Cell, Vol. 112, 765–777, March 21, 2003, Copyright ©2003 by Cell Press

Chromosomal Cohesin Forms a Ring

Stephan Gruber,[1] Christian H. Haering,[1] and Kim Nasmyth*
Research Institute of Molecular Pathology
Dr. Bohr-Gasse 7
1030 Vienna
Austria

Summary

The cohesin complex is essential for sister chromatid cohesion during mitosis. Its Smc1 and Smc3 subunits are rod-shaped molecules with globular ABC-like ATPases at one end and dimerization domains at the other connected by long coiled coils. Smc1 and Smc3 associate to form V-shaped heterodimers. Their ATPase heads are thought to be bridged by a third subunit, Scc1, creating a huge triangular ring that could trap sister DNA molecules. We address here whether cohesin forms such rings in vivo. Proteolytic cleavage of Scc1 by separase at the onset of anaphase triggers its dissociation from chromosomes. We show that N- and C-terminal Scc1 cleavage fragments remain connected due to their association with different heads of a single Smc1/Smc3 heterodimer. Cleavage of the Smc3 coiled coil is sufficient to trigger cohesin release from chromosomes and loss of sister cohesion, consistent with a topological association with chromatin.

Introduction

The segregation of sister chromatids to opposite poles of a cell depends on the attachment of their centromeres to microtubules of the mitotic spindle with opposing orientations (bi-orientation). This is made possible by some form of "cohesion" holding sister chromatids together for long after DNA replication is complete. By resisting the tendency of microtubules to split bi-oriented chromosomes, sister chromatid cohesion ensures that centromeric chromatin comes under tension, which is thought to stabilize the attachment of kinetochores to microtubules. In addition to facilitating bi-orientation, cohesion prevents sister chromatid splitting while bi-orientation of other chromosomes takes place. Only when every chromosome has bi-oriented (a state known as metaphase) is cohesion between sister chromatids destroyed, which triggers their disjunction to opposite poles of the cell, known as anaphase.

The physical nature of sister chromatid cohesion has long been elusive. Genetic studies have recently made this problem amenable to study by identifying a multisubunit "cohesin" complex necessary to maintain chromatid cohesion from chromosome duplication until the onset of anaphase (reviewed by Nasmyth, 2001). Cohesin contains four core subunits, Scc1 (Mcd1), Scc3, Smc1, and Smc3, all of which are conserved from yeast to humans (Guacci et al., 1997; Michaelis et al., 1997; Losada et al., 1998; Toth et al., 1999). A less tightly associated subunit called Pds5 is also necessary for cohesion. Scc1 is replaced by a related protein called Rec8 during yeast meiosis (Klein et al., 1999; Buonomo et al., 2000).

In yeast, cohesin is associated with chromosomes from S phase until anaphase in a manner that is resistant to high salt (1.6 M KCl) concentrations. Its loading onto chromosomes depends on a different complex, which is composed of the Scc2 and Scc4 proteins (Ciosk et al., 2000). Though cohesin can be loaded onto chromosomes throughout the cell cycle, it is only capable of generating cohesion between sister chromatids when present during DNA replication (Uhlmann and Nasmyth, 1998). Cohesin is finally released from chromosomes after all chromosomes have bi-oriented. Activation of the APC/C ubiquitin protein ligase by Cdc20 (Zachariae and Nasmyth, 1999) causes release of a thiol protease called separase from its inhibitory chaperone called securin. Separase induces cleavage of cohesin's Scc1 subunit, which is both necessary and sufficient for the destruction of sister chromatid cohesion and for the onset of anaphase (Uhlmann et al., 1999; Uhlmann et al., 2000; Hauf et al., 2001). This is accompanied by the dissociation of at least Scc1 (Michaelis et al., 1997) and Scc3 (Toth et al., 1999) from chromosomes.

Understanding cohesin's structure may provide insight into how it associates so tightly with chromosomal DNA, how it connects chromatids after DNA replication, and how both chromosomal association and sister chromatid cohesion are destroyed by separase. Previous work has led to the notion that the N- and C-terminal halves of all SMC proteins, including Smc1 and Smc3, fold back on themselves to form long (up to 50 nm) stretches of intra-molecular (Haering et al., 2002) and anti-parallel (Melby et al., 1998) coiled coils. This brings N- and C-terminal domains together to form one half of an ABC-like ATPase, with the other half coming from a second SMC protein. At the center of the folding axis of SMC proteins lies a domain whose dimerization joins two SMC proteins to form a V shaped molecule with ATPases at the apex of each arm and a donut-shaped dimeric structure at its base. Most if not all bacteria produce SMC homodimers created by homotypic interactions between their dimerization domains. Cohesin instead forms heterodimers through a heterotypic interaction between the dimerization domains of Smc1 and Smc3 (Haering et al., 2002).

Electron micrographs suggest that the non-SMC subunits of cohesin (i.e., Scc1 and Scc3) may be located in the vicinity of SMC head domains (Anderson et al., 2002). Actual contacts between these subunits and the Smc1/Smc3 heterodimers have been detected following coexpression of yeast cohesin subunits in insect cells. This showed that Smc3's head domain associates with the N-terminal Scc1 cleavage fragment while Smc1's head domain associates with its C-terminal fragment. Scc3 binds to the complex by virtue of its association with Scc1's C-terminal fragment.

*Correspondence: nasmyth@nt.imp.univie.ac.at
[1]These authors contributed equally to this work.

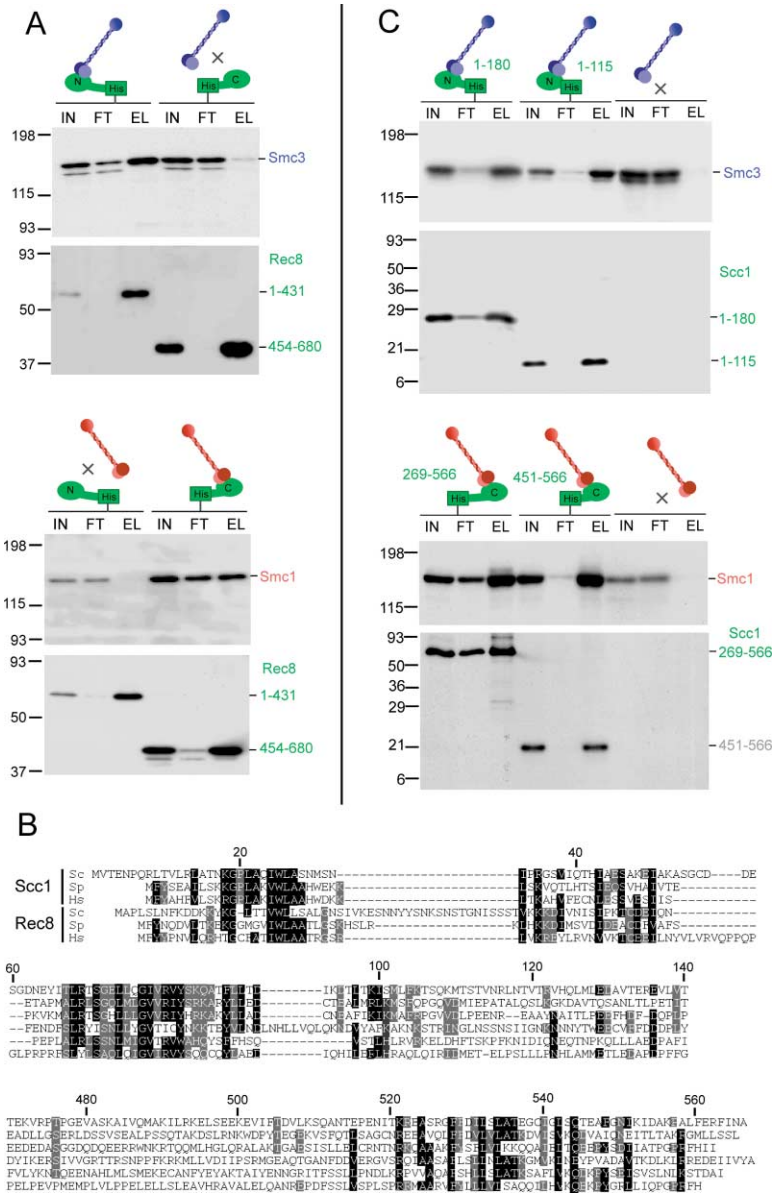

Figure 1. Smc1 and Smc3 Bind to Conserved N- and C Termini of Scc1 and Rec8

(A) Smc3 binds to the N-terminal and Smc1 to the C-terminal fragment of Rec8. HA_3Smc3 or HA_3Smc1 were co-expressed with either N-terminal (aa 1–431) or C-terminal (aa 454–680) Rec8 cleavage fragments fused to FLAG and His_6 epitopes from recombinant baculoviruses in insect cells. Rec8 fragments were bound to Ni^{2+}-NTA and eluted with imidazole. Copurification of HA_3Smc1 or HA_3Smc3 was tested by immunoblotting against HA_3 and FLAG epitopes in input (IN), flowthrough (FT), and imidazole eluate (EL) fractions.

(B) Scc1 and Rec8 homolog sequences are conserved at their N- and C termini. Amino acid sequence alignment of N- and C-terminal parts of Scc1 and Rec8 homologs from *Saccharomyces cerevisiae* (Sc), *Schizosaccharomyces pombe* (Sp), and *Homo sapiens* (Hs) using CLUSTAL X (Thompson et al., 1997). Numbers refer to amino acid residues of Sc Scc1; gray and black boxes indicate conserved residues. For a comprehensive alignment, see Schleiffer et al. (2003).

(C) Scc1's conserved N- and C-terminal regions bind Smc1 and Smc3. HA_3Smc3 expressed alone or co-expressed with N-terminal Scc1 fragments (aa 1–180 or aa 1–115) and HA_3Smc1 expressed alone or co-expressed with C-terminal Scc1 fragments (aa 269–566 or aa 451–566) in insect cells was tested for copurification on Ni^{2+}-NTA as in (A).

The failure to detect copurification of more than one molecule of each of cohesin's subunits in yeast implies that a single Scc1 subunit connects the two head domains of a single Smc1/Smc3 heterodimer, creating a large proteinaceous ring (Haering et al., 2002). This notion is consistent with the finding that some electron micrographs of soluble cohesin complexes do indeed have the appearance of rings (Anderson et al., 2002). Such images cannot, however, by themselves establish whether contacts are really made between different subunits nor do they address the state of cohesin when bound to chromosomes. Because a ring structure has the potential to trap DNA strands, which could provide a mechanism by which cohesin binds to and holds chromatids together, it was of paramount importance to establish whether chromosomal cohesin does indeed form rings and if so, whether its association with chromosomes depends on their integrity.

Because no current cytological or structural technique seemed capable of addressing this issue, we have adopted a strictly biochemical approach in which contacts between subunits have been detected by measuring the degree to which cohesin subunits or fragments thereof copurify with other subunits in a highly specific manner in affinity purification. The principle finding described in this paper was inspired by the notion that interactions between Scc1's termini and SMC heads might survive not only Scc1's cleavage by separase but also its subsequent dissociation from chromosomes. If so, then the N- and C-terminal fragments released from chromosomes at the onset of anaphase should remain associated by virtue of their being bound to different apices of the same Smc1/Smc3 heterodimer. We have not only detected this linkage but also demonstrated that it is broken by severing Smc3's coiled coil. To do this, we developed an SMC protein whose coiled coil

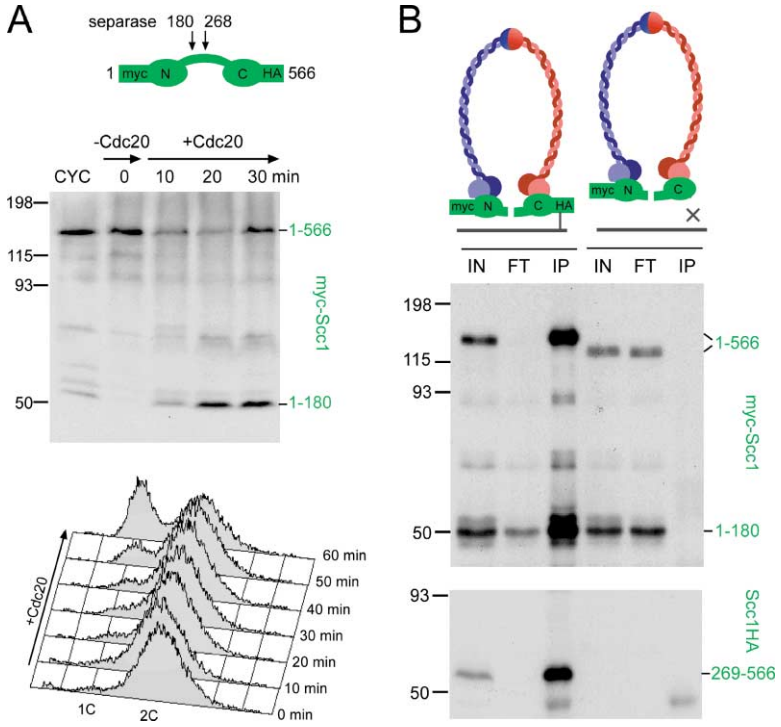

Figure 2. Separase Cleavage Fragments of Scc1 Coimmunoprecipitate

(A) Yeast strain K10005 (*MATa myc$_9$-SCC1-HA$_6$*, Δ*cdc20*, *GAL1-10-CDC20*, and Δ*ubr1*) grown to logarithmic phase in medium containing galactose (CYC) was transferred to galactose free media for metaphase arrest. Synchronous expression of Cdc20 was induced by readdition of galactose (time point zero) and samples were taken every ten minutes for preparation of whole-cell extract. Cleavage of myc$_9$-Scc1-HA$_6$ was followed by probing whole-cell extracts against the myc epitope on immunoblots. FACScan analysis shows that cells were arrested and released efficiently.

(B) Immunoprecipitations with antibodies against the HA-epitope from whole-cell extracts of strains K10005 and K10040 (*MATa, myc$_9$-SCC1*, Δ*cdc20*, *GAL1-10-CDC20*, and Δ*ubr1*) prepared 20 min after Cdc20 induction were probed against the myc-epitope at the amino terminus and the HA-epitope at the carboxyl terminus of Scc1 in input (IN), flow-through (FT), and immunoprecipitated (IP) fractions.

can be cleaved by the TEV protease. We show that cleavage of Smc3's coiled coil causes cohesin's release from chromatin and the destruction of sister chromatid cohesion. Our data imply that chromosomal cohesin does indeed form a ring and suggest that cohesin's tight association with chromosomes may be due to the passage of DNA through this ring.

Results

Smc1 and Smc3 Associate with Conserved N- and C termini of Scc1 and Its Meiotic Counterpart Rec8

If the asymmetric linkage of cohesin's head domains by Scc1-like proteins were a general feature, then it should be conserved in complexes in which Scc1 is replaced by its meiotic counterpart Rec8. We therefore tested the binding of Smc1 and Smc3 to N- and C-terminal Rec8 cleavage fragments after co-expression in insect cells. Smc1 bound the C- but not N-terminal Rec8 fragment while Smc3 bound the N- but not C-terminal fragment (Figure 1A). Alignment of Scc1 and Rec8 sequences from a wide variety of eukaryotes identified amino acid sequence conservation only at the extreme N- and C termini of these proteins (Figure 1B). A polypeptide containing only the conserved N-terminal 115 amino acids of Scc1 also bound efficiently to Smc3, as did a polypeptide containing the most C-terminal 115 amino acids to Smc1 (Figure 1C). This suggests that the conserved stretches at the N- and C termini of Scc1 and Rec8 proteins are responsible for binding the Smc3 and Smc1 heads, respectively.

N- and C-Terminal Scc1 Cleavage Fragments Remain Linked after Anaphase Onset

If cohesin's Smc1 and Smc3 heads were connected by Scc1 when bound to metaphase chromosomes, then N- and C-terminal Scc1 fragments should remain bound to the same Smc1/Smc3 heterodimer following their dissociation from chromosomes induced by separase cleavage. To test this, we created a yeast strain that expresses a functional Scc1 protein whose N- and C termini are attached to 9 myc and 6 HA epitopes, respectively (Figure 2A). The APC/C-activator Cdc20 was under control of the inducible *GAL1-10* promoter (Lim et al., 1998). Cells were arrested in metaphase by depletion of galactose from the medium. Its readdition caused induction of Cdc20 and synchronous activation of separase (Uhlmann et al., 2000). Most Scc1 was cleaved 15–20 min after Cdc20 induction (Figure 2A).

The HA-tagged C-terminal Scc1 fragment dissociates from chromatin under these circumstances (Uhlmann et al., 1999) but is thereafter targeted for degradation by the Ubr1 ubiquitin protein ligase (Rao et al., 2001), most being destroyed by 30 min. The myc-tagged N-terminal fragment is more stable and lingers until cells enter S phase in the next cell cycle (not shown). We prepared whole-cell extracts from cells 20 min after Cdc20 induction and immunoprecipitated the C-terminal Scc1 fragment using antibodies against its HA$_6$-epitope. The myc-tagged N-terminal fragment was efficiently coprecipitated despite cleavage of Scc1 at two positions, but only when Scc1 carried HA epitopes at its C terminus (Figure 2B). The N- and C-terminal Scc1 fragments created by separase clearly remain tightly linked.

An Smc3 Protein with Cleavable Coiled Coils

The link between N- and C-terminal Scc1 fragments could be caused either by direct affinity between the two fragments or by their association with the same Smc1/Smc3 heterodimer. In the latter case, association should depend on integrity of the coiled coils that connect the Smc1 and Smc3 head domains. To test this,

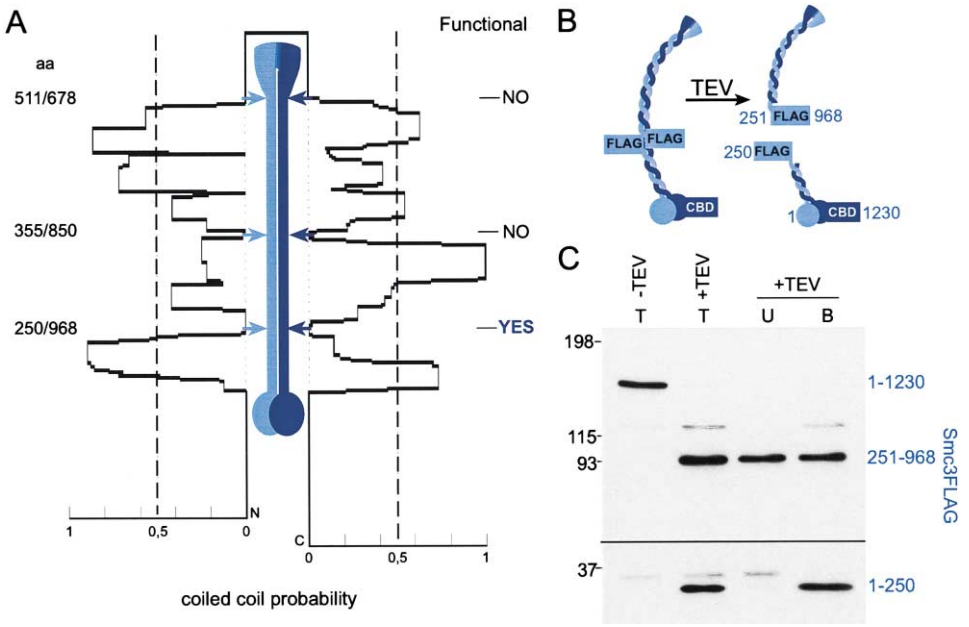

Figure 3. Generation of a Cleavable Smc3 Protein

(A) Coiled-coil probability for the primary sequence of *S. cerevisiae* Smc3 was predicted using PAIRCOIL (Berger et al., 1995) and aligned along the intra-molecular coiled coil of Smc3. Target sequences for the TEV protease preceded by a FLAG epitope sequence have been inserted in pairs at the sites indicated by arrows, following the amino acid positions shown at the left side. The chitin binding domain (CBD) was fused to the carboxy termini of the three constructs. Only the insertions closest to the Smc3 head domain (250/968) produced a functional protein in vivo. Three copies of the TEV recognition sequence replaced the single copies for more efficient cleavage. This construct is referred to as Smc3(TEVs).

(B) Scheme for the cleavage of Smc3(TEVs).

(C) Cleared cell extract was prepared from yeast strain K10331 (*MATa*, Δ*smc3*, and *SMC3(TEVs)*) and Smc3(TEVs) protein was purified on chitin beads. Beads were incubated without or with recombinant TEV protease (−TEV or +TEV). Reactions were either loaded directly (total, T), or supernatant (unbound, U) was separated from the beads (B) and loaded individually. Samples were analyzed by Western blotting against the FLAG epitopes.

we created an Smc3 protein whose coiled coil could be cleaved by the TEV protease. Analysis of Smc3's primary sequence indicates several short stretches of low-coiled coil probability (gaps) within the two arms of the protein. We reasoned that recognition sites for the tobacco etch virus (TEV) protease might be inserted into these gaps and be accessible to the protease without perturbing the proper folding of the coils and disrupting Smc3 function (Figure 3A). Interestingly, the largest gaps on one strand of the coiled coil (light blue) coincide with those on its partner (dark blue), if we assume that SMC proteins form intra-molecular anti-parallel coiled coils (Haering et al., 2002). If it were possible to insert TEV sites into opposing gaps, then cleavage at both sites should fragment the coiled coil, leaving one half associated with Smc3's head domains and the other associated with its dimerization domain (Figure 3B).

We engineered TEV recognition sequences into the gaps closer to the head domain (aa positions 250 and 968), closer to the dimerization domain (aa positions 355 and 850), or directly at the dimerization domain (aa positions 511 and 678). TEV sites were preceded by FLAG epitopes to permit detection of cleavage products. For the purpose of affinity purification, we fused a chitin binding domain to Smc3's C terminus. When introduced into yeast, only the construct with TEV sites within the gaps closest to the head domain (aa 250 and 968; henceforth called Smc3(TEVs)) complemented a deletion of the endogenous *SMC3* gene in vivo (not shown). To test cleavage of Smc3(TEVs), the protein was affinity purified from yeast extracts on chitin beads and incubated in the presence of recombinant TEV protease. Cleavage was inefficient (not shown). However, we obtained efficient cleavage in both strands when we inserted at each position three instead of one copy of the TEV recognition sequences, as judged by the detection of aa 1–250 and aa 251–968 fragments on immunoblots (Figure 3C). The construct containing triple TEV sites also complemented the deletion of the endogenous *SMC3* gene under normal growth conditions.

Strikingly, about half of the Smc3(TEVs) cleavage fragment containing the dimerization domain (aa 251–968) was released from the chitin beads during TEV treatment (Figure 3C, unbound), while all of the smaller N-terminal fragment (aa 1–250) remained bound to the beads, presumably through its interaction with the C-terminal fragment containing the chitin binding domain (Figure 3C, bound). We conclude that proteolytic cleavage of Smc3(TEVs) at sites 250 and 968 does indeed cleave the coiled coils into two fragments. The portion of the large Smc3(TEVs) cleavage product (aa 251–968) that remains bound to the beads presumably does so due

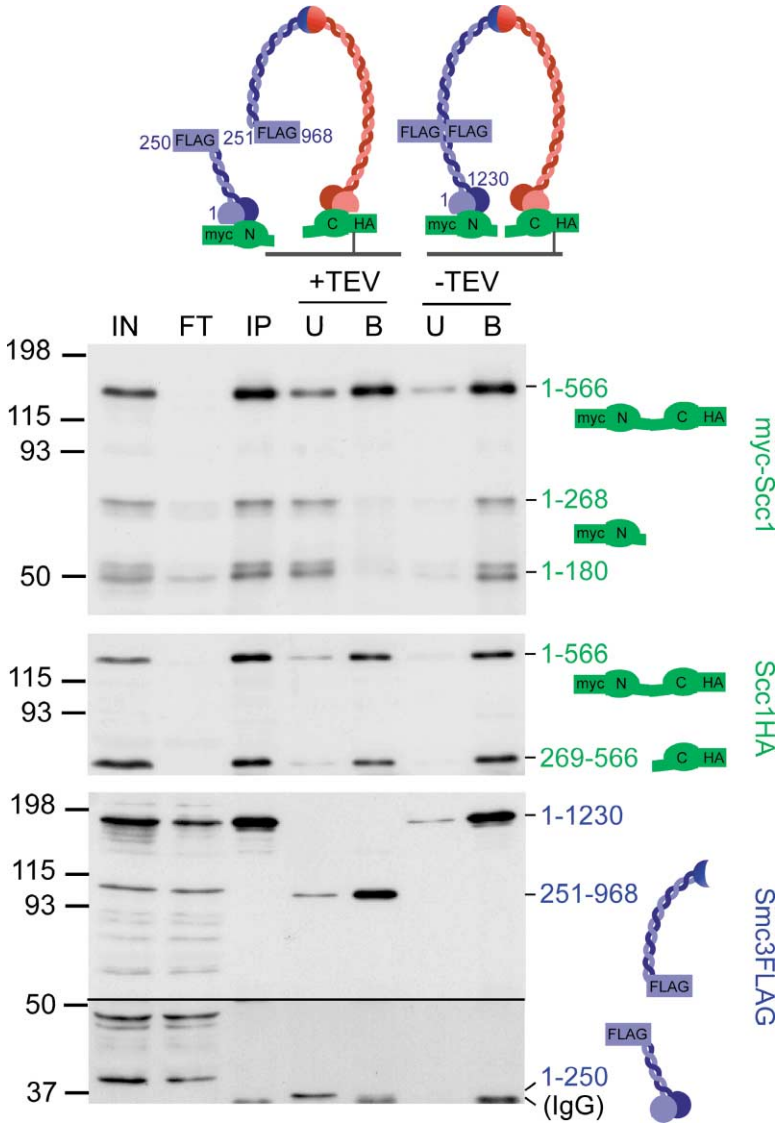

Figure 4. Coimmunoprecipitation of Scc1 Fragments Depends on Intact Smc3

Yeast strain K10632 (*MATa, myc$_9$-SCC1-HA$_6$*, Δ*cdc20*, *GAL1-10-Cdc20*, Δ*smc3*, and *SMC3(TEVs)*) was arrested in metaphase and Cdc20 expression was induced by readdition of galactose as in Figure 2. Cleared extracts were prepared 15 min after Cdc20 induction (IN) and used in immunoprecipitations against the HA-epitope at the carboxyl terminus of Scc1 (FT, flowthrough). Immunoprecipitation beads were aliquoted into three parts. One aliquot was loaded directly onto immunoblots (IP), while the other two aliquots were incubated with or without TEV protease and supernatant (unbound, U) was separated from the beads (B) before loading. Immunoblots were probed against the myc-, HA-, and FLAG-epitopes.

to its linkage to the smaller C-terminal cleavage product (aa 969–1230) via Smc1 and Scc1 (see below). These results confirm that the Smc3 protein does indeed form intra-molecular coiled coils under physiological conditions in vivo.

Linkage of N- and C-Terminal Scc1 Cleavage Fragments Depends on Integrity of the Smc1/Smc3 Heterodimer

With a cleavable Smc3 protein in hand, we were now in a position to address whether coimmunoprecipitation of Scc1's N- and C-terminal separase cleavage fragments depends on the integrity of Smc3's coiled coil. We created a strain that expresses both Smc3(TEVs) and the N- and C terminally tagged Scc1 and prepared extracts from cells collected 15 min after Cdc20 induction. Cohesin was immunoprecipitated with antibodies against the HA$_6$ epitope at Scc1's C terminus and aliquots of the immunoprecipitation beads were subsequently incubated in the presence or absence of recombinant TEV protease. Protein released from the beads (unbound-U) was separated from protein bound to the beads (bound-B).

Remarkably, both N-terminal myc tagged Scc1 separase cleavage fragments (aa 1–268 and 1–180) were completely released from the beads after incubation with TEV protease but remained tightly bound when incubated in cleavage buffer alone (Figure 4, top image). The HA tagged C-terminal Scc1 fragment, in contrast, remained bound independently of whether TEV protease was present (middle image). Crucially, the central Smc3(TEVs) fragment (aa 251–968) largely remained bound to the beads while the small Smc3(TEVs) N-terminal fragment (aa 1–250) was completely released (bottom image). These results are consistent with the ring model, which predicts that following cleavage of both Scc1 and Smc3 the central Smc3(TEVs) fragment (aa 251–968) should remain associated with the C-terminal Scc1 fragment due to its association with Smc1, while the head-containing Smc3(TEVs) fragment (aa 1–250) should be released together with the N-terminal Scc1 fragment. It is

remarkable that neither cleavage of Smc3 nor that of Scc1 alters the interactions made by subdomains of these proteins with each other or with Smc1.

In Vitro Cleavage of Scc1 Releases Cohesin from Chromatin with N- and C-Terminal Fragments Bound to Smc3 and Smc1 Heads, Respectively

To confirm these conclusions in a more defined system in vitro, we investigated whether N- and C-terminal Scc1 cleavage fragments produced by cleavage of cohesin bound to chromosomes in vitro are released from chromatin, even though both are bound to the two heads of an Smc1/Smc3 heterodimer. To do this, we used a yeast strain expressing an N- and C terminally tagged Scc1 protein (myc_9-Scc1(TEVs)-HA_3), one of whose separase cleavage sites has been replaced by the recognition sequence for the TEV protease (Uhlmann et al., 2000). We prepared chromatin from cells arrested in a metaphase-like state after incubation for several hours in nocodazole and incubated aliquots of the chromatin pellets either with or without recombinant TEV protease. Chromatin pellets (CP) were then separated from the soluble supernatant (SO) by centrifugation. While uncleaved Scc1 remained associated with chromatin, most of the amino-terminal (Figure 5A) and carboxy-terminal (not shown) TEV cleavage fragments of Scc1 were released into the soluble fraction. Cleavage of Scc1 is therefore sufficient to release both its N- and C-terminal cleavage fragments from chromatin.

We next asked whether the Scc1 fragments released from chromatin in this manner are linked to each other due to their attachment to the same Smc1/Smc3 heterodimer. We prepared chromatin from nocodazole-arrested cells that expressed myc_9-Scc1(TEVs)-HA_3 either with or without Smc3(TEVs) and incubated it with recombinant TEV protease. Scc1 cleavage was efficient and caused release of the myc-tagged N-terminal fragment from chromatin (Figure 5B). Cleavage of Smc3(TEVs) was less efficient. Less than one quarter of the molecules were cleaved at both sites at this stage (see IN Figure 5C). The solubilized proteins were then immunoprecipitated in the presence of TEV protease using antibodies against the HA_3 tag on Scc1's C terminus. Cleavage at both sites of Smc3(TEVs) was largely completed during the immunoprecipitation (see IP Figure 5C). Scc1's C-terminal fragment (aa 269–566) was efficiently pulled down (Figure 5C). Strikingly, the myc tagged N-terminal fragment was coimmunoprecipitated when Smc3 remained intact, but not when Smc3(TEVs) had been cleaved by the TEV protease. The central Smc3(TEVs) fragment containing its dimerization domain (aa 251–968) was largely bound to the immunoprecipitation beads, presumably due to interaction with Smc1 bound to the C-terminal Scc1 fragment. We conclude that N- and C-terminal Scc1 cleavage fragments are connected via the Smc1/Smc3 heterodimer after their cleavage from chromatin in vitro as well as in vivo. Because these reactions take place at rather low protein concentrations, it is inconceivable that coprecipitation of N- and C-terminal fragments could arise from rearrangements following their release from chromatin. The linkage must therefore reflect the state of cohesin bound to chromatin before its cleavage.

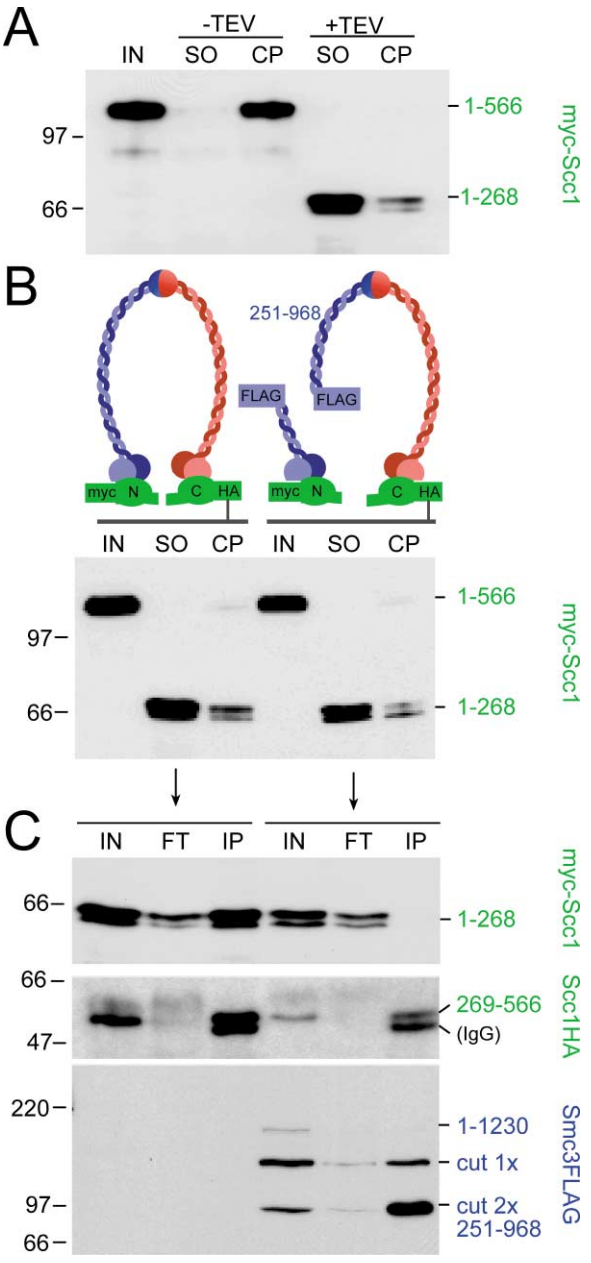

Figure 5. Scc1 Cleavage Fragments Are Linked by the Smc1/Smc3 Heterodimer in Cohesin Released from Chromatin In Vitro

(A) In vitro cleavage of Scc1 releases it from chromatin. Chromatin was prepared from cells of strain K10428 (*MAT*α, Δ*scc1*, and *myc_9-SCC1(TEVs)-HA_3*) arrested in metaphase with nocodazole. Chromatin was incubated with or without recombinant TEV protease. Soluble fraction (SO) and chromatin pellets (CP) were separated and analyzed by Western blotting against the myc-epitope at the N terminus of Scc1.

(B) Strains K10428 and K10427 (*MAT*α, Δ*scc1*, *myc_9-SCC1(TEVs)-HA_3*, Δ*smc3*, and *SMC3(TEVs)*) were arrested with nocodazole, chromatin pellets were prepared and incubated with TEV protease to release the cohesin complex from chromatin (CP) into solution (SO). Efficient cleavage of Scc1(TEVs) on chromatin and release from chromatin is shown by probing against the myc-epitope on Scc1.

(C) Chromatin-released fractions were used in immunoprecipitations against the HA-epitope at the carboxyl terminus of Scc1 and coimmunoprecipitation of the N-terminal Scc1 and Smc3 cleavage fragments was analyzed by probing for myc-, HA- and FLAG-epitopes.

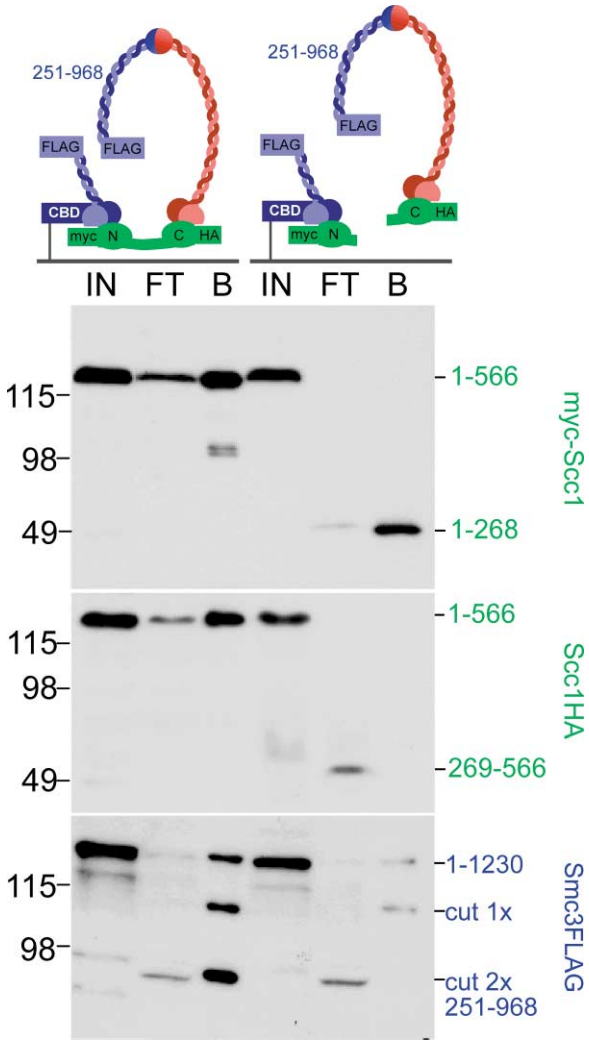

Figure 6. The N-Terminal Scc1 Cleavage Fragment Is Bound to the Head of Smc3

Extracts were prepared from asynchronously growing yeast strains K10470 (*MAT*α, *myc*$_9$*-SCC1-HA*$_3$, Δ*smc3*, and *SMC3(TEVs)*) and K10427 and incubated with recombinant TEV protease. The C-terminal Smc3 fragment was bound to chitin beads and copurification of Scc1 (fragments) and Smc3 fragments in input (IN), flowthrough (FT), and chitin bound (B) fractions were probed with antibodies against the myc-, HA- and FLAG-epitopes as illustrated.

Smc3's Heads and Dimerization Domains Are Linked by Scc1 Following Cleavage of Its Coiled Coil

The ring model not only predicts that Scc1 fragments should be held together by virtue of their attachment to the heads of the same Smc1/Smc3 heterodimer, it also predicts that the two halves of an Smc3 molecule whose coiled coil has been cleaved by TEV should be held together by an intact molecule of Scc1. To test this prediction, we prepared soluble extracts from asynchronous cultures of yeast strains expressing Smc3(TEVs) along with either myc$_9$-Scc1-HA$_3$ or myc$_9$-Scc1(TEVs)-HA$_3$. We affinity purified the C terminus of Smc3(TEVs) on chitin beads while simultaneously cleaving with recombinant TEV protease. Uncleaved Scc1 (aa 1–566; Figure 6A, top and middle left) copurified with the Smc3 head as did the myc-tagged N-terminal TEV cleavage fragment produced from Scc1(TEVs) (aa 1–268; Figure 6, top right). The HA$_3$-tagged C-terminal fragment of Scc1(TEVs) (aa 269–566), in contrast, did not bind to the chitin beads (Figure 6, middle right). The central fragment of Smc3(TEVs) (aa 251–968) produced by cleavage of both TEV sites copurified with its chitin binding head when Scc1 remained intact but not when Scc1 was also cleaved by the TEV protease (Figure 6C, bottom image). This demonstrates a connection between Smc3's head and its dimerization domain independent of its coiled coil, a connection that is dependent on the integrity of Scc1. Thus, not only are Scc1 cleavage fragments linked in an Smc3 dependent manner but also Smc3's head and dimerization domains are linked in an Scc1 dependent manner. Because this series of experiments was performed with soluble complexes, it is likely that cohesin forms a ring even when not bound to chromosomes.

Cleavage of Smc3(TEVs) Releases Cohesin from Chromatin

Might the trapping of chromosomal DNA within cohesin's ring be responsible for its tight association with chromosomes? If so, opening of the ring at any position should release cohesin from chromatin. Cleavage of Scc1 by separase clearly does this (Uhlmann et al., 1999), but what about cleavage of Smc3? To test this, TEV protease was expressed from the *GAL1-10* promoter in yeast strains bearing either Smc3 with TEV sites only within its N-terminal helical strand (aa 250), only within its C-terminal helical strand (aa 968), within both strands (aa 250 and 968; i.e., Smc3(TEVs)), or wild-type Smc3. Strains with wild-type Smc3 and with TEV sites in only one of Smc3's helical strands formed normal sized colonies on medium-containing galactose (Figure 7A). In contrast, most cells of the strain with TEV cleavage sites in both strands were unable to form colonies on galactose medium and ceased dividing soon after TEV induction. To check whether Smc3 proteins with TEV sites in only one strand were efficiently cleaved in vivo, we prepared extracts from cells of strains with and without the *GAL1-10 TEV* construct grown in the presence of galactose. Smc3 protein was affinity purified from extracts using the C-terminal chitin binding domain. Immunoblotting showed that the Smc3 protein was efficiently cleaved at either aa250 or aa968 site (Figure 7B). We conclude that Smc3 remains functional when cleavage of either one or the other strand of its coiled coil is cleaved, i.e., when it is nicked, but not when both are cleaved, i.e., when the coiled coil is completely severed. Remarkably, neither insertion of more than 30 amino acids containing TEV sites into Smc3's helical axis nor the "nicking" of just one strand greatly interfered with Smc3's function in vivo. Smc3's coiled-coil should therefore be rather viewed as an elastic chain than a stiff force-transmitting pole.

We next used chromosome spreads to measure association of cohesin's Scc1 subunit (HA$_3$ tagged) with chromatin after cleavage of Smc3(TEVs) in vivo. Cells were first arrested in a metaphase-like state with nocodazole and two hours later galactose was added to induce TEV protease expression from the *GAL1-10* promoter.

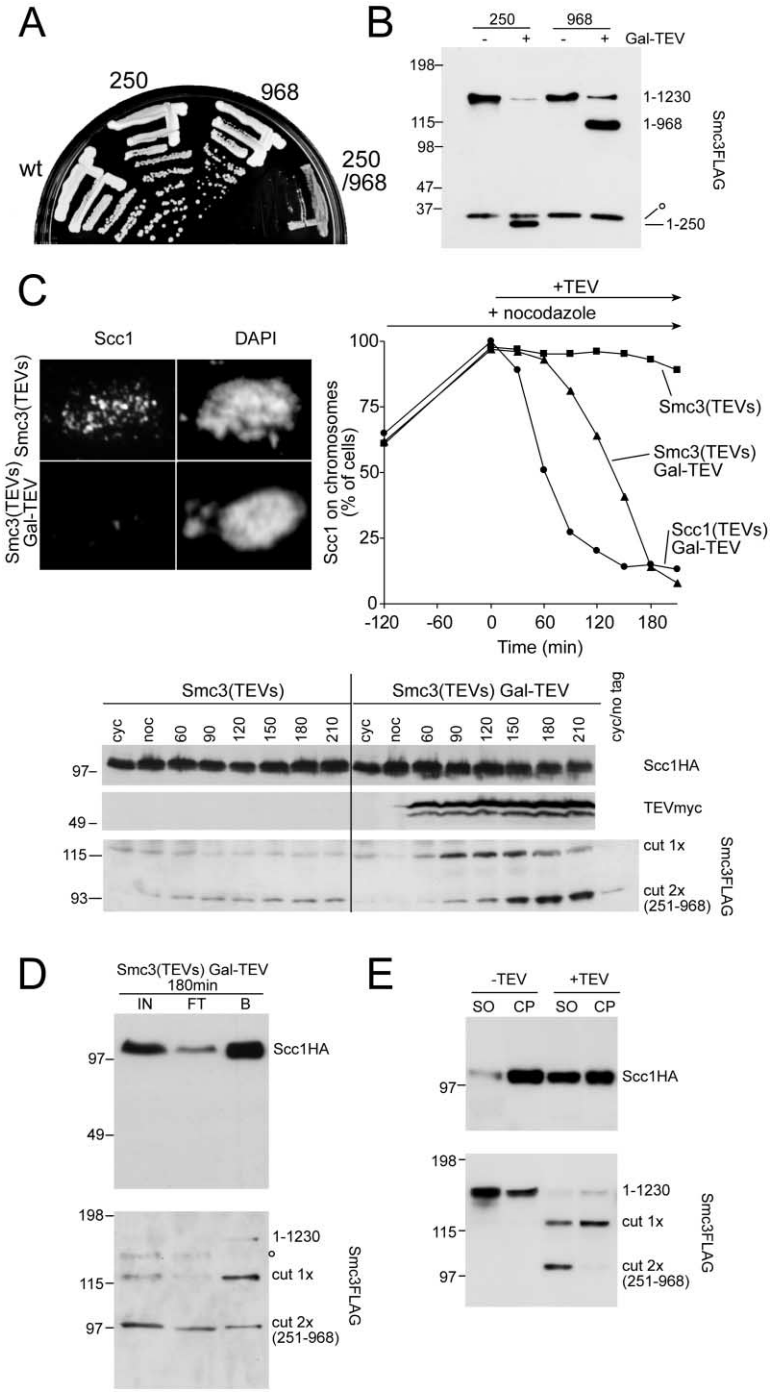

Figure 7. Cleavage of the Smc3 Coiled Coil Releases the Cohesin Complex from Chromatin

(A) In vivo cleavage of both Smc3 coiled coil strands is lethal. Yeast strains K9872 (*MATa* and *GAL-NLS-myc$_9$-TEV-NLS2x10*), K10783 (*MATa*, *Δsmc3*, *SMC3(nTEVs)*, and *GAL-NLS-myc$_9$-TEV-NLS2x10*), K10784 (*MATa*, *Δsmc3*, *SMC3(cTEVs)*, and *GAL-NLS-myc$_9$-TEV-NLS2x10*) and K10874 (*MATa*, *Δsmc3*, *SMC3 (TEVs)*, and *GAL-NLS-myc$_9$-TEV-NLS2x10*) were streaked on YEP plates containing 2% raffinose and galactose.

(B) Cells of strains K10738 (*MATα*, *Δsmc3*, *SMC3(nTEVs)*), K10783, K10739, *MATa*, *Δsmc3*, and *SMC3(cTEVs)*) and K10784 were grown in YEP medium containing 2% raffinose. Expression of TEV protease was induced by addition of 2% galactose for 6 hr. Extracts were prepared and incubated with chitin beads. Proteins were eluted from the beads and analyzed for the FLAG epitope tag on Smc3. A flag crossreacting band is marked by "°".

(C) Strains K8758 (*MATα, Δscc1*, *SCC1(TEVs)-HA$_3$*, and *GAL-NLS-myc$_9$-TEV-NLS2x10*), K10375 (*MATα*, *Δsmc3*, *SMC3(TEVs)*, and *Scc1-HA$_3$*) and K10336 (*MATa*, *Δsmc3*, *SMC3(TEVs)*, *GAL-NLS-myc$_9$-TEV-NLS2x10*, and *Scc1-HA$_3$*) were grown in YEP medium containing 2% raffinose and arrested in metaphase with nocodazole for two hours. Then, 2% galactose was added to induce the expression of TEV protease in cells of strains K8758 and K10336. Aliquots of the cultures were harvested and analyzed by chromosome spreading. The pictures shown are from cells at 3 hr after induction of TEV expression by galactose addition. Western blots against HA on Scc1, myc on TEV protease and FLAG on Smc3(TEVs) was performed on whole-cell extracts from cells taken during the time course. The double cleaved Smc3(TEVs) band comigrates with a FLAG-crossreacting band in the yeast extracts (see no tag control).

(D) Soluble extracts from cells of strain K10336 at three hours after induction with galactose were prepared and incubated with chitin beads. Pull down efficiency of Scc1 by the chitin binding domain on Smc3 was determined by Western blotting. A flag crossreacting band in the yeast crude extract is marked by "°".

(E) Strain K10375 was grown in YPD and arrested for two hours in nocodazole. Chromatin was purified from these cells and incubated with and without recombinant TEV protease. Supernatant and chromatin pellet were collected and analyzed on Western blots.

In cells lacking the *GAL-TEV* expression cassette, Scc1 remained bound to chromatin for at least three hours after mock induction (Figure 7C). In contrast, more than 90% of the cells expressing TEV protease lost Scc1 staining on chromosome spreads within this time period. The loss of Scc1 from chromosome spreads cannot be due to degradation of the protein, because immunoblotting showed that the total amount of Scc1 protein in whole-cell extracts remained constant throughout the experiment (Figure 7C). Crucially, disappearance of Scc1 from chromosomes coincided with the appearance of the doubly cleaved aa 251–968 Smc3 fragment. Dissociation of Scc1 caused by cleavage of Smc3(TEVs) was slower than that caused by cleavage of Scc1 with TEV (Figure 7C), presumably because two cuts are necessary in the case of the former.

To test whether cohesin's dissociation from chromatin might be due to disassembly of the complex caused by Smc3's cleavage in vivo, we affinity purified Smc3's head domain on chitin beads from soluble extracts pre-

pared 180 min after TEV protease induction. Both Scc1 and the central aa 251–968 fragment containing Smc3's dimerization domain copurified with its head (Figure 7D). This implies that interactions between cohesin subunits remain largely unperturbed by severance of Smc3's coiled coil also in vivo. A fraction of Smc3's central aa 251–968 fragment was not bound to the chitin matrix, presumably because a portion of the Smc1/Smc3 heterodimer is not bound to Scc1 (see Figure 3C).

To investigate whether cleavage of Smc3(TEVs) also releases cohesin from chromatin in vitro, we incubated a crude chromatin fraction prepared from nocodazole-arrested cells in the presence or absence of recombinant TEV protease. Cleavage of Smc3(TEVs) caused dissociation of all of the doubly cleaved aa 251–968 Smc3 fragment and about half of Scc1HA_3 from the chromatin pellets (Figure 7E), which corresponds to the fraction of Smc3(TEVs) that had been doubly cleaved. In summary, cleavage of Smc3's coiled coil dissociates cohesin from chromatin in vitro as well as in vivo.

Cleavage of Smc3's Coiled Coil Destroys Sister Chromatid Cohesion

Cleavage of cohesin's Scc1 subunit destroys cohesion between sister chromatids and is sufficient to trigger metaphase-arrested cells to undergo anaphase (Uhlmann et al., 2000). To address whether cleavage of Smc3 also disrupts cohesion, we used yeast strains in which the TEV protease could be induced from the *GAL1-10* promoter. Their sole source of Cdc20 was under the control of the methionine repressible *MET3* promoter, which allowed us to arrest cells in metaphase by shifting them into medium supplemented with methionine and then to induce TEV by addition of galactose. These strains contained TEV sites either in Scc1 (Scc1(TEVs)) or in Smc3 (Smc3(TEVs)).

Separation of sister chromatids was monitored by visualizing DNA sequences at the *URA3* locus 35 kb away from *CEN5* marked by the green fluorescent protein (GFP) (Michaelis et al., 1997). Cells with TEV cleavable Scc1 arrested in metaphase as large-budded cells with a single DNA mass at the bud neck. Sister chromatids are so close together that they appear as a single green spot. Upon induction of TEV protease, more than 90% of the cells separated sister chromatids, as seen by the appearance of two GFP dots within a single cell (Figure 8A). Cells with TEV cleavable Smc3 also arrested in metaphase as large-budded cells with a single DNA mass at the bud neck. However, in about 25% of the arrested cells, two GFP dots were seen within a single nucleus. This indicates that cells containing Smc3(TEVs) have a modest cohesion defect. The fraction of cells in this state did not further increase during the arrest as long as TEV protease was not expressed. In contrast, induction of TEV protease caused the fraction of cells with separated sister DNA sequences to rise from 25 to 75% (Figure 8A). Induction of TEV protease under these conditions did not cause sister chromatid separation in cells whose cohesin lacks any TEV sites.

In contrast to cells with a TEV-cleavable Scc1 protein (Uhlmann et al., 2000), those with TEV-cleavable Smc3 did not complete anaphase chromosome segregation. The DNA masses of Smc3(TEVs) cells rarely segregated to opposite poles of the cell (Figure 8A). Smc3 cleavage did cause stretching of chromosomal DNA within the bud neck and disassembly of mitotic spindles (Figure 8B), which is presumably due to spindle elongation in the absence of APC/C function (Uhlmann et al., 2000; Severin et al., 2001). Spindle instability as well as incomplete and asynchronous double cleavage may therefore be responsible for the poor chromosome segregation in Smc3(TEVs) cells.

Discussion

Cohesin Forms an Asymmetric Ring Containing One of Each of Its Subunits

We show here that the preferential association of Scc1's N-terminal fragment with Smc3's head and its C-terminal one with that of Smc1 is conserved in its meiotic counterpart Rec8. Scc1 and Rec8 share very little sequence homology except within their first and last 100 amino acids, which are generally conserved amongst Scc1 and Rec8 homologs from a wide variety of eukaryotes. These conserved terminal sequences must contain the SMC head interaction domains because fragments containing the first 115 amino or the last 115 amino acids of Scc1 formed complexes specifically with Smc3 or Smc1, respectively. There is no apparent similarity between Scc1's Smc3 binding N-terminal 115 amino acids with its Smc1 binding 115 C-terminal ones. This asymmetry presumably corresponds to an asymmetry of the Smc1/3 heterodimer's two heads, to which these sequences bind. The recent finding that these N- and C-terminal domains of Scc1 and Rec8 are homologous to those of ScpA proteins in bacteria and barren subunits of condensin (Schleiffer et al., 2003) implies that the heads of all SMC-like proteins may be connected in a similar manner. It is for this reason that we suggest that members of this family are called kleisins (from the Greek word for closure: kleisimo). Cohesin's asymmetry is presumably shared by condensin, which contains an Smc2/Smc4 heterodimer but contrasts with the symmetry of bacterial SMC proteins. If the latter bind the ScpA, as is currently suspected, then the molecular symmetry of the SMC dimer would predict that they bind at least two molecules of ScpA in a symmetric fashion.

This image of Scc1-SMC interactions, inferred merely from co-expression of different subunits and their fragments in insect cells, is in full agreement with our analysis of cohesin released from yeast chromosomes after cleavage by separase in vivo. Thus, both Scc1's N-terminal cleavage fragment and a fragment containing Smc3's head domain are released from the rest of separase-cleaved cohesin by severance of Smc3's coiled coil, while the C-terminal Scc1 fragment remains associated with the central domain severed from its heads, presumably due to the latter's association with Smc1. Soluble yeast cohesin appears to possess the same fundamental geometry; namely Scc1's N-terminal half is connected with Smc3's head while Scc1's C-terminal half is connected to Smc3′s central domain via its interaction with Smc1.

The ring hypothesis postulates that Scc1, Smc1 and Smc3 form a triangle and predicts that all three subunits and fragments therefore should remain associated with

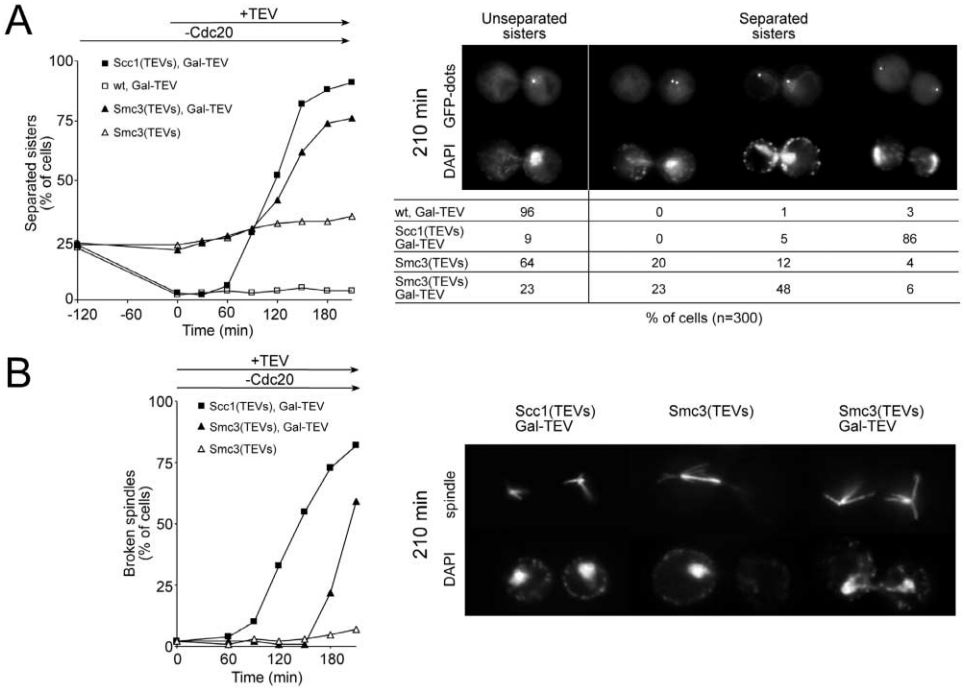

Figure 8. Cleavage of Smc3 Destroys Cohesion between Sister Chromatids

Cells of strains K9027 (*MAT*α, *Met3-Cdc20*, Δ*scc1*, *SCC1(TEVs)-HA*$_3$, *GAL-NLS-myc*$_9$*-TEV-NLS2x10*, *tetOs*, and *tetR*), K9128 (*MATa*, *Met3-Cdc20*, *GAL-NLS-myc*$_9$*-TEV-NLS2x10*, *tetOs*, and *tetR*), K10429 (*MAT*α, *Met3*-Cdc20, Δsmc3, SMC3(TEVs), GAL-NLS-myc$_9$-TEV-NLS2x10, tetOs, and tetR) and K10611 (*MAT*α, *Met3-Cdc20*, Δ*smc3*, *Smc3(TEVs)*, *tetOs*, and *tetR*) were grown in synthetic medium lacking methionine. Cultures were arrested in metaphase by shifting cells to YEPD media supplemented with 2 mM methionine. After 2.5 hr, 2% galactose was added to induce expression of TEV protease. Samples were taken every 30 min after induction of TEV expression during the time course.
(A) Separation of sister chromatids was monitored by looking at separation of GFP dots on the arm of chromosomes. Cells from 210 min after TEV induction were put into four categories according to the positions of sister chromatids within the cell.
(B) Spindles were visualized by immunostaining against tubulin using monoclonal antibody YOL1/34 (Serotec). Sample pictures shown are from 210 min after induction of TEV protease.

each other when any single side of the triangle has been broken irrespective of where this break is. Our finding that Smc3 holds together N- and C-terminal cleavage fragments of Scc1 while Scc1 holds together the two halves of Smc3 when its arm has been severed by double cleavage with the TEV protease clearly confirms this prediction. It is remarkable that breakage of any one the triangle's sides destroys its closure and hence its potential for trapping DNA without affecting its three corners, i.e., subunit interactions.

Though these findings together with the electron micrographs of soluble cohesin (Anderson et al., 2002) are consistent with cohesin being an asymmetric monomeric complex, they do not by themselves rule out the possibility that it forms dimers when bound to chromatin, especially after DNA replication when cohesin complexes on different sister chromatids could in principle interact to generate cohesion. Available evidence is, however, inconsistent with this notion. For example, differently tagged versions of the same cohesin subunit cannot be coimmunoprecipitated from cohesin released from chromatin by micrococcal nuclease digestion (Haering et al., 2002). One specific dimer model would have it that the two Scc1 molecules within a cohesin dimer connect Smc1 and Smc3 heads not from the same but from different heterodimers. If this were so, then one might predict that cleavage of just one of the Scc1 molecules might be sufficient to release cohesin dimers from chromatin. We found that TEV protease treatment of chromatin from a heterozygous diploid strain carrying wild-type and TEV cleavable Scc1 released Scc1 fragments but not intact Scc1 (data not shown). We therefore favor the notion that both soluble and chromatin bound cohesin form an asymmetric monomeric complex.

Does the Cohesin Ring Embrace Chromosomal DNA?

An important but yet unresolved issue is the mechanism by which the cohesin and other SMC protein-containing complexes bind to chromosomes. One model predicts that each of the two SMC head domains binds to one sister chromatid (Anderson et al., 2002). It has been suggested on the basis of electron spectroscopic images of condensin complexes bound to DNA that the double helix might be wrapped around the SMC head domains (Bazett-Jones et al., 2002). It is, however, unclear from the existing crystal structure of these domains (Lowe et al., 2001) how DNA could conceivably be wrapped round a domain whose dimensions are much smaller than those of a nucleosome.

Our finding that chromosomal cohesin forms a closed ring raises the possibility that cohesin's interaction with DNA is topological and not chemical in nature. By passing through cohesin's ring, chromosomal DNA could be topologically trapped by cohesin (Haering et al., 2002).

This model makes no prediction as to the actual path of DNA as it passes through cohesin's ring and does not exclude the possibility of DNA being wrapped around the ring as opposed to simply passing once through it. A topological interaction of this nature would explain the resistance to high salt concentrations of cohesin's association with chromosomes (Ciosk et al., 2000) as well as its resistance to DNA intercalating agents such as ethidium bromide (data not shown). It would also explain why the ring's severance, be it within Scc1 or Smc3, is sufficient to release cohesin from chromosomes either in vitro or in vivo without apparently affecting any of cohesin's subunit interactions. If cohesin embraces chromosomal DNA in this manner, then sister chromatid cohesion could arise from the passage of sister DNA molecules through the same cohesin ring. Sister DNA molecules could also conceivably pass through different but interacting cohesin rings; though this would predict hitherto undetected interactions between cohesin complexes. Further experiments will be required to establish whether DNA really passes inside cohesin's ring.

Though our experiments are consistent with DNA's passage through cohesin's ring, they shed little or no insight into the mechanism by which transient opening of the ring permits entry of the double helix. Such a process would be analogous to the entry of a climbing rope into a carabiner, which is a ring with a gate. In this model, Scc1 could be considered cohesin's gate and the binding of ATP to the SMC head domains and/or its hydrolysis might regulate the opening and shutting of cohesin's gate. A key question is whether Scc1 connects the head domains of Smc1 and Smc3 when they are bound to ATP and have thereby themselves "dimerized" (Hopfner et al., 2000) or whether it only connects the two heads after ATP has been hydrolyzed. In the latter case, cohesin could switch between two types of ring: one in which the heads are bound to each other but the Scc1 gate is open and another in which Scc1 alone connects the heads. Such a system would permit DNA strands to enter cohesin's ring without pre-existing strands exiting. Even if this hypothesis is correct, it remains a mystery how some gates can apparently be reopened without destroying Scc1, as presumably occurs during prophase in metazoan cells, while others can only be opened by cleavage of Scc1 at anaphase onset (Waizenegger et al., 2000).

Given the similar structure of Scc1, barren, ScpA, and the other members of the kleisin superfamily, it is hard not to believe that all complexes composed of SMC proteins operate using a similar topological principle to that proposed for cohesin, namely passage of DNA inside two arms held together by Scc1-like bridges (kleisins), which can be opened and shut. Such devices are presumably indispensable for regulating the packing of DNA within cells because unlike nucleosomes, they clearly existed in the common ancestor of all life forms on this planet and have been retained in almost all organisms ever since. Cohesin may be unique in holding sister DNAs within a single ring whereas condensin may have the ability to pass the same DNA molecule more than once through its ring, thereby acting as a DNA coil securing device.

Experimental Procedures

Yeast Strains

All strains are derivatives of W303. Genotypes used in the individual experiments are given in the figure legends.

Binding Assays of Baculovirus-Expressed Cohesin Subunits

DNA sequences encoding parts of yeast genes *SCC1* or *REC8* were cloned by PCR into the Bac-to-Bac (Gibco Life Technologies) pFASTBAC1 baculovirus expression vector adding His_6 epitopes at the indicated positions. Plasmids for expression of HA_3Smc1 or HA_3Smc3 and generation of recombinant baculoviruses were described previously. Cohesin subunits were co-expressed by co-infecting HighFive insect cells with respective viruses and binding assays on Ni^{2+}-NTA were performed as explained before (Haering et al., 2002).

Coimmunoprecipitation of Scc1 Fragments Cleaved In Vivo

Yeast strains containing *GAL1-10-CDC20* Δ*cdc20* grown at 25°C to mid-log phase in YEP media containing 2% raffinose and 2% galactose (YEPRG) were collected by filtration, washed in YEP media containing 2% raffinose (YEPR), and arrested in YEPR for 4 hr. Synchronous Cdc20 expression was induced by adding back galactose to 2%. To prevent degradation of the carboxy-terminal Scc1 cleavage product by the N-end rule pathway, we initially used strains deleted for the *UBR1* gene. We then found that this fragment was stable even in an *UBR1* background when extracts were prepared 15 min after Cdc20 induction. Protein extracts were prepared by breaking $\sim 6 \times 10^5$ cells with glass beads in EBX buffer (50 mM HEPES-KOH [pH 7.5], 100 mM KCl, 2.5 mM $MgCl_2$, 1 mM DTT, and 0.25% Triton X-100) containing protease inhibitors. Extracts were cleared by centrifugation at 16,000 × g and 100,000 × g and precleared on protein G Sepharose before immunoprecipitation against the HA epitope with 16B12 monoclonal antibody (BAbCO).

Cloning of Smc3(TEVs) Constructs

The *SMC3* coding sequence plus 350bp upstream and 282bp downstream sequences were cloned into the integrative yeast vectors YIplac128 and YIplac211 (Gietz and Sugino, 1988). At the C terminus of the *SMC3* open reading frame a chitin binding domain was added (New England Biolabs). Overlap extension PCR was used to insert following pairs of peptide sequences containing an FLAG tag and a TEV cleavage site into the *SMC3* coding sequence: 250/968: 250D-YKDDDDK-AS-ENLYFQ-251G/968D-YKDDDDK-TS-ENLYFQG-969F; 355/850: 355I-DYKDDDDK-AS-ENLYFQG-356L/850D-YKDDDDK-TS-ENLYFQG-851L; and 511/678: 511M-DYKDDDDK-AS-ENLYFQG-512S/678D-YKDDDDK-TS-ENLYFQG-679Q. Constructs were transformed into strain K10165 (*MAT a/α, SMC3/Δsmc3*). Stable transformants were sporulated, tetrads were dissected, and individual spores checked for their viability on YEPD plates. The Smc3 (TEVs) construct complemented the deletion of the endogenous Smc3 gene at 25°C and 30°C but not at 37°C. The Smc3 construct containing triple TEV sites was created by insertion of DNA oligos into the existing single TEV sites: the final sequence was: 250D-YKDDDDK-AS-ENLYFQG-PR-ENLYFQG-AS-ENLYFQ-251G/968D-YKDDDDK-TS-ENLYFQG-PR-ENLYFQG-AS-ENLYFQG –969F. Constructs with triple TEV sites in only one of the coiled-coil strand (Smc3(nTEVs) and Smc3(cTEVs)) were produced by replacing the region with TEV insertions by wild-type Smc3 sequences.

Purification of Cohesin from Soluble Yeast Extracts Using Chitin Beads

200 ml culture of Smc3(TEVs) strains were grown in YEPD medium at 25°C, and optionally arrested in nocodazole for two hours. Cells were harvested and protein extracts were prepared by spheroblasting and detergent lysis (Liang and Stillman, 1997). Extracts were cleared by centrifugation. A total of 350 μl of cleared extract was loaded onto 75 μl chitin beads equilibrated in buffer EBX and 10 μl rTEV protease (Invitrogen) was added. For efficient binding and cleavage of Smc3, chitin beads were rotated for 12 hr at 4°C. Supernatant was collected by centrifugation, and beads were washed three times in 500 μl EBX. Alternatively, Smc3(TEVs) was first purified and then cleaved on beads by TEV protease; cell lysates were sup-

plemented by 1 μg/ml DNaseI (Roche Mol. Biochem.) and kept on ice for ten minutes. After clearing the lysates by centrifugation, 350 μl extracts were loaded onto 75 μl chitin beads. Binding was performed by rotating the beads in extracts for two hours at 4°C. Beads were washed three times in 1 ml EBX and once in 1 ml TEV buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.5 mM EDTA, and 1 mM DTT). Beads were divided into three aliquots and resuspended in 30 μl TEV buffer. Cleavage was performed by adding 2 μl rTEV protease and incubating for 2.5 hr at 16°C.

Cleavage of Scc1(TEVs) and Smc3(TEVs) on Chromatin Fractions

Crude chromatin fractions from nocodazole-arrested cells containing myc_9-Scc1(TEVs)-HA_3 were prepared as described (Liang and Stillman, 1997). Chromatin obtained from 100 μl cell lysate was washed once in 100 μl EBX and resuspended in 50 μl TEV buffer. Scc1(TEVs) was cleaved by adding 2 μl of rTEV protease and incubating at 25°C for 20 min. Chromatin was separated form supernatant by centrifugation and washed once in 100 μl TEV buffer. For immunoprecipitation experiments, chromatin from 800 μl crude yeast lysate was prepared in eight aliquots. After washing, chromatin was resuspended in 500 μl TEV buffer and incubated with 16 μl rTEV protease for 15 min at 25°C. Supernatant was recovered by centrifugation and precleared on protein G Sepharose. The supernatant was then supplemented by 5 μl of 16B12 ascites fluid (BAbCO) and incubated for 1 hr at 4°C before the addition of 150 μl protein G Sepharose slurry. The immunoprecipitation was stopped after 1 hr by washing 3 times in 1 ml TEV buffer. To check release of Scc1 from chromatin by cleavage of Smc3(TEVs), cells having Smc3(TEVs) and a HA_3-tagged version of Scc1 were arrested in nocodazole and chromatin was prepared as above. Cleavage took place in 100 μl TEV buffer with or without 3 μl of rTEV protease at 25°C for 20 min.

Cleavage of Smc3(TEVs) by TEV Protease In Vivo

Smc3(TEVs) strains expressing the TEV protease gene from the *GAL1-10* promoter were generated by crossing to K9873 (*MAT*α, *GAL 1-10-NLS-myc*$_9$*-TEV-NLS2x10*). Viability of strains expressing TEV protease cleavable Smc3 and TEV protease from the *GAL1-10* promoter was tested by streaking the strains on YEPRG and incubating them at 25°C for 2 days. Efficiency of in vivo cleavage of Smc3(nTEVs) and Smc3(cTEVs) was determined 6 hr after addition of galactose to a culture of cycling cells. Lysates were prepared and TEV cleavable Smc3 proteins were purified via the chitin binding domain from DNaseI treated extracts as described above. To test the release of cohesin from chromatin by cleavage of Smc3, we crossed the strain K10331 to either K9127 or K8266 (*MAT*α, *Scc1-HA*$_3$) to get HA_3-tagged Scc1 strains with or without the TEV protease gene under the *GAL1-10* promoter. Cells of these strains were grown overnight in YEPR at 25°C. Exponentially growing cells were arrested in nocodazole for two hours and the expression of the TEV protease was induced by addition of 2% galactose. Samples were taken for chromosomal spreads and Western blotting analysis every 30 min after induction of TEV protease until the 210 min time point. At the 180 min time point, 200 ml of culture from the TEV protease expressing cells were harvested, extracts were prepared by spheroblasting and detergent lysis of the cells. Cohesin was purified from these extracts using the chitin binding domain on Smc3(TEVs) as described above. Strains K9128 and K10336 were crossed to obtain strain K10429 and K10611 used in the experiments. Cells were arrested in metaphase by depletion of Cdc20 and expression of TEV protease was induced.

Acknowledgments

We are grateful to Frank Uhlmann, whose unpublished data first indicated the release of cohesin from chromatin by in vitro cleavage of Scc1 with TEV protease and Alexander Schleiffer for communicating unpublished results on the homology of Scc1/Rec8. We thank Jan M. Peters and Michael Glotzer for critical reading of the manuscript. We also thank Doris Schoffnegger for help in creating recombinant Rec8 baculoviruses and the members of the Nasmyth laboratory for helpful discussions. This research was supported by Boehringer Ingelheim, the Austrian Industrial Research Promotion Fund (FFF) and the Austrian Science Fund (FWF).

Received: December 4, 2002
Revised: January 27, 2003

References

Anderson, D.E., Losada, A., Erickson, H.P., and Hirano, T. (2002). Condensin and cohesin display different arm conformations with characteristic hinge angles. J. Cell Biol. *156*, 419–424.

Bazett-Jones, D.P., Kimura, K., and Hirano, T. (2002). Efficient supercoiling of DNA by a single condensin complex as revealed by electron spectroscopic imaging. Mol. Cell *9*, 1183–1190.

Berger, B., Wilson, D.B., Wolf, E., Tonchev, T., Milla, M., and Kim, P.S. (1995). Predicting coiled coils by use of pairwise residue correlations. Proc. Natl. Acad. Sci. USA *92*, 8259–8263.

Buonomo, S.B., Clyne, R.K., Fuchs, J., Loidl, J., Uhlmann, F., and Nasmyth, K. (2000). Disjunction of homologous chromosomes in meiosis I depends on proteolytic cleavage of the meiotic cohesin Rec8 by separin. Cell *103*, 387–398.

Ciosk, R., Shirayama, M., Shevchenko, A., Tanaka, T., Toth, A., Shevchenko, A., and Nasmyth, K. (2000). Cohesin's binding to chromosomes depends on a separate complex consisting of Scc2 and Scc4 proteins. Mol. Cell *5*, 243–254.

Gietz, R.D., and Sugino, A. (1988). New yeast-*Escherichia coli* shuttle vectors constructed with in vitro mutagenized yeast genes lacking six-base pair restriction sites. Gene *74*, 527–534.

Guacci, V., Koshland, D., and Strunnikov, A. (1997). A direct link between sister chromatid cohesion and chromosome condensation revealed through analysis of MCD1 in *S. cerevisiae*. Cell *91*, 47–57.

Haering, C.H., Lowe, J., Hochwagen, A., and Nasmyth, K. (2002). Molecular architecture of SMC proteins and the yeast cohesin complex. Mol. Cell *9*, 773–788.

Hauf, S., Waizenegger, I., and Peters, J.M. (2001). Cohesin cleavage by separase required for anaphase and cytokinesis in human cells. Science *293*, 1320–1323.

Hopfner, K.P., Karcher, A., Shin, D.S., Craig, L., Arthur, L.M., Carney, J.P., and Tainer, J.A. (2000). Structural biology of Rad50 ATPase: ATP-driven conformational control in DNA double-strand break repair and the ABC-ATPase superfamily. Cell *101*, 789–800.

Klein, F., Mahr, P., Galova, M., Buonomo, S.B.C., Michaelis, C., Nairz, K., and Nasmyth, K. (1999). A central role for cohesins in sister chromatid cohesion, formation of axial elements, and recombination during yeast meiosis. Cell *98*, 91–103.

Liang, C., and Stillman, B. (1997). Persistent initiation of DNA replication and chromatin-bound MCM proteins during the cell cycle in cdc6 mutants. Genes Dev. *11*, 3375–3386.

Lim, H.H., Goh, P.Y., and Surana, U. (1998). Cdc20 is essential for the cyclosome-mediated proteolysis of both Pds1 and Clb2 during M phase in budding yeast. Curr. Biol. *8*, 231–234.

Losada, A., Hirano, M., and Hirano, T. (1998). Identification of *Xenopus* SMC protein complexes required for sister chromatid cohesion. Genes Dev. *12*, 1986–1997.

Lowe, J., Cordell, S.C., and van den Ent, F. (2001). Crystal structure of the SMC head domain: an ABC ATPase with 900 residues antiparallel coiled-coil inserted. J. Mol. Biol. *306*, 25–35.

Melby, T.E., Ciampaglio, C.N., Briscoe, G., and Erickson, H.P. (1998). The symmetrical structure of structural maintenance of chromosomes (SMC) and MukB proteins: long, antiparallel coiled coils, folded at a flexible hinge. J. Cell Biol. *142*, 1595–1604.

Michaelis, C., Ciosk, R., and Nasmyth, K. (1997). Cohesins: chromosomal proteins that prevent premature separation of sister chromatids. Cell *91*, 35–45.

Nasmyth, K. (2001). Disseminating the genome: joining, resolving, and separating sister chromatids during mitosis and meiosis. Annu. Rev. Genet. *35*, 673–745.

Rao, H., Uhlmann, F., Nasmyth, K., and Varshavsky, A. (2001). Degra-

dation of a cohesin subunit by the N-end pathway is essential for chromosome stability. Nature *410*, 955–959.

Schleiffer, A., Kaitna, S., Maurer-Stroh, S., Glotzer, M., Nasmyth, K., and Eisenhaber, F. (2003). Kleisins: a superfamily of bacterial and eukaryotic SMC protein partners. Mol. Cell, in press.

Severin, F., Hyman, A.A., and Piatti, S. (2001). Correct spindle elongation at the metaphase/anaphase transition is an APC-dependent event in budding yeast. J. Cell Biol. *155*, 711–718.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., and Higgins, D.G. (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. *25*, 4876–4882.

Toth, A., Ciosk, R., Uhlmann, F., Galova, M., Schleifer, A., and Nasmyth, K. (1999). Yeast cohesin complex requires a conserved protein, Eco1p (Ctf7), to establish cohesion between sister chromatids during DNA replication. Genes Dev. *13*, 320–333.

Uhlmann, F., and Nasmyth, K. (1998). Cohesion between sister chromatids must be established during DNA replication. Curr. Biol. *8*, 1095–1101.

Uhlmann, F., Lottspeich, F., and Nasmyth, K. (1999). Sister chromatid separation at anaphase onset is promoted by cleavage of the cohesin subunit Scc1p. Nature *400*, 37–42.

Uhlmann, F., Wernic, D., Poupart, M.A., Koonin, E., and Nasmyth, K. (2000). Cleavage of cohesin by the CD clan protease separin triggers anaphase in yeast. Cell *103*, 375–386.

Waizenegger, I., Hauf, S., Meinke, A., and Peters, J.M. (2000). Two distinct pathways remove mammalian cohesin from chromosome arms in prophase and from centromeres in anaphase. Cell *103*, 399–410.

Zachariae, W., and Nasmyth, K. (1999). Whose end is destruction: cell division and the anaphase-promoting complex. Genes Dev. *13*, 2039–2058.