Architecture of the Smc5/6 Complex of *Saccharomyces cerevisiae* Reveals a Unique Interaction between the Nse5-6 Subcomplex and the Hinge Regions of Smc5 and Smc6

Xinyuan Duan, Yan Yang, Yu-Hung Chen, Jacqueline Arenz, Gurish K. Rangi, Xiaolan Zhao, and Hong Ye

From the 4Brown Cancer Center, University of Louisville School of Medicine, Louisville, Kentucky 40202, the 5Molecular Biology Program, Memorial Sloan-Kettering Cancer Center, New York, New York 10065, and the 6Programs in Biochemistry, Cell, and Molecular Biology, Cornell University Graduate School of Medical Sciences, New York, New York 10021

The evolutionarily conserved structural maintenance of chromosome (SMC) proteins forms the core structures of three multimeric complexes as follows: cohesin, condensin, and the Smc5/6 complex. These complexes play crucial roles in different aspects of chromosomal organization, duplication, and segregation. Although the architectures of cohesin and condensin are better understood, that of the more recently identified Smc5/6 complex remains to be elucidated. We have previously shown that the Smc5/6 complex of *Saccharomyces cerevisiae* contains Smc5, Smc6, and six non-SMC elements (Nse1–6). In this study, we investigated the architecture of the budding yeast Smc5/6 complex employing the yeast two-hybrid assay as well as *in vitro* biochemical approaches using purified recombinant proteins. These analyses revealed that Smc5 and Smc6 associate with each other at their hinge regions and constitute the backbone of the complex, whereas the Nse1–6 subunits form three distinct subcomplexes/entities that interact with different regions of Smc5 and Smc6. The Nse1, -3, and -4 subunits form a stable subcomplex that binds to the head and the adjacent coiled-coil region of Smc5. Nse2 binds to the middle of the coiled-coil region of Smc5. Nse5 and Nse6 interact with each other and, as a heterodimer, bind to the hinge regions of Smc5 and Smc6. These findings provide new insights into the structures of the Smc5/6 complex and lay the foundation for further investigations into the mechanism of its functions.

In eukaryotic cells, the structural maintenance of chromosome (SMC)4 protein family contains six members, Smc1–6, and is an important group of proteins that can actively tether and fold DNA in different ways. All SMC proteins have modular structures consisting of two globular domains at the N and C termini, a middle hinge domain and two coiled-coil regions located between the hinge and the globular domains (1–3). Each SMC protein is believed to bind at its hinge domain such that the two coiled-coil domains associate with each other, resulting in a filamentous structure (called the arm). Bending at the hinge domain also leads to the association of the two globular domains to form the head domain that processes ATPase activity (1–3). SMC proteins function in three pairs, namely Smc1/Smc3, Smc2/Smc4, and Smc5/Smc6. Each pair of SMC proteins associate with each other and bind to a unique set of non-SMC proteins as follows: Smc1/Smc3 binds to Scc1 (Mcd1) and Scc3 to form cohesin; Smc2/Smc4 binds to Ycs4, Brn1, and Ycg1 to form condensin; and Smc5/Smc6 interacts with six recently identified proteins, Nse1–6 (non-SMC elements), to form the Smc5/6 complex (1–7).

These three SMC complexes play distinct roles in chromosomal organization and functions. A key feature of these complexes is that they act as protein linkers that support specific connections between chromatin and chromatids. Cohesin tethers sister chromatids together and functions in sister chromatid cohesion, double strand break repair, and transcriptional regulation (1–3). Condensin tethers different regions of the same chromatid and is required for chromosome compaction and single strand break repair (1–3). The action of the Smc5/6 complex is implicated in several chromosomal activities, including double strand break repair, replication fork restart, and telomere maintenance; however, the mechanisms underlying its actions are poorly understood (8–11). Although Smc5 and Smc6 share overall sequence homology with Smc1–4 and are thus believed to adopt similar structures, the Smc5/6 complex exhibits properties distinct from those of cohesin and condensin. The non-SMC subunits of cohesin and condensin are known to provide structural roles; however, the Smc5/6 complex contains a SUMO ligase subunit, Nse2 (also called Mms21), that facilitates the addition of the small protein modifier SUMO to specific target proteins, such as Yku70 of budding yeast and shelterin subunits in human cells (4, 12–14). The presence of such enzymatic activity, known to regulate proper-

Gal4 activation domain; GBD, Gal4 DNA-binding domain; SUMO, small ubiquitin-like modifier.
ties of its target proteins, suggests that the Smc5/6 complex, in addition to being a protein linker, could also function as a molecular switch. Furthermore, the sequences of the hinge domains of Smc5 and Smc6 are divergent from those of Smc1–4 (15, 16), suggesting that the architecture and functions of the Smc5/6 complex could be unique compared with the other SMC complexes.

Genetic studies in yeasts and human cells revealed that the roles of this complex are evolutionarily conserved; however, it is not clear how the complex carries out its diverse functions. To answer this question, it is critical to know the biochemical and biophysical properties of this complex. We have previously shown that the eight subunits of the Smc5/6 complex can be co-purified as a stable complex from budding yeast (4). Each of the eight subunits of the budding yeast Smc5/6 complex is required for viability, and mutants containing hypomorphic alleles exhibit similar defects, suggesting that they function together (4, 17–19). The Smc5/6 complex in fission yeast has also been shown to be composed of eight subunits (5, 7). Interestingly, although six of its subunits share sequence homology with their budding yeast counterparts, Nse5 and Nse6 do not; furthermore, unlike in budding yeast, fission yeast Nse5 and Nse6 are nonessential (5, 7). The variations between the Smc5/6 complex in the two yeasts are also reflected in their different chromosomal localization patterns, sumoylation targets, and stability of the complex (4, 5, 7, 14, 20, 21). These observations predict that the architecture and functions of the Smc5/6 complex in the two yeasts share significant similarities yet exhibit some divergence. An in-depth understanding of the functions of the Smc5/6 complex requires a detailed characterization of the complex in both yeasts. Although subunit interactions of the fission yeast Smc5/6 complex have been characterized, those of the budding yeast complex have not been examined.

Here we describe our study of the architecture of the budding yeast Smc5/6 complex. Using yeast two-hybrid assays, we systematically examined the pairwise interactions among the eight subunits of the Smc5/6 complex as well as the interactions between each NSE subunit and different regions of Smc5 and Smc6. Results from these analyses were further verified by in vitro binding experiments with purified recombinant subunits. These experiments revealed several features of the budding yeast Smc5/6 complex. First, Smc5 and Smc6 bind to each other at their hinge regions. Second, the Nse1, Nse3, and Nse4 subunits form a stable subcomplex that interacts with the head and adjacent coiled-coil regions of Smc5. Third, the SUMO ligase subunit, Nse2/Mms21, binds to the middle portion of the coiled-coil region of Smc5. Finally, Nse5 and Nse6 associate as a stable heterodimer that binds to the hinge regions of Smc5 and Smc6. This study is the first to reveal important aspects of the architecture of the budding yeast Smc5/6 complex. The difference and similarities between the two yeast complexes and with cohesin and condensin will be discussed.

**EXPERIMENTAL PROCEDURES**

**Plasmids Constructs**—The hinge, head, and coiled-coil regions of Smc5 and Smc6 were determined using the secondary structure prediction program PredictProtein (PHD) and the homologous alignment of these proteins with other SMC proteins. Full-length or regions of the subunits of the Smc5/6 complex were PCR-amplified and cloned into a pET24D (Novagen), pGEX4T3 (GE Healthcare), or pCDDFduet (Novagen) vector for expression in *Escherichia coli* and were also cloned into pOBD and pOBD vectors for two-hybrid tests. The detailed amino acid positions of each fragment and all the constructs used in this study are listed in Table 1. All constructs used for this work were verified by DNA sequencing. Primer sequences used for cloning are available upon request.

**Expression and Purification of Recombinant Proteins from E. coli**—All proteins were expressed in Rosetta BL21(DE3) (Novagen) cells. The cells were grown at 37 °C to an A600 of 0.5 and induced by 0.4 mM isopropyl β-D-thiogalactopyranoside for 16 h at 20 °C. The cells were harvested and lysed in buffer containing 100 mM Tris-HCl (pH 8.0) and 200 mM NaCl. The proteins containing the His6 tag were purified by nickel-affinity chromatography, and those containing the GST tag were purified by glutathione-affinity chromatography using standard protocols.

**GST Pulldown Assay**—50–100 µg of GST or GST protein were added to 10 µl of glutathione-Sepharose beads and incubated for 10 min. The beads were washed with 1 ml of lysis buffer. Next, 50–100 µg of testing proteins were added to the beads and incubated for 30 min at 4 °C with rotation. The beads were washed three times with 1 ml of phosphate-buffered saline solution and eluted with buffer containing 15 mM glutathione, 100 mM Tris-HCl (pH 8.0). The eluted samples were analyzed by SDS-PAGE.

**Gel Filtration Assay**—Recombinant proteins, expressed alone or with their corresponding binding partners, were first purified using a glutathione column (for GST proteins) or a Ni-NTA column (for His6-tagged proteins) and then injected onto a Superdex 200 gel filtration column using AKTA-FPLC (GE Healthcare). Eluate fractions were subjected to SDS-PAGE and stained with Coomassie Blue.

**Two-hybrid Tests**—Two-hybrid tests were performed as described previously (22, 23). In brief, two-hybrid strain pLE9–4a was transformed with GAD vector (pOAD) or GAD fusion plasmids; transformants were selected and grown on SC-LEU medium. Similarly, two-hybrid strain pLE9–4a was transformed with GBD vector (pOBD) or GBD fusion plasmids and was selected and grown on SC-TRP medium. Both pOAD and pOBD are CEN plasmids and present 1–3 copies/cell. The resulting transformants were then mated, and the diploid cells containing both the GAD and GBD constructs were selected on SC-TRP-LEU plates. The activation of reporters was scored by replica plating the diploid cells to SC-TRP-LEU-HIS and SC-TRP-LEU-ADE medium. Positive interactions rendered cell growth on one or both media. Each construct was tested with vector alone as a control.

**RESULTS**

**Pairwise Two-hybrid Tests between Subunits of the Smc5/6 Complex Reveals Three NSE Entities That Bind to Smc5**—As an initial step to study the architecture of the budding yeast Smc5/6 complex, we systematically examined the interactions between all the subunits of the Smc5/6 complex. Studies of
other multiple protein complexes (such as the spindle pole body and the nuclear pore complexes) suggest that pairwise two-hybrid tests can reveal interactions between close neighbors, despite the fact that all subunits can be co-purified as holocomplexes (24–26). Thus, we used this strategy to map interactions between subunits. For this purpose, each of the eight subunits of the budding yeast Smc5/6 complex was cloned into two-hybrid vectors allowing them to be fused with either a GAD (Gal4 activation domain) or a GBD (Gal4 DNA-binding domain). Using a mating procedure, 64 pairwise two-hybrid tests involving all possible combinations of the subunits were performed along with 16 control pairs.

Positive interactions recovered from these tests are summarized in Fig. 1A. Several conclusions can be drawn from these results. First, none of the subunits exhibited self-association. Second, Nse1, Nse3, and Nse4 interacted in a pairwise fashion, but not with other NSE subunits (Fig. 1, A and B), suggesting that Nse1, -3, and -4 can form a subcomplex. Third, Nse5 and Nse6 interacted with each other but not with any of the other subunits in two-hybrid. Fourth, Nse2/Mms21 interacted with Smc5, but not with any of the other NSE subunits or with Smc6 (Fig. 1A). This is consistent with our earlier observation that these two proteins interact in vitro (4). Fifth, Nse4 and Nse6 also bind to Smc5, although none of the NSE subunits were found to bind Smc6 (Fig. 1A–C). Taken together, these results suggest that there may be three NSE subcomplexes/entities, each of which can bind to Smc5. It is worth noting that although two-hybrid
Architecture of the Smc5/6 Complex

A FullLength
hinge
CC-a
CC-b
CC-c
CC
Head
Head-CC-b
Hinge-CC-a

B Smc5

| FL  | CC-c | head-CC-b | hinge |
|-----|------|-----------|-------|
| Nse2| +    | +         |       |
| Nse4| +    | +         |       |
| Nse6| +    | +         |       |
| Smc6-hinge | + | + |       |

C GAD-Smc6 constructs

-TRP-LEU
-TRP-LEU-HIS
-TRP-LEU-ADE

D GAD constructs

-TRP-LEU
-TRP-LEU-HIS
-TRP-LEU-ADE

E

-TRP-LEU
-TRP-LEU-HIS

FIGURE 2. Interactions of non-SMC subunits and Smc6 with different fragments of Smc5 in two-hybrid tests. A, schematic representation of Smc5 and Smc6 fragments used in this study. The amino acid positions of each fragment are listed in Table 1. These fragments were used in two-hybrid tests and in vitro binding experiments (Figs. 2–5). The hypothetical shapes of the protein fragments are depicted. B, summary of the interactions between Nse2, Nse4, Nse6, and Smc6 with the full length (FL) or fragments of Smc5 (named as in A). C, hinge regions of Smc5 and Smc6 interact with each other. A GBD domain fused with either the Smc5 hinge or the Smc6 hinge was tested against a GAD-fused Smc6-hinge, Smc6-CC-a, or full-length Smc6 (FL). White squares highlight the interaction between the Smc5-hinge and the Smc6-hinge. Selected negative interactions show that the Smc6-hinge does not bind to full-length Smc6 or its own hinge and that the Smc3-hinge does not bind to the coiled-coil domain of Smc6. D, Nse2/Mms21 interacts with a portion of the coiled-coil region of Smc5. Two coiled-coil regions of Smc5, namely the middle section (CC-c) and the half of the coiled-coil region closer to the head domain (CC-b), were fused to GBD and tested against with a GAD fusion containing Nse1, Nse2, or Nse3. White squares highlight the interaction between Nse2/Mms21 and the middle section of the Smc5 coiled-coil region. E, Nse4 interacts with the Smc5-head-CC-b region, but not with the Smc5 head, CC-b region, or hinge alone. GAD-fused Nse4 was tested against GBD constructs containing the Smc5 hinge, full-length Smc5 coiled-coil region (CC), Smc5-head-CC-b, or the Smc5 head alone. White squares highlight the interaction between Nse4 and Smc5-head-CC-b.

tests can reveal substantial amounts of information of protein interactions, they do not exclude other possible interactions. However, the unique interactions between specific subunits recovered from this test strongly suggest that this is a valid method to initially map interactions between subunits, which can be further tested using biochemical approaches (see below).

Nse1-3-4 and the Nse5-6 Subcomplexes Can Be Purified in Vitro—Next, we used purified recombinant proteins expressed from E. coli to examine the interactions revealed by the two-hybrid tests. We previously demonstrated that when expressed from E. coli, Nse2/Mms21 and Smc5 can be co-purified to homogeneity in the absence of any of the other subunits (4). We then examined the formation of the Nse1-3-4 subcomplex. Full-length Nse1, Nse3, and Nse4-His6 were co-expressed in E. coli. Soluble cell lysates were subjected to chromatography on a nickel-affinity column. Elution with buffer containing imidazole resulted in the co-elution of these three proteins (data not shown). The eluted complex was pooled and then subjected to a gel filtration column. Each collected fraction was examined by SDS-PAGE and a single peak of co-migrating Nse1, -3, and -4 proteins was found, demonstrating that Nse1, -3, and -4 form a heterotrimeric complex (Fig. 1D).

We also examined whether Nse5 and Nse6 form a complex. Full-length Nse5 and a His6-tagged Nse6 were co-expressed in E. coli. A two-step purification scheme using nickel-affinity chromatography followed by a gel-filtration column revealed that Nse5 and Nse6 co-purified as a single peak (Fig. 1E). Examination of eluted fractions by SDS-PAGE showed that the two proteins co-migrate, demonstrating that they form a complex.

Interactions between the NSE Subunits and Fragments of Smc5 and Smc6 in Two-hybrid Assays—As mentioned above, Smc5 and Smc6 are modular proteins, each composed of hinge, arm, and head regions (Fig. 2A). To understand how Smc5 and Smc6 interact with each other and with the NSE subunits, each
region of Smc5 and Smc6 was cloned into both GAD and GBD two-hybrid vectors. The constructs containing the head region of each protein were made by linking the N- and C-terminal global domains with a flexible linker. Constructs across the arm were made by linking the N- and C-terminal coiled-coil domains predicted to be associated with each other (see “Experimental Procedures” and Table 1). Besides the full-length coiled-coil regions of Smc5 and Smc6, constructs containing different arm segments were also made to further dissect the roles of this region (Fig. 2A). Additional constructs were made by combining the hinge and adjacent coiled-coil region (hinge-CC-a) and by combining the head and adjacent coiled-coil region (head-CC-b) (Fig. 2A).

The two-hybrid constructs containing the various regions of Smc5 and Smc6 were then tested for intra- and intermolecular interactions in all possible combinations using 196 pairwise tests. In addition, the interactions between these constructs and each NSE subunit were examined in 224 pairwise tests. In each case, a control test with the appropriate vector was included. Positive interactions from these tests are summarized in Fig. 2B. Among all constructs examined, interactions between Smc5 and Smc6 were only detected with constructs containing hinge domains (Fig. 2, B and C, and data not shown); the minimal binding regions of both proteins were thus defined as the hinge alone of both proteins. In addition, we determined that the middle segment of the Smc5 coiled-coil (CC-c) is the minimal region that binds Nse2/Mms21 (Fig. 2D). The minimal region of Smc5 needed to bind Nse4 included both its head and adjacent coiled-coil region (head-CC-b), as Nse4 did not bind to either the Smc5 head or its coiled-coil region separately (Fig. 2E). These results suggest that the Nse1-3-4 subcomplex can interact with the head and adjacent coiled-coil region of Smc5 via the Nse4 subunit.

In Vitro Studies Confirm the Interactions between the Hinges of Smc5 and Smc6 and between Mms21 or Nse4 and Different Fragments of Smc5—To examine the interactions revealed by the two-hybrid assays described above, we first tested whether the hinge regions of Smc5 and Smc6 can bind each other in vitro. The Smc5-hinge was tagged with His6 tag and co-expressed with the hinge region of Smc6 (not tagged) in E. coli. The proteins were purified by Ni-NTA chromatography followed by a gel filtration separation. As shown in Fig. 3A, Smc5-hinge and Smc6-hinge fragments co-eluted as a single peak from the gel filtration column. The molecular mass of the complex peaked at a size of 44 kDa, matching the predicted molecular mass of a stoichiometric complex. This is consistent with the similar intensity of Coomassie Blue staining of the two bands.

Next, we tested whether Nse2/Mms21 binds directly to the middle of the Smc5 coiled-coil region (Smc5-CC-c). This region of Smc5 and His6-tagged Nse2/Mms21 was co-expressed in E. coli and was purified by nickel-affinity chromatography. We found that Smc5-CC-c co-purified with Nse2/Mms21 (data not shown), indicating that these two proteins directly interact with each other.

Finally, we examined whether Nse4 directly binds to the Smc5-head plus its adjacent coiled-coil region (Smc5-head-CC-b) in vitro. GST-tagged Nse4 or GST alone was co-expressed with Smc5-head-CC-b in E. coli. As shown in Fig. 3B, Smc5-head-CC-b co-purified with GST-Nse4, but not with GST. The intensity of the Coomassie Blue staining showed that the ratio of GST-Nse4 and Smc5-head-CC-b in the eluate is about 1:1. This result demonstrates that Nse4 directly binds to the Smc5 head and adjacent coiled-coil region.

The Nse5-6 Subcomplex Interacts with the Hinge Regions of Smc5 and Smc6 in Vitro—The two-hybrid assays identified regions required for the interaction between Smc5 and Smc6, between Nse4 and Smc5, and between Nse2/Mms21 and Smc5. However, they did not define how Nse5 or Nse6 interacts with Smc5 or Smc6. One explanation for this is that a multiprotein interface is needed for these interactions, which can not be assayed by two-hybrid test. Thus, we employed in vitro approaches to examine protein interactions with more than two proteins. We found that when retained on the glutathione

### TABLE 1

| Constructs                  | Amino acid position | E. coli vectors | GAD constructs | GBD constructs |
|-----------------------------|---------------------|----------------|----------------|---------------|
| Nse1                        | Full-length         | pET24D         | pXZ170         | pXZ198        |
| Nse2/Mms21                  | Full-length         | pCDFDuet/pGex4T3 | pXZ89         | pXZ255        |
| Nse3                        | Full-length         | pET24D/pGex4T3 | pXZ110        | pXZ191        |
| Nse4                        | Full-length         | pET24D         | pXZ122        | pXZ196        |
| Nse5                        | Full-length         | pET24D/pGex4T3 | pXZ188        | pXZ199        |
| Nse6                        | Full-length         | pET24D/pGex4T3 | pXZ166        | pXZ193        |
| Smc5-FL                     | Full-length         | pET24D         | pXZ189        | pXZ194        |
| Smc6-FL                     | Full-length         | pET24D/pGex4T3 | pXZ171        | pXZ195        |
| Smc5-hinge                  | 471–670             | pET24D         | pXZ167        | pXZ175        |
| Smc5-CC                     | 149–369 and 733–944 | pET24D        | pXZ149        | pXZ141        |
| Smc5-head                   | 1–148 and 945–1093  | pET24D         | pXZ148        | pXZ141        |
| Smc5-head-CC-a              | 1–301 and 814–1093  | pET24D         | pXZ172        | pXZ178        |
| Smc5-CC-a                   | 302–470 and 660–813 | pET24D         | pXZ150        | pXZ143        |
| Smc5-CC-b                   | 149–301 and 814–944 | pET24D         | pXZ151        | pXZ144        |
| Smc5-CC-c                   | 226–373 and 681–836 | pET24D         | pXZ174        | pXZ180        |
| Smc5-CC-c*                  | 302–369 and 733–813 | pET24D         | pXZ149        | pXZ142        |
| Smc5-hinge-CC-a             | 323–751             | pET24D         | pXZ154        | pXZ147        |
| Smc5-hinge-CC-a*            | 430–679             | pET24D         | pXZ154        | pXZ147        |
| Smc6-head                   | 486–732             | pCDFDuet       | pXZ168        | pXZ176        |
| Smc6-CC-a                   | 1–199 and 988–1114  | pET24D         | pXZ173        | pXZ179        |
| Smc6-CC-b                   | 350–485 & 733–837   | pET24D         | pXZ226        | pXZ227        |
| Smc6-hinge-CC-a             | 350–837             | pCDFDuet       | pXZ228        | pXZ229        |
| Smc6-hinge-CC-a*            | 350–787             | pCDFDuet       | pXZ210        | pXZ211        |
| Smc6-head-CC-b              | 1–349 and 838–1114  | pGex4T3        | pXZ206        | pXZ207        |
| Smc6-CC                     | 200–485 and 733–917 | pET24D         | pXZ208        | pXZ209        |

Architecture of the Smc5/6 Complex
resin, the GST-Nse6/Nse5 complex, but not GST alone, can efficiently pull down the hinge complex of Smc5/6 (Fig. 3C).

Similar results were obtained when we used a GST-Nse5/Nse6 complex retained on the resin (Fig. 3D). These results demonstrate that the Nse5-6 subcomplex interacts with the hinge region of Smc5-6.

We then examined whether the Nse5-6 subcomplex interacts with other regions of Smc5 or Smc6. To this end, regions of Smc5 and Smc6 as depicted in Fig. 2A were fused with a His6 tag and purified from E. coli using a Ni-NTA column. We found that the GST-Nse6/Nse5 complex did not bind to either Smc5-head-CC-b or a coiled-coil region of Smc5 adjacent to CC-b (CC-c*, see Table 1 and Fig. 4, A and B). However, it bound the Smc5-hinge associated with a small segment of its neighboring coiled-coil (Smc5-hinge-CC-a*; Fig. 4C). In addition, GST-Nse5/Nse6, but not GST, pulled down the Smc5-hinge attached to its adjacent coiled-coil region (Smc5-hinge-CC-a) (supplemental Fig. 1A). Taken together, these results suggest that the Nse5-6 subcomplex interacts with the Smc5 hinge and that the small adjacent coiled-coil region of the hinge domain may facilitate its binding; however, this subcomplex does not bind to the head or most of the coiled-coil region.

Using a similar approach, we examined which regions of Smc6 bind to the Nse5-6 subcomplex. As shown in Fig. 5A, GST-Nse5/Nse6, but not GST, when retained on glutathione resin, efficiently pulled down the His6-tagged Smc6-hinge region associated with a small segment of the adjacent coiled-coil (Smc6-hinge-CC-a*). The presence of Smc6 was confirmed by Western blot (Fig. 5A, right panel). Similarly, GST-Nse5/Nse6, but not GST, efficiently pulled down the Smc6-hinge associated with a larger segment of adjacent coiled-coil (Smc6-hinge-CC-a; supplemental Fig. 1B). In contrast, GST-Nse5/Nse6 did not pull down the Smc6 coiled-coil region adjacent to the hinge (CC-a; Fig. 5B). Furthermore, the Nse5-6 subcomplex did not bind to the Smc6-head and its adjacent coiled-coil region (Smc6-head-CC-b; Fig. 5C). Taken together, these
The architecture of the Smc5/6 complex interacts with each other at their hinges but not at the head or coiled-coil regions. This is similar to the interaction between Smc1–3 and Smc2–4 in cohesin and condensin. In addition, there are three NSE entities found within the Smc5/6 complex as follows: the Nse1–3–4 subcomplex, the Nse5–6 subcomplex, and Nse2/Mms21. Interestingly, these three NSE entities bind to different regions of Smc5 and Smc6 as follows: the Nse1–3–4 subcomplex binds to the head and adjacent coiled-coil region of Smc5; the Nse5–6 subcomplex binds to the Smc5 and Smc6 hinge; and Nse2/Mms21 binds to the middle of the coiled-coil region of Smc5. The binding of Nse1–3–4 to Smc5 is likely mediated by the Nse4 subunit, whereas the binding of Nse5–6 to Smc5–6 hinges can be mediated by both subunits. These features of the Smc5/6 complex are summarized in Fig. 5D, and their implications are discussed below.

**Similarities and Differences of the Smc5/6 Complex Architecture between Two Yeasts**—During the course of this work, interactions among subunits of the fission yeast Smc5/6 complex have been studied using protein pulldown assays and two-hybrid assays (5, 7, 27). Comparing the Smc5/6 complexes in the two yeasts, it is clear that they share significant similarities, but each possesses distinct features. In both yeasts, Smc5 and Smc6 interact solely at the hinges. In addition, both complexes contain the same NSE entities, namely the Nse1–3–4 subcomplex, the Nse5–6 subcomplex, and Nse2/Mms21. Moreover, in both yeasts, Nse2/Mms21 binds to the arm of Smc5, whereas the Nse1–3–4 subcomplex associates with the head region of Smc5.

The most striking difference of the two Smc5/6 complexes is that the budding yeast Nse5–6 subcomplex binds to the hing of Smc5 and Smc6, whereas that of the fission yeast binds to the head regions of Smc5 and/or Smc6. It is possible that such differences reflect structural and functional differences in the Smc5/6 complexes between the two species. As mentioned earlier, unlike other NSE subunits, Nse5 and Nse6 from the two yeasts display the following different properties. 1) They do not share sequence homology. 2) Although both are essential in budding yeast, neither is required for viability in fission yeast (the other subunits are). 3) They bind tightly to the rest of the complex in budding yeast but not in fission yeast (4, 5, 7). Thus, the differential binding of the Nse5–6 subcomplex may provide a molecular basis for the functional differences of the two subunits in the two organisms. However, one cannot rule out the possibility that the difference is partly the result of the different interactions within a multisubunit complex. When two-hybrid assays are performed in a systematic manner, the recovered interactions can be comprehensive. Results from such a study can then guide and facilitate in vitro binding assays or purification. As all the proteins used in the in vitro assays in this work were purified from *E. coli*, which does not contain homologs of the Smc5/6 complex, the detected interactions reflect direct bindings.

The results from these two approaches largely agree with each other and enable the identification of several key features of the budding yeast Smc5/6 complex. First, Smc5 and Smc6 interact with each other at their hinges but not at the head or coiled-coil regions. This is similar to the interaction between Smc1–3 and Smc2–4 in cohesin and condensin. In addition, there are three NSE entities found within the Smc5/6 complex as follows: the Nse1–3–4 subcomplex, the Nse5–6 subcomplex, and Nse2/Mms21. Interestingly, these three NSE entities bind to different regions of Smc5 and Smc6 as follows: the Nse1–3–4 subcomplex binds to the head and adjacent coiled-coil region of Smc5; the Nse5–6 subcomplex binds to the Smc5 and Smc6 hinge; and Nse2/Mms21 binds to the middle of the coiled-coil region of Smc5. The binding of Nse1–3–4 to Smc5 is likely mediated by the Nse4 subunit, whereas the binding of Nse5–6 to Smc5–6 hinges can be mediated by both subunits. These features of the Smc5/6 complex are summarized in Fig. 5D, and their implications are discussed below.

**Architecture of the Budding Yeast Smc5/6 Complex**—Using a combined approach of two-hybrid assays and in vitro biochemical analyses, we have identified interactions among the NSE subunits, between Smc5 and Smc6, and between the NSE subunits and regions of Smc5 and Smc6. These two approaches have different strengths. The two-hybrid assay addresses the interaction between two subunits and regions of Smc5 and Smc6, whereas that of the fission yeast binds to the head regions of Smc5 and/or Smc6. It is possible that such differences reflect structural and functional differences in the Smc5/6 complexes between the two species. As mentioned earlier, unlike other NSE subunits, Nse5 and Nse6 from the two yeasts display the following different properties. 1) They do not share sequence homology. 2) Although both are essential in budding yeast, neither is required for viability in fission yeast (the other subunits are). 3) They bind tightly to the rest of the complex in budding yeast but not in fission yeast (4, 5, 7). Thus, the differential binding of the Nse5–6 subcomplex may provide a molecular basis for the functional differences of the two subunits in the two organisms. However, one cannot rule out the possibility that the difference is partly the result of the different interactions within a multisubunit complex. When two-hybrid assays are performed in a systematic manner, the recovered interactions can be comprehensive. Results from such a study can then guide and facilitate in vitro binding assays or purification. As all the proteins used in the in vitro assays in this work were purified from *E. coli*, which does not contain homologs of the Smc5/6 complex, the detected interactions reflect direct bindings.

The results from these two approaches largely agree with each other and enable the identification of several key features of the budding yeast Smc5/6 complex. First, Smc5 and Smc6 interact with each other at their hinges but not at the head or coiled-coil regions. This is similar to the interaction between Smc1–3 and Smc2–4 in cohesin and condensin. In addition, there are three NSE entities found within the Smc5/6 complex as follows: the Nse1–3–4 subcomplex, the Nse5–6 subcomplex, and Nse2/Mms21. Interestingly, these three NSE entities bind to different regions of Smc5 and Smc6 as follows: the Nse1–3–4 subcomplex binds to the head and adjacent coiled-coil region of Smc5; the Nse5–6 subcomplex binds to the Smc5 and Smc6 hinge; and Nse2/Mms21 binds to the middle of the coiled-coil region of Smc5. The binding of Nse1–3–4 to Smc5 is likely mediated by the Nse4 subunit, whereas the binding of Nse5–6 to Smc5–6 hinges can be mediated by both subunits. These features of the Smc5/6 complex are summarized in Fig. 5D, and their implications are discussed below.

**Discussion**

Architecture of the Budding Yeast Smc5/6 Complex—Using a combined approach of two-hybrid assays and in vitro biochemical analyses, we have identified interactions among the NSE subunits, between Smc5 and Smc6, and between the NSE subunits and regions of Smc5 and Smc6. These two approaches have different strengths. The two-hybrid assay addresses the interaction between two subunits and regions of Smc5 and Smc6, whereas that of the fission yeast binds to the head regions of Smc5 and/or Smc6. It is possible that such differences reflect structural and functional differences in the Smc5/6 complexes between the two species. As mentioned earlier, unlike other NSE subunits, Nse5 and Nse6 from the two yeasts display the following different properties. 1) They do not share sequence homology. 2) Although both are essential in budding yeast, neither is required for viability in fission yeast (the other subunits are). 3) They bind tightly to the rest of the complex in budding yeast but not in fission yeast (4, 5, 7). Thus, the differential binding of the Nse5–6 subcomplex may provide a molecular basis for the functional differences of the two subunits in the two organisms. However, one cannot rule out the possibility that the difference is partly the result of the different interactions within a multisubunit complex. When two-hybrid assays are performed in a systematic manner, the recovered interactions can be comprehensive. Results from such a study can then guide and facilitate in vitro binding assays or purification. As all the proteins used in the in vitro assays in this work were purified from *E. coli*, which does not contain homologs of the Smc5/6 complex, the detected interactions reflect direct bindings.

The results from these two approaches largely agree with each other and enable the identification of several key features of the budding yeast Smc5/6 complex. First, Smc5 and Smc6 interact with each other at their hinges but not at the head or coiled-coil regions. This is similar to the interaction between Smc1–3 and Smc2–4 in cohesin and condensin. In addition, there are three NSE entities found within the Smc5/6 complex as follows: the Nse1–3–4 subcomplex, the Nse5–6 subcomplex, and Nse2/Mms21. Interestingly, these three NSE entities bind to different regions of Smc5 and Smc6 as follows: the Nse1–3–4 subcomplex binds to the head and adjacent coiled-coil region of Smc5; the Nse5–6 subcomplex binds to the Smc5 and Smc6 hinge; and Nse2/Mms21 binds to the middle of the coiled-coil region of Smc5. The binding of Nse1–3–4 to Smc5 is likely mediated by the Nse4 subunit, whereas the binding of Nse5–6 to Smc5–6 hinges can be mediated by both subunits. These features of the Smc5/6 complex are summarized in Fig. 5D, and their implications are discussed below.

**Summary**

The Nse5–6 subcomplex interacts with the hinge but not with the head or coiled-coil regions of Smc5. Purified GST-Nse6/Nse5 (as shown in Fig. 3C, left panel) or GST retained on the glutathione resin was mixed with different proteins that were fused with a His<sub>6</sub> tag and purified by Ni-NTA (input). A, input protein is the Smc5-head and its adjacent coiled-coil region (Smc5-head-CC-b). B, input protein is the coiled-coil region of Smc5 adjacent to CC-b (Smc5-CC-c*). C, input protein is the Smc5 hinge and its adjacent coiled-coil domain (Smc5-hinge-CC-a*). In each case, eluted samples (E) and the last wash (W) were examined by SDS-PAGE and the silver-stained gel image is shown. The Nse5-6 subcomplex can pull down Smc5-hinge-CC-a* (C), but not Smc5-head-CC-b (A) or Smc5-CC-c* (B). As Smc5-head-CC-b runs at the same position as a background band, the absence of this Smc5 fragment in the eluate was confirmed by Western blot (WB) using anti-His<sub>6</sub> antibody (A, lower panel).
Architecture of the Smc5/6 Complex

FIGURE 5. Nse5-6 subcomplex interacts with the hinge but not with the head or coiled-coil regions of Smc6. A and B, Nse5-6 subcomplex was purified by glutathione beads (left panel, A). As in Fig. 4, different fragments of Smc6 were fused with a His6 tag, purified by Ni-NTA (I, input), and added to the glutathione resin containing either GST-Nse5-Nse6 (A) or GST-Nse6-Nse5 (B). As controls, the same input proteins were added to glutathione resin containing purified GST protein. The input protein for the middle and right panels of A is the Smc6 hinge and a small adjacent coiled-coil region (Smc6-hinge-CC-a*) and that for B is the Smc6 coiled-coil region adjacent to the hinge (Smc6-CC-a). In each case, eluted samples (E) and the last wash (W) were examined by SDS-PAGE, and the silver stained gel image is shown. The Nse5-6 subcomplex can pull down Smc6-hinge-CC-a* (A) but not Smc6-CC-a (B). As the Smc6-hinge-CC-a* runs at the same position as a background band, the presence of this Smc6 fragment in the eluate was confirmed by Western blot (WB) using anti-His6 antibody (A, right panel). C, Smc6 head and adjacent coiled-coil region (Smc6-head-CC-b) was fused with a GST tag and purified by glutathione beads, whereas the Nse5-6 subcomplex (with Nse6 fused to a His6 tag) was purified by Ni-NTA resin (I, input). The Nse5-6 subcomplex was mixed with glutathione resin containing either GST-Smc6-head-CC-b or GST alone. Eluted samples (E) and the last wash (W) were examined by SDS-PAGE and the silver-stained image is shown. Neither Nse5 nor Nse6 was visible in the final eluate. D, model for the architecture of the budding yeast Smc5/6 complex is depicted based on the results from this study (see text for detail).

Methodologies used in studying protein interactions. Although we used purified recombinant protein in binding assays, work of fission yeast proteins used protein mixtures from in vitro transcription-translation reticulocyte lysates (27). The interactions observed in such a study may therefore be complicated by the presence of endogenous proteins.

Another difference between the two yeasts lies in the relationship between Smc6 and the Nse1-3-4 subcomplex. We did not detect any interaction of Nse1, -3, or -4 with either the full-length or segments of Smc6 in two-hybrid assays. Studies using fission yeast counterparts produced by in vitro transcription-translation have detected binding between Nse3/Nse4 and the Smc6 head (27). However, another study using the fission yeast Nse1-3-4 subcomplex expressed in insect cells did not detect any interaction with Smc6 (7). Thus, future work is needed to clarify whether the Nse1-3-4 subcomplex can bind to Smc6. Such interactions will be important in understanding how the head regions of Smc5 and Smc6 are regulated. In addition, it will be interesting to determine whether the architecture of the Smc5/6 complex in both yeasts is dynamic and changes during the cell cycle or under DNA-damaging conditions.

Comparison of the Structures of the Smc5/6 Complexes and Those of Cohesin and Condensin—The structures of the budding yeast cohesin and condensin are similar. In both complexes, the two SMC proteins interact with each other at the hinge region, forming the backbones of the ring-shaped structures of these complexes (1–3). All non-SMC proteins bind to the head regions of the SMC proteins. Furthermore, the kleisin protein of each complex can bridge the two SMC heads. In cohesin, the kleisin (Mdc1/Scc1) is cleaved by a protease called separase during mitosis, leading to the loss of cohesion and subsequent sister-chromatid separation (28, 29). In contrast, the kleisin subunit in condensin (Brn1) is not cleaved, and it is not clear whether it can regulate the association of the SMC proteins.

As mentioned earlier, one similarity between the Smc5/6 complexes and cohesin and condensin is that all of the SMC proteins interact at the hinges but not at other regions. In addi-
tion, Nse4, whose structure was predicted to share certain similarities with the kleisin proteins (1), binds to the head region of Smc5. It remains to be determined if Nse4, like the kleisins, can bridge the head regions of Smc5 and Smc6, or if it diverges to associate only with the head region of Smc5.

The binding of the other NSE subunits to Smc5 and Smc6 exhibits two features that have not been observed in cohesin or condensin as follows: the binding of Nse2/Mms21 to the middle of the arm region of Smc5 and the interactions between the Nse5-6 subcomplex with the hinges of Smc5-6. The special placement of Nse2/Mms21 on the coiled-coil region is conserved in fission yeast (5), suggesting that it plays an important role. As Nse2/Mms21 is a SUMO ligase, it is possible that its unique position in the complex can facilitate its interaction with the SUMO conjugating enzyme and/or with substrates. However, these cannot be the only functions for such interaction, because the SUMO ligase activity of Nse2/Mms21 is not essential, whereas the Mms21 protein is required for cell viability (4). Therefore, the binding of this subunit to the arm of Smc5 must serve other purposes, such as regulating the flexibility, stability, or shape of Smc5. Future work is needed to examine these possibilities.

The binding of Nse5 and Nse6 to the hinge region of Smc5-6 is also unique to the Smc5/6 complex. As indicated, this feature may not be conserved in the fission yeast. Although the biological significance of the different positioning of Nse5-6 in the complex remains to be determined, it is interesting to note that the hinge-hinge interaction of cohesin is dynamic. This leads to the opening and closing of the complex and subsequently may result in the entering and exiting of DNA (30). It is thus possible that Nse5-6 can regulate the dynamics of the hinges of Smc5/6. Recently, it was shown that Pds5, an essential protein required for cohesion in budding yeast, can bind to the hinges of cohesin (31). It is possible that the hinge regulation is a common theme for SMC complexes, but the mechanisms may be different depending on the context, and await further investigation.

Acknowledgments—We thank Ken Marians, Jerry Hurwitz, Hao Wu, Lisa Hang, Prabha Sarangi, and Andrew Marsh for reading the manuscript and providing helpful discussions. We also thank William Pierce and Jian Cai at Mass Spectrometry, University of Louisville, Louisville, KY.

REFERENCES
1. Nasmyth, K., and Haering, C. H. (2005) Annu. Rev. Biochem. 74, 595–648
2. Hirano, T. (2006) Nat. Rev. 7, 311–322
3. Onn, I., Heidinger-Pauli, J. M., Guacci, V., Unal, E., and Koshland, D. E. (2008) Annu. Rev. Cell Dev. Biol. 24, 105–129
4. Zhao, X., and Blobel, G. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 4777–4782
5. Sergeant, J., Taylor, E., Palecek, J., Foutoumi, M., Andrews, E. A., Sweeney, S., Shinagawa, H., Watts, F. Z., and Lehmann, A. R. (2005) Mol. Cell. Biol. 25, 172–184
6. Taylor, E. M., Copsey, A. C., Hudson, J. J., Vidot, S., and Lehmann, A. R. (2008) Mol. Cell. Biol. 28, 1197–1206
7. Pebernard, S., Wohlschlegel, J., McDonald, W. H., Yates, J. R., and Boddy, M. N. (2006) Mol. Cell. Biol. 26, 1617–1630
8. Strom, L., and Sjogren, C. (2007) Curr. Opin. Cell Biol. 19, 344–349
9. Murray, J. M., and O’Connell, M. J. (2008) DNA Repair 5, 138–141
10. Lehmann, A. R. (2005) DNA Repair 4, 309–314
11. Potts, P. R., and Yu, H. (2005) Mol. Cell. Biol. 25, 7021–7032
12. Potts, P. R., Porteus, M. H., and Yu, H. (2006) EMBO J. 25, 3377–3388
13. Andrews, E. A., Palecek, J., Sergeant, J., Taylor, E., Lehmann, A. R., and Watts, F. Z. (2005) Mol. Cell. Biol. 25, 185–196
14. Beasley, M., Xu, H., Warren, W., and McKay, M. (2002) Genome Res. 12, 1201–1209
15. Cobbe, N., and Heck, M. H. (2004) Mol. Biol. Evol. 21, 332–347
16. Hu, B., Liao, C., Millson, S. H., Mollapour, M., Prodromou, C., Pearl, L. H., Piper, P. W., and Panaretou, B. (2005) Mol. Microbiol. 55, 1735–1750
17. Torres-Rosell, J., Machin, F., Farmer, S., Jarmuz, A., Eydmann, T., Dalgaard, J. Z., and Aragon, L. (2005) Nat. Cell Biol. 7, 412–419
18. Cost, G. J., and Cozzarelli, N. R. (2006) Genetics 172, 2185–2200
19. Lindroos, H. B., Strom, L., Itoh, T., Katou, Y., Shirahige, K., and Sjogren, C. (2006) Mol. Cell. Biol. 25, 1197–1206
20. Pebernard, S., Schaffer, L., Campbell, D., Head, S. R., and Boddy, M. N. (2006) EMBO J. 27, 3011–3022
21. Golemis, E. A., and Khazak, V. (1997) Methods Mol. Biol. 63, 197–218
22. James, P., Halladay, J., and Craig, E. A. (1996) Genetics 144, 1425–1436
23. Jaspersen, S. L., and Winey, M. (2004) Annu. Rev. Cell Dev. Biol. 20, 1–28
24. Alber, F., Forster, F., Korkin, D., Topf, M., and Sali, A. (2008) Annu. Rev. Biochem. 77, 443–477
25. Yu, H., Braun, P., Yildirim, A. M., Lemmens, I., Venkatesan, K., Sahali, J., Hirozane-Kishikawa, T., Gebreab, F., Li, N., Simonis, N., Hao, T., Rual, J. F., Dricot, A., Vazquez, A., Murray, R. R., Simon, C., Tardivo, L., Tam, S., Svrzikapa, N., Fan, C., de Smet, A. S., Motyl, A., Hudson, M. E., Park, I., Xin, X., Cusick, M. E., Moore, T., Boone, C., Snyder, M., Roth, F. P., Barabasi, A. L., Tavernier, J., Hill, D. E., and Vidal, M. (2008) Science 322, 104–110
26. Palecek, J., Vidot, S., Feng, M., Doherty, A. J., and Lehmann, A. R. (2006) J. Biol. Chem. 281, 36952–36959
27. Ciosk, R., Zachariae, W., Michaelis, C., Shevchenko, A., Mann, M., and Nasmyth, K. (1998) Cell 93, 1067–1076
28. Uhlmann, F., Lottspeich, F., and Nasmyth, K. (1999) Nature 400, 37–42
29. Grober, S., Arumugam, P., Katou, Y., Kuglitsch, D., Helmhart, W., Shirahige, K., and Nasmyth, K. (2006) Cell 127, 523–537
30. Mc Intyre, J., Muller, E. G., Weitzer, S., Snydsman, B. E., Davis, T. N., and Uhlmann, F. (2007) EMBO J. 26, 3783–3793

MARCH 27, 2009•VOLUME 284•NUMBER 13
JOURNAL OF BIOLOGICAL CHEMISTRY 8515