Splicing mutation in *Sbf1* causes nonsyndromic male infertility in the rat

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Abstract

In the inbred SHR/OlaIpcv rat colony, we identified males with small testicles and inability to reproduce. By selectively breeding their parents, we revealed the infertility to segregate as an autosomal recessive Mendelian character. No other phenotype was observed in males, and females were completely normal. By linkage using a backcross with Brown Norway strain, we mapped the locus to a 1.2 Mbp segment on chromosome 7, harboring 35 genes. Sequencing of candidate genes revealed a G to A substitution in a canonical 'AG' splice site of intron 37 in *Sbf1* (SET binding factor 1, alias myotubularin-related protein 5). This leads to either skipping exon 38 or shifting splicing one base downstream, invariably resulting in frameshift, premature stop codon and truncation of the protein. Western blotting using two anti-Sbf1 antibodies revealed absence of the full-length protein in the mutant testis. Testicles of the mutant males were significantly smaller compared with SHR from 4 weeks, peaked at 84% wild-type weight at 6 weeks and declined afterward to 28%, reflecting massive germ cell loss. Histological examination revealed lower germ cell number; latest observed germ cell stage were round spermatids, resulting in the absence of sperm in the epididymis (azoospermia). SBF1 is a member of a phosphatase family lacking the catalytic activity. It probably modulates the activity of a phosphoinositol phosphatase MTMR2. Human homozygotes or compound heterozygotes for missense *SBF1* mutations exhibit Charcot–Marie–Tooth disease (manifested mainly as progressive neuropathy), while a single mouse knockout reported in the literature identified male infertility as the only phenotype manifestation.

Reproduction (2016) **152** 215–223

Introduction

Infertility is a widespread health problem, affecting approximately 8–12% couples (Agarwal *et al.* 2015) or about 70 million of couples worldwide (Boivin *et al.* 2007). Male factor is responsible for about half of the cases (Agarwal *et al.* 2015). Substantial proportion of male infertility cases remain idiopathic despite advances in clinical diagnostics; a large proportion of the latter is thought to be genetic in origin (Hotaling & Carrell 2014, Krausz & Chianese 2014). Most prevalent genetic cause of male infertility is Klinefelter syndrome, about 8% in azoospermic males (Vincent *et al.* 2002) and the azoospermia factor (AZF) deletions in the male-specific region of the Y chromosome, reaching 11% in azoospermic males (Foresta *et al.* 2001). Other genetic factors that cause spermatogenic failure in human are suspected, but their identification has been challenging. Study of genetically infertile animal models thus provided most of the information about genes required for proper

spermatogenic function of the testis. According to the available models, most often targeted mutations in the mouse, azoospermia resulted from a block in germ cell development, most often in pachytene spermatocytes, but also in other stages of meiosis, in postmeiotic round spermatids and spermatogonia. Sertoli cell dysfunction is another reason for spermatogenesis failure with subsequent azoospermia (Liška 2003).

SBF1 (SET binding factor 1, SET being $\text{Su}(\text{var})3-9$, Enhancer of zeste and Trithorax domain, also MTMR5) is a myotubularin family protein with catalytically inactive phosphatase domain. Highest level of SBF1 expression was observed in testis and central nervous system. In testis, it is expressed both in germ cells and in Sertoli cells (Firestein *et al.* 2002). SBF1 heterodimerizes with and stimulates the activity of MTMR2 (myotubularin-related protein 2), also from the myotubularin family, but an active phosphoinositide lipid phosphatase, specific for position 3 in phosphatidylinositol-3-monophosphate (PtdIns3P) and phosphatidylinositol-3,

5-bisphosphate (PtdIns3,5P₂). PtdIns3P is in turn concentrated in early endosomes and is an important regulator of endosome trafficking (Laporte *et al.* 2003, Nicot & Laporte 2008). Mice lacking SBF1 are viable, with isolated male infertility due to spermatogenesis failure resulting in azoospermia (Firestein *et al.* 2002). Human homozygotes or compound heterozygotes for damaging missense mutations develop Charcot–Marie–Tooth demyelinating peripheral neuropathy (Nakhro *et al.* 2013, Alazami *et al.* 2014). Here, we report a splicing site mutation leading to truncation of the rat SBF1 ortholog, which closely resembles the null mice with male infertility as principal pathological finding.

Materials and methods

Animals

Animal experiments were performed in conformity with the Animal Protection Law of the Czech Republic and were approved by the Ethical Committee of the First Faculty of Medicine, Charles University in Prague. Adult rat males were held under temperature- and humidity-controlled conditions on 12-h light:12-h darkness cycle. At all times, the animals had free access to food (standard chow) and water. Tail biopsy for DNA extraction was performed in local anesthesia. For tissue samples, animals were killed using isoflurane overdosing. The mutation was discovered in SHR/Ola^{lpcv} inbred rats and after isolation maintained by crossing the mutant females to heterozygous males. We named the mutation *ifm* (infertile males), Rat Genome Database assigned it identification number 10002782 and standard designation, after discovery of the gene mutation, *Sbf1*^{*mtlpcv*}.

Linkage mapping

Mutant homozygous *ifm/ifm* fertile females were crossed to BN/Cub wild-type males. F1 males were crossed to the mutant females to generate backcross progeny. DNA was isolated from tail biopsy using phenol–chloroform extraction and ethanol precipitation. Polymorphic microsatellites were amplified by PCR using primers with sequences retrieved from Rat Genome Database (RGD, <http://rgd.mcw.edu/>). Additional microsatellites for fine mapping were identified using Tandem Repeat Finder (Benson 1999) track in UCSC Genome Browser (<http://www.genome.ucsc.edu/>) and primers were designed using Primer 3 (Untergasser *et al.* 2012). The primers are listed in Supplementary Table 1, see section on supplementary data given at the end of this article. Standard PCR was performed on backcross population, using parental strains as controls. The PCR products were separated on agarose (3–4%) or polyacrylamide (7–10%) gels and detected in UV light after ethidium-bromide staining using Syngene G:Box (Syngene, Cambridge, UK).

RT-PCR

Testes were snap-frozen in liquid nitrogen and stored at –80°C. Tissue fragments were grinded in liquid nitrogen

and the powder homogenized in Trizol (Invitrogen). After addition of chloroform, the water phase was applied to RNeasy columns (Qiagen) and purified according to manufacturer's instructions. Total RNA was converted to cDNA using SuperScript III reverse transcriptase and oligo-dT primer (Invitrogen). Specific primers for amplification and sequencing were designed by Primer3 and are listed in Supplementary Table 1.

Microarrays

We selected four control samples and four mutants aged 6 weeks. Double-stranded complementary DNA (cDNA) was synthesized from 50 ng total RNA. The labeled and fragmented cDNA was hybridized to Affymetrix GeneChip Rat Gene 2.0 ST Arrays (Affymetrix). The whole hybridization procedure including DNA adjustment was performed according to the protocol recommended by Affymetrix.

qPCR

To validate microarray gene expression data, quantitative real-time PCR (SYBR-Green) was used. Total RNA (1 µg) was reverse-transcribed with oligodT primers using the SuperScript III reverse transcriptase (Invitrogen). Primers were designed using Primer-Blast (Ye *et al.* 2012) or Primer3 (Supplementary Table 1). Real-time PCR reaction was performed in triplicate with EXPRESS SYBR GreenER qPCR SuperMix with Premixed ROX Kit according to the manufacturer's protocol (Invitrogen). Reactions were set up in 384 well plates using epMotion 5075 liquid handling workstation (Eppendorf, Hamburg, Germany). PCR amplification was performed using Applied Biosystems 7900HT Real-Time PCR System (Applied Biosystems). Each reaction contained cDNA amount corresponding to 5 ng of initial total RNA. We used cyclophilin A (*Ppia*) as endogenous control.

Statistical and pathway analyses

Transcriptomic data: after evaluation of the hybridization, the quality control and the data normalization by robust multiarray analysis (RMA), the gene expression was compared between the SHR and the SHR *ifm/ifm* utilizing PARTEK Genomics Suite 6.6 (Partek, St. Louis, MO, USA). The transcripts found to be differentially expressed with raw *P* value < 0.005 and by more than 20% between the two groups were included in the gene enrichment and pathway analyses, which were performed using Ingenuity Pathway Analysis software (application build 364062M, content version 26127183). qPCR: statistical comparison was performed on ddCt values (normally distributed) by unpaired Student's *t*-test using Statistica 12 (StatSoft, Tulsa, OK, USA).

Sequencing

PCR or RT-PCR products were directly sequenced using BigDye Cycle Sequencing Kit v1.1 (Applied Biosystems). Multiple alternatively spliced RT-pcr products of mutants were first cloned into pCRII (Zero Blunt TOPO Cloning Kit,

Invitrogen) and the clones sequenced using M13 primers and BigDye Cycle Sequencing Kit v1.1 (Applied Biosystems).

Western blotting

N- and C-terminal rabbit monoclonal SBF1 antibodies, EPR12566 and EPR12567 respectively were purchased from Abcam. Testis lysates were run on SDS-PAGE (8% separating gel); proteins were blotted onto PVDF membranes Immobilon P (EMD Millipore). Membranes were incubated overnight at 4°C with anti-SBF1 antibodies at final dilution 1:5000, secondary HRP-conjugated antibody was from GE Healthcare and signal was detected using ECL Prime chemiluminiscent detection kit (GE Healthcare). Control mouse monoclonal β -actin antibody was purchased from Abgent (San Diego, CA, USA).

Histological examination

After removal of tunica albuginea, testicular tissue was cut in cca 3×3 mm pieces and fixed in Bouin solution. Standard procedures for paraffin embedding, sectioning and hematoxylin–eosin staining were employed. Slides were examined with a Nikon Eclipse E 600 light microscope (Nikon). All samples were evaluated in a blinded fashion. All microphotographs were made using Nikon NIS-Elements AR 3.1 morphometric software (Nikon). All samples were evaluated in a blinded fashion.

Immunohistochemistry

For immunohistochemical studies, 4 μ m-thick paraffin sections were subjected to heat-induced epitope retrieval by immersion in an antigen retrieval solution (pH 6.0, Dako) for 30 min at 98°C. Endogenous peroxidase was blocked by a 20-min treatment with 3% hydrogen peroxide in absolute methanol. Staining with rabbit monoclonal anti-SBF1 antibody (EPR12567, Abcam) diluted 1:800 in ChemMate antibody diluent (Dako) was performed for 1 h at room temperature. Staining with rabbit polyclonal anti-SCF1 antibody (C-Terminus) (LS-C2911, LSBio, Seattle, WA, USA) diluted 1:400 in ChemMate antibody diluent (Dako) was performed for 1 h at room temperature. The Envision Kit (Dako) was used to visualize sections incubated with primary antibodies. The chromogen 3,3'-diaminobenzidine was applied to all sections and counterstaining was performed with Mayer's hematoxylin. Optimal staining conditions of any given antibody were determined using appropriate positive and negative controls.

Results

ifm is mapping to rat chromosome 7

In our inbred spontaneously hypertensive rat (SHR/Ola^{pcv}) colony, several males from the same family were unable to reproduce and had strikingly small testicles. By breeding the parents and sisters, we were able to isolate the trait and ascertain that it was inherited in Mendelian fashion, showing autosomal recessive pattern. Only males were infertile; there was

no discernible phenotypic abnormality in females. We named the mutation '*ifm*'. To ascertain the fertility of the *ifm* males, we cohoused 15 *ifm/ifm* males with wild-type females (who were proven fertile with wild-type males) for at least 1 month. Despite normal sexual behavior of the males, we did not observe any pregnancy.

Using 54 backcross males, we performed genetic mapping of the mutation with 212 microsatellite polymorphisms distributed over the 20 rat autosomes, and we revealed linkage of the mutation to rat chromosome 7. Fine mapping with additional 325 males specified a nonrecombinant region comprising 1.2 Mbp (coordinates chr7:127047128–128250751, rat genome Baylor version 3.4, Fig. 1A). The region contains 34 protein coding and one miRNA gene. We excluded most of the genes as functional candidates based on the available information about their expression pattern and molecular function. Therefore, we sequenced four 'functional candidate' genes with possible involvement in spermatogenesis and male fertility: *Mov10l1*, *Sbf1*, *Odf3b* and *Syce3* using testicular cDNA. *Mov10l1*, *Odf3b* and *Syce3* coding sequences did not contain any sequence variation between SHR and *ifm* homozygotes.

Sbf1 contains a mutation in acceptor splice site

Amplification of cDNA from testes of the mutants with primers flanking exon 38 (SBF1 structure see Fig. 1B) resulted in altered band pattern compared with the expected product size amplified from control cDNA. The altered pattern was also observed in heterozygotes, albeit with lower intensity. Sequencing of the mutant RT-PCR products (74 clones) recovered eight variants with different splicing (Fig. 1C). There was, in general, either skipping of exon 38 or retainment of intron 37. In products with 'correct' splicing of intron 37 and exon 38 first base of exon 38 was missing. All these events are causing frame shifting and are predicted to result in SBF1 protein truncation. The reason for the aberrant splicing, as we uncovered by sequencing genomic DNA, was transition from G to A in position -1 of intron 37 (Fig. 1D), disrupting the canonical AG acceptor splice site. Consequently, as the first base of the exon 38 is G, a new acceptor splice site was created just 1 bp downstream compared with wild type. The new splice site was less efficient, as predicted by lower NNSPLICE score (Reese *et al.* 1997, Fig. 1D) and resulted in increased frequency of failure to splice out intron 37 or skipping exon 38. Expression level of *Sbf1* mRNA was significantly lower in mutant testes, but also in some other organs, presumably due to nonsense-mediated decay of transcripts containing premature stop codons (Supplementary Fig. 1A). Sensitive qPCR assay identified SBF1 expression in all tested organs; however, Western blotting with C-terminal SBF1 antibody revealed full-length SBF1 expression in brain, adrenals and male reproductive tract; weak expression was also observed

Figure 1 (A) Nonrecombinant region of mutation on chromosome 7. Map is based on UCSC Genome Browser view, rat genome version 3.4. Top track – all polymorphic markers that manifested complete linkage with the mutant allele. Middle – genes in the region, *SBF1* encircled. Bottom – conservation and simple repeats; asterisks highlight positions of additional completely linked ‘custom’ microsatellite polymorphisms. (B) Structure of 3’ portion of *Sbf1* gene and position of the mutation at the intron 37/exon 38 boundary (middle red vertical line). Left and right red vertical lines depict forward and reverse primer positions respectively. (C) RT-PCR of mutant testis RNA demonstrates multiple aberrant splicing events with either skipping exon 38 or retaining some of the introns. Aberrant spliced-in introns are in green, standard exons in blue. The number on right represents the number of cDNA clones sharing identical structure from total 74 clones. (D) Sanger sequencing electropherogram showing the intron 37 mutation c.5152_-1G>A. This shifts the acceptor splice site one nucleotide downstream, resulting in frameshift at protein position 1718 and premature termination codon at position 1745 (p.G1718fsX1745). Additionally, NNSPLICE score for each splice site is shown in green.

in kidneys and liver (Supplementary Fig. 1B). Using immunohistochemistry (IHC), we confirmed SBF1 expression in testes (see below) and brain, where we observed granular cytoplasmic positivity of SBF1 within neurons (Supplementary Fig. 2A and B). In agreement with Western blotting experiments, we additionally observed similar granular cytoplasmic positivity in kidneys – within the tubular compartment, no staining was apparent in glomeruli (Supplementary Fig. 2C and D). Heart and liver did not express SBF1 by IHC. However, nonspecific positivity within the endothelium was present in all organs.

ifm/ifm homozygotes lack full-length *Sbf1* protein

Western blotting of lysates from testes of the mutant homozygotes compared with wild type using the C-terminal antibody (Fig. 2A) revealed the lack of full-length SBF1 protein (expected molecular weight 211 kDa, apparent molecular weight 220 kDa, Fig. 2B). Interestingly, the C-terminal antibody also detected some lower molecular weight species in both mutant and wild-type samples. These protein bands may result from alternative transcripts skipping the mutated splicing site inframe. Such transcripts are theoretically possible; however, by analysis of 74 cDNA clones, we found all transcripts to contain frameshift upstream of the antibody binding site. It is, therefore, more likely that the additional bands likely represent cross-reactivity with other proteins. Using N-terminal antibody, we confirmed the lack of full-length SBF1 in mutant homozygotes

(Fig. 2C). This antibody also detected additional lower molecular weight protein bands. One of them, around 210 kDa, was also detected by the C-terminal antibody, making it more plausible that the protein band represents real SBF1 variant expressed also in mutants.

Azoospermia in *ifm/ifm* males

Testis weight in mutant homozygotes falls behind the wild type at 4 weeks, peaks at 6 weeks (still below wild type), then drops down to one-third to one-quarter of wild type in 4-month adults (Fig. 3A). Epididymides of the mutants were devoid of any sperm; the rats were fully azoospermic.

Blocked spermiogenesis in *ifm/ifm* males

During the postnatal development, first evidence of abnormality is slightly lower cellularity of the tubules accompanied by vacuolization of the Sertoli cells (32 days, Fig. 4A and B). In adults, the mutant testes did not contain elongated spermatids (60 days, Fig. 4C and D), only diminished numbers of round spermatids could be detected (arrow in Fig. 4D). Lower amount of germ cells and enlarged vacuoles of Sertoli cells were evident (Fig. 4D and F compared with C and E).

Differential gene expression in *ifm/ifm* males

We performed transcriptome analysis in testes of 6-week-old *Sbf1* mutant homozygotes and wild-type

Figure 2 (A) Domain structure of Sbf1 protein as retrieved from NCBI-conserved domain database (Marchler-Bauer *et al.* 2015). Compare also with (Laporte *et al.* 2003). Mutation *ifm* leads to loss of C-terminal pleckstrin homology domain. (B) Western blotting of testicular lysates using C-terminal SBF1 antibody (see A). Full-length Sbf1 (apparent molecular weight approximately 220 kDa, expected molecular weight 211 kDa) is completely missing in mutants. However, the antibody also detects shorter species (210, 45, and 43 kDa) in all samples. They may represent other cross-reacting proteins. (C) Western blot using N-terminal Sbf1 antibody confirms the lack of full-length Sbf1 protein in mutants. The shorter 210 kDa species is also detected by this antibody. Additionally, a 90 kDa band is visible in all samples. (D) β -actin was used as endogenous control.

Figure 3 (A) During the first wave of spermatogenesis, the testis weight in mutants grew on a lower level compared with wild-type SHR. After a peak at 6 weeks of age, testis weight declined sharply, reflecting massive germ cell loss. In the wild-type SHR, testis weight reached plateau in at the same time (8–12 weeks). Data are mean $\pm$ s.d. Minimum number of animals per group was 3. Statistical significance: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (Student's *t*-test with Bonferroni correction for multiple comparison). (B) Genes differentially expressed between wild-type and *Sbf1* mutant testes. Data represent means, error bars standard errors. Wild-type expression was set to 1. Both microarray and qPCR values are depicted, with the exception of *Sbf1*, which was not represented in the rat microarray.

controls ($n=4$ for each group) using Affymetrix microarrays. We selected that age since the first wave of spermatogenesis should already produce haploid spermatids while the seminiferous epithelium was not yet degenerated. 494 transcripts had raw P values < 0.005 and 308 corresponded to known genes (Supplementary Table 2 for all known genes with raw P values < 0.005). The comparison did not reach whole-genome significance using Benjamini–Hochberg correction; however, we were able to confirm 11 genes to be differentially expressed using qPCR (Fig. 3B). Genes expressed in spermatids were strikingly downregulated in mutants compared with wild type; these included *Acrv1* (Golden *et al.* 1993), *Akap4* (Brown *et al.* 2003), *Gapdh* (Welch *et al.* 1995), *Odf1* (Yang *et al.* 2012), *Smcp* (Nam *et al.* 1997) and many other genes not validated by qPCR, confirming the histopathological findings. Conversely, at least some Sertoli cell-specific

Figure 4 Histological examination of testis. (A and B) Juvenile seminiferous tubules of *ifm/ifm* homozygotes (32 days, B) show lower cellularity and vacuoles formed in Sertoli cells compared with wild type (A). (C and D) Mature tubules also demonstrate complete absence of elongated spermatids in mutants (D) compared with wild type (C). Spermatogenesis is blocked in round spermatid stage, resulting in azoospermia (absence of sperm in the epididymis, not shown). (E and F) Low magnification of samples C + D. Sertoli cell vacuoles are prominent, germ cells strikingly reduced. (G and H) Immunohistochemical detection of *Sbf1* in testis using the C-terminal antibody. Highest expression was observed in basal layer of the seminiferous epithelium comprising spermatogonia, and in primary spermatocytes. Mutants show diminished signal in the seminiferous epithelium. High-density signal in the interstitial compartment is not specific (as it was observed in negative control preparations). Note hypertrophy of the interstitial (Leydig cell) compartment in mutants. (I and J) Immunohistochemical detection of SCP1. Control sample shows nuclear positive signal in primary spermatocytes in second from basal layer of the seminiferous epithelium. In mutants, positive spermatocytes in prophase of meiosis I are also observed, but they are situated also in the adluminal compartment, as the epithelium is disorganized (arrow). Scale bars 20 μ m, in panels E and F 250 μ m.

transcripts were upregulated, from which we validated *Testin* (Cheng *et al.* 1989) and *Itgb1* (Mulholland *et al.* 2001, Beardsley *et al.* 2006). The reason of Sertoli cell-specific transcript upregulation could be the relative increase in Sertoli cell compartment due to missing spermatids in mutants. In testin case, it can be also due to a lacking feedback from germ cells (Cheng *et al.* 1989). β 1-integrin (coded by *Itgb1*) upregulation can point to impairment of the ectoplasmic specializations between Sertoli cells and (diminished) germ cells. A modest increase (1.4-fold according to qPCR) in steroidogenic enzyme 17 α -hydroxylase/C17-20 lyase transcript *Cyp17a1* (Payne & Youngblood 1995) can indicate an increased proportion or hyperplasia of Leydig cells. Another group of genes upregulated in mutants represented molecules involved in inflammatory response: *Tlr8* (Dasari *et al.* 2008), *Il6r* (Rival *et al.* 2006) and *Il1r1* (Lysiak 2004). Genes specifically expressed during different developmental stages of spermatogonia (such as *Id4*, *Cdh1*, *Zbtb16*, *Kit*) were not differentially expressed (raw *P* values > 0.05). Similarly, there was no difference between ifm mutants and wild-type controls in the expression of meiosis-specific or primary spermatocyte genes (e.g. *Scp2*, *Prdm9*, *Rad51*, *Brca1*, *Brca2*). Ingenuity Pathway Analysis also did not indicate enrichment of apoptosis genes in the 308 known putatively differentially expressed genes (Supplementary Fig. 3).

Immunohistochemical analysis of SBF1

To confirm the SBF1 expression at the protein level and explore its distribution pattern, we examined *Sbf1* by IHC in testes. Strong cytoplasmic positivity was observed in spermatogonia, pachytene spermatocytes, Leydig and Sertoli cells during normal spermatogenesis at different postnatal days. We found only weak cytoplasmic positivity in postmeiotic spermatids. Interestingly, the SBF1 signal was not absent in samples from mutants, only somewhat weaker and wild-type rats exhibited more positive spermatogonia compared with mutants (Fig. 4G and H). This finding is consistent with the possibility that minor alternative forms of SBF1 protein are still expressed in mutants, as suggested by Western blots.

We used antibody against SCF1 (major component of the transverse filaments of synaptonemal complexes) as a marker of primary spermatocytes to confirm their presence in mutant males. As expected from the lack of differential expression of spermatocyte-specific genes, primary spermatocytes were found in mutants. However, they were not retained in the second to basal compartment of the seminiferous epithelium like in controls, but were found also closer to the adluminal compartment, suggesting impairment of the germ cell–Sertoli cell junctions (Fig. 4I and J).

Discussion

Targeted *Sbf1* null mutation was reported (Firestein *et al.* 2002). The mice lacking *Sbf1* exhibited failure of spermatogenesis and azoospermia. The mutation described here recapitulates all major findings of the null mouse, namely the vacuolization of the Sertoli cells as the first histopathological sign, progress of the first wave of spermatogenesis to round spermatid stage, but not further, and progressive degeneration of the germ cells in older animals. The rat mutation can be predicted as null too, due to the splicing disruption and protein truncation predicted from transcription data. However, the mutant transcripts are present, although the expression level is lower. Moreover, the Western blotting and IHC experiments suggest possible presence of non-full-length or truncated SBF1 variants in mutants. The functional impact of these shorter SBF1 variants is doubtful, e.g. observed normal phenotype of heterozygotes would be less likely in presence of a truncated protein that could disrupt the protein complexes, presumably MTMR2/Sbf2, that require SBF1. It was shown recently that human homozygotes or compound heterozygotes for missense SBF1 mutations exhibit hereditary progressive neuropathy Charcot–Marie–Tooth disease type 4B3 (Nakhro *et al.* 2013, Alazami *et al.* 2014). There is no neurological impairment even in old SHR *ifm/ifm* rats, nor there was reported any such phenotype for the knockout mice (Firestein *et al.* 2002). It remains to be investigated, whether there is any subclinical neurological impairment in the rat or mouse mutants. In the rat mutation described here, it could be a compensatory effect of the shorter SBF1 variants still produced in mutants. On the other hand, the point mutations described in CMT4B3 patients may exert a different mode of action, i.e. they can represent ‘gain of function’. Since it can be predicted that the proteins are expressed and could potentially bind to their interaction partners, they can block the normal function of the respective protein complexes. In human unaffected heterozygotes, some of the complexes may be normal, depending on stoichiometry, which may be enough for phenotype compensation. In the null mice (or mutant rats), there could also be a compensation achieved by different member of SBF family, like SBF2 (MTMR13) in neurons but not in male germ cells. Strikingly, *Mtmr2* knockout mice develop CMT4B and azoospermia (Bolino *et al.* 2004); however, in male cases of CMT4B3 with *Sbf1* mutations, spermatogenesis was not evaluated (Bohlega *et al.* 2011) leaving open the possibility that it could be also abnormal. Despite forming a complex, MTMR2 and SBF1 functions in spermatogenesis may not be identical; there is a phenotype difference between *Mtmr2* mutant mice whose major pathology seems to be shedding of germ cells into lumen, which is not observed in *Sbf1* mutants. Although it is not known whether Sertoli cell function or germ cell function of SBF1 is required

for spermatogenesis, some mouse mutants which disrupt genes believed to be Sertoli cell-specific have some phenotypic resemblance to the SBF1 mutation (Liška 2003). Sertoli cell involvement can be also supported by a speculative mechanism, involving the upregulated inflammatory genes. Sbf1 may participate in blood–testis barrier formation and recycling (Mruk & Cheng 2011). The functional blood–testis barrier precludes the immune system attacking the shielded germ cells, but in the mutant, they can become more exposed. As IL6 and its receptor are involved in autoimmune orchitis (Rival *et al.* 2006), this mechanism could be plausible. However, as TLR8 and IL6R are abundant in leukocytes, it can also only be an increased relative blood amount in the mutant testis due to lower spermatid number or some other anomaly. Involvement of cellular junctions between Sertoli cells and between Sertoli and germ cells in the pathogenesis of male infertility in the rat Sbf1 mutants is also corroborated by the finding of β 1-integrin transcripts upregulation, Sertoli cell vacuolization and disorganization of the seminiferous epithelium with spermatocytes in adluminal compartment in mutants. Interestingly, β 1-integrin (*Itgb1*) and also Testin transcripts were upregulated as a response to testicular toxicity of 1,3-dinitrobenzene (Matsuyama *et al.* 2011), suggesting common mechanisms of Sertoli cell dysfunction.

In conclusion, we identified a truncating mutation of *Sbf1* in the rat that recapitulates exactly spermatogenic arrest and azoospermia in the *Sbf1* null mice, confirming thus the necessary role of *Sbf1* in mammalian spermatogenesis.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-16-0042>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported by the Ministry of Health of the Czech Republic grant No. NT/12269 (F L) and the Czech Science Foundation grant No. 16-06548S (M P).

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Received 23 January 2016

First decision 23 February 2016

Revised manuscript received 25 May 2016

Accepted 17 June 2016