Ndc10 is a platform for inner kinetochore assembly in budding yeast

Uhn-Soo Cho & Stephen C Harrison

Kinetochores link centromeric DNA to spindle microtubules and ensure faithful chromosome segregation during mitosis. In point-centromere yeasts, the CBF3 complex Skp1–Ctf13–(Cep3)2–(Ndc10)2 recognizes a conserved centromeric DNA element through contacts made by Cep3 and Ndc10. We describe here the five-domain organization of Kluyveromyces lactis Ndc10 and the structure at 2.8 Å resolution of domains I–II (residues 1–402) bound to DNA. The structure resembles tyrosine DNA recombinases, although it lacks both endonuclease and ligase activities. Structural and biochemical data demonstrate that each subunit of the Ndc10 dimer binds a separate fragment of DNA, suggesting that Ndc10 stabilizes a DNA loop at the centromere. We describe in vitro association experiments showing that specific domains of Ndc10 interact with each of the known inner-kinetochore proteins or protein complexes in budding yeast. We propose that Ndc10 provides a central platform for inner-kinetochore assembly.

Kinetochores are tightly regulated, multicomponent complexes that assemble on centromeres and effect chromosome attachment to mitotic-spindle microtubules. They are the loci of force generation necessary to establish bipolar attachment of paired sister chromatids and the source of spindle checkpoint signals to prevent entry into anaphase until all chromatid pairs are correctly attached. The kinetochore of budding yeasts, with ‘point centromeres’ of roughly 150 base pairs (bp) and a single microtubule attachment, appears to correspond to a single module of the multimodular kinetochores in higher eukaryotes, which have ‘regional centromeres’ of up to several Mb and 30–40 microtubule attachments. Various lines of evidence—for example, protein sequence conservation, quantitative fluorescence microscopy and the similarity of the CENP-A–H4 chaperone structures from point and regional centromeres strongly suggest conservation of basic kinetochore architecture across all eukaryotes.

An assembled kinetochore comprises a set of proteins that interact directly with centromeric DNA (‘inner kinetochore’ proteins), a set of proteins that contact the attached microtubule(s) (‘outer kinetochore’ proteins) and an intermediate array of linkers. Proteins of the inner kinetochore conserved throughout evolution include a variant histone H3, known as Cse4 in budding yeast and as CENP-A in higher eukaryotes, and a transcription-factor-like protein called Mif2, which binds DNA weakly but specifically, with contacts that include a CCG element on AT-rich sequences. The Skp1–Ctf13 heterodimer is probably a structural homolog of the higher eukaryotic Skp1–Skp2 complex, a component of the Skp1–Cull1–F-box protein (SCF) ubiquitin ligase. What relationship, if any, the ubiquitin ligase–like structure of this heterodimer has to its organizational role in kinetochore assembly is yet to be determined.

The components of CBF3 are a Skp1–Ctf13 heterodimer, a Cep3 homodimer and an Ndc10 homodimer. There is some in vitro evidence for a second Ndc10 homodimer assembling with the DNA-bound complex. A Skp1–Ctf13–(Cep3)2 subassembly, which we will call here the CBF3 core, is stable in vitro. The positions of various components with centromeric DNA have been mapped in vitro by DNA modification methods and in vivo by high-resolution chromatin immunoprecipitation (ChIP). Cep3 is a DNA-binding protein with an N-terminal, GAL4-like ‘Zn cluster’. It binds DNA weakly but specifically, with contacts that include a CCG element on one side of an approximately palindromic sequence. Ndc10 binds DNA strongly but nonspecifically (other than with a preference for AT-rich sequences). The Skp1–Ctf13 heterodimer is probably a structural homolog of the higher eukaryotic Skp1–Skp2 complex, a component of the Skp1–Cul1–F-box protein (SCF) ubiquitin ligase. What relationship, if any, the ubiquitin ligase–like structure of this heterodimer has to its organizational role in kinetochore assembly is yet to be determined.

Although its initial characterization was as a component of CBF3 (refs. 25,34), Ndc10 may also have centromere-independent functions.
The chromosomal passenger complex (Ipl1–Sli15–Bir1–Nbl1 in yeast; Aurora B–INCENP–Survivin–Borealin in higher eukaryotes) and Ndc10 colocalize with interpolar microtubules at the spindle midzone in late anaphase\(^{38}\). The interaction of Ndc10 with the chromosomal passenger complex is through a contact with Bir1 (ref. 39).

We report here an analysis of the structure and interactions of Ndc10 from \textit{Kluyveromyces lactis}, a budding yeast with six point-centromeric chromosomes\(^{40}\). The sequence characteristics of CDEI and CDEII are conserved between \textit{K. lactis} and \textit{S. cerevisiae}, but the AT-rich CDEII sequence is twice as long in the former (~160 bp) as in the latter (~80 bp)\(^{41}\).

We find that the 85-kDa polypeptide chain of \textit{K. lactis} Ndc10 has five domains, which we have defined either by direct structural analysis or by proteolytic dissection. We have mapped interactions of the Ndc10 domains with all the other known DNA-proximal kinetochore components, including Scm3, a Cse4 specific chaperone. The crystal structure of a large fragment comprising domains I and II in complex with DNA shows that the protein has an unexpected structural relationship to tyrosine DNA recombinases and that each subunit of an Ndc10 dimer is likely to bind a distinct DNA element, rather than closely spaced elements on a single DNA duplex. We have confirmed this latter conclusion in \textit{vitro}. These results suggest that Ndc10 may create or stabilize a loop within centromeric DNA, and together with the data on Ndc10 interactions with other kinetochore proteins, they suggest that Ndc10 is a central organizing hub of the inner kinetochore.

**RESULTS**

**Domains of \textit{K. lactis} Ndc10**

In an effort to overcome problems associated with bacterial expression of \textit{S. cerevisiae} Ndc10, we cloned and expressed Ndc10 from other point-centromere yeasts. Ndc10 is highly diverged in these species, but the AT-rich CDEII sequence is twice as long in the former (~160 bp) as in the latter (~80 bp)\(^{41}\). In addition to the full-length protein, we purified fragments of \textit{K. lactis} CEN1 CDEIII and found that optimum binding required 25–30 bp of DNA (Supplementary Fig. 1d). We also tested sequence specificity using 30-mers with different G–C content and found that binding was essentially independent of G–C content and base sequence (Supplementary Fig. 1e).

**DNA binding by Ndc10**

The complete \textit{S. cerevisiae} CBF3 complex—comprising Skp1–Ctf13, (Cep3)\(_2\) and (Ndc10)\(_2\)—covers about 57 bp of CDEIII\(^{35,42}\). In the absence of the specialized nucleosome, Ndc10 can also bind CDEII \textit{in vitro}\(^{36}\). We tested the affinity of full-length \textit{K. lactis} Ndc10 for various lengths of the \textit{K. lactis} CEN1 CDEIII and found that optimum binding required 25–30 bp of DNA (Supplementary Fig. 1d). We also tested sequence specificity using 30-mers with different G–C content and found that binding was essentially independent of G–C content and base sequence (Supplementary Fig. 1e).

**Structure of a \textit{K. lactis} Ndc10–DNA complex**

We crystallized a complex of \textit{K. lactis} Ndc10 DI–II (residues 1–403) with a 30-bp DNA fragment of \textit{K. lactis} CEN1 CDEIII, and we defining four fragments that we designate as domains I–II (the further division is based on the three-dimensional structure; see below), III, IV and V (Fig. 1a–c). In addition to the full-length protein, we purified fragments of \textit{K. lactis} Ndc10 corresponding to DI–II (residues 1–403), DI–III (residues 1–534), and C-terminal fragments DIV–V (residues 552–736) and DV (residues 631–736). By sedimentation equilibrium analytical ultracentrifugation, we found that DI–II is monomeric and DI–III is dimeric (Supplementary Fig. 1b,c), indicating that DIII is the principal dimerization domain.

![Figure 1](https://example.com/figure1.png)

**Figure 1** Domains of \textit{K. lactis} Ndc10 and crystal structure of DI–II. (a) Domain organization of Ndc10; numbers show residues at the domain boundaries and are derived either from limited proteolysis or from the crystal structure. (b) Structure of \textit{K. lactis} Ndc10 (DI–II; 1–402) with 30-bp poly(dA–dT) DNA. Domain I (N domain, residues 1–100) is in cyan, and domain II (DNA-binding domain, residues 101–402) is in dark blue. Dashed lines represent disordered residues 36–39 and 283–292. A second, symmetry-related, 15-bp DNA fragment is shown in gray. The DNA has been modeled as poly(dA–dT) (see text), with the sequences of 5′-TTAACCTTTAATT-3′ (1–15) and 5′-AAATTTTATAA-3′ (1′–15′), as indicated. (c) Sequence conservation of Ndc10 among point-centromere yeasts. Location of insertions (red) in \textit{S. cerevisiae} Ndc10 DI–II with respect to \textit{K. lactis} Ndc10 DI–II, shown on a schematic representation of the primary sequence and on a ribbon representation of the structure. All molecular illustrations were made with PyMOL (Delano Scientific).
Table 1 Data collection and refinement statistics for *K. lactis* Ndc10 DI-II in complex with CDEIII DNA

|                              | Native 1 | SAD (SeMet) | Native 2 |
|------------------------------|----------|-------------|----------|
| **Data collection**          |          |             |          |
| Space group                   | C222₁    | C222₁       | P2₁2₁2₁  |
| Cell dimensions               | a, b, c (Å) | 117.29, 147.51, 95.63 | 113.37, 148.20, 93.1, 99.7, 125.9 |
| Wavelength                    | 0.9795   | 0.9795      | 0.9795   |
| Resolution (Å)               | 50–2.8   | 50–3.0      | 50–3.6   |
| Rsym                         | 0.113 (0.717) | 0.127 (0.603) | 0.104 (0.912) |
| t / σf                        | 23.8 (2.0) | 15.5 (2.0)  | 6.4 (1.4) |
| Completeness (%)             | 92.6 (74.2) | 91.7 (66.8) | 99 (99.9) |
| Redundancy                    | 14.7     | 5.2         | 3.4      |
| **Refinement**               |          |             |          |
| Resolution (Å)               | 50–2.8   | 50–3.6      |          |
| No. reflections              | 17,663   | 13,133      |          |
| Rwork / Rfree                | 19.3 / 25.1 | 28.6 / 33.1 |          |
| No. atoms                    | Protein  | 3,196       | 6,392    |
|                              | DNA      | 615         | 1,211    |
| **B-factors**                |          |             |          |
| Protein                      | 110.87   | 122.43      |          |
| DNA                          | 159.20   | 142.26      |          |
| R.m.s. deviations            | Bond lengths (Å) | 0.005        | 0.009    |
|                              | Bond angles (°) | 1.147        | 1.466    |

Values in parentheses are for the highest-resolution shell.

determined the structure to a resolution of 2.8 Å by SAD with selenomethionine (SeMet)-substituted protein (Fig. 1b and Table 1). The final model contains residues 1–402 of Ndc10, except for disordered loops from 36 to 39 and 283 to 292, and the DNA (built as poly(dA·dT); see below). The crystals are in space group C222₁, with one Ndc10 subunit and 15 bp of DNA in the asymmetric unit. The DNA fragments stack end-to-end, parallel to the c axis (which is equal in length to one 30-bp DNA duplex). Because there is only half of the 30-bp DNA fragment in one asymmetric unit, the observed electron density is the two-fold average of the two halves of the DNA. We therefore modeled the DNA as poly(dA·dT). A single Ndc10 subunit contacts about 25 bp of DNA (consistent with the results in Supplementary Fig. 1d); overlapping contacts from adjacent Ndc10 subunits on opposite faces of each DNA account for the half-frAGMENT asymmetric unit (Supplementary Fig. 11).

Ndc10 (residues 1–402) contains two closely associated globular domains—an N domain (domain I, residues 1–100) and a DNA-binding domain (domain II, residues 101–402). The N domain is a bundle of five α-helices and the DNA-binding domain is mainly helical, with a small β-sheet. The domain I–domain II interface has well-packed hydrophobic residues, suggesting that the two domains constitute a single globular unit (Supplementary Fig. 11). This conclusion is consistent with our failure to detect any tryptic cleavage at lysine 97, which lies just at the domain boundary.

The contacts between Ndc10(1–402) and DNA are essentially all hydrogen bonding, with one Ndc10 subunit and 15 bp of DNA in the asymmetric unit. The well-packed hydrophobic interface between domains I and II are also different. Domains I in Cre and Flp wrap as it lacks the relevant active site residues, nor does it have any base-pair binding by EMSA; none of mutants we examined retained detectable affinity for DNA (Fig. 2c).

The S. cerevisiae Ndc10 polypeptide chain is 220 residues longer than that of *K. lactis*. The insertions are distributed among a number of discrete segments. Of the incremental residues, 129 are in domains I and II. They form insertions into surface loops (Fig. 1c), and none of them are in DNA-contacting regions. The expansions in the S. cerevisiae Ndc10 sequence also tend to coincide with positions of poor conservation among the entire group of point-centromere yeast Ndc10 orthologs (Supplementary Fig. 2a,b).

Structural alignment with DNA recombinases

A search for three-dimensional structures related to Ndc10 using both a distance-matrix alignment program (Dali)44 and a secondary-structure-matching program (SSM)45 yields the DNA recombinases Flp46 (PDB 1M6X, Fig. 3a,b) and Cre47 (PDB code 1CRX), members of the λ-integrase family of tyrosine-based, site-specific DNA recombinases. Similarity of Ndc10 and λ-integrase is detectable from sequence relationships, but only after a large number of iterations of PSI-BLAST48. Also among PSI-BLAST-identified relatives are the crypton-encoded recombinase domains49, which, in addition to the recombinase region, have a C-terminal domain related to Ndc10 domain V.

Direct structure comparison shows that domains I and II of Ndc10 have essentially the same folds as do the corresponding domains of Cre and Flp, and when domain II of Ndc10 is superposed on domain II of either recombinase, the positions of bound DNA overlap (Supplementary Fig. 3a). Ndc10 has no catalytic activity, however, as it lacks the relevant active site residues, nor does it have any base-pair sequence specificity (Supplementary Fig. 3b). The relative orientations of domains I and II are also different. Domains I in Cre and Flp wrap back against the DNA, whereas in Ndc10, domain I projects in the opposite direction. The well-packed hydrophobic interface between domains I and II in Ndc10 probably rules out any large-scale reorientation toward a configuration resembling the one in the recombinase-DNA complexes. The additional domains of Ndc10, which mediate dimerization and partner interactions, have no counterparts in Cre and Flp.

An Ndc10 dimer

Ndc10 dimerization is mainly enforced by domain III, as demonstrated by the data described above (Supplementary Fig. 1b,c). Nonetheless, the two-fold rotational axis along b in space group C222₁ generates a relatively extended interface (~800 Å²) between two crystallographically related Ndc10 subunits (Fig. 4a and Supplementary Fig. 4a), suggesting that this contact may be present in the physiologically relevant dimer. Moreover, the C-terminal residues of the molecules...
Figure 2 Surface charge distribution and DNA contacts of Ndc10 DI–II. (a) Two views of the surface charge distribution of Ndc10 DI–II; bound DNA is shown in worm representation. (b) Sugar-phosphate backbone interactions. Residues involved in DNA contacts are labeled and shown in stick representation. Colors as in Figure 1b. (c) EMSA of wild-type and mutant Ndc10 (10% (w/v) TBE acrylamide gel stained sequentially with ethidium bromide and Coomassie blue).

Figure 3 Structural alignment of K. lactis Ndc10 DI–II with Flp recombinases. (a) Monomer structure of Flp (PDB 1M6X) aligned with the K. lactis Ndc10 DI–II. The N domain and the DNA-binding domain of Flp recombiantase are colored in orange and yellow, respectively. In Flp, the DNA structure of the Holliday junction was replaced by 30-bp CDEIII DNA for simple comparison. (b) Folding diagrams of K. lactis Ndc10 DI–II and Flp recombinase. Secondary-structure elements are labeled according to their position in the polypeptide chain; domains are colored as in panel a.

related by this dyad are about 30 Å from each other, at one end of domain II, as might be expected if these residues were to connect into a pair of dimerized domains III. Supporting the relevance of this dimer interface is the presence of the same interface in a different crystal form of Ndc10(1–402) bound to a 30-bp DNA fragment. These crystals, in space group P2₁2₁2₁, have an asymmetric unit that contains the Ndc10 dimer discussed above and two noncontiguous segments of 15 bp of DNA (Supplementary Fig. 4b,c). Thus, the stability of this interface is independent of crystal packing. In the following section, we describe experiments that support the dimer assignment, showing that in solution, a dimer of Ndc10(1–534), which contains domains I–III, indeed binds two independent DNA segments. We note, however, that amino acid residues participating in the dimer interface are not well conserved among budding-yeast Ndc10 proteins and that domain III is clearly the principal determinant of dimerization.

Ndc10 dimer interacts with two independent DNA segments
To test whether an Ndc10 dimer binds a single extended site on one segment of DNA or more compact sites on each of two independent segments of DNA, we carried out EMSA experiments with 30-bp CDEIII DNA and either dimeric Ndc10(1–534), containing domains I–III, or monomeric Ndc10(1–403), containing domains I and II only. We monitored the DNA mobility shifts as a function of DNA concentration, keeping the protein concentration fixed. If an Ndc10(1–534) dimer binds two independent 30-bp DNA fragments noncooperatively (as the flexible linkage of the dimerization domain suggests), then as the DNA concentration increases, an initially observed band, corresponding to a single 30-bp DNA duplex shifted by an Ndc10 dimer, will decrease in strength, in favor of a more rapidly migrating band, corresponding to binding of a second DNA fragment. The Ndc10(1–403) monomer should yield only a single shifted band at all DNA concentrations. The results shown in Figure 4b are fully consistent with these predictions.

To confirm these results by an alternative method, we tested whether an Ndc10 dimer can bind two differentially labeled DNA fragments in solution (Fig. 4c). We incubated a mixture of biotin-modified CDEIII DNA and 32P-labeled CDEIII DNA, at equal concentrations, with either dimeric Ndc10(1–534) or monomeric Ndc10(1–403). After affinity pulldown with streptavidin-coated resin, the reaction with dimeric Ndc10(1–534) yielded substantial amounts of 32P-labeled DNA in the bead eluate, but the reaction with monomeric Ndc10(1–403) yielded no more label than a control containing no protein.

We also measured the ratio of Ndc10 to bound CDEIII in solution by size-exclusion chromatography and isothermal titration calorimetry (ITC). In the former experiments, a fixed quantity of Ndc10 dimer (1–534) was incubated with 30-bp CDEIII DNA in various molar ratios, then applied to an analytical size-exclusion column. The amounts of protein–DNA complex and unbound free DNA were monitored by absorption at 260 nm. Consistent with our other experiments, the binding capacity of the protein saturated a 1:2 molar ratio of protein-dimer:DNA, and peaks corresponding to unbound DNA appeared when larger amounts of DNA were used (Fig. 4d,e). In the ITC measurements (Supplementary Fig. 5), Ndc10 monomer (1–403) bound a 30-bp DNA fragment in a 1:1 molar ratio, and Ndc10 dimer (1–534) bound the same fragment in about a 1:2 molar ratio (Ndc10 dimer:DNA). These results further substantiate our conclusion that one Ndc10 dimer binds two separate DNA fragments in solution.
Interactions with other kinetochore components

Identification of structurally meaningful domain boundaries has allowed us to carry out interaction experiments with well defined domains of Ndc10. We conducted pull-down experiments with His-tagged, biochemically well-characterized proteins or protein complexes as bait, and in vitro translated, 35S-labeled proteins as prey. In these experiments, we used the K. lactis homologs of all the proteins in question, and previous results with yeast two-hybrid methods on some of the S. cerevisiae counterparts support our findings.

His-tagged CBF3 core (Skp1–Ctf13–(Cep3) 2 with a His-tag on K. lactis Cep3) pulled down full-length Ndc10, a domain I–III fragment and a domain I fragment, but not fragments that lacked domain I (Fig. 5a,b).

The critical contact with the CBF3 core thus appears to be in domain I. A reverse experiment, using His-tagged, full-length K. lactis Ndc10 and in vitro translated CBF3 core components, resulted in a weak interaction with Cep3 and no interactions with the remaining two components (data not shown). Stronger Ndc10 association with Cep3 might therefore require the complete CBF3 core complex.

The DNA-binding region of Cbf1, residues 229–359, a basic helix-loop-helix-zipper (bHLHZ) element, is at the C terminus of the molecule, and residues 1–228 are probably mainly unstructured. The bHLHZ region of Cbf1 interacts with Ndc10 DI–II (see data in Fig. 5c). Analytical size-exclusion chromatography with purified recombinant proteins confirms that Cbf1 associates with the CBF3 holocomplex but not with the CBF3 core (Supplementary Fig. 6).

CBF3 recruits the chromosomal passenger complex (in yeast, Ipl1, Bir1 and Sli15) through an interaction between Ndc10 and Bir1 (ref. 39). This association may have a centromere-independent function, as Ndc10 and the chromosomal passenger complex move together to the spindle midzone in late anaphase.

We carried out pull-down experiments with in vitro translated K. lactis Bir1 and His-tagged K. lactis Ndc10 and found that domains I–III of Ndc10 were sufficient for association with Bir1 and that the N-terminal half of Bir1 (residues 1–328) contains the Ndc10 target site (Fig. 5d).

N-terminal region of Scm3 interacts with Ndc10 domain IV

The 200-residue protein, Scm3, binds Ndc10 and links CBF3 with the Cse4-containing nucleosome. In yeast with regional centromeres, which lack a clearly identified CBF3 equivalent, orthologs of Scm3 have additional domains appended to the C terminus, which may have DNA-recognition properties of their own. The Scm3 ortholog...
in higher eukaryotes, HJURP\textsuperscript{30,31}, is similarly a larger protein with
an N-terminal Scm3-like region\textsuperscript{31}. The structures of mammalian
HJURP–CENP-A–H4 and yeast Scm3–Cse4–H4 show conserved
recognition of the centromeric histone complex by its chaperone\textsuperscript{2,8}. We
found that an N-terminal, 115-residue fragment of Scm3 (which
includes the Cse4 binding domain, residues 41–115)\textsuperscript{2} interacts with
a DIV–V fragment of Ndc10 but not with domain V alone (Fig. 6a).

Amylose pull-down experiments with various maltose-binding protein
(MBP)-tagged Scm3 fragments showed that residues 1–28 are
sufficient for Ndc10 binding (Fig. 6b). Size exclusion chromatography
confirmed that a fragment containing residues 1–115 of Scm3, but
not the fragment spanning residues 29–115, forms a stable complex
with Ndc10 domain IV–V (Supplementary Fig. 6).

DISCUSSION

Ndc10 is a multidomain, dimeric protein. We have located boundaries
that define five distinct domains, through use of limited proteolysis
and by determining a high-resolution structure of the fragment
comprising residues 1–402. Domains I and II form a DNA-binding
module, domain III stabilizes the dimer, and all of the five domains
(with the possible exception of domain V) interact with other kinetochore components. Pull-down experiments suggest that there are
detectable contacts with the CBF3 core, with Scm3 and with Cbf1—in
short, with all the other known DNA-contacting or nucleosome-contacting proteins in the yeast inner kinetochore (Fig. 6c). Thus, Ndc10
appears to be a central scaffold for inner kinetochore assembly.

The crystal structure of DNA-bound Ndc10(1–402) suggests that
the two subunits in a single Ndc10 dimer bind independent DNA
fragments, rather than adjacent sites along a single fragment. Our
experiments on DNA-binding in solution confirm this suggestion. For
assembly of a kinetochore in vivo, we can entertain three possibilities:
One is that an Ndc10 dimer bridges between sister chromatids; because
sister kinetochores can be widely separated even before anaphase onset,
such a state could only occur during a limited part of the cell cycle.

A second possibility is that one of the two DNA contacts remains
unsatisfied. A third possibility—the one that impresses us as most
probable—is that centromeric DNA is looped and that Ndc10 contacts
both the well-characterized site in CDEIII and a site (perhaps less
precisely defined) upstream of CDEI or downstream of CDEIII (Fig. 7).

A further conclusion from the crystal structure is that the fragment
containing domains I and II closely resembles tyrosine recombinases
but has no endonuclease activity. The complex common
fold of these proteins is not one that seems likely to have arisen
independently, and the local structural relationships between Ndc10
and the Cre and Flp recombinases are quite extensive (Fig. 4 and
Supplementary Fig. 4). Moreover, the homologous surfaces of
domain II make contact with DNA. We suggest that the two groups
of proteins are distant evolutionary cousins, but the functional
implications, if any, of the homology between Ndc10 and recombinases
are not apparent from the information currently in hand.

The notion that Ndc10 binding might introduce or stabilize a loop in
yeast centromeric DNA, as suggested by the finding that the partners

Figure 6 Interaction of Ndc10 domain IV–V with N-terminal Scm3. (a) Ni\textsuperscript{2+}
affinity pulldown of \textsuperscript{35}S-labeled, in vitro translated Ndc10 proteins with purified, His-tagged Scm3 proteins,
analyzed by SDS-PAGE and visualized by phosphoimaging. (b) In vitro amylose pulldown of
purified MBP-tagged Scm3 proteins with Ndc10 domain IV–V. (c) Schematic overview of domain
interaction of Cbf1 with Ndc10
association of K. lactis Ndc10 with other kinetochore proteins. Ndc10 DI interacts with CBF3 core;
Ndc10 DI–II, with Cbf1 (229–359) and Bir1p (1–328). Scm3 N (1–28) associates with Ndc10 DIV–V but not with DIV. Interaction of Cbf1 with Ndc10
was confirmed by analytical size-exclusion chromatography using purified proteins (Supplementary Fig. 6).

Figure 7 Schematic model of Ndc10 interactions on budding yeast
centromeres. Cbf1 and CBF3 core recognize CDEI and CDEIII, respectively.
Ndc10 does not have sequence-specific DNA contacts, but it binds in
defined register through its interactions with Cbf1 and CBF3 core. We
propose that these contacts bring CDEI and CDEIII together to form a
loop. Two potential loop configurations are shown. The Scm3–Cse4–H4
heterotrimeric complex can be recruited through Scm3–Ndc10 interaction.
in an Ndc10 dimer bind two distinct DNA sites rather than one extended site, is consistent with our interaction data from pull-down experiments with in vitro translated targets. Ndc10, which contacts DNA at the 3' end of the consensus centromere10, also contacts Cbf1, which binds at the 5' edge (Fig. 7). Moreover, incorporation of Ndc10 at centromeric DNA will recruit the Scm3–Cse4–H4 complex through the Ndc10–Scm3 interaction (Figs. 6b and 7). Between the Ndc10 footprint in CDEIII and DNA-bound Cbf1 is CDEII, which is coiled around the Cse4-containing nucleosome20. In K. lactis and A. gossypii, CDEII is exactly twice the length (80 bp) of CDEII in S. cerevisiae2. In either case, this wrapping will bring CDEI and CDEIII close enough (r.m.s. deviation = 0.7 Å for C_H4 complex superposes well on that of the Scm3–Cse4–H4 complex corresponding to the regions that bind Ndc10 and Cse4, respectively from point-centromeric yeasts identified two conserved regions, the H4 binding region (N-terminal segment (residues 1–28), which does not overlap the Cse4–H4 binding region (Fig. 6b). Multiple alignment of Scm3 sequences from point-centromeric yeasts identified two conserved regions, corresponding to the regions that bind Ndc10 and Cse4, respectively (Supplementary Fig. 8). A crystal structure of the HJURP–CENP-A–H4 complex superposes well on that of the Scm3–Cse4–H4 complex (r.m.s. deviation = 0.7 Å for C atoms of 176 overlapping residues). Recognition of recruitment of CENP-A–H4 by its chaperone are thus the same for both point and regional centromeres2,8.

The structure and interaction data reported here suggest that Ndc10 contributes to the formation and stability of a compact structure for the inner kinetochore. Ndc10 is a platform for initial kinetochore assembly. By recruiting the Scm3–Cse4–H4 complex into centromeres, it initiates formation of an elaborate superstructure. Because the specialized nucleosome is a universally conserved architectural feature of any kinetochore, whatever the length and organization of its centromere, the way in which Ndc10 scaffolds centromere compaction in budding yeast is likely to have counterparts in all other eukaryotes.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/nsmb/.

Accession codes. Coordinates and structure factors for the reported crystal structures have been deposited into the Protein Data Bank under accession codes 3SQI (C222, form) and 3T79 (P2_12_1 form).

Note: Supplementary information is available on the Nature Structural & Molecular Biology website.

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AUTHOR CONTRIBUTIONS

U.-S.C. designed and conducted experiments, determined and refined the structures, analyzed data and wrote the manuscript; S.C.H. directed the project, analyzed data and wrote the manuscript.

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ONLINE METHODS

Cloning, expression and protein purification. Full-length and truncated \textit{K. lactis} Ndc10 constructs, and point-mutants of residues involved in DNA contacts, were cloned in \textit{E. coli} and purified as described in Supplementary Methods.

Limited trypsin proteolysis and mass spectrometry. To identify distinct domains of Ndc10, purified full-length \textit{K. lactis} Ndc10 was treated with trypsin at 23 °C for 10 min (1:50 (w/w)) and submitted for ion-trap mass-spectrometry analysis at the Howard Hughes Medical Institute Mass spectrometry laboratory at the University of California at Berkeley. We identified three trypsin-sensitive loops in the protein from trypsin cleavage sites C-terminal to Lys410, Lys541 and at the University of California at Berkeley. We identified three trypsin-sensitive

Electrophoretic mobility shift assay. To identify binding partners of Ndc10, target proteins were translated \textit{in vitro} using TNT T7-coupled reticulocyte lysate (Promega) with \textsuperscript{35}S-labeled methionine (Roche). \textit{In vitro} translated target proteins were incubated with His-tagged bait proteins at 4 °C for 1 h. Ni-NTA agarose beads (Qiagen) pre-equilibrated with washing buffer (buffer A plus 0.1% NP-40 (w/v)) were mixed and incubated at 4 °C for 30 min with rotation. The beads were washed three times with washing buffer, and bead-bound proteins were separated by SDS-PAGE. Dried gels were analyzed by overnight exposure of a phosphorimager plate.

Amylose affinity pull-down assay. Ndc10 domains IV-V constructs were cloned, expressed in \textit{E. coli} and purified as described in Supplementary Methods. Ndc10 domains IV-V constructs were incubated in a 1:1 molar ratio with each of the MBP-tagged Scm3 proteins for 1 h at 4 °C. Amylose beads (New England Biolab) pre-equilibrated with washing buffer (buffer A plus 0.1% NP-40 (w/v)) were added and the mixtures incubated at 4 °C for 30 min with rotation. The beads were washed three times with washing buffer, and bead-bound proteins were separated by SDS-PAGE.

DNA-binding of mutant Ndc10. Site-directed mutagenesis of residues at the DNA contact seen in the crystal structure followed a PCR extension strategy. All mutant constructs of the \textit{K. lactis} Ndc10 DI–III were confirmed by DNA sequencing. Mutant proteins were purified as described in Supplementary Methods.

To determine binding, 20 pmol DNA (\textit{K. lactis} CEN1 CDEIII) was incubated with a comparable molar quantity of wild-type or mutant Ndc10 in 30 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM TCEP (buffer A), and the mixture was treated by EMSA as described in the preceding paragraph.

Ndc10:DNA ratio in bound complexes. EMSA: 20 pmol of \textit{K. lactis} Ndc10 DI–III was incubated in buffer A with different molar ratios (1:0.5, 1:1, 1:1.5, 1:2, 1:2.5, 1:3, 1:3.5 and 1:4) of 30-bp \textit{K. lactis} CEN1 CDEIII DNA. Electrophoresis and staining were done as described above.

Differentially labeled DNA pull-down assay: Biotinylated 30-bp CDEIII DNA was synthesized using MWG-Biotech AG, and \textsuperscript{32}P-labeled CDEIII DNA was prepared by \textit{in vitro} labeling using [\gamma-\textsuperscript{32}P]ATP (Roche) and T4 polynucleotide kinase (New England Biolabs). Ndc10 DI–III dimer (20 pmol) was incubated in buffer A with 25 pmol of biotin-labeled DNA, 5 pmol \textsuperscript{32}P-labeled DNA and 20 pmol unlabeled DNA. Biotinylated CDEIII was separated with Dynabeads M-280 Streptavidin (Invitrogen), using a magnetic separator (Invitrogen). Beads were washed at least five times with washing buffer (buffer A plus 0.1% NP-40 (w/v)) and eluted with SDS-PAGE sample buffer containing 300 mM imidazole. Dried gels were scanned with a phosphorimager (Bio-Rad). The same protocol was used with Ndc10 DI-II and with DNA alone, as negative controls.

Size-exclusion chromatography of protein–DNA complexes: A fixed amount of Ndc10 DI–III dimer (5 µM) was mixed with 30-bp CDEIII DNA in various molar ratios (1:0, 1:0.5, 1:1, 1:1.5, 1:2, 1:2.5, 1:3, 1:3.5 and 1:4) and applied to a Superdex200 10/300 GL size-exclusion column (GE Healthcare) pre-equilibrated with buffer A. Elution profiles were monitored at 260 nm wavelength to detect both the DNA–protein complex and unbound free DNA. The column profile of protein-free DNA was also obtained.

Ni\textsuperscript{2+}-affinity pull-down assay. To identify binding partners of Ndc10, target proteins were translated \textit{in vitro} using TNT T7-coupled reticulocyte lysate (Promega) with \textsuperscript{35}S-labeled methionine (Roche). \textit{In vitro} translated target proteins were incubated with His-tagged bait proteins at 4 °C for 1 h. Ni-NTA agarose beads (Qiagen) pre-equilibrated with washing buffer (buffer A plus 0.1% NP-40 (w/v)) were mixed and incubated at 4 °C for 30 min with rotation. The beads were washed three times with washing buffer, and bead-bound proteins were separated by SDS-PAGE. Dried gels were analyzed by overnight exposure of a phosphorimager plate.

Amylose affinity pull-down assay. Ndc10 domain IV-V (residues 532–736) and MBF-tagged Scm3 constructs were cloned, expressed in \textit{E. coli} and purified as described in Supplementary Methods. Ndc10 domains IV-V constructs were incubated in a 1:1 molar ratio with each of the MBP-tagged Scm3 proteins for 1 h at 4 °C. Amylose beads (New England Biolab) pre-equilibrated with washing buffer (buffer A plus 0.1% NP-40 (w/v)) were added and the mixtures incubated at 4 °C for 30 min with rotation. The beads were washed three times with washing buffer, and bead-bound proteins were separated by SDS-PAGE.

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