SPOC1 (PHF13) is required for spermatogonial stem cell differentiation and sustained spermatogenesis

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Summary
SPOC1 (PHF13) is a recently identified protein that has been shown to dynamically associate with somatic chromatin, to modulate chromatin compaction and to be important for proper cell division. We report on the expression of SPOC1 in promyelocytic leukaemia zinc finger (PLZF)-positive undifferentiated spermatogonial stem cells (SSCs) of the mouse testis. To investigate further the biological function of SPOC1 in germ cells we generated Spoc1 mutant mice from a gene-trap embryonic stem cell clone. Postpubertal homozygous Spoc1−/− animals displayed a pronounced progressive loss of germ cells from an initially normal germ epithelium of the testis tubules leading to testis hypoplasia. This loss first affected non-SSC stages of germ cells and then, at a later time point, the undifferentiated spermatogonia. Remarkably, successive loss of all germ cells (at >20 weeks of age) was preceded by a transient increase in the number of undifferentiated A aligned (As) spermatogonia in younger mice (at >10 weeks of age). The number of primary Spoc1−/− gonocytes, the proliferation of germ cells, and the initiation and progression of meiosis was normal, but we noted a significantly elevated level of apoptosis in the Spoc1−/− testsis. Taken together, the data argue that SPOC1 is indispensable for stem cell differentiation in the testis and for sustained spermatogenesis.

Key words: Spermatogonial stem cell, Differentiation, SPOC1 (PHF13), Spermatogenesis

Introduction
Stem cells are fundamental for the regenerative capacity of organ systems (Dick, 2008). They are characterized by having the potential to self-renewal and to differentiate. The mechanisms regulating the balance between these processes remain poorly understood. An impressive example for the capacity of self-renewal and differentiation of stem cells comes from spermatogenesis, where millions of spermatogonia are produced each day in the postpubertal male. Thus, spermatogenesis is one of the most efficient cell-producing systems in the adult mammalian organism (Sharpe et al., 2003), making it an excellent model system to elucidate the general mechanisms of stem cell renewal and differentiation.

It is suggested that, in non-primate mammals, spermatogonial stem cells (SSCs) are single cells (Asingle, Aa) attached to the basement membrane of the seminiferous tubules. Depending on whether the cells undergo self-renewal or differentiation, the Aa spermatogonia either divide into two separate (Aa) cells or into a pair of spermatogonia (Apair, Aa), respectively. The Aa enters additional rounds of cell divisions after which the spermatogonia form chains and are called Aaligned (As) spermatogonia. Aa and Aa spermatogonia are still considered undifferentiated (Nakagawa et al., 2007). The Aa cells then further differentiate into the A1–A4 spermatogonia, intermediate and B spermatogonia, which undergo a final round of replication and enter the first meiotic prophase, resulting in primary spermatocytes. After completion of first meiotic prophase, a reductional division (meiosis I) and a subsequent mitosis-like division (meiosis II) lead to the formation of haploid spermatids that develop into spermatozoa (Aponte et al., 2005; de Rooij, 2001; He et al., 2009; Olive and Cuzin, 2005; Russell et al., 1990). During differentiation, the germ cells migrate from the basal lamina into the adluminal compartment of the seminiferous epithelium by traversing the blood-testis barrier (BTB) formed by Sertoli cells. This process requires extensive interaction between Sertoli cells, as well as between Sertoli and germ cells. This tight coordination between germ cell movement and differentiation is required in order to avoid arrest of spermatogenesis and apoptosis during this passage (reviewed by Mruk and Cheng, 2004). The basal compartment provides a niche assuring a specialized microenvironment capable of generating the necessary balance between self-renewal and differentiation. This process is regulated by extrinsic niche stimuli secreted by the Sertoli cells, as well as by intrinsic gene expression in the SSCs. Although several extrinsic stimuli have been described (Chen et al., 2005; Hofmann, 2008; Oatley and Brinster, 2008) little is known about the intrinsic factors that regulate SSC self-renewal, proliferation and differentiation. In undifferentiated SSCs (A−,Aa), some core transcription factors are known (e.g. OCT4 and SOX2) that are of general importance for the maintenance of the pluripotency of stem cells (Dann et al.,...
results

the differentiation of the SSC pool. Thus, SPOC1 represents a new disturbed differentiation of SSCs, where SPOC1 is normally that this is not caused by defects in meiosis, but rather by a process of apoptosis of pachytene spermatocytes. We provide evidence by a gene-trap insertion.

We next performed quantitative real-time PCR (qRT-PCR) with a probe corresponding to the junction of exon 2 and 3 of Spoc1 cDNA. Accordingly, RNA was extracted from the testes of homozygous mice and their wild-type littermates at various ages (Fig. 1E). We observed a low expression of Spoc1 mRNA in homozygous animals (Fig. 1D), whereas heterozygous animals expressed Spoc1 at levels similar to those observed in wild-type animals. The residual Spoc1 transcripts are probably due to skipping of the gene-trap construct in some transcripts. Nevertheless, western blot analysis of testis tissue from homozygous mice demonstrated that, at the protein level, no detectable Spoc1 was observed at different ages (5, 10 and 20 weeks). By contrast, a strong signal for SPOC1 was observed in testes of homozygous mice and their wild-type littermates at various ages (Fig. 1E). The absence of detectable Spoc1 transcripts is consistent with the immunohistochemical studies described below (Fig. 2).

Results

Spoc1 gene disruption by a gene-trap insertion

Embryonic stem cells (ESCs) carrying a mutant Spoc1 (Phf13) locus were obtained from a library of ESC clones generated by random insertional mutagenesis. The Spoc1 locus in the selected ESC clone was disrupted by a gene-trap vector containing intronic sequences and a splice acceptor site of the en12 gene, as well as β-geo, a fusion of β-galactosidase and neomycin phosphotransferase II (BayGenomics, CA). The resulting insertional mutation leads to a fusion transcript containing sequences from gene-specific exons upstream of the insertion joined to the β-geo marker and terminated by a poly(A) site. As a result, expression of the trapped gene can be detected by lacZ staining and its sequence can easily be determined by 5′ rapid amplification of cDNA ends (5′ RACE). In the ESC clones Xb691 and Xa022, the 5′ RACE product contained sequences from exon 1 [nucleotides 303 to 354 of the Spoc1 (Phf13) cDNA (GenBank accession number NM_172705)] of the Spoc1 gene, indicating an insertion of the gene-trap vector into intron 1 (Fig. 1A). We further mapped the integration sites to between intron numbers +917 and +918 (Xb691) and +876 and +877 (Xa022) of intron 1 relative to the first base of intron 1. On the RNA level this insertion leads to the termination of Spoc1 gene transcripts after exon 1, which contains the translation start site.

Mutant mice were generated from both ESC clones by injection into blastocysts from C57BL/6 mice. Subsequent intercrosses of heterozygous animals generated a mixed genetic background. The phenotypes were identical for both mice strains and therefore the data are presented only for the mouse strain generated from ESC clone Xb691. For PCR genotyping (Fig. 1B) of mutant Spoc1, primers were generated to both the β-gal cassette and to intron 1, as shown in Fig. 1A. To determine the effect of the gene-trap insertion on splicing of Spoc1, RT-PCR was performed on RNA isolated from tail cuts of heterozygous and homozygous mice using primers corresponding to the β-galactosidase sequence of the gene-trap vector, as well as from exon 1 of Spoc1. Sequencing of the resulting RT-PCR products revealed splicing of exon 1 to the splice acceptor site of the gene-trap cassette and to intron 1, as shown in Fig. 1A. To confirm the efficiency of the gene-trap insertion, we performed quantitative PCR (qRT-PCR) with probes corresponding to the junction of exon 1 and 2 of Spoc1 (Fig. 1C). In the ESC clone Xa022, the 5′ untranslated region (5′-UTR) was disrupted by a gene-trap insertion containing sequences from gene-specific exons upstream of the insertion joined to the β-geo marker and terminated by a poly(A) site. As a result, expression of the trapped gene can be detected by lacZ staining and its sequence can easily be determined by 5′ rapid amplification of cDNA ends (5′ RACE). In the ESC clones Xb691 and Xa022, the 5′ RACE product contained sequences from exon 1 [nucleotides 303 to 354 of the Spoc1 (Phf13) cDNA (GenBank accession number NM_172705)] of the Spoc1 gene, indicating an insertion of the gene-trap vector into intron 1 (Fig. 1A). We further mapped the integration sites to between intron numbers +917 and +918 (Xb691) and +876 and +877 (Xa022) of intron 1 relative to the first base of intron 1. On the RNA level this insertion leads to the termination of Spoc1 gene transcripts after exon 1, which contains the translation start site.

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In order to prove the efficient knockdown of endogenous SPOC1 in vivo, several approaches were undertaken. First, northern blot analysis of testis tissue (mice at 18 weeks of age) was performed with a probe corresponding to the Spoc1 transcript ranging from 2 to 14% in the testes of homozygous mice and their wild-type littermates at various ages (Fig. 1E). We observed a low expression of Spoc1 mRNA in homozygous animals (Fig. 1D), whereas heterozygous animals showed an ~50% reduction of Spoc1 expression in comparison to wild-type animals. Nevertheless, western blot analysis of testis tissue from homozygous mice demonstrated that, at the protein level, no detectable SPOC1 was observed at different ages (5, 10 and 20 weeks). By contrast, a strong signal for SPOC1 was observed in testes of homozygous mice and their wild-type littermates at various ages (Fig. 1E). We observed a low expression of persisting wild-type Spoc1 transcript ranging from 2 to 14% in the homozygous mutant mice compared with wild-type tissue in the different animals. The residual Spoc1 transcripts are probably due to skipping of the gene-trap construct in some transcripts. Nevertheless, western blot analysis of testis tissue from homozygous mice demonstrated that, at the protein level, no detectable SPOC1 was observed at different ages (5, 10 and 20 weeks). By contrast, a strong signal for SPOC1 was observed in testes of homozygous mice and their wild-type littermates at various ages (Fig. 1E). We observed a low expression of persisting wild-type Spoc1 transcript ranging from 2 to 14% in the homozygous mutant mice compared with wild-type tissue in the different animals. The residual Spoc1 transcripts are probably due to skipping of the gene-trap construct in some transcripts. Nevertheless, western blot analysis of testis tissue from homozygous mice demonstrated that, at the protein level, no detectable SPOC1 was observed at different ages (5, 10 and 20 weeks). By contrast, a strong signal for SPOC1 was observed in testes of homozygous mice and their wild-type littermates at various ages (Fig. 1E). We observed a low expression of persisting wild-type Spoc1 transcript ranging from 2 to 14% in the homozygous mutant mice compared with wild-type tissue in the different animals. The residual Spoc1 transcripts are probably due to skipping of the gene-trap construct in some transcripts. Nevertheless, western blot analysis of testis tissue from homozygous mice demonstrated that, at the protein level, no detectable SPOC1 was observed at different ages (5, 10 and 20 weeks). By contrast, a strong signal for SPOC1 was observed in testes of homozygous mice and their wild-type littermates at various ages (Fig. 1E). We observed a low expression of persisting wild-type Spoc1 transcript ranging from 2 to 14% in the homozygous mutant mice compared with wild-type tissue in the different animals. The residual Spoc1 transcripts are probably due to skipping of the gene-trap construct in some transcripts. Nevertheless, western blot analysis of testis tissue from homozygous mice demonstrated that, at the protein level, no detectable SPOC1 was observed at different ages (5, 10 and 20 weeks). By contrast, a strong signal for SPOC1 was observed in testes of homozygous mice and their wild-type littermates at various ages (Fig. 1E). We observed a low expression of persisting wild-type Spoc1 transcript ranging from 2 to 14% in the homozygous mutant mice compared with wild-type tissue in the different animals. The residual Spoc1 transcripts are probably due to skipping of the gene-trap construct in some transcripts. Nevertheless, western blot analysis of testis tissue from homozygous mice demonstrated that, at the protein level, no detectable SPOC1 was observed at different ages (5, 10 and 20 weeks). By contrast, a strong signal for SPOC1 was observed in testes of homozygous mice and their wild-type littermates at various ages (Fig. 1E). We observed a low expression of persisting wild-type Spoc1 transcript ranging from 2 to 14% in the homozygous mutant mice compared with wild-type tissue in the different animals. The residual Spoc1 transcripts are probably due to skipping of the gene-trap construct in some transcripts.
SPOC1 is coexpressed with PLZF in undifferentiated spermatogonia (A–Aal)

SPOC1 expression and localization was investigated by X-gal staining of Spoc1+/− testis, which expresses β-galactosidase under the control of the Spoc1 promoter (Fig. 2A). X-gal staining showed a granular signal in a few peritubular basal cells in most tubules, consistent with the position of spermatogonia (Buaas et al., 2004; Costoya et al., 2004; Dadoune, 2007). A similar spermatogonia-specific pattern was observed when testes showed a granular signal in a few peritubular basal cells in most tubules, consistent with the position of spermatogonia (Buaas et al., 2004; Costoya et al., 2004; Dadoune, 2007). A similar spermatogonia-specific pattern was observed when testes stained with the monoclonal rat anti-SPOC1 antibody (Fig. 2B). Spoc1−/− gonocytes were PLZF positive (staining not shown) but were negative for SPOC1 (Fig. 2C), demonstrating the specificity of the antibody, as well as the knockdown of SPOC1, in the mouse model. The higher number of β-galactosidase-positive cells in comparison with cells stained with anti-SPOC1 antibody might be due to a greater sensitivity of the enzyme-linked β-galactosidase staining, to post-transcriptional degradation of Spoc1 in some Spoc1-RNA-containing cells or to diffusion of X-gal, and remains to be investigated.

To demonstrate further that SPOC1 is expressed in early spermatogonia, we evaluated immunohistochemically the coexpression of SPOC1 with PLZF. PLZF is a marker of undifferentiated A-type spermatogonia (As–Aal) and is a known intrinsic factor involved in SSC self-renewal (Buaas et al., 2004; Costoya et al., 2004; Payne and Braun, 2006). We observed the expression of both proteins in the nuclei of gonocytes, the progenitors of SSCs, in testis 3 days post partum (dpp) (Fig. 2C), as well as in spermatogonia of 10-week-old mice (Fig. 2D,E), demonstrating that SPOC1 is expressed in undifferentiated A-type spermatogonia. Quantitative analysis over a developmental window (postnatal day (P) P1 to 10 weeks) demonstrated that there was coexpression of PLZF in 77–100% of SPOC1-positive cells, whereas SPOC1 was found to be coexpressed in 97–100% of PLZF-positive spermatogonia (supplementary material Table S1 and Fig. S1). Furthermore, whole-mount immunofluorescence confirmed SPOC1 localization in seminiferous tubules of adult mice (12 weeks of age) and revealed that SPOC1 was expressed in undifferentiated A-type spermatogonia (As–Aal) and is a known intrinsic factor involved in SSC self-renewal (Buaas et al., 2004; Costoya et al., 2004; Payne and Braun, 2006). We observed the expression of both proteins in the nuclei of gonocytes, the progenitors of SSCs, in testis 3 days post partum (dpp) (Fig. 2C), as well as in spermatogonia of 10-week-old mice (Fig. 2D,E), demonstrating that SPOC1 is expressed in undifferentiated A-type spermatogonia. Quantitative analysis over a developmental window (postnatal day (P) P1 to 10 weeks) demonstrated that there was coexpression of PLZF in 77–100% of SPOC1-positive cells, whereas SPOC1 was found to be coexpressed in 97–100% of PLZF-positive spermatogonia (supplementary material Table S1 and Fig. S1). Furthermore, whole-mount immunofluorescence confirmed SPOC1 localization in seminiferous tubules of adult mice (12 weeks of age) and revealed that SPOC1 was expressed in single (Aa), paired (Apa) and aligned (Aal) spermatogonia (supplementary material Fig. S2). Taken together these results demonstrate that SPOC1 is expressed, in a manner similar to PLZF, in undifferentiated spermatogonial stem cells.

SPOC1−/− mice have smaller testes and a reduced number of germ cells

Spoc1−/− mice were indistinguishable from wild-type littermates on the basis of appearance and breeding capacity. Intercrosses of...
the heterozygous animals produced ~14% Spoc1<sup>−/−</sup> animals, which deviates from the expected Mendelian ratio of 25% (supplementary material Fig. S3). Preliminary observations indicate intrauterine death [at embryonic day (E) E13.5–E15.5] as well as peri- and early postnatal lethality, explaining the divergent Mendelian ratio.

Gross phenotypic examination of Spoc1<sup>−/−</sup> mice revealed that there was initially no visible abnormalities. However, homozygous males rapidly (at 5 weeks and older) developed a reproductive defect and required a substantially prolonged period of time to impregnate the females, whereas heterozygous animals bred normally (supplementary material Fig. S4). A more detailed examination of Spoc1<sup>−/−</sup> mice at different ages revealed a substantially reduced testis size (Fig. 3A). Already at 5 weeks of age the relative testis weight (testis weight to body weight ratio, GSI) was dramatically reduced (56%) compared with wild-type animals (Fig. 3B). Correspondingly, we found a substantial reduction in the cross-sectional area of seminiferous tubules in Spoc1<sup>−/−</sup> mice (Fig. 3C). Sperm number was decreased to 28.6% (5 weeks), 9.3% (10 weeks) and 10.2% (20 weeks) of the wild-type number, respectively. Histological investigation of testis from aged mice (59 weeks) revealed a complete germ cell loss in Spoc1<sup>−/−</sup> animals, whereas heterozygous (Spoc1<sup>+/−</sup>) and wild-type mice appeared normal (Fig. 3D). Together, these data indicate a vast breakdown of spermatogenesis, which explains the early infertility of the Spoc1<sup>−/−</sup> mice.

The observed phenotype is solely caused by the absence of Spoc1 and not by additional non-specific gene-trap integrations. This can be concluded from the following observations: (i) mice generated from two independent ESC clones (Xb691 and Xa022) show similar phenotypes; (ii) the β-galactosidase expression pattern in Spoc1<sup>+/−</sup> mice matches the expression of Spoc1, as determined by RNA in situ hybridization and immunohistochemical detection; and (iii) in the mixed genetic background we observed a 100% penetrance between the Spoc1<sup>−/−</sup> genotype and the observed phenotypic changes.

Abnormal spermatogenesis in Spoc1<sup>−/−</sup> mice

In contrast to Spoc1<sup>+/−</sup> testis, which appeared phenotypically normal and indistinguishable from those in wild-type mice even after >12 months (Fig. 3D), histological examination of Spoc1<sup>−/−</sup> testes revealed severe testis atrophy with profoundly...
altered spermatogenesis in a large number of tubules. Detailed histological investigation of Spoc1−/− testes from mice at 5, 10 and 20 weeks of age revealed an increase in the number of seminiferous tubules that showed a reduced number of germ cells with progressing age. In 5-week-old animals spermatogenesis appeared normal despite a reduced cross-sectional area of seminiferous tubules (Fig. 3C, Fig. 4A), whereas tubules from 10-week-old animals were disorganized (Fig. 4A). In 20-week-old animals the number of tubules with abnormal spermatogenic epithelium had further increased and the testis became completely atrophic. Unfortunately, inter-individual variation prevented an exact quantification of this phenotype. Nevertheless, tubules with a normal germinal epithelium were rarely present at this age. Remarkably, several tubules (per testis) predominantly contained Sertoli cells and a few spermatozoa but no developmentally younger germ cells, whereas other tubules contained Sertoli cells together with chains of Aal spermatogonia (Fig. 7B) and still other tubules (25% of tubules at 20 weeks of age) showed a Sertoli-cell-only phenotype (Fig. 4A,B and Fig. 5B). A gross quantification of these changes is depicted in supplementary material Fig. S5. The Spoc1−/− Sertoli cells were similar in number and size to those of wild type (Fig. 4) and expressed the Sertoli-specific protein GATA1 (Fig. 4C). When we determined the number of GATA1-positive cells per seminiferous tubule in sections from 10-week-old animals, there were no significant differences between wild-type and Spoc1−/− testes (data not shown).

In summary, our histological data demonstrate a normal onset of spermatogenesis in juvenile animals followed by a successive loss of spermatogonial cells from the germ cell epithelium after 5-weeks post partum. The loss of germ cells in early developmental stages, but the presence of later developmental stages of germ cells, as was observed in older animals, indicates an ongoing process of depletion of germ cells possible owing to a failure in stem cell renewal. Accumulation of Aal spermatogonia in the absence of more progressed germ cell stages might arise through a failure in their differentiation and/or their entry into meiosis.
Normal numbers of gonocytes and Sertoli cells are present in the gonadal anlage of Spoc1\(^{-/-}\) mice

To exclude that the phenotypes observed are simply caused by a reduced number of gonocytes before the formation of SSCs, histological evaluation of Spoc1\(^{-/-}\) and wild-type testes (3 dpp) was performed and these testes were stained for PLZF and SPOC1, respectively. These experiments demonstrated that Spoc1\(^{-/-}\) and wild-type animals displayed similar numbers of gonocytes (Fig. 2C), strongly arguing that the observed loss of germ cells in the postpubertal testis is a result of impaired SSC self-renewal and/or differentiation.

**Spoc1\(^{-/-}\) testes show increased apoptosis and normal proliferation of germ cells**

The observed reduction in testis size and seminiferous tubule cross-sectional area indicate either a reduced proliferation of germ cells or increased apoptosis. To address these possibilities we performed TUNEL assays and immunohistochemical stainings of apoptosis and proliferation markers. The TUNEL assays revealed a substantially increased overall rate of apoptosis in tubules from 5-week- and 20-week-old Spoc1\(^{-/-}\) animals (Fig. 5A–C), which was confirmed by staining of activated caspase 3 (supplementary material Fig. S6). Apoptosis was strongly increased in prophase I cells, especially in the pachytene stage at seminiferous epithelial stages IV to VII (Fig. 5D–G). Stage X tubuli served as internal positive controls for the assay because these tubuli always contain TUNEL-positive and TUNEL-negative cells (Voet et al., 2003). Next, testis sections were stained with antibodies against PCNA and Ki67, two widely used proliferation markers that are expressed in a subset of A-, In-, and B-spermatogonia, as well as in prophase spermatocytes (Costoya et al., 2004; Wrobel et al., 1996). PCNA staining for mice at 5, 10 and 20 weeks of age revealed a slightly reduced appearance in the mutant being significant only at 20 weeks (\(P<0.034\)) (supplementary material Fig. S7). By contrast, Ki67 staining revealed no significant difference (\(P>0.16\)) between Spoc1\(^{-/-}\) and Spoc1\(^{+/+}\) mice at 5, 10 and 20 weeks of age (supplementary material Fig. S8). At 1 week of age no significant difference in the number of PCNA- or Ki67-positive cells was apparent (data not shown). These results strongly indicate that the demise of spermatogenesis is driven by enhanced apoptosis in the knockout.

Arrest of spermatogenesis can be elicited by the absence of numerous factors, some of which are required for passage through prophase I (Cooke and Saunders, 2002; Scherthan, 2003). To determine whether Spoc1 deficiency causes defects in spermatocyte development, we studied the course of prophase I by following meiotic differentiation through markers for double-strand break (DSB) formation and repair (phosphorylated H2AX, known as γH2AX) (Mahadevaiah et al., 2001; Meyer-Ficca et al., 2005) and the homologous recombination repair protein MRE11 that is expressed at high levels in meiotic cells (Eije et al., 2000). Determination of testes tubule stages was performed as described previously (Russell et al., 1990).

Normal patterns of γH2AX formation and localization were detected in prophase I cells of testis sections from 10-week-old
Spoc1<sup>−/−</sup> mice, with strong γH2AX signals being present in leptotene and zygote nuclei and at the XY body of pachytene-diplotene spermatocytes (Fig. 6A,B). A physiological chromatin signal (Meyer-Ficca et al., 2005) was also seen in spermatids of Spoc1<sup>−/−</sup> and wild-type animals (Fig. 6A,B). Strong expression of the DNA DSB repair protein Mre11 was present in early meiotic cells and at the XY body of pachytene spermatocytes of knockout (Fig. 6C) and wild-type (not shown) testes, which is consistent with previous studies on wild-type mice (Eijpe et al., 2000).

Next, we investigated meiotic and mitotic cell division by immunostaining for phosphorylated histone H3 serine 10, which labels condensing mitotic and meiotic chromosomes (Cobb et al., 1999; Hendzel et al., 1997). Meiotic metaphase I and II cells were identified in stage XII tubules, pachytene cells in stage I–X, and ‘others’ represents cell death at the tubule periphery (i.e. spermatogonia or spermatocytes near the periphery). Usually this category includes cells with advanced apoptosis, complicating assessment of cell type. Cell death was more abundant in Spoc1<sup>−/−</sup> cells in pachytene and ‘other cells’ located at the tubule periphery. (E–G) Details of apoptosis in knockout tubules.

Fig. 5. Increased apoptosis in Spoc1<sup>−/−</sup> testis. (A,B) Apoptosis (TUNEL, green) in testis section of Spoc1<sup>−/−</sup> and wild-type mice at 20 weeks of age. Many Sertoli-cell-only tubules are visible (*). Nuclei are stained with DAPI (grey, colour-inverted from blue). Leydig cells between tubules display fixation-induced unspecific green autofluorescence. TUNEL-stained wild-type testis sections at the same age display only a few apoptotic cells. (C) Overall number of apoptotic cells per tubule in wild-type and knockout mice at 5, 10 and 20 weeks of age. (D) Frequency of apoptotic cells per Spoc1<sup>−/−</sup> and wild-type testis cross-section (20 weeks of age). Metaphase I cells were identified in stage XII tubules, pachytene cells in stage I–X, and ‘others’ represents cell death at the tubule periphery (i.e. spermatogonia or spermatocytes near the periphery). Usually this category includes cells with advanced apoptosis, complicating assessment of cell type. Cell death was more abundant in Spoc1<sup>−/−</sup> cells in pachytene and ‘other cells’ located at the tubule periphery. (E–G) Details of apoptosis in knockout tubules.
formation, was found to be normal by γH2AX and SYCP3 (Liebe et al., 2006) staining of spreads of wild-type and Spoc1−/− spermatocytes (Fig. 6D). Together, these findings suggest that DNA repair and chromosome pairing progress normally in Spoc1−/− mice.

The spatial distribution of PLZF-positive undifferentiated spermatogonia is significantly altered in Spoc1−/− mice

Because the histological data indicate a defect in SSC self-renewal or differentiation, we specifically investigated the distribution of undifferentiated spermatogonia over time in the Spoc1−/− testes. PLZF is a marker for undifferentiated spermatogonia (Aα, Aβ and Aβd) and is co-expressed with the stem cell markers OCT4 (also known as POU5F1) and GDNF family receptor z1 (GFRz1) (Buas et al., 2004; Costoya et al., 2004; Payne and Braun, 2006). Using a mouse anti-PLZF antibody, we examined testis sections from wild-type and Spoc1−/− mice at P0 (neonates) and 20 weeks of age. Tubules without PLZF-positive cells [measured according to Buas et al. (Buas et al., 2004)] and PLZF-positive cells per tubulus were counted, respectively. In adult testes, only strongly PLZF-expressing cells at the periphery of the tubules (thus representing Aα−Aβd spermatogonia) were scored (Fig. 7). The data obtained clearly demonstrated that the number of tubules with PLZF-positive cells was unchanged between neonatal (P0) Spoc1+/+ and Spoc1−/− mice, whereas there was a highly significant reduction (P<0.0001) in adult mice (Fig. 7A,C). Unexpectedly, the average number of PLZF-positive cells per tubule remained unchanged in both neonatal and adult testis (Fig. 7D). This result can be attributed to tubules exclusively present in Spoc1−/− testis that showed a strong increase in the number of PLZF-positive cells (>10) clustered in the periphery of a few mutant tubules (Fig. 7B). Quantification revealed 3.9% of tubules with >10 PLZF-positive cells in Spoc1−/− and 0.1% in wild-type testis in mice at 5 weeks of age. At 10 weeks of age, the values were even more divergent, with 6.7% in Spoc1−/− compared with 0.1% in wild-type testis. This accumulation of cells represents Aβd spermatogonia and can be found in tubules that in addition contain only Sertoli cells.

Because Spoc1−/− mice with a mixed 129 and C57BL/6 genetic background were used throughout most of the experiments, we sought to verify this unexpected result in a congenic gene-trap line in order to exclude genetic background effects. We therefore generated a congenic mouse strain by successive backcrossing of Spoc1−/− animals with C57BL/6 wild-type animals. Testes from knockout mice of generation N12 were investigated. The testis showed a comparable, but slightly more severe phenotype than in the mixed genetic background (data not shown). Again, PLZF-positive cells per tubule were counted in testis sections from mice at 5, 10 and 20 weeks of age (Fig. 8). The result clearly demonstrated an almost complete loss of PLZF-positive spermatogonia at 20 weeks of age in Spoc1−/− mice (Fig. 8C). Remarkably, this was preceded by an increase in the number of tubules with extended chains of spermatogonia and...
Thus, an elevated number of A\textsubscript{al} spermatogonia, which was already visible at 5 weeks of age (Fig. 8A,B). This result confirms our previous findings and demonstrates that although further differentiated germ cells are almost completely lost, undifferentiated spermatogonia still proliferate and accumulate, generating extended chains of A\textsubscript{al} spermatogonia. With progressive aging these cells also eventually disappear, leading to a Sertoli-cell-only phenotype. The results in the congenic strain strongly indicate a profound defect in differentiation (and not in proliferation or self-renewal) of the SSCs, leading to the observed transient accumulation of undifferentiated spermatogonia in mutant tubules of older mice.

**Discussion**

Here, we have shown that SPOC1 is essential for the maintenance of spermatogenesis in the postpubertal testis and is a prerequisite for sustained spermatogenesis and normal fertility in adult mice. Spoc1\textsuperscript{-/-} mice displayed a wild-type number of gonocytes and a normal first wave of spermatogenesis in juvenile animals, but a progressive loss of germ cells was initiated several weeks after puberty. This loss first affected non-SSC stages of germ cells and then, at a later time point, the undifferentiated spermatogonia, which finally resulted in a large number of Sertoli-cell-only seminiferous tubules and a nearly complete atrophy of the testes in animals older than 20 weeks. The testes tubules of younger Spoc1\textsuperscript{-/-} animals displayed an altered distribution of undifferentiated spermatogonia in tubules, with a transient increase in A\textsubscript{al} spermatogonia and a successive loss of all germ cells with increasing age. Furthermore, there were substantially more apoptotic germ cells overall, but specifically in the pachytene stage of Spoc1\textsuperscript{-/-} testis, whereas there was no significant difference in the number of PCNA- or Ki67-positive (proliferating) cells or the number of primary gonocytes. Immunostainings of molecular markers for initiation and progression of meiosis were also normal. These results indicate that the seminiferous epithelium gradually loses its ability to start and/or complete new waves of differentiation, whereas the mutant epithelium has a compromised capacity to support the transit of spermatocytes I through the pachytene stage.

Overall, our findings strongly indicate that SPOC1 represents a new intrinsic regulating factor in undifferentiated and PLZF-positive spermatogonia (A\textsubscript{al}-A\textsubscript{ol}) that is responsible for maintaining proper and sustained differentiation of cells from the self-renewing stem cell pool. This conclusion is based on several observations. First, the initial colonization of the seminiferous tubules by germ cells appears to be normal, given that the number of gonocytes after birth does not differ between the seminiferous tubules of Spoc1\textsuperscript{-/-} and wild-type animals. Second, morphological and histological analysis, as well as immunohistochemical staining indicates that the observed testis atrophy is not caused by a failure in supporting cells or in hormone status because Sertoli cell number and seminal vesicles were normal. Third, the biologically active SSCs first appear in male mice of ~3–4 dpp (McLean et al., 2003). It has been suggested that two different populations of gonocytes are present in the postnatal period. In the first postnatal week gonocytes directly develop into Kit-
species expresses and, therefore, differentiating spermatogonia. These cells are the starting point of a first round of spermatogenesis. They do not undergo self-renewal and are therefore rapidly depleted (de Rooij, 1998; de Rooij and Russell, 2000; Yoshida et al., 2007). By contrast, a second subpopulation of gonocytes develops into a pool of spermatogonia, which undergo self-renewal and thus provides a stem cell population for all subsequent rounds of spermatogenesis (de Rooij, 1998; de Rooij and Russell, 2000; Yoshida et al., 2007). The fact that the testes of 5-week-old Spoc1−/− mice show seminiferous tubules with almost unaffected spermatogenesis (although reduced in diameter) indicates that the first round of spermatogenesis is unaffected and the ensuing meiosis and spermiogenesis can proceed normally. Consequently, our data suggest a specific defect in the differentiation of spermatogonia descending from the second subpopulation of gonocytes, which is characterized by a self-renewing capability. Forth, we observed a consecutive loss of spermatogenic substages, with a loss of spermatocytes I preceding the loss of spermatids, and finally spermatozoa, eventually leading to Sertoli-cell-only tubules in animals >20 weeks of age. This sequential abrogation of spermatogenic stages, together with the transient increase in Aal spermatogonia and the subsequent loss of all undifferentiated spermatogonia, strongly suggests a failure of SSCs to transit to differentiating spermatogonia. Hyperproliferation of undifferentiated spermatogonia leading to a transient alignment of undifferentiated spermatogonia is not probable because PCNA- and Ki67-positive cells were not increased in Spoc1−/− testis.

It is tempting to speculate that the observed transient clustering of PLZF-positive spermatogonia along the periphery in some mutant tubules indicates an abrogated differentiation and migration of spermatagonia, given that both processes are tightly coupled (reviewed by Lie et al., 2009; Mruk and Cheng, 2004). Finally, we noted an increased apoptotic rate in germ cells in the pachytene stage during stages IV to VII of the seminiferous epithelial cycle and in spermatogonia. However, most of the spermatocytes managed to complete prophase I and spermatogenesis. This agrees with the normal expression and localization of Mre11 and γH2AX (prophase I recombination markers), phosphorylated histone H3 (a meiotic and somatic metaphase marker) and the presence of a normal XY-body and normal chromosome pairing during prophase I in the Spoc1−/− and wild-type mice. These data suggest that a pachyten (recombination) checkpoint at stage IV of the seminiferous cycle (Ashley et al., 2004; Barchi et al., 2005) is not responsible for the increased cell death of Spoc1−/− spermatocytes. Because, in mitotic cells, SPOC1 is important for prophase condensation and chromosome alignment in metaphase (Kinkley et al., 2009), and given that prophase I of meiotic cells is equivalent to the G2 phase of mitotic cells and involves chromosome condensation into pachytene, it seems probable that SPOC1 could also play a role in meiotic prophase I chromatin function during pachytene chromosome condensation. Hence, the absence of SPOC1 in the knockout might underlie the elevated levels of pachytene apoptosis. The surviving spermatocytes do not seem to have defects in haploid (spermatid) development because spermatids and spermatozoa are the last form to occur before Sertoli-cell-dependent spermiogenesis can proceed normally. Consequently, our data suggest a specific defect in the spermatid differentiation defect of SSCs, this might explain the fast demise of spermatogenesis in the knockout.

An alternative explanation for the observed phenotype would be an altered differentiation and migration process in mutant spermatogonia and carry over of alterations that cause cell death in some pachytene spermatocytes. It is known that even the slightest deregulation affecting either the ability of the germ cell to traverse through the BTB or its migration through the adluminal compartment can cause arrest of spermatogenesis leading to germ cell degeneration and apoptosis (reviewed by Lie et al., 2009; Mruk and Cheng, 2004). Such a scenario in the Spoc1−/− testes could feasibly result in a deregulation of the differentiation processes responsible for proper spermatogenesis.

![Figure 8. PLZF-positive cells in testis from wild-type and Spoc1−/− mice with a congenic C57BL/6 background. Number of tubules without PLZF-positive cells from 5- (A), 10- (B) and 20-week-old (C) animals (wild-type, grey; Spoc1−/−, black). In total, 697 (+/+) and 552 (−/−) for A; 774 (+/+) and 912 (−/−) for B; and as 758 (+/+) and 933 (−/−) for C tubules were scored, respectively.](image)
and thus trigger a subsequent apoptosis during or right after traverse through the BTB or migration through the adluminal compartment. Of note, this process would also not affect the first wave of spermatogenesis because the BTB forms later during puberty (reviewed by Cheng et al., 2009). This explanation would be consistent with the expression of the SPOC1 protein in undifferentiated, PLZF-positive spermatogonia and with the altered (clustered) distribution of these cells even in tubules with an otherwise Sertoli-cell-only phenotype.

PLZF is a known intrinsic transcription factor important for the self-renewal of SSCs. Interestingly, PLZF and SPOC1 are coexpressed in undifferentiated SSCs and the phenotype of the Spoc1−/− mice is very similar to the testis phenotype of Plzf−/− (luxoid) mice (Buaas et al., 2004; Costoya et al., 2004). PLZF belongs to the BTB–POZ–ZF (for broad complex, tramtrack, bric–à-brac or poxxviruses and zinc finger) family of transcription factors. It has been suggested that this group of proteins represents transcriptional repressors that act by epigenetically modifying chromatin (Kelly and Daniel, 2006) and that PLZF probably influences the epigenetic program of spermatogonial cells (Buaas et al., 2004). Similarly, SPOC1 is a PHD-finger-containing protein that is predominantly chromatin-associated and is involved in regulating chromatin structure and in mitotic chromosome condensation (Kinkley et al., 2009). Therefore, owing to the similarities between SPOC1 and PLZF, and because PHD-containing proteins are repeatedly being identified as new transcriptional repressors that act by epigenetically modifying chromatin, it is tempting to speculate that SPOC1 also affects stem cell differentiation in a manner similar to PLZF by altering the epigenetic program. Interestingly, another PHD finger protein (PYGO2) has recently been shown to modulate chromatin and to be involved in spermatogenesis (Nair et al., 2008). Of note, the reduced number of Spoc1−/− offspring (compared with the expected Mendelian ratio) indicates a profound effect on viability with reduced penetrance, possibly due to the variation of epigenetic factors.

In summary, we have identified a new intrinsic factor in undifferentiated PLZF-positive spermatogonia that is required for the differentiation of self-renewing spermatogonial stem cells into spermatocytes, possibly through a chromatin-mediated effect on differentiation-inducing genes. A further study will thus be conducted to identify the effect of SPOC1 disruption at the expression level.

Materials and Methods

Generation of Spoc1 gene-trap mutant mice

All animal experiments were performed according to approved guidelines. To develop a Spoc1-deficient mouse strain we used ESC lines, Xb691 and Xa022, which have a gene-trap insertion in the Spoc1 gene between exon 1 and 2 (Bay Genomics, San Francisco, CA; http://www.genetrap.org). According to JAX convention the gene-trap allele in these mice is very similar to the testis phenotype of Plzf−/−;4 (Scherthan et al., 2011).

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Western analysis

Protein extracts were prepared from the ovaries and testes of 18-week-old mice by homogenizing in RIPA buffer (~300 µl per 5 mg tissue; 50 mM Tris-HCl pH 8, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS; freshly prepared with Complete Mini Protease Inhibitor from Roche). The proteins were immunoblotted and probed with a rat monoclonal anti-SPOC1 antibody (Kinkley et al., 2009) at a concentration of 1:1000 and a rabbit polyclonal anti-actin antibody (sc-1616, Santa Cruz Biotechnology) at a dilution of 1:3000. The membranes were further probed with secondary horseradish peroxidase (HRP)-conjugated rabbit anti-rat-IgG antibody (1:15,000) (P0450, Dako) or swine anti-rabbit-IgG antibody (1:15,000) (P0399, Dako) antibodies, respectively. The protein bands were visualized using ECL reagents (Pierce) as described by the manufacturer.

Histological processing

After perfusion, the testes were fixed in 10% formalin solution for at least 12 hours, washed in cacodylic buffer for 3 hours and incubated for 2 hours in 1% osmium tetroxide in cacodylic buffer. After washing in cacodylic buffer, the testes were dehydrated in ascending ethanol concentrations (70, 80, 90 and 100%) and embedded through a 1% osmium tetroxide in cacodylic buffer for 3 hours and incubated for 2 hours in 1% osmium tetroxide (1:1) and then epox for 2 hours. The epon was hardened for 24 hours at 60°C and then 48 hours at 90°C. The testis was sagittally cut using an Ultracut microtome (Reichert-Jung, Germany) assembled with a diamond knife (Drukker, Germany). The sections were stained with Toluidine Blue for 1 minute at 50°C, washed for 10 minutes in Xylene and mounted with Entellan (Merck, Germany). Sections were analyzed with an Olympus IX70 microscope.

Immunohistochemistry

Testes were fixed in 10% formalin overnight, washed with tap water for 24 hours, embedded in paraffin and sectioned at 5-µm thickness. Immunohistochemical staining was performed on deparaffinized and rehydrated sections subjected to antigen retrieval using 10 mM citrate buffer at pH 6.5 for 20 minutes. After blocking of endogenous peroxidase (30 minutes in 0.3% H2O2, 10% methanol in PBS at room temperature) and three 5-minute washing steps in PBS, a permeabilization step (20 minutes in 0.5% Triton X-100 in PBS) was performed. After an additional three 10-minute washing steps in PBS, the further steps are executed with Vectastain Elite ABC Kit (Vector Laboratories).

Sections were incubated with rat monoclonal anti-SPOC1 antibody (1:10) (Kinkley et al., 2009) rabbit polyclonal anti-Mre11 antibody (1:200, Novus Biologicals, Littleton, CO), rabbit polyclonal anti-H2AX antibody (1:300, Biomol, Hamburg), rat monoclonal anti-GATA1 antibody (1:200, Santa Cruz Biotechnology, California, USA), anti-phosphorylated histone H3 (serine 10) antibody, mouse polyclonal anti-PLZF antibody (1:250, Santa Cruz Biotechnology), mouse monoclonal anti-PCNA antibody (PC10 Calbiochem) or rabbit polyclonal anti-Ki67 antibody (Bethyl Laboratories) overnight at 4°C. Fluorescence detection of primary antibodies was performed as described (Barrionuevo et al., 2009). Nuclei were counterstained with hemalaun or DAPI. Sections were analyzed using Olympus IX70 (peroxidase staining) or a Zeiss Axioplan 2 microscope, equipped with fluorescence filters for red, green and blue excitation (Chroma) (fluorescent staining).

TUNEL assays

TUNEL assays were performed using the In Situ Cell Death Detection Kit and Fluorescein (Roche) on paraffin-embedded tissue following the instructions of the manufacturer.

Preparation of meiotic chromosome spreads

Preparation of spermatocyte spreads was performed as described previously (Scherthan et al., 2011).
X-gal staining

Tissue was fixed in 4% paraformaldehyde for 2 hours at 4°C. After three washing steps in PBS (4°C), tissue was incubated in 30% sucrose in PBS at 4°C with slight agitation. For cryosections, the tissue was embedded in Tissue-Tek (Sakura, Staufen, Germany). Sections were washed in detergent solution (1 × PBS, 2 mM MgCl₂, 0.02% NP-40 and 0.01% Na-deoxycholate in water) for 15 minutes before incubation with X-gal solution (1 × PBS, 20 mM Tris HCl, pH 7.5, 2 mM MgCl₂, 0.02% NP-40, 0.01% Na-deoxycholate, 5 mM K₄Fe(CN)₆, 5 mM K₃Fe(CN)₆, 0.75 mM β-galactosidase in DMSO) under optical control. To stop the staining reaction, sections were washed three times in post-washing solution (1 × PBS, 5 mM EDTA, pH 8 in water). Sections were mounted with Crystal Mount (Sigma-Aldrich, St. Louis, Mo.), Prolong Gold (Invitrogen) and Clarion (Biomeda). Sections were analyzed using the Olympus IX 70 microscope.


References


Further references...


**Fig. S1. Co-expression of SPOC1 and PLZF in the testes of mice aged between 1 day and 10 weeks:** Testis sections were co-stained with rat monoclonal anti-SPOC1 antibody 6F6 (red) and a mouse monoclonal anti-PLZF antibody (green). DNA (blue) was stained with DraQ5. Scale bar, 10 μm.
Fig. S2. Whole-mount immunofluorescence localization of SPOC1 in seminiferous tubules of an adult mouse (12 wks): Intact seminiferous tubules were stained with rat monoclonal anti-SPOC1 antibody. Tubuli were prepared as described in Buaas et al. (Nat. Genet. 36, 2004, 647-652).
Fig. S3. Distribution of the genotypes from 13 heterozygous matings. Genotypes show a deviation from the Mendelian ratio with a reduced number of homozygous (Spoc1<sup>+/−</sup>) offspring.
Fig. S4. Litter sizes of Spoc1+/− x Spoc1+/− and Spoc1+/- x Spoc1−/− matings at various ages. In total, 11 breeding pairs were evaluated, respectively. Since fertility of Spoc1−/− females mated to Spoc1+/− or Spoc1+/+ males is almost normal (data not shown), the reduction in litter size can be attributed to a defect in male fertility.
Fig. S5. Histological changes in seminiferous tubuli of Spoc1<sup>-/-</sup> mice. Hematoxylin and eosin-stained histological cross-sections through the testes of Spoc1<sup>+/+</sup> (n=2) and Spoc1<sup>-/-</sup> (n=2) mice at 5, 10 and 20 weeks of age were investigated. Tubules were divided into three categories depending on their grade of disorganization (A) and counted (B). 5 wks (n=371wt/295ko); 10 wks (n=278wt/262ko); 20 wks (n=348wt/397ko).
Fig. S6. Increased apoptosis in Spoc1−/− testis. A) Immunofluorescent staining of activated Caspase 3 in the testis of Spoc1+/+ (n=2) and Spoc1−/− (n=2) mice at the age of 5, 10 and 20 wks was performed and the numbers of active Caspase 3-positive cell per tubule cross-section was counted. The data shown for 5, 10 and 20 week old mice represents the mean value +/- SD.
Fig. S7. PCNA expression in *Spoc1<sup>+/+</sup>* and *Spoc1<sup>-/-</sup>* testis. A) Immunofluorescent staining of PCNA in the testis of *Spoc1<sup>+/+</sup>* and *Spoc1<sup>-/-</sup>* mice at the age of 5, 10 and 20 wks. Testis sections were stained with a mouse monoclonal anti-PCNA antibody. Scale bar, 50 μm. B) The number of PCNA positive cells was scored in 40-50 tubule cross sections for two (5, 10, 20 wks) different mice. The data shown for 5, 10 and 20 week old mice represent the mean value +/- SD.
Fig. S8. Ki67 expression in Spoc1+/+ and Spoc1−/− testis. A) Immunofluorescent co-staining of Ki67 and PLZF in the testis of Spoc1+/+ and Spoc1−/− mice at the age of 10 wks, demonstrating proliferation of most PLZF-positive spermatogonia. Examples of PLZF-expressing spermatogonia with Ki67 (yellow arrow) and without Ki67 (green arrow) expression are shown. B) The number of Ki67-positive cells was scored in 20-30 tubule cross-sections for two (5, 10, 20 wks) different mice. The data shown for 5, 10 and 20 week old mice represent the mean value +/- SEM.
Table S1. Co-expression of SPOC1 and PLZF in the testes of mice aged between 1 day and 10 weeks

<table>
<thead>
<tr>
<th>Age</th>
<th>% of SPOC1 expressing spermatogonia co-expressing PLZF</th>
<th>% of PLZF expressing spermatogonia co-expressing SPOC1</th>
</tr>
</thead>
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<tr>
<td>P1</td>
<td>100 ± 0.0</td>
<td>100 ± 0.0</td>
</tr>
<tr>
<td>P6</td>
<td>98 ± 2.1</td>
<td>94 ± 2.8</td>
</tr>
<tr>
<td>P8</td>
<td>84 ± 0.6</td>
<td>97 ± 0.1</td>
</tr>
<tr>
<td>P10</td>
<td>82 ± 4.9</td>
<td>99 ± 0.7</td>
</tr>
<tr>
<td>P12</td>
<td>84 ± 3.7</td>
<td>99 ± 0.1</td>
</tr>
<tr>
<td>P15</td>
<td>77 ± 0.0</td>
<td>98 ± 2.0</td>
</tr>
<tr>
<td>P18</td>
<td>95 ± 3.4</td>
<td>98 ± 0.6</td>
</tr>
<tr>
<td>P23</td>
<td>84 ± 4.9</td>
<td>99 ± 0.3</td>
</tr>
<tr>
<td>10 weeks</td>
<td>85 ± 0.9</td>
<td>100 ± 0.5</td>
</tr>
</tbody>
</table>

Average percentage (±s.d.) from duplicate stainings; \( n=30–50 \) tubule cross-sections.