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

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Received March 31, 2010; Revised May 27, 2010; Accepted May 28, 2010

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 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.

| 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::URA4c pRS415-smc6-1             | This study             |
| CCG1052     | BY4741 SMC6-3HA::His3MX6 bar1::URA4c                          | This study             |
| AS499       | Mata bar13 leu2-3,112 ura3-32 hid3-200 trp1-63 adel2-1 lys2-801 pep4 | (10)                   |
| CCG1761     | AS499 smc6-9:natMX4                                           | (10)                   |
| YTR506      | AS499 his3::pHIS3-lacI-NLS-GFP:HIS3 CEN3::256xclacO:URA3      | This study             |
| YTR516      | AS499 his3::pHIS3-lacI-NLS-GFP:HIS3 CEN3::256xclacO: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                                           | This study             |
| CCG3227     | AS499 ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 GAL psi+ ura3::URA3/GPD-TK/7x) | This study             |
|             | RAD5 p45-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) | This study             |
|             | RAD5 p45-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             |

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.
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 permeabilize 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 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 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 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-(Nmorpholino) 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 mms21AC 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, re-budding (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.
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 and studied for chromosome segregation phenotype for the indicated strains after a pulse of MMS in G1 (Table 2).

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

### 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 nuclease |  |
| apn1A | Wild type |
| apn2A | Wild type |
| NER nuclease |  |
| rad1A | Wild type |
| rad10A | Wild type |
| rad2A | Wild type |
| Other DNA nuclease |  |
| exo1A | Wild type |
| slx1A | Wild type |
| slx4A | Wild type |
| mms4A | Wild type |
| mss81A | Wild type |
| rad27A | Wild type |
| mre11A | Wild type |
| sae2A | Wild type |
| dna2-1 (37°C) | Wild type |
| yen1A | Wild type |
| Topoisomerases |  |
| top1A | Wild type |
| top3A | Wild type |
| top2-4 (25°C and 30°C) | Wild type |
| Helicases |  |
| mph1A | Wild type |
| sgs1A | Wild type |
| srs2A | Wild type |
| rrm3A | Wild type |
| Smc5/6 complex |  |
| smc6-9 | Chromosome missegregation |
| smc6-1 | Chromosome missegregation |
| mms21Ac | 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.
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 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). 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.

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 mms21AC 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 misregulation 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 linkages, i.e. proteinaceous, topological and DNA-mediated. However, our current results indicate that the function of Smc5/6 on chromatid 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.

ACKNOWLEDGEMENTS
We thank Sónia Rius and Adam Jarmuz for their excellent technical assistance; Marti Aldea, Carme Gallego, Eloi Gari, Siba Al Medawar and all members in L.A. and J.T-R. labs for critically reading the manuscript; Michael Lisby, Mitsuhiro Yanagida and Tim Formosa for reagents and strains.

FUNDING
Spanish Ministry of Science and Innovation (FPU fellowship to M.B.); Medical Research Council (MRC) of the UK (to L.A.); Spanish Ministry of Science and Innovation (BFU2006-06423, BFU2009-08808, Colsolider-Ingenio2010 and Ramón y Cajal to J.T-R.). Funding for open access charge: Grants BFU2009-08808, Colsolider-Ingenio2010 and Ramón y Cajal from the Spanish Ministry of Science and Innovation (to J.T-R.).

Conflict of interest statement. None declared.

REFERENCES
1. Onn, I., Heidinger-Pauli, J.M., Guacci, V., Unal, E. and Koshland, D.E. (2008) Sister chromatid cohesion: a simple concept with a complex reality. *Annu. Rev. Cell Dev. Biol.*, 24, 105–129.
2. Diaz-Martiner, L.A., Gimenez-Abian, J.F. and Clarke, D.J. (2008) Chromosome cohesion - rings, knots, orcs and fellowship. *J. Cell Sci.*, 121, 2107–2114.
3. Sundin, O. and Varshavsky, A. (1980) Terminal stages of SV40 DNA replication proceed via multiply intertwined catenated dimers. *Cell*, 21, 103–114.
4. Losada, A. and Hirano, T. (2005) Dynamic molecular linkers of the genome: the first decade of SMC proteins. *Genes Dev.*, 19, 1269–1287.
5. Nasmuth, K. and Haering, C.H. (2005) The structure and function of SMC and kleisin complexes. *Annu. Rev. Biochem.*, 74, 595–648.
6. Ulrich, H.D. (2005) The RAD6 pathway: control of DNA damage bypass and mutagenesis by ubiquitin and SUMO. *ChemBiochem.*, 6, 1735–1743.
7. Wu, L. and Hickson, I.D. (2003) The Bloom’s syndrome helicase suppresses crossing over during homologous recombination. *Nature*, 426, 870–874.
8. Liberi, G., Maffioletti, G., Luca, C., Chiolo, I., Baryshnikova, A., Cotta-Ramusino, C., Lopes, M., Pellicer, A., Haber, J.E. and Foiani, M. (2005) Rad51-dependent DNA structures accumulate at damaged replication forks in sgs1 mutants defective in the yeast ortholog of BLM RecQ helicase. *Genes Dev.*, 19, 339–350.
9. Zhao, X.L. and Blobel, G. (2005) A SUMO ligase is part of a nuclear multiprotein complex that affects DNA repair and chromosomal organization. *Proc. Natl Acad. Sci. USA*, 102, 4777–4782.
10. Torres-Rosell, J., Machin, F., Farmer, S., Jarmuz, A., Eydmann, T., Dalgaard, J.Z. and Aragon, L. (2005) SMC5 and SMC6 genes are required for the segregation of repetitive chromosome regions. *Nat. Cell Biol.*, 7, 412–419.
11. Cost, G.J. and Cozzarelli, N.R. (2006) Smc5p promotes faithful chromosome transmission and DNA repair in Saccharomyces cerevisiae. *Genetics*, 172, 2185–2200.
12. Brautzi, D., Sollier, J., Liberi, G., Zhao, X.L., Maeda, D., Seki, M., Enomoto, T., Ohta, K. and Foiani, M. (2006) Ubc9 and mms21 mediated sumoylation counteracts recombinogenic events at damaged replication forks. *Cell*, 127, 509–522.
13. Sollier, J., Driscoll, R., Castellucci, F., Foiani, M., Jackson, S.P. and Brautzi, D. (2009) The Saccharomyces cerevisiae Esc2 and Smc5-6 proteins promote sister chromatid junction-mediated intra-S repair. *Mol. Biol. Cell*, 20, 1671–1682.
14. Ampatzidou, E., Irmisch, A., O’Connell, M.J. and Murray, J.M. (2006) Smc5/6 is required for repair at collapsed replication forks. *Mol. Cell Biol.*, 26, 9387–9401.
15. Irmisch, A., Ampatzidou, E., Mizuno, K., O’Connell, M.J. and Murray, J.M. (2009) Smc5/6 maintains stalled replication forks in a recombination-competent condition. *EMBO J.*, 28, 144–155.
16. Torres-Rosell, J., Sunjavicar, L., De Piccoli, G., Sacher, M., Eckert-Boulet, N., Reid, R., Jentsch, S., Rothstein, R., Aragon, L. and Lisby, M. (2007) The Smc5-Smc6 complex and SUMO modification of Rad52 regulates recombinational repair at the ribosomal gene locus. *Nat. Cell Biol.*, 9, 923–931.
17. Torres-Rosell, J., Machin, F. and Aragon, L. (2005) Smc5-Smc6 complex preserves nuclear integrity in S.cerevisiae. *Cell Cycle*, 4, 868–872.
18. Gallego, C., Gari, E., Colomina, N., Herrero, E. and Aldea, M. (1997) The Cin3 cyclin is down-regulated by translational repression and degradation during the G1 arrest caused by nitrogen deprivation in budding yeast. *EMBO J.*, 16, 7196–7206.
19. Torres-Rosell, J., De Piccoli, G., Cordon-Preciado, V., Farmer, S., Jamuz, A., Machin, F., Pasero, P., Lisby, M., Haber, J.E. and Aragon, L. (2007) Anaphase onset before complete DNA replication with intact checkpoint responses. *Science*, 315, 1411–1415.
20. Lengronne, A., Pasero, P., Bensimon, A. and Schwob, E. (2001) Monitoring S phase progression globally and locally using BrdU incorporation in Tk(+) yeast strains. *Nucleic Acids Res.*, 29, 1433–1442.
21. Tercero, J.A. and Diffley, J.F. (2001) Regulation of DNA replication fork progression through damaged DNA by the Mec1/Rad53 checkpoint. *Nature*, 412, 553–557.
22. Behlke-Steinert, S., Touat-Todeschini, L., Skoufias, D.A. and Margolis, R.L. (2009) SMC5 and MMS21 are required for...
chromosome cohesion and mitotic progression. *Cell Cycle*, 8, 2211–2218.

23. Ciosk, R., Zachariae, W., Michaelis, C., Shevchenko, A., Mann, M. and Nasmyth, K. (1998) An Esp1/Pds1 complex regulates loss of sister chromatid cohesion at the metaphase to anaphase transition in yeast. *Cell*, 93, 1067–1076.

24. Uhlmann, F., Lottspeich, F. and Nasmyth, K. (1999) Sister-chromatid separation at anaphase onset is promoted by cleavage of the cohesin subunit Scc1. *Nature*, 400, 37–42.

25. Luke, B., Versini, G., Jaquenoud, M., Zaidi, I. W., Kurz, T., Pintard, L., Pasero, P. and Peter, M. (2006) The cullin Rtt101p promotes replication fork progression through damaged DNA and natural pause sites. *Curr. Biol.*, 16, 786–792.

26. Outwin, E. A., Irmisch, A., Murray, J. M. and O’Connell, M. J. (2009) Smc5-Smc6-dependent removal of cohesin from mitotic chromosomes. *Mol. Cell Biol.*, 29, 4363–4375.

27. Baxter, J. and Diffley, J. F. X. (2008) Topoisomerase II inactivation prevents the completion of DNA replication in budding yeast. *Mol. Cell*, 30, 790–802.

28. Holm, C., Stearns, T. and Botstein, D. (1989) DNA topoisomerase II must act at mitosis to prevent nondisjunction and chromosome breakage. *Mol. Cell Biol.*, 9, 159–168.

29. Hwang, J. Y., Smith, S., Ceschia, A., Torres-Rosell, J., Aragon, L. and Myung, K. (2008) Smc5-Smc6 complex suppresses gross chromosomal rearrangements mediated by break-induced replications. *DNA Repair*, 7, 1426–1436.

30. Andrews, E. A., Palecek, J., Sergeant, J., Taylor, E., Lehmann, A. R. and Watts, F. Z. (2005) Nse2, a component of the Smc5-6 complex, is a SUMO ligase required for the response to DNA damage. *Mol. Cell Biol.*, 25, 185–196.

31. Potts, P. R. and Yu, H. T. (2005) Human MMS21/NSE2 is a SUMO ligase required for DNA repair. *Mol. Cell Biol.*, 25, 7021–7032.

32. Pebernard, S., Perry, J. J., Tainer, J. A. and Boddy, M. N. (2008) Nse1 RING-like domain supports functions of the Smc5-Smc6 holocomplex in genome stability. *Mol. Biol. Cell.*, 19, 4099–4109.

33. Chan, K. L., Palmai-Pallag, T., Ying, S. and Hickson, I. D. (2009) Replication stress induces sister-chromatid bridging at fragile site loci in mitosis. *Nat. Cell Biol.*, 11, 753–760.

34. Naim, V. and Rosselli, F. (2009) The FANC pathway and BLM collaborate during mitosis to prevent micro-nucleation and chromosome abnormalities. *Nat. Cell Biol.*, 11, 761–768.

35. Potts, P. R. and Yu, H. (2007) The Smc5/6 complex maintains telomere length in ALT cancer cells through SUMOylation of telomere-binding proteins. *Nat. Struct. Mol. Biol.*, 14, 581–590.

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

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

38. De Piccoli, G., Cortes-Ledesma, F., Ira, G., Torres-Rosell, J., Uhle, S., Farmer, S., Hwang, J. Y., Machin, F., Ceschia, A., McAleenan, A. et al. (2006) Smc5-Smc6 mediate DNA double-strand-break repair by promoting sister-chromatid recombination. *Nat. Cell Biol.*, 8, 1032–1034.

39. Potts, P. R., Porteus, M. H. and Yu, H. T. (2006) Human SMC5/6 complex promotes sister chromatid homologous recombination by recruiting the SMC1/3 cohesin complex to double-strand breaks. *EMBO J.*, 25, 3377–3388.

40. Lindroos, H. B., Strom, L., Itoh, T., Katou, Y., Shirahige, K. and Sjogren, C. (2006) Chromosomal association of the Smc5/6 complex reveals that it functions in differently regulated pathways. *Mol. Cell*, 22, 755–767.