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The Smc5/6 complex is required for dissolution of DNA-mediated sister chromatid linkages

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ABSTRACT

Mitotic chromosome segregation requires the removal of physical connections between sister chromatids. In addition to cohesin and topological entrapments, sister chromatid separation can be prevented by the presence of chromosome junctions or ongoing DNA replication. We will collectively refer to them as DNA-mediated linkages. Although this type of structures has been documented in different DNA replication and repair mutants, there is no known essential mechanism ensuring their timely removal before mitosis. Here, we show that the dissolution of these connections is an active process that requires the Smc5/6 complex, together with Mms21, its associated SUMO-ligase. Failure to remove DNA-mediated linkages causes gross chromosome missegregation in anaphase. Moreover, we show that Smc5/6 is capable to dissolve them in metaphase-arrested cells, thus restoring chromosome resolution and segregation. We propose that Smc5/6 has an essential role in the removal of DNA-mediated linkages to prevent chromosome missegregation and aneuploidy.

INTRODUCTION

Faithful chromosome segregation depends on correct establishment of sister chromatid cohesion to allow chromosome biorientation in mitosis (1,2). Tight control of sister chromatid cohesion dissolution is also crucial in order to separate sister chromatids. Sister chromatids can be linked not only by proteins, but also by catenations and other types of DNA-mediated links. DNA catenation was the first cohesion mechanism to be proposed (3). Condensin, a structural maintenance of chromosomes (SMC) complex, has been proposed to collaborate with topoisomerase II in decatenating sister chromatids (4). Protein linkages are established by the SMC complex cohesin, a multiprotein ring-shaped structure that topologically entraps the two replicated sister chromatids (5). Proteolytic cleavage of the cohesin ring provides an irreversible and quick mean of dissolving sister chromatid cohesion at the metaphase to anaphase transition.

The presence of DNA-mediated linkages can also prevent chromosome segregation. Formation of these structures may be due to the exchange of DNA strands or topological links involving only one of the two strands (hemicatenanes). The presence of unreplicated regions in a chromosome can also physically connect almost fully duplicated sister chromatids. Therefore, the DNA replication process *per se* and activation of DNA repair pathways can both contribute to the formation of DNA-mediated linkages, a hazardous structure for chromosome segregation. For example, formation of sister chromatid junctions (SCJs) is required to bypass certain types of DNA damage that block replication fork progression, such as those caused by the alkylating agent methyl methane sulfonate (MMS) (6). Although DNA-mediated linkages arise during a normal S phase, and accumulate in response to DNA damage, there is no known essential mechanisms dedicated to its detection and removal before anaphase. The final step in removal of the covalent DNA links most probably requires the action of enzymes with DNA cleavage activity (nucleases, topoisomerases) to remove the links holding the two sister chromatids together, or helicases to remove strand pairing. The combined action of a helicase (Sgs1) and a topoisomerase (Top3) has been proposed to dissolve some types of DNA linkages (7) and accumulation of SCJs has been observed in *sgs1Δ* and *top3Δ*mutants during continuous MMS damage (8). However, their requirement for chromosome segregation after SCJ generation is currently unknown.

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Apart from condensin and cohesin, eukaryotic organisms possess a third essential SMC complex, known as Smc5/6, which is involved in an undefined step during recombinational repair (4). Mutations in the Smc5/6 complex render cells sensitive to MMS (9–11). Inactivation of the Smc5/6 complex leads to the accumulation of SCJs in the rDNA locus during a normal cell cycle (10) and at other genomic loci in response to MMS treatment (12,13). Current hypothesis propose that the Smc5/6 complex would prevent homologous recombination at replication forks (12,14) or that Smc5/6 would maintain stalled replication forks in a recombination-competent conformation (15). These functions might be also subjected to compartmentalization, since the Smc5/6 complex is required to prevent access of the recombination machinery to the nucleolus (16). Overall, these results have lead to the proposal that Smc5/6 has an active role in aborting unscheduled recombination events thereby preventing generation of DNA junctions. However, it has not been tested if Smc5/6 also has a more direct role in the removal of such linkages, after their formation.

Here, we show that the Smc5/6 complex plays an important role in the removal of DNA-mediated linkages. Smc5/6 reactivation is able to dissolve SCJs, indicating an active role in their removal rather than a function in preventing their accumulation. This function is essential in order to prevent chromosome missegregation and the appearance of aneuploid cells. Moreover, Smc5/6 can perform this function in metaphase-arrested cells, suggesting that its window of operation ranges from S phase until the time of chromosome segregation.

MATERIALS AND METHODS

Yeast strains and plasmids

A list of the strains used in this study is provided in Table 1. The *smc6-9* and allele has been described previously (10). The *smc6-1* temperature-sensitive allele was generated by random mutagenesis PCR. The *SMC6* sequence was re-amplified in five parallel reactions using the GeneMorph PCR mutagenesis Kit (Stratagene). The mutagenized PCR products were pooled and cloned into pRS415. A library of mutagenized plasmids was used to transform an *smc6::kanMX4* pRS416-*SMC6* strain. Transformants were selected for growth in FOA plates lacking leucine at 25°C to counterselect for the *URA3* marker in pRS416 and to select for cells that bear the pRS415 *LEU2* plasmid. *ura3-LEU2+* cells, containing only mutagenized *SMC6* derivates in pRS415 were tested for temperature sensitivity at 37°C. Temperature sensitive colonies were tested for rescue of the *ts* phenotype by the pRS416-*SMC6* plasmid, carrying the wild-type gene. For the *smc6-1* reversibility experiments, the pRS416-*smc6-1* plasmid was recovered from yeast and transformed into a *GAL-SMC6* strain (17). The *mms21ΔC* allele codes for a protein truncated by a Cys183-STOP mutation and contains a deletion of the *MMS21* C-terminal domain.

Growth and cell cycle conditions

Yeast cells were grown in YPD at 25°C except if otherwise stated. For G1 synchronization, exponentially growing cultures were treated with 10^{-8} M alpha factor at 25°C for 2 h, or until >95% of cells were arrested in G1. Cells were shifted to 36°C for 30 min to inactivate temperature-sensitive alleles. Except stated, cells were treated with 0.01% MMS (SIGMA) for 30 min to induce a pulse of alkylation damage. To release from cell cycle block and remove MMS, cells were washed twice with prewarmed medium and resuspended in YPD plus 0.1 mg/ml pronase E (SIGMA). Nocodazole (SIGMA) was used at a final concentration of 15 μg/ml in the presence of 1% DMSO from a 6 mg/ml stock. For prolonged metaphase arrests, the concentration of nocodazole was raised to 22.5 μg/ml. For nocodazole release, cultures were washed twice and resuspended with prewarmed medium containing 1% DMSO.

Table 1. List of strains used in this study

Strain	Genotype	Reference
BY4741	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	EUROSCARF
YTR53	BY4741 *smc6-9:His3MX6*	This study
YTR608	BY4741 *smc6::kanMX4 pRS415-smc6-1*	This study
YTR633	BY4741 *GAL-3HA-SMC6 HIS bar1::URAca pRS415-smc6-1*	This study
CCG1052	BY4741 *SMC6-3HA:HisMX6 bar1::URAca*	This study
AS499	*Mata bar1Δ leu2-3,112 ura3-52 his3-Δ200 trp1-Δ63 ade2-1 lys2-801 pep4*	(10)
CCG1761	AS499 *smc6-9:natMX4*	(10)
YTR506	AS499 *his3::pHIS3-lacI-NLS-GFP:HIS3 CEN3::256xlacO:URA3*	This study
YTR516	AS499 *his3::pHIS3-lacI-NLS-GFP:HIS3 CEN3::256xlacO:URA3 smc6-9:natMX4*	This study
YMB539	AS499 *nse2ΔC::hphMX4*	This study
CCG1800	AS499 *rad52::kanMX4*	(10)
CCG1804	AS499 *smc6-9:His3MX6 rad52::kanMX4*	(10)
YTR493	AS499 *smc6-9:natMX4 TUB1-GFP:URA*	This study
CCG3227	*MATa ade2-1 trp1-1 can1-100 leu2-3, 112 his3-11,15 GAL psi+ ura3::URA3/GPD-TK(7x) RAD5 p415-ADH hENT1::LEU2 bar1::natMX4*	(19)
CCG3229	*MATa ade2-1 trp1-1 can1-100 leu2-3, 112 his3-11,15 GAL psi+ ura3::URA3/GPD-TK(7x) RAD5 p415-ADH hENT1::LEU2 smc6-9:natMX4 bar1::hphMX4*	(19)
YTR1154	AS499 *SCC1-GFP:KanMX4*	This study
YTR1156	AS499 *SCC1-GFP:KanMX4 smc6-9:natMX4*	This study

Cell extracts and western blotting

Yeast protein extracts were prepared under denaturing conditions as described (18). Protein extracts were ran in 7.5% SDS–PAGE gels. Rad53 was detected with α-Rad53 antibody (SC-6749; Santa Cruz) at a dilution of 1:5000.

Microscopy

Yeast cells carrying GFP or YFP-tagged proteins were analyzed by fluorescence microscopy after DNA staining. DNA was stained using 4,6,-Diamidino-2-phenylindole (DAPI) at 1 μg/ml final concentration in the presence of mounting solution and 0.4% Triton to permeablize cells. For fluorescence microscopy, series of *z*-focal plane images were collected with a DP30 monochrome camera mounted on an upright BX51 Olympus fluorescence microscope. Image stacks were z-projected using ImageJ software.

DNA techniques

The 2D gel electrophoresis was performed as described (19). For Pulse field gel electrophoresis (PFGE), a volume of cell culture containing 7 OD of yeast was taken at time 0. The same volume was taken for each of the following samples of the time-course experiment to reflect the same number of cells. Chromosomal DNA was purified as described (20). PFGE was carried out in a 1.0% agarose gel in 0.5× TBE buffer run for 24 h at 6 V/cm with initial switching time of 60 s, final of 120 s. Gels were stained with ethidium bromide. Quantification of stained PFG images was done with ImageJ software. In Figure 4D, E and F a band corresponding to a single chromosome was quantified and made relative to time 0. For DNA combing experiments, samples were collected when >95% of cells had arrested in G2/M phase. Cells were embedded in agarose plugs and genomic DNA was purified as for PFGE. Chromosomal DNA was then resuspended in 50 mM 2-(*N*morpholino) ethanesulfonic acid pH 5.5 at a concentration of 200 ng/ml in a Teflon reservoir. Silanized coverslips were dipped into the DNA solution for 5 min and pulled out at a constant speed (0.3 mm/s). Coverslips were baked at 60°C overnight, mounted on microscope slides and stored at −20°C until use. Coverslips were incubated for 30 min in 1 M NaOH to denature the DNA and neutralized in PBS pH 7.5. Coverslips were washed with 2× SSC-50% formamide and 2× SSC, and subsequently were processed for immunofluorescence. Bromodeoxyuridine (BrdU) was detected with a rat monoclonal antibody (AbCys, clone BU1/75) and a secondary antibody-coupled Alexa 488 (Molecular Probes). DNA molecules were counterstained with an anti-guanosine antibody (Argene) and an anti-mouse IgG coupled to Alexa 546 (Molecular Probes). Replication gaps were scored as regions positive for the anti-guanosine signal but negative for the anti-BrdU one.

RESULTS

Smc6 promotes resolution of SCJs

In order to test if Smc5/6 has a direct role in the removal of SCJs, we designed an experiment to reactivate the Smc5/6 complex after generation of sister chromatid linkages. Smc5/6 reactivation will help to eliminate DNA-mediated linkages only if the complex is required for their dissolution. A region of chromosome 3 containing the *ARS305* origin of replication was used to study the presence of SCJs by 2D electrophoresis (Figure 1A and B). We first analyzed if different hypomorphic *smc6* mutants accumulate SCJs. As shown in Figure 1C, *smc6-1* and *smc6-9* mutant cells accumulate SCJs during MMS treatment, similarly to what has been described for other mutants in the Smc5/6 complex (12,13). Double *smc6-1 Gal-SMC6* cells were arrested in G1 and released into S phase in the presence of 0.033% MMS. After 180 min, a sample was taken as control for SCJ accumulation. The culture was then split into two, and galactose was added to one half to induce expression of wild-type *SMC6*. Samples were taken 2 and 3 h after galactose addition and processed for 2D gel electrophoresis. As shown in Figure 1D, reactivation of Smc6 allows the removal of the SCJs previously accumulated in *smc6-1* cells. In contrast, SCJs remain in cells that do not express wild-type *SMC6* (glucose conditions). Quantification of the X-shaped molecules (relative to the 1N spot) shows that the level of SCJs is 2.5 times lower in the induced culture, compared to the glucose control, at the two time points tested after Smc6 reactivation. These results indicate that, similarly to Sgs1 and Top3, Smc6 is involved in the removal of DNA-mediated linkages.

Smc5/6 mutants display genome segregation defects after low-dose MMS treatment

Mutations that severely impair sensing or removal of DNA-mediated linkages are expected to display defects in chromosome segregation after MMS treatment. Wild-type cells go through a very long and slow S phase when replicating in the presence of MMS (21) and activation of the DNA damage checkpoint further arrests cells in G2/M. To monitor anaphase progression after induction of MMS lesions, we set up a more physiological approach based on a pulse (30 min) of low levels (0.01%) of MMS while cells are arrested in G1. Before the release from the G1 arrest, cells are washed thoroughly and enter S phase in the absence of MMS. Under these conditions, wild-type cells do not experience a detectable delay in cell cycle progression (Figure 2A and B) and do not activate the DNA damage checkpoint (Figure 2C). Surprisingly, nuclear segregation is unaffected in *sgs1* or *top3* mutants after an MMS pulse (Table 2). This finding suggests that the Sgs1–Top3 pair does not have an essential role in the removal of DNA-mediated linkages, at least under our experimental conditions. Nuclear segregation was also unaffected in several other helicase, topoisomerase and nuclease mutants (Table 2). This analysis indicates that these genes are either redundant or not essential for the removal of DNA links. On the other hand, cultures of

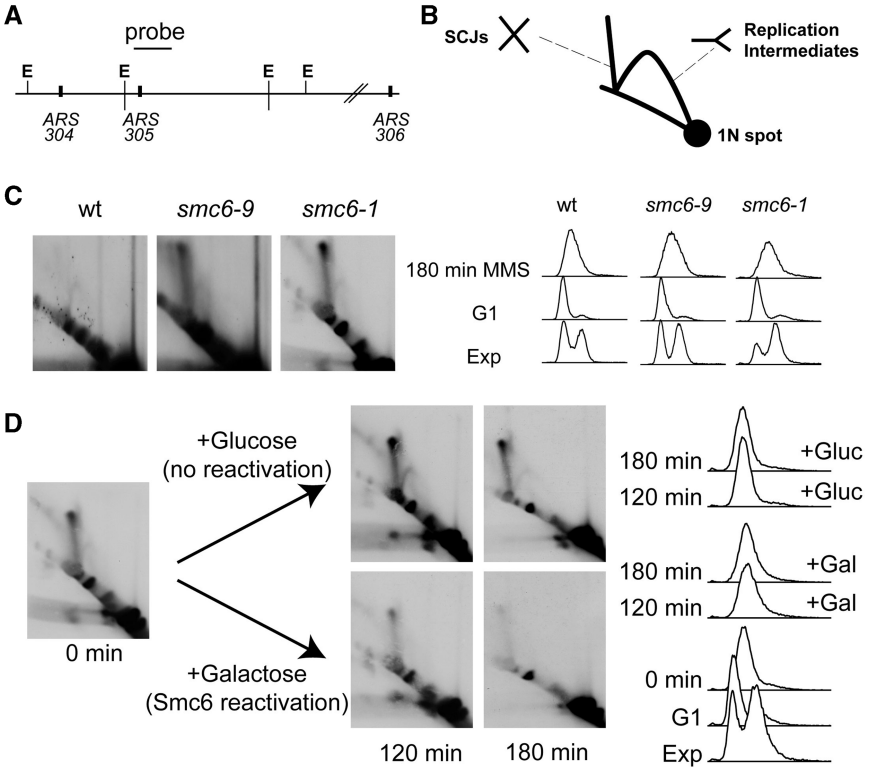

Figure 1. Smc6 promotes resolution of SCJs. (**A**) Genomic region containing *ARS305* origin of replication and the probe used for hybridization. E indicates EcoRV restriction site used. (**B**) Schematic representation of structures visualized by 2D gel electrophoresis. (**C**) Exponentially growing cultures of wild-type, *smc6-9* and *smc6-1* mutant cells were arrested in G1 and released into S phase at the restrictive temperature in the presence of 0.033% MMS for 180 min. Samples were taken and processed for 2D gel and FACS analysis. Note the accumulation of SCJs in both *smc6* mutant cultures. (**D**) *smc6-1 Gal-SMC6* cells were synchronized in G1 in YP raffinose and released into S phase with 0.033% MMS for 3 h. Galactose was then added to one half of the culture to induce expression of wild-type *SMC6*. Glucose was added to the other half as a control for no *SMC6* expression. Samples were taken at the indicated times and processed for 2D gel and FACS analysis. Note that *SMC6* reactivation allows removal of SCJs.

smc6 mutant cells display an abnormally high proportion of anaphase cells and are defective in generation of binucleated cells after an MMS pulse (Figure 2D), suggesting chromosome disjunction problems. Moreover, at the time of entry into the second cell cycle (80 min onwards) *smc6* mutant cells display massive nuclear segregation defects: ~30% of the total *smc6-9* and *smc6-1* population display missegregated nuclei at time 120 min, and ~70% of those that grow a second bud from 80–120 min have distributed unequal masses of DNA between mother and daughter cells, or have simply failed to fission the nucleus (Figure 2D). We have observed a similar phenotype for the *smc5/6* and *mms21ΔC* alleles, affected on other subunits of the Smc5/6 complex (see below). This indicates that this function requires both SMC proteins in the complex and is probably not related to a direct role in sister chromatid cohesion (22). Importantly, the defects we observe are dependent on the MMS pulse [data not shown; (10)]. Analysis of Rad53 phosphorylation in *smc6* mutant cells shows that the DNA damage checkpoint is not activated during the first S phase after the MMS pulse, which is in accordance with the absence of G2/M arrest (Figure 2C). Rad53 phosphorylation is detected at later time points in *smc6-9* cells. However, the appearance of the slower mobility forms of Rad53 coincides with entry into the second cell cycle. FACS analysis also reveals the presence of cells with less than 1N DNA content at later time points in the cell cycle, indicative of gross chromosome segregation defects (Figure 2E). A similar phenotype has been described for mutants defective in cohesion removal, such as separase mutants or cells expressing uncleavable versions of cohesin (23,24). To evaluate the chromosome segregation defects in more detail, we analyzed the fate of a chromosomal tag inserted close to centromere 3. Centromeres 3, 5 and 12 are faithfully segregated after Smc5/6 inactivation in the absence of DNA damage [data not shown; (10)]. In contrast, the centromere 3 tag frequently fails to segregate in *smc6-9* mutant cells after a G1 MMS pulse (Figure 2F, red area). Overall, these findings suggest that *smc6* mutant cells suffer from deficiencies to remove chromosome linkages after a G1 MMS pulse.

Smc5/6 mutant cells accumulate SCJs and unfinished replication intermediates after MMS treatment

We next studied the nature of the linkages that were preventing chromosome segregation in *smc6* mutants. The segregation defects indicated that these structures should

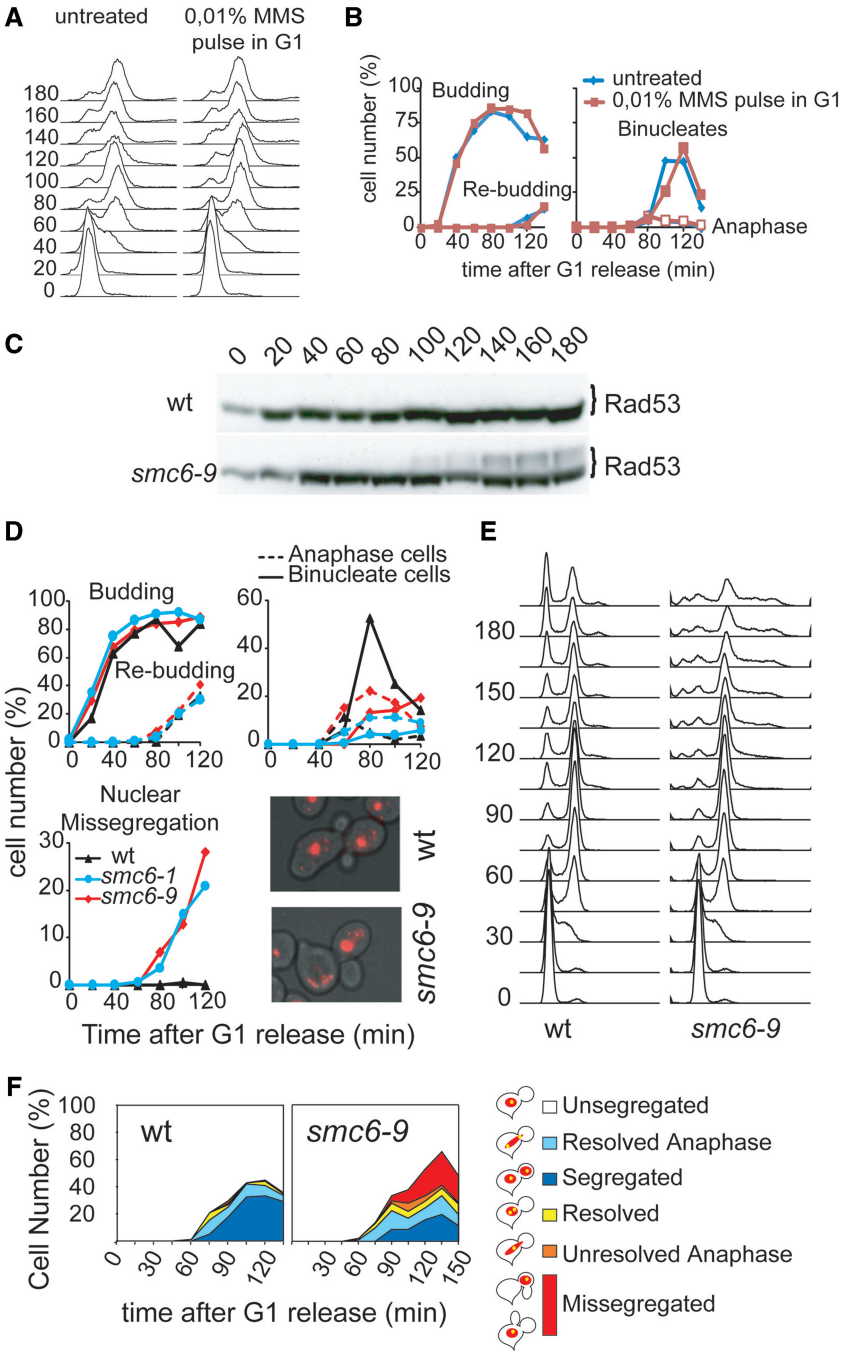

Figure 2. *Smc6* mutant cells suffer from nuclear segregation defects after induction of SCJs. (**A**) and (**B**) Wild-type cells were arrested in G1 with alpha factor at 25°C. The arrested culture was split into two and one half was treated with 0.01% MMS for 30 min. Both cultures were subsequently washed and allowed to enter the cell cycle in the absence of MMS. Samples were taken for FACS (A) and DAPI staining (B). (**C**) Wild-type and *smc6-9* mutant cells were arrested in G1 at 25°C. Arrested cells were shifted to 36°C to inactivate *smc6-9* and simultaneously treated with 0.01% MMS for 30 min. Cells were then allowed to enter the cell cycle in the absence of MMS and samples were taken for western blot analysis of Rad53. Note that wild-type cells do not phosphorylate Rad53, indicative of no checkpoint activation. *smc6-9* cells activate Rad53 after mitosis, but not during the first cell cycle. (**D**) Wild-type, *smc6-1* and *smc6-9* mutant cells were arrested in G1 at 25°C with alpha factor. Arrested cells were shifted to 36°C to inactivate *smc6-9* and simultaneously treated with 0.01% MMS for 30 min. Cells were then allowed to enter the cell cycle in the absence of MMS, and samples taken for microscopy. Cells were scored for budding, rebudding (re-bud), anaphase nucleus and two nuclei (binucleates) after DAPI staining. Bottom right corner: representative micrographs of wild-type and *smc6-9* cells at time 120 min after G1 release (DAPI-stained nuclei false-colored in red); *smc6* cells entering a second cell cycle, as evidenced by appearance of a new bud, display unequal segregation of DNA masses. (**E**) Wild-type and *smc6-9* cells were treated as in (A) and samples were taken for FACS analysis. Note the appearance of *smc6-9* mutant cells with less than 1N DNA content from 120 min onwards. (**F**) Wild-type and *smc6-9* cells carrying a centromere 3 tags were treated as in (A). Samples were taken at different time points after the G1 release, stained with DAPI and microscopically examined for localization of chromosome tags. Data are presented as stacked percentages of cells in each category. Note that very few *smc6-9* mutant cells complete a successful anaphase and many mutant cells display aberrant morphologies.

Table 2. Nuclear segregation phenotype for the indicated strains after a pulse of MMS in G1

	Strain	Phenotype after 0.01% MMS G1 pulse
BER nucleases	*apn1Δ*	Wild type
	apn2Δ	Wild type
NER nucleases	*rad1Δ*	Wild type
	rad10Δ	Wild type
	rad2Δ	Wild type
Other DNA nucleases	*exo1Δ*	Wild type
	slx1Δ	Wild type
	slx4Δ	Wild type
	mms4Δ	Wild type
	mus81Δ	Wild type
	rad27Δ	Wild type
	mre11Δ	Wild type
	sae2Δ	Wild type
	dna2-1 (37°C)	Wild type
	yen1Δ	Wild type
Topoisomerases	*top1Δ*	Wild type
	top3Δ	Wild type
	top2-4 (25°C and 30°C)	Wild type
Helicases	*mph1Δ*	Wild type
	sgs1Δ	Wild type
	srs2Δ	Wild type
	rrm3Δ	Wild type
Smc5/6 complex	*smc6-9*	Chromosome missegregation
	smc6-1	Chromosome missegregation
	mms21Δc	Chromosome missegregation
	smc5-6	Chromosome missegregation

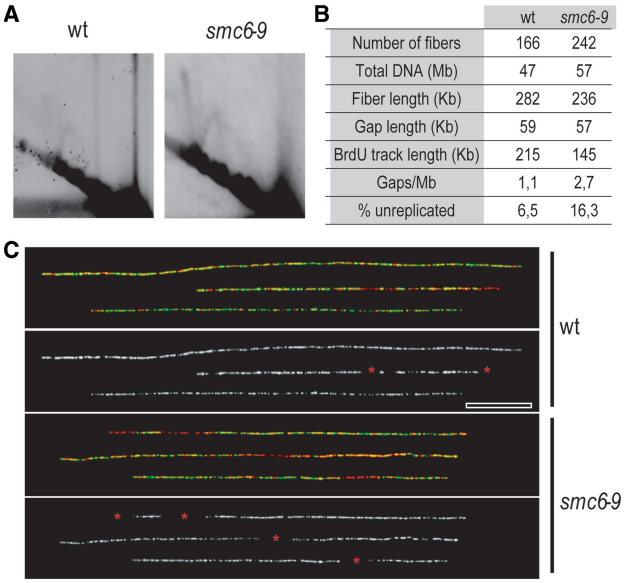

Figure 3. *Smc6* mutant cells accumulate SCJs and unfinished replication intermediates after MMS damage. Wild-type and *smc6-9* mutant cells were treated as in Figure 2D. Cells were subsequently blocked in metaphase with nocodazole. (**A**) Samples were taken for 2D gel analysis and probed for the same region studied in Figure 1. Note the presence of replication intermediates and SCJs in *smc6-9* cells. (**B**) Cells were treated as in (A) except that they were released from the G1 block in the presence of BrdU to label replicated DNA. Once arrested in G2/M, samples were taken for DNA combing. Absolute values for number of examined fibers and total DNA examined; mean values for length of fibers, gaps, BrdU tracks and gaps per megabase. (**C**) Representative fibers from wild-type and *smc6-9* cells. Red, DNA; green, BrdU; white, BrdU channel alone; asterisk, unreplicated gaps. Bar, 50 kb.

still be present at the time of anaphase onset. We therefore treated G1 arrested cells with a pulse of MMS and studied their DNA after G1 release into a metaphase block. Under these conditions, the levels of replication and recombination intermediates are almost undetectable in wild-type cells by 2D gel electrophoresis (Figure 3A). In contrast, *smc6-9* mutant cells display low but readily detectable levels of X-shaped DNA and replication forks in the *ARS305* region on chromosome 3. This result indicates that the MMS pulse conditions used in this study are inducing the accumulation of SCJs in *smc6-9* cells. Chromosome linkages can also be generated by the presence of replication forks. In fact, *smc6-9* mutant cells accumulate both unfinished replication and SCJs in the rDNA array at the time of chromosome segregation (10,19). Therefore, we checked replication of individual chromosomes in *smc6* cells by DNA combing. Wild-type and *smc6-9* mutant cells were treated with a pulse of MMS in G1. Cells were then released into S phase in the presence of BrdU to label replicated DNA. Nocodazole was added to arrest cells in metaphase. Intact chromosomal DNA was prepared and DNA fibers were treated as described (25). The number of unreplicated gaps (BrdU-negative) was scored and the overall percentage of DNA replication was calculated on the basis of the number and length of gaps. It should be noted that the gap frequency in wild-type cells is not affected by the MMS pulse; besides, unchallenged *smc6-9* cells display the same frequency of replication gaps as wild-type cells (data not shown). In contrast, *smc6-9* mutant cells display ~2.5 times increase in the number of unreplicated gaps relative to wild-type cells after a G1 pulse of MMS (Figure 3B and C). The estimated failure in DNA replication completion increases from 6.5% in wild-type cells to 16.3% in *smc6-9* mutant cells. Additionally, the average length of chromosome fibers is significantly shorter in *smc6-9* cells, suggesting that *smc6-9* fibers are more fragile. This could be due to the presence of sister chromatid linkages, which could also minimize the calculated amount of unreplicated chromosomal DNA. It is worth noting that, although the percentages of unreplicated gaps are relatively high in both strains, most of the chromosomes have been replicated in the metaphase arrest. We conclude that the Smc5/6 complex is required for completion of genome replication in response to a pulse of alkylation DNA damage. Therefore, the persistent chromosomal links in *smc6* cells arises from, at least, two different types of structures, i.e. incomplete replication and X-shaped DNA structures.

DNA linkages accumulated in smc5/6 mutants are protein- and catenation-independent

The chromosome segregation defects in *smc6* mutant cells can not only stem from defects in dissolution of DNA-mediated linkages, but also from defects in the removal of catenanes or protein-based cohesion. It has been recently proposed that the Smc5/6 complex has a role in cohesin removal in *Schizosaccharomyces pombe* (26). A GFP-tagged version of the Scc1 cohesin subunit

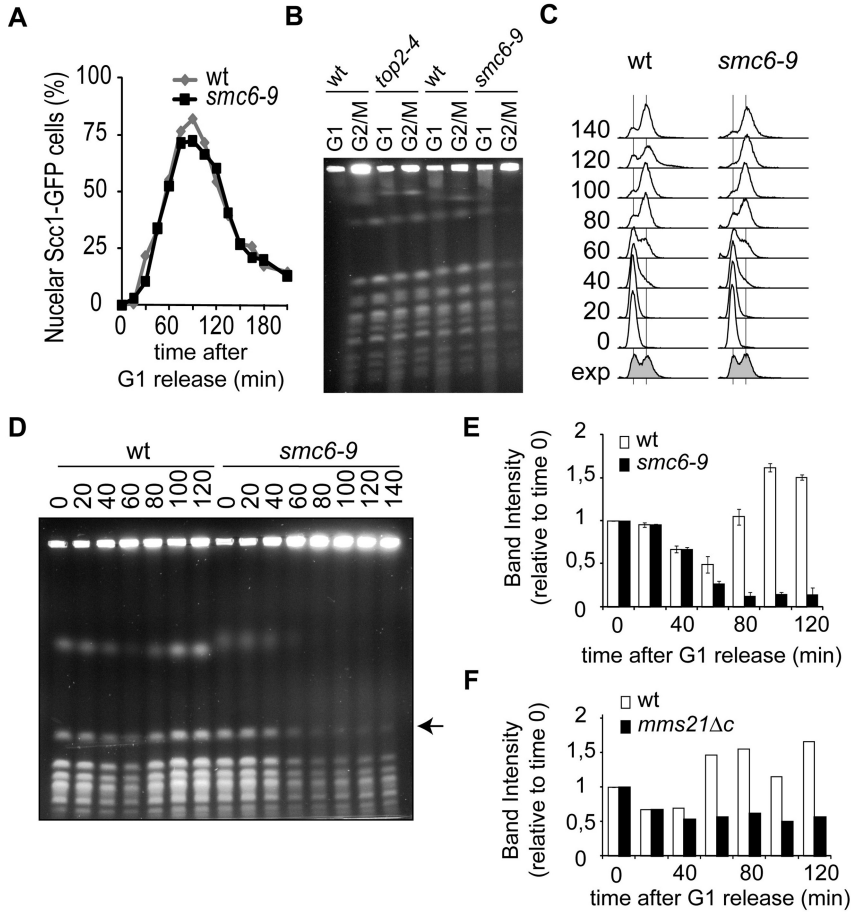

Figure 4. Chromosomes from *smc5/6* mutant cells accumulate DNA-dependent protein/topological-independent linkages after MMS damage. (**A**) Wild-type and *smc6-9* mutant cells expressing a GFP-tagged version of Scc1 were treated as in Figure 2D. Samples were taken after G1 release and microscopically examined for nuclear Scc1-GFP signal. Note the parallel disappearance of nuclear Scc1-GFP signal in both cultures. (**B**) Wild-type, *top2-4* and *smc6-9* mutant cells were treated as in Figure 2D. After release from G1, cultures were arrested in G2/M with nocodazole. Samples from G1 and G2/M arrests were processed for PFG. Note that chromosomes from *top2-4* cells enter the gel in G2/M, while those from *smc6-9* cells are defective. (**C**) FACS and (**D**) PFGE analysis of wild-type and *smc6-9* mutant cells treated as in Figure 2D. The *smc6-9* cells start replication on time but fail to resolve chromosomes after S phase (note that DNA fails to re-enter into the PFG after S phase and stays in the well). Arrow points to chromosome used for quantification. (**E**) Data from three independent experiments as those shown in (D) was used for quantification. Bars represent average; lines on bars are standard deviation. (**F**) Wild-type and *mms21ΔC* cells were treated as in Figure 2D, except that cultures were released from the G1 arrest at 30°C. Data from two independent experiments were used for quantification as in (E).

disappears from *smc6-9* nuclei with similar kinetics to wild-type cells (Figure 4A), suggesting that chromosome links in *smc5/6* mutant cells are cohesin-independent. However, and in order to discard linkages mediated by other proteins, we prepared intact chromosomes in agarose plugs and ran them in a PFGE. Sister chromatids that are glued by proteins are resolved from each other during preparation of chromosomes: yeast chromosomes from wild-type G2/M arrested cells are fully loaded with cohesin, yet they can be resolved in a PFG (Figure 4B). Catenated sister chromatids are also separated during migration and reorientation of chromosomes in PFGE (27,28) (Figure 4B). Therefore, only sister chromatids that are linked by replication intermediates or SCJs fail to migrate into the gel when subjected to a pulsed electric field. Wild-type and *smc6* mutant cells were treated with a pulse of MMS in G1. Figure 4D shows that there is a decrease in the amount of wild-type chromosomes that enter the PFG during DNA replication. This is due to the presence of branched replication intermediates. Following S phase completion (Figure 4C), chromosomes from wild-type cells re-enter into the gel (100 min onwards). In contrast, chromosomes from *smc6-9* mutant cells fail to enter the gel even at later time points (Figure 4C and D). Quantification of bands shows that chromosomes replicated in *smc6-9* cells suffer a drastic reduction in its ability to re-enter into the gel after S phase. Similar results were observed for metaphase-arrested *smc6-9* cells (Figure 4B) and for the *smc6-1* allele (data not shown). *mms21ΔC* mutant cells, lacking the Mms21 SUMO-ligase domain, display defects in nuclear segregation after a G1 pulse of MMS (data not shown) and in resolution of chromosomes in a PFGE (Figure 4F). We conclude that SUMOylation by Mms21, together with a functional Smc5/6 complex, are required to prevent the accumulation of chromosomal linkages at the time of chromosome segregation. Analysis of double *smc6-9 rad52Δ* mutant cells

progressing synchronously in the cell cycle indicates that the DNA linkages arising in *smc6* mutant cells are due, at least in part, to HR-dependent processes; equivalent results were obtained after arrest of cultures in G2/M (data not shown). Therefore, some of the DNA-mediated linkages that compromise chromosome resolution in *smc5/6* mutants are dependent on the homologous recombination machinery.

Smc6 is required for the timely removal of DNA-mediated linkages

If, as expected from Figure 1, Smc5/6 has a role in the timely removal of DNA-mediated linkages, its reactivation should allow chromosome resolution and segregation. To test this idea, we designed an experiment to reactivate the Smc5/6 complex after induction of DNA-mediated linkages by a pulse of MMS. Double *GAL-SMC6 smc6-1* mutant cells were arrested in G1 and treated with 0.01% MMS before release from G1 into a metaphase block. Once arrested in G2/M, the culture was split into two. The *GAL* promoter was induced in one half by addition of galactose, and repressed in the other by glucose. Both cultures were kept arrested in nocodazole for the rest of the experiment (data not shown). Western blot indicates that the Smc6 protein is detected 20 min after the induction of the *GAL* promoter, and accumulates above wild-type levels after 40 min of expression (Figure 5A). Repression of the wild-type copy of *SMC6* does not alter the amount of chromosomes resolution during the 70 min time course (Figure 5B). In contrast, samples from cultures incubated in galactose show re-entry of chromosomes into the PFGE 20 min after induction of *SMC6* expression, indicating resolution of DNA-mediated linkages (Figure 5B and C). The re-entry is most obvious for the two more retarded chromosomes in the gel, most probably chromosomes XII and IV, which can barely be detected and quantified in the non-induced culture conditions. Quantification of bands in the PFGE shows active linkage resolution for all medium-sized chromosomes (~3 times more after 30 min than at time 0). If Smc5/6 reactivation in a metaphase block can induce removal of DNA-mediated linkages, it is expected that *smc6-1* mutant cells will display improved nuclear segregation after expression of *SMC6* from the *GAL* promoter. To test this idea, we repeated the same procedure, except that both cultures were released from the nocodazole block into anaphase. Microscopic analysis indicates that both cultures complete mitosis and enter a new cell cycle with similar kinetics (data not shown). As expected from Figure 2D, *GAL-SMC6 smc6-1* cells that progress into anaphase in raffinose media display nuclear segregation defects (Figure 5D), since most of the cells that rebud display unequal separation of nuclei. As expected from Figure 2E, these failures are paralleled by the appearance of cells with less than 1N DNA content by FACS (Figure 5E). In contrast, *smc6-1* cells that progress into anaphase after expression of wild-type *SMC6* display virtually no defects in nuclear segregation (Figure 5D and E). The reversibility of chromosome segregation failures

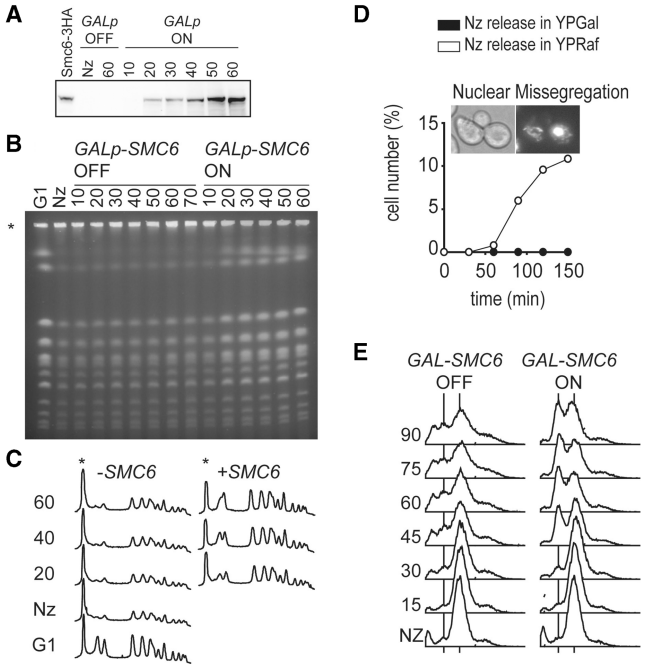

Figure 5. Smc6 removal of MMS-induced DNA linkages is required for chromosome segregation. **(A)** and **(B)** *smc6-1 GalSMC6* cells growing in YPRaf were arrested and treated as in Figure 2D. Cells were then arrested in G2/M with nocodazole. Once blocked in G2/M, galactose or glucose was added to 2% final concentration and samples were taken for western blot and PFGE analysis at the indicated times after induction of the *GAL* promoter. **(C)** Lane profiles of the indicated time points from the gel shown in (B). Note that only *SMC6* reactivation allows re-entry of chromosomes into the gel. Asterisk denotes signal from well. **(D)** *smc6-1 GalSMC6* were treated as in (A), except that cells were released from the metaphase block and allowed to enter anaphase. Twenty minutes before the nocodazole release, galactose was added to one half of the culture to induce expression of *SMC6* from the *Gal* promoter, while the other half was kept in raffinose. Cells were stained with DAPI and scored for cell cycle progression and nuclear missegregation. **(E)** FACS analysis of the nocodazole release; note that *SMC6* reactivation in the metaphase arrest prevents nuclear missegregation and the appearance of cells with less than 1N DNA content.

confirms that, rather than a function in preventing the appearance of sister chromatid DNA-linkages, Smc5/6 has a role in their resolution. Smc5/6 probably triggers a fast reaction, since chromosome resolution is also a relatively quick event after *SMC6* reactivation (20 min onwards). It is worth noting that although such reaction might be carried out in S phase during a normal cell cycle, Smc5/6 can perform its function even in metaphase-arrested cells.

DISCUSSION

The ability to connect sister chromatids is essential for chromosome biorientation and segregation. However, failure to remove these connections can also threaten the integrity of the genome, a hallmark of many cancer cells. Sister chromatid linkages can be generated by: (i) proteins, such as those provided by cohesin, and resolved by separase at the metaphase to anaphase transition; (ii)

catenations, generated by transcription and replication, and resolved by topoisomerase and condensin in G2/M; and (iii) DNA-mediated links, which arise during DNA replication and repair. Here, we have shown that the Smc5/6 complex is a central element in the removal of DNA-mediated linkages between sister chromatids.

Two dimensional gel electrophoresis indicates that *smc6* mutants accumulate SCJs during MMS-induced damage (Figure 1) (12,13). Therefore, Smc5/6 is not required for the generation of these structures. These X-shaped DNA structures are dependent on the Homologous Recombination (HR) pathway, and double *smc5/6 rad52* mutants do not accumulate SCJs (12,13). It was important to test whether the accumulation of SCJs in *smc5/6* mutants is dependent upon the inability to prevent their appearance (i.e. anti-recombinogenic) or to dissolve them. The results presented here (Figures 1 and 5) show that the physical linkages can be dissolved upon reactivation of Smc6, indicating that the Smc5/6 complex must have an active role in their removal. Therefore, the Smc5/6 complex operates downstream of the HR machinery to promote resolution of recombination-dependent structures. Our study indicates that the linkages preventing chromosome segregation in *smc5/6* mutants are generated not only by SCJs, but also by unfinished replication structures (Figure 3). We have collectively referred to these connections as DNA-mediated linkages, since they are not mediated by proteins or catenations (Figure 4). It has been recently described that Smc6 could also have an additional role in loading of Rad52 at forks stalled by HU in the rDNA (15). It is therefore possible that Smc6 might have different roles at different times during recombinational repair, specially within special loci such as the rDNA.

Alkylation damage by MMS is normally used as a model to induce blocks in replication fork progression and to generate homologous recombination-dependent SCJs. However, the effects of MMS-induced linkages on chromosome segregation had not been previously addressed because activation of the DNA damage checkpoint precludes anaphase entry. Here, we have used a pulse of MMS in G1 to generate DNA-mediated linkages under conditions that do not activate the DNA damage checkpoint (Figure 2). In contrast to all other mutants tested in this study (Table 2), *smc5/6* mutant cells cannot separate sister chromatids after a pulse of MMS in G1. As a consequence, they suffer from chromosome missegregation, leading to the appearance of aneuploid cells (Figure 2). It is important to note that the role of Smc5/6 in chromosome segregation is not restricted to the rDNA array, the major binding site for the Smc5/6 complex in budding yeast (10,19), and even centromeric regions can experience resolution defects during anaphase in *smc6* mutant cells (Figure 2F). The nuclear segregation failures in *smc5/6* mutants are observed in response to MMS treatment, and not in unchallenged mutant cells, presumably because MMS increases the frequency of replication fork stalling and SCJs formation. The higher incidence of gross chromosomal rearrangements in *smc5/6* mutants (29) most probably reflects the inability to dissolve naturally arising DNA-mediated linkages.

Smc5/6 probably triggers a fast reaction, since chromosome resolution is also a relatively quick event after *SMC6* reactivation (20 min onwards; Figure 5). It is worth noting that although such reaction might be carried out in S phase during a normal cell cycle, Smc5/6 can perform its function even in metaphase-arrested cells. Sequence prediction and homology searches indicate that there is no subunit in the Smc5/6 complex with helicase or DNA cleavage activity (data not show). This suggests that Smc5/6 most probably detects and signals removal of DNA linkages but does not catalyze the resolution reaction. In support for this, some subunits of the Smc5/6 complex have signaling activity: Nse2/Mms21 is an E3 SUMO-ligase, capable of SUMOylating substrates *in vitro* and *in vivo* (9,30,31); and the RING-finger domain in the Nse1 protein is compatible with ubiquitin-ligase activity (32). Thus, differently to cohesin and condensin, and in addition to its structural role on DNA, we propose that the Smc5/6 complex assists dissolution of DNA linkages through SUMOylation of other proteins. In fact, we have found that *mms21ΔC* mutants display similar defects to *smc6* mutant cells, indicating that SUMO-modification is also important for removing DNA-mediated linkages (Figure 4F). The most obvious Smc5/6 targets for the removal of DNA linkages are enzymes with DNA cleavage activity or helicase–topoisomerase pairs. Single mutation in most yeast DNA nucleases, helicases and topoisomerases lead to no obvious nuclear missegregation phenotype after a G1 MMS pulse (Table 2). These results indicate that these genes are either not essential or cooperate in the removal of SCJs. It should be noted that our experimental procedures were not sensitive enough to detect small but significant contributions to dissolution of chromosome links, since only the bulk of the DNA masses were analyzed by microscopy. A more detailed analysis of individual loci resolution will be required to conclusively ascertain their requirement in segregation of damaged chromosomes. On the other hand, the removal of unfinished replication structures might require either reactivation of stalled replisomes or unzipping of the unreplicated dsDNA region. The latter could be achieved through uncoupling of the replicative helicase and the DNA synthesis machinery. Non-replicative DNA helicases could also have a role in resolution of unreplicated regions. In support for this, it has been recently proposed that FANC and the BLM helicase could mediate resolution of unfinished replication intermediates during anaphase by unzipping the unreplicated dsDNA (33,34). It will be interesting to examine a possible role for Smc5/6 in elimination of anaphase bridges in higher eukaryotes, and any putative regulation of BLM or FANC by the Smc5/6 complex. However, it should be noted that the yeast homologue of BLM (Sgs1) appears not to be defective in gross nuclear separation (under our experimental approach; Table 2), suggesting that it is very unlikely to be regulated by Smc5/6, at least in budding yeast. Besides, Sgs1 is not SUMOylated by Mms21 in yeast (12). Given the plethora of telomere proteins SUMOylated by Mms21 in humans (35), it is highly probable that the Smc5/6–Mms21 complex will also regulate more than one target for the removal of chromosome linkages. Further work will be required to

identify the relevant targets of Smc5/6 in chromosome resolution.

Despite recent findings indicating new roles for the cohesin complex in gene regulation, their subunits were discovered by their very specialized role in keeping the two replicated sister chromatids together (36,37). In contrast, the Smc5/6 complex was identified mainly by an ill-defined role in DNA damage repair. Therefore, while it is well established that the cohesin complex works by tethering sister chromatids together, the function of the Smc5/6 complex has remained largely unknown. Here, we have shown that the Smc5/6 complex is required for resolution of DNA-mediated sister chromatid linkages. Therefore, loading of the Smc5/6 complex at double strand breaks or collapsed forks (38–40) would not influence DNA repair *per se*, but would be required to maintain the identity of each sister chromatid during the replication/repair processes. This could be important to later promote dissolution of the connections, and to prevent chromosome missegregation, gross chromosomal rearrangements and loss of heterozygosity (11,29). The three eukaryotic SMC complexes, cohesin, condensin and Smc5/6, probably share basic mechanisms of interaction and association with DNA. Besides, each complex is most probably devoted to one of the three different forms of sister chromatid linakges, i.e. proteinaceous, topological and DNA-mediated. However, our current results indicate that the function of Smc5/6 on chromatin will be more close to a 'resolvin' than to a cohesin. Further experiments and more mechanistic details on this cryptic complex will be required to test this hypothesis.

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