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# The SMC5/6 Complex Is Involved in Crucial Processes During Human Spermatogenesis[1]

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## ABSTRACT

**Genome integrity is crucial for safe reproduction. Therefore, chromatin structure and dynamics should be tightly regulated during germ cell development. Chromatin structure and function are in large part determined by the structural maintenance of chromosomes (SMC) protein complexes, of which SMC5/6 recently has been shown to be involved in both spermatogonial differentiation and meiosis during mouse spermatogenesis. We therefore investigated the role of this complex in human spermatogenesis. We found SMC6 to be expressed in the human testis and present in a subset of type $A_{dark}$ and type $A_{pale}$ spermatogonia, all spermatocytes, and round spermatids. During human meiosis, SMC5/6 is located at the synaptonemal complex (SC), the XY body, and at the centromeres during meiotic metaphases. However, in contrast to mouse spermatogenesis, SMC6 is not located at pericentromeric heterochromatin in human spermatogenic cells, indicating subtle but perhaps important differences in not only SMC5/6 function but maybe also in maintenance of genomic integrity at the repetitive pericentromeric regions. Nonetheless, our data clearly indicate that the SMC5/6 complex, as shown in mice, is involved in numerous crucial processes during human spermatogenesis, such as in spermatogonial development, on the SC between synapsed chromosomes, and in DNA double-strand break repair on unsynapsed chromosomes during pachynema.**

*chromatin, human reproduction, human spermatogenesis, male infertility, meiosis, meiotic recombination, SMC5/6, spermatogenesis, spermatogonial differentiation, synaptonemal complex*

## INTRODUCTION

Spermatogenesis involves three major developmental stages: mitotic stem cell self-renewal, proliferation, and differentiation (spermatogonia); meiosis (spermatocytes); and haploid cell development and maturation (spermatids) [1]. All three stages are characterized by continuous changes in composition and function of chromatin.

Proper chromatin structure and dynamics are crucial for genomic integrity, influence replication and transcription, and drive the mitotic and meiotic cell cycles. Failure in the spatiotemporal organization of chromatin will cause incorrect chromosome segregation, chromosomal aberrations, and aneuploidy. This, in turn, can result in spermatogonial apoptosis, meiotic arrest, or gametes with genomic instability, leading to either infertility or congenital malformation of the offspring [2, 3]. Tight regulation of chromatin architecture and genome integrity maintenance is therefore crucial for safe reproduction.

The structural maintenance of chromosomes (SMC) protein complexes, called cohesin (SMC1/3), condensin (SMC2/4), and SMC5/6, are known to control cell-cycle progression, differentiation, DNA damage repair, and the structure and dynamics of chromatin [4–6]. Of these, the SMC5/6 complex has been most directly and exclusively described to be involved in DNA damage repair and genomic integrity maintenance [7–10]. Like the other SMC complexes, the SMC5/6 complex consists of two SMC proteins, SMC5 and SMC6, and several non-SMC elements (for review, see [11]).

Initially, in yeast cells, the SMC5/6 complex was described to promote repair of DNA double-strand breaks (DSBs) by facilitating sister chromatid recombination and recruitment of cohesin to the site of damage [7–10]. Moreover, the complex was found to facilitate alternative lengthening of telomeres in mammalian cancer cells by promoting intratelomeric recombination [10, 12]. In contrast, the SMC5/6 complex is also thought to maintain genomic stability by preventing aberrant recombination between repetitive DNA sequences, such as heterochromatin or rDNA [10, 13–15]. Besides recombination, yeast SMC5/6 has also been found to provide structural organization and topological stress relief during replication in mitotically dividing cells [13, 16–23] and is perhaps involved in regulating gene transcription [24].

The SMC5/6 complex in yeast is required during meiosis for correct chromosome segregation, and abrogation of components of the complex can lead to unresolved DSB-dependent recombination intermediates [25–27]. In addition, very recently in budding yeast, the SMC5/6 complex was determined to play a key role in destabilizing early recombination intermediates, maintaining the interhomologue recombination bias, and resolving a subset of joint molecules that would otherwise lead to the inability to segregate chromosomes during meiosis [28–30].

Recently, two studies investigated the role of SMC5/6 in mouse spermatogenesis [31, 32]. These studies revealed that SMC6 is expressed as soon as undifferentiated type A spermatogonia differentiate into $A_1$ spermatogonia and commit toward meiosis (often referred to as differentiating spermato-

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gonia) [32]. In contrast to the mouse, differentiating type A spermatogonia have not been defined for the human. Furthermore, human type A spermatogonia can be morphologically subdivided in $A_{dark}$ and $A_{pale}$ spermatogonia that represent quiescent and actively dividing pools of spermatogonia, respectively [33–35]. In the mouse, SMC6 is strongly present during meiosis and disappears before spermatid elongation [32]. During the meiotic prophase I, SMC5/6 complex proteins were found at several structures that are crucial for meiotic chromatin dynamics and genome integrity. First, SMC5 and SMC6 were found at the synaptonemal complex (SC), where they might facilitate pairing of homologous chromosomes, stabilize the SC, or regulate meiotic DSB repair and recombination [31]. Second, SMC5 and SMC6 were found at the XY body during pachynema, suggesting that it might mediate the chromatin conformation changes occurring at this site or the silencing of unsynapsed sex chromosomes [31]. Third, SMC6 was shown to be present at the pericentromeric heterochromatin and was postulated to protect the repetitive centromeric regions against aberrant recombination [31, 32]. During the meiotic metaphases, SMC6 localization is restricted to the centromeres [31, 32], indicating, as found in yeast, functions in sister-chromatid centromere cohesion [36, 37] or proper chromosome segregation during anaphase [13].

In humans, spermatogenesis is a complex and tightly regulated process, and failure in any stage will lead to sub- or infertility, caused by, for example, complete spermatogonial stem cell depletion or meiotic arrest. Even though spermatogenic failure accounts for a large proportion of male infertility cases, surprisingly little is known about the responsible molecular mechanisms. To better understand human spermatogenesis, spermatogenic arrest mechanisms, and genomic maintenance, we investigated the role of SMC5/6 in human spermatogenesis and meiosis. Based on previous mouse studies, we asked ourselves the following questions: At which stage of human spermatogenesis does the SMC5/6 complex start to be expressed? Is human SMC5/6 involved in spermatogonial differentiation? Can SMC5/6 play a role in pericentromeric heterochromatin during human spermatogenesis? Could SMC5/6 be involved in human SC formation and/or meiotic DSB repair?

## MATERIALS AND METHODS

### Patient Material and Ethical Approval

For immunohistochemistry, testicular material was donated after oral informed consent by three patients undergoing bilateral orchidectomy as part of prostate cancer treatment (patients URO0038, URO077, and URO0126). According to Dutch law, approval of the ethics committee was not required, because anonymized tissue samples were used. None of the patients had previously received chemotherapy or radiotherapy, and the morphology of the testes showed normal spermatogenesis in all cases. Testis biopsies were fixated in 4% paraformaldehyde and embedded in paraffin.

For meiotic spread preparation, we used testicular material of six individuals (patients URO0063, URO0159, URO0165, URO0166, AMC1587, and AMC1801) who underwent testicular sperm extraction (TESE) and signed informed consent to donate their spare TESE-material for research. In all patients, TESE yielded motile spermatozoa. After sperm extraction, the leftover material was used for research fresh or was cryopreserved in 8% dimethyl sulfoxide (Sigma-Aldrich) and 20% fetal calf serum (Invitrogen) in Minimum Essential Medium (Invitrogen) and stored at −196°C for later research use.

### Western Blot Analysis

A custom-made human tissue blot was received from ProSci (Poway) containing 15 μg of total cellular proteins from testis, ovary, spleen, skeletal muscle, small intestine, liver, skin, and brain. The staining protocol was performed as described previously [32] using guinea pig anti-SMC6 (GP anti-SMC6; 1:200; custom made), rabbit anti-SMC6 (1:1000; ab18039; Abcam), and rabbit anti-SMC5 (1:200; sc-134544; Santa Cruz) as primary antibodies. As secondary antibodies, goat anti-rabbit and donkey anti-guinea pig IRDye 800CW (1:15 000; LI-COR Biosciences) were used. As a loading control, a primary goat anti-GAPDH antibody (1:1000; ab9483; Abcam) was used. Image acquisition and quantification were done with the Odyssey Infrared Imaging System (LI-COR Biosciences).

### Immunochemistry

Immunohistochemical staining on human testis sections (thickness, 5 μm) was performed as described previously [32]. Meiotic spread preparations were made according to the method described by De Vries et al. [38], with the first spin of 20 sec at $40 \times g$, the second spin of 7 min at $260 \times g$, and the third spin of 15 min at $9 \times g$. Per TESE pellet, a sufficient amount of germ cells was isolated to prepare 20–25 slides. Coimmunofluorescent stainings were performed as described previously [32] using the primary and secondary antibodies listed in Supplemental Tables S1 and S2 (Supplemental Data are available online at www.biolreprod.org).

### Cot-1 DNA Fluorescence In Situ Hybridization

To visualize repetitive DNA sequences and proteins in the same sample, meiotic spread slides were first subjected to the standard immunofluorescence protocol as described above, after which they underwent a Cot-1 DNA-fluorescence in situ hybridization (FISH) protocol. Human Cot-1 DNA (Roche) was biotin labeled by nick translation and used as a probe, diluted in a 60% formamide hybridization mix (60% formamide, 2× sodium chloride/sodium citrate solution [SSC], and 0.02 M sodium phosphate buffer) to a final concentration of 2 ng/μl. Meiotic spread slides were treated with 100 μg/ml of RNase A (Roche) in 2× SSC for 1 h at 37°C, after which they were washed three times for 2 min each time with 2× SSC at 37°C. Slides were postfixed in 0.4% formaldehyde for 5 min at 4°C, followed by two 5-min washes in PBS at room temperature and a dehydration series using 70%, 96%, and 100% ethanol. After air-drying the slides, placing the probe on the slides, and denaturing the slides at 85°C for 6 min followed by placing them on ice for at least 1 min, the slides were hybridized with the probe overnight at 37°C. The next day, the slides were washed three times for 5 min each time with 50% formamide in 2× SSC at 42°C, twice for 5 min each time in 2× SSC at 42°C, and once for 5 min in 4× SSC/0.05% Tween-20 at room temperature. The slides were blocked in TNB (0.1 M Tris [Sigma], 0.15 M NaCl [Merck], 0.02% Thimerosal [Sigma], and 0.05% blocking reagent [Roche]) for 10 min at room temperature and incubated for 20 min at 37°C in avidin-Cy3 (Jackson ImmunoResearch). Finally, the slides were counterstained with 4′,6-diamidino-2-phenylindole, mounted in Prolong Gold (Cell Signaling Technology), and analyzed as described below.

### Microscopy

Bright-field microscopy images were acquired at room temperature using an Olympus BX41 microscope equipped with an Olympus DP20 color camera. Fluorescence microscopy images were acquired at room temperature using a Plan Fluotar 100×/1.30 oil objective on a Leica DM5000B microscope equipped with a Leica DFC365 FX CCD camera. Images were analyzed using Leica Application Suite Advanced Fluorescence software. Figures were constructed using Adobe Photoshop CS5 version 12.0.

## RESULTS

### SMC6 Protein Is Present in the Human Testis

To investigate whether the SMC5/6 complex has the same relatively high expression in the human testis as described in the mouse [32], we stained a Western blot containing several human organs, including the testis, for SMC6. An intense band of the expected size (just below 150 kDa) representing SMC6 was clearly expressed in the testis and ovary and, to a much lesser extent, in skeletal muscle (Fig. 1A). The other tissues, especially the liver, displayed several bands of varying sizes not corresponding with the expected size of SMC6.

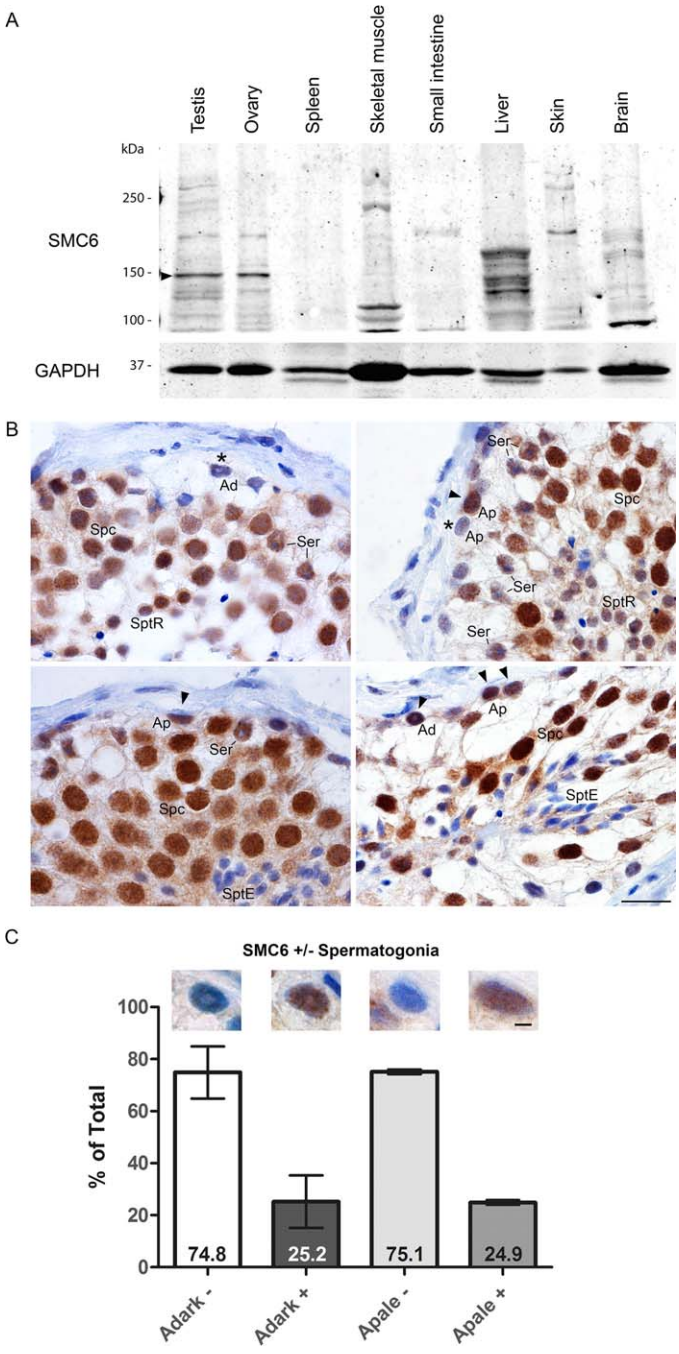


FIG. 1. Characterization of SMC6 in the human testis. **A**) Western blot analysis of SMC6 in human organs, using the GP anti-SMC6 antibody. The band corresponding with the expected size of SMC6 is indicated with an arrowhead. GAPDH was used as a loading reference. **B**) Immunohistochemical localization of SMC6 in human testis sections using the antibody against the SMC6 C-terminus. SMC6 (in brown) is expressed in a subpopulation of the spermatogonia, spermatocytes, and round spermatids. SMC6-positive (arrowhead) and -negative (asterisk) $A_{dark}$ and $A_{pale}$ spermatogonia (Ad and Ap, respectively), spermatocytes (Spc), round spermatids (SptR), elongated spermatids (SptE), and Sertoli cells (Ser) are indicated. Bar = 20 μm. **C**) Quantification of SMC6-positive and -negative A spermatogonia. Cells were counted in three different patients, with a minimum of 12 seminiferous tubules per patient. A representative cell is shown for each group. Data are presented as the mean ± SEM. Bar = 2 μm.

### *Human SMC6 Is Present in a Distinct Spermatogonial Subpopulation, During Meiosis, and in Early Haploid Cells*

We then studied the localization of SMC6 in the different cell types present in the human testis by immunohistochemistry (Fig. 1B). Consistent with what was recently found in the mouse [32], SMC6 was present in a subpopulation of the spermatogonia. Because SMC6 protein expression was shown to specifically mark differentiating spermatogonia in the mouse [32], we further characterized and quantified the SMC6-negative and -positive spermatogonia (Fig. 1C). Three different patients were examined, from which a total of 126 $A_{dark}$ spermatogonia and 676 $A_{pale}$ spermatogonia were counted from a minimum of 12 different tubules per patient (Supplemental Table S3). Interestingly, we found that approximately 75% of the human spermatogonia were SMC6-negative and 25% were SMC6-positive. Moreover, despite the variation between patients concerning the amount of $A_{dark}$ spermatogonia, the SMC6-positive spermatogonia were equally distributed among the $A_{dark}$ and $A_{pale}$ spermatogonia (Fig. 1C and Supplemental Table S3). Furthermore, comparable to the mouse, SMC6 was strongly expressed in spermatocytes, decreased in round spermatids, and absent in elongated spermatids (Fig. 1B).

### *During Human Meiosis, But Not Mitosis, SMC6 Is Located at the Centromeres at Metaphase*

Consistent with data from mitotically dividing cells in culture [22, 39] and mouse spermatogonia [32], we found SMC6 to be absent from the chromosomes during the M-phase in human mitotically dividing spermatogonia (Fig. 2A). However, also in line with recent mouse studies [31, 32], SMC6 was strongly present on the chromosomes during the meiotic metaphases (Fig. 2B).

Because only the meiotic metaphases contained SMC6 (Fig. 2A), we were able to identify meiotic metaphases in spread human testicular cells and determine the localization of SMC6 in these cells in more detail. Coimmunostaining with CREST serum to mark the centromeres clearly revealed that SMC6 was located at or adjacent to the centromeres during meiotic metaphases (Fig. 2C).

### *SMC5/6 Is Located at the Synaptonemal Complex During Meiotic Prophase I*

To further characterize the localization of the SMC5/6 complex during meiosis, we immunodetected SMC5 and SMC6 on meiotic spread preparations. Prophase stages were determined based on the expression pattern of the SC components STAG3 or SCP3 and CREST serum to mark the centromeres. Consistent with SMC5 expression in mouse prophase I spermatocytes [31], SMC5 localized to the SC at zygotene when homologous chromosomes initiate synapsis (Fig. 3A), leaving the unsynapsed axes negative for SMC5 (Fig. 3). In addition to its presence along the synapsed SC axes, SMC5 aggregated in areas referred to as polycomplexes [40] (Fig. 3A). When stained with our GP anti-SMC6 antibody, SMC6 appeared present on the SC, being most clearly visible during pachytene, when formation of the SC is most pronounced (Fig. 3B).

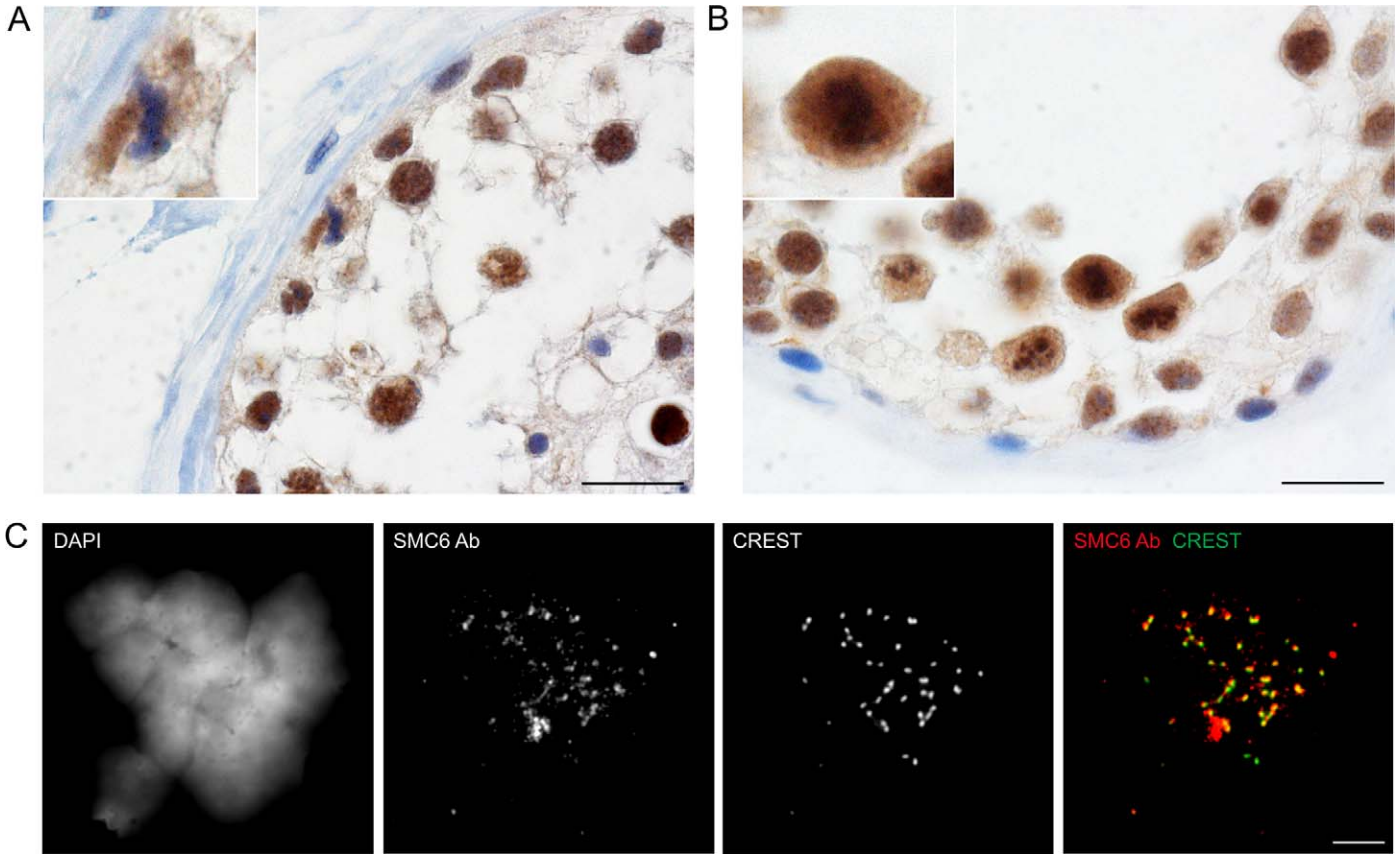


FIG. 2. SMC6 is located at the centromeres during meiosis but not during mitosis. **A**) During mitosis, SMC6 (immunohistochemical staining in brown) always appears absent from metaphase chromosomes. **B**) All meiotic metaphases show SMC6 to be located at the chromosomes. Bar = 20 μm. **C**) Meiotic chromatin spread preparation of a metaphase I nucleus. SMC6 is located at the centromeres (marked by CREST serum) during metaphase I. Bar = 5 μm.

### *Suppression of Meiotic Recombination in Pericentromeric Heterochromatin in Human Spermatocytes Does Not Require SMC6*

To investigate whether SMC6 might play a role in pericentromeric heterochromatin during human meiosis, as previously suggested in a mouse study [32], we stained human spermatocytes using the antibody against the SMC6-C-terminus, which has been shown to mark SMC6 in these chromosomal regions in the mouse [31, 32], in combination with the heterochromatin marker H3K9me3 [41, 42] or a Cot-1 DNA FISH to visualize repetitive DNA sequences. In contrast to the mouse, SMC6 did not appear to localize specifically to pericentromeric heterochromatin and/or repetitive DNA sequences (Fig. 4A). To check whether the repetitive DNA sequences stained by Cot-1 FISH are actually part of pericentromeric heterochromatin in human spermatocytes, we costained these cells with our markers for heterochromatin, centromeres, and Cot-1 DNA. Colocalization of these markers showed that Cot-1-stained repetitive DNA sequences are indeed contained in pericentromeric heterochromatin (Fig. 4B).

In the mouse, sites of meiotic recombination marked by Rad51 are never present in pericentromeric heterochromatin domains marked by SMC6 or H3K9me3 [32]. Because SMC6 has been shown to inhibit aberrant recombination in heterochromatin [10, 13–15], the lack of SMC6 in human pericentromeric heterochromatin raised the question whether these chromatin domains might be less stringently protected against potentially damaging recombination events and thus contain recombination sites. However, despite the absence of SMC6 in these regions, we still found Rad51 [43] to be excluded from pericentromeric heterochromatin during human meiosis (Fig. 4C).

### *During Human Meiosis, SMC6 Is Located at the XY Body and Unsynapsed Chromosomes Where DSBs Persist During Pachynema*

In pachytene spermatocytes, the antibody against the SMC6 C-terminus stained the largely unsynapsed X and Y chromosomes, marked by γH2AX (Figs. 4B and 5A) and colocalized with DMC1 (Fig. 5B). It recently became clear that the formation of meiotic DSBs is continued or reintroduced on unsynapsed chromosomes during pachynema [44]. We therefore studied human pachytene-like cells that partially failed to synapse their homologous autosomes. We found that these unsynapsed autosomes, in addition to the sex chromosomes, contained both DMC1 foci indicating DSB repair and SMC6 (Fig. 5C).

Because DSBs and their repair foci—marked by γH2AX and DMC1, respectively—are usually present during zygonema, we additionally studied zygotene spermatocytes to determine the localization of SMC6 during this stage. Importantly, SMC6 did not appear to be present at the multiple sites of DSBs that mark meiotic recombination during zygonema (Fig. 5, D and E) but rather to localize at random within the nucleus.

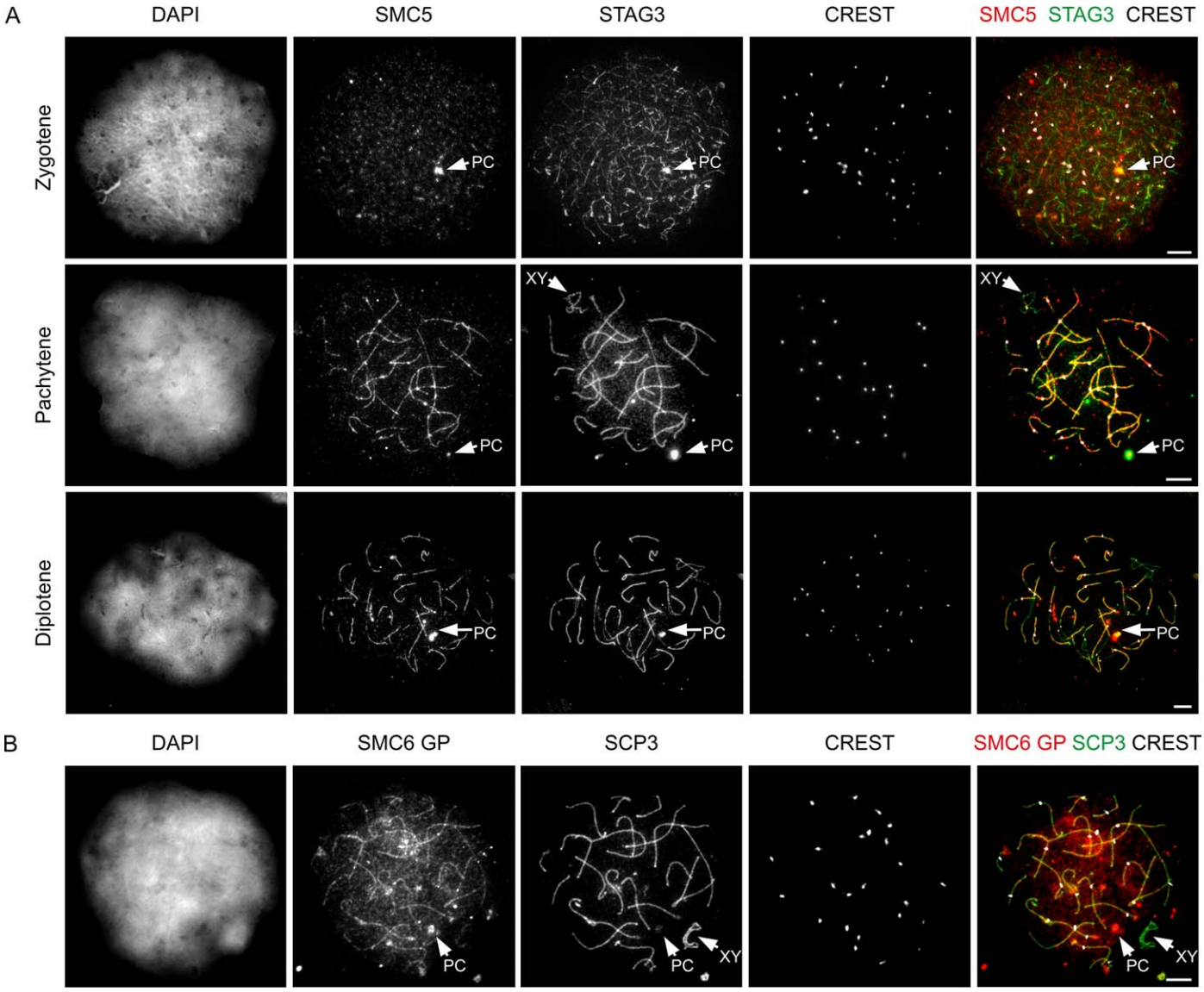


FIG. 3. SMC5 and SMC6 are located at the synaptonemal complex during meiotic prophase. Meiotic chromatin spread preparations show immunofluorescence staining for SMC5, SMC6, the synaptonemal complex (STAG3 and SCP3), and centromeres (CREST). **A**) SMC5 is located at the synaptonemal complex between synapsed chromosomes and in additional polycomplexes (PC). **B**) Like SMC5, SMC6 is located at the synaptonemal complex and PC during pachynema. X and Y chromosomes are indicated with XY. Bar = 5 μm.

## DISCUSSION

As shown previously [31], different SMC5 or SMC6 antibodies show different expression patterns. As previously seen in the mouse, the Abcam anti-SMC6 antibody detects an additional band that is not detected by the GP anti-SMC6 antibody (Supplemental Fig. S1). One explanation for these different expression patterns could be that multiple (iso-)forms or posttranslational modifications of SMC6 are present in different chromatin structures and thus display different expression patterns. Alternatively, the specific epitopes recognized by our antibodies are not as easily accessible in each structure. SMC6 has a complex protein structure (it folds in the middle via a hinge domain and coils around itself, bringing together its N- and C-terminus) and is part of a protein complex involving many other proteins and protein interactions. In addition, the DNA-binding characteristics of the entire complex have not been analyzed. Taken together, it is very plausible that the epitopes recognized by specific antibodies can be masked by configuration or interactions with DNA or other proteins. We therefore regard a compilation of expression patterns (though acquired using different antibodies) to be essential to provide a more complete picture of the localization of the SMC5/6 complex during spermatogenesis. As an overview, the different staining patterns of the different antibodies used in the present study are summarized in Table 1.

During human spermatogenesis, the SMC6 protein is first evident in spermatogonia, is strongly present throughout meiosis, and decreases upon spermatid elongation. As described recently for the mouse [32], two spermatogonial subpopulations can be distinguished in humans based on the presence of SMC6: the SMC6-negative spermatogonia that account for approximately 75% of these cells and the remaining 25% that contain SMC6. Interestingly, we have recently shown that LIN28, a marker for undifferentiated spermatogonia, and SMC6 are mutually exclusive in the mouse testis [32]. Moreover, we have shown that spermatogonia in the testes of vitamin A-deficient rats, which are arrested before

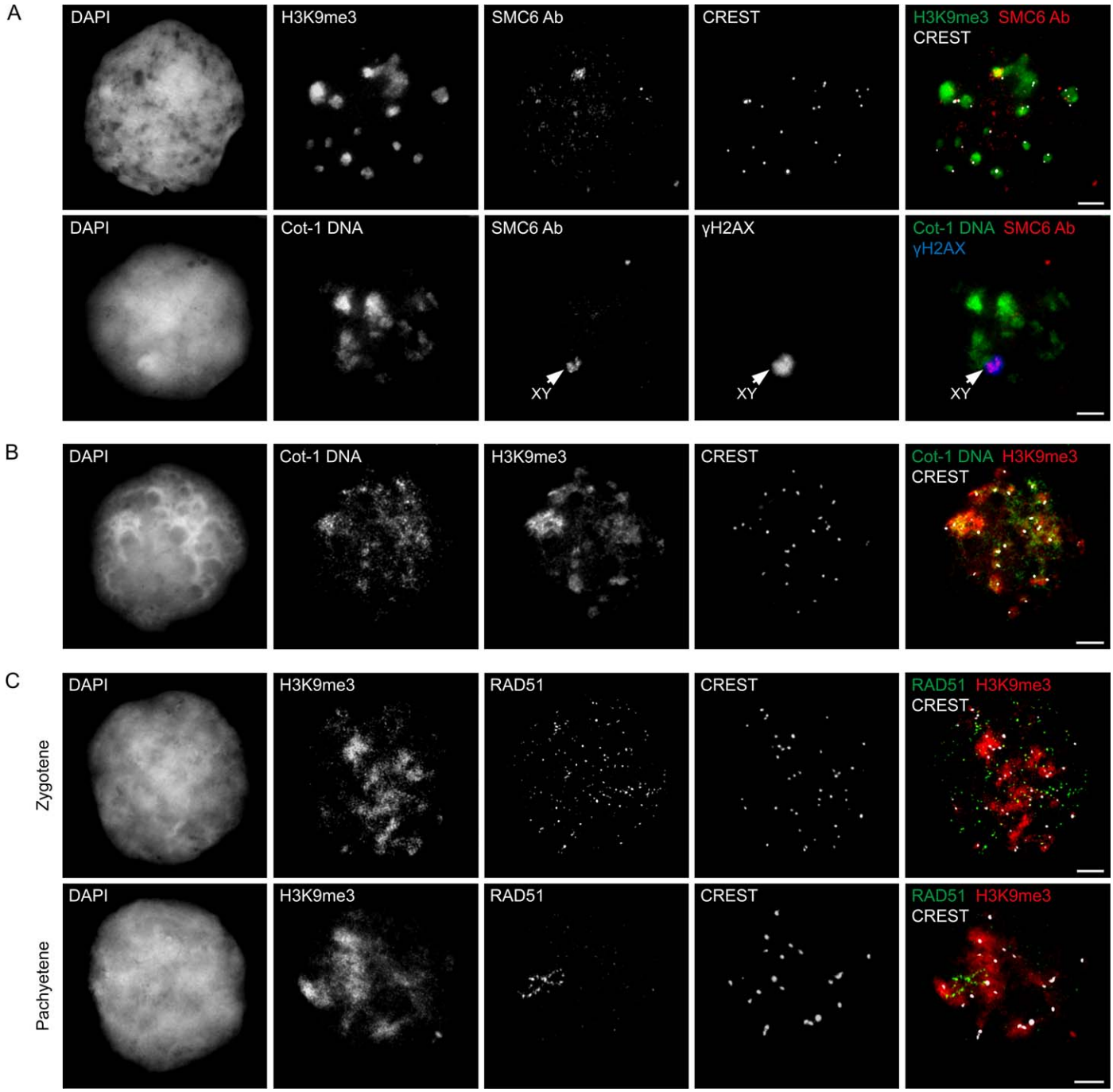


FIG. 4. Suppression of meiotic recombination in pericentromeric heterochromatin in human spermatocytes does not require SMC6. **A**) Coimmunofluorescence staining for SMC6 (Ab), H3K9me3, and CREST (**top**) and staining for SMC6 (Ab) and γH2AX with Cot1-DNA FISH (**bottom**). SMC6 does not colocalize with repetitive sequences or heterochromatin in human pachytene spermatocytes but does localize at the XY body marked by γH2AX. **B**) Coimmunofluorescence staining of H3K9me3 combined with Cot1-DNA FISH shows that human pericentromeric heterochromatin contains repetitive sequences. **C**) DSB-repair foci marked by RAD51 are not present in pericentromeric heterochromatin marked by H3K9me3. Bar = 5 μm.

spermatogonial differentiation, do not yet contain SMC6. Only when spermatogonial differentiation was enabled by the administration of retinoic acid were SMC6-positive spermatogonia found [32]. These experiments show that the presence of SMC6 marks differentiating spermatogonia in rodents [32]. In contrast to the undifferentiated spermatogonia, differentiating spermatogonia are irreversibly committed to eventually enter meiosis and spermiogenesis. Moreover, this spermatogonial differentiation step and subsequent spermatogenesis are tightly orchestrated and follow the strictly regulated stages of the seminiferous epithelium [1, 45]. In the mouse, differentiating spermatogonia are recognizable by a more condensed chromatin [46, 47]. In contrast, all human type A spermatogonia look very similar, and human spermatogonial differentiation markers are lacking [48]. Therefore, a subgroup of human differentiating spermatogonia has not been previously observed. However, in combination with the evidence we recently obtained for rodent spermatogonial differentiation [32], it is very plausible that human differentiating spermatogonia can be distinguished by the presence of SMC6. Our results would then

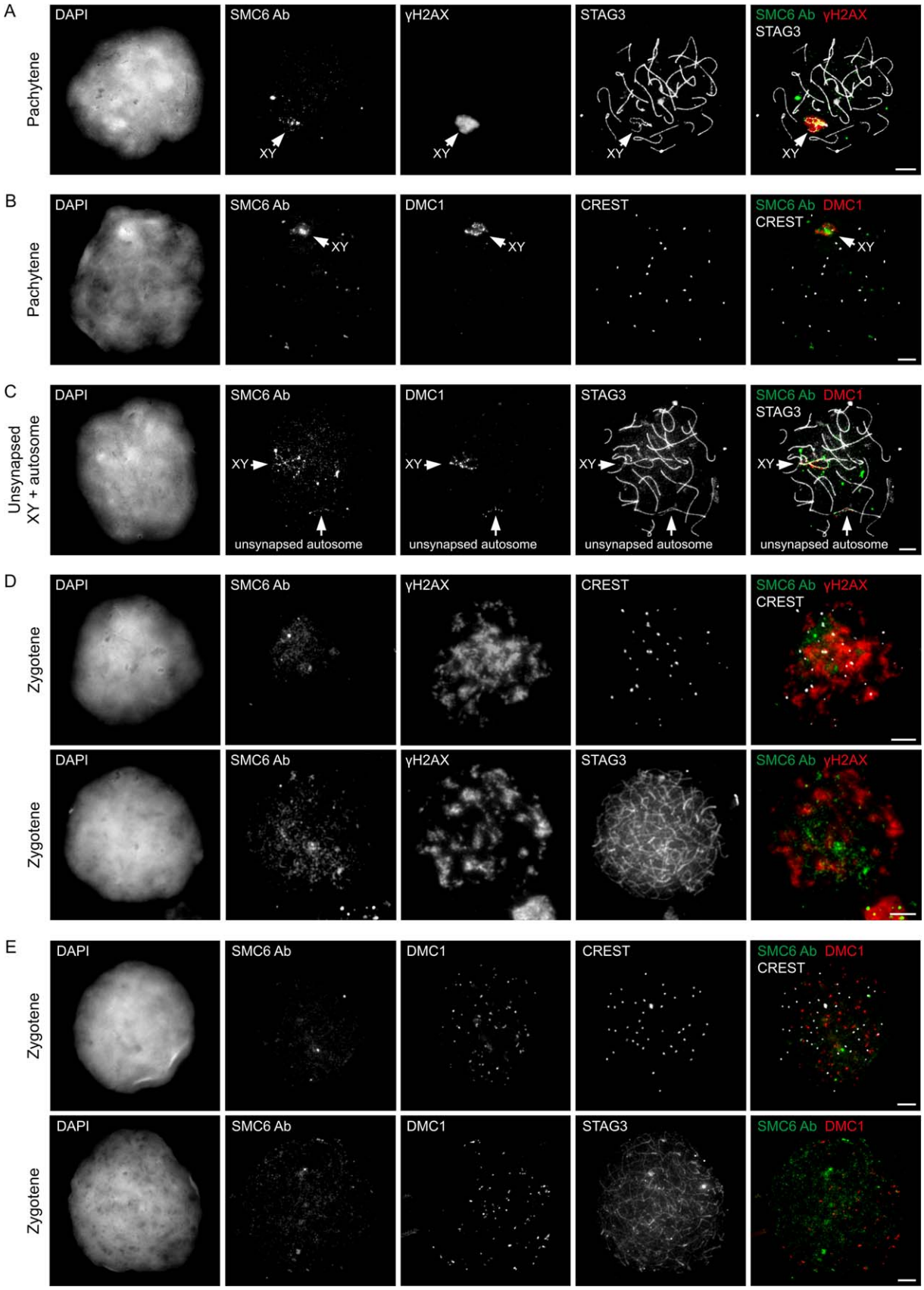


FIG. 5. SMC6 foci are present at unsynapsed axes during pachynema, including X and Y. Coimmunofluorescence staining of SMC6 (Ab) with DMC1 to mark DSBs, γH2AX to mark the XY body (pachynema) and DSB-repair (zygonema), and STAG3 to visualize the SC and CREST serum as a centromere marker are shown. **A**) During pachynema, SMC6 is located in foci at the X and Y chromosomes. **B** and **C**) During pachynema, SMC6 and DMC1 localize in distinct foci at the X and Y chromosomes (**B**) and at autosomes that fail to synapse (**C**). **D** and **E**) During zygonema, before chromosome synapsis is completed, SMC6 does not colocalize with γH2AX (**D**) or DMC1 (**E**). Bar = 5 μm.

TABLE 1. Overview of expression patterns observed with immunochemistry using different antibodies against SMC5 or SMC6.

| | Spermatogonia | | Spermatocytes | | | |
|---|---|---|---|---|---|---|
| Antibody | Interphase | Metaphase | Metaphase | Synaptonemal complex axes | Pericentromeric heterochromatin | XY-chromosomes/ XY-body |
| SMC6 Ab | Even nuclear (Fig. 1B) | Absent from chromosomes (Fig. 2A) | Centromeres (Fig. 2, B and C) | Foci on unsynapsed axes (Fig. 5) | No (Fig. 4) | Foci on unsynapsed XY (Fig. 4A, Fig. 5A) |
| SMC6 GP | Not suitable for IHC | Not suitable for IHC | Not observed | Synapsed axes (Fig. 3B) | No (Fig. 3A and B) | No (Fig. 3, A and B) |
| SMC5 | Not suitable for IHC | Not suitable for IHC | Not observed | Synapsed axes (Fig. 3A) | No (Fig. 3, A and B) | No (Fig. 3, A and B) |

indicate, to our knowledge for the first time, that 25% of the human spermatogonia are differentiating. Interestingly, these potentially differentiating spermatogonia are equally distributed amongst the quiescent $A_{dark}$ and actively dividing $A_{pale}$ spermatogonia. Because the $A_{dark}$ spermatogonia are quiescent, one could expect that these spermatogonia are undifferentiated. However, because 25% of both the $A_{dark}$ and $A_{pale}$ spermatogonia contain SMC6, it seems that both undifferentiated and differentiating $A_{pale}$ spermatogonia can become $A_{dark}$ spermatogonia without losing their differentiation status. Because all $A_{dark}$ spermatogonia can convert into $A_{pale}$ spermatogonia when the actively dividing spermatogonia become depleted [1], such as due to irradiation or other genotoxic stress, having a pool of differentiating (and thus SMC6-expressing) $A_{dark}$ spermatogonia readily available would lead to a faster recovery of spermatogenesis.

Similar to what has been shown previously for mitotically dividing cells in culture [39] and spermatogonia in the mouse [32], we find SMC6 to be absent from the chromosomes of mitotically dividing spermatogonia. In contrast, we observe SMC6 to be located at the centromeres of meiotically dividing spermatocytes. These results are in line with those of recent mouse studies [31, 32], suggesting that SMC6 might regulate sister-chromatid centromere cohesion [31].

In contrast to what we previously found in the mouse [32], we did not observe SMC5 and SMC6 localization at pericentromeric heterochromatin, a region of condensed chromatin surrounding the centromeres present in both mouse and human spermatocytes. The densely packed, repetitive DNA makes these regions vulnerable to errors in recombination, a process therefore suppressed at these sites [32, 49]. SMC5/6 has been found to play an important role in the protection of heterochromatin in *Drosophila* sp. and yeast by preventing aberrant recombination within heterochromatin and by subsequent relocation of the DSBs to euchromatic regions [10, 13–15]. In accordance with these findings, mouse SMC5/6 is located at the pericentromeric heterochromatin during meiosis, whereas DSB-repair foci are excluded from these regions [31, 32]. Surprisingly, we did not find SMC6 to be present on the human pericentromeric heterochromatin. Because we show that recombination foci marked by Rad51 are still excluded from human heterochromatin, and assuming that the Abcam antibody against SMC6 is also able to detect SMC6 in human heterochromatin, we postulate that an alternative mechanism inhibits homologous recombination within these domains in humans. Following this thought, the question arises why the observed difference between human and mouse pericentromeric heterochromatin composition exists. In humans, pericentromeric sequences consist of type I, II, and III short satellite repeats that comprise approximately 4% of the genome, the size of the total region varying between chromosomes [50]. In mice, pericentromeric sequences contain a repetition of (AT-rich alpha) ''major'' satellite motifs, forming a total region of approximately 6 Mb on each chromosome [50, 51]. It could thus be possible that due to this difference in sequence and size, protection mechanisms of these regions vary between the two species. Another explanation could be the location of the centromere within the chromosome. In contrast to the mouse telocentric chromosomes (i.e., the centromere is located at the end of the chromosome), human chromosomes are (sub)metacentric (i.e., the centromere is located in or near the middle) or acrocentric (i.e., the centromere is located almost at the end of the chromosome). Depending on the location of the centromeres within the chromosomes, recombination errors might result in diverse problems. For instance, Robertsonian translocations in the human only occur between acrocentric chromosomes and are characterized by aberrant recombination between the two acrocentric centromeres, leading to fusion of the two long arms and leaving the two smaller arms to be lost [50]. This can subsequently result in meiotic segregation errors, leading to meiotic arrest and subsequent failure of gametogenesis, or aneuploidy and embryo loss [52]. All mouse chromosomes, except Y, are telocentric [53], and the regions between the centromere and the telomere in mouse chromosomes consist of repeats sharing a high sequence identity of more than 99% between nonhomologous chromosomes. This makes mouse chromosomes extremely vulnerable to aberrant recombination and subsequent translocations, segregation errors leading to meiotic arrest, or congenital malformations in the offspring. In contrast, only a minority of the human chromosomes are acrocentric, and the α-satellites located at the centromeres show less sequence homology between nonhomologous chromosomes [51, 53]. These differences might explain why mouse chromosomes would need more stringent protection against aberrant recombination in pericentromeric regions. However, it must be noted that SMC5/6 has not been functionally proven to actually prevent aberrant recombination in mouse pericentromeric heterochromatin, as it does in *Drosophila* sp. and yeast, and some reservations toward this hypothesis remain in place.

As in the mouse [31], we also find human SMC5 and SMC6 to be located at the SC on synapsed homologous chromosomes. The longitudinal localization pattern along the synapsed SC axes of SMC5 and SMC6 suggests that the complex could facilitate SC assembly and/or chromosome synapsis. Furthermore, both SMC5 and SMC6 can be found in aggregates, supposedly polycomplexes of SC components that are not in use [54]. In addition, we find human SMC6 to be located on the sex chromosomes. Previous work in the mouse indicated that SMC5/6 might contribute to structural and epigenetic changes required for meiotic sex chromosome inactivation [31, 55]. However, in contrast to the mouse, we do not observe human SMC6 to mark the entire XY body but only smaller regions or foci on the unsynapsed X and Y chromosomes, as well as on unsynapsed autosomes when still present during

pachynema. Moreover, we find that these unsynapsed chromosomes contain DSBs marked by DMC1. This can be explained by recent findings that failure in synapsis allows the continued induction of DSBs at these sites throughout pachynema [44]. Because it has been widely accepted that the SMC5/6 complex is essential in DNA repair by facilitating homologous recombination [7–10], our data could indicate that SMC5/6 mediates intersister recombination at late sites of pachytene-induced DSBs. In accordance with this idea, SMC6 does not colocalize with DMC1 during zygonema, when DSBs required for meiotic recombination are induced. Our data thus indicate that the SMC5/6 complex is not part of the regular meiotic recombination process but might come into action when DNA repair proteins are required to resolve DSBs induced at unsynapsed chromosomes later during meiosis.

In conclusion, we observe a subpopulation of human type A spermatogonia that contains SMC6, which we propose to be differentiating spermatogonia, a spermatogonial subgroup that, to our knowledge, has not been described before in humans. In contrast to the mouse, we do not find any evidence that the human SMC5/6 complex plays a role at pericentromeric heterochromatin during meiosis. However, the complex does seem to be involved in SC formation and late meiotic DSB repair or silencing of unsynapsed chromosomes during pachytene. The differences we find between mouse and human SMC6 function during meiosis illustrate that the molecular mechanisms safeguarding the genome in reproductive cells in the mouse might not necessarily directly translate to the spermatogenic arrest phenotypes found in humans. Therefore, one should always aspire to verify findings obtained from animal models using human tissues and cells in order to acquire better understanding of chromatin dynamics in human reproductive cells. Because the spatiotemporal localization of human SMC5/6 points to several crucial roles in spermatogenesis that coincide with the timing of spermatogenic arrest phenotypes that are often observed in infertile men [48], we propose that SMC5/6 plays a role in genomic integrity maintenance during human germ cell development.

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