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Maize NDC80 is a constitutive feature of the central kinetochore

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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.

Introduction

Kinetochores are large proteinacious 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, McCleland *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, McCleland *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 G_1 but appears as centromeric dots from prophase to anaphase (Chen *et al*. 1997, Wigge & Kilmartin 2001, McCleland *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-TACAAGGTCACCCGCTCCG CACTC and R-AACATACCAACTACTACCT CACCAG).

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, McCleland *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

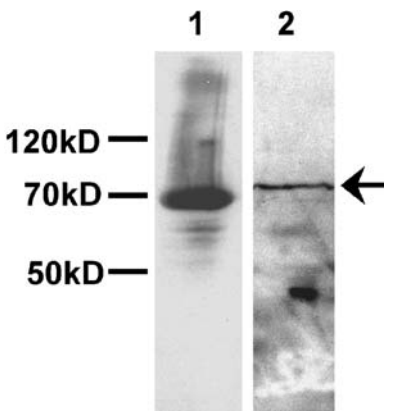

Figure 1. ZmNDC80 protein blot. Lane 1: a 6X histidine-ZmNDC80 fusion protein expressed in bacteria. Lane 2: protein extract from root tips. A single protein with an apparent mass of 75 kDa (arrow) was identified in maize root tissue.

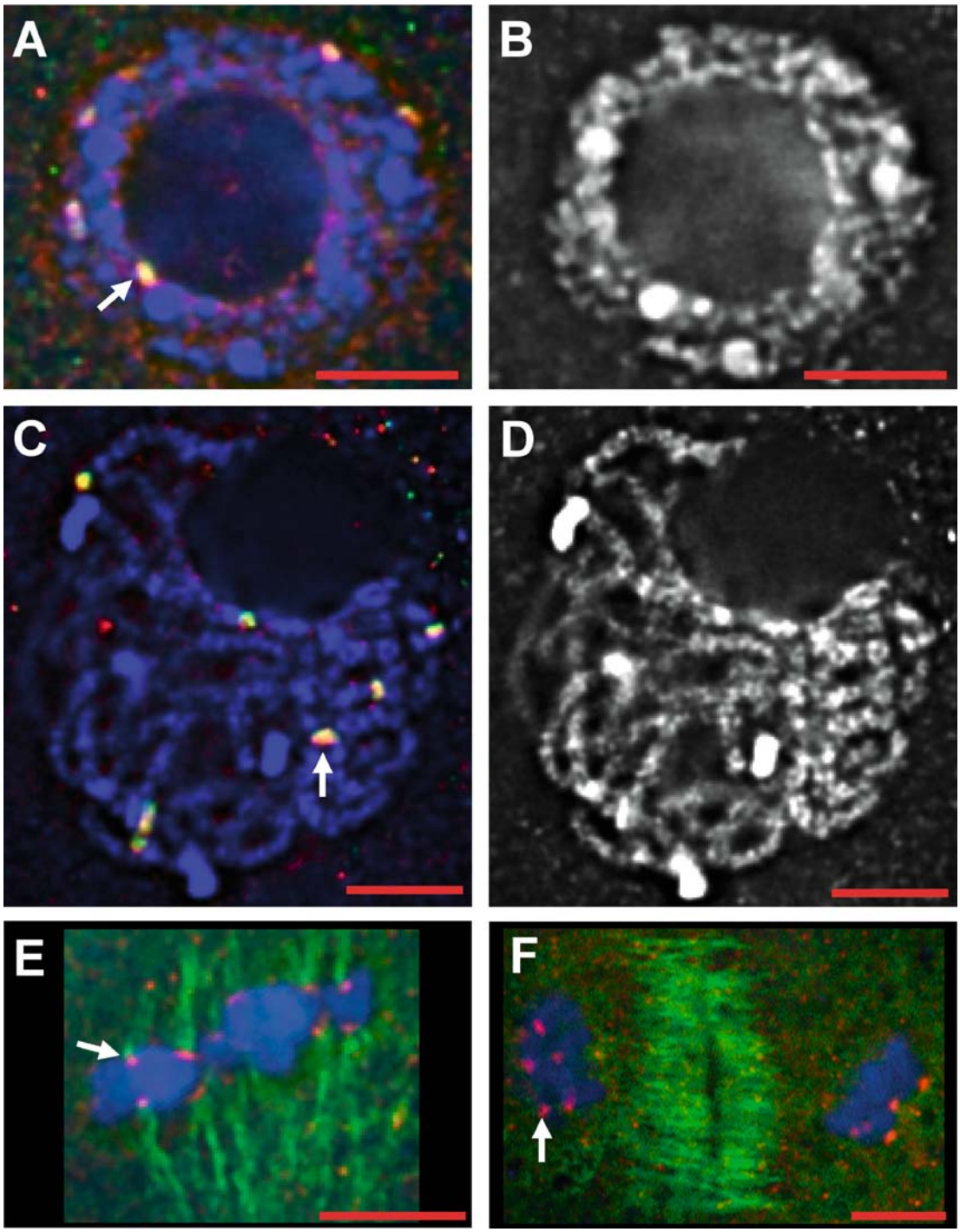

Figure 2. Immunolocalization of ZmNDC80 in meiotic cells. Images are single optical sections; only a subset of the kinetochores is visible. Arrows highlight kinetochore locations. (A) Leptotene, showing overlay of CENP-C and NDC80. DNA is in blue, CENP-C in green, and ZmNDC80 in red. (B) Chromosomes 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 μm.

(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 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 post-translational 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 2A,B). Later in pachytene, all kinetochores are paired and the centromere/kinetochore complexes are at their largest (Figure 2C,D; containing the kinetochores from four chromatids). NDC80 stained brightly in all subsequent stages, including metaphase (Figure 2E), late anaphase, and early telophase I (Figure 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 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.

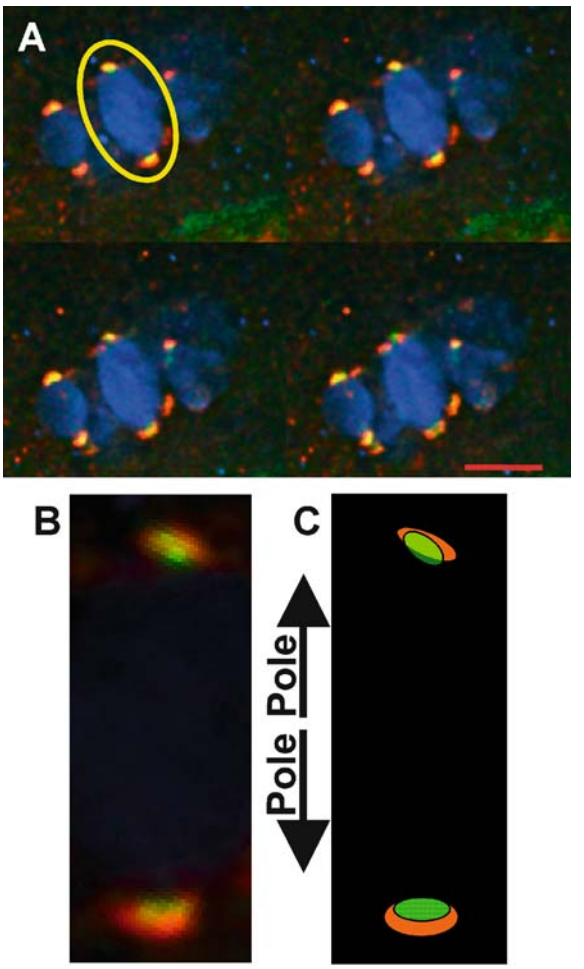

Figure 3. ZmNDC80 localizes outside of CENP-C at metaphase II. (A) Four consecutive sections of a prometaphase II cell co-stained for CENP-C (green) and ZmNDC80 (red). (B) An enlarged region (circled in A), showing two kinetochores with red signal (ZmNDC80) on the poleward side of the green signal (CENP-C). (C) A cartoon of (B). Bar = 5 μm.

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 4A,B), prophase (Figure 4C), metaphase (Figure 4D), and late telophase (Figure 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 G_2 of interphase (e.g., Hori *et al*. 2003). Since root tips are actively growing structures, they contain cells in

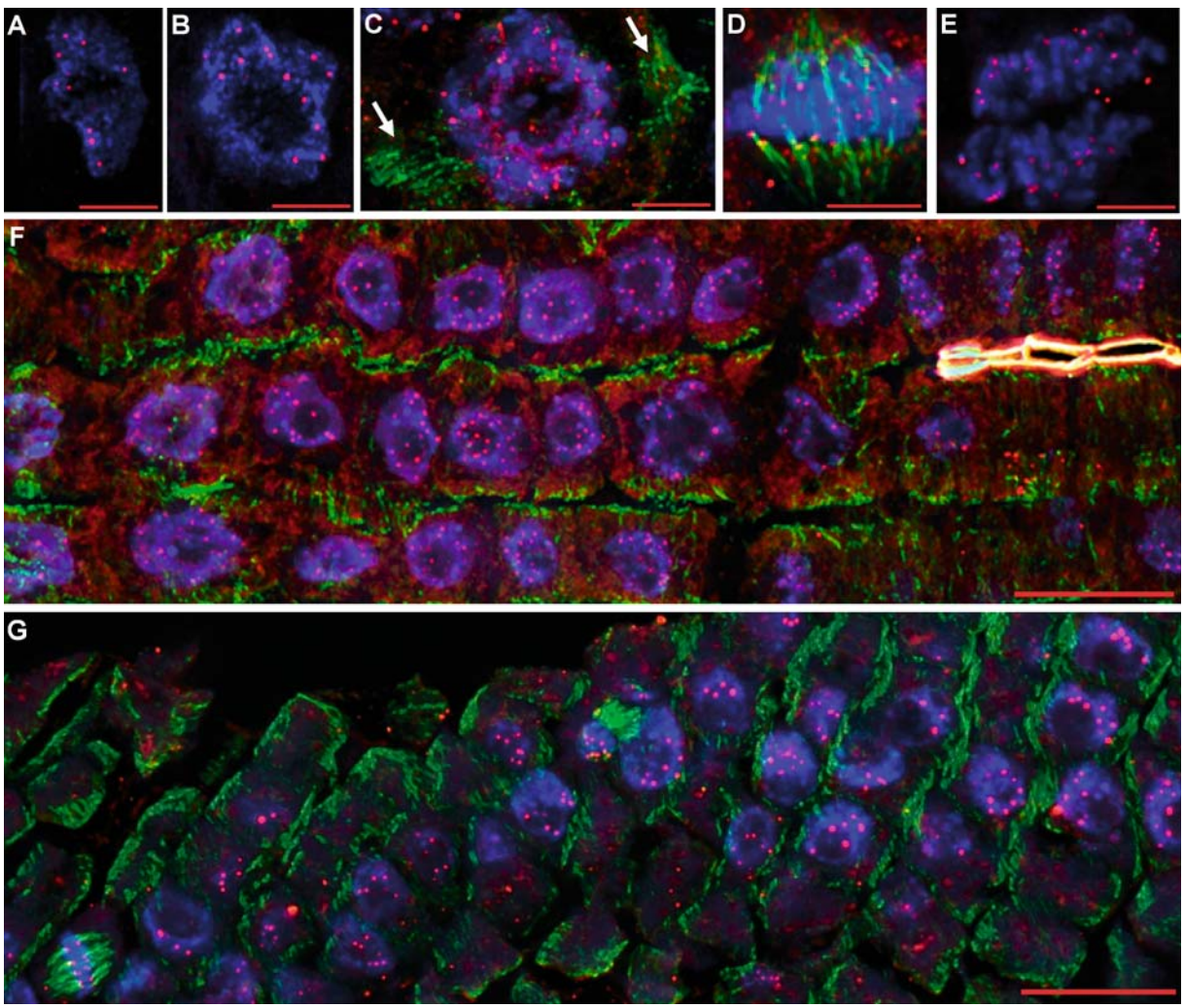

Figure 4. Immunolocalization of ZmNDC80 and CENP-C in mitotic cells. DNA is shown in blue, tubulin is shown in green, and ZmNDC80 (A–F) or CENP-C (G) is shown in red. The images in (A–E) are partial projections. (A) A G_1 cell. (B) A G_2 cell. (C) A prophase cell with the remnant of a preprophase band (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).

all stages of interphase, i.e., G_1, S, and G_2 stages. To calibrate the expected staining intensity at G_1 we used telophase nuclei (which, having just separated their chromosomes, have unreplicated DNA). To calibrate the expected staining for G_2,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 4C; Granger & Cyr 2001). There was an excellent correlation between nuclear diameter and DNA staining intensity, with small nuclei (G_1; similar in size to telophase) having about half the DNA staining as the large nuclei (G_2; often with variously-staged preprophase bands).

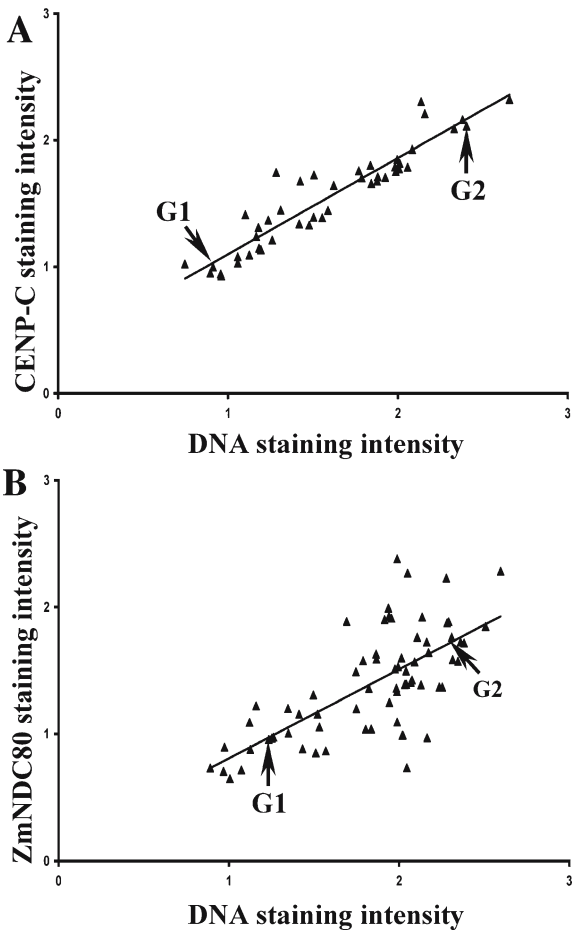

Figure 5. Linear relationship between DNA, CENP-C, and ZmNDC80 staining intensity. G_1 and G_2 cells are expected to differ in DNA content by a factor of 2. Known G_1 and G_2 cells are indicated: G_1 was scored by the presence of a remnant phragmoplast between two neighboring cells (indicating very recent 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.

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 4F,G). As shown in Figure 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 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 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, McCleland *et al*. 2003). In *S. pombe*, the kinetochores detach from spindle poles during karyogamy and coincidently 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 G_2 (as is the case for *Arabidopsis* CENH3; Lermontova *et al*. 2006), we can say with confidence that both proteins are present in abundance at G_1 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 KNl-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.

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