and TA-cloned into pCR4 vector (Invitrogen). 156 bp and 67 bp DNAs were generated by PCR using the

primers: forward 5'-GGTTCGGTGGCAAAAACCTCGTGC-3' and reverse 5'-ATTTCTTCGTTTTTCGCAACGAAC-3' for 156bp DNA and 5'-GCACGTCACCCATTCTGAAAACGG-3' for 67bp DNA. The PCR products were purified and quantified. Given the known sequences, the shorter single-stranded DNA was synthesized (Integrated DNA Technologies) as an oligonucleotide and if needed, annealed with a complementary oligonucleotide to form a duplex. For example, 44 bp, 24 bp, and 24 nt CentC DNA; 44 bp Ndc80 DNA (Du and Dawe, 2007); 48 nt GT polynucleotides. Then DNA used as probe was end-labeled with ³³P-ATP using T4 polynucleotide kinase (Invitrogen).

Preparation of RNA probes and competitors

Long single-stranded RNA (167nt RNA) was transcribed *in vitro* using Sp6 RNA polymerase (New England Biolabs) and Riboprobe kit (Promega). To minimize sequence other than native repeats that is transcribed, centromeric repeat monomer synthesized from the annealing of two long primers (see DNA preparation) serves as a template and Sp6 promoter (5'-ATTAGGTGACACTATAGAAGAG-3') was added right upstream the centromeric repeat sequence. Transcripts were purified and quantitated. To label RNA, ³³P-labeled UTP was added to the transcription reaction and incorporated into the transcripts. For small RNA such as 24 nt RNA and 10 nt RNA, oligonucleotide synthesis was carried out and products were purified (Integrated DNA Technologies). The sequence for 24 nt RNA was known from the hits against CentC in a maize small RNA library (V. Sundaresan from UC-Davis). Then synthesized RNA was end-labeled with ³³P-ATP using T4 polynucleotide kinase (Invitrogen) for RNA gel shift.

Electrophoretic mobility shift assays

Radiolabelled DNA or RNA probe was incubated with various amounts of CENPC or CENPC derivative on ice for 20 min in a 20 μ l solution comprising 10 mM Tris (pH 7.5), 50 mM NaCl, 0.5mM dithiothreitol, 0.5 mM EDTA, 1mM MgCl₂, 4% glycerol. Unlabeled DNA or RNA was also added in some case. For competition experiments, equal length of different unlabeled DNA was added to the solution of the CENPC-DNA complex in excess. In case of facilitation experiments, unlabeled RNA was added to the solution of the complex. The reaction mixture was loaded and run on a 5% polyacrylamide gel. The labeled probes were detected by a PhosphorImager.

Generation of different YFP-tagged CENPC constructs

CENPC, delCENPC, and intCENPC sequences with their natural stop codon were amplified from the vectors previously used for *in vitro* binding by PCR using Accu Taq polymerase (Sigma) and the following primers: forward 5'-CACCATGGACGCCGCCGACCCCCTCT-3' and reverse 5'-TCAGTACCTTGCAGATTTAGCAACAAGATCTG-3'. The PCR products were cloned into the pENTR/D-TOPO vector (Invitrogen). Entry clones containing CENPC, delCENPC, and intCENPC sequences (pENTR-CENPC, pENTR-delCENPC, and pENTR-intCENPC) were linearized with MluI to avoid subsequent transformation of entry vectors into *E. coli*. The DNA fragments of the CENPC/delCENPC/intCENPC sequences were recombined into the pEarleyGate 104 plasmids (Earley et al., 2006) using LR clonase (Invitrogen) to form an N-terminal YFP fusion. Recombined plasmids were then transformed into *E. coli* TOP10 cells. Positive clones were selected using Kanamycin. Recombinant plasmids with sequence

confirmed were transformed into Agro bacterium strain C58C1 for subsequent maize transformation.

Plant transformation and analysis of YFP expression *in vivo*

Agrobacterium-mediated transformation of maize Hi Type II hybrid was accomplished by Plant Transformation Facility at Iowa State University (Frame et al., 2002). Immature zygotic embryo was used for transformation with pEarleyGate vectors. Transgenic YFP-CENPC-containing progenies were selected using Bialaphos. Transgenic plantlets were received and grown to maturity in the greenhouse. Tester lines were prepared to serve as silk recipient for transgenic pollen. Crossed seeds were grown in 30°C incubator and root tips transgenic for YFP-CENPC were obtained. YFP fluorescent signals were observed under microscope *in vivo*.

The YFP-CENPC, YFP-delCENPC, and YFP-intCENPC plasmids were also used for particle bombardment of maize callus. Transient transformation was performed with plasmid DNA coated 0.6µ gold particles using a PDS1000 system (Bio-Rad). After bombardment, calluses were cultured for 18h under dark conditions before YFP localization via fluorescence microscopy.

Image capture, processing and intensity analysis

3D data sets for YFP localization of all three constructs (YFP-CENPC, YFP-delCENPC, YFP-intCENPC) were acquired using an Intelligent Imaging Innovations (Denver, CO, USA) Everest Digital Microscope Workstation. Conditions (temperature, light) of image capture were

identical and signals were below saturation point. Images were further processed by SlideBook 4.0 (Intelligent Imaging Innovations) software package.

The sum intensities and volumes of YFP signals at kinetochore and nucleoplasm were measured for individual cells using SlideBook 4.0 software. To remove background, the average of four random cytoplasmic areas were calculated and subtracted from the intensity data set of the corresponding cells. For each cell analyzed, the ratio of mean intensities at kinetochore and nucleoplasm were calculated to quantitatively evaluate the efficiency of YFP-fused CENPC (or delCENPC or intCENPC) loading at kinetochore.

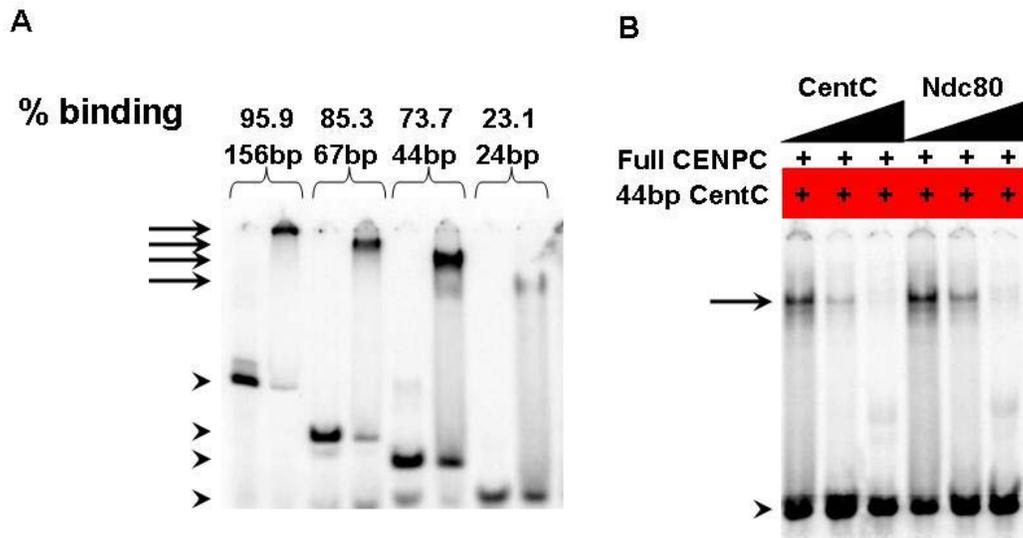


Figure 3.1. Basic gel shift properties of full length CENPC.

(A) Purified full CENPC and decreasing lengths of radiolabeled DNA (length indicated at the top) were mixed, electrophoresed in a 5% native acrylamide gel, and visualized by a PhosphorImager. Proteins are absent in lanes 1, 3, 5, 7 for each length of DNA. % binding = intensity of bound DNA/(intensity of bound DNA + intensity of free DNA).

(B) Competition experiments with DNA. Lanes were loaded with the mixture of radiolabeled 44 bp CentC DNA and full CENPC protein and competed with excess unlabeled 44 bp CentC or Ndc80 DNA. The triangles represent identical reactions with increasing amounts of competitor DNA.

The free DNA (arrowhead) and bound DNA (arrow) are indicated at left.

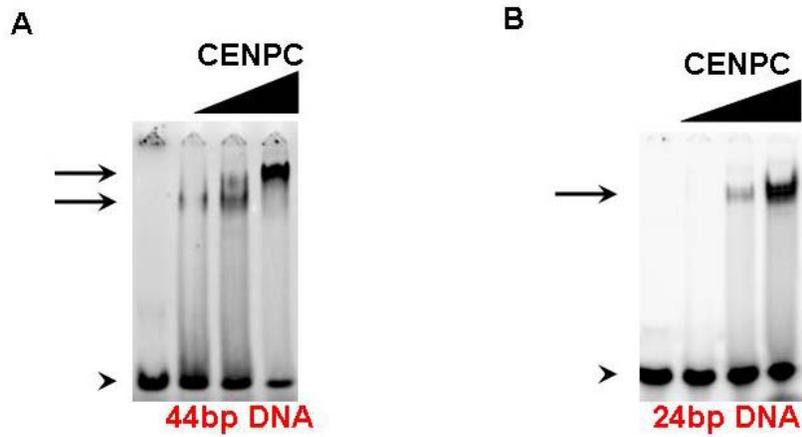


Figure 3.2. High concentrations of CENPC cause a supershift on 44 bp but not 24 bp DNA.

(A) Radiolabeled 44bp DNA was incubated with increasing amounts of full CENPC.

(B) Radiolabeled 24bp DNA was incubated with increasing amounts of full CENPC.

The triangles represent identical reactions with increasing amounts of proteins. The free DNA (arrowhead) and bound DNA (arrow) are indicated at left.

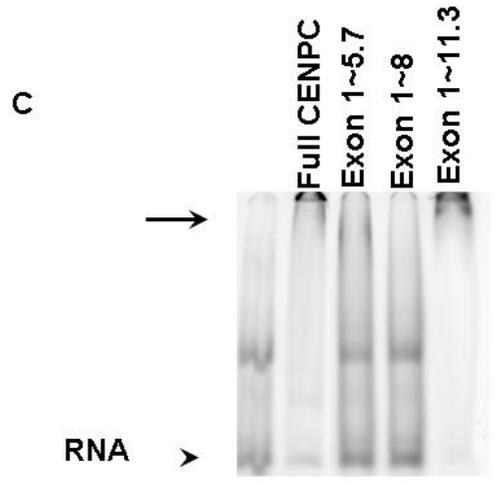
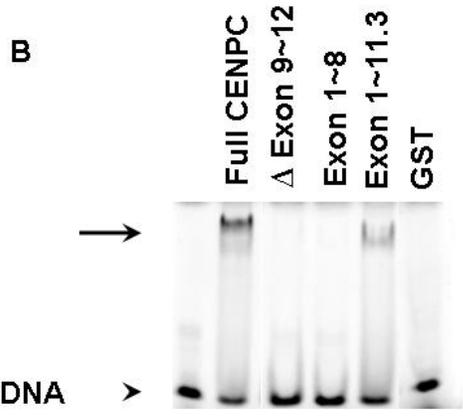
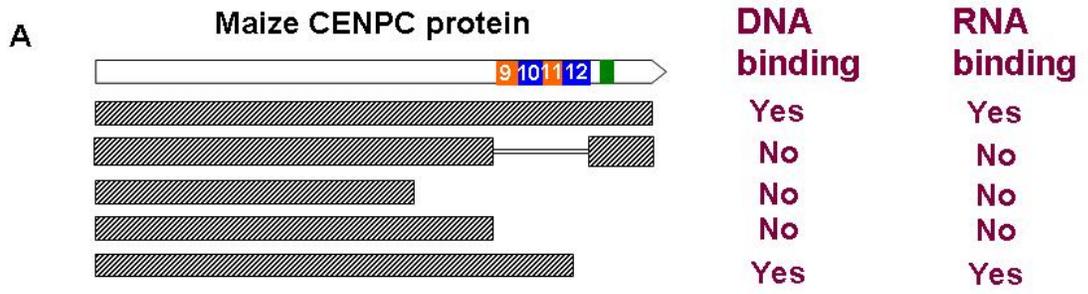
Figure 3.3. DNA and RNA binding localize to exon 9-12.

(A) Schematic representation of the selected CENPC constructs for which DNA- or RNA-binding results are shown in **(B)** or **(C)**: Full CENPC (1-701 aa), Δ exon 9-12 (1-502 aa + 625-701 aa), exon1-5.7 (1-399 aa), exon1-8 (1-502 aa), and exon1-11.3 (1-601 aa). Yes and No indicate positive and negative DNA or RNA binding (see **B** and **C**).

(B) Radiolabeled 44 bp DNA was incubated with equal amounts of the different CENPC fragments. A shifted DNA band (arrow) was seen for Full CENPC and exon1-11.3 (but not Δ exon 9-12 and exon1-8), indicating a major DNA-binding property for exon 9-12. As a negative control, GST (Glutathione S-transferase) was tested and no band shift was seen.

(C) Radiolabeled 167 nt RNA was incubated with equal amounts of the different CENPC fragments. A band shift of RNA (arrow) was seen for Full CENPC and exon1-11.3 (but not exon1-5.7 and exon1-8), indicating a major RNA-binding property for exon 9-12.

The free DNA/RNA (arrowhead) and bound DNA/RNA (arrow) are indicated at left.



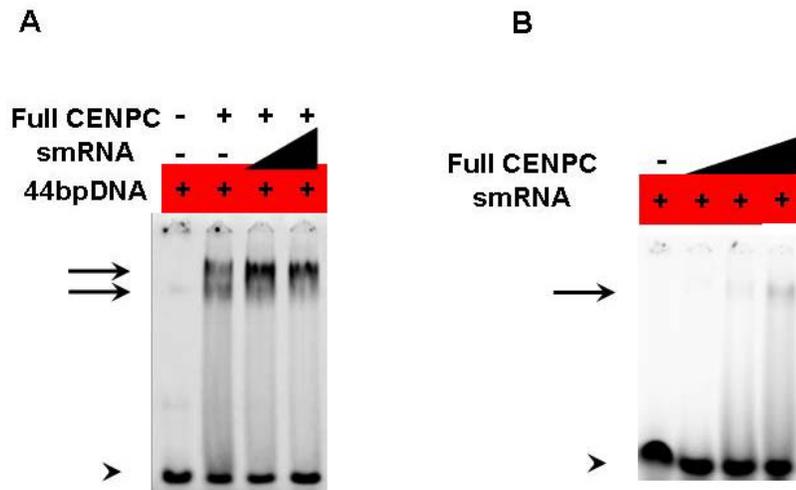


Figure 3.4. RNA promotes the CENPC supershift.

(A) Radiolabeled 44 bp CentC DNA was incubated without (lane 1) or with (lane 2) full CENPC.

Then increasing amount of unlabeled 24 nt small RNA was added to the mixture of CENPC and DNA (lanes 3 and 4). The triangles represent identical reactions with increasing amounts of small RNA.

(B) Radiolabeled 24 nt small RNA was incubated with increasing amounts of full CENPC. The triangles represent identical reactions with increasing amounts of proteins. Full CENPC binds small RNA alone very weakly, supporting the idea that there is more protein/DNA in the supershift.

The free DNA/RNA (arrowhead) and bound DNA/RNA (arrow) are indicated at left.

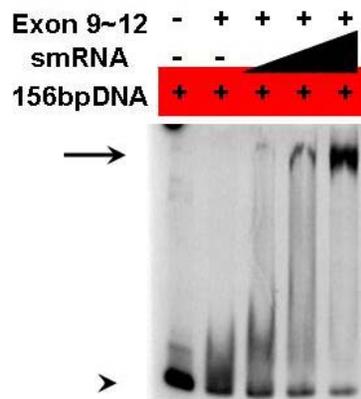


Figure 3.5. The purified exon 9-12 domain is dependent on RNA for DNA binding.

Radiolabeled 156 bp CentC DNA was incubated without (lane 1) or with exon 9-12 (lane 2).

Then increasing amount of unlabeled 24 nt small RNA was added to the mixture of exon 9-12 and DNA (lanes 3, 4, and 5). The triangle represents identical reactions with increasing amounts of small RNA.

The free DNA (arrowhead) and bound DNA (arrow) are indicated at left.

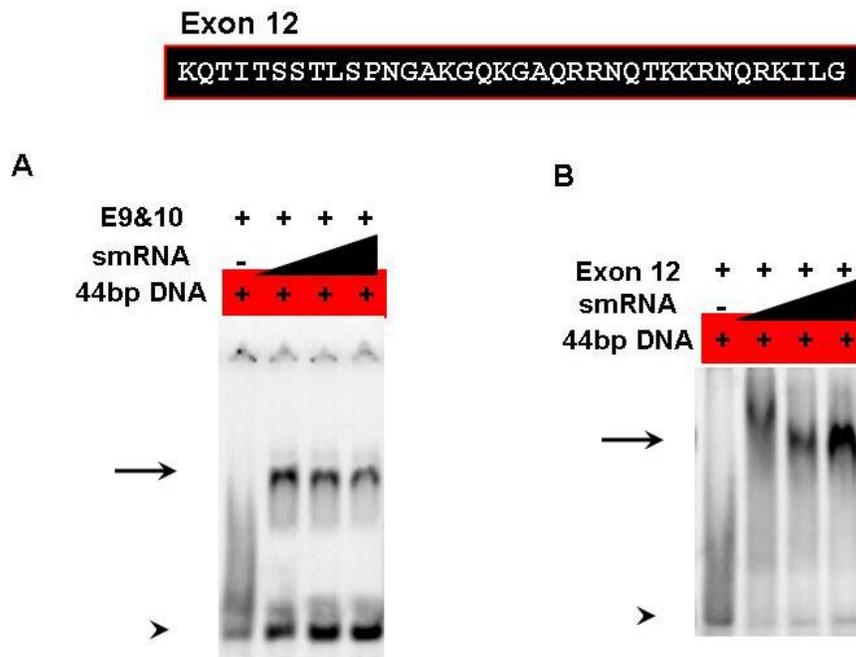


Figure 3.6. Exon 12 alone can support RNA-dependent DNA binding.

(A) Radiolabeled 44 bp CentC DNA was incubated with exon 9&10 (lane 1). Then an increasing amount of unlabeled 24 nt small RNA was added to the mixture of exon 9&10 and DNA (lanes 2, 3, and 4). The triangles represent identical reactions with increasing amounts of small RNA.

(B) Radiolabeled 44 bp CentC DNA was incubated with exon 12 (lane 1). Then increasing amount of unlabeled 24 nt small RNA was added to the mixture of exon 12 and DNA (lanes 2, 3, and 4). The triangles represent identical reactions with increasing amounts of small RNA.

The free DNA (arrowhead) and bound DNA (arrow) are indicated at left.

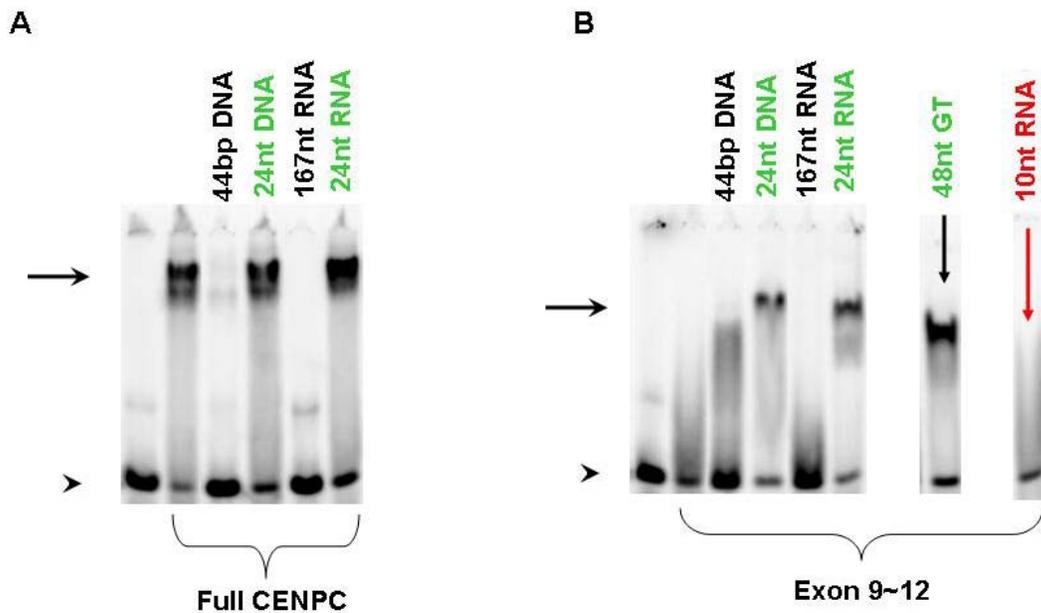


Figure 3.7. Only small single-stranded molecules promote CENPC-DNA binding.

(A) Radiolabeled 44 bp CentC DNA was incubated without (lane 1) or with (lane 2) full CENPC. Then unlabeled DNA or RNA was added to the mixture of CENPC and DNA (lanes 3-6). The stabilization of CENPC-DNA complex (arrow) was seen for unlabeled 24 nt DNA and 24 nt RNA (but not unlabeled 44 bp DNA and 167 nt RNA).

(B) Radiolabeled 44 bp CentC DNA was incubated without (lane 1) or with (lane 2) exon 9-12. Then unlabeled DNA or RNA was added to the mixture of exon 9-12 and DNA (lanes 3-8). The stabilization of exon 9-12-DNA complex (arrow) was seen for unlabeled 24 nt DNA, 24 nt RNA, and 48 nt GT (but not unlabeled 44 bp DNA, 167 nt RNA, and 10 nt RNA).

The free DNA (arrowhead) and bound DNA (arrow) are indicated at left.

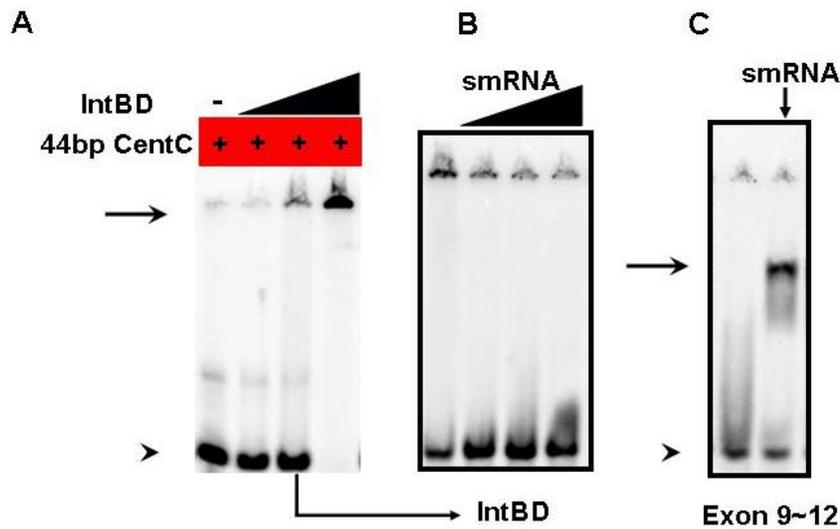


Figure 3.8. HIV integrase DNA binding domain can replace the DNA-binding activity of exon 9-12, but does not exhibit stabilization by RNA.

(A) Radiolabeled 44 bp DNA was incubated with increasing amounts of IntBD (HIV integrase Binding domain). The triangles represent identical reactions with increasing amounts of proteins.

(B) Radiolabeled 44 bp CentC DNA was incubated with IntBD (lane 1). Then increasing amount of unlabeled 24 nt small RNA was added to the mixture of IntBD and DNA (lanes 2, 3, and 4). The triangles represent identical reactions with increasing amounts of small RNA.

(C) Radiolabeled 44 bp CentC DNA was incubated with exon 9-12 (lane 1). Then unlabeled 24 nt small RNA was added to the mixture of exon 9-12 and DNA (lane 2).

The free DNA (arrowhead) and bound DNA (arrow) are indicated at left.

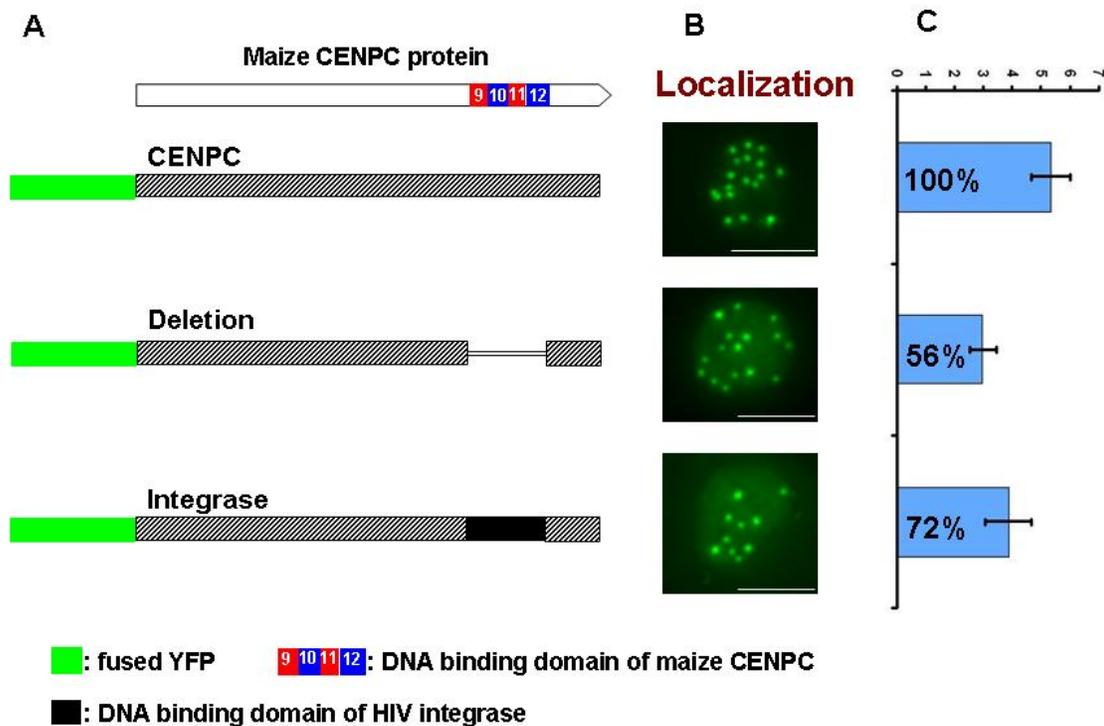


Figure 3.9. Reduction of centromere localization w/o exon 9-12 (transient).

(A) Schematic representation of YFP-CENPC constructs for maize transformation.

(B) *In vivo* YFP signals in a maize nucleus with the transient expression of YFP-CENPC (top), YFP-delCENPC (middle), and YFP-intCENPC (bottom) respectively. Bar = 5 μ m.

(C) The ratio of YFP mean intensity at the centromere and in the nucleoplasm transiently transformed with YFP-CENPC (top), YFP-delCENPC (middle), and YFP-intCENPC (bottom) constructs. The control (YFP-CENPC) is scaled to 100%. There is a significant difference among three treatments ($n > 25$, $P < 0.05$).

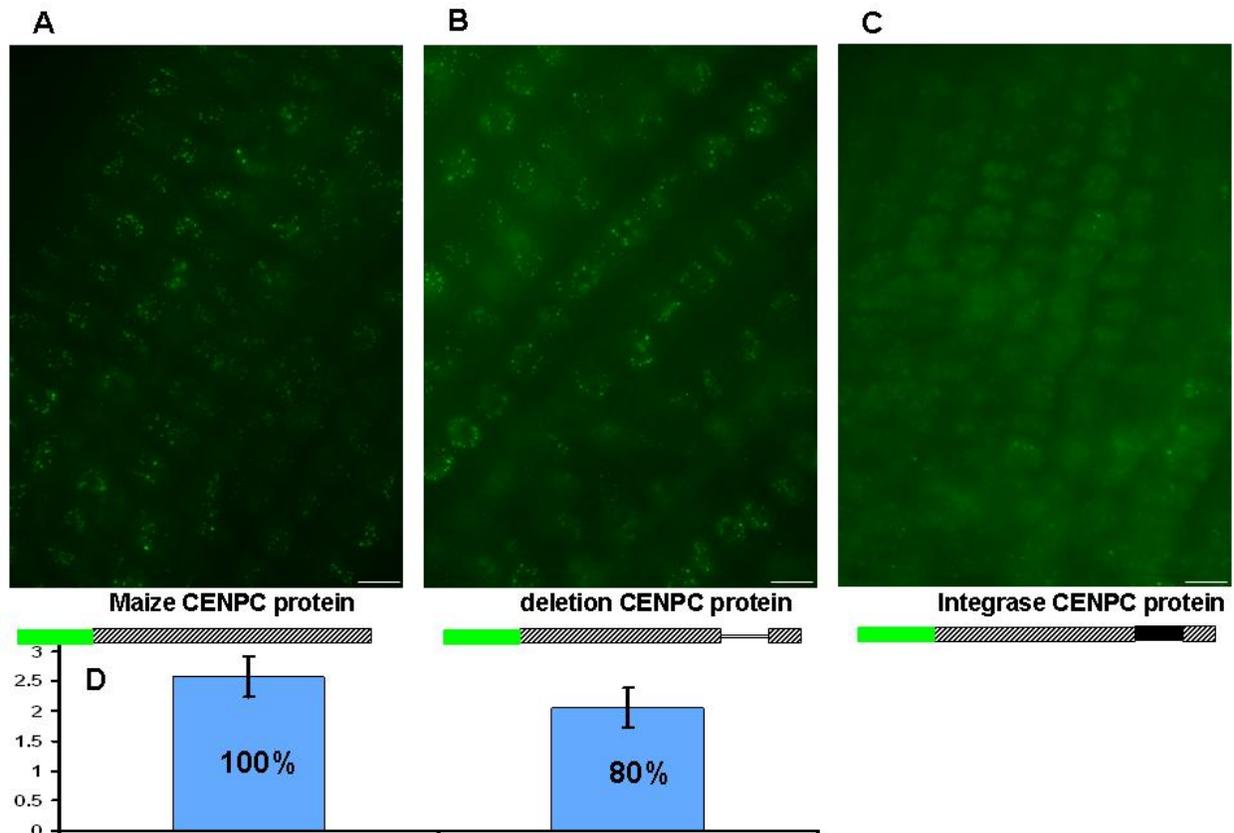


Figure 3.10. Reduction of centromere localization w/o exon 9-12 (mitosis).

(A) Multiple root tip cells with *in vivo* YFP-CENPC signals. Bar = 10 μm.

(B) Multiple root tip cells with *in vivo* YFP-delCENPC signals. Bar = 10 μm.

(C) Multiple root tip cells with *in vivo* YFP-intCENPC signals. Bar = 10 μm.

(D) The ratio of YFP mean intensity at the centromere and in the nucleoplasm of root tip cells transformed with YFP-CENPC (left) and YFP-delCENPC (middle) constructs (three events for each constructs, three individuals for each event). The control (YFP-CENPC) is scaled to 100%.

There is a significant difference among the treatments ($P < 0.05$).

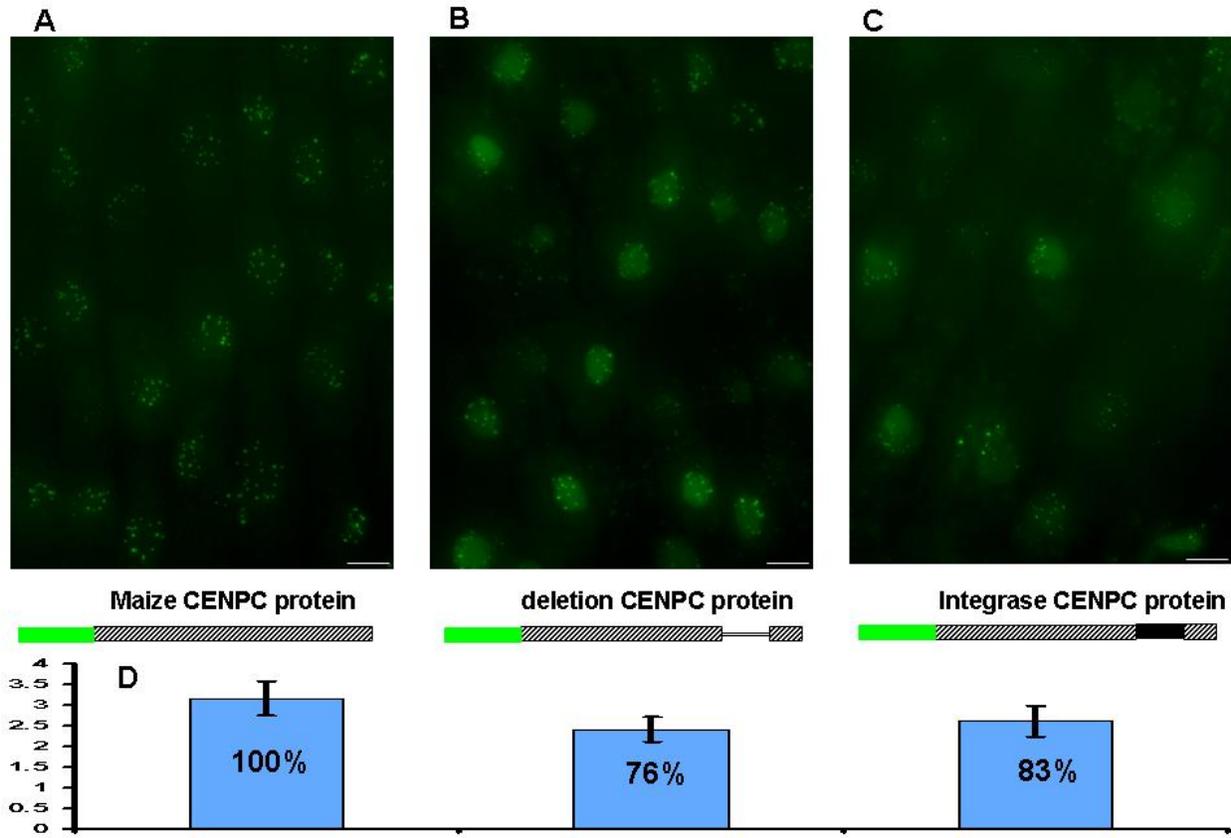


Figure 3.11. Reduction of centromere localization w/o exon 9-12 (elongation).

(A) Multiple cells at the elongation zone with *in vivo* YFP-CENPC signals. Bar = 10 μ m.

(B) Multiple cells at the elongation zone with *in vivo* YFP-delCENPC signals. Bar = 10 μ m.

(C) Multiple cells at the elongation zone with *in vivo* YFP-intCENPC signals. Bar = 10 μ m.

(D) The ratio of YFP mean intensity at the centromere and in the nucleoplasm of cells at the zone of elongation transformed with YFP-CENPC (left), YFP-delCENPC (middle), and YFP-intCENPC (right) constructs (three events for each constructs, three individuals for each event).

The control (YFP-CENPC) is scaled to 100%. There is a significant difference among the three treatments ($P < 0.05$).

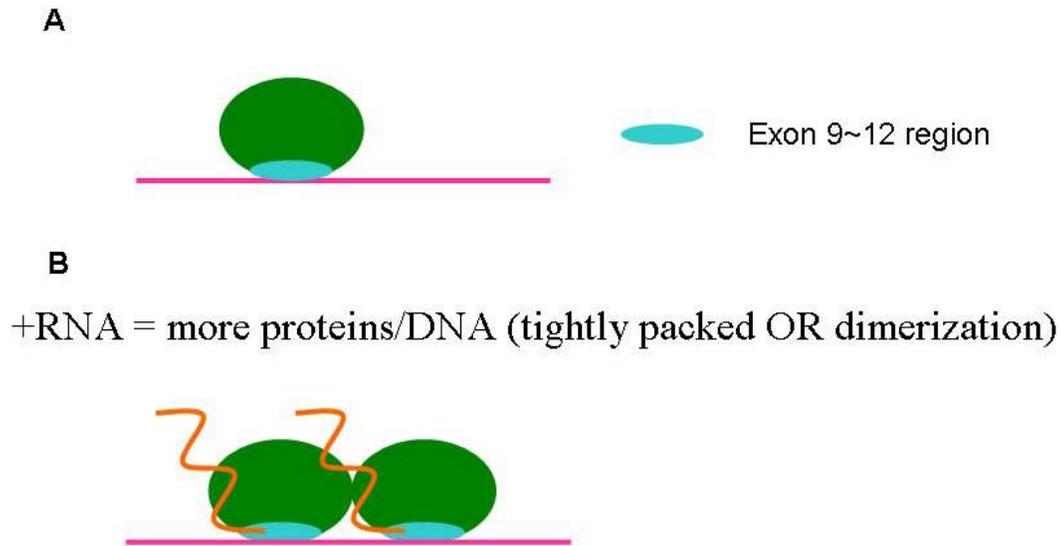
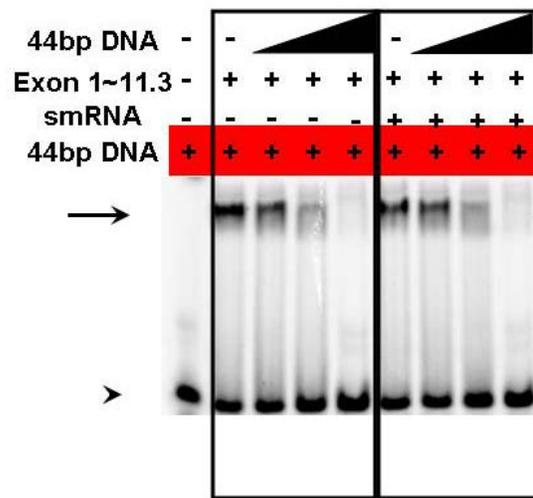


Figure 3.12. A model for how RNA facilitates CENPC-DNA interaction.

(A) CENPC randomly targets to the chromosome by its non sequence-specific DNA binding.

The surface that includes exon 9-12 is where CENPC contacts DNA.

(B) When CENPC encounters the centromeric region where centromeric RNA is abundant, RNA promotes folding or accumulation of CENPC onto centromeric DNA via exon 9-12.



Supplemental figures

The mixture of radiolabeled 44 bp CentC DNA and exon1-11.3 was incubated without (lanes 2-5) or with unlabeled 24 nt small RNA (lanes 6-9). Then unlabeled 44 bp DNA was added for competition experiments. The triangles represent identical reactions with increasing amounts of competitor DNA. The free DNA (arrowhead) and bound DNA (arrow) are indicated at left.

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CHAPTER 4

CONCLUSION

In an effort to understand kinetochore functions in securing equal distribution of chromosomes during cell division, I have made contributions in the following areas: (1) kinetochore structure, (2) kinetochore components and their respective functions, (3) kinetochore assembly/recruitment. The results have furthered our understanding of plant kinetochores by demonstrating a new component at the central kinetochore and revealing a mechanism for how RNA is involved in kinetochore recruitment. Meanwhile, many questions have been raised by my work and possible ways to address them will be discussed.

NDC80 – a new central kinetochore protein in plant

In order to identify NDC80 in the maize kinetochore, I have looked for cytological evidence that shows NDC80 is localized at centromeres. Using peptide antibodies against the protein sequence, NDC80 was found at maize centromeres (Chapter 2), making us believe for the first time that a kinetochore protein NDC80 functions in plants. According to previously established subdomains (Yu et al., 1999, Dawe et al., 2005), NDC80 is best placed in the region that bridges the inner kinetochore (i.e., CENPC) with the outer kinetochore (i.e., MAD2). The fact that maize NDC80 is a central kinetochore protein supports functional conservation of this protein (De Wulf et al., 2003, DeLuca et al., 2005, Meraldi et al., 2006), and provides strong

support for the idea of a conserved kinetochore structure that is widespread throughout the eukaryotes.

NDC80 – a constitutive component?

Interestingly, NDC80 can be detected at centromeres at all stages of the cell cycle, suggesting it is a constitutive component of the kinetochore (Chapter 2). This is different from other species where NDC80 comes on and off kinetochores as the cell cycle proceeds. The NDC80 interacting partners may be involved in the assembly and constitutive maintenance of this protein. No DNA-binding properties were found for this protein (my unpublished data), so centromere DNA is not associated directly. MIS12, another constitutive component, was suggested to be a NDC80-binding partner (De Wulf et al., 2003, Westermann et al., 2003) and has been characterized in *Arabidopsis* (Sato et al., 2005) and maize (Li & Dawe, unpublished). In recent years, several lines of evidence have shown that NDC80, together with MIS12, participates in forming the microtubule attachment site and that NDC80 binds to microtubules (He et al., 2001, DeLuca et al., 2005, Cheeseman et al., 2006, DeLuca et al., 2006, Wei et al., 2007, Wilson-Kubalek et al., 2008). If maize NDC80 has the same function in microtubule attachment, centromeres may be continuously capable of attaching to microtubules. There are four components (NDC80, NUF2, SPC24, SPC25) in the NDC80 complex with the NDC80/NUF2 dimer binding to microtubules and the SPC24/SPC25 dimer facing centromeric chromatin (Cheeseman and Desai, 2008). Isolation and functional studies of these proteins will help to understand how NDC80 functions in plants.

NDC80 – function?

Functions of NDC80 can be further investigated by gene mutation. Since the *Ndc80* gene was shown to be essential (McClelland et al., 2003), viable mutants can only be obtained by reducing gene expression or partially destroying protein function. RNA interference has been successfully applied to the functional study of MIS12, another kinetochore protein in maize (Li & Dawe, unpublished). TILLING, a PCR-based method to screen EMS-induced mutants of a gene, may also be a good approach to obtain the knockdown phenotype because maize has only one copy of *Ndc80* gene (my unpublished data). Given that NDC80 is required for assembly of outer kinetochore proteins and may play a role in spindle checkpoint signaling (Martin-Lluesma et al., 2002, De Wulf et al., 2003, Hori et al., 2003, McClelland et al., 2003, Stucke et al., 2004, DeLuca et al., 2005, Lin et al., 2006), the phenotypes may include disassembly of the outer kinetochore proteins, mitotic delay, increasing rate of chromosome missegregation, and cells with multiple micronuclei. Li's studies on *Mis12* RNAi mutants indicate that NDC80 works with MIS12 in ensuring the togetherness of two sister chromatids during segregation of homologous chromosomes at meiosis I. It is likely that pre-separation of sister chromatids at meiosis I will be seen in the *Ndc80* mutants as it is in the *Mis12* mutants.

CENPC – targets centromeres by a novel DNA/RNA interaction

Combining *in vitro* and *in vivo* approaches, I have revealed a new mechanism for how CENPC assembles at centromere via RNA facilitation. Different subdomains of maize CENPC including the whole protein were produced *in vitro* for DNA- and RNA-binding assays. By comparing the affinity difference, I have mapped the DNA/RNA binding domain to a region that has been repeatedly duplicated in grasses. This is a new DNA/RNA binding domain first

characterized by my work. The *in vitro* results have been further tested *in vivo* by centromere assembly of the exogenous CENPC variants that have the binding domain deleted or not. These results, along with published information from human cells (Sugimoto et al., 1994, Yang et al., 1996, Sugimoto et al., 1997, Politi et al., 2002, Trazzi et al., 2002, Wong et al., 2007), suggest that a combined DNA/RNA binding properties are conserved in CENPC from non-plants to plants. I found that short RNA molecules facilitate the CENPC-DNA interaction *in vitro*, providing a biochemical mechanism for the role of centromere RNA.

CENPC – role of DNA binding domain in CENPC targeting?

Although CENPC DNA binding has been well characterized in human and yeast (Sugimoto et al., 1994, Yang et al., 1996, Sugimoto et al., 1997, Politi et al., 2002, Trazzi et al., 2002, Cohen et al., 2008), no information is available in plants. Low protein similarity among CENPCs makes it impossible to predict the DNA-binding domain from one species to another. An exon duplication event was discovered in grass CENPCs during a search for adaptive evolution in the coding sequences (Talbert et al., 2004). Similar duplication events have occurred repeatedly during evolution and have been suggested to be involved in the function of proteins such as substrate-binding (Sunita et al., 2007). It is possible that exon duplication plays a role in the DNA binding of CENPC. In Chapter 3, our biochemical studies provide empirical evidence that maize CENPC binds to DNA and RNA, and that the binding domain maps to the exon duplication region. Furthermore, the exon binding domain was shown *in vivo* to be required for efficient centromere localization. Reduction instead of total abolishment of CENPC localization after the binding domain was deleted suggests that mechanisms other than DNA binding may be involved in kinetochore recruitment. Dimerization is one possible mechanism.

CENPC was shown to form a dimer in human and yeast (Sugimoto et al., 1997, Cohen et al., 2008), so the rule may be generalized to maize. Because native CENPC is still present in the maize transgenic lines, dimerization may help to recruit the deletion and substitution CENPC variants I tested. The interactions between CENPC and other kinetochore proteins may contribute to CENPC recruitment as well. In yeast, the MIS12 complex co-purifies with CENPC, and deletion of the human MIS12 complex reduces CENPC accumulation at centromeres (Meluh and Koshland, 1997, Westermann et al., 2003, Kline et al., 2006). With *Mis12* mutants available in our lab, the efficiency of CENPC recruitment can be measured, and the interaction between proteins can be assayed.

CENPC – function of RNA in CENPC targeting?

There are several mechanisms proposed for the role of centromere RNA (Maison et al., 2002, Dawe, 2003, Jiang et al., 2003, Zappulla and Cech, 2004, Dawe and Henikoff, 2006). The possibility that RNAi-regulated chromatin modifications specify the centromere was discussed, but no small RNA (the product of RNAi) has been seen or isolated from centromeres; instead the centromere is enriched in long single-stranded RNA. In budding yeast, telomerase RNA was suggested to provide a flexible scaffold for its binding proteins. Perhaps centromeres employ a similar mechanism involving RNA. When centromere RNA was found to be tightly associated with CENH3 (Topp et al., 2004), it was suggested that RNA may guide inner kinetochore proteins to centromeres (Jiang et al., 2003). Supporting the idea is a study in human showing that CENPC delocalizes from centromeres after RNA digestion (Wong et al., 2007). However, no mechanism was proposed to explain this observation. The results from my biochemical studies show that RNA directly facilitates the binding of CENPC to DNA, probably regulating

centromere specification and CENPC recruitment (Chapter 3). In addition, I carried a series of biochemical tests of the interaction between CENPC and RNA. The data revealed that only single-stranded RNA larger than 10 nt conferred stabilization, consistent with the characteristic of centromere RNA found *in vivo*. However, it remains unknown whether RNA is a stable feature of the CENPC-DNA (or serves a catalytic role). One test would be to see whether RNA can be precipitated with CENPC by anti-CENPC antibodies. Nevertheless, we are now limited by the methods used. Probably the best approach will be some form of structural analysis by techniques such as crystallography or NMR.

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APPENDICES

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Dear Yaqing Du,

Thank you for your kind mail. As a maize biologist myself (I studied Paramutation), it is always good to read interesting science on this model system.

Of course you have permission to use your paper in your dissertation. Please refer to Chromosome Research as the place to find the published version.

All the best finishing your dissertation, and best wishes,

Max