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Evidence that Loading of Cohesin Onto Chromosomes Involves Opening of Its SMC Hinge

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SUMMARY

Cohesin is a multisubunit complex that mediates sister-chromatid cohesion. Its Smc1 and Smc3 subunits possess ABC-like ATPases at one end of 50 nm long coiled coils. At the other ends are pseudosymmetrical hinge domains that interact to create V-shaped Smc1/Smc3 heterodimers. N- and C-terminal domains within cohesin's kleisin subunit Scc1 bind to Smc3 and Smc1 ATPase heads respectively, thereby creating a huge tripartite ring. It has been suggested that cohesin associates with chromosomes by trapping DNA within its ring. Opening of the ring due to cleavage of Scc1 by separase destroys sister-chromatid cohesion and triggers anaphase. We show that cohesin's hinges are not merely dimerization domains. They are essential for cohesin's association with chromosomes, which is blocked by artificially holding hinge domains together but not by preventing Scc1's dissociation from SMC ATPase heads. Our results suggest that entry of DNA into cohesin's ring requires transient dissociation of Smc1 and Smc3 hinge domains.

INTRODUCTION

By resisting spindle forces, sister-chromatid cohesion makes possible the tension that is thought to stabilize kinetochore-microtubule attachments. Cohesion depends on a highly conserved multisubunit complex called cohesin, which consists of a heterodimer formed between two SMC proteins, Smc1 and Smc3, that forms a complex with two non-SMC proteins, Scc1 and Scc3 (Nasmyth and Haering, 2005). In yeast, cohesin is loaded onto chromosomes slightly before DNA replication with the assistance of a separate Scc2/Scc4 protein complex (Ciosk et al., 2000). Cohesin holds sister chromatids together until initiation of anaphase, when cleavage of its Scc1 subunit by separase releases cohesin from chromosomes and destroys sister-chromatid cohesion (Uhlmann et al., 2000).

SMC proteins form 50 nm long intramolecular antiparallel coiled coils with a dimerization domain at one end and one half of an ABC-type ATPase ("head") at the other (Gruber et al., 2003; Haering et al., 2002; Melby et al., 1998). Heterotypic interaction between Smc1's and Smc3's dimerization domains creates a huge V-shaped structure with ABC ATPase heads at its two apices and dimerization domains at its central "hinge." ATP bound to Walker A and Walker B motifs within Smc1's head binds to a signature motif within Smc3's head and vice versa, creating at least transiently a bipartite ring that hydrolyzes both ATP molecules sandwiched between its two heads (Haering et al., 2004). Cohesin's Scc1 (kleisin) subunit binds via a C-terminal winged-helix domain to Smc1's head and via N-terminal sequences predicted to form a winged-helix or helix-turn-helix domain to the Smc3 head of the same Smc1/Smc3 heterodimer. This creates a huge tripartite ring with a diameter of about 40 nm, which is opened by cleavage of Scc1's central region by separase (Gruber et al., 2003; Haering et al., 2002, 2004).

It has been suggested that cohesin grasps chromosomes topologically, by trapping DNA molecules inside its ring. If so, cohesin rings must either assemble de novo around chromatin fibers or (more likely) possess a gate that transiently opens to admit entry of DNA. Using the yeast *S. cerevisiae* as a model system, we show that linkage of Smc1's and Smc3's hinge domains by rapamycin-dependent dimerization of FKBP12 and Frb hinders de novo association of cohesin with DNA and blocks establishment, but not maintenance, of sister-chromatid cohesion. Our findings are consistent with the notion that DNA entry depends on transient dissociation of cohesin's Smc1 and Smc3 hinge domains.

RESULTS

Fusion of Scc1 to Either Smc1 or Smc3 Heads Does Not Prevent Establishment of Sister-Chromatid Cohesion

Two observations suggest that preassembled cohesin rings can associate with chromosomes. First, cohesin subunits stored in *Xenopus* eggs are preassembled into complexes but are nevertheless capable of associating with chromatin (Losada et al., 1998). Second, most cohesin in animal cells that dissociates from chromosomes during prophase in the absence of any cleavage by separase exists as a soluble complex during mitosis and reassociates with chromosomes during telophase (Waizenegger et al., 2000). If cohesin associates with chromosomes by trapping DNA, then its ring must have an entry gate. We envision four possibilities (Figure 1A): Scc1 dissociates transiently from the Smc1 or Smc3 head (carabiner models A and B), Scc1 dissociates transiently from both heads (the bicycle lock model), or Smc1 and Smc3 hinge domains dissociate transiently (the clothes peg model).

To investigate whether Scc1's C-terminal winged-helix domain must dissociate from Smc1's ATPase head, we expressed from an ectopic chromosomal site a myc-tagged Scc1-Smc1 fusion in which Scc1's C terminus was connected to Smc1's N terminus (Figure 1B). The fusion protein was absent in cells arrested in G1 by α factor but present in cycling and nocodazole-arrested cells (Figure 1B). The latter contained only full-length (240 kDa) protein, while cycling cells also contained a 200 kDa separase fragment. Upon sporulation, diploid yeast cells heterozygous for *SCC1* and *SMC1* gene deletions and *SCC1-SMC1* often produced tetrads in which three or four spores gave rise to colonies, some of which contained deletions of both *SCC1* and *SMC1* (Figure 1C). Strains that expressed the Scc1-Smc1 fusion protein but neither Scc1 nor Smc1 grew at 30°C (Figure 1D) and 37°C (data not shown).

If the Scc1-Smc1 fusion were only functional because its Scc1 moiety bound to a Smc1 head from a separate fusion protein, then cells should remain sensitive to overexpression of Scc1's C-terminal cleavage fragment (CScc1), which competes with full-length Scc1 (Rao et al., 2001). Fusion of Scc1 to Smc1 in fact overrides toxicity caused by CScc1 (Figure 1E). Scc1 must therefore interact with the Smc1 protein to which it is fused. This was confirmed by the finding that the fusion suppresses the temperature sensitivity (data not shown) caused by a mutation (Q544K) in Scc1's C-terminal winged-helix domain that reduces its affinity for Smc1's head (Haering et al., 2004). Scc1's detachment from Smc1's ATPase head cannot be necessary for generating sister-chromatid cohesion.

To explore whether detachment of Scc1's N terminus from Smc3's head is necessary, Smc3's C terminus was connected to Scc1's N terminus. Spores expressing Smc3-Scc1 instead of individual Smc3 and Scc1 proteins also formed colonies (Figures 1F and 1G). This fusion is not fully functional, as cells grow more slowly than wild-type at 37°C (data not shown). Importantly, fusion of Scc1 to Smc3 but not to Smc1 suppressed the lethality (data not shown) caused by mutations (I24K, A47K, V81K, and D92K) in Scc1's N terminus that reduce its interaction with Smc3's head (Arumugam et al., 2006), implying that the fusion's Scc1 moiety binds to the Smc3 head with which it is joined. We conclude that detachment of Scc1's N terminus from Smc3's ATPase head is not necessary for cohesin function.

Connection of Scc1 to Both of Its SMC Heads Does Not Destroy Cohesin Function

To exclude the possibility that Scc1 detaches from both Smc1 and Smc3 ATPase heads, we created a Smc3-Scc1-Smc1 fusion protein. This protein was not functional even in the presence of TEV protease that cleaves the peptide sequences connecting Scc1 with Smc3 and Smc1. This could be for trivial reasons. The fusion might not fold correctly due to topological problems (see Figure S5 in the Supplemental Data available with this article online), and/or, due to its length, it might not be expressed rapidly enough at the onset of S phase. We therefore used rapamycin-dependent dimerization of human FKBP12 and Frb to link Scc1 to Smc1 in a conditional manner (Ho et al., 1996) in a cohesin ring containing a Smc3-Scc1 fusion protein (Figure 2C). For these experiments, we used yeast strains carrying the *TOR1-1* mutation, which confers rapamycin resistance, and a deletion of the *FPR1* gene, which eliminates the most abundant yeast FKBP12-like protein and reduces binding of "free" yeast FBKP12-like proteins to the Frb moiety fused to Scc1. Fusion of human FKBP12 to Smc1's C terminus, fusion of Frb to Scc1's C terminus, and the presence of both fusions all failed to affect yeast growth in either the absence or presence of 100 nM rapamycin (Figure 2A). Interestingly, when cells expressed both Smc1-FKBP12 and Scc1-Frb, rapamycin suppressed the lethality caused by two *scc1* mutations (S525N and Q544K) that weaken its binding to Smc1's head (Figure 2B), implying that rapamycin does indeed induce connection of Scc1 with Smc1. The N terminus of the Scc1-Frb fusion protein was next fused to Smc3's C terminus. Cells expressing this Smc3-Scc1-Frb fusion along with Smc1-FKBP12 (as the sole sources of Smc1, Smc3, and Scc1) were viable in absence of rapamycin and even formed colonies in its presence, albeit more slowly than in its absence (Figure S1A). Importantly, introduction of the *scc1* Q544K mutation created a strain that grew only in the presence of rapamycin (Figure S1B).

To investigate the effect of this alteration in cohesin's topology on sister-chromatid cohesion, we synchronized cells in G1 by growing unbudded cells isolated by centrifugal elutriation in the presence of α factor and then transferred them to fresh medium with or without rapamycin. Neither DNA replication nor the onset of nuclear division was affected by rapamycin (Figure S1C). This suggests that the drug did not greatly compromise establishment of sister-chromatid cohesion because a major defect

would have activated the spindle assembly checkpoint and delayed degradation of securin by 20–30 min (Michaelis et al., 1997). Separate aliquots of the synchronized cells were released (with and without rapamycin) into medium containing nocodazole, which blocks nuclear division. Fluorescence in situ hybridization (FISH) revealed that sister *CEN4* sequences were split in 24% of cells in the absence of rapamycin (Figure 2D), which is more frequent than in wild-type. Crucially, rapamycin caused only a very modest increase in the frequency of split *CEN4* sequences, from 24 to 28% (Figure 2D). To ensure that this protocol could have detected deleterious effects of a rapamycin-mediated interconnection, we repeated these experiments with a yeast strain in which FKBP12 and Frb interconnect Smc1's and Smc3's hinge domains in the presence of rapamycin (see below). Rapamycin both delayed nuclear division by 20 min (Figure S1D) and caused the frequency of split *CEN4* sequences to rise from 12 to 49% (Figure 2D). These data imply that hindering a hypothetical disconnection of Scc1's C terminus from Smc1's ATPase head has no major deleterious effect on cohesin's ability to establish sister-chromatid cohesion, even when its N terminus is fused to Smc3's ATPase head.

To test whether blocking dissociation of Scc1 from SMC heads has any effect on cohesin's association with chromosomes, we used quantitative PCR to measure association of a myc-tagged Scc3 subunit with specific DNA sequences following chromatin immunoprecipitation (ChIP). Some Scc3 was associated with chromatin in G1 synchronized cells, which was unexpected. A similar result was also obtained with wild-type cells (data not shown). More important, the increase in Scc3's association with centromeric and pericentric sequences as cells enter S phase was unaffected by rapamycin (Figure 2E). Thus, connecting Smc3-Scc1-Frb with Smc1-FKBP12 using rapamycin has little discernable effect on cohesin's ability to load onto chromosomes. These data are difficult to reconcile with the bicycle lock model.

MP1 and p14 Interact Tightly Enough to Resist Spindle Forces

We next addressed whether dissociation of cohesin's hinge domains is necessary. If their sole function were to hold Smc1 and Smc3 together, then it should be possible to replace them by a different pair of heterodimer-forming protein domains. This would not be possible if SMC hinges also served as DNA entry gates. To test whether candidate heterodimers were capable of keeping cohesin rings closed tightly enough to maintain sister-chromatid cohesion, we inserted into Scc1's central region pairs of protein domains connected by three tandem TEV protease cleavage sites (Figure 3A) and measured whether TEV protease induction (from the *GAL* promoter) would trigger nuclear division in cells arrested in metaphase due to Cdc20 depletion. Tightly interacting protein pairs should hold N- and C-terminal Scc1 cleavage fragments together and block sister-chromatid disjunction. Cells in which FKBP12 and Frb flanked TEV sites underwent anaphase as efficiently as cells lacking these domains in the absence of rapamycin, whereas in its presence, the onset of anaphase was delayed by about 30 min but was not blocked (data not shown). The dissociation constant of FKBP12 and Frb in the presence of rapamycin is in the low nanomolar range, but the half-life of the complex is relatively short ($\tau \approx 30$ s) (Banaszynski et al., 2005). Our data suggest that the complex is not stable enough to resist spindle pulling forces over extended periods of time. Two tandem copies of FKBP12 and Frb were capable of resisting anaphase onset in the presence of rapamycin (data not shown), but such a combination could not be used to replace Smc1 and Smc3 hinges.

We next tested a pseudosymmetric protein complex consisting of MP1 and p14 ($\tau \approx 20$ min) (Kurzbauer et al., 2004). This complex blocked sister-chromatid disjunction despite complete Scc1 cleavage (Figure 3A) but did not do so after activation of separase (Figure 3A, +Cdc20), which cleaves to the left and right of MP1 and p14. Remarkably, cells with MP1 and p14 surrounding TEV sites proliferated vigorously upon continuous TEV protease induction (Figure 3B). We conclude that the MP1/p14 complex holds N- and C-terminal halves of Scc1 together in a manner stable enough to support mitosis. An important corollary is that disconnection of Scc1's N-terminal half from its C-terminal half rather than cleavage of Scc1 per se destroys sister-chromatid cohesion.

Hinge-Substituted Smc1/Smc3 Heterodimers Form Rings with Scc1 and Hydrolyze ATP

Structural information (Figure 4A) about the MP1/p14 complex (PDB ID code 1VET) and the bacterial homodimeric SMC hinge (PDB ID code 1GXL) was used to design eight different hybrids that varied in the transition between Smc1's N-terminal amphipathic α helix and the p14 domain and between p14 and Smc1's C-terminal amphipathic α helix. Western blotting following immunoprecipitation showed that two Smc1p14 hybrids (myc9-tagged) interacted with Scc1 (Pk6-tagged) as efficiently as a hingeless version of Smc1 that, like Smc1p14, has no SMC binding partner (data not shown). Efficient binding of Scc1 implies that the ATPase heads of the hybrid proteins are folded correctly. The other six hybrids interacted with Scc1 only poorly (data not shown).

We then replaced Smc3's hinge domain by mouse MP1. Five different HA3-tagged Smc3MP1 hybrids were introduced into a yeast strain expressing a Pk6-tagged version of Scc1 (Scc1-Pk6) and one of the "good" myc9-tagged Smc1p14 hybrids. Three out of five Smc3MP1 hybrid proteins coprecipitated with both Scc1 and Smc1p14 with an efficiency comparable to that between Smc3 and Scc1 or Smc3 and Smc1 (data not shown). In all three cases, dimerization between Smc3MP1 and Smc1p14 increased the efficiency with which Scc1 bound to Smc1p14 (data not shown). These data imply that Smc1p14 forms a heterodimer with Smc3MP1 and that Scc1's C-terminal winged helix binds to Smc1's ATPase head.

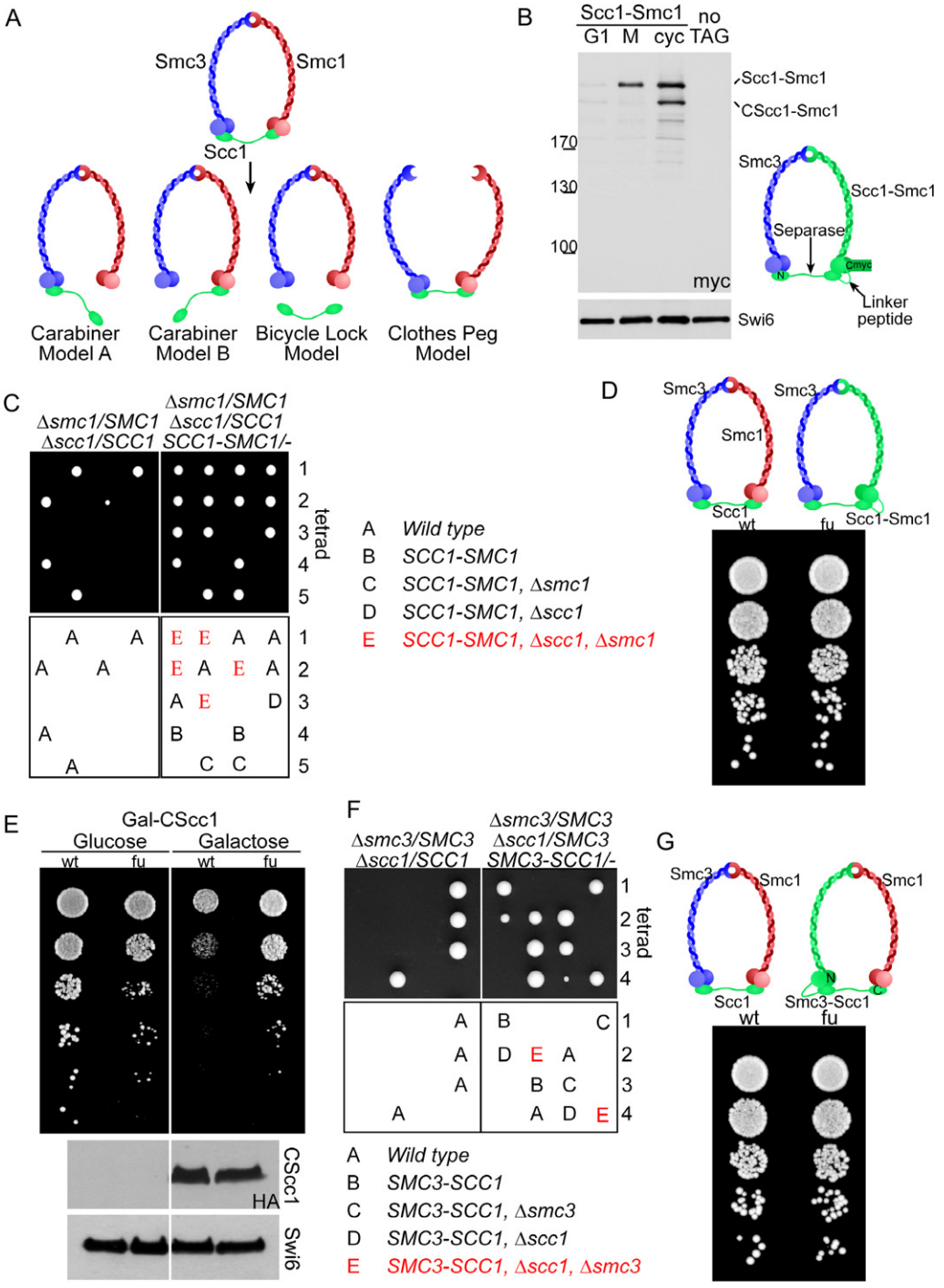

Figure 1. Connection of Scc1 with Either SMC Head Permits Cohesion

(A) Models for cohesin ring opening.

(B) Scc1-Smc1 fusion protein expression. Cells of strains K699 (*MAT***a**) and K11680 (*MAT***a** P^{SCC1}*SCC1-TEV3-SMC1-myc18* Δ*scc1* Δ*smc1*) were grown in exponential phase (cyc) and arrested in metaphase using nocodazole (M) or in G1 phase by addition of α factor peptide (G1). Cell extracts were prepared and analyzed by blotting for myc and Swi6 protein. For detailed genotypes, see Table S1.

(C) *SCC1-SMC1* is functional. Genotypes of spores (germinated on YPD plates at 30°C) from diploid yeast strains K12314 (*MAT***a**/α *SCC1*/Δ*scc1 SMC1*/Δ*smc1*) and K13630 (*MAT***a**/α *SCC1*/Δ*scc1 SMC1*/Δ*smc1 leu2/leu2::*P^{SCC1}*SCC1-TEV3-SMC1::LEU2*) are shown.

(D) *SCC1-SMC1* supports cell growth. Five-fold serial dilutions of strains K699 (wt) and K12385 (*MAT***a** Δ*scc1* Δ*smc1* P^{SCC1}*SCC1-TEV3-SMC1*) (fu) were plated onto YPD and grown at 30°C.

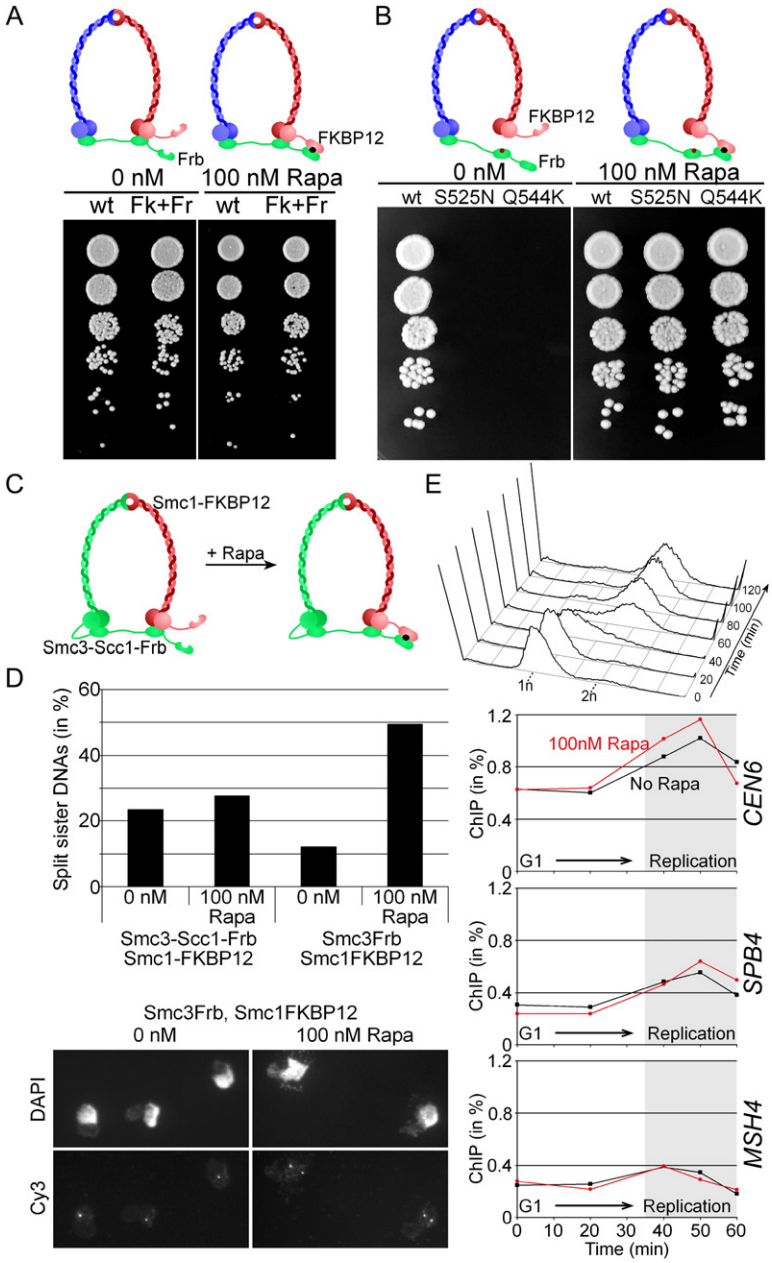

Figure 2. Linking Scc1 to Both SMC Heads Permits Sister-Chromatid Cohesion

(A) Linking Scc1's C terminus with Smc1's head by rapamycin-dependent dimerization permits cohesin function. Strains K14708 (*MAT*α Δ*fpr1 TOR1-1*) (wt) and K14195 (*MAT***a** Δ*fpr1 TOR1-1 SCC1-Frb SMC1-FKBP12*) (Fk+Fr) were plated on YPD containing 0 or 100 nM rapamycin. Plates were incubated at 30°C.

(B) Linking Scc1's C terminus with Smc1's head suppresses mutations in Scc1's C terminus. Strains K14195 (wt), K14196 (*MAT***a** Δ*fpr1 TOR1-1 scc1(S525N)-Frb SMC1-FKBP12*) (S525N), and K14197 (*MAT***a** Δ*fpr1 TOR1-1 scc1(Q544K)-Frb SMC1-FKBP12*) (Q544K) were grown in the presence of 100 nM rapamycin and plated onto YPD containing 0 or 100 nM rapamycin.

(C and D) Linking Scc1 to both SMC heads permits sister-chromatid cohesion. Cells of strains K30016 (*MAT***a** Δ*fpr1 TOR1-1* Δ*scc1* Δ*smc3* P^{SCC1}*SMC3-TEV3-SCC1-Frb SMC1-FKBP12*) and K14668 (*MAT***a** Δ*fpr1 TOR1-1* Δ*smc3 SMC3Frb SMC1FKBP12*) were synchronized by centrifugal elutriation and incubation with α factor for 3 hr at 25°C. Cells were released from the arrest and incubated for 3 hr in nocodazole with or without rapamycin. Cells were fixed and subjected to fluorescence in situ hybridization (FISH) using a Cy3-labeled DNA probe covering a 16.6 kb region around the centromere on chromosome IV.

(E) Linking Scc1 to both SMC heads does not block cohesin's DNA association. Cells of strain K14829 (*MAT***a** Δ*fpr1 TOR1-1* Δ*scc1* Δ*smc3* P^{SCC1}*-SMC3-TEV3-SCC1-Frb SMC1-FKBP12 SCC3-myc18*) were synchronized by centrifugal elutriation and arrested with α factor for 3.5 hr at 25°C. Cells were released by washing with YPD medium and incubated in the presence or absence of rapamycin. Replication timing was monitored by flow cytometry. The levels of cohesin associated with DNA were determined by chromatin immunoprecipitation (ChIP) and quantitative PCR using primer pairs specific to three different regions of chromosome VI (see below).

To test whether Scc1's N terminus binds to the Smc3 head of the hinge-substituted SMC dimer (hsSMC), we expressed either wild-type Smc1/Smc3 or hinge-substituted versions in cells that express a TEV-cleavable version of Scc1 that is tagged at both N and C termini with myc epitopes (Figure 4B) (Arumugam et al., 2003). Sepharose beads containing immunoprecipitated HA3-tagged Smc3 or Smc3MP1 proteins were either directly boiled

(E) *SCC1-SMC1* cells are resistant to CScc1. Strains K11969 (*MAT***a** *YCp-*P^{GAL1}*CScc1-HA3*) (wt) and K11970 (*MAT***a** Δ*scc1* Δ*smc1* P^{SCC1}*SCC1-TEV3-SMC1-myc18 YCp-*P^{GAL1}*CScc1-HA3*) (fu) were plated on −TRP plates containing either 2% glucose or 2% raffinose plus galactose and grown at 25°C. Expression of CScc1 (aa 268–566) was confirmed by detecting HA epitopes in blots from extracts of cells shifted for 3 hr from raffinose to glucose (−CScc1) or galactose (+CScc1). The fusion strain grows significantly more slowly than wild-type on −Trp medium, possibly because SCC1-SMC1-myc18 cells more frequently lose the centromeric plasmid.

(F) *SMC3-SCC1* is functional. Genotypes of spores from diploid yeast strains K12542 (*MAT***a**/α *SCC1*/Δ*scc1 SMC3*/Δ*smc3*) and K13638 (*MAT***a**/α *SCC1*/Δ*scc1 SMC3*/Δ*smc3 ura3/ura3::*P^{SCC1}*SMC3-TEV3-SCC1::URA3*) after dissection on YPD at 30°C.

(G) *SMC3-SCC1* supports cell growth. Serial dilutions of strains K699 (wt) and K12988 (*MAT*α Δ*scc1* Δ*smc3* P^{SCC1}*SMC3-TEV3-SCC1*) (fu) plated onto YPD and grown at 30°C are shown.

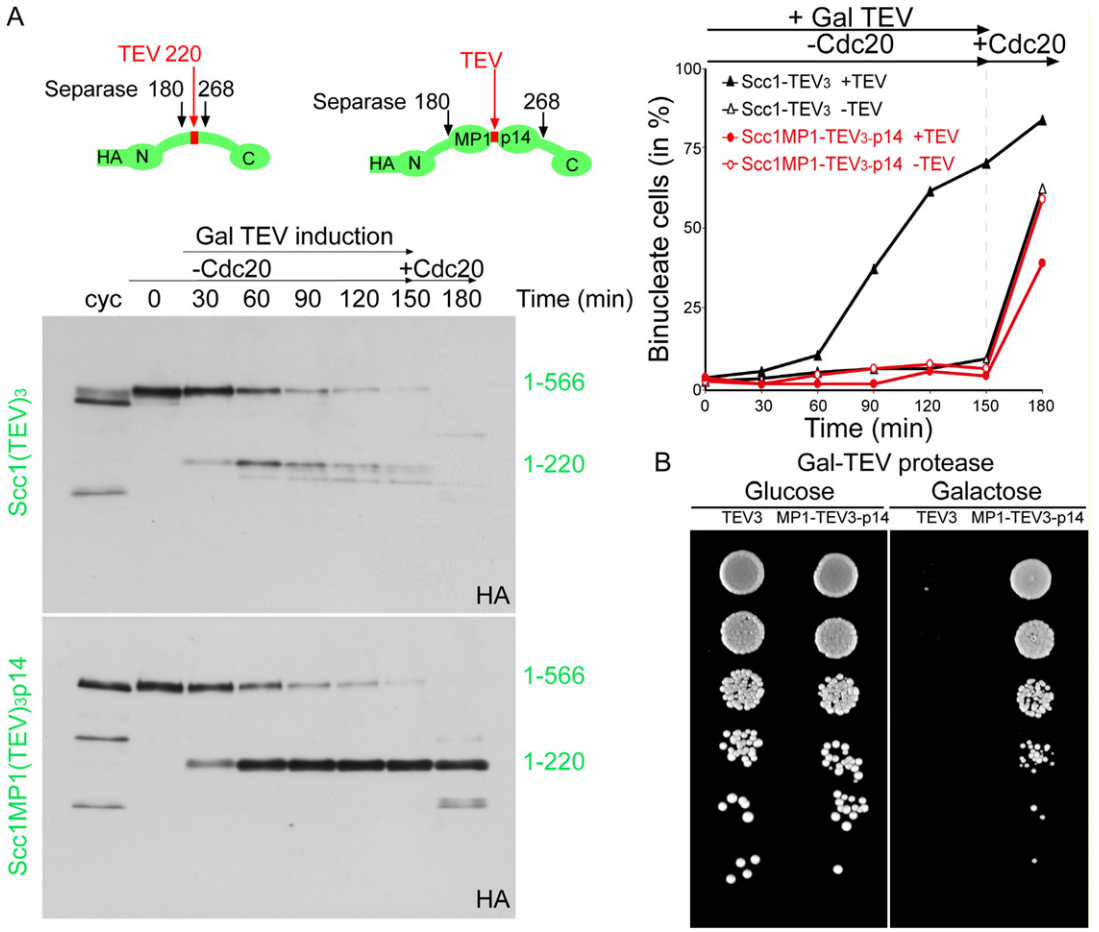

Figure 3. MP1 and p14 Interact Tightly Enough to Resist the Mitotic Spindle

(A) Yeast strains K12822 (*MATα* P^{MET3}*CDC20 YEp* Δ*scc1 HA6-SCC1(TEV220)*), K12823 (*MATα* P^{MET3}*CDC20 YEp-*P^{GAL1}*TEV* Δ*scc1 HA6-SCC1(TEV220)*), K12824 (*MAT***a** P^{MET3}*CDC20 YEp* Δ*scc1 HA6-SCC1MP1-(TEV220)-p14*), and K12825 (*MAT***a** P^{MET3}*CDC20 YEp-*P^{GAL1}*TEV* Δ*scc1 HA6-SCC1MP1-(TEV220)-p14*) were grown to log phase in synthetic medium lacking methionine (cyc) and arrested for 2 hr in YEP medium supplemented with 2 mM methionine and 2% raffinose (0 min). Expression of TEV protease was induced by addition of 2% galactose (0–150 min). Cells were transferred back to synthetic medium lacking methionine at 150 min (+Cdc20). Mono- and binucleate cells were counted after staining with DAPI. Protein extracts were prepared and probed by immunoblotting for HA epitopes.

(B) Dilution of cells of strains K13653 (*MATα* Δ*scc1 HA6-SCC1(TEV220) YEp-*P^{GAL1}*TEV*) and K13654 (*MAT***a** Δ*scc1 HA6-SCC1MP1-(TEV220)-p14 YEp-*P^{GAL1}*TEV*) were plated on synthetic medium lacking tryptophan with glucose or raffinose and galactose. Plates were incubated at 30°C.

in SDS buffer (IP) or incubated with or without TEV protease for 45 min at 16°C, liberating some protein into the supernatant (SN) but leaving some associated with the beads (B). Smc3 and Smc3MP1 as well as their respective partners Smc1 and Smc1p14 remained bound to the beads even after TEV protease treatment (Figure 4B, middle and right panels). Both full-length Scc1 (1–566) and its N-terminal separase cleavage fragment (1–180) were collected on the beads by HA3-tagged Smc3 (middle panel) and Smc3MP1 (right panel) proteins with equal efficiency but not by untagged Smc3 protein (left panel). The C-terminal separase cleavage fragment (180–566) is more unstable, and less was detected with both sets of SMC proteins. Cleavage of Scc1 by TEV generates a novel set of cleavage fragments, namely an N-terminal (1–268) and a C-terminal (269–566) fragment. Crucially, both fragments remained on the beads, whether they contained Smc3 or Smc3MP1. These observations indicate that Scc1's N-terminal and C-terminal domains associate with the Smc3 and Smc1 heads respectively, forming tripartite rings, even when the Smc1/Smc3 hinge has been replaced by MP1/p14.

We next tested whether the ATPase heads of Smc1p14/Smc3MP1 heterodimers can hydrolyze ATP. Scc1's C-terminal winged-helix domain stimulates the ATPase activity of purified SMC heterodimers in vitro (Arumugam et al., 2006). Both wild-type and hinge-substituted SMC preparations had very low ATP hydrolysis activities on their own (Figure 4C, black lines), but addition of Scc1's C-terminal domain enhanced the activities of both

samples (red lines). Importantly, mutation of Smc3MP1's ATP binding pocket (K38I) abolished the stimulation of ATP hydrolysis by Scc1. These data imply that the Smc1 and Smc3 heads of hinge-substituted heterodimers are folded properly and cooperate to hydrolyze ATP in a manner similar to wild-type Smc1/Smc3 heterodimers.

Hinge-Substituted SMC Dimers Cannot Associate with Chromosomes

Tetrad dissection of asci from diploids heterozygous for *SMC1* and *SMC3* deletions showed that ectopic hinge-substituted *SMC* genes could not support growth of spores lacking endogenous *SMC1* and *SMC3* genes (Figure S6A). To address whether hsSMCs are loaded onto chromosomes in vivo, we analyzed chromosome spreads from nocodazole-arrested cells. Smc1-myc9, but very little Smc1p14-myc9, colocalized with DAPI-staining material (Figure S6B). To measure this more quantitatively, we used real-time PCR and ChIP to detect association with four loci on chromosome VI. Smc1-myc9 protein precipitated about 1.5% to 2.5% of the input material at two pericentric loci (*MSH4* and *SPB4*), at the centromere (*CEN6*), and at a cohesin-rich arm site (*MET10-SMC2*) (Figure 4D, middle). In contrast, Smc1p14-myc9 precipitated few, if any, of these sequences (Figure 4D). We also performed chromatin immunoprecipitations with antibodies against an HA3 epitope tag on Smc3 and Smc3MP1 and hybridized the immunoprecipitated DNA to a tiled oligonucleotide array representing the entire length of chromosome VI (Figure 4E). This confirmed that wild-type Smc3 protein accumulates at centromeric and pericentromeric DNA sequences as well as at specific sites along the chromosome arms. This pattern was not observed with Smc3MP1 protein, which did not precipitate any specific region of chromosome VI (Figure 4E, bottom panel). We conclude that hsSMC proteins do not associate stably with chromosomes despite being transported normally into the nucleus (data not shown). This implies that the hinge domains of Smc1 and Smc3 have a role in cohesin's association with chromosomes that extends beyond merely holding Smc1 and Smc3 together.

Artificial Connection of Cohesin's Smc1 and Smc3 Hinge Domains Prevents Establishment, but Not Maintenance, of Sister-Chromatid Cohesion

Having excluded DNA entry through a gate created by dissociation of Scc1 from cohesin's ATPase heads, hinge opening would appear to be the only realistic alternative. According to this notion, the MP1/p14 interaction cannot substitute for the Smc1/Smc3 hinge because the former cannot be opened in a manner that permits DNA entry. This hypothesis makes the key prediction that interconnection of Smc1 and Smc3 hinges by artificial means should hinder trapping of DNA by cohesin and, as a consequence, should destroy its ability to establish sister-chromatid cohesion. We therefore asked whether it is possible to insert FKBP12 and Frb into Smc1 and Smc3 hinges respectively without compromising cohesin function and, if so, whether the interconnection of FKBP12 and Frb by rapamycin inactivates cohesin.

The hinge domains of cohesin, condensin, and most bacterial SMC proteins are highly conserved throughout their entire length. There nevertheless exist short stretches whose amino acid sequences and length are variable. Such regions form loops on the surface of the *Thermotoga maritima* Smc crystal structure with a high temperature factor that is indicative of structural flexibility. This raises the possibility of inserting the small globular domains of FKBP12 and Frb into one or another of these loops without greatly altering the overall structure of the SMC hinge domain. Remarkably, insertion of FKBP12 via 10 amino acid long linker peptides into Smc1's hinge domain at two out of three positions (Figure 5A, pink arrows) did not compromise Smc1's function. Likewise, insertion of Frb at analogous positions within Smc3's hinge (purple arrows) did not perturb the function of Smc3. Even more remarkable, certain combinations of Smc1FKBP12 and Smc3Frb hybrids produced functional Smc1/Smc3 heterodimers. Thus, a heterodimer formed between Smc1 with FKBP12 inserted at position L597 (horizontal pink arrow) and Smc3 with Frb inserted at position S606 (horizontal purple arrow) supported wild-type-like growth of yeast colonies at 30°C (Figure 5B, left panel) and 37°C (data not shown).

To test the sensitivity of this *SMC1FKBP12 SMC3Frb* strain to rapamycin, we introduced the *TOR1-1* mutation to confer rapamycin-resistant growth and a deletion of *FPR1* (Δ*fpr1*) to minimize binding of Frb to rapamycin bound to yeast FKBP12-like proteins. One hundred nanomolar rapamycin inhibited the growth of *SMC1FKBP12 SMC3Frb* cells (Fk + Fr), but not that of wild-type (WT), *SMC1FKBP12* (Fk), or *SMC3Frb* strains (Fr) (Figure 5B, right panel), implying that interaction between FKBP12 inserted into Smc1's hinge and Frb inserted into Smc3's hinge does indeed inactivate cohesin function. We observed a similar effect when FKBP12 was inserted into Smc1 at position I647 (vertical pink arrow) instead of position L597 (data not shown).

It is possible that the rapamycin-dependent lethality is caused not by the interconnection of Smc1 and Smc3 half-hinges per se but rather by the formation of a bulky Frb-rapamycin-FKBP12 complex in their vicinity. To exclude this possibility, we tested whether the rapamycin-dependent lethality is suppressed by the presence of yeast Fpr1, whose complexes with rapamycin should compete with rapamycin-FKBP12 complexes associated with Smc1 for binding to Smc3Frb. Crucially, the presence of Fpr1 fully relieved the inhibition by rapamycin of *SMC1FKBP12 SMC3Frb* cell growth (Figure 5C). If Fpr1 suppressed lethality merely by titrating rapamycin, then increasing the drug concentration would kill cells. This is not the case. *SMC1FKBP12 SMC3Frb TOR1-1* cells lacking Fpr1 are sensitive to 10 nM rapamycin, while cells containing Fpr1 are completely resistant to 10 μM rapamycin (Figure S4). Moreover, Fpr1 is only about ten times more abundant than Smc1 (Newman et al., 2006).

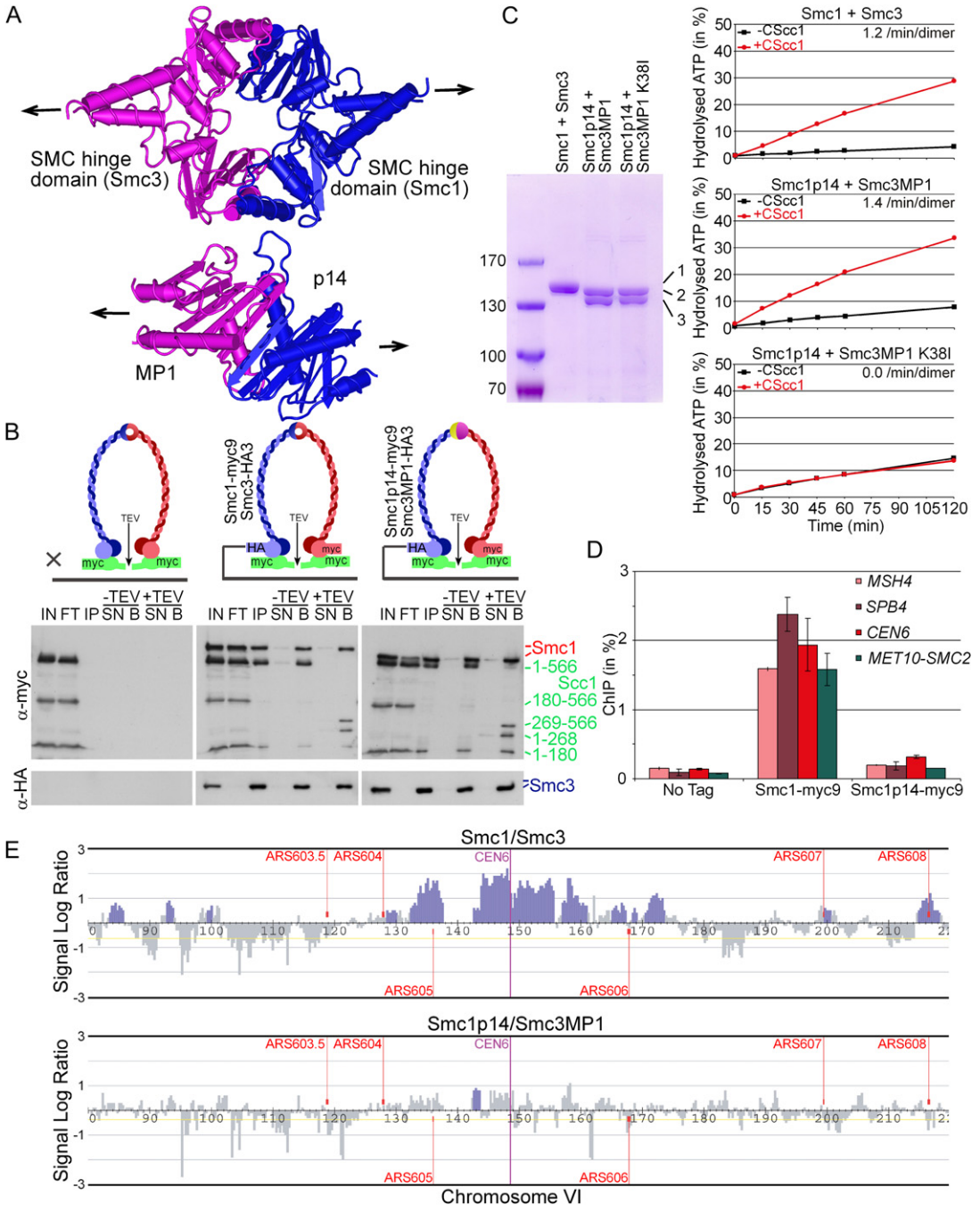

Figure 4. Hinge-Substituted SMC Heterodimers Form Rings with Scc1 and Hydrolyze ATP with Wild-Type Kinetics but Cannot Associate with Chromosomes

(A) Ribbon diagrams of the structures of a SMC hinge homodimer from *T. maritima* (top) and the MP1-p14 complex from mouse (bottom). Diagrams were produced by Cn3D using the PDB ID codes 1GXL and 1VET, respectively.

(B) Hinge-substituted SMC heterodimers form tripartite rings with Scc1. Yeast strains K14084 (*MAT***a**/α *myc9-SCC1TEV268-myc9/ -P^{GAL1}SCC1/SCC1*), K14081 (*MAT***a**/α *myc9-SCC1TEV268-myc9/(SMC1-myc9+SMC3-HA3) P^{GAL1}SCC1/SCC1*), and K14082 (*MAT***a**/α *myc9-SCC1TEV268-myc9/(smc1p14-myc9+smc3MP1-HA3) P^{GAL1}SCC1/SCC1*) were grown to log phase in YPD medium. Cleared extracts (IN) were immunoprecipitated with anti-HA antibodies, and supernatants were removed (FT). The beads were washed (IP) and incubated with or without TEV protease for 45 min at 16°C. Supernatant fractions were collected (SN) and proteins eluted from the beads using SDS (B). Samples were analyzed by immunoblotting using α-HA and α-myc antibodies.

(C) Hinge-substituted SMC heterodimers hydrolyze ATP. (Left) Purified SMC heterodimers (5 μg per lane) were separated on a 6% SDS polyacrylamide gel and stained with Coomassie brilliant blue R-250 (1, Smc1 + Smc3; 2, Smc3MP1; 3, Smc1p14). (Right) ATP hydrolysis assays were

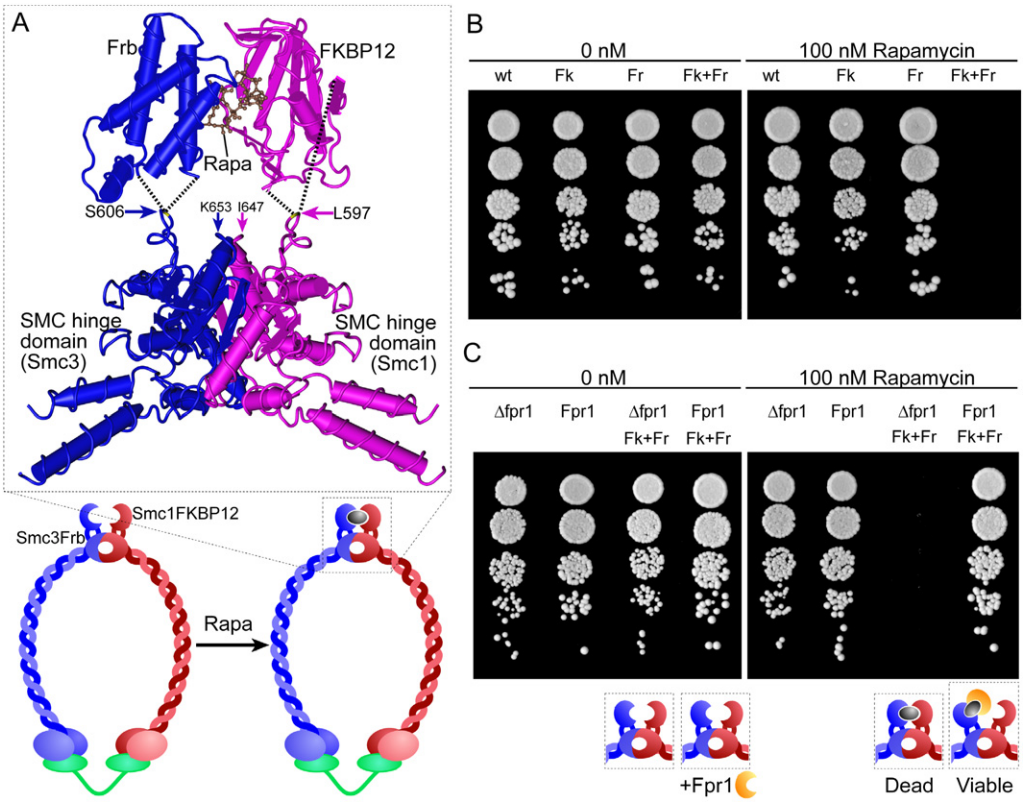

Figure 5. Linking SMC Hinge Domains by the FKBP12-Frb-Rapamycin Complex Is Lethal

(A) Ribbon diagrams of the structures of a FKBP12-rapamycin-Frb complex and a bacterial SMC hinge-domain dimer. Dotted lines represent 10 aa linker peptides that insert FKBP12 into Smc1's hinge domain and Frb into Smc3's hinge domain.

(B) Rapamycin-dependent lethality of hinge-inserted SMCs. Yeast strains K14708 (*MATα Δfpr1 TOR1-1*) (wt), K14645 (*MATα Δfpr1 TOR1-1 SMC1FKBP12*) (Fk), K14644 (*MAT***a** *Δfpr1 TOR1-1 Δsmc3 SMC3Frb*) (Fr), and K14643 (*MATα Δfpr1 TOR1-1 Δsmc3 SMC3Frb SMC1FKBP12*) (Fk+Fr) were grown for 2 days on YPD plates in the absence or presence of 100 nM rapamycin.

(C) Fpr1 rescues rapamycin-dependent lethality. Yeast strains K11607 (*MAT***a** *TOR1-1*), K14708, K14642 (*MATα TOR1-1 Δsmc3 SMC3Frb SMC1FKBP12*), and K14643 were grown for 2 days on YPD plates in the absence or presence of 100 nM rapamycin.

To test whether rapamycin perturbs establishment of sister-chromatid cohesion in *SMC1FKBP12 SMC3Frb* cells, we measured association of sister DNA sequences at the *URA3* locus 35 kb away from *CEN5* that were marked by green fluorescent protein (GFP) (Michaelis et al., 1997). *MAT***a** *SMC1FKBP12 SMC3Frb* cells whose APC/C activator Cdc20 was under the control of the methionine-repressible *MET3* promoter were first arrested in G1 phase by addition of α factor and then released in the presence or absence of rapamycin into medium

performed using the same SMC preparations at 0.5 μM and 250 μM ATP in the absence (black curve) or presence (red curve) of 6 μM C-terminal Scc1 fragment. γ^{32}P-labeled ATP and $^{32}P_i$ were resolved by thin-layer chromatography, and ratios of P_i to P_i + ATP were plotted for each time point. Estimated CScc1-dependent hydrolysis rates are given as molecules of ATP hydrolyzed per minute per SMC heterodimer at 250 μM ATP. The variable levels of activity in the absence of CScc1 presumably stem from different expression and purification efficiencies and represent background activity from impurities.

(D) ChIP of wild-type and hinge-substituted SMC proteins. Yeast strains K11990 (*MAT***a** *Scc1-Pk6*), K14022 (*MAT***a** *Scc1-Pk6 (SMC1-myc9+SMC3-HA3)*), and K14024 (*MAT***a** *Scc1-Pk6 (smc1p14-myc9+smc3MP1-HA3)*) were grown in log phase and fixed with 3% formaldehyde. Samples were processed for ChIP analysis using α-myc antibodies. Efficiency of chromatin immunoprecipitation (ratio of input versus IP) was measured by real-time PCR using four primer pairs (for positions on chromosome VI, see below). Error bars indicate standard deviations from the arithmetic mean calculated from two independently processed samples.

(E) Distribution of wild-type and hinge-substituted SMCs around the chromosome VI centromere. Yeast strains K13581 (*MAT***a** *SMC1::(SMC1+SMC3-HA3)*) and K13585 (*MAT***a** *SMC1::(smc1p14+smc3MP1-HA3)*) were arrested in mitosis using benomyl. Cells were then processed for ChIP-on-chip analysis using α-HA antibodies. The blue shading represents the binding ratio of loci that show significant enrichment in the immunoprecipitated fraction. The yellow line indicates the average signal ratio of loci that are not enriched in the immunoprecipitated fraction. The scale of the vertical axis is $\log_2$. The horizontal axis shows kilobase units (kb). Original ChIP-on-chip data can be accessed from the GEO database under the accession number GSE4827.

supplemented with methionine, which allowed one synchronous round of DNA replication followed by arrest in metaphase. α factor-arrested cells contain a single GFP dot, but chromosome duplication in the absence of rapamycin creates a pair of GFP dots that are so close together in most cells that they still appear as a single GFP dot, even when cells enter metaphase (Figure 6A, black curve, no rapamycin). DNA replication in the presence of rapamycin, in contrast, caused duplicated GFP dots in 60% of cells to split soon (20–40 min) after completion of S phase (Figure 6A, red curve, 100 nM rapamycin). DNA replication in the presence of rapamycin also caused the appearance of nuclei whose chromosomal DNA did not remain in the bud neck, with chromosomal DNA segregating to different poles of the cell (Figure 6A). We conclude that rapamycin severely perturbs the establishment of sister-chromatid cohesion in *SMC1FKBP12 SMC3Frb* cells when added prior to DNA replication. Experiments that measured sister-chromatid cohesion in nocodazole-arrested cells using FISH confirmed this conclusion (Figure 2D).

To address whether interconnection of Smc1FKBP12 and Smc3Frb hinges by rapamycin compromises sister-chromatid cohesion that has already been established, we repeated the above experiment but added rapamycin only after DNA replication had been completed, namely 80 min after release from α factor (Figure 6A, right panel). Under these conditions, addition of rapamycin caused no splitting of duplicated GFP dots, implying that linkage of Smc1FKBP12 and Smc3Frb hinges by rapamycin hinders establishment, but not maintenance, of sister-chromatid cohesion. Lastly, interconnection of hinges by rapamycin in metaphase cells had no effect on their ability to undergo anaphase (Figure S4C).

Connection of Smc1 and Smc3 Hinge Domains Hinders Cohesin's Association with Chromosomes

To test whether rapamycin affects cohesin's association with chromosomes, we used real-time PCR to measure coprecipitation with Pk9-tagged Scc1 of the core centromere (*CEN6*), two pericentric regions (*MSH4* and *SPB4*), and a cohesin-rich arm site (*MET10-SMC2*) on chromosome VI (Figure 6B, bottom panel) as *MAT***a** *SMC1FKBP12 SMC3Frb* cells enter S phase in the presence or absence of rapamycin. Scc1 protein is largely absent from G1-arrested cells and is resynthesized only shortly before S phase (Michaelis et al., 1997). In the absence of rapamycin, Scc1's association with the core centromere commenced slightly before and peaked during the middle of S phase (25 min after release), at which point 5% of input DNA coprecipitated with Scc1 (Figure 6B, black lines). Accumulation of cohesin at the centromere-proximal pericentric site (*SPB4*) had a similar pattern, but accumulation at *MSH4*, which is further away from the core centromere, was less pronounced and took place more slowly. Meanwhile, accumulation within the interval between *MET10* and *SMC2* was slower still, peaking only after completion of S phase. Crucially, rapamycin greatly reduced/delayed cohesin's association with *CEN6* and *SPB4* and had a similar but less pronounced effect at *MSH4* and at *MET10-SMC2* (Figure 6B, red lines).

DISCUSSION

The experiments described in this paper were predicated on the notion that trapping of DNA inside cohesin's tripartite ring is essential for its stable association with chromosomes (Ivanov and Nasmyth, 2005). If preassembled rings trap DNA, then at least one of the three interaction interfaces between Smc1, Smc3, and Scc1 must be transiently broken. We tested first whether Scc1's N- or C-terminal domains must dissociate from Smc3 and Smc1 heads respectively by fusing the C terminus of Smc3 to the N terminus of Scc1 or the C terminus of Scc1 to the N terminus of Smc1. Because neither fusion inactivated cohesin, we conclude that DNA does not enter through a gate created by the transient dissociation of just one end of Scc1 from a SMC head.

During the course of these studies, we found that fusion of Scc1 to Smc3's head suppresses mutations in Scc1's N-terminal domain that compromise its association with the Smc3 head. Likewise, fusion of Scc1 to Smc1's head suppresses some, but not all, mutations in Scc1's C-terminal winged helix that compromise its association with a Smc1 head. These observations therefore confirm that the interaction of Scc1's N- and C-terminal domains with the ATPase heads of Smc3 and Smc1 respectively is essential for cohesin function.

The above experiments did not exclude the possibility that a gate is created by the transient dissociation of both N- and C-terminal domains of Scc1 from SMC heads. We tested this bicycle lock model by fusing Smc3's C terminus to Scc1's N terminus and using rapamycin to clamp together Frb and FKBP12 fused to the C termini of Scc1 and Smc1, respectively. Crucially, rapamycin did not exacerbate the nonlethal cohesion defects of cells expressing Smc3-Scc1-Frb along with Smc1-FKBP1 and had no discernable effect on the loading of cohesin onto chromosomes. In the course of these studies, we also found that loading of Scc1 onto centromeres and pericentric regions was largely unaffected by rapamycin-dependent dimerization of Smc1 and Smc3 heads in vivo (Figure S7).

If dissociation of Scc1 from SMC heads is not obligatory, then either DNA enters the ring between SMC hinges or our premise that cohesin traps DNA must be wrong. To test the former possibility, we replaced the hinges of Smc1 and Smc3 by a separate pair of proteins that form pseudo-symmetrical heterodimers, namely MP1 and p14. There were two potentially grave stumbling blocks to this approach. The interaction between MP1 and p14 might not be tight enough to create a tripartite ring capable of trapping DNA for long periods of time, and, even if it were, the MP1/p14 hinge might not permit formation of coiled coils and folding of the SMC ATPase heads. We addressed the former problem by showing that the MP1/p14 interaction is tight enough to hold together the N- and C-terminal

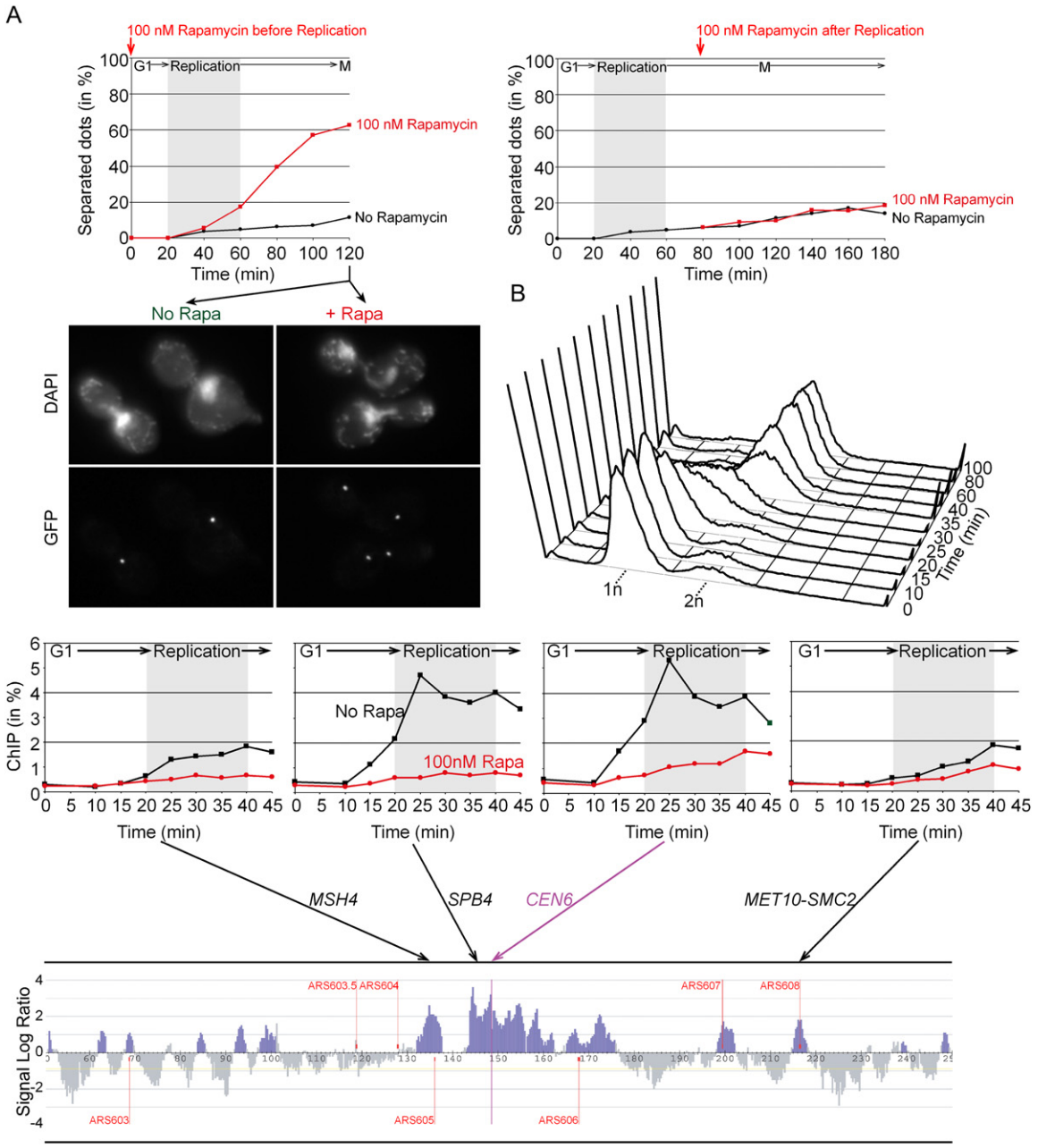

Figure 6. SMC Hinge Interconnection Hinders Establishment of Cohesion and Cohesin's Association with Chromosomes

(A) Hinge connection hinders establishment, but not maintenance, of cohesion. Cells of strain K14690 (*MAT***a** Δ*fpr1 TOR1-1* Δ*smc3 SMC3Frb SMC1FKBP12* P^{MET3}*-CDC20 112x tetOs TetR-GFP*) were grown in medium lacking methionine and arrested in G1 phase with α factor. Cells were released from the arrest (0 min) into medium supplemented with 2 mM methionine. Replication timing was monitored by flow cytometry (Figure S4B). Rapamycin was added at time point 0 (left graph) or 80 min later (right graph). Cells were stained with DAPI, and sister-chromatid cohesion was monitored by detecting fused or split GFP dots.

(B) Hinge connection hinders association of cohesin with chromosomes. Cells of strain K14697 (*MAT***a** Δ*fpr1 TOR1-1* Δ*smc3 SMC3Frb SMC1FKBP12 SCC1-Pk9*) were grown in YPD medium and arrested in G1 phase with α factor. Cells were released and incubated in YPD medium in the absence or presence of 100 nM rapamycin. Samples were taken every 5 min, and cellular DNA contents were measured by flow cytometry (top panel). Sample aliquots were subjected to chromatin immunoprecipitation using α-Pk-tagged antibodies. Input and ChIP DNA samples were analyzed by real-time PCR using four different primer pairs. Efficiency of pull-down (% chromatin IP) is plotted for each time point. Positions of the primer pairs are indicated in the bottom panel, which shows Scc1 distribution on the central part of chromosome VI in nocodazole-arrested wild-type cells.

halves of Scc1 in a functional manner—that is, we converted cohesin's tripartite ring into a quaternary one without compromising its function.

Two pieces of evidence suggest that substitution of Smc1/Smc3 hinges by MP1/p14 did not compromise folding of SMC heads. First, hinge-substituted SMC heterodimers still formed tripartite rings—that is, the heads of Smc3 and Smc1 still bind tightly to Scc1's N- and C-terminal domains, respectively. Second, hinge substitution had little or no effect on the stimulation of ATPase activity due to binding of Scc1's C-terminal domain to Smc1 heads. Because this activity depends on ATP bound to the Smc3 head as well as Scc1 bound to the Smc1 head (Arumugam et al., 2006), it suggests that both Smc1 and Smc3 ATPase heads of hinge-substituted Smc1/Smc3 heterodimers must be correctly folded. Despite forming tripartite rings that enter nuclei, hinge-substituted cohesin complexes fail to associate stably with chromosomes and establish sister-chromatid cohesion. These data imply that Smc1 and Smc3 hinges are not merely dimerization domains and are consistent with the notion that they might also act as DNA gates.

To test this, we inserted FKBP12 and Frb into the hinges of Smc1 and Smc3 respectively and asked whether the connection of these domains by rapamycin would inactivate cohesin. Remarkably, cohesin modified in this manner is fully functional in the absence of rapamycin but inactive in its presence. Rapamycin hinders cohesin's association with chromosomes and the establishment of sister-chromatid cohesion during S phase. Because rapamycin has no effect on pre-established sister-chromatid cohesion, it must inhibit an aspect of cohesin function that occurs only transiently prior to or during DNA replication. What might this be? We suggest that this function is the entry of DNA inside cohesin's ring, that this requires transient ring opening, that the ring does not open by releasing Scc1 from SMC heads and must therefore involve transient dissociation of Smc1 and Smc3 hinge domains, that hinge substitution creates a cohesin complex that cannot be opened, and that artificial linkage of these two hinges by the formation of FKBP12-rapamycin-Frb complexes either blocks the passage of DNA between hinges that have transiently opened or prevents them from opening sufficiently to let DNA pass through.

We cannot fully exclude at this stage the possibility that rapamycin-mediated linkage of Smc1 and Smc3 hinges prevents loading of cohesin onto chromosomes by a mechanism other than preventing the creation of a DNA entry gate. It is conceivable that SMC hinges do not in fact open and merely provide an interaction surface for either DNA (Hirano and Hirano, 2006; Yoshimura et al., 2002) or factors like Scc2/Scc4 that promote cohesin's engagement with chromosomes. However, this notion fails to explain why cohesin's engagement with chromosomes is blocked by linking its two hinges and not by formation of Frb-FKBP12 (Fpr1) complexes at Smc3 hinge domains together with insertion of FKBP12 within Smc1's hinge domain. Moreover, if we accept the notion that cohesin functions by trapping DNA within its ring and accept that this does not come about through dissociation of Scc1 from SMC heads, then it must involve dissociation of its hinge domains. We therefore favor the notion that SMC hinges serve as DNA entry gates because this seems to be the most parsimonious way of explaining both how cohesin associates with DNA using a topological principle and the fact that artificial interconnection of its hinge domains hinders association with chromosomes. Even if this explanation proves incorrect, our findings have demonstrated an essential function for this highly conserved SMC domain during the establishment of sister-chromatid cohesion besides dimerization of SMC molecules.

The notion that Smc1 and Smc3 hinges transiently dissociate to permit DNA entry raises a number of interesting problems. These domains have a very high affinity for each other, with a K_D in the low nanomolar range (Haering et al., 2002). How could such a tight interaction be disrupted? Two features of cohesin could be relevant. The first is that the hinges interact with each other using two independent interfaces, which creates a small hole in the middle of the hinge-domain dimer. We suggest that opening of the hinge would be greatly facilitated if only a single hinge-hinge interface needed to be opened at any one stage. A predicted dissociation constant for half of a hinge-hinge interaction would be in the higher micromolar range (about 70 μM). The hole inside a fully closed hinge is too small to accommodate double-helical DNA, but if one of its two interfaces were broken while the other remained intact, then twisting of the hinge domains out of plane (or in plane) could create a pocket large enough to accommodate DNA (see Figure 7B). If the opened interface were now to shut and the unopened one were to open in a concerted manner, then DNA's departure from the pocket would allow both interfaces to shut, and DNA would be trapped inside the cohesin ring. By opening one interface at a time and by doing so using twisting forces, the energy required to open the hinge could be greatly reduced. An out-of-plane twisting mechanism has recently been proposed for the opening of PCNA rings by the RFC clamp loading complex based on EM picture analysis and molecular dynamics simulations (Barsky and Venclovas, 2005). Interestingly, the arrangement of secondary structural elements at PCNA interfaces resembles that in SMC hinge interfaces (T. Nishino, personal communication). Remarkably, the SMC hinges are actually twisted open in such a manner in one of the two crystal forms of the homodimeric hinges of *T. maritima* (see Figure 7A) (Haering et al., 2002).

Another feature of cohesin that may be germane to the problem of how its SMC hinges might open is the observation that cohesin's stable association with chromosomes requires hydrolysis of ATP bound to its Smc1 and Smc3 heads (Arumugam et al., 2003; Weitzer et al., 2003). We suggest that the energy created either by ATP binding or by hydrolysis might be used to open the hinge. It is conceivable that conformational changes to Smc1 and

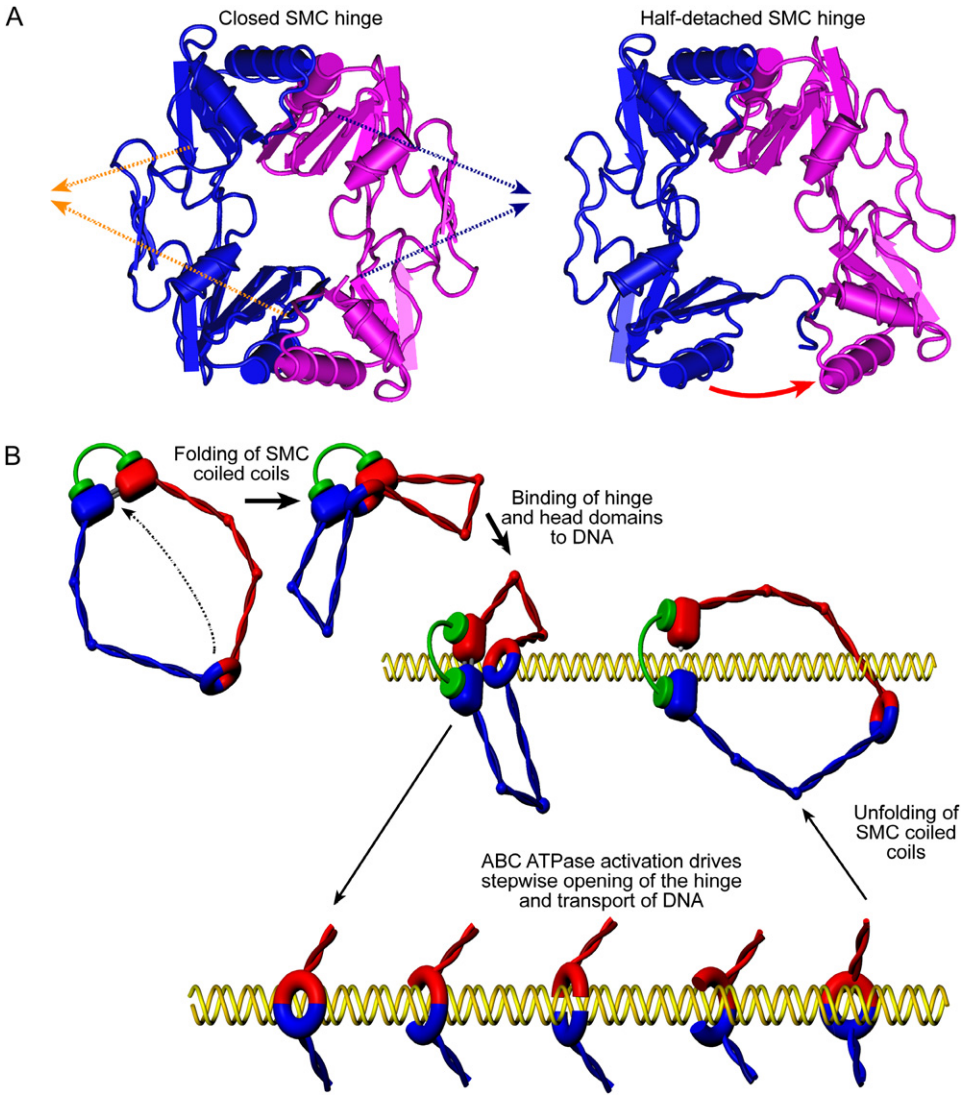

Figure 7. A Model for the Transport of DNA through SMC Hinges

(A) Crystal structure analysis of the bacterial SMC hinge dimer from *T. maritima* revealed a closed (left; PDB ID code 1GXK) and a half-open conformation (right; PDB ID code 1GXJ) (Haering et al., 2002). Arrows indicate the directions of the amphipathic helices.

(B) A model for transporting DNA into a cohesin ring. Folding of the Smc1 and Smc3 coiled coils might enable interactions of SMC hinge and head domains from the same cohesin ring, possibly creating a DNA binding surface formed by sequences of both hinge and head domains. Upon contact with DNA, binding to or hydrolysis of ATP by the ABC ATPase generates a conformational change in the SMC heads that is transmitted to hinge domains via the coiled coils, the DNA double helix, and/or direct interactions between head and hinge domains, causing disruption of one of the two hinge-hinge contact sites. Entry of DNA into the central channel of the half-open hinge might drive transient detachment of the other SMC hinge-domain interface. Reclosing of the SMC hinge after exit of the DNA double helix would finally trap DNA inside cohesin's ring.

Smc3 ATPase heads brought about by the ATP hydrolysis cycle are somehow transmitted to their hinges via the long coiled coils that connect their head and hinge domains. One problem with this notion is that either one but not both of the two polypeptide chains that are part of Smc3's coiled coil can be severed without impairing cohesin function (Gruber et al., 2003). It is difficult, albeit not impossible, to imagine forces being transmitted from heads to hinges along a single polypeptide chain. Rad50 proteins share a similar architecture with SMC proteins but use zinc hooks rather than hinge domains for dimerization. Based on atomic force microscopy (AFM), it was proposed that the conformations of Rad50 coiled coils change drastically upon binding of their ABC ATPase heads to DNA so that formation of a zinc hook within the Rad50 dimer is blocked. This supposedly leads to intercomplex interactions (Moreno-Herrero et al., 2005). However, this model is challenged by the finding that Rad50 hook domains can

be substituted by other dimerization domains without eliminating its DNA repair function (Wiltzius et al., 2005).

An alternative is that the coiled coils of SMC proteins actually fold into sections and thereby enable hinge domains to interact directly with the ATPase heads that facilitate hinge opening (Figure 7B). Electron micrographs of intact cohesin or condensin complexes after rotary shadowing show no signs of such a foldback structure (Anderson et al., 2002). However, such structures were seen by AFM with Smc2/Smc4 heterodimers when lightly fixed with glutaraldehyde (Yoshimura et al., 2002). We have observed similar foldback structures with wild-type but not with hinge-substituted Smc1/Smc3 heterodimers under the same conditions in which they were seen for Smc2/Smc4 heterodimers (data not shown). Direct interactions between hinge domains and ATPase heads might also explain how certain mutations in the SMC hinge domains of bacterial SMCs affect ATP hydrolysis by the SMC heads (Hirano and Hirano, 2006).

The notion that cohesin's ring can be opened and shut in a highly regulated manner has a number of implications. If DNA gains entry to the cohesin ring by hinge opening, then the process could easily be reversed—that is, DNA that has previously been trapped by cohesin could escape due to hinge opening. In animal cells, cohesin complexes at centromeres remain on chromosomes until the metaphase→anaphase transition, whereupon cleavage by separase is essential for their removal, while cohesin complexes on chromosome arms dissociate from chromosomes in a separase-independent manner in response to phosphorylation of their Scc3/SA subunit (Hauf et al., 2005). If the latter had also associated with chromosomes in the first place by trapping DNA within their rings, then Scc3/SA phosphorylation must somehow cause ring opening, which might occur due to hinge-hinge dissociation. If this scenario is correct, then a key question must be why cohesin rings on chromosome arms can be reopened at their hinges while others at centromeres cannot and must therefore be opened irreversibly by cleavage of Scc1 by separase.

The amino acid sequences of cohesin's Smc1 and Smc3 hinge domains are highly conserved. They are present in bacterial SMC proteins as well as Smc2 and Smc4 from condensin and Smc5 and Smc6. If, as we propose, cohesin associates with chromosomes by passing DNA between its Smc1 and Smc3 hinge domains, then this property is presumably shared by all SMC proteins. Transient dissociation of hinge domains permitting passage of DNA inside a ring structure might therefore be a feature that is fundamental to the activity of all complexes containing SMC proteins.

EXPERIMENTAL PROCEDURES

All strains were derivatives of W303 (K699). For sequences of constructs and detailed genotypes, see Supplemental Data. The open reading frames of the *SCC1*, *SMC1*, *SMC3*, and *FPR1* genes were deleted by one-step PCR disruption. *TOR1-1* was transferred into the W303 background by transformation with a PCR product and selection for rapamycin-resistant growth. The endogenous *SMC1* open reading frame was fused via a flexible linker (ESGGGGGSGGGSGGGGLE) to the *FKBP12* coding sequence by one-step tagging to produce Smc1-FKBP12 proteins. The *SMC3* and *SCC1* genes were C-terminally tagged with the Frb domain using the linker peptides TSGGGGSGGGSGGGGAS and ASGGGGGSGGGSGGGGAS, respectively, resulting in Smc3-Frb and Scc1-Frb. Mutations in *SCC1*'s C terminus were incorporated during the tagging process. All genome modifications were confirmed by DNA sequencing. Strains were grown in full medium (YEP) with 2% glucose, 2% raffinose, or 2% raffinose plus galactose at 30°C. For G1 phase arrest, cells were incubated in 5 μg/ml α factor peptide for 90 min starting at OD_{600} = 0.2 unless otherwise stated. Strains with the *CDC20* gene under control of the *MET3* promoter were grown in minimal medium lacking methionine and arrested in mitosis in YEP supplemented with 2 mM methionine for 120 min. Centrifugal elutriation was performed as described in Schwob and Nasmyth (1993). Rapamycin was dissolved in DMSO at 1 mM concentration. Chromosome spreads and DNA content analysis (flow cytometry) were performed as in Michaelis et al. (1997). Coimmunoprecipitation (ring formation assay) was performed as described in Arumugam et al. (2003), but without arresting and temperature shifting cells. FISH analysis was done as described in Lorenz et al. (2003). WT and hsSMC proteins were expressed and purified as described in Arumugam et al. (2006) with some modifications (see Supplemental Data). For the ChIP protocol and qPCR primer sequences, see Supplemental Data. ChIP-on-chip analysis was performed as described in Katou et al. (2003).

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, Supplemental References, seven figures, and one table and can be found with this article online at http://www.cell.com/cgi/content/full/127/3/523/DC1/.

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