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Germ cell expression of the transcriptional co-repressor TIF1β is required for the maintenance of spermatogenesis in the mouse

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SUMMARY

The gene for transcriptional intermediary factor 1β (TIF1β) encodes a transcriptional co-repressor known to play essential roles in chromatin remodeling as well as in early embryonic development. During spermatogenesis, TIF1β is preferentially associated with heterochromatin structures of Sertoli cells and round spermatids, as well as with meiotic chromosomes. Its expression is tightly regulated within spermatocyte and spermatid populations, and it is undetectable in spermatogonia. Spatiotemporally controlled ablation of TIF1β by using a germ cell lineage-specific CreER^T/loxP system leads to testicular degeneration. This degeneration is not due to impairment of chromatin remodeling processes during meiosis and spermiogenesis, as TIF1β-deficient spermatocytes are able to complete their differentiation into spermatozoa. It rather occurs as a consequence of shedding of immature germ cells (spermatocytes and spermatids), and disappearance of stem spermatogonia. These results indicate that TIF1β has important functions in the homeostasis of the seminiferous epithelium, and probably plays a crucial role in the network of paracrine interactions between germ cell subpopulations and/or Sertoli cells.

Key words: Heterochromatin protein 1, Transcriptional silencing, KRAB zinc-finger proteins, Testis-specific conditional knockout, Cellular interactions, Mouse

INTRODUCTION

Transcriptional regulation of gene expression in eukaryotes in response to developmental and other environmental signals is a multi-step process that requires the concerted action of many cellular factors. Central players in this elaborate process are sequence-specific transcription factors that activate and/or repress transcription through interactions with co-activators and co-repressors, whose ultimate function is to remodel chromatin structure (reviewed by Hassan et al., 2001; Muller and Leutz, 2001), to stimulate or inhibit (pre)initiation complex formation (reviewed by Hampsey and Reinberg, 1999; Rachez and Freedman, 2001) or to associate target genes with specialized nuclear compartments (Cockell and Gasser, 1999; Francastel et al., 2000).

Mammalian transcriptional intermediary factor 1β (TIF1β; Trim28 – Mouse Genome Informatics) (also called KAP-1 or KRIP-1), which was originally identified as a co-repressor for the large family of KRAB domain-containing zinc-finger proteins (Friedman et al., 1996; Kim et al., 1996; Moosmann et al., 1996), has also been defined as a member of an emerging family of transcriptional regulators that includes TIF1α and TIF1γ in mammals (Le Douarin et al., 1995; Le Douarin et al., 1996; Venturini et al., 1999), and Bonus in *Drosophila* (Beckstead et al., 2001). The domain structure that characterizes these proteins consists of an N-terminal RBCC (RING finger, B boxes, coiled coil) motif and a C-terminal bromodomain preceded by a PHD finger (Le Douarin et al., 1996). All TIF1 family members have been reported to repress basal and activated transcription when tethered to DNA through fusion to an heterologous DNA-binding domain. In the case of TIF1β, an epigenetic mechanism of control has been suggested by the finding of an association with members of the heterochromatin protein 1 (HP1) family (Nielsen et al., 1999; Ryan et al., 1999), a class of non-histone chromosomal proteins with a well-established function in heterochromatin-mediated silencing (reviewed by Eissenberg and Elgin, 2000). TIF1β has been shown to colocalize with members of the HP1 family in interphase nuclei of several mammalian cell lines (Nielsen et al., 1999; Ryan et al., 1999). In vitro, TIF1β interacts with and phosphorylates the HP1 proteins (Nielsen et al., 1999). This interaction is required for the TIF1β-mediated repression of transcription (Nielsen et al., 1999; Ryan et al., 1999) and for its association with pericentromeric heterochromatin in cultured cells (Matsuda et al., 2001) (F. C., M. Oulad-Abdelghani, J. L. Vonesch, P. C. and R. L., unpublished). A mechanistic link between TIF1β repression and histone modification has also been established, with the

demonstration that deacetylase inhibitors such as Trichostatin A can interfere with TIF1β-mediated repression in transient transfection assays (Nielsen et al., 1999; Schultz et al., 2001). In agreement with this result, TIF1β has recently been reported to be an intrinsic component of a novel histone deacetylase complex, called N-CoR-1 (Underhill et al., 2000), and to interact both physically and functionally with the subunit Mi-2α of the nucleosome remodeling and deacetylation (NuRD) complex (Schultz et al., 2001). Thus, TIF1β may exert its co-repressor function via the assembly and/or maintenance of transcriptionally inactive, higher order chromatin structures through histone deacetylation and heterochromatinization.

We have recently shown that mice devoid of *Tif1β* expression die at the egg cylinder stage, prior to the onset of gastrulation (Cammas et al., 2000). Analysis of the *Tif1β*-null embryos has revealed a reduced cell number in the ectoderm, morphological alterations of the visceral endoderm and absence of mesoderm formation (Cammas et al., 2000). This phenotype indicates that TIF1β exerts essential functions in early embryogenesis. However, the lethal outcome of this null mutation precludes the analysis of the roles of TIF1β in later developmental and cell differentiation processes. Spermatogenesis is a cyclic cell differentiation process that includes spermatogonia self-renewal and their differentiation towards spermatozoa. We now show that during spermatogenesis, TIF1β is expressed in a finely regulated pattern and is preferentially associated with heterochromatin. To investigate whether TIF1β has a role in spermatogenesis, we have generated a conditional germline-specific *Tif1β* mutation in mice by using the tamoxifen-inducible Cre-ER^{T}/loxP recombination system (Metzger and Chambon, 2001). Mice homozygous for a conditional allele of *Tif1β* (TIFβ$^{L2/L2}$), an allele in which essential coding exons are flanked by loxP sites (Cammas et al., 2000), were crossed with a transgenic PrP-Cre-ER$^{T(tg/0)}$ hemizygous line, in which tamoxifen selectively induces DNA excision in spermatogonia and spermatocytes (P. W., C. G., M. M., D. M. and P. C., unpublished). Analysis of the testes of tamoxifen-treated TIF1β$^{L2/L2}$:PrP-Cre-ER$^{T(tg/0)}$ mice reveals that TIF1β plays a key role in the maintenance of spermatogenesis.

MATERIALS AND METHODS

Mice

Mice allowing conditional inactivation of *Tif1β* were obtained by crossing *Tif1β$^{L3/L3}$* mice (Cammas et al., 2000) with CMV-Cre transgenic mice (Dupé et al., 1997). Offspring harboring the partially excised L2 allele of the *Tif1β* gene without the antibiotic resistance cassette were identified by a PCR screen performed on DNA of tail biopsies using primers YD208, VR211 and TV210 as described (Cammas et al., 2000). Positive *Tif1β$^{L2/+}$* mice were bred with wild-type mice and offspring which had lost the CMV-Cre transgene, but not the *Tif1β* L2-allele, were intercrossed to generate homozygous *Tif1β$^{L2/L2}$* mice. *Tif1β$^{L2/L2}$* mice were viable, and did not show any phenotypic abnormalities.

Transgenic mice harboring a testis-restricted expression of the ligand-inducible Cre-ER^{T} (Feil et al., 1996) under control of the murine Prion (PrP)-promoter (PrP-Cre-ER^{T} line 28.8) are described elsewhere (P. W., C. G., M. M., D. M. and P. C., unpublished). *Tif1β$^{L2/L2}$* males with (PrP-Cre-ER$^{T(tg/0)}$) or without (PrP-Cre-ER$^{T(0/0)}$) the testis-specific transgene were generated and injected intra-peritoneally (IP) at 4 weeks of age for 5 consecutive days with tamoxifen (1 mg/day) (Metzger and Chambon, 2001). Experimental (TIF1β$^{L2/L2}$:PrP-Cre-ER$^{T(tg/0)}$) and control (TIF1β$^{L2/L2}$:PrP-Cre-ER$^{T(0/0)}$) males were sacrificed 1 day, and 2, 4, 6, 7 and 8 weeks after tamoxifen treatment (i.e. after the last tamoxifen injection).

Immunohistochemical detection of TIF1β in mouse testes

Rabbit antisera were raised against two peptides (PF64 and PF65) corresponding to the N-terminal sequence (amino acids 140-154 and 66-80, respectively) of the TIF1β protein and purified on a sulfolink coupling gel (Oulad-Abdelghani et al., 1996). Cellular localization of TIF1β in testis of TIF1β$^{L2/L2}$:PrP-Cre-ER$^{T(0/0)}$ mice was performed on 10 μm thick cryosections hydrated in phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde (PFA) in PBS (for 10 minutes at 4°C). Sections were rinsed in PBS containing 0.1% Triton X-100 (PBST; 3×5 minutes at room temperature), then saturated with 5% normal goat serum (NGS) in PBST (30 minutes at room temperature), and incubated with the antibody against the PF64 and PF65 peptides (4 μg/ml; 2 hours at room temperature). Sections were washed in PBST (3×5 minutes), then PBS (5 minutes), incubated with the secondary antibody (Cy3-coupled donkey anti-rabbit diluted at 1/400 in PBS, Jackson Laboratories) (1 hour at room temperature), washed in PBST and mounted in Vectashield (Vector) containing DAPI at 10 μg/μl. In another set of immunohistochemical experiments, testis of TIF1β$^{L2/L2}$:PrP-Cre-ER$^{T(tg/0)}$ (experimental) and TIF1β$^{L2/L2}$:PrP-Cre-ER$^{T(0/0)}$ (control) mice were fixed in 4% PFA in PBS (16 hours at 4°C) and embedded in paraplast. Sections (7 μm) were dewaxed, hydrated, rinsed in PBS, then placed into 5 mM sodium citrate buffer pH 6.0 and exposed to a microwave treatment (power output 800 W; 2×2.5 minutes) (Balaton et al., 1993). After cooling down to room temperature, sections were rinsed in PBST, treated with 5% NGS in PBST (30 minutes) to block nonspecific antibody binding to the tissue sections and incubated for 16 hours at 4°C with the anti-PF64 and PF65 antibodies. Sections were then washed, incubated with the secondary antibody (1 hour at room temperature) and mounted as described for cryosections.

Preliminary experiments indicated that the antibodies against PF64 and PF65 labeled the same cells in the seminiferous epithelium. However, the signal was more intense with the later antibody, which was therefore used in subsequent immunostaining experiments. In immunohistochemical experiments, some differences were observed in the nuclear localization of TIF1β and intensities of the signals in germ cell and Sertoli cell between frozen and microwave-treated paraffin wax-embedded sections. These differences were reproduced with both anti-PF64 and anti-PF65 antibodies. They could not be accounted by prolonged fixation in paraformaldehyde before paraffin wax embedding (i.e. 16 hours versus 10 minutes after cryosectioning) nor by the microwave treatment. Indeed, prolonged post-fixation of cryosections for 16 hours resulted in a global decrease of the anti-TIF1β immunoreactivity in all cell types within the seminiferous epithelium. Likewise, microwave treatment globally restored the immunostaining that was decreased in all cell types upon a 16 hours stay of the tissue in paraformaldehyde. These differences in immunostaining may result from diffusion of the TIF1β protein within the chromatin during the paraffin wax embedding process. As negative controls of the immunostaining procedure, histological sections were incubated either with non-immune rabbit IgGs (5 μg/ml) or with a mixture of the primary antibody and 12-fold excess of the immunizing peptide (10 μg/ml).

Histological analysis and detection of proliferating and apoptotic cells

For histological analysis, testes of tamoxifen-treated TIF1β$^{L2/L2}$:PrP-Cre-ER$^{T(tg/0)}$ and TIF1β$^{L2/L2}$:PrP-Cre-ER$^{T(0/0)}$ mice were fixed in Bouin's fluid. Paraffin wax embedded sections (7 μm) were stained with Hematoxylin and Mallory's trichrome (Mark et al., 1993). Detection of apoptotic cells on sections from PFA-fixed and paraffin wax-embedded testes was performed by TdT-mediated dUTP nick

end labeling (TUNEL), according to the manufacturers instructions (In Situ Cell Death Detection Kit, Fluorescein, Roche); sections were counterstained with DAPI. To identify proliferating cells, adult TIF1β$^{L2/L2}$:PrP-Cre-ER$^{T(0/0)}$ mice were injected intra-peritoneally four times at intervals of 2 hours with 50 mg/kg of BrdU. Testis were collected 2 hours after the last BrdU injection, fixed in 4% PFA in PBS (16 hours; 4°C). Paraffin wax-embedded sections were incubated with an antibody against BrdU (Boehringer Mannheim) diluted 1/100 in 0.1% NGS/PBS (16 hours; 4°C), revealed with Cy3-conjugated donkey anti-rabbit IgG, and mounted in Vectashield medium containing DAPI.

Statistical analysis

The number of seminiferous tubules showing abnormal expression of TIF1β (i.e. partial staining along the circumference of spermatocyte and/or spermatid layers in a given tubular cross section, instead of staining along the whole circumference of these layers) was scored on testes from TIF1β$^{L2/L2}$:PrP-Cre-ER$^{T(tg/0)}$ and TIF1β$^{L2/L2}$:PrP-Cre-ER$^{T(0/0)}$ animals 2 weeks after tamoxifen treatment. The number of degenerating tubules (i.e. tubules showing signs of vacuolation) was scored on histological sections from testes of TIF1β$^{L2/L2}$:PrP-Cre-ER$^{T(tg/0)}$ and TIF1β$^{L2/L2}$:PrP-Cre-ER$^{T(0/0)}$ 8 weeks after tamoxifen treatment. Testicular sections of three individuals were analyzed for each genotype and experimental group. Statistical analysis of data was performed using ANOVA and the Fischer's protected least significant difference test (Fischer's PLSD).

RESULTS

TIF1β is associated with heterochromatin and expressed in a finely regulated pattern during spermatogenesis

The expression pattern of TIF1β was analyzed by immunohistochemistry on sections from frozen and paraffin wax-embedded TIF1β$^{L2/L2}$:PrP-Cre-ER$^{T(0/0)}$ testes (referred hereafter as control testes). On frozen sections, fluorescent nuclear signal for TIF1β was present in all Sertoli cells (S; Fig. 1A-C), round spermatids (RS; Fig. 1A-C) and spermatids at early stages of elongation (ES-9; Fig. 1D-F). In all these cells, the intensity of the immunostaining paralleled that of the DNA-specific DAPI counterstain (arrowheads in Fig. 1A-F) (Barcellona and Gratton, 1990). A stippled signal was detected in the nuclei from large pachytene spermatocytes (P and arrows in Fig. 1D-F), whereas other meiotic cells as well as spermatogonia and elongated spermatids appeared negative (e.g. ES-13 in Fig. 1A-C and data not shown).

Confocal microscopy was used to assess the subnuclear localization of TIF1β further. Single optical sections counterstained with DAPI showed that the TIF1β staining was consistently more intense in heterochromatin than in euchromatin in both Sertoli cells and round and early elongating (step 9) spermatids. In Sertoli cells, TIF1β was predominantly localized in nucleolar satellites (Sa in Fig. 1G-I) (Russell et al., 1990); in round spermatids, it was concentrated within the chromocenter, a structure formed from the association of the centromeric heterochromatin of all chromosomes (confocal data not shown, see arrowheads in Fig. 1D-F) (Pardue and Gall, 1970; Hoyer-Fender et al., 2000). Confocal microscopic analysis also showed a specific association of TIF1β with chromosomes in pachytene spermatocytes (Ch in Fig. 1J-L).

Immunostaining with the anti-TIF1β antibody of microwave-treated paraffin wax-embedded sections from control testes revealed an intense fluorescent signal in many spermatocytes (P in Fig. 2A-C) and round spermatids (RS).

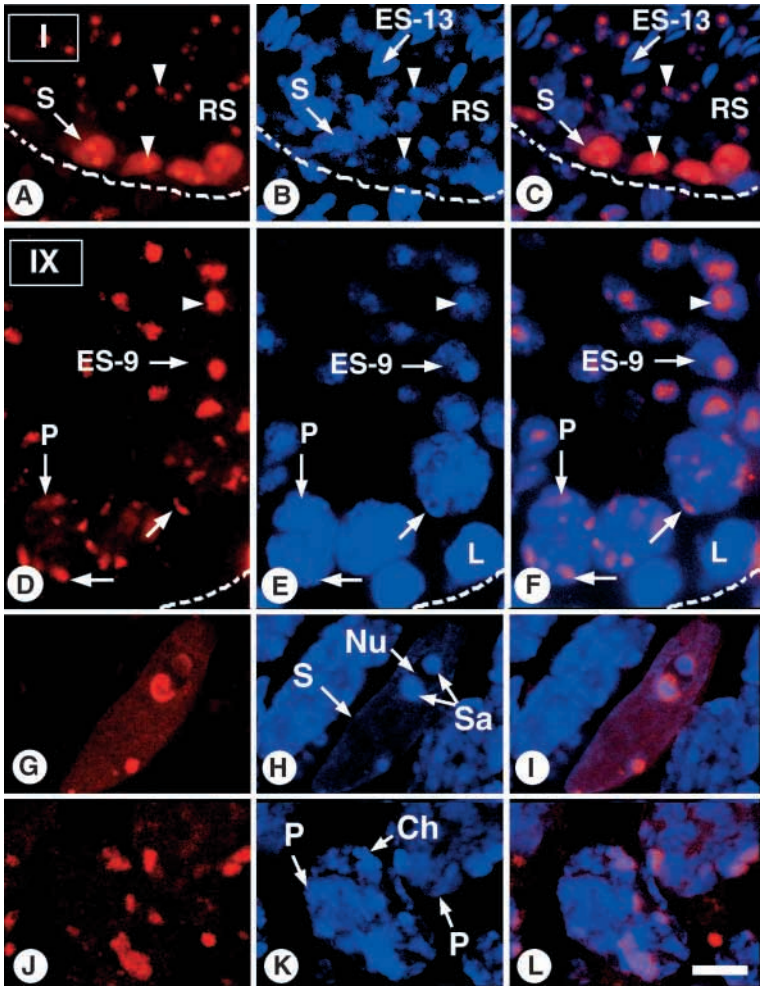

Fig. 1. Immunolocalization of TIF1β in post-pubertal wild-type testis. Frozen histological sections were incubated with the anti-TIF1β antibody whose binding to cell structures was then revealed with a Cy3-conjugated secondary antibody (red signal) and nuclei were counterstained with DAPI (blue signal). (A,D,G,J) Cy3 and (B,E,H,K) DAPI labeling.(C,F,I,L) Superimposition of the two fluorochromes. (G-L) Single optical sections of confocal microscopic analysis. Roman numerals refer to stages of the seminiferous epithelium cycle (Russell et al., 1990). Each stage is defined by a specific association of germ cell types. The cycle corresponds to the series of changes occurring at a given level of the seminiferous tubule between two successive appearances of the same cell association. In normal mice, there are 12 stages, designated I to XII, each corresponding to one of the first 12 steps of spermatid maturation. ES-9 and ES-13 correspond to steps in spermatid maturation. Ch, meiotic chromosomes; L, leptotene spermatocytes; P, pachytene spermatocytes; Nu, nucleolus of Sertoli cell; RS, round spermatids; S, Sertoli cells; Sa, satellite nucleolar heterochromatin of the Sertoli cell. The arrowheads and arrows in A-F indicate heterochromatin- and chromosome-associated TIF1β, respectively. The broken lines indicate the contours of seminiferous tubules. Scale bar: 30 μm in A-C; 15 μm in D-F; 3 μm in G-L.

The signal detected in Sertoli cells (S) was in general weaker than that observed on frozen sections. Moreover, in both germ cells and Sertoli cells, this immunostaining was exclusively nuclear, but was evenly distributed in the nucleus, instead of being associated with heterochromatin, as in the case of frozen sections (see Materials and Methods). Detection of TIF1β in germ cells was dependent on their state of maturation and, therefore, on the stage of the seminiferous epithelium cycle (Russell et al., 1990) (see legend of Fig. 1). Immunostaining was undetectable in spermatogonia (SG) and young (preleptotene, leptotene, zygotene and early pachytene) spermatocytes (PR and L) (Fig. 2A-C and data not shown). It was intense in growing pachytene and in diplotene spermatocytes populating stage VI-XI tubules (P, Fig. 2A-C), at all steps of round spermatid maturation (e.g. RS in Fig. 2A,C) and in early elongating spermatids (i.e. step 9 spermatids, data not shown). Step 10 spermatids were only faintly immunostained (ES-10, Fig. 2B and data not shown), and spermatids at later stages of elongation (i.e. steps 11-16) were negative (ES-16 in Fig. 2A,C and data not shown). Unmasking of the epitope after microwave-induced disruption of heterochromatin structures in germ cells is likely to account for the discrepancies in patterns and intensities of immunostaining between sections from frozen and paraffin wax-embedded testes. These discrepancies were reproduced with antibodies raised against two distinct peptides derived from TIF1β, indicating that they were not caused by auto-antibodies that might contaminate rabbit antisera. Note also that no immunostaining could be found when the primary antibody was replaced by non-immune IgG or a mixture of the primary antibody and immunizing peptide (data not shown).

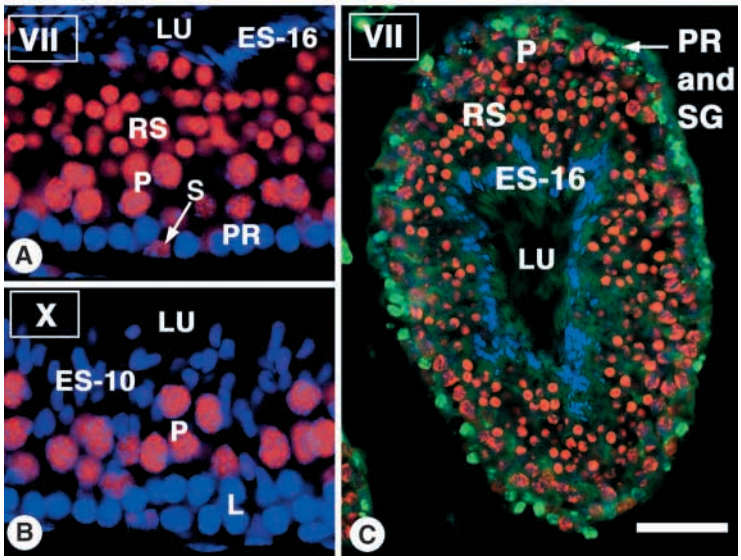

Fig. 2. Immunolocalization of TIF1β in post-pubertal wild-type testis. Histological sections from paraffin wax-embedded testes were incubated with the anti-TIF1β antibody, whose binding to cell structures was then revealed with a Cy3-conjugated secondary antibody (red signal) and nuclei were counterstained with DAPI (blue signal). BrdU incorporation in the tubular cross section shown in C was detected using a green fluorochrome. Roman numerals refer to stages of the seminiferous epithelium cycle. ES-10 and ES-16 correspond to steps in spermatid maturation. L, leptotene spermatocytes; LU, lumen of the seminiferous tubules; P, pachytene spermatocytes; PR, preleptotene spermatocytes; RS, round spermatids; S, Sertoli cells; SG, spermatogonia. Scale bar: 25 μm in A,B; 55 μm in C.

To further investigate the possibility that some spermatogonia might express TIF1β, control mice were injected with BrdU, which incorporates into the nuclei of spermatogonia and preleptotene spermatocytes. In double immunostaining experiments, colocalization of TIF1β and BrdU was never observed (Fig. 2C and data not shown). Altogether, these data indicate that within the seminiferous epithelium, TIF1β is present in all Sertoli cells, as well as in specific subpopulations of meiotic cells (i.e. mid-pachytene spermatocytes) and post-meiotic cells (i.e. step 1-10 spermatids). By contrast, this protein is undetectable in proliferating germ cells (i.e. spermatogonia), in preleptotene and early pachytene spermatocytes, as well as in condensing (step 12-14) and condensed (step 15-16) spermatids (Baarends et al., 1999).

PrP-Cre-ER^T mediates inactivation of the floxed *TIF1β* gene in spermatogonia and spermatocytes

PrP-Cre-ER^T transgenic mice (line 28.8), which enable a tamoxifen-induced time-controlled and tissue-specific DNA excision in germ cells are described elsewhere (P. W., C. G., M. M., D. M. and P. C., unpublished). Before tamoxifen treatment, TIF1β$^{L2/L2}$:PrP-Cre-ER$^{T(tg/0)}$ mice harboring both *Tif1β* floxed alleles and the PrP-Cre-ER^T transgene (referred hereafter as experimental animals) were normal with respect to fertility and testicular morphology (data not shown). The feasibility of spatiotemporal inactivation of TIF1β in the testis was assessed by following up the disappearance of the TIF1β protein on histological sections of experimental animals at different time points after tamoxifen treatment. For the sake of simplicity, only observations of tubular cross-sections in stage VII (and beginning of stage VIII) will be reported, unless otherwise mentioned. Stage VII (and the beginning of stage VIII) are characterized by the alignment of elongated spermatid nuclei at the luminal side of the seminiferous epithelium, immediately before their release, as spermatozoa, into the lumen of the seminiferous tubules. Histological sections of TIF1β control (TIF1β$^{L2/L2}$:PrP-Cre-ER$^{T(0/0)}$) testes displayed a normal TIF1β signal in all seminiferous tubules one day and 2, 4, 6 and 8 weeks after tamoxifen treatment (Fig. 3A and data not shown). By contrast, experimental (TIF1β$^{L2/L2}$:PrP-Cre-ER$^{T(tg/0)}$) testes displayed an abnormal pattern of TIF1β distribution as early as 1 day after the end of the tamoxifen treatment: all pachytene spermatocytes were negative for TIF1β (Fig. 3B). This finding indicates that the 'induced' Cre can rapidly and efficiently mediate disruption of the floxed *Tif1β* gene in most (possibly all) spermatocytes. Experimental testes analyzed 2, 4 and 6 weeks after tamoxifen treatment contained only histologically normal seminiferous tubules, which were classified into three categories based on TIF1β expression patterns: (1) tubules expressing TIF1β specifically in pachytene spermatocytes and round spermatids, which were undistinguishable from their counterparts in controls (Fig. 3C); (2) tubules devoid of TIF1β positive germ cells (Fig. 3D); (3) tubules showing an abnormal, mosaic, expression of

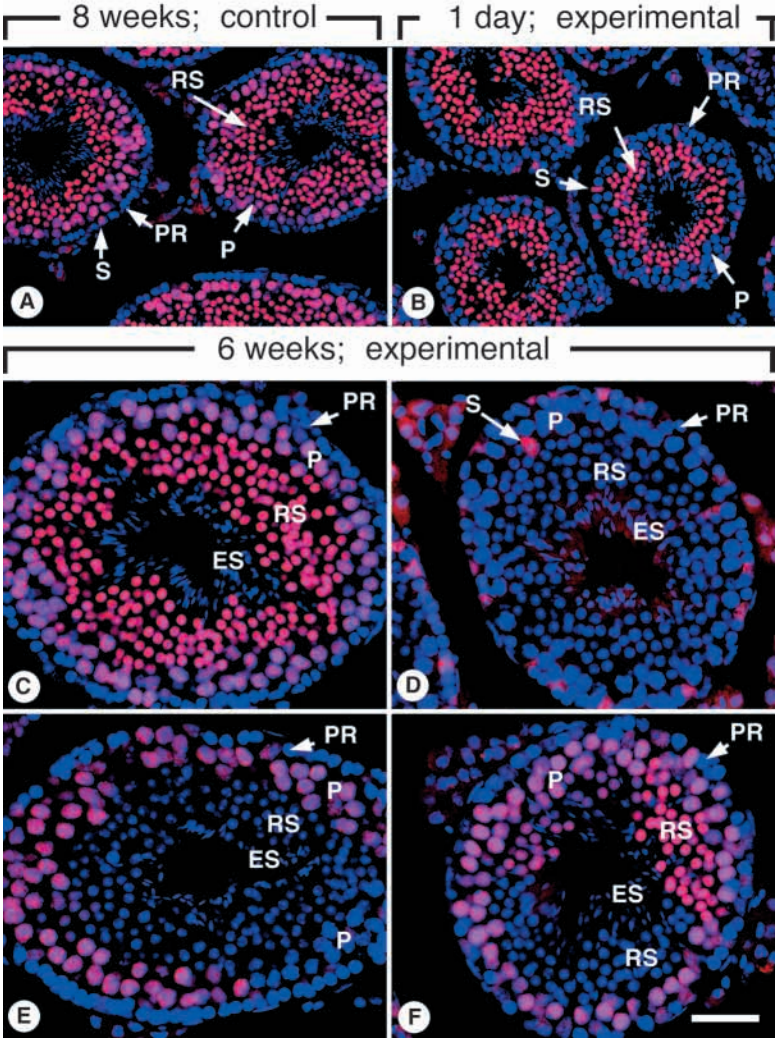

Fig. 3. Distribution of the TIF1β protein in control ($TIF1\beta^{L2/L2}$:PrP-Cre-$ER^{T(0/0)}$) and experimental ($TIF1\beta^{L2/L2}$:PrP-Cre-$ER^{T(tg/0)}$) testes, 1 day (B), 6 weeks (C-F) and 8 weeks (A) after tamoxifen treatment. All the seminiferous tubules displayed here correspond to histologically normal stage VII tubules. C-F are from the same experimental testis. ES, elongated spermatids; P, pachytene spermatocytes; PR, preleptotene spermatocytes; RS, round spermatids; S, Sertoli cells. Immunostaining for TIF1β (pink and red signals) with DAPI counterstain (blue signal). Scale bar: 100 μm in A,B; 50 μm in C-F.

TIF1β in pachytene spermatocytes and/or round spermatids (Fig. 3E,F).

In the mouse, proceeding from spermatogonia to spermatozoa takes about 35 days (Oakberg, 1956a; Oakberg, 1956b). Therefore, the presence of TIF1β-negative tubules in testes analyzed more than 40 days after tamoxifen treatment demonstrates that Cre-mediated DNA excision can occur in stem spermatogonia, in accordance with our previous observations (P. W., C. G., M. M., D. M. and P. C., unpublished). It is clear, however, that not all stem spermatogonia underwent excision, as indicated by the reappearance of TIF1β-positive spermatocytes 2 weeks after tamoxifen treatment (Fig. 3C,E,F and data not shown). Moreover, in experimental testes analyzed 2 weeks after tamoxifen treatment about half of the tubules (53.17±11.82%) showed an abnormal TIF1β expression in the spermatocyte layers (e.g. Fig. 3E,F and data not shown), instead of expression in tubules (0.76±0.16%) of similarly treated control mice ($P<0.0001$) (Fig. 4). Eight weeks after tamoxifen treatment, about half of the seminiferous tubules in experimental testes (52.92±3.19%) exhibited histological signs of degeneration (i.e. vacuolation of the seminiferous epithelium (V in Fig. 5E,F) instead of only 2.70±0.68% in similarly treated control mice ($P<0.0001$) (Fig. 4). The similarity in the percentages of tubules exhibiting immunohistochemical and histo-pathological abnormalities strongly suggests that Cre-mediated DNA excision in spermatogonia actually had occurred only in approximately half of the seminiferous tubules. Importantly, upon excision in germ cells, immunostaining of Sertoli cell nuclei remained very strong on frozen sections and was also unaltered on paraffin wax-embedded sections (Fig. 3B,D, Fig. 5G,H and data not shown). Moreover, tamoxifen treatment did not affect the expression of TIF1β in other organs of $TIF1\beta^{(L2/L2)}$:PrP-Cre-$ER^{T(tg/0)}$ mice (data not shown).

Cre-mediated disruption of *Tif1β* in spermatogonia causes testicular degeneration

Histological analysis of experimental and control testes was performed 1 day, and 2, 4, 6, 7 and 8 weeks after tamoxifen treatment. Experimental testes were histologically normal up to 6 weeks after treatment (data not shown). Histological sections through testes of experimental mice 7 and 8 weeks after treatment showed a mosaic of seminiferous tubules

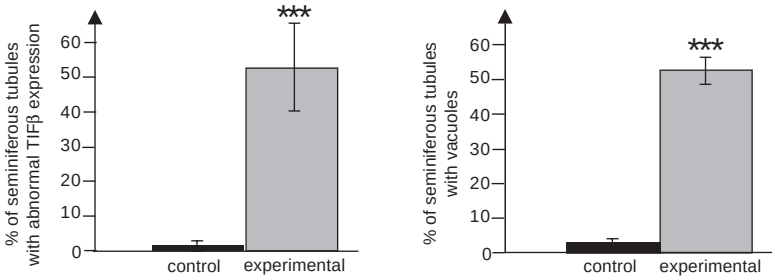

Fig. 4. Percentages of seminiferous tubules exhibiting an abnormal expression pattern of TIF1β or displaying vacuoles in $TIF1\beta^{L2/L2}$:PrP-Cre-$ER^{T(0/0)}$ (control) and $TIF1\beta^{L2/L2}$:PrP-Cre-$ER^{T(tg/0)}$ (experimental) mice. The percentage of seminiferous tubules cross sections showing abnormal expression of TIF1β (i.e. partial staining of spermatocyte and/or spermatid layers along the circumference of a given tubule, instead of staining along its whole circumference) was determined by immunohistochemistry 2 weeks after tamoxifen treatment (left). The percentage of degenerating (vacuolated) versus normal tubules was determined 8 weeks after tamoxifen treatment (right). Results are mean±s.e.m. from at least three animals of each genotype and age (***$P<0.0001$ according to Fischer's PLSD).

displaying normal germ cell associations (NT in Fig. 5B and data not shown) and of morphologically abnormal tubules exhibiting different patterns and extents of germ cell loss (e.g. ST in Fig. 5B). Interestingly, all abnormal tubular cross-sections were devoid of TIF1β expression in germ cells (Fig. 5G,H). Some of the abnormal tubules contained apparently normal populations of spermatogonia and spermatocytes but lacked spermatids, probably reflecting their premature detachment from the seminiferous epithelium. By contrast, other tubules contained healthy round spermatids and Sertoli cells without mitotic and meiotic germ cells (compare Fig. 5C with 5D, and 5E with 5G). In the third category of abnormal tubules, germ cell depletion yielded Sertoli cell-only seminiferous tubules (Fig. 5B,F,H) whose epithelium was reduced to a row of Sertoli cells (showing extensive intercellular vacuolation; V in Fig. 5F) and to a few elongated spermatids (ES, Fig. 5F,H), but was devoid of spermatogonia. TUNEL analysis did not reveal any increase in apoptotic cell death in the epithelium of the degenerating tubules (Fig. 6A-D). The abnormal DNA fragmentation detected in nuclei from mature elongated spermatids (Fig. 6B,D), probably reflects their phagocytosis and degradation by Sertoli cells (Kastner et al., 1996). Germ cell depletion resulted in part from sloughing off of apparently healthy immature germ cells (mainly round spermatids) into the lumen of the tubules (arrowhead in Fig. 5E), probably yielding large and irregular vacuoles in the epithelium (V, in Fig. 5E,F). In accordance with this observation, the number of round spermatids was markedly increased in the lumen of the epididymis from experimental males examined 7 and 8 weeks after tamoxifen treatment (arrowheads in Fig. 6E,F). No sign of testicular degeneration was observed in age-matched tamoxifen-treated control mice (Fig. 5A,C).

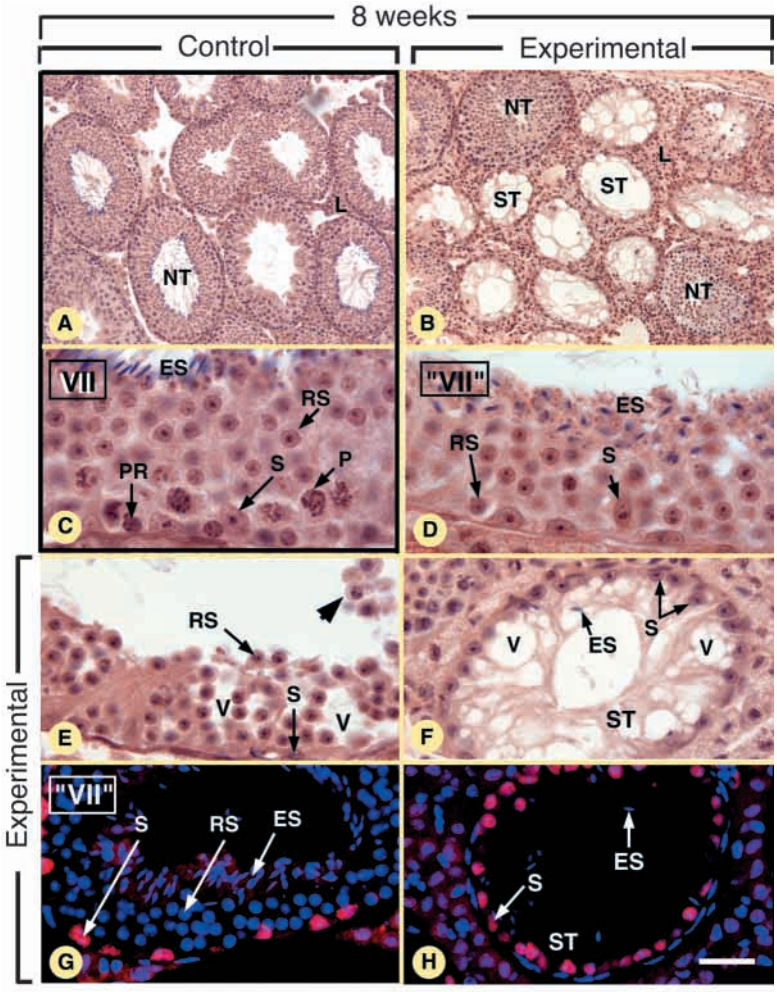

Fig. 5. Histological aspect of TIF1β($^{L2/L2}$):PrP-Cre-ER$^{T(0/0)}$ control testis (A,C) and TIF1β$^{L2/L2}$:PrP-Cre-ER$^{T(tg/0)}$ experimental testis (B,D-H), 8 weeks after tamoxifen treatment. The seminiferous tubule in C is at stage VII. The abnormal seminiferous tubules in D,G are difficult to stage because of the complete absence of spermatocyte populations; however, they have features in common with a stage VII tubule ('VII'), namely the aspect of acrosomes in round spermatids (not visible at this magnification) and/or alignment of elongated spermatids. Groat Hematoxylin and Malloy's trichrome (A-F) or immunolabeling for TIF1β and DAPI counterstaining (G,H). L, Leydig cells; NT, tubules showing normal germ cell associations; P, pachytene spermatocytes; PR, preleptotene spermatocytes; RS, round spermatids; S, Sertoli cells; ST, Sertoli cell-only seminiferous tubules. The arrowhead in E indicates exfoliated round spermatids. Scale bar: 100 μm (A,B), 15 μm (C,D,F) and 20 μm (E,G,H).

DISCUSSION

Spermatogenesis includes spermatogonial stem cell self-renewal and the differentiation of spermatogonia. Proliferative spermatogonia generate differentiating spermatogonia that are irreversibly committed towards the production of spermatozoa. The most mature spermatogonia divide to form preleptotene spermatocytes that replicate their DNA, then initiate the meiotic process leading to round spermatids. Round spermatids undergo spermiogenesis, which involves nuclear shaping and chromatin compaction as well as major cytoplasmic transformations, to yield mature spermatozoa (Brinster and Avarbock, 1994; Brinster and Zimmermann, 1994; Kistler et al., 1996). In mice, the entire developmental process takes 35 days and is regulated by a complex signaling network involving endocrine, paracrine and autocrine factors (Grootegoed et al., 2000), as well as multiple transcriptional regulators (Sassone-Corsi, 1997).

We have shown here that the TIF1β protein is expressed in germ cells during a restricted window of time corresponding to the maturation of mid-pachytene spermatocytes into elongating (i.e. step 10) spermatids, and is associated with heterochromatin structures in these cells. TIF1β is localized preferentially with the chromocenter of round (i.e. step 1 to step 9) spermatids indicating that it might repress expression of specific genes by sequestering them in this subnuclear compartment (Francastel et al., 2000). However, as TIF1β expression is turned off prior to the onset of spermatid condensation, it is unlikely to be necessary for the protamine-dependent DNA compaction process which is characteristic of spermiogenesis (Kistler et al., 1996; Sassone-Corsi, 1997; Baarends et al., 1999). Moreover, although it is expressed in

pachytene spermatocytes, TIF1β may also be dispensable for chromatin remodeling processes during meiosis, as TIF1β-deficient spermatocytes can generate morphologically normal TIF1β-deficient round spermatids, which in turn yield terminally differentiated condensed spermatids. Whether this reflects a possible functional redundancy with other members of the TIF1 family remains to be determined. Indeed, based on biochemical data, both TIF1α and TIF1β have previously been shown to interact directly with HP1 proteins (Le Douarin et al., 1996). However, in the case of TIF1α, the biological significance of the HP1 interaction is unclear, as TIF1α does not require HP1 binding for repression in a transfection assay (Nielsen et al., 1999), and no significant subnuclear colocalization of TIF1α and HP1α has been observed in cultured cells (Remboutsika et al., 1999). Moreover, TIF1β, but neither TIF1α nor TIF1γ, has been reported to interact with and act as a co-repressor for KRAB domains (Abrink et al., 2001), supporting the view that members of the TIF1 family may be functionally distinct.

TIF1β has nevertheless important physiological functions in the maintenance of the structural integrity of the seminiferous epithelium, as its loss in spermatocytes and round spermatids results in testicular degeneration with complete disappearance of germ cells. Spermatogenesis is crucially dependent on intimate contacts and paracrine interactions between Sertoli cells and germ cells. Sertoli cells support and nurture the germ cells (Russell et al., 1990; Sharpe, 1993; Griswold, 1998). Spermatocytes and spermatids can, in turn, influence Sertoli cell functions and gene expression, as demonstrated in models of germ cell depletion in vivo and in co-cultures of Sertoli and germ cells (Jegou, 1993; Boujrad et al., 1995; Syed and Hecht, 1997; Wright et al., 1995; Griswold, 1995; Yomogida et al., 1994). The disappearance of TIF1β in $TIF1\beta^{L2/L2}$:PrP-Cre-$ER^{T(tg/0)}$ spermatocytes after tamoxifen treatment and the subsequent generation, within the next 10 days (Oakberg, 1956a), of TIF1β-deficient round spermatids from these TIF1β-less spermatocytes, indicates that TIF1β is not cell-autonomously required for their survival and differentiation. Thus, the timing of degeneration of the TIF1β-less seminiferous epithelium rather suggests that *Tif1β* expression is required for short-range cellular interactions in this epithelium. For example, TIF1β present in round spermatids could, indirectly, regulate the expression of a Sertoli cell-derived factor(s) that mediate cell adhesion; the generation of TIF1β-deficient round spermatids, as a consequence of *Tif1β* disruption, may progressively result in depletion of this Sertoli cell factor, leading to immature germ cells detachment. However, neither shedding of immature germ cells, nor germ cell apoptosis can account for the observed selective depletion in spermatogonia and spermatocytes in TIF1β-deficient degenerating seminiferous tubules still containing spermatids. Rather, this 'window of missing germ cells' may reflect an absence of spermatogonial proliferation or a failure of self-renewing stem spermatogonia to maintain their normal, undifferentiated, state. In any event, as TIF1β is not detectable in wild-type spermatogonia at any stage of the cycle of the seminiferous epithelium, the deleterious effects of its ablation on this cell type must be mediated through paracrine mechanisms possibly involving Sertoli cells. Accordingly, it is interesting to note that BMP8B secreted by spermatocytes and round spermatids is thought to play an important role in the paracrine regulation of spermatogonial self-renewal and/or differentiation (Zhao et al., 1996). Similar functions in the regulation of spermatogonial cell fate decision have been ascribed to Sertoli cell-derived GDNF (Meng et al., 2000), but it is not known whether secretion of GDNF is under germ cell control.

In conclusion, we have demonstrated that TIF1β is localized preferentially in heterochromatin of round spermatids, indicating that it might repress expression of specific genes in these cells. Moreover, the observation that seminiferous tubules in which *Tif1β* is specifically disrupted in spermatogonia are initially normal, while they subsequently

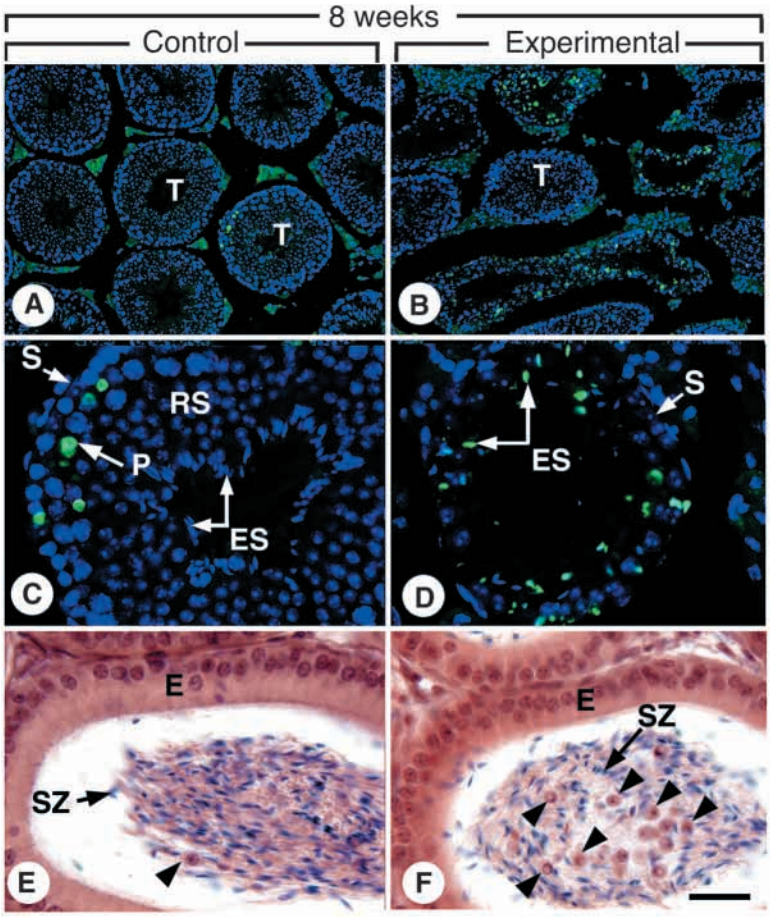

Fig. 6. Tunnel labeling (A-D) of the testis and histological detection (E,F) of immature germ cells in the epididymis of TIF1βL2/L2:PrP-Cre-ERT(0/0) control and TIF1βL2/L2:PrP-Cre-ERT(tg/0) experimental males 8 weeks after tamoxifen treatment. The green fluorescent signal corresponds to nuclei containing DNA fragments. Arrowheads indicate exfoliated round spermatids. E, epithelium of the cranial portion of the epididymis; ES, elongated spermatids; P, pachytene spermatocytes; RS, round spermatids; S, Sertoli cells; SZ, spermatozoa; T, seminiferous tubules. Scale bar: 100 μm in A,B; 20 μm in C-F.

degenerate, indicates that TIF1β has important functions in the homeostasis of the seminiferous epithelium, and probably plays a crucial role in the network of paracrine interactions between germ cell subpopulations and/or Sertoli cells.

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REFERENCES

Abrink, M., Ortiz, J. A., Mark, C., Sanchez, C., Looman, C., Hellman, L., Chambon, P. and Losson, R. (2001). Conserved interaction between distinct Krüppel-associated box domains and the transcriptional intermediary factor 1 β. *Proc. Natl. Acad. Sci. USA* **98**, 1422-1426.

Baarends, W. M., Hoogerbrugge, J. W., Roest, H. P., Ooms, M., Vreeburg, J., Hoeijmakers, J. H. and Grootegoed, J. A. (1999). Histone ubiquitination and chromatin remodeling in mouse spermatogenesis. *Dev. Biol.* **207**, 322-333.

Balaton, A. J., Ochando, F. and Painchaud, M. H. (1993). Use of microwaves for enhancing or restoring antigens before immunohistochemical staining. *Ann. Pathol.* **13**, 188-189.

Barcellona, M. L. and Gratton, E. (1990). The fluorescence properties of a DNA probe. 4'-6-Diamidino-2-phenylindole (DAPI). *Eur. Biophys. J.* **17**, 315-323.

Beckstead, R., Ortiz, J. A., Sanchez, C., Prokopenko, S. N., Chambon, P., Losson, R. and Bellen, H. (2001). Bonus, a Drosophila homolog of TIF1 proteins, interacts with nuclear receptors and can inhibit βFTZ-F1-dependent transcription. *Mol. Cell* **7**, 753-765.

Boujrad, N., Hochereau-de Reviers, M. T. and Carreau, S. (1995). Evidence for germ cell control of Sertoli cell function in three models of germ cell depletion in adult rat. *Biol. Reprod.* **53**, 1345-1352.

Brinster, R. L. and Avarbock, M. R. (1994). Germline transmission of donor haplotype following spermatogonial transplantation. *Proc. Natl. Acad. Sci. USA* **91**, 11303-11307.

Brinster, R. L. and Zimmermann, J. W. (1994). Spermatogenesis following male germ-cell transplantation. *Proc. Natl. Acad. Sci. USA* **91**, 11293-11302.

Cammas, F., Mark, M., Dollé, P., Dierich, A., Chambon, P. and Losson, R. (2000). Mice lacking the transcriptional corepressor TIF1β are defective in early postimplantation development. *Development* **127**, 2955-2963.

Cockell, M. and Gasser, S. (1999). Nuclear compartments and gene regulation. *Curr. Opin. Genet. Dev.* **9**, 199-205.

Dupé, V., Davenne, M., Brocard, J., Dollé, P., Mark, M., Dierich, A., Chambon, P. and Rijli, F. M. (1997). In vivo functional analysis of the Hoxa-1 3′ retinoic acid response element (3′RARE). *Development* **124**, 399-410.

Eissenberg, J. C. and Elgin, S. C. R. (2000). The HP1 protein family: getting a grip on chromatin. *Curr. Opin. Genet. Dev.* **10**, 204-210.

Feil, R., Brocard, J., Mascrez, B., LeMeur, M., Metzger, D. and Chambon, P. (1996). Ligand-activated site-specific recombination in mice. *Proc. Natl. Acad. Sci. USA* **93**, 10887-10890.

Francastel, C., Schubeler, D., Martin, D. I. and Groudine, M. (2000). Nuclear compartmentalization and gene activity. *Nat. Rev. Mol. Cell Biol.* **1**, 137-143.

Friedman, J. R., Fredericks, W. J., Jensen, D. E., Speicher, D. W., Huang, X.-P., Neilson, E. G. and Rauscher, F. J., III (1996). KAP-1, a novel corepressor for the highly conserved KRAB repression domain. *Genes Dev.* **10**, 2067-2078.

Griswold, M. D. (1995). Interactions between germ cells and Sertoli cells in the testis. *Biol. Reprod.* **52**, 211-216.

Griswold, M. D. (1998). The central role of Sertoli cells in spermatogenesis. *Semin. Cell Dev. Biol.* **9**, 411-416.

Grootegoed, J. A., Siep, M. and Baarends, W. M. (2000). Molecular and cellular mechanisms in spermatogenesis. *Bailliere's Clin. Endocrinol. Metab.* **14**, 331-343.

Hampsey, M. and Reinberg, D. (1999). RNA polymerase II as a control panel for multiple coactivator complexes. *Curr. Opin. Genet. Dev.* **2**, 132-139.

Hassan, A. H., Neely, K. E., Vignali, M., Reese, J. C. and Workman, J. L. (2001). Promoter targeting of chromatin-modifying complexes. *Front. Biosci.* **6**, 1054-1064.

Hoyer-Fender, S., Singh, P. B. and Motzkus, D. (2000). The murine heterochromatin protein M31 is associated with the chromocenter in round spermatids and is a component of mature spermatozoa. *Exp. Cell Res.* **254**, 72-79.

Jégou, B. (1993). The Sertoli-germ cell communication network in mammals. *Int. Rev. Cytol.* **147**, 25-96.

Kastner, P., Mark, M., Leid, M., Gansmuller, A., Chin, W., Grondona, J. M., Decimo, D., Krezel, W., Dierich, A. and Chambon, P. (1996). Abnormal spermatogenesis in RXR beta mutant mice. *Genes Dev.* **10**, 80-92.

Kim, S.-S., Chen, Y.-M., O'Leary, E., Witzgall, R., Vidal, M. and Bonventre, J. V. (1996). A novel member of the RING finger family, KRIP-1, associates with the KRAB-A transcriptional repressor domain of zinc finger proteins. *Proc. Natl. Acad. Sci. USA* **93**, 15299-15304.

Kistler, W. S., Henriksen, K., Mali, P. and Parvinen, M. (1996). Sequential expression of nucleoproteins during rat spermiogenesis. *Exp. Cell Res.* **225**, 374-381.

Le Douarin, B., Zechel, C., Garnier, J.-M., Lutz, Y., Tora, L., Pierrat, B., Heery, D., Gronemeyer, H., Chambon, P. and Losson, R. (1995). The N-terminal part of TIF1, a putative mediator of the ligand-dependent activation function (AF-2) of nuclear receptors, is fused to B-raf in the oncogenic protein T18. *EMBO J.* **14**, 2020-2033.

Le Douarin, B., Nielsen, A. L., Garnier, J.-M., Ichinose, H., Jeanmougin, F., Losson, R. and Chambon, P. (1996). A possible involvement of TIF1α and TIF1β in the epigenetic control of transcription by nuclear receptors. *EMBO J.* **15**, 6701-6715.

Mark, M., Lufkin, T., Vonesch, J. L., Ruberte, E., Olivo, J. C., Dollé, P., Gorry, P., Lumsden, A. and Chambon, P. (1993). Two rhombomeres are altered in Hoxa-1 mutant mice. *Development* **119**, 319-338.

Matsuda, E., Agata, Y., Sugai, M., Katakai, T., Gonda, H. and Shimizu, A. (2001). Targeting of Krüppel-associated box-containing zinc finger proteins to centromeric heterochromatin. Implication for the gene silencing mechanisms. *J. Biol. Chem.* **276**, 14222-14229.

Meng, X., Lindahl, M., Hyvönen, M. E., Parvinen, M., de Rooij, D. G., Hess, M. W., Raatikainen-Ahokas, A., Sainio, K., Rauvala, H., Lakso, M. et al. (2000). Regulation of cell fate decision of undifferentiated spermatogonia by GDNF. *Science* **287**, 1489-1493.

Metzger, D. and Chambon, P. (2001). Site- and time-specific gene targeting in the mouse. *Methods* **24**, 71-80.

Moosmann, P., Georgiev, O., Le Douarin, B., Bourquin, J.-P. and Schaffner, W. (1996). Transcriptional repression by RING finger protein TIF1β that interacts with the KRAB repression domain of KOX1. *Nucleic Acids Res.* **24**, 4859-4867.

Muller, C. and Leutz, A. (2001). Chromatin remodeling in development and differentiation. *Curr. Opin. Genet. Dev.* **11**, 167-174.

Nielsen, A. L., Ortiz, J. A., You, J., Oula-Abdelghani, M., Khechumian, R., Gansmuller, A., Chambon, P. and Losson, R. (1999). Interaction with members of the heterochromatin protein 1 (HP1) family and histone deacetylation are differentially involved in transcriptional silencing by members of the TIF1 family. *EMBO J.* **18**, 6385-6395.

Oakberg, E. F. (1956a). Duration of spermatogenesis in the mouse and timing of stages of the cycle of the seminiferous epithelium. *Am. J. Anat.* **99**, 507-516.

Oakberg, E. F. (1956b). A description of spermiogenesis in the mouse and its use in analysis of the cycle of the seminiferous epithelium and germ cell renewal. *Am. J. Anat.* **99**, 391-414.

Oulad-Abdelghani, M., Bouillet, P., Chazaud, C., Dollé, P. and Chambon, P. (1996). AP-2.2: a novel AP-2-related transcription factor induced by retinoic acid during differentiation of P19 embryonal carcinoma cells. *Exp. Cell Res.* **225**, 338-347.

Pardue, M. L. and Gall, J. G. (1970). Chromosomal localization of mouse satellite DNA. *Science* **168**, 1356-1358.

Rachez, C. and Freedman, L. P. (2001). Mediator complexes and transcription. *Curr. Opin. Cell Biol.* **13**, 274-280.

Remboutsika, E., Lutz, Y., Gansmuller, A., Vonesch, J. L., Losson, R. and Chambon, P. (1999). The putative nuclear receptor mediator TIF1α is tightly associated with euchromatin. *J. Cell Sci.* **112**, 1671-1683.

Russell, L. D., Hikim, A. P. S., Ettlin, R. A. and Clegg, E. D. (1990). *Histological and Histopathological Evaluation of the Testis*. Clearwater: Cache River Press.

Ryan, R. F., Schultz, D. C., Ayyanathan, K., Singh, P. B., Friedman, J. R., Fredericks, W. J. and Rauscher III, F. J. (1999). KAP-1 corepressor protein interacts and colocalizes with heterochromatic and euchromatic HP1 proteins: a potential role for Krüppel-associated box-zinc finger proteins in heterochromatin-mediated gene silencing. *Mol. Cell. Biol.* **19**, 4366-4378.

Sassone-Corsi, P. (1997). Transcriptional checkpoints determining the fate of male germ cells. *Cell* **88**, 163-166.

Schultz, D. C., Friedman, J. R. and Rauscher, F. J., III (2001). Targeting histone deacetylase complexes via KRAB-zinc finger proteins: the PHD and bromodomains of KAP-1 form a cooperative unit that recruits a novel isoform of the Mi-2α subunit of NuRD. *Genes Dev.* **15**, 428-443.

Sharpe R. (1993). Experimental evidence for Sertoli-germ cell and Sertoli-Leydig cell interactions. In *The Sertoli Cell* (ed. Russell and Griswold), pp. 391-418. Clearwater: Cache River Press.

Syed, V. and Hecht, N. B. (1997). Up-regulation and down-regulation of genes expressed in cocultures of rat Sertoli cells and germ cells. *Mol. Reprod. Dev.* **47**, 380-389.

Underhill, C., Qutob, M. S., Yee, S.-P. and Torchia, J. (2000). A novel nuclear receptor corepressor complex, N-CoR, contains components of the mammalian SWI/SNF complex and the corepressor KAP-1. *J. Biol. Chem.* **275**, 40463-40470.

Venturini, L., You, J., Stadler, M., Galien, R., Lallemand, V., Koken, M. H. M., Mattei, M. G., Ganser, A., Chambon, P., Losson, R. and de Thé, H. (1999). TIF1γ, a novel member of the transcriptional intermediary factor 1 family. *Oncogene* **18**, 1209-1217.

Wright, W. W., Zabludoff, S. D., Penttilä, T. L and Parvinen, M. (1995). Germ cell-Sertoli cell interactions: regulation by germ cells of the stage-specific expression of CP-2/cathepsin L mRNA by Sertoli cells. *Dev. Genet.* **16**, 104-113.

Yomogida, K., Ohtani, H., Harigae, H., Ito, E., Nishimune, Y., Engel, J. D. and Yamamoto, M. (1994). Developmental stage- and spermatogenic cycle-specific expression of transcription factor GATA-1 in mouse Sertoli cells. *Development* **120**, 1759-1766.

Zhao, G. Q., Deng, K., Labosky, P. A., Liaw, L. and Hogan, B. L. M. (1996). The gene encoding bone morphogenetic protein 8B is required for the initiation and maintenance of spermatogenesis in the mouse. *Genes Dev.* **10**, 1657-1669.