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Murine and Human Spermatids Are Characterized by Numerous, Newly Synthesized and Differentially Expressed Transcription Factors and Bromodomain-Containing Proteins[1]

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ABSTRACT

Much of spermatid differentiation takes place in the absence of active transcription, but in the early phase, large amounts of mRNA are synthesized, translationally repressed, and stored. Most nucleosomal histones are then degraded, and chromatin is repackaged by protamines. For both transcription and the histone-to-protamine transition in differentiating spermatids, chromatin must be opened. This raises the question of whether two different processes exist. It is conceivable that for initiation of the histone-to-protamine transition, the already accessible, actively transcribed chromatin regions are utilized or vice versa. We analyzed the enrichment of different canonical TATA-box-binding, protein-associated factors and their variants in murine spermatids, diverse bromodomain-containing proteins, and components of the Polycomb repressive complexes PRC1 and PRC2 using quantitative PCR. We compared the enrichment of corresponding proteins in human and murine spermatids and analyzed the time frame of postmeiotic transcription and expression of histones, transition proteins, and protamines in human and murine spermatids using immunohistology. We correlated the expression of different transcription factors and bromodomain-containing proteins and the pattern of acetylated histones to active transcription and to the histone-to-protamine transition in both human and murine spermatids. Our findings suggest that differentiating spermatids use both common and specific features to open chromatin first for transcription and subsequently for histone-to-protamine transition.

chromatin, differentiation, epigenetics, sperm maturation, transcription, transition protein

INTRODUCTION

In the course of spermatogenesis, male germ cells pass through a series of differentiation processes to develop into highly specialized sperm cells. Spermatogenesis starts with a mitotic amplification phase, followed by a meiotic and a postmeiotic phase. These different phases rest upon a tightly controlled gene expression program.

The postmeiotic phase, also known as spermiogenesis, is characterized by extensive morphological changes of the haploid cells that require numerous different gene products. In mice, transcripts of 1652 genes (about 20% of which are turned on specifically in the testis) markedly increase during or after meiosis [1]. Large amounts of active RNA polymerase II are detected in haploid round spermatids up to steps 8–9 in mice and up to step 3 in humans [2]. The proteins needed in later spermatids are made from translationally repressed and stored mRNAs synthesized in round spermatids at the latest [3]. Several studies indicate that specialized transcription mechanisms exist in male germ cells, especially in meiotic spermatocytes and round spermatids [4, 5]. In many cases, transcriptional initiation starts with the binding of the TFIID complex to gene promoters. The TFIID complex consists of the TATA-box-binding protein (TBP) and about 13 TBP-associated factors (TAFs) [6]. Beside the canonical transcription factors, paralogs, such as TBP-like factor (TLF), which is also known as TBP-related factor 2 (TRF2), TAF4B, and TAF7L, are expressed in spermatocytes and/or round spermatids [7–9]. Recent data indicate that TAF7L cooperates with TRF2 to regulate a subset of postmeiotically activated genes [10].

In most cases, the acetylation of histone tails is correlated with an open chromatin structure. These residues can be recognized by bromodomain-containing proteins, which are involved in diverse biological functions, for example, transcription or ATP-dependent chromatin remodeling [11–13]. The germ-cell-specific bromodomain-containing protein BRDT is expressed in both spermatocytes and round spermatids [14]. BRDT can bind to acetylated histones and is involved in gene expression in round spermatids [15–17]. The bromodomain-containing protein BRD2 is highly expressed in both spermatocytes and round spermatids. *Brd3* transcripts are enriched in round spermatids, and BRD4 is mainly expressed in spermatogonia [18].

The mechanisms of gene activation in round spermatids are only poorly understood, and hardly anything is known about gene silencing in round spermatids. A highly conserved silencing mechanism is mediated by the Polycomb repressive

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complexes PRC1 and PRC2 [19]. In the male germline, these complexes play essential roles in mitotic and meiotic phases [20–22]. Transcriptional silencing in haploid spermatids could be caused by the histone-to-protamine transition that occurs during spermiogenesis in elongating spermatids after the bulk of transcription [23]. During this transition, almost all nucleosomal histones are replaced by transition proteins and later by protamines [24]. Large amounts of acetylated histones accompany the initial steps of the histone-to-protamine transition [25, 26]. However, little is known about the underlying mechanisms of the histone-to-protamine transition. Consequently, haploid spermatid nuclei have to deal with two different processes, namely transcription and histone-to-protamine transition, and both require mechanisms to open and subsequently pack the genome.

To obtain a better understanding of how gene expression and the histone-to-protamine transition are achieved, haploid spermatids must be examined in more detail and chromatin changes in spermatids must be considered as a whole. Here, we examined haploid spermatids in mice and humans and analyzed TAFs, bromodomain-containing proteins, PRC1 and PRC2 components, and unmodified and modified histones. We present a detailed description of the stage-specific distribution of the different proteins and discuss their role in gene regulation and/or the histone-to-protamine transition. Our results demonstrate 1) which TAFs, bromodomain-containing proteins, and PRC1 and PRC2 components are enriched in haploid spermatids, 2) the distribution of these factors in relation to gene activity and the histone-to-protamine transition, 3) a detailed temporal picture of individual histone disappearance and transition protein and protamine incorporation, and 4) a detailed temporal picture of individual histone modifications.

MATERIALS AND METHODS

Murine Testicular Tissue

These studies have been approved by the animal welfare officer of the Philipps-Universität Marburg. For quantitative PCR (qPCR) and immunohistochemical analyses, C57BL/6NCrl mice (Charles River Laboratories) were used. For qPCR, testes from mice 7 days postpartum (dpp), 16 dpp, and 25 dpp were frozen in liquid nitrogen and stored at −80°C until further processing. The use of testes from juvenile mice allows the analysis of specific developmental phases of spermatogenesis (7 dpp: testes contain only spermatogonia; 16 dpp: testes contain spermatogonia and spermatocytes; 25 dpp: testes contain spermatogonia, spermatocytes, and spermatids). We validated the germ cell compound in testes of different ages using qPCR of specific marker genes. In addition, testes from mice 7, 16, and 25 dpp were fixed overnight in Bouin solution and embedded in paraffin using standard techniques. Sections (4 μm) were stained with hematoxylin, and the germ cell compound was analyzed. For immunohistochemical analyses, testes from 12-wk-old mice were fixed overnight in 4% formaldehyde in PBS (F-PBS) at 4°C or overnight in Bouin solution at room temperature and were then embedded in paraffin. Sections of 4 μm were prepared.

Human Testicular Tissue

This study has been approved by the ethics committee of the Justus-Liebig-Universität Gießen (decision 75/00). After informed consent was obtained, we used testicular biopsy specimens of three different patients (35, 41, and 70 yr old) that showed normal spermatogenesis. The testicular biopsy of all three patients yielded the same results. Specimens were routinely fixed by immersion in Bouin fixative and embedded in paraffin using standard techniques. Sections of 7 μm were mounted on slides coated with aminopropyltriethoxysilane (Sigma).

RNA Isolation and Quantitative PCR

Frozen testes from mice 7, 16, and 25 dpp were thawed on ice. RNA was isolated using TRIzol (Life Technologies). RNA of testes from three mice of each age was pooled to avoid individual differences. RNA was treated with DNase (Promega) according to the manufacturer's protocol. RNA (1 μg) was reverse transcribed using Transcriptor First Strand cDNA Synthesis Kit (Roche). The qPCR was performed with a Sybrgreen platform on a Bio-Rad CFX Cycler. Primer sequences are given in Supplemental Table S1 (Supplemental Data are available online at www.biolreprod.org).

Statistical Analysis

In order to obtain statistical significance, qPCRs were performed three times with each sample. The values were normalized to the expression of *β-actin* as an internal control. Expression in testes at 7 dpp was set to 1 for comparison to expression in testes at 16 and 25 dpp. Normalized values were used for the calculation of the *P* value via *t*-test.

Antibodies and Immunohistochemistry

Immunohistochemical analyses were carried out with minor modifications as previously described [27, 28]. Briefly, sections were deparaffinized and rehydrated. For heat-mediated antigen retrieval, citrate buffer (pH 7.6) was used. Endogenous peroxidase was inactivated using 3% H_2O_2. Afterward, sections were blocked in 1.4% bovine serum albumin. The primary antibody (Supplemental Table S2) was incubated with the sections at 4°C overnight, followed by incubation with biotinylated secondary antibody (Supplemental Table S2) at room temperature for 1 h, and then with avidin-biotin complex (Vectastain ABC Elite Kit, Vector Laboratories) with 3,3′-diaminobenzidine as the chromogen at room temperature for 45 min. Sections were partially counterstained with hematoxylin to label cell nuclei. The sections were staged according to [29, 30]. Sections were analyzed using a Zeiss Axioplan light microscope equipped with a Zeiss AxioCam MRm digital camera. Each antibody showed the same results in at least three independent samples of human or mouse.

RESULTS

The Transcripts of Numerous TAFs, Bromodomain-Containing Proteins, and PRC1/PRC2 Components Accumulate in Postmeiotic Spermatids

To obtain an overview of which TAFs, bromodomain-containing proteins, and PRC1/PRC2 components are enriched in haploid male germ cells, we analyzed cDNA from testes of mice at different ages (7, 16, and 25 dpp) using qPCR. Testes of mice 7 dpp contain spermatogonia as the only germ cell type, testes of mice 16 dpp contain spermatogonia and spermatocytes, and testes of mice 25 dpp contain spermatogonia, spermatocytes, and spermatids (Supplemental Fig. S1). As a control, we analyzed the distribution of *Trf2* and *Brdt* transcripts, which are known to increase during testicular development [17, 31]. These transcripts were significantly higher in testes of mice 16 dpp than at 7 dpp and highest in testes of mice 25 dpp, which contained spermatogonia, spermatocytes, and postmeiotic spermatids (Fig. 1A). Similar results were obtained for transcripts encoding general TAFs (TAF2, TAF5, TAF6, TAF9, TAF10, and TAF12) (Fig. 1B) and the TAF paralogs TAF5L and TAF6L (Fig. 1C). These data indicated that these TAFs are highly active during mouse spermiogenesis. By contrast, transcripts of the TAF paralogs TAF4B, TAF7L, and TAF9B were enriched in testes of mice 16 dpp (Fig. 1C). *Taf7* transcript levels did not significantly change during testicular development (Fig. 1B). The transcripts of *Brd1, Brd2, Brd3, Brd7,* and *Brd8* were significantly enriched in testes of mice 25 dpp (Fig. 1D). The same was demonstrated for transcripts encoding the bromodomain-containing proteins PCAF, SMARCA2, and TRIM24 (Fig. 1E). By contrast, *Brd4* and *Brd9* transcripts were detected throughout testicular development (Fig. 1D). Transcripts of the PRC1 components RING1, BMI1, and SCMH1, and the PRC2 component EZH2, significantly increased in testes of mice 25 dpp (Supplemental Fig. S2). By contrast, *Cbx2* and *Cbx8* (PRC1) transcripts were enriched in testes of mice 16 dpp

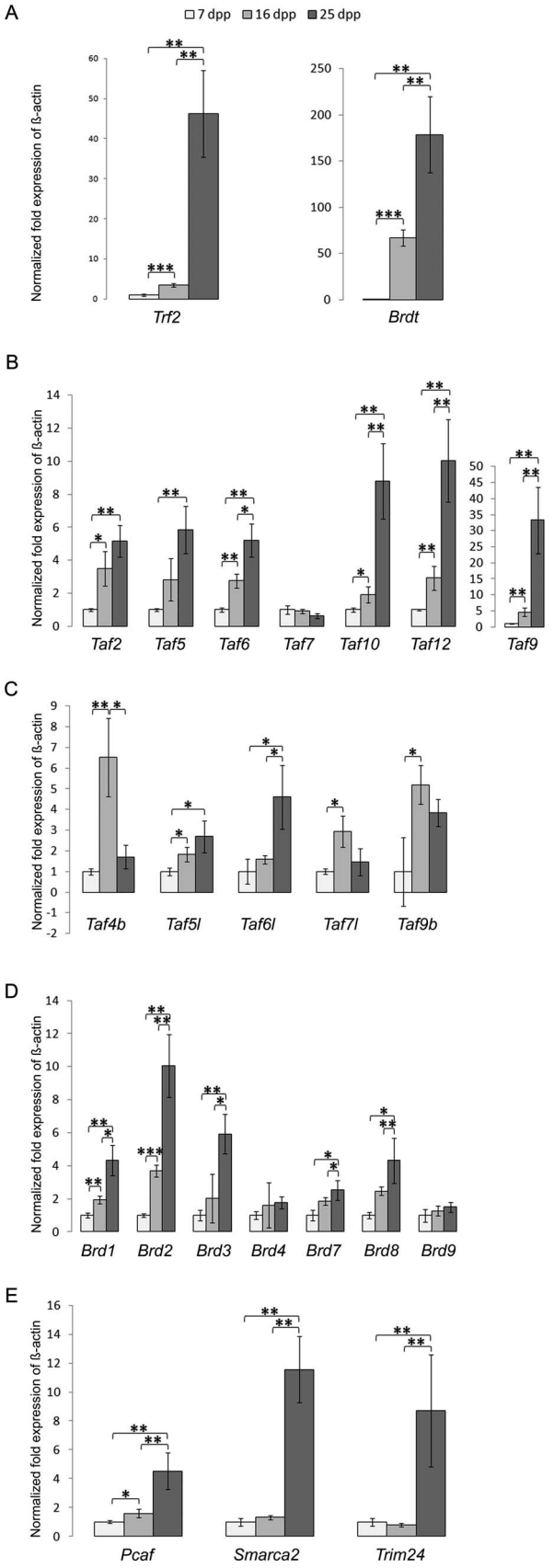

FIG. 1. Transcripts of several transcription factors and bromodomain-containing proteins are enriched during spermiogenesis. **A**–**E**) Analysis of murine transcripts as indicated by qPCR using cDNA from testes of mice 7, 16, and 25 dpp. The values were normalized to *β-actin* expression. *P* values for significance were validated via *t*-test. $*P \leq 0.05$, $**P \leq 0.01$, and $***P \leq 0.001$.

(Supplemental Fig. S2A). *Eed* and *Suz12* (PRC2) transcripts were detected throughout testicular development (Supplemental Fig. S2B).

Several Different TAFs and Bromodomain-Containing Proteins Occur in Elongating Spermatids

Our qPCR data revealed that transcripts of many TAFs, bromodomain-containing proteins, and PRC1/PRC2 components accumulate in haploid spermatids. Consequently, we concentrated on the postmeiotic phase of spermatogenesis and studied the protein distribution of these factors in relation to that of active RNA polymerase II in mouse (Figs. 2 and 3) and human sections (Fig. 4 and Supplemental Fig. S4). In mice, active RNA polymerase II was observed in each germ cell up to steps 8–9 spermatids but not in later spermatids (Fig. 2A) [2]. TAF2 was detected in the nuclei of spermatocytes and spermatids up to step 9 (Fig. 2B). From step 10 spermatids onward, TAF2 was distributed in the cytoplasm (Fig. 2B). TAF5 exhibited a nuclear expression pattern from spermatogonia up to step 8 spermatids (Fig. 2C). By contrast, TAF6 showed a broader pattern from spermatogonia up to steps 9–10 spermatids (Fig. 2D). Surprisingly, the PRC2 component EZH2 was detected in the nuclei of spermatids in steps 1–3 up to steps 7–8 but not in earlier germ cells (Fig. 2E). The bromodomain proteins BRD2 (Fig. 3A) and TRIM24 (Fig. 3D) were both detected in nuclei of spermatids up to steps 9–10. BRD8 was observed in nuclei of spermatocytes and spermatids up to steps 7–8 (Fig. 3B). BRDT was detected in nuclei of spermatocytes and spermatids up to step 12 (Fig. 3C). PCAF was mainly distributed in the cytoplasm of spermatocytes (Fig. 3E and Supplemental Fig. S3). In postmeiotic cells, PCAF was restricted to the nuclei of steps 1–9 spermatids (Fig. 3E).

In human sections, active RNA polymerase II was detected from spermatogonia up to step 3 spermatids (Supplemental Fig. S4A) [2]. TAF2 seemed to be weakly expressed in spermatocyte nuclei up to step 5 spermatids (Supplemental Fig. S4B). TAF5 was observed from spermatogonia until step 3 spermatids (Supplemental Fig. S4C). TAF6 was detected in spermatogonia and in nuclei of steps 1–4 spermatids (Supplemental Fig. S4D). The PRC2 component EZH2 was expressed in spermatocyte nuclei until step 4 spermatids (Supplemental Fig. S4E). The bromodomain proteins BRD8 and BRDT were detected from spermatocyte nuclei up to step 4 spermatids (Fig. 4, A and B). Interestingly, in germ cell nuclei, BRD3 (Fig. 4C) and SMARCA2 (Fig. 4D) were specifically observed in step 4 spermatids. PCAF was detected in spermatogonia, early spermatocytes, and in steps 1–2 spermatids (Fig. 4E).

The Histone-to-Protamine Transition Revised

Because some of the above-analyzed factors were detected longer than active RNA polymerase II, we reanalyzed the timing of the histone-to-protamine transition. In mice, the core histones H2A, H2B, H3, and H4 were detected up to step 12 spermatids (Fig. 5A and Supplemental Fig. S5). Expression of the transition protein TNP1 was restricted to spermatids of steps 9–12 (Fig. 5B). Protamine PRM1 was observed from step 12 spermatids onward (Fig. 5C), and protamine PRM2 was detected from late step 15 spermatids onward (Fig. 5D). Surprisingly, human histones H2A and H2B were hardly detectable in spermatogonia, in spermatocytes, and in spermatids of steps 1–3 (Supplemental Fig. S6, A and B). However, strong signals were visible in step 4 spermatids (Supplemental Fig. S6, A and B). Histones H3 and H4 were detected in all

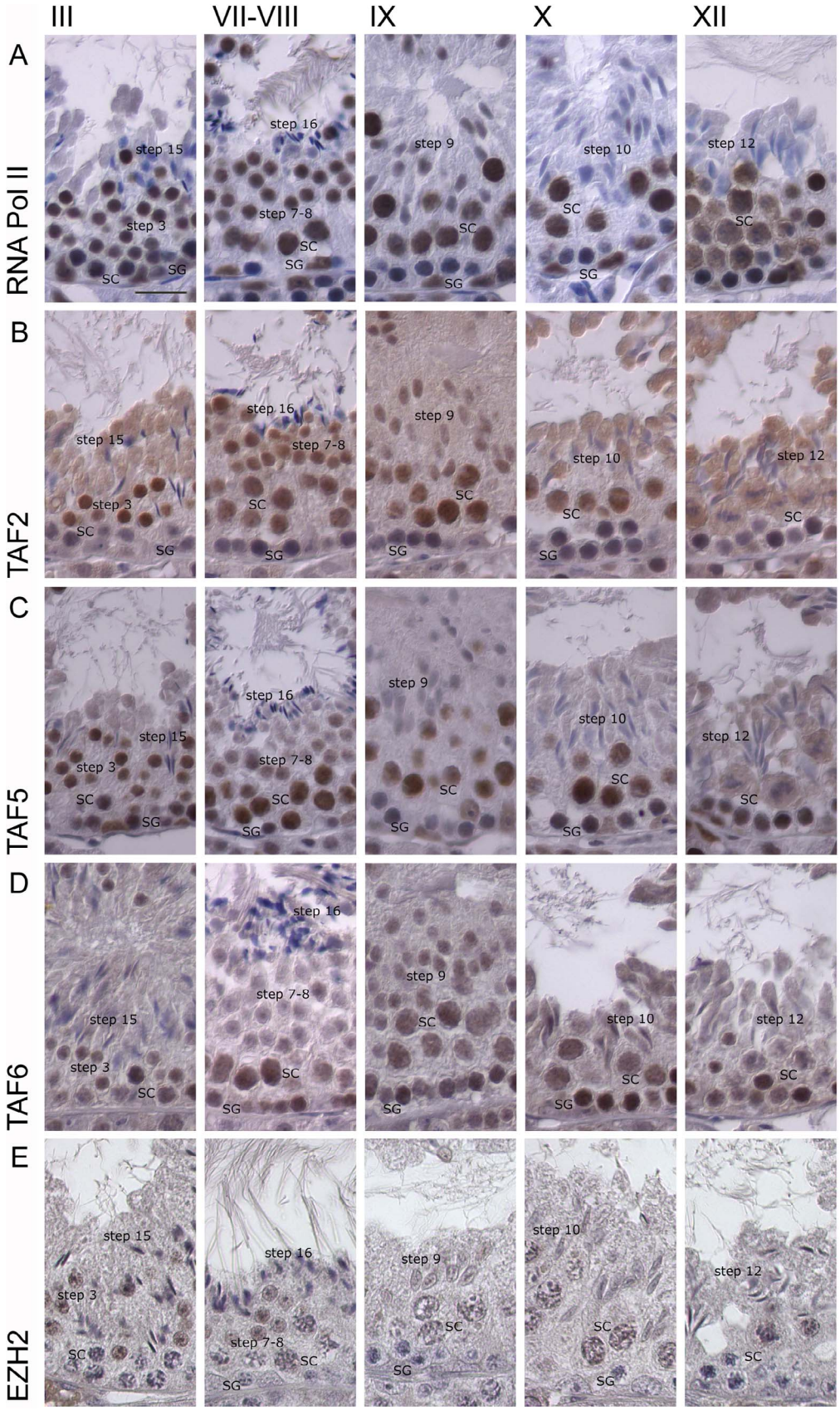

FIG. 2. Active RNA polymerase II, TAF2, TAF5, TAF6, and EZH2 are detectable in early murine spermatids. **A**–**E**) Mouse testicular tissue sections stained with antibodies against active RNA polymerase II (**A**), TAF2 (**B**), TAF5 (**C**), TAF6 (**D**), and EZH2 (**E**). Roman numerals indicate stages of the seminiferous epithelial cycle. Bar = 20 μm. SG, spermatogonia; SC, spermatocytes; step, steps of spermatid differentiation.

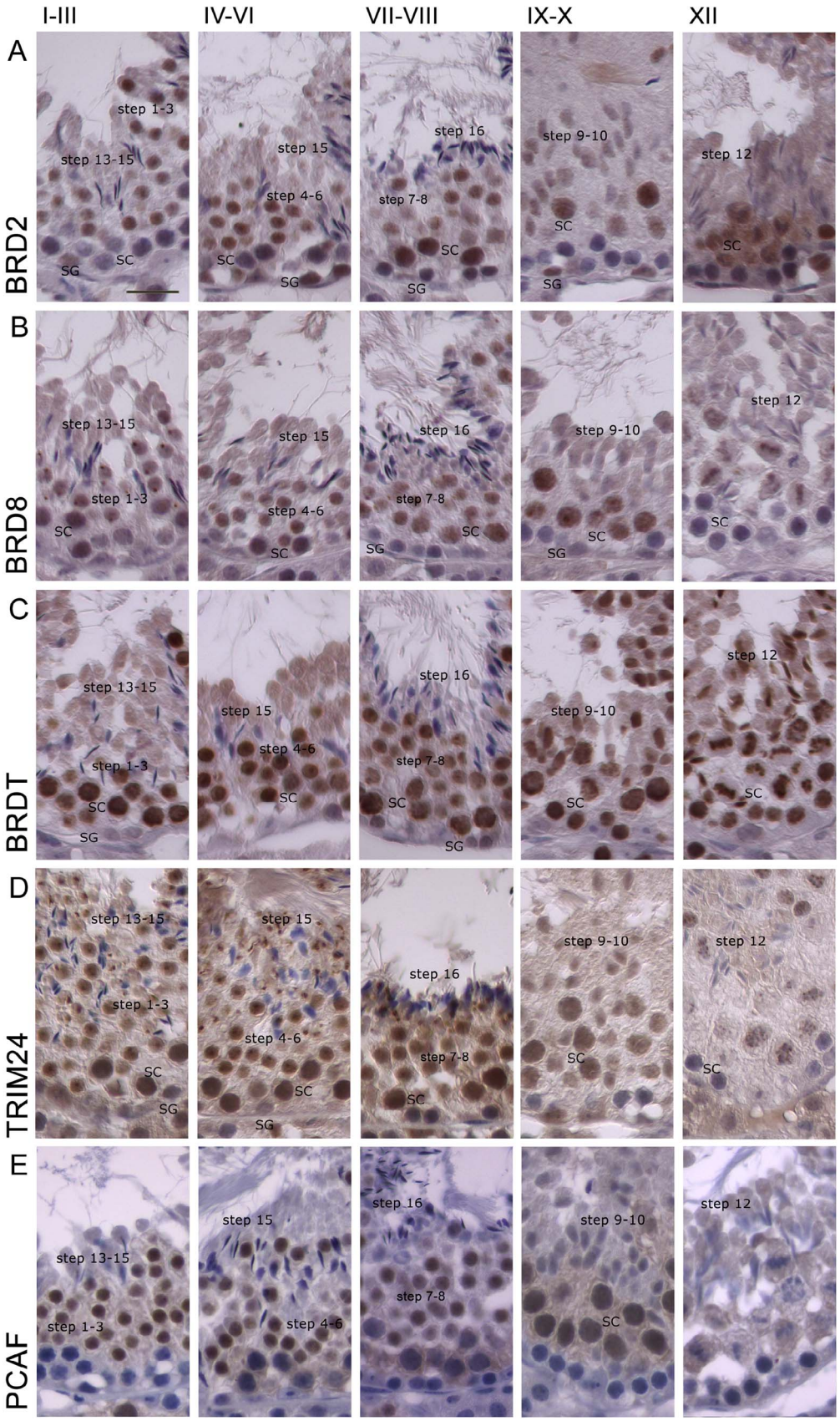

FIG. 3. Bromodomain-containing proteins BRD2, BRD8, BRDT, TRIM24, and PCAF are expressed in murine spermatids. **A**–**E**) Mouse testicular tissue sections stained with antibodies against BRD2 (**A**), BRD8 (**B**), BRDT (**C**), TRIM24 (**D**), and PCAF (**E**). Roman numerals indicate stages of the seminiferous epithelial cycle. Bar = 20 μm. SG, spermatogonia; SC, spermatocytes; step, steps of spermatid differentiation.

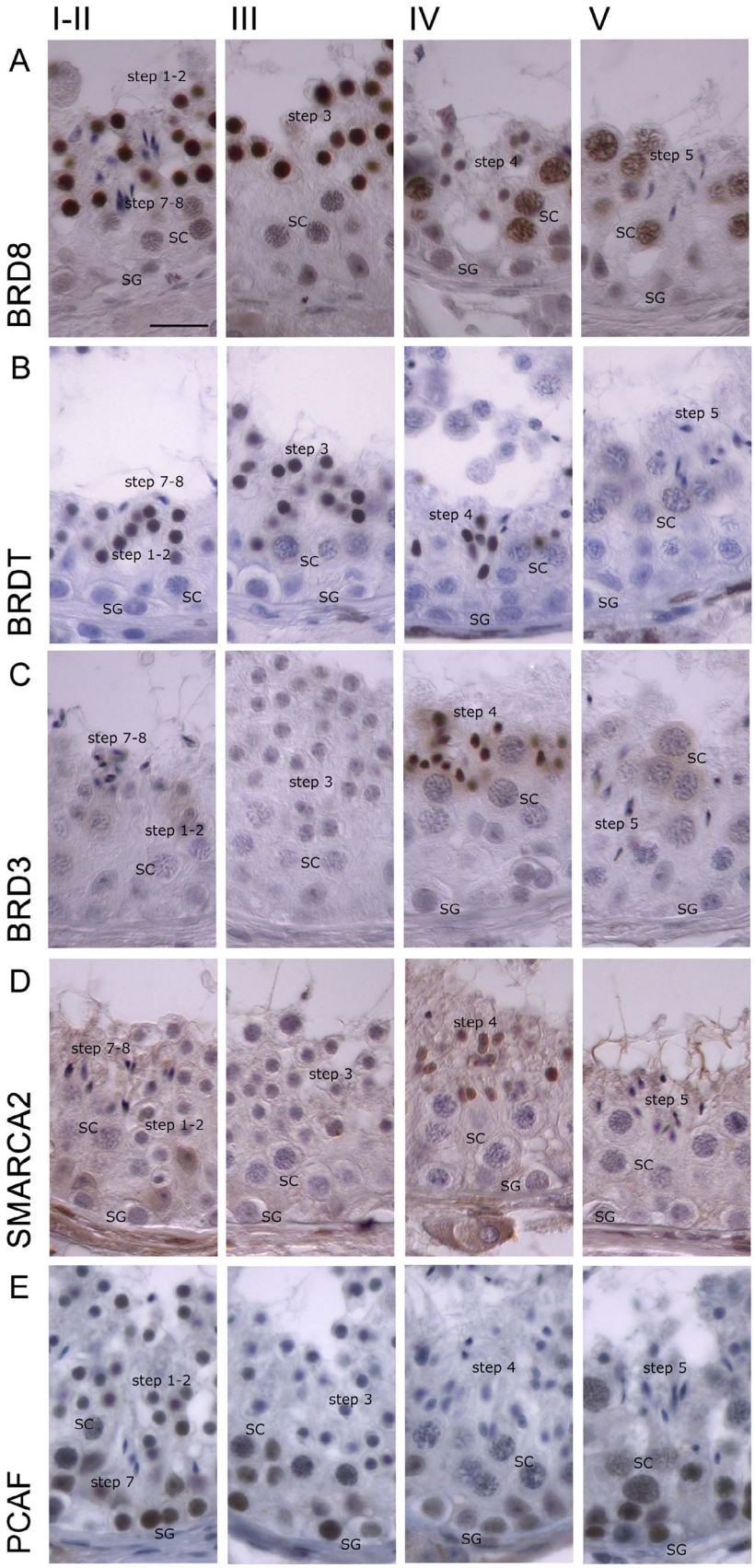

FIG. 4. BRD3 and SMARCA2 expression is restricted to step 4 human spermatids. **A**–**E**) Human testicular tissue sections stained with antibodies against BRD8 (**A**), BRDT (**B**), BRD3 (**C**), SMARCA2 (**D**), and PCAF (**E**). Roman numerals indicate stages of the seminiferous epithelial cycle. Bar = 20 μm. SG, spermatogonia; SC, spermatocytes; step, steps of spermatid differentiation.

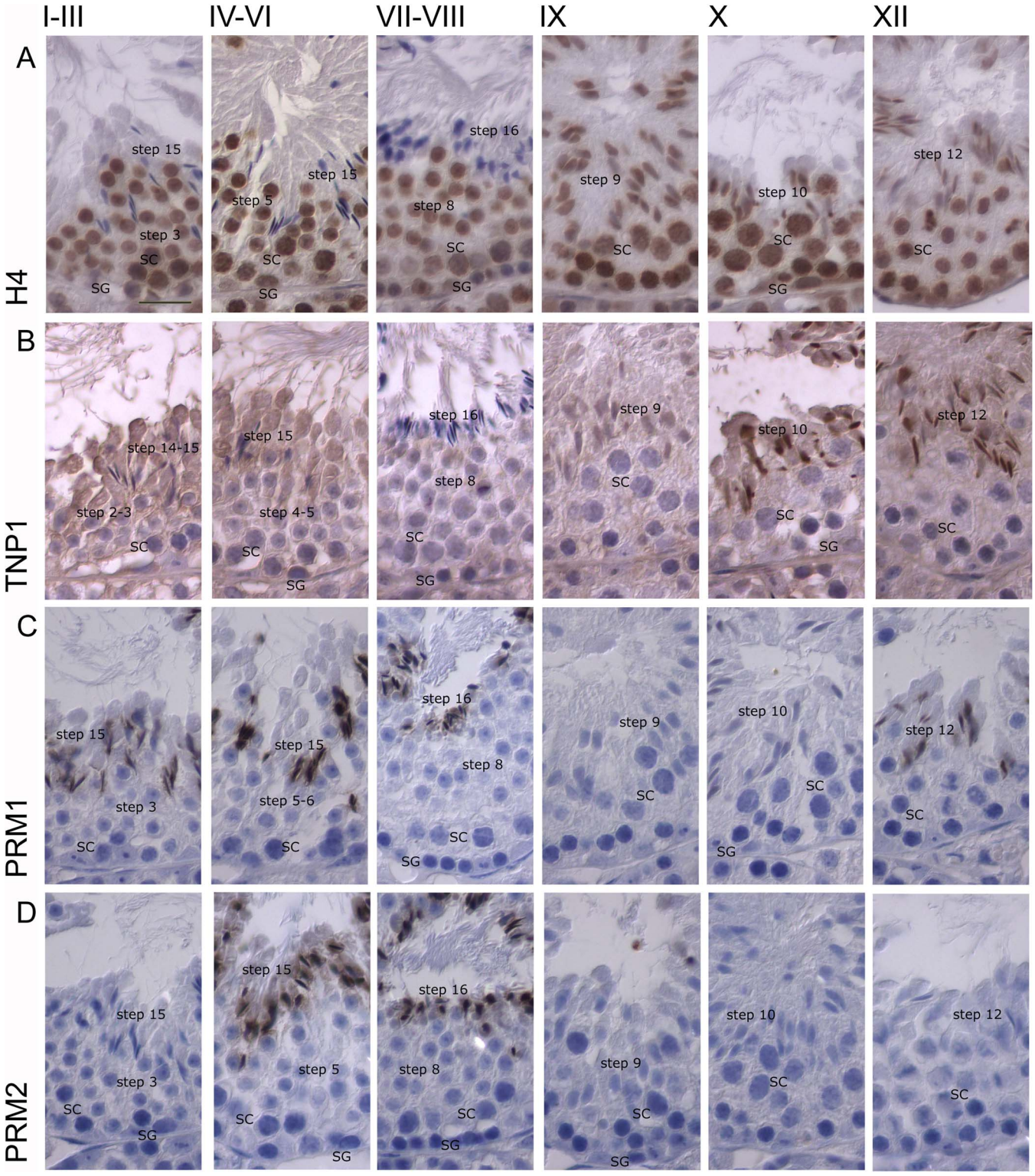

FIG. 5. Expression of proteins in the histone-to-protamine transition in murine spermatids. **A**–**D**) Mouse testicular tissue sections stained with antibodies against histone H4 (**A**), transition protein TNP1 (**B**), protamine PRM1 (**C**), and protamine PRM2 (**D**). Roman numerals indicate stages of the seminiferous epithelial cycle. Bar = 20 μm. SG, spermatogonia; SC, spermatocytes; step, steps of spermatid differentiation.

germ cells up to step 4 spermatids (Supplemental Fig. S6, C and D). Expression of the transition protein TNP1 was specific for steps 4 and 5 spermatids (Supplemental Fig. S6E). Both PRM1 and PRM2 were detected from step 5 onward (Supplemental Fig. S6F and data not shown).

Chromatin Changes in Spermatids Are Accompanied by Several Epigenetic Marks

As described above, haploid spermatids expressed a range of bromodomain-containing proteins known to act as epigenetic reader proteins. Hence, we checked whether antibodies

against acetylated histones H3 and H4 stained spermatids (Fig. 6 and Supplemental Fig. S7). In mice, acetylated histone H3 (H3ac) was observed in spermatids up to steps 12–13 (Fig. 6A). Acetylated histone H4 (H4ac) was strongly detected in steps 1–4 and steps 9–14 spermatids but only weakly in steps 5–8 (Fig. 6B). A similar pattern was observed for H4 acetylated at lysine 12 (H4K12ac), which was detected in spermatids of steps 1–3 and steps 9–14 but not in steps 4–8 spermatids (Fig. 6E). H4 acetylated at lysine 5 (H4K5ac) was detected in spermatids up to steps 14–15 (Fig. 6C); H4 acetylated at lysine 8 (H4K8ac; Fig. 6D) and H4 acetylated at lysine 16 (H4K16ac; Fig. 6F) were detected in spermatids up to step 12. In humans, H4ac was detected in steps 4 and 5 spermatids (Supplemental Fig. S7A), H4K5ac and H4K8ac were detected in spermatids up to step 5 (Supplemental Fig. S7, B and C), H4K12ac was detected in spermatids up to step 4 (Supplemental Fig. S7D), and H4K16ac was detected only in step 4 spermatids (Supplemental Fig. S7E).

DISCUSSION

Late phases of spermiogenesis are based on translationally repressed and stored mRNAs synthesized in earlier phases. After the meiotic divisions, transcriptional activity massively increases and then later decreases during the course of chromatin compaction [4]. Our observations that active RNA polymerase II was detected up to steps 8–9 spermatids in mice (Fig. 7, green) and up to step 3 in human spermatids (Fig. 8, green) are in agreement with these changes in transcriptional activity, both of which indicate that much of spermiogenesis occurs in the absence of active gene transcription. A long-standing hypothesis is that the histone-to-protamine transition causes transcriptional silencing in haploid spermatids [23]. However, we showed that histones were clearly detected up to step 12 mice spermatids (Fig. 7, yellow) and up to step 4 human spermatids (Fig. 8, yellow). These results indicate that the bulk of transcription ceases before histone removal and protamine loading, in both human and murine spermatids.

Parts of the sperm DNA retain a histone-based nucleosomal structure (about 15% in human sperm; about 1% mice sperm) [32, 33]. We believe that the method we used here is not sensitive enough to detect residual histones in highly compacted sperm chromatin. Nevertheless, histones H2A and H2B in human sections, in contrast to histones H3 and H4, were surprisingly hardly detectable in germ cells before step 4 spermatids (Fig. 8). The reason for this might be that the antigen is simply not accessible in spermatogonia, spermatocytes, and early spermatids. In step 4 spermatids, the antigen probably becomes temporarily accessible owing to a more open chromatin structure in the course of transition protein (TNP1) incorporation to the chromatin (Fig. 8, red). In mice, histones H2A and H2B were more clearly detected when sections from testes fixed in 4% F-PBS were analyzed instead of sections fixed in Bouin solution. Fixation with 4% F-PBS in many cases allows a more sensitive immunohistochemical detection of proteins. However, it was not possible to test different fixatives for the human samples due to limited material.

Our qPCR data showed that several TAFs and TAF variants are enriched in spermatids, which indicates that many of the canonical TAFs are involved in postmeiotic gene transcription. Interestingly, TAF5, TAF5L, TAF6, and TAF6L seem to be present together within spermatids. Hence, in round spermatids, TFIID complexes of different compositions might each regulate a subset of genes. Interestingly, in both human and murine spermatids, TAF6 was detected even longer than active RNA polymerase II (Figs. 7 and 8). It is possible that TAFs play a role outside of the TFIID complex in spermatids. Indeed, TAFs have not only been identified in the TFIID complex but also in several other protein complexes, for example, yeast SAGA and human PCAF, both of which are able to modify the chromatin structure [34]. We found that the bromodomain-containing histone acetyltransferase (HAT) PCAF accumulates in human and murine spermatid nuclei in the course of postmeiotic transcription initiation (Figs. 7 and 8). PCAF acts as a coactivator of transcription and builds together with more than 20 other proteins the so-called PCAF complex [35, 36]. This complex shares the histonelike subunits TAF9, TAF10, and TAF12 with the transcription factor complex TFIID and contains a histone octamerlike structure [36]. TAF5L and TAF6L are also part of the PCAF complex [37]. All these TAFs and TAF variants were enriched in postmeiotic spermatids and might be part of both the PCAF and TFIID complexes.

For transcription, not only are general transcription factors needed, but also mechanisms that facilitate DNA accessibility and subsequently gene expression. In round spermatids, several histone variants are expressed and diverse histone modifications have been identified [38]. Histone variants contribute in marking specific genomic regions and thereby regulate chromatin structure and dynamics [39, 40]. Histone modifications can alter the charge of the respective histones, which can lead to structural chromatin changes. In addition, modified histones can bind different proteins and thereby allow downstream events on the chromatin [41, 42].

The HAT PCAF acetylates histones H3 and H4 and the general transcription factors TFIIEβ and TFIIF [43]. In most cases, acetylation of histone tails is correlated with active gene transcription. Here we showed that in murine spermatids, the beginning of postmeiotic transcription is accompanied by hyperacetylation of histone H4 (H4ac). The level of H4ac decreases as transcription proceeds and increases again when active RNA polymerase II starts to become undetectable (Fig. 7), which is in keeping with the well-known hyperacetylation of histone H4 prior to histone removal [25, 26]. We observed a similar pattern for H4K12ac, which probably reflects the initiation of massive transcription in spermatids and then initiation of the histone-to-protamine transition (Fig. 7). H4K8ac and H4K16ac were detected from step 1 spermatids up to step 12 spermatids and H4K5ac even up to steps 14–15 spermatids (Fig. 7). However, active RNA polymerase II was no longer detected already in steps 8–9 spermatids. Hence, these histone modifications might be involved in both transcription and the histone-to-protamine transition. The data presented here are contradictory to our recently published data [2] and to other published data showing that histones are unacetylated in most round spermatids [25]. This difference in our current observations could be explained by the use of 4% F-PBS to fix testes, which, as mentioned above, in many cases allows a more sensitive immunohistochemical detection of proteins. Recently, a dynamic pattern of H4K5ac, H4K8ac, and H4K12ac has been shown in immunofluorescent stainings of murine male germ cells; high levels of H4K5ac, H4K8ac, and H4K12ac are detected during meiosis and in steps 9–12 spermatids, and low to moderate levels are found only in a portion of steps 1–8 spermatids [44]. Our observations that the levels of H4ac and H4K12ac are low in steps 4–8 spermatids and high in steps 9–12 spermatids are consistent with these results. However, our observation that H4K8ac and H4K5ac were detected up to step 12 and steps 14–15 spermatids, respectively, is not consistent with the earlier results.

In human spermatids, H4K5ac and H4K8ac were detected in steps 1–5 spermatids, and H4K12ac was detected in steps 1–

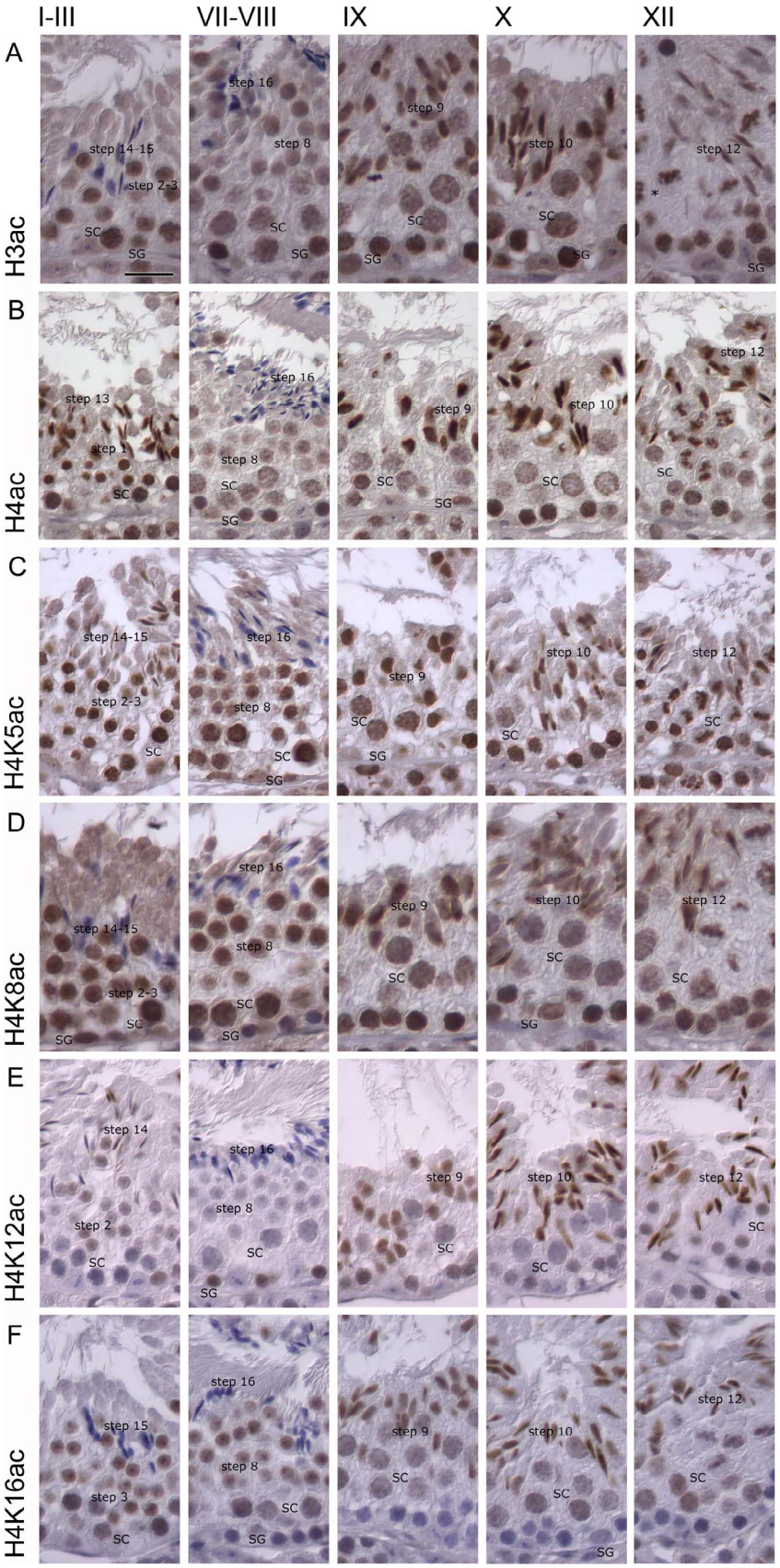

FIG. 6. Histone modifications in murine spermatids. **A**–**F**) Mouse testicular tissue sections stained with antibodies against acetylated histone H3 (**A**), acetylated histone H4 (**B**), Lys5-acetylated histone H4 (**C**), Lys8-acetylated histone H4 (**D**), Lys12-acetylated histone H4 (**E**), and Lys16-acetylated histone H4 (**F**). Roman numerals indicate stages of the seminiferous epithelial cycle. Bar = 20 μm. SG, spermatogonia; SC, spermatocytes; step, steps of spermatid differentiation.

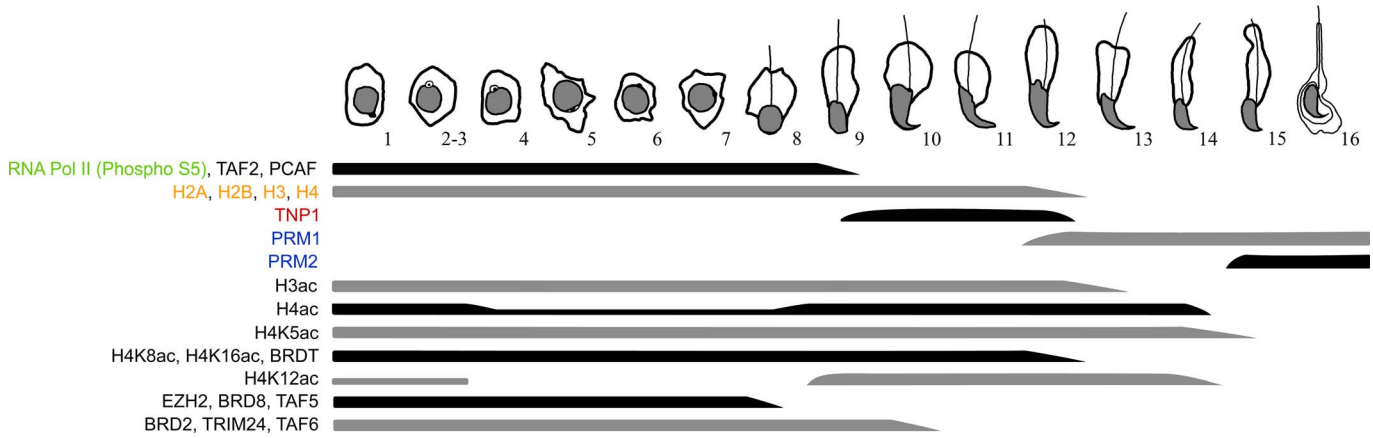

FIG. 7. Detection of epigenetic markers and proteins during murine spermatid differentiation in comparison to that of active transcription (green), histones (yellow), transition proteins (red), and protamines (blue). The spermatid stages are based on those of Hess and de Franca [61].

4 spermatids (Fig. 8). Hence, as in murine spermatids, these modifications seem to be required for both gene transcription and the initial steps of histone-to-protamine transition. By contrast, H4K16ac occurred only in step 4 spermatids, when transcription had already ceased (Fig. 8). We cannot exclude that using a different fixative would allow detection of this modification in earlier spermatids. However, based on our results, H4K16ac seems to play a role in histone-to-protamine transition rather than in transcription.

Acetylated histones can be recognized by bromodomain-containing proteins, for example, TAF1 and PCAF [11, 45]. Besides PCAF, several other bromodomain-containing proteins were enriched in spermatids. In murine spermatids, BRD2 and TRIM24 (also known as TIF1α) were detected longer than active RNA polymerase II (Fig. 7) and might have a function also in TNP1 loading. TRIM24 plays a role in multiple signaling pathways and can act as both corepressor and co-activator [46]. BRD2 belongs to the bromodomain and extraterminal (BET) family [47]. BRD2 binds to H4K12ac and is part of a multiprotein transcription complex in different cell types [48–50]. It has been proposed that BRD2 recruits transcription factors, HATs, and chromatin-remodeling proteins to gene promoters by providing a scaffold on the chromatin [49, 51–53]. In murine spermatids, BRD2 could fulfill a similar role in promoting transcription and initial steps of the histone-to-protamine transition. Also in murine spermatids, transcripts of BET family members, for example, *Brd2* and *Brdt*, are enriched [54]. In murine spermatids, the presence of two further bromodomain-containing proteins, BRD8 and BRDT, overlaps with that of active RNA polymerase (Fig. 7). BRDT exhibited a broader expression pattern and was detected until TNP1 vanished and the first protamines were loaded. BRDT is the best-characterized bromodomain-containing protein in spermatogenesis; it is involved in gene regulation processes and later in the histone-to-protamine transition [17, 55]. The function of BRD8 has been hardly described, but it seems to be required for the incorporation of the histone variant H2A.Z and gene activation during adipogenesis [56]. In human spermatids, the presence of BRD8 and BRDT overlaps with that of both active RNA polymerase II and TNP1 (Fig. 8); these proteins might be involved in both transcription and histone-to-protamine transition. In human spermatids, the bromodomain-containing protein BRD3 and the chromatin remodeler SMARCA2 (also known as Brahma) were specifically detected in step 4 spermatids when histones become replaced by transition proteins (Fig. 8). By contrast, in mice, SMARCA2 has been detected in spermatids until step 8 [57] and might be involved in transcription rather than histone-to-protamine transition.

Previously, it has been postulated that BRD4 plays an essential role in postmeiotic spermatids [58]. However, we (data not shown) and others [18] detected BRD4 solely in

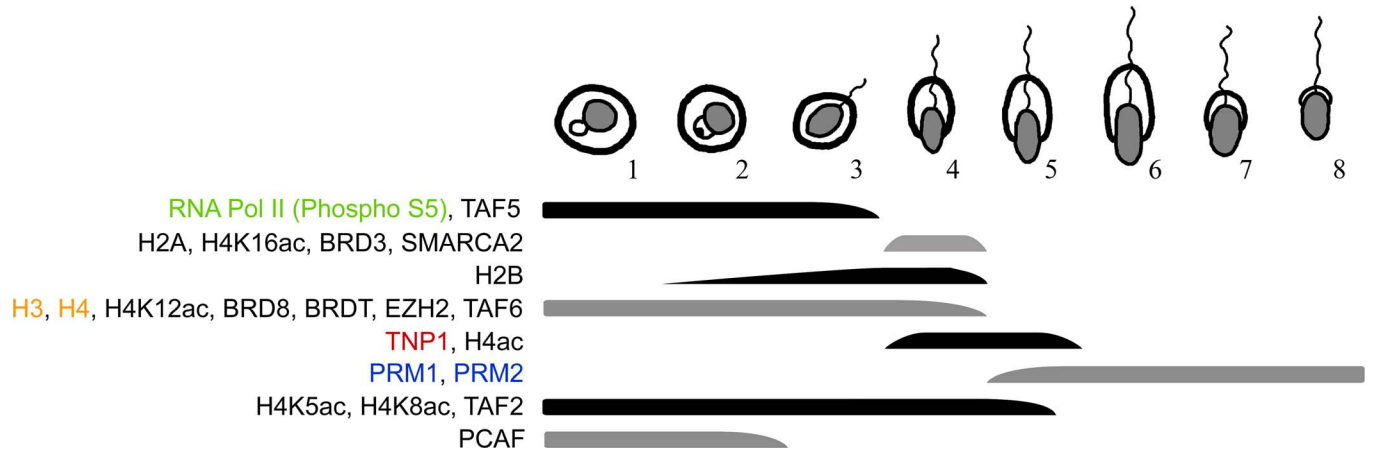

FIG. 8. Detection of epigenetic markers and proteins during human spermatid differentiation in comparison to that of active transcription (green), histones (yellow), transition proteins (red), and protamines (blue). The spermatid stages are based on those of Bergmann and Kliesch [62].

spermatogonia. Recently, it has been shown that the dual bromodomain-containing protein BRWD1 is involved in transcription of about 300 genes, most of which are activated in spermatids [59].

Transcripts of the PRC1 and PRC2 subunits RING1 and EZH2 were enriched in murine spermatids, which indicated a new synthesis of both in postmeiotic stages. The histone methyltransferase EZH2 has been mainly detected in the perinuclear acrosome region in round spermatids [60]. By contrast, we observed EZH2 in the nucleus in steps 1–8 spermatids in mice (Fig. 7) and in steps 1–4 in human spermatids (Fig. 8). Hence, PRC2 might play a role in shutting down transcription in differentiating spermatids. However, we cannot exclude that EZH2 fulfills a role outside the PRC2 in spermatids. Further studies are required to analyze the biological function of PRC1 and PRC2 components in differentiating spermatids.

Based on our results, we propose that in humans and mice, the chromatin in spermatids is opened by common and specific features first for transcription and subsequently for the histone-to-protamine transition. We are convinced that our detailed descriptive work offers an excellent starting point for future research on male fertility disorders.

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