The Saccharomyces cerevisiae Smc2/4 Condensin Compacts DNA into (+) Chiral Structures without Net Supercoiling* §

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Smc2/4 forms the core of the Saccharomyces cerevisiae condensin, which promotes metaphase chromosome compaction. To understand how condensin manipulates DNA, we used two in vitro assays to study the role of SMC (structural maintenance of chromosome) proteins and ATP in reconfiguring the path of DNA. The first assay evaluated the topology of knots formed in the presence of topoisomerase II. Unexpectedly, both wild-type Smc2/4 and an ATPase mutant promoted (+) chiral knotting of nicked plasmids, revealing that ATP hydrolysis and the non-SMC condensins are not required to compact DNA chirally. The second assay measured Smc2/4-dependent changes in linking number (Lk). Smc2/4 did not induce (+) supercoiling, but instead induced broadening of topoisomer distributions in a cooperative manner without altering Lk. To explain chiral knotting in substrates devoid of chiral supercoiling, we propose that Smc2/4 directs chiral DNA compaction by constraining the duplex to retrace its own path. In this highly cooperative process, both (+) and (−) loops are sequenced (about one per kb), leaving net writhes and twist unchanged while broadening Lk. We have developed a quantitative theory to account for these results. Additionally, we have shown at higher molar stoichiometries that Smc2/4 prevents relaxation by topoisomerase I and nick closure by DNA ligase, indicating that Smc2/4 can saturate DNA. By electron microscopy of Smc2/4-DNA complexes, we observed primarily two protein-laden bound species: long flexible filaments and uniform rings or “doughnuts.” Close packing of Smc2/4 on DNA explains the substrate protection we observed. Our results support the hypothesis that SMC proteins bind multiple DNA duplexes.

SMC (structural maintenance of chromosome) proteins are the central components of several multiprotein complexes that help to organize chromosomes throughout the cell cycle (1–5). Understanding the SMC proteins may illuminate their essential function in cohesion, condensin, “compensin,” repair complexes containing Smc5/6, and recombination complexes (6–10). Disruption of SMC function in prokaryotes and eukaryotes alike leads to chromosome instability, defects in repair and recombination, and death under conditions of rapid growth (11–15). The budding yeast Smc2 and Smc4 proteins form a heterodimeric ATPase (16) that, in combination with the non-SMC proteins Brn1, Ycg1, and Ycs4, comprises the holo-condensin enzyme. Holo-condensin drives global chromosome condensation at mitosis (4, 17, 18) and specific condensation of the rDNA locus at anaphase (19). The Xenopus 8 S condensin FF and the Schizosaccharomyces pombe Cut3/14 complex are examples of other stable condensin SMC pairs whose properties have been studied in some detail (20–23).

The SMC proteins are large (~150 kDa) and contain five structural motifs that include N- and C-terminal globular domains, a central hinge domain (H), and two long α-helices (αN and αC) as follows: N-αN-H-αC-C. Coiled-coil pairing of αN and αC folds a single SMC domain symmetrically, placing the hinge at one end and the co-folded N- and C-terminal SMC “head” domains at the other (24, 25). These flexible ~45-nm SMC rod structures assemble into pairs and appear throughout nature as homodimers in prokaryotes and heterodimers in eukaryotes (26, 27). The N/C-terminal head domains dimerize within pairs when ATP binds to form the bipartite ATPEase active site of the enzyme (28, 29). Nucleotide binding does not, however, lead to stable SMC pairing, as some hinge mutants and hingeless variants of the Bacillus subtilis SMC protein remain monomeric even in the presence of ATP (24). More stable association of the SMC head domains is thought to require the non-SMC subunit known as kleisin (Brn1 in Saccharomyces), which interacts with both heads and completes the tripartite SMC ring (30, 31). The space bounded by the coiled-coils and kleisin is in theory large enough to hold multiple DNA duplexes (25, 32, 33).

As members of the ATP-binding cassette family of ATPases, all SMC proteins bear the signature sequence -LSGGK-, in addition to the Walker A and Walker B nucleotide-binding motifs (34–37). ATP binding and hydrolysis by the SMC proteins are essential for yeast viability, and mutation of the phosphate-binding loop or ATP-binding cassette signature motif of a single protomer of an SMC pair prevents hydrolysis by the wild-type partner (16). The essentiality of ATP for condensin function is clear, but its precise role in the rearrangement of DNA is not. In vitro studies of DNA reconfiguration by condensin were first performed with affinity-purified 13 S holo-condensin and the 8 S SMC heterodimer isolated from mitotic and interphase Xenopus egg extracts (38–40). These early studies revealed two intriguing ATP-dependent condensin activities: DNA (+) knotting and (+) supercoiling in the presence of topoisomerases. Both the interphase and, to a much greater degree, the mitotic 13 S holoenzymes promote topoisomerase (topo) II-dependent (+) chiral knotting of nicked plasmids. However, only the mitotic condensin fosters topo I-dependent (+) supercoiling of relaxed plasmids. The Xenopus 8 S condensin lacks both activities. A complex from Caenorhabditis elegans containing the Smc4 homolog SMC-4 paired with SMC homolog MIX-1 or DPY-27 was also found to intro-

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3 The abbreviations used are: topo, topoisomerase; Lk, linking number; ATPγS, adenosine 5′-[(γ-thio)triphasate]phosphate; AMPPCP, adenosine 5′-[(β,γ-methylene)triphosphate]phosphate; AMPPNP, adenosine 5′-[(β,γ-imido)triphosphate]phosphate; EM, electron microscopy.
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duce (+) supercoils, although knotting was not tested (41). The reciprocal situation exists for the human condensin (chromosome-associated proteins hCAP-C, -D2, -E, -G, and -H), which promotes knotting of nicked plasmids, but whose supercoiling activity remains untested (42). The interpretation is that condensin stabilizes the global positive writhe of DNA, leading to the formation of (+) chiral knots by topo II. In contrast to the inactivity of the Xenopus 8 S condensin, yeast Smc2/4 is able to promote topo II-dependent knotting of nicked plasmids, but does not induce net supercoiling (16).

In this study, we examine directly the topology of knots produced by Smc2/4. We found that knots formed with the wild-type enzyme and an ATP binding/hydrolysis-deficient mutant enzyme were predominantly (+) in sign. Smc2/4 binding did not stabilize (+) supercoils, but caused a marked increase in the variance of topoisomer distributions \( (\sigma^2) \). We developed a model to explain these results. We show that chiral knots can be formed in substrates with minimum writhe when a DNA duplex is constrained to retrace its own path. Chiral geometries so formed can strongly bias the sign of knots formed without changing the average linking number \( (\ell_k) \) upon relaxation. We explain \( \ell_k \) broadening by is constrained to retrace its own path. Chiral geometries so formed can strongly bias the sign of knots formed without changing the average linking number \( (\ell_k) \) upon relaxation. We explain \( \ell_k \) broadening by

**EXPERIMENTAL PROCEDURES**

**Protein Purification and Antibody Generation**—The expression and purification of *Saccharomyces cerevisiae* wild-type Smc2/4 and the ATPase double mutant Smc2-P/4-P were performed as described previously (16). The purity of enzymes used in this study was > 95% as judged by Coomassie Blue and silver staining of proteins separated by SDS-PAGE (supplemental Fig. S1). Topoisomerase I was purified from raw wheat germ (43). Phage T4 topo II was generously provided by Wai-Mun Huang (University of Utah). Anti-Smc2 antiserum was raised in rabbits immunized against highly purified recombinant Smc2, additionally separated, and excised from SDS-polyacrylamide gels. Total IgG was purified by protein A affinity chromatography, and the Smc2-specific IgG fraction was isolated by elution from immobilized Smc2. Antibodies were dialyzed against 10 mM Tris-Cl (pH 7.9), 5% glycerol, 75 mM KCl, and 0.2 mM EDTA and stored at \(-80^\circ\)C.

**DNA Supercoiling and Knotting Reactions**—Measurements of DNA supercoiling were made with the 3-kb plasmid pBluescript\textsuperscript{TM} SK(+) (Stratagene) or the easily nicked 3-kb derivative pJES131, which bears a BbvCI site in the pBluescript\textsuperscript{TM} SK(+) multiple cloning site. Nicks generated with N.BbvCI-A or N.BbvCI-B (New England Biolabs Inc.) were efficiently sealed with T4 or *Escherichia coli* DNA ligase. To eliminate all (+) supercoiled DNA, which would have biased knot chirality or been a source of error in LK analysis by nick closure, it was necessary to use nicking conditions that generated some linear DNA. Reactions (25 \( \mu \)l) were assembled on ice in 10 mM Na-HEPES (pH 7.9); 75 mM KCl; 5% glycerol; 0.2 mM EDTA; 0.2 mM EGTA; 1 mM dithiothreitol; 3 mM Mg(OAc)\textsubscript{2}; 100 \( \mu \)g/ml bovine serum albumin; or 0.1 mM ATP, ADP, ATP\( \gamma \)S, AMPPCP, or AMPPNP; and 10 ng (0.2 nM) of substrate plasmid. Smc2/4 (0–512 nM) was added next, and the reactions were incubated for 30 min at 30°C. DNA substrates were relaxed or ligated by adding 20 ng of wheat germ topo I for relaxed plasmids or 0.4 units of T4 DNA ligase or 0.1 unit of *E. coli* DNA ligase for nicked plasmids and incubated for 30 min at 30°C. For ligation with *E. coli* DNA ligase, 100 \( \mu \)M NAD\textsuperscript{+} was added at time 0. Reactions were quenched by adding 25 \( \mu \)l of 50 mM Tris-Cl (pH 7.3), 10 mM EDTA, 1% SDS, 10 \( \mu \)g/ml yeast tRNA, and 100 \( \mu \)g/ml proteinase K and digested for 30 min at 37°C. DNA was extracted with phenol/chloroform and recovered by ethanol precipitation. Resolved topoisomers were analyzed by Southern blotting. For preparative scale knotting, reactions were performed in 5 \( \times \) 300-\( \mu \)l volumes containing 200 ng (2.6 nM) of a 7.2-kb plasmid singly nicked with DNase I in the presence of ethidium bromide. To this was added a 64-fold molar excess (170 nM) of wild-type or ATPase mutant Smc2/4, and reactions were incubated for 30 min at 25°C. Bacteriophage T4 topo II (20 ng) was then added, and incubation was continued for 15 min at 30°C. Reactions were quenched and processed as described for supercoiling assays. Recovered DNA was separated on Tris acetate/EDTA (TAE) 0.8% agarose gels (see Fig. 4, A and B) or in TAE containing 0.03% SDS (see Fig. 4C) as described (44). DNA was detected by ethidium bromide staining or by Southern blotting. Knoted DNA for electron microscopy (EM) analysis was purified from agarose gels as described (45).

**Analysis of LK Broadening— Autoradiographic analyses were analyzed using a STORM\textsuperscript{TM} PhosphorImager (Amersham Biosciences). Measured topoisomer intensities were fit to gaussian distributions using MicroCal Origin\textsuperscript{TM} 3.78 software by nonlinear least-square minimization: \( y = (A/(2/\pi))^{1/2}/w) \exp\{-(2(x-xc)^2)/w^2\} + y_o \), where \( y \) is the band intensity, \( A \) is the area under the curve, \( w \) is the distribution width, \( x \) is the band position (\(-2, -1, 0, +1, +2, \text{etc.}\)), \( xc \) is the distribution center, and \( y_o \) is the base-line offset term for band intensity. The variance of the topoisomer distribution \( (\sigma^2) \) equals \( w^2/4 \).

**EM Analysis of Knot DNA Chirality**—Knotted DNA was prepared for EM to visualize DNA crossings as described previously (45). Briefly, purified nicked DNA knots were denatured with glyoxal, coated with RecA protein in the presence of ATP, fixed with 0.2% glutaraldehyde for 15 min, and purified by size exclusion over Sepharose CL-4B. RecA-coated DNA was deposited onto glow-discharged, carbon-coated Formvar grids (Ted Pella, Inc.) and stained for 30 s in 2% uranyl acetate prior to rotary and directional shadowing with tungsten. Knots were photographed at a magnification of \( \times50,000 \), and the topological sign of the crossings was determined.

**EM of Smc2/4-DNA Complexes**—Smc2/4 (15, 50, or 300 nM) was incubated in the presence of 2.7-kb relaxed, 7.2-kb nicked circular, or 7-kb linearized DNA (0.5 nM). Reactions (30 \( \mu \)l) contained 10 mM Na-HEPES (pH 7.6), 50 mM NaCl, 5 mM MgCl\textsubscript{2}, 1.5 mM ATP, 1 mM dithiothreitol, and 10% glycerol. After 30 min at 25°C, glutaraldehyde was added to a final concentration of 0.2%. Cross-linking was quenched after 15 min by the addition of 4 \( \mu \)l of 50 mM glycine in 10 mM Na-HEPES (pH 7.6). Smc2/4-DNA complexes were separated from unbound proteins by Sepharose CL-2B chromatography, applied to grids, and processed as described above.

**RESULTS**

**DNA Knotting Is Promoted by Smc2/4 and the ATPase Mutant Smc2/4-Dependent knotting of circular DNA, indicating DNA compaction** (16). However, we could not determine whether this activity was ATP-dependent because the type II topoisomerases also required ATP. Here, we tested the ATPase double mutant Smc2-P/4-P (16), in which the sequence GKS in the phosphate-binding loop of each SMC protein was mutated to AAA, inactivating ATP hydrolysis. In UV cross-linking studies, we saw relatively weak cross-linking of ATP to the wild-type enzyme and reproducibly diminished cross-linking to the P-loop.
mutant enzymes. High background labeling, possibly due to ATP binding elsewhere in the enzymes, prevented us from concluding that we had ablated ATP binding in the active site of the mutant enzyme. Although the double P-loop mutant is incapable of hydrolyzing ATP, it still promoted knotting much like the wild-type enzyme (Fig. 1, A and B). The yield of knots in replicate experiments with each enzyme was variable. For example, Fig. 1C shows a highly efficient knotting reaction in which Smc2-P/4-P directed the conversion of nearly 50% of the substrate to knots. This inter- and intra-assay variability makes it impossible to conclude that ATP hydrolysis stimulates knotting. As with the Xenopus holo-condensin, Smc2/4 promoted the creation of far fewer four-noded knots than trefoil knots and favored the formation of five-noded knots over four-noded knots (Fig. 1C). This is most easily judged by comparing the distribution in Fig. 1C with the twist knot ladder, for which the abundance of knots diminishes exponentially with each additional crossing (46). The knotting pattern is fully consistent with our model for chiral structures configured by Smc2/4, as explained under “Discussion.” Our results show that DNA binding alone, without the requirement for ATP hydrolysis, is sufficient for Smc2/4 to fold DNA and promote knotting.

**Chiral Knotting Does Not Require ATP Hydrolysis by Smc2/4**—The type and chirality of knots are reporters of the conformation that DNA molecules are in at the time of topo II action. We determined the topology of DNA knots formed by T4 topo II in the presence of Smc2/4 and Smc2-P/4-P by EM. We examined 77 knots produced using the wild-type enzyme and 130 knots produced with the ATPase mutant enzyme. The type and chirality of knots are reporters of the conformation that DNA molecules are in at the time of topo II action. We determined the topology of DNA knots formed by T4 topo II in the presence of Smc2/4 and Smc2-P/4-P by EM. We examined 77 knots produced using the wild-type enzyme and 130 knots produced with the ATPase mutant enzyme.
Examples of these knots are presented in Fig. 1D. A summary of the topology and type of knots identified is presented in TABLE ONE. For the wild-type enzyme, 89% of the three-noded (trefoil) knots scored (+/−H11001). This is comparable with the percentage of (+/−H11001) trefoils (95%) seen with the mitotic *Xenopus* condensin (40). Trefoils formed with the mutant enzyme were also predominantly positive (72%). Statistical analysis suggests that the wild-type and mutant distributions are highly significantly different from random (p < 10−5) and significantly different from one another (p = 0.012). We conclude that, although there is no absolute ATP requirement for (+) chiral knotting, nucleotide hydrolysis may aid Smc2/4 in chirally reconfiguring DNA. Surprisingly, chiral knotting by Smc2/4 did not require the non-SMC condensin proteins or mitotic modification.

Knots containing more than three nodes also exhibited a bias toward (+) crossings. For example, all 10 of the five-noded torus knots obtained with wild-type and mutant Smc2/4 were (+) (Fig. 1D, panels S and 6). The simple knots, like the trefoil and four-noded knot, or twist knots containing multiple crossings, like those in Fig. 1D (panels 2–4) with five, six, and seven nodes each, can be formed by a single duplex passage. More complex twist knots and higher order torus knots require multiple, sequential transport of one DNA segment through another. For example, the granny knot requires two duplex passages, and the five- and seven-noded torus knots require two and three, respectively (Fig. 1D, panels 5–8) (47). In our reactions, topo II catalyzed multiple passage events and, like the *Xenopus* condensin, resulted in the Smc2/4-dependent formation of largely (+) complex knots.

**FIGURE 2.** Smc2/4 induces Lk broadening and protects DNA from relaxation by topo I at high molar ratios. A, increasing molar ratios of wild-type Smc2/4 were incubated with 10 ng of 2.7-kb relaxed plasmid DNA and 1.5 mM ATP for 30 min at 25 °C. Wheat germ (WG) topo I was added, and the incubation was continued for 25 min. Product topoisomers were resolved on a 1% agarose gel run in TAE containing 0.3 µg/ml chloroquine, in which the most positive topoisomers in the Lk distribution migrate the fastest. Topoisomers of 5.4-kb plasmid dimers are also visible. The bands were detected by Southern hybridization and quantified with a Storm PhosphorImager. An autoradiograph of the gel is shown. NC, nicked circular DNA; Lin, linear DNA; (+−SC), (+−) supercoiled. B, the center and variance (σ²) of the topoisomer distributions shown in A were determined by least-squares fitting to a gaussian distribution. Fits (solid lines) were normalized by area. The calculated variance and Smc2/4:plasmid ratios are listed to the left and right, respectively, of each curve. The dotted line indicates Lk₀ for plasmids relaxed in the absence of Smc2/4 (A, lane 6), and the dashed line indicates Lk₀ for the unreacted starting substrate (A, lanes 5 and 16). C, the variance of each distribution was plotted against the log₂ of the Smc2/4:plasmid ratios. Inhibition of topo I relaxation occurred at ratios >128:1. D, Lk₀ calculated for each distribution was plotted against the log₂ of Smc2/4:plasmid ratios, and the data were fit to a sigmoid function.
Effect of Smc2/4 on Lk Distributions—The generation of (+) knots by Smc2/4 led us to expect that supercoiling assays would reflect this chiral preference as well. Supercoiling by Smc2/4 was analyzed quantitatively with relaxed DNA, increasing molar equivalents of Smc2/4, and wheat germ topo I under conditions identical to those used for knotting (Fig. 2A). We obtained three significant results. First, Smc2/4 caused the width of topoisomer distributions (the variance, \( \sigma^2 \)) to increase without the net accumulation of (+) or (−) supercoils (Fig. 2A, lanes 8–11). The latter result had been observed previously (16). This lack of effect on Lk was initially puzzling in light of our (+) knotting results; however, the increase in Lk variance suggested an explanation. The spread in topoisomers arises naturally from random thermal fluctuations in DNA writhe and twist. To quantify changes in this base-line width, band intensity data from Fig. 2A were fit to gaussian distributions to obtain the variance for each distribution. The fitted data are presented in Fig. 2B. Maximum broadening occurred at a protein:DNA ratio of 128:1: a 1.7-fold increase in \( \sigma^2 \) compared with DNA relaxed in the absence of protein (3.2 versus 1.9). The apparent negative shift in Lk at ratios above 256:1 is not due to the action of Smc2/4, but rather the inability of topo I to relax the starting substrate (see below).

The second significant result was the aforementioned lack of shift in Lk at ratios above 256:1. Despite the marked increases in variance at high protein ratios (Fig. 2B, C), the fitted center of distributions remained unchanged (Fig. 2D). This constancy of Lk suggests that there is no net change in writhe
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FIGURE 4. Lk broadening is efficiently induced by the ATPase double mutant enzyme Smc2-P/4-P. A, 3-kb singly nicked plasmid DNA (10 ng) was incubated for 30 min at 30 °C with increasing molar ratios of the ATPase mutant Smc2-P/4-P or wild-type Smc2/4 in reactions containing 1 mM ATP or the nucleotide analogs listed. E. coli DNA ligase was added, and the incubation was continued for 15 min. Ligation products were resolved on 1.4% agarose gels run in TAE containing 0.9 μg/ml chloroquine. ADP·PC, AMPPCP; ADP·NP, AMPPNP. B, topoisomer band intensities (points) were fit (solid lines) to a sigmoid function. The squares depict the Lk variance obtained for wild-type Smc2/4 at a ratio of 256:1 in the presence of ATP or the nucleotide analogs listed in A for lanes 15-20 (white bars) compared with starting substrate in lane 1 (shaded bar). D, Lk0 was determined for Smc2/4 (circles) or Smc2-P/4-P (squares) at the various protein:DNA ratios tested.

A

Smc2P/4P

Smc2/4

ATP: ++++++++ ++++++++ ADP

2/4 plasmid: 32 64 128 256 512

Gaussian fits of Smc2P/4P

C

Smc2P/4P or 2P/4P : plasmid

D

Smc2P/4P or 2P/4P : plasmid

or twist in DNA circles bound by Smc2/4. However, the existence of knotting and Lk broadening shows that the lack of change was not due to the absence of DNA binding or compaction by Smc2/4.

Third, as mentioned, we detected an Smc2/4-dependent inhibition of topo I relaxation at or above a ratio of 256:1 (Fig. 2A, lanes 12–14). This was easy to detect because Lk0 for the starting substrate was shifted by approximately one (~) topoisomer relative to the relaxed products (Fig. 2A, lane 6 versus lane 5, 15, or 16). Inhibition was total at the highest ratios (Fig. 2D). Relaxation by vaccinia virus topo I, an enzyme one-third the mass of the wheat germ topo I, was also inhibited (data not shown). The impenetrable quality of Smc2/4-DNA complexes emerged when the molar excess of Smc2/4 to DNA approached one Smc2/4 complex per ~30 bp. We suggest that substrate protection signals a binding change in which condensed DNA becomes saturated by Smc2/4.

Neither Smc2/4 nor the ATPase Mutant Smc2-P/4-P Shifts Lk0 upon Ligation ofNicked Plasmids—To confirm the unexpected increase in topoisomerase variance, the lack of shift in Lk, and the occlusion of topo I, we measured Smc2/4 supercoiling by a complementary and more quantitative method: nick ligation. In this assay, a singly nicked plasmid was ligated in the presence of Smc2/4 using either T4 (Fig. 3) or E. coli DNA ligase. The chief advantage of this method over topo I assays is that relaxation cannot be incomplete, and any unligated DNA is easily resolved from the relaxed topoisomer distribution. The results of a typical assay and the corresponding Gaussian fits are presented in Fig. 3 (A and B, respectively). Changes in σ2 could be detected at ratios as low as 16:1. In this particular experiment, the variance increased nearly 3-fold between 8:1 and 32:1 (Fig. 3A, lanes 4 and 5) and became maximum at higher stoichiometries (Fig. 3C). In six independent assays, Lk broadening rose sharply between a 16- and 64-fold molar excess of Smc2/4 to plasmid. The maximum Lk broadening was 5.5 ± 0.6 topoisomers: more than three times the σ2 we found for naked DNA (1.4 ± 0.16) (Fig. 3D). Our value for naked DNA is in close agreement with previous estimates (48).

The double mutant Smc2-P/4-P also induced Lk broadening (Fig. 4, A–C). Broadening was likewise observed in the presence of the wild-type enzyme without added nucleotide or in the presence of ADP, ATP·γS, AMPPCP, or AMPPNP (Fig. 4, A, lanes 16–20; and C, inset). These results show that topoisomerase broadening requires only the ATP-independent binding of Smc2/4 to DNA.

To rule out the possibility that these effects were promoted by a contaminant common to both the wild-type and mutant enzyme preparations, Lk broadening was inhibited with highly purified anti-Smc2 IgG. Supplemental Fig. S2 (A and B) shows that Lk broadening was reduced to near background levels (σ2 ~ 1.3) by the addition of total or affinity-purified anti-Smc2 IgG. A slight decrease was observed with preimmune IgG at the highest ratio and is likely due to cross-reactive
IgG. The neutralizing potential of affinity-purified anti-Smc2 IgG strongly suggests that topoisomer broadening (and by extension, DNA knotting) is promoted specifically by Smc2/4 and not a contaminant. As with topo I, Lk broadening captured by nick ligation occurred without shifting Lk0 (Figs. 3E and 4D). Substrate protection was seen with the wild-type and mutant enzymes as well. This is indicated by the presence of unligated DNA in Figs. 3A and 4A (lanes 7–9). Inhibition of ligation appeared suddenly and progressed to near completion at ratios coincident with those that blocked relaxation by topo I (Fig. 3F). Blocking the activity of four dissimilar enzymes (wheat germ and vaccinia virus type I topoisomerases and E. coli and T4 DNA ligases) suggests that Smc2/4 acts to protect DNA from modification rather than inhibiting enzymes directly.

EM of Smc2/4-DNA Complexes Reveals Compact Structures and Coated Filaments—To investigate the nature of Smc2/4-DNA interactions further, we analyzed the products of DNA binding reactions by EM. Relaxed, nicked, and linear DNAs were incubated with a 30-, 100-, or 600-fold molar excess of Smc2/4 under conditions used for knotting and supercoiling, except that bovine serum albumin was omitted. Samples were cross-linked with glutaraldehyde, and free protein was separated from free DNA and Smc2/4-bound DNA by gel filtration. The DNA containing fraction was applied to EM grids, negatively stained, and shadowed with tungsten. We observed two types of structures: rings or “doughnuts” and long flexible filaments. Neither was seen if DNA was left out of the binding reactions. Doughnuts were observed 25–50 times more often than filaments. Unlike filaments, which assem-
bled only in the presence of linear DNA, doughnuts were recovered when either circular or linear DNA was used (Fig. 5A). The dimensions of the doughnuts were remarkably uniform (Fig. 5B and supplemental Table S2), irrespective of the length or topologic form of the DNA or the concentration of Smc2/4 in the binding reactions. This might be explained if the overall shape of doughnuts is determined primarily by interactions between Smc2/4 bound to the DNA. The outer diameter of the doughnuts was ~120 nm, and the wall thickness was ~35 nm (Fig.

**FIGURE 6.** Chiral knot formation through near zero writhe/twist intermediates. A, a two-loop overlay model is shown in which loops of opposite sign are placed one on top of the other in either a left-over-right (L/R)-handed or right-over-left (R/L)-handed manner. Strand passage at node II generates a trefoil knot, and passage at any other node returns a circle. B, the overlay between two loops is topologically equivalent to the simplified standard geometry. By migrating the crossings at the base of the loops, one can smoothly deform the starting geometry into the simpler representation that is independent of the sign of the migrated nodes. C, shown is the standard geometry held in the left/right or right/left configuration. When the net sign of crossings within the DNA region between the crossings (I–VI) is zero as shown, only (+) trefoils are formed from the left/right geometry, and only (−) trefoils are formed from the right/left geometry. D, the ribbon diagram shows two chiral geometries with near zero writhe and zero change in twist. E, shown is the conversion of the R/L configuration of the standard geometry to a (+) trefoil knot following strand passage at node II.

**FIGURE 7.** Models for DNA binding and reconfiguration by Smc2/4. A: the sequential capture of two DNA duplexes (black helices) by Smc2/4 (blue and green molecules) between the coiled coils by passage through separated head domains. Stable binding or “nucleation” occurs when a sufficient number of Smc2/4 dimers assemble on the DNA. Once established, cooperative spreading of Smc2/4 can occur bidirectionally. B: upper, potential schemes for two-duplex capture by Smc2/4. (Proteins were omitted for clarity.) The left scheme (Model I) shows binding at a single site and the spread of Smc2/4 without trapping crossings in the core of the structure. The center scheme (Model II) depicts binding that also begins at one site but that traps plectonemic crossings at random as protein loads. The right scheme (Model III) shows the establishment of multiple binding sites. As proteins load, they cause DNA to be looped out as a result of capturing different lengths of DNA between sites of binding initiation. Lower, models for resulting Smc2/4-DNA complexes with expected broadening and percentage of (+) trefoil knots indicated. C, model showing the sequestration of a free supercoil (or bulge) by asymmetric duplex capture. Loops or bulges would be extruded as a natural consequence of collision between advancing Smc2/4 fronts.
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In this work, we have demonstrated that, in the absence of accessory proteins, the yeast Smc2/4 complex shapes the path of DNA. This is an important step in the functional dissection of an essential SMC-containing complex. Elucidating the activities of the Smc2/4 complex provides the groundwork for understanding how the non-SMC components may modulate the activity of the condensin holoenzyme. We have shown that Smc2/4 binding directs the formation of (+) chiral knots in nicked DNA substrates when topo II is present, independent of ATP hydrolysis (Fig. 1). This was not, however, accompanied by the accumulation of (+) supercoiling observed with the Xenopus 13 S condensin. Instead, binding of Smc2/4 to nicked and relaxed plasmids led to the broadening of topoisomer distributions without changing Lk0 (Figs. 2–4). From this we infer that Smc2/4 introduces an unbiased accumulation of (+) and (−) DNA supercoils when it binds. We present below a model to explain chiral knotting in the absence of net supercoiling that invokes the cooperative manner in which Smc2/4 binds DNA. Using EM, we have directly shown that Smc2/4 can coat DNA cooperatively, as evidenced by the assembly of some DNA into doughnuts (Fig. 5) and filaments (Fig. 5 and supplemental Fig. S5) while other DNA molecules remain naked. This effect was seen at an Smc2/4:DNA ratio of 100:1, well below the ratio that occludes substrate (Figs. 2–4). Condensins cluster at chromosomal foci in vivo in both eukaryotes and prokaryotes such that the local density must be very high (41, 49–53). Our results are consistent with such high density condensin packing. We anticipate that the non-SMC condensin subunits modulate Smc2/4-dependent DNA manipulation in a holoenzyme-specific fashion. Our initial results indicate that the yeast non-SMC condensin subunits do not affect the level of knotting, but do influence supercoiling.4

Explaining Chiral Knotting in the Absence of Chiral Supercoiling—Efficient knotting of DNA requires stabilization of crossings in circular substrates, followed by topo II-mediated passage of one DNA duplex through another. To create the three-noded trefoil knot, three crossings must be stabilized, and duplex passage at the intersection of segments must leave three crossings of the same sign: (+) or (−). The easiest way for a protein to accomplish this feat is to stabilize supercoils of the same sign. This is the model for (+) supercoiling and formation of (+) DNA knots by the Xenopus 13 S condensin (40) and the condensins from C. elegans and humans (41, 54). It cannot, however, be the correct model for (+) knotting by the yeast condensin SMC proteins because Lk0 remains unchanged at all protein ratios tested. If an equivalent number of (+) and (−) supercoils are stabilized by Smc2/4, then, in the absence of further directing influences, knots formed by topo II must also be equally (+) and (−). How does a (+) chiral bias insert itself into Smc2/4?

4 J. E. Stray, J. E. Lindsley, and N. R. Cozzarelli, unpublished data.
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4-directed DNA knotting? We find a solution by postulating that crossings stabilized by Smc2/4 distribute equally between (+) and (−), but that the protein-DNA complex itself adopts a (+) chiral geometry, which places bound DNA and sequestered loops into a unique spatial arrangement.

Although a bit counterintuitive, we found it is indeed possible to obtain such superhelical relationships without introducing appreciable net writhe. This is required because ΔLk in our experiments was always zero. Fig. 6A outlines how this is possible using a presentation similar to the global writhe model of Kimura et al. (40), but for which the supercoils have opposite sign and cancel. Inversion of crossings at nodes I, III, and IV has no effect on topology. However, inverting the crossing at node II forms a trefoil knot whose sign is dependent on the overlay handedness of the loops (supplemental Fig. S3). Fig. 6A illustrates clearly how loop overlay can determine knot chirality in substrates having loops that cancel. But for further exposition of the model, it is convenient to present the DNA in a more simplified “standard geometry,” the path to which is shown in Fig. 6B. Fig. 6C shows the symmetry of the left- and right-handed conformations of the standard geometry. Nodes I–IV remain topologically equivalent in all figures (supplemental Fig. S3). Fig. 6E illustrates how the standard geometry converts to a (+) trefoil knot following sign reversal at node II.

The standard geometry illustrates two important concepts key to the loop overlay model: that overlay handedness determines the sign of the resultant trefoil knots and that writhe in chiral structures can be very close to zero. Writhe approaches exactly zero when a closed curve is planar or when it exactly retraces its own path, as illustrated in Fig. 6D (55). The (+) writhe that develops as the ribbon rises above the plane is exactly cancelled by the (−) writhe gained on its return. We readily see for the ribbons in Fig. 6D that their net writhe is nearly zero, that their change in twist is zero, and that both are chiral.

Model for DNA Binding and Reconfiguration by Smc2/4—How could the condensin SMC proteins stabilize such a DNA structure? One possibility is that when Smc2/4 binds, it sequesters not just one DNA segment but pairs of duplexes (Fig. 7A), thereby constraining circular DNA to retrace its own path. This maintains writhe neutrality as the DNA is folded. When DNA retraces its own path, writhe changes that may accumulate in the protein-constrained portion of the molecule do not produce extra writhe or twist in the protein-free portion of the DNA. Thus, there would be no shift in Lk upon relaxation with topo I or ligation of a nick (56).

There are additional topological data that we now incorporate into this working model: broadening of topoisomer distributions. As we argue below, broadening most likely results from the random trapping of (+) and (−) supercoils. We depict in Fig. 7A DNA binding by duplex capture between the coiled-coils of an SMC pair. This mode of binding has been suggested for cohesin and other SMC-containing complexes (14, 16, 33, 57, 58). We suggest that protein-DNA and overriding protein-protein interactions provide the ternary stability necessary to induce chiral helicity and so create handedness. Fig. 7B (upper) shows three ways that protein binding could initiate the capture of two DNA duplexes at once. Each leads to a hypothetical end point that traps no supercoils (left), only plectonemes (middle) or purely solenoidal supercoils (right). If no supercoils are trapped (Model I), there will be chiral knots but no Lk broadening. Trapping of only plectonemic supercoils (Model II) results in Lk broadening, but rapidly degrades the expected chiral bias in knotted products (see supplemental “Experimental Procedures”). Even a single plectonemic interwind causes the fraction of (+) trefoils expected for right-handed overlays to fall from 100 to 67% (TABLE TWO, Part C). Our broadening data indicate the random trapping of between four and five supercoils per bound plasmid (see below), which would degrade the expected bias even further to 56%. The lack of plectonemic supercoils is further supported by the fact that so few four-noded knots were observed (Fig. 1C). For the substrates shown in TABLE TWO (Part A), we note that the number of (+) trefoils begins to equal the number of four-noded knots when more than one plectonemic superturn is introduced.

An alternative explanation for the observed broadening could be protein-induced random bending of the DNA. Bends introduced at random decrease the apparent persistence length of DNA, which consequently increases the variance in writhe (59) and hence the breadth of topoisomer distributions. However, it would not lead to chiral knotting.

Model III depicts Smc2/4 bound to plasmid in a manner that is most consistent with our data. Solenoidal windings do not affect knot chirality and thus do not degrade knotting bias, which makes this the more plausible model for the DNA conformation held by Smc2/4. The solenoidal path includes multiple points of binding initiation. This would lead to the extrusion of (+) or (−) loops or bulges in the DNA and could explain the trapping of solenoidal loops by protein binding alone. Fig. 7C depicts how the longer of two DNA segments is naturallylooped out as protein binding saturates and competing fronts collide. This model was developed to explain chiral knotting without Lk changes. It is mindful of the fact that any route to compact DNA geometries must 1) lead to chiral knotting, 2) not bias the net writhe or change in twist in the substrate, 3) lead to the broadening of topoisomer distributions and, 4) saturate DNA to a density of about one Smc2/4 heterodimer per 100 bp.

The chiral overlap Models II and III can be easily modified to explain the behavior of the Xenopus 13 S condensin (38, 40). By sequestering only (+) supercoils, plectonemic or solenoidal, both (+) knotting and (+) supercoiling are predicted. This satisfying aspect of the model leaves open the possibility that, in the presence of the non-SMC proteins, the yeast condensin may also support (+) supercoiling. However, our preliminary results have yet to show this.

The topology, chirality, and relative abundance of more complicated knots formed with Smc2/4 fit several predictions of our model as well. First, the scarcity of four-noded knots relative to trefoils (Fig. 1 (A–C) and TABLE ONE) is predicted by the stabilization of solenoidal supercoils in substrates lacking plectonemic interwinds (TABLE TWO, Part A). Using the standard geometry, a four-noded knot can be formed by a single duplex passage in plectonemically interwound substrates (TABLE TWO, Part A). Trefoil knots can be formed in substrates lacking plectonemic crossings or loops and readily in substrates with solenoidal supercoils. Second, our model suggests an explanation for why all of the five-noded torus knots were (+) (TABLE ONE) and why there were more five-noded knots than four-noded knots. These more complex knots form mostly by a second duplex passage in an already formed knot, rather than by one passage in structures with plectonemic crossings in the core, as this would neutralize the observed chiral bias. Our model predicts that the (+) pentafoil or (+) five-noded torus will be the most abundant knot formed if a second passage is applied to a (+) trefoil that has only one or no plectonemic crossings. This is illustrated by supplemental Fig. S4, which shows that the (+) five-noded torus is the only knot created by reversal of any new nodes formed in loops previously targeted by topo II. Secondary sign reversal at the original nodes I–III unties the trefoil knot and returns a circle. Subsequent passage at node IV preserves the trefoil knot. Finally, because the trefoil is formed most often, it becomes the next most abundant substrate for topo II. This contributes further to the formation of five-noded knots in the presence of Smc2/4.
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**Interpreting Lk Broadening**—We can link changes in Lk variance to the number of bound crossings in the following way. For an unbiased random walk with a discrete step size, the total number of steps is equivalent to the variance in the positional distribution. By analogy, the number of crossings created upon protein binding is directly tied to the variance in topoisomer distributions. One DNA crossing stabilized by Smc2/4 loading is equivalent to one stepwise change in Lk. The additional Lk variance is thus equivalent to the total number of crossings of either sign trapped,

\[
N = \langle \sigma_0 \rangle^2 - \langle \sigma_0 \rangle^2 \tag{Eq. 1}
\]

where \(N\) is the number of crossings, \(\langle \sigma_0 \rangle^2\) is the variance of the topoisomer distribution in the presence of protein, and \(\langle \sigma_0 \rangle^2\) is the “base-line” variance due to thermal fluctuation of DNA held inside a protein-DNA complex. At one extreme, protein binding could completely dampen thermal fluctuations and reduce the intrinsic DNA variance, \(\langle \sigma_0 \rangle^2\), to zero. At the other extreme, protein binding could have no effect at all, in which case, \(\langle \sigma_0 \rangle^2\) would equal that of naked DNA: 1.4 ± 0.16 topoisomers (Fig. 3D). For \(\langle \sigma_0 \rangle^2\), we obtained a maximum broadening of 5.5 ± 0.6 topoisomers (Fig. 3D). Thus, the estimated number of extra crossings introduced by Smc2/4 binding falls between four and six. The extra crossings are not plectonemic, but are solenoidal (Models II and III, respectively) (Fig. 7) because solenoidal loops preserve the chiral knotting bias (TABLE TWO, Part C).

The Nature of DNA Binding and Substrate Protection by Smc2/4—In both our topological trapping assays and EM studies, we observed that Smc2/4-bound DNA in a cooperative manner at ~30–100 protein complexes per DNA in the reaction. At ratios above 256:1, Smc2/4 limited or blocked access of modifying enzymes to the DNA, suggesting saturation of the substrate. However, the effects we saw for Smc2/4 binding on DNA structure, topoisomer broadening, and knotting occurred before saturation of the DNA. The requirement for large ratios of protein to DNA in our experiments is not unique to the yeast Smc2/4 complex. Indeed, all condensin effects reported in vitro require a large number of proteins per DNA to be seen (38, 40–42, 53, 60). Why is so much condensin needed to reconfigure DNA? It does not seem that condensins from Xenopus or yeast load poorly because direct measures of protein binding in vitro show that ~50% of the enzyme is associated with DNA (16, 61). Although one study found that ATP-dependent supercoiling could be promoted by a single plasmid-bound condensin (62), this seems not to be the rule. The limiting step for measurable condensin effects appears to be the establishment or nucleation of DNA binding. Broadening and DNA binding are strongly correlated, and both are highly cooperative processes. Indeed, knotting, supercoiling, and DNA re-annealing activities have all been shown to correlate with DNA binding in gel shift assays (24, 38, 60, 63, 64).

Our expectation that Smc2/4-bound DNA would have a tightly packed arrangement was substantiated by the EM observations of protein-coated DNA doughnuts and filaments (Fig. 5 and supplemental Fig. S4). We conclude that doughnuts represent a compact form of DNA, as evidenced by the DNA exiting these structures (Fig. 5, A and C, arrows). Using filament packing as a guide, we estimate that one Smc2/4 heterodimer is bound for every 100 bp, in reasonable agreement with solution estimates for the Xenopus condensin, for which ~36 condensins bind to each 3-kb plasmid (39, 40).

Filaments were observed only in the presence of linear DNA. Some of the filaments were several times longer than the substrate DNA, which suggests that Smc2/4 mediates lateral coupling between linear duplexes. To accomplish this, Smc2/4 could either grasp more than one DNA duplex at a time or cause association of individually coated duplexes by protein-protein interaction. We do not know the architecture of the proteins, but favor the model diagramed in Fig. 5D (panel S), in which two DNAs are held together at a region of overlap concealed in the interior of a filament. This is most consistent with our DNA competition studies (16).

Although we observed complete DNA coating by Smc2/4 in our in vitro studies, it is unlikely to be the case in vivo, where the condensin:DNA ratio is lower and the DNA is bound by histones and a host of other proteins. Yet close packing of condensin complexes over short regions of DNA, perhaps involving nucleosomal linker DNA (58), could be enough to induce helical bending of the DNA locally and contribute to global chromosome compaction in vivo (41, 42, 50–53, 65).

It will also be important to explore the function of the non-SMC subunits alone, in combination with Smc2/4, and as part of the condensin holoenzyme. Our results shed new light on the DNA activity of the yeast Smc2/4 pair. Given their structural conservation, it is perhaps reasonable to expect a shared “general” mechanism for the Smc2/4, Smc1/3, and Smc5/6 pairs. The fact that the same SMC pair can assist with different sets of non-SMC proteins to assume different cellular roles, such as condensins I and II in Xenopus and humans and the Smc5/6 complexes (50, 66), suggests that a common mechanism may underlie SMC function. Our results demonstrate that the yeast condensin SMC pair has intrinsic DNA reconfiguring activity that is not entirely dependent on its ATPase activity. The same may hold for other SMC pairs.

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The *Saccharomyces cerevisiae* Smc2/4 Condensin Compacts DNA into (+) Chiral Structures without Net Supercoiling

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