Overexpression of Lis1 in Different Stages of Spermatogenesis Does Not Result in an Aberrant Phenotype

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Abstract
Previous studies showed that in the mouse mutant Lis1 GT/GT gene trap integration in intron 2 of Lis1 gene leads to male infertility in homozygous Lis1 GT/GT mice. We further analyzed this line and could confirm the suggested downregulation of a testis-specific Lis1 transcript in mutant animals in a quantitative manner. Moreover, we analyzed the gene trap mutation on different genetic backgrounds in incipient congenic animals and could exclude a genetic background effect. To gain further insights into the role and requirement of LIS1 in spermatogenesis, 3 transgenic lines were generated, that overexpress Lis1 under control of the testis-specific promoters hEF-1α, which is exclusively active in spermatogonial cells, PGK2, which is active in pachytene spermatocytes and following stages of spermatogenesis, and Tnp2 which is active in round spermatids and following stages of spermatogenesis, respectively. All 3 transgenic lines remained fertile and testis sections displayed no abnormalities. To overcome the infertility of Lis1 GT/GT males, these transgenic Lis1-over-expressing animals were mated with Lis1 GT/GT mice to generate ‘rescued’ Lis1 GT/GT/Lis1 Tpos males. ‘Rescued’ animals from all transgenic lines remained infertile, thus overexpression of Lis1 in different stages of spermatogenesis could not rescue the infertility phenotype of homozygous gene trap males.

The PAFAH1B1 (LIS1) gene has been identified in the genomes of all eukaryotic organisms studied, and comparison of amino acid and nucleotide sequence has revealed a high level of sequence conservation during evolution. There is only one single amino acid difference between mouse and human LIS1 [Peterfy et al., 1994] and 3 amino acid differences between human and bovine LIS1 [Hattori et al., 1995].

LIS1 is an important protein that is involved in numerous protein-protein interactions and cellular processes. Besides its involvement in regulation levels of platelet-activating factor (PAF) as the noncatalytic subunit of the heterotrimeric complex type I platelet-activating factor...
acetylhydrolase (PAF-AH (I)) in mammals, it functions as a microtubule-associated protein involved in cell proliferation, intracellular transport and neuronal migration [Leventer et al., 2001]. The regulation of dynein motor function by LIS1 and microtubule organization is conserved in mammalian cells. Especially the role of LIS1 in dynein-mediated neuronal migration in diverse mammalian species has been explicitly studied [reviewed by Kerjan and Gleeson, 2007; Wynshaw-Boris, 2007]. LIS1 protein is accumulated in regions of high microtubule density in neurons and fibroblasts, especially at the centrosome and microtubule-organizing center (MTOC). Overexpression of Lis1 in cultured mammalian cells interferes with spindle orientation and progression into mitosis, whereas blocking of LIS1 interferes with attachment of chromosomes to the metaphase plate and leads to chromosome loss [Faulkner et al., 2000]. Moreover, overexpression of Lis1 results in peripheral redistribution of microtubules and tighter packing of the Golgi complex around the nucleus, whereas reduction of LIS1 in heterozygous knockout mice leads to enrichment of microtubules near the nucleus and looser packing of the Golgi complex [Smith et al., 2000].

Heterozygous mutations in the human LIS1 gene result in the well-known disease lissencephaly, which is characterized by a smooth cerebral surface, broad or absent gyri, abnormally thick cortex, reduced or abnormal lamination and diffuse neuronal heterotopia. Patients suffer from severe mental retardation, epileptic seizures and an early death [Friede, 1989; Kato and Dobyns, 2003]. Heterozygous disruption of the LIS1 gene causes type 1 lissencephaly (classical lissencephaly). Postmortem cytohistological studies indicate that neurons are aberrantly positioned in affected regions, which is caused by abnormal neuronal migration during early embryogenesis. Mice with one inactive Lis1 allele display cortical, hippocampal and olfactory bulb disorganization resulting from delayed neuronal migration by a cell-autonomous neuronal pathway. Mice with further reduction of Lis1 activity display more severe brain disorganization as well as cerebellar defects, suggesting an essential, dosage-sensitive neuronal-specific role for PAFAH1B1 in neuronal migration throughout the brain [Hirotsume et al., 1998; Gambello et al., 2003]. Homozygous deletion of the Lis1 gene (Lis1–/–) in mouse results in early embryonic lethality immediately after the implantation stage, demonstrating an essential role for Lis1 in early embryonic development [Hirotsume et al., 1998; Cahana et al., 2003].

The functional role of Lis1 in organs other than brain has not been determined, especially because of early embryonic lethality of Lis1-null mutants. The gene trap mouse line Lis1GT/GT carries a mutagenic insertion within the Lis1 gene [Nayernia et al., 2003]. Briefly, integration of the gene trap vector in intron 2 of Lis1 gene resulted in female infertility of homozygous mutant mice, while females reproduced normally. Testes of these males were about 50% smaller than testes of wild-type males, and histological analysis revealed that epididymides contained essentially no spermatozoa. In seminiferous tubules round and elongating spermatids were released prematurely from the epithelium and found inside the lumen of the tubules. Moreover, only few spermatozoa were found in the testis with a disorganized tubular structure, lacking the epithelial architecture and a clearly visible lumen. Expression analysis in mouse had revealed that the Lis1 gene is expressed in all adult tissues, but certain splicing and polyadenylation variants are differentially expressed in adult brain, heart and testis [Peterfy et al., 1998]. In testis an alternatively spliced transcript is expressed that contains an additional exon 2a as part of the 5′-untranslated region. This testis-specific Lis1 transcript was shown to be downregulated in the homozygous gene trap mice by Northern blotting. RT-PCR experiments confirmed the testis specificity of the transcript and the downregulation in homozygous gene trap males. Expression of transcripts lacking exon 2a were unaffected by the mutation.

In this work we have analyzed the downregulation of Lis1 transcripts in the gene trap mouse in detail and examined the effect of genetic background on phenotype of Lis1GT/GT animals. Moreover, our goal was to determine the role of LIS1 in different stages of spermatogenesis in vivo ‘gain of function’ transgenic models and to use these transgenic mice for a genetic rescue of the infertile gene trap line Lis1GT/GT.

Materials and Methods

Generation of Transgenic Animals and Backcross of the Gene Trap Line Lis1GT/GT

Three transgenic lines were generated, which express Lis1 cDNA (lacking exon 2a) under the control of the testis-specific promoters Tnp2, PGK2 and hEF-1a. Generation of transgenic mice was performed by ‘Transgenic Service’ of Max Planck Institute for Experimental Medicine in Goettingen by injection of purified DNA into pronuclei of one-cell mouse embryos obtained from FVB/N mouse strain. The injected embryos were transferred into FVB/N pseudopregnant foster mothers. Transgenic animals were identified by PCR as previously described [Hogan et al., 1986] and positive founders were mated with non-transgenic FVB/N female and male mice, respectively. Primer
sequences can be found in online supplementary table 1 (www.karger.com/doi/10.1159/000329482) and an example of each genotyping PCR can be found in online supplementary figure 1. Crossings did not show deviation from mendelian ratio (data not shown).

For Tnp2 (transition nuclear protein 2)-controlled Lis1 overexpression, 525 bp of 5′ flanking region of rat Tnp2 gene and 920 bp of 3′ flanking region of rat Tnp2 gene were used [Nayernia et al., 2001]. A 1.5-kb fragment of Lis1 cDNA was cloned into a Tnp2 flanking region containing vector [TPCATI; Nayernia et al., 2001] using BamHI and XbaI restriction sites (fig. 3A). The final construct was released by SphI digestion. From 2 transgenic founders, 2 mouse lines were established and analyzed. Genotyping was done with primers Lis1-F and Lis1-R.

For PGK2 (phosphoglycerate kinase-2)-controlled Lis1 overexpression, a 1.4-kb fragment containing the PGK2 promoter found in 2 mouse lines were established and analyzed. Genotyping PCR can be found in online supplementary figure 1. Crossings did not show deviation from mendelian ratio (data not shown).

For Lis1/20 sense, Lis1/21 antisense and 2A53 antisense. All experiments with animals were performed at the Animal Care and Use Committee of the University of Goettingen.

Histology and Analysis of Impaired Spermatogenesis

Tests and epididymides from mice of different postnatal ages were fixed in Bouin’s solution for 1 day at room temperature. The fixative was removed with 70% ethanol for 2–3 days, and tissues were embedded in paraffin. Sections (4–6 mm) were deparaffinized, rehydrated, and stained with hematoxylin and eosin (H&E).

To analyze putative spermatogenic defects in ‘rescued’ mice and LisiGGGT mice tests cross-sections of NMRI wild type, LisiGGGT, Lis1GGGT/PGK2/L3Tpos, Lis1GGGT/PGK2/L3Tpos and LisiGGGT/bEF-1α/L15Tpos animals were analyzed. Tests of three 46-day-old animals were fixed in Bouin’s solution for each genotype, cut in half and embedded in paraffin. Five 3-μm sections were transferred onto 1 glass slide with the following 7 sections were discarded before again 5 sections were collected and so forth. Ten glass slides were prepared for each genotype. Three slides (no. 1, no. 6 and no. 10) per animal were stained with H&E prior to analysis. Two cross-sections per glass slide were viewed with a fluorescence microscope and 2 images per cross-section were collected at 100× magnification (one right and one left from the center of the section).

All in all 12 images per animal and 36 images per genotype were analyzed. Firstly, the total number of tubules was determined with subsequent assignment to the following 4 classes: class 1: normal spermatogenesis; class 2: mild hypospermatogenesis (normal tubule architecture, germ cell number reduced, few elongated spermatids, no giant or degenerated germ cells); class 3: medium hypospermatogenesis (tubule architecture disturbed, few degenerated germ cells, few giant cells, reduced amount of haploid cells, premature release of immature germ cells); class 4: severe hypospermatogenesis (severe disturbance of tubule architecture, degenerated germ cells, multinucleated giant cells, severe vacuolization, few haploid cells, premature release of immature germ cells). An example for each of the 4 classes is shown in online supplementary figure 2.

RT-PCR and qRT-PCR Analysis

Total RNA from tissues was isolated using TriFast Reagent (Pqlab, Erlangen, Germany). Five microgram of isolated RNA was treated with DNase I (Sigma, Deisenhofen, Germany), and was then reverse-transcribed using Superscript II CDNA synthesis kit (Invitrogen, Karlsruhe, Germany) according to the manufacturer’s instructions.

RT-PCR was carried out with 0.5 μl of cDNA, 10 pmol each of forward and reverse primers, and 3 units of Taq polymerase (Invitrogen). Cycling conditions were as follows: 33 cycles of 94°C (30 s), 60°C (30 s), and 72°C (1 min). Hprt (hypoxanthin-phosphoribosyltransferase) was used as internal control.

Real-time quantitative PCR was performed using an ABI Prism 7900 HT Fast Detection System (Applied Biosystems Inc., Foster City, Calif., USA) as previously described [Kurrasch et al., 2004]. Each 10-μl reaction was performed in 384-well format using HYBRgreen PCR Master Mix (Qiagen, Hilden, Germany) and 9 μl of each PCR primer. All reactions were performed in triplicate and repeated 3 times with different RNA preparations. Results were compared by Mann-Whitney U test and p values <0.05

Overexpression of Lis1 in Spermatogenesis

Experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Goettingen.
were considered statistically significant. Levels of mRNA expression were normalized to those of the mouse housekeeping gene Sdhα (succinate dehydrogenase). Oligonucleotide primers for qRT-PCR were obtained from Operon Technologies (Cologne, Germany). Primer sequences used for gene amplification can be found in the online supplementary material (online suppl. table 1).

Tissue Preparation for Cryopreservation and Immunohistochemistry

For cryopreservation of tissues, animals were perfused with 4% paraformaldehyde (PFA) prior to dissection. Appropriate tissue was embedded in tissue freezing medium (Leica Microsystems) and stored at −80°C. Embedded tissue was cut into 5–6-μm-thick slices and transferred to SuperFrost® slides. Sections were washed 3 times in DPBS and were then incubated with a 5% blocking solution (3% BSA, 1% Triton X-100 or Tween-20 in DPBS) for 1 h at room temperature. Testis sections were incubated with anti-mouse IgG-peroxidase conjugated secondary antibody (Sigma-Aldrich). Protein bands were visualized using enhanced chemiluminescence as described above. Following antibodies were used: mouse monoclonal anti-c-Myc tag antibody (Milipore, Schwalbach/Ts., Germany) (1:50–1:200) in a 1.5% blocking solution (1.5% BSA, 1% Triton X-100 or Tween-20 in DPBS) overnight at 4°C. Sections were then rinsed 3 times in DPBS and subsequently incubated with anti-mouse IgG Cy3 conjugated antibody (Sigma-Aldrich, Deisenhofen, Germany) (1:500 in 1.5% blocking solution) for 1 h at room temperature. After incubation with secondary antibody, sections were washed again in DPBS and the nuclei were counterstained with VectaShield, a DAPI-containing mounting medium (Linearis, Hamburg, Germany).

Analysis of Fertility and Sperm Count in Caudae epididymidis

Reproductive capacity of males was determined by breeding with wild-type or mutant females. Females were checked for vaginal plugs every day, positive females were sacrificed and uterus and oviducts dissected in M2 medium (Sigma-Aldrich), flushed out and sperm number was determined using a Neubauer counting chamber. Caudae epididymidis of mice were dissected under aseptic conditions and put into 0.4 ml of IVF medium. Spermatzoa were allowed to swim out of caudae epididymidis for 1 h at 37°C, 5% CO2 and then counted.

Western Blot Analysis

For protein isolation from different tissues, about 30 mg of tissue was homogenized in lysis buffer (10 mM Tris/HCl, pH 8.1 mM EDTA, 2.5% SDS) containing 1 mM phenylmethanesulfonylfluoride and proteinase inhibitors and were sonicated. Protein extracts were denatured at 70°C in NuPage SDS sample buffer (Invitrogen) containing 0.1 mM dithiothreitol (DTT), separated on NuPage 10% Bis-Tris Gel (Invitrogen) and transferred on a Hybond-C extra membrane (GE Healthcare Europe, Freiburg, Germany). Blots were blocked for unspecified binding and were incubated overnight at 4°C with primary and for 1 h at 4°C with secondary HRP-conjugated antibody. Protein bands were visualized using enhanced chemiluminescence as described by the manufacturer (Santa Cruz Biotechnology, USA). The following antibodies were used: mouse monoclonal anti-α-tubulin (T5168; Sigma-Aldrich) (dilution 1:5,000), mouse monoclonal anti-LIS1 (G-3; Santa Cruz Biotechnology, Heidelberg, Germany), mouse monoclonal anti-c-Myc tag antibody (Milipore) and anti-mouse IgG-peroxidase conjugated secondary antibody (Sigma-Aldrich).

Results

Decreased Expression of Lis1 Transcripts in Testis of Lis1GT/GT Males

The gene trap integration in Lis1GT/GT animals abrogates expression of a testis-specific Lis1 exon 2a containing transcript (‘Ex2a/3’) and was suggested to be the reason of the infertility of Lis1GT/GT males [Nayernia et al., 2003]. To analyze Lis1 expression pattern in testes of homozygous Lis1GT/GT males during testicular development, quantitative RT-PCR analysis was performed on testicular cDNA obtained from 10-, 15-, 20-, 25- and 36-day-old and adult (2-month-old) Lis1GT/GT, Lis1GT−/− and NMRI wild-type males. Three animals per age and genotype were investigated in independent experiments. Two Lis1 splice variants were analyzed, the testis-specific Lis1 ‘Ex2a/3’ transcript and the ubiquitously expressed Lis1 ‘Ex2/3’ transcript (fig. 1). The relative expression level of both splicing forms did not differ significantly in NMRI wild-type males. The expression of Lis1 ‘Ex2a/3’ transcript was significantly reduced in homozygous males of all analyzed stages, while the Lis1 ‘Ex2/3’ transcript was significantly reduced in 25-day-old mice and following stages but not as strong as the Lis1 ‘Ex2a/3’ transcript. In adult animals the relative expression of Lis1 ‘Ex2a/3’ transcript appeared to be reduced to approximately 35% of the wild-type level, the relative expression of Lis1 ‘Ex2/3’ is reduced to approximately 65% of wild-type level. These results suggest that the infertility of homozygous gene trap males is caused by a strong decrease of Lis1 transcripts.

The Phenotype of Lis1GT/GT Mice Is Not Altered by Different Genetic Backgrounds

There is increasing evidence that the phenotype of a given single gene mutation in mice is modulated by the genetic background of the inbred strain in which the mutation is maintained [reviewed by Montaguelli, 2000]. To analyze the effects of genetic background on the phenotype of Lis1GT/GT/NMRI in mice, incipient congenic strains were generated. Heterozygous Lis1GT−/– males on NMRI outbred were crossed with females of C57BL/6J, FVB/N, CD-1 or 129/Sv/Ola genetic background. Backcrossing was done for 7 generations before heterozygous animals were crossed to produce homozygous incipient congenic strains. Females and heterozygous gene trap males were fertile, while breeding of 10 homozygous males of different genetic backgrounds did not lead to any pregnancy and no sperm could be recovered from uterus or oviduct of females positive for vaginal plugs. The testes size of ho-
Germ Cell-Specific Overexpression of Lis1 in Different Stages of Spermatogenesis

As decreasing Lis1 transcripts in testis lead to infertility in male mice, we wondered whether an increase in LIS1 protein level would also have an impact on spermatogenesis. For this, 3 transgenic lines were generated, which express Lis1 under control of testis-specific promoters. The promoters used are (i) hEF-1α promoter, a promoter exclusively active in spermatogonial cells, (ii) PGK2 promoter, a promoter active in pachytyne spermatocytes and following stages of spermatogenesis, and (iii) Tnp2 promoter, a promoter active in round spermatids and following stages of spermatogenesis. With these transgenic lines all stages of spermatogenesis are covered (fig. 3B).

Postmeiotic overexpression of Lis1 was obtained by cloning Lis1 cDNA between 5’ and 3’ regions of rat Tnp2 gene.

To obtain overexpression of Lis1 in pachytyne spermatocytes and following stages, a construct was gener-
ated that includes 5’ flanking region of human PGK2 gene followed by Lis1 cDNA and 6 c-Myc tags followed by SV40 poly(A) (from now on referred to as PGK2 construct). Three lines from different founders were established and analyzed. These lines were named PGK2/L3, PGK2/L5 and PGK2/L9.

Spermatogonia-specific expression was obtained by generating a construct that includes 5’-flanking region of human hEF-1α gene followed by Lis1 cDNA and 6 c-Myc tags followed by SV40 poly(A) (from now on referred to as hEF-1α construct). Five lines from different founders were established and analyzed. The 2 lines discussed here were named hEF-1α/L15 and hEF-1α/L19.

Expression of the fusion transcripts was confirmed by RT-PCR (fig. 3C) and Northern blot (data not shown). All transgenic animals were fertile with expected litter sizes and genotype contribution. To evaluate the influence of germ cell-specific overexpression of Lis1 on testis morphology, testis sections of adult transgenic males were stained with H&E (fig. 3D). Testis sections of transgenic males of all lines displayed no morphological abnormalities. Full spermatogenesis could be observed in the males.

To confirm expression of LIS1-c-Myc tag on protein level and to analyze its expression pattern in seminiferous tubules, Western blotting and immunohistochemistry analyses on transgenic animals were performed (fig. 4). The fusion protein was expressed specifically in testes of transgenic mice, while all other tissues lacked LIS1-c-Myc tag expression (fig. 4A). Cryosections of transgenic testes and brain were immunostained with monoclonal c-Myc tag antibody. The expression in PGK2/L9 (fig. 4C) is restricted to some meiotic and postmeiotic cells, with a particularly strong staining in haploid spermatids, which
Overexpression of Lis1 in Germ Cells of Infertile Lis1GT/GT Males Has No Effect on Spermatogenesis

As Lis1GT/GT males show a markedly decrease in LIS1 level, we wondered whether the overexpression of Lis1 in germ cells of transgenic animals could rescue the infertility phenotype of Lis1GT/GT males. For this genetic rescue approach, Lis1GT/GT females were mated with Tnp2/L7, PGK2 (PGK2/L3 and PGK2/L9) and hEF-1α (hEF-1α/L15 and hEF-1α/L19) transgenic males, respectively. Hetero-

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Fig. 3. Transgenic constructs, expression analysis and morphology of testis of transgenic mice. D Sections through testes of transgenic males revealed normal spermatogenesis.

Fig. 4. Determination of LIS1 protein in transgenic animals on FVB/N background. A Exemplary Western blots of protein extracts from different tissues of transgenic animals (PGK2/L3 and hEF-1α/L15) using a c-Myc tag antibody show testis-specific expression of the 59-kDa LIS1-c-Myc tag fusion protein. Tissues analyzed were testis (T), heart (H), lung (Lu), kidney (Ki), brain (Br), liver (Li) and spleen (Sp). The α-tubulin protein (50 kDa) is shown as a control for protein loading (M: prestained protein marker, Invitrogen; pos: testis lysate of a L3 male; neg: testis lysate of a Lis1<sup>GT/GT</sup> male). Although the amount of protein from different tissues varies, expression of the fusion protein could only be detected in the low concentrated testicular lysates of transgenic animals and not in any other tissue.
zygous F₁ animals were mated to produce Lis¹GT/GT/transgenicTpos males. Fertility of these ‘rescued’ males was tested. Matings yielded vaginal plugs, but no sperm cells could be recovered from uteri and no pregnancies were detected. To evaluate the influence of Lis¹ overexpression in testes of Lis¹GT/GT males, testis and epididymis sections of adult ‘rescued’ males and heterozygous siblings were stained with H&E (fig. 5A–F). The histological analysis revealed the same extensive degeneration of a large fraction of seminiferous tubules as observed in Lis¹GT/GT males of different genetic backgrounds (fig. 2). Tubules of epididymides of ‘rescued’ animals were mainly devoid of spermatozoa, but some contained few prematurely released germ cells or single sperm cells.

To confirm expression of the transgenic fusion protein LIS1-c-Myc tag, immunohistochemistry was performed (fig. 5G–J). As seen in the transgenic line PGK2/L3 (fig. 4B), the c-Myc tag antibody detected the fusion protein in cytoplasm of meiotic and postmeiotic cells of Lis¹GT/PGK2/L3Tpos mice, while LIS1 antibody detects LIS1 protein in all testicular cells. Moreover, the over-expression of LIS1 in ‘rescued’ males compared to Lis¹GT/GT males was confirmed by Western blot (an exemplary analysis is shown in fig. 5K for Lis¹/hEF-1α/L15 animals).
Fig. 5. Histology and immunohistochemistry of ‘rescued’ mice on mixed FVB/N/NMRI background. A–F H&E staining of testis and epididymis sections of adult ‘rescued’ mice. Sections through testes of heterozygous Lis1<sup>GT/–</sup>/PGK2/L3<sup>Tpos</sup> (B) mice revealed normal spermatogenesis, while testis sections of ‘rescued’ Lis1<sup>GT/–</sup>/PGK2/L3<sup>Tpos</sup> (A), Lis1<sup>GT/–</sup>/hEF-1α/L15<sup>Tpos</sup> (C) and Lis1<sup>GT/–</sup>/TNP2/L7<sup>Tpos</sup> (D) mice demonstrated severe impairment of spermatogenesis in most tubules. Sections through epididymides revealed a high number of spermatozoa in heterozygous males of Lis1<sup>GT/–</sup>/hEF-1α/L15<sup>Tpos</sup> (F), whereas most tubules of epididymides of ‘rescued’ males were devoid of spermatozoa, with some showing few prematurely released germ cells or single sperm cells (marked with an arrow) (E) (original magnifications A, B ×100; C, D ×200; E, F ×600). G–J Expression of endogenous LIS1 (H, J, LIS1 antibody (green)) and LIS1-c-Myc tag fusion protein (G, I, c-Myc tag antibody (red)) in testis sections of Lis1/PGK2/L3 mice using immunohistochemistry. The expression of the fusion protein in Lis1<sup>GT/–</sup>/PGK2/L3<sup>Tpos</sup> mice (G) is restricted to meiotic and postmeiotic cells, while endogenous LIS1 is expressed in all testicular cells (H). LIS1-c-Myc tag protein in Lis1<sup>GT/–</sup>/PGK2/L3<sup>Tpos</sup> mice (I) was detected mainly in haploid cells with a clear cytoplasmic localization, while endogenous LIS1 is expressed in all testicular cells (J). All pictures are dual-image overlays of Cy3 and DAPI and Cy2 and DAPI fluorescence, respectively. K Western blot of testicular protein extracts from Lis1<sup>GT/–</sup>/hEF-1α/L15 and Lis1<sup>GT/–</sup> mice confirmed an increase in LIS1 protein level in transgenic mice compared to Lis1<sup>GT/–</sup> animals.
Lis1 cued mice did not differ from that in meiotic cells in testes of the gene trap line together, the overexpression of seen in ‘Rescued’ mice showed severe spermatogenesis defects as were not statistically significant. tubules were more severely affected, but these differences that looked morphologically normal (the average number were analyzed) a low number of spermatozoa was found on mixed FVB/N/NMRI background), NMRI wild-type and were analyzed. No significant difference was found in NMRI Spermatogenesis Fig. 6. (sections of three 46-day-old animals per genotype to severe hypospermatogenesis (class 4) (fig. 6). As ex-

suppl. fig. 2) ranging from normal spermatogenesis (class 1) to severe hypospermatogenesis (class 4) (fig. 6). As expected no significant difference in spermatogenesis in NMRI wild-type and Lis1GT/GT NGT/GT (lacking exon2a) un-
downregulation of the ‘Ex2a/3’ transcript, which might have an influence on the expression of the other splicing form. To compare spermatogenesis defects in ‘rescued’ Lis1GT/GT/PKG2/L3pos, Lis1GT/GT/hEF-1α/L15pos and Lis1GT/GT in a more quantitative manner, testis cross-sections of three 46-day-old animals per genotype (Lis1GT/GT, Lis1GT/GT/PKG2/L3pos, Lis1GT/GT/hEF-1α/L15pos, Lis1GT/GT/hEF-1α/L15pos and NMRI wild-type) were analyzed. Total number of tubules was determined and tubules were assigned to 4 different classes (for an example for each of the 4 classes see online suppl. fig. 2) ranging from normal spermatogenesis (class 1) to severe hypospermatogenesis (class 4) (fig. 6). As expected no significant difference in spermatogenesis in NMRI wild-type and Lis1GT/GT/PKG2/L3pos was found. ‘Rescued’ mice showed severe spermatogenesis defects as seen in Lis1GT/GT males. Some tubules of ‘rescued’ mice showed mild hypospermatogenesis, while in Lis1GT/GT all tubules were more severely affected, but these differences were not statistically significant.

As single sperm cells were found in epididymal sections of Lis1GT/GT/hEF-1α/L15pos males (fig. 5E), we counted sperm cells in cauda epididymides of Lis1GT/GT/NMRI, Lis1GT/GT/FVB/N, Lis1GT/GT/129/Sv/Ola and ‘rescued’ animals Lis1GT/GT/PKG2/L3pos, Lis1GT/GT/hEF-1α/L15pos and Lis1GT/GT/hEF-1α/L15pos. In all animals (3 per genotype were analyzed) a low number of spermatozoa was found that looked morphologically normal (the average number was 104 spermatozoa). The amount of spermatozoa in ‘rescued’ mice did not differ from that in Lis1GT/GT mice. Taken together, the overexpression of Lis1 in meiotic and postmeiotic cells in testes of the gene trap line Lis1GT/GT had no influence on spermatogenesis of these animals.

Discussion

Analysis of the Gene Trap Line Lis1GT/GT

It is known that in mouse multiple Lis1 transcripts are expressed. In testis a Lis1 transcript containing an additional exon 2a as part of the 5’ untranslated region is present [Pitergy et al., 1998]. Northern blotting and RT-PCR analysis revealed a downregulation of this testis-specific Lis1 ‘Ex2a/3’ transcript in homozygous gene trap mice [Nayernia et al., 2003]. To investigate the testicular expression pattern of Lis1 transcripts in a quantitative fashion, qRT-PCR analysis was performed on mutant and wild-type animals from postnatal days 10–60 (fig. 1). A strong, significant downregulation of Lis1 ‘Ex2a/3’ splicing form from 10 days on could be detected in Lis1GT/GT males. With this result the insight on Lis1 expression pattern in testis was extended. The downregulation of Lis1 ‘Ex2a/3’ transcript could be confirmed. Ten-day-old mice already display a significant downregulation of the Lis1 ‘Ex2a/3’ transcript. This result raises the possibility that Lis1 is expressed in early stages of spermatogenesis. Moreover, a significant downregulation of Lis1 ‘Ex2a/3’ splicing form was detected from 25 days on in homozygous mice which has not been shown before. It is not clear whether this is due to the integration of the gene trap vector or the downregulation of the ‘Ex2a/3’ transcript, which might have an influence on the expression of the other splicing form.

Germ Cell-Specific Overexpression of Lis1

To gain further insights into the role and requirement of LIS1 in spermatogenesis, 3 transgenic mouse lines were generated, which overexpress Lis1 (lacking exon2a) under the control of testis-specific promoters. Polypeptide chain elongation factor 1α (EF-1α) is the eukaryotic counterpart of E. coli EF-Tu which promotes the GTP-dependent binding of an aminoacyl-tRNA to ribosomes. It is one of the most abundant proteins in eukaryotic cells and expressed in almost all kinds of mammalian cells. It was shown that a 1.2-kb fragment of human elongation factor-1α (hEF-1α) promoter drives a testis-specific spermatogonial expression of reporter genes [Furuchi et al., 1996; Meng et al., 2000]. In our group this fragment was used to overexpress PwiiI-2 specifically in spermatogonia [JH Lee, personal communication]. Phosphoglycerate kinase-2 (Pgk2) is an autosomal gene expressed in a testis-specific manner exclusively in the late stages of spermatogenesis [Kramer and Erickson, 1981]. Pgk2 transcription is first seen in pachytene spermatocytes with message levels increasing during later stages of spermatogenesis.
Transgenic approaches have been used previously to demonstrate that a 1.4-kb 5′-flanking region of human PGK2 gene is sufficient to confer spermatocyte-specific expression of a CAT reporter gene [Robinson et al., 1989]. The same promoter sequence was also used in our group to examine the susceptibility of spermatocytes to transformation by targeted expression of SV40 tag in spermatocytes of transgenic mice [Tascou et al., 2001]. Transition nuclear protein 2 (Tnp2) is involved in chromatin condensation during spermiogenesis in the mouse [Kleene et al., 1987]. It is expressed postmeiotically in male germ cells. Transcription of the Tnp2 mRNA starts in round spermatids and is then stored for about 6 days before translation of the protein starts in elongating spermatids. It was shown in our group, that 525 bp of 5′- and 920 bp of 3′-flanking sequences of rat Tnp2 gene could direct chloramphenicol acetyltransferase gene expression to the postmeiotic male germ cells of transgenic mice. During male germ cell differentiation, the first transgene transcripts were observed in round spermatids and translation started 6 days later in elongating spermatids, which is an evidence for posttranscriptional regulation of transgene expression [Nayernia et al., 2001].

Integration of the transgenic construct is a random event, so different transgenic founder lines have different sites of integration. Thus, it is important to compare the phenotype of mice from different founder lines to determine whether the phenotype is linked to the transgene integration site or the construct itself. Three PGK2-Lis1 transgenic lines, 5 hEF-1α-Lis1 lines and 2 Tnp2-Lis1 lines were analyzed. All analyzed lines transmitted the transgene and no phenotypical abnormalities were found. Western blot analysis confirmed testis specificity of the fusion transcripts (fig. 4A). Immunohistochemical staining of testis sections of transgenic animals revealed the expected staining pattern for hEF-1α-Lis1/L15 and hEF-1α-Lis1/L19 in spermatogonial cells (fig. 4D, E) and for PGK2/L3 and PGK2/L9 in meiotic and postmeiotic cells (fig. 4B, C). These results indicate that expression of the LIS1-c-Myc tag fusion protein is under control of the designed promoters (hEF-1α and PGK2, respectively). Nevertheless, slight differences were seen in PGK2-overexpressing lines, where line 9 displayed strong staining in haploid, elongating spermatids while in line 3 staining was more widely distributed in meiotic and postmeiotic cells. The integration sites might have an additional effect on the expression due to regulatory elements specific for the integration site [for an example, see Weis et al., 1991]. Transgenic constructs usually integrate in multiple copies into the genome in a head-to-tail orientation [Tinkle and Jay, 2002]. This copy number can affect the resulting phenotype [for an example, see Alexander et al., 2004]. All transgenic lines generated in this study have a single integration site confirmed by qPCR (data not shown), but copy numbers were not determined. Nevertheless, to look for a possible dose-dependent effect, homozygous animals (in which the transgene copy number is doubled) were analyzed, but no effect was found. All males were fertile and histological analysis of testis and epididymis sections did not display abnormalities (data not shown). The downregulation or deficiency of Lis1 is studied in detail, but not much is known about upregulation of Lis1. Bi et al. [2009] could show that in contrast to PAFAHIB1 haploinsufficiency, which causes lissencephaly, submicroscopic duplication in 17p13.3 including PAFAHIB1 and/or YWHAE genes causes milder brain structural abnormalities, moderate to severe developmental delay and failure to thrive. It would be of interest to analyze whether patients with this milder phenotype have any problems with fertility. Moreover, a transgenic mouse model was analyzed, which overexpresses Lis1 in the developing brain. Animals had a decreased brain size, an increase in apoptotic cells and a distorted cellular organization in the ventricular zone [Bi et al., 2009]. Again, this phenotype is milder than in mice with decreased levels of LIS1.

LIS1 forms complexes with the 2 catalytic subunits α1 and α2 of PAFAHIB1. Both catalytic subunits are expressed in testis, and mutants lacking α2 or α1 and α2 subunits are infertile with no other apparent phenotype. According to Koizumi et al. [2003], α1 subunit-deficient mice are indistinguishable from wild-type mice, whereas α2 subunit-deficient mice show a reduction in testis size, but are fertile. However, α1−/−/α2−/− males are infertile and exhibit a significant reduction in spermatocytes beyond the pachytene stage and round spermatids. Moreover, elongated spermatids are rare and the remaining spermatids have deformed nuclei. This phenotype is more severe in older mice, where an increased depletion of spermatocytes and spermatogonia is observed. In contrast to this, the observation by Yan et al. [2003] showed that α1 subunit-deficient mice have normal fertility, while α2-deficient mice are infertile. Spermatogenesis is disrupted at mid- or late pachytene stages of meiosis or early spermiogenesis. α1−/−/α2−/− males exhibit an earlier disturbance of spermatogenesis with an onset at preleptotene or leptotene stages of meiosis. Both studies associate the testis phenotype with altered expression levels of Lis1 in mutant mice. While Koizumi et al. [2003] found a significant
downregulation of LIS1 protein in α2+/– and α1+/–/α2+/-
males (suggested to be due to post-transcriptional control, as mRNA levels of Lis1 are unaltered in α2+/– mice), Yan et al. [2003] found
an increase in LIS1 protein expression. This discrepancy could be explained by the age of the analyzed animals. While Koizumi et al. analyzed adult males, Yan et al. analyzed 12-day-old mice. The reduction of LIS1 protein might be due to a severe depletion of meiotic and postmeiotic germ cells in adult testis, while in 12-day-old animals only pachytene spermatocytes and earlier germ cells are present which are not affected in α1+/–/α2+/– mutants. Interestingly, the infertility phenotype of the double mutant mice (α1+/–/α2+/–) could be rescued by generating triple mutant mice (α1+/–/α2+/–/Lis1+/-). These mice exhibit full spermatogenesis, although some degenerating germ cells and multinucleated giant cells were observed. These results suggest that LIS1 levels influence the testis phenotype in mutant mice.

Heterozygous Lis1+/- males are fertile, but display a reduction in testis weight, reduction in tubule diameter and degenerating germ cells [Yan et al., 2003]. These results are in line with the analysis of the gene trap line Lis1GT/GT, where heterozygous males (Lis1GT/-) with a reduced expression of Lis1 transcripts are fertile (fig. 2). Only a further reduction of Lis1 expression in homozygous mice leads to infertility of the males. Overexpression of the α2 subunit in cultured mammalian CHO cells was found to induce centrosomal amplification and microtubule disorganization, while overexpression of the α1 subunit leads to a less prominent phenotype [Yamaguchi et al., 2007]. Interestingly, these phenotypical changes were not found in cells overexpressing a mutant form of the α2 subunit that cannot bind LIS1, suggesting that binding of the α2 subunit and LIS1 is a prerequisite for structural cell abnormality and that the quantitative balance of α2 and LIS1 is important for LIS1 functions in testis [Yamaguchi et al., 2007]. Taken together, it is clear that a certain LIS1 protein level is important for proper function of spermatogenesis. Cells, including germ cells, are sensitive to an increase or decrease of LIS1 protein level. The upregulation of LIS1 protein in testis of transgenic mice should be analyzed further in a quantitative manner, to study the amount of upregulation and to be able to compare different LIS1 protein levels.

Transgenic Rescue Experiment
‘Rescued’ males of all analyzed lines in this study (Lis1GT/GT/PKG2/L3, Lis1GT/GT/PKG2/L9, Lis1GT/GT/hEFE-1α/L15 and Lis1GT/GT/hEFE-1α/L19) remained fertile. A detailed analysis of spermatogenesis defects in ‘rescued’ males and gene trap males revealed no significant differences (fig. 6). Several successful transgenic rescue experiments have been reported [for examples, see Ksiazek et al., 2007; Woldeyesus et al., 1999; Kumar et al., 1998]. Interestingly, the infertility phenotype of the double mutant mice α1+/–/α2+/– (catalytic subunits of PAFAH1b) could be rescued by generating triple mutant mice (α1+/–/α2+/–/Lis1+/-), which had a decreased LIS1 level [Yen et al., 2003, discussed above]. ‘Rescued’ males of all analyzed lines in our study remained infertile. A detailed analysis of spermatogenesis defects in ‘rescued’ males and gene trap males revealed no significant differences (fig. 6). Also sperm number in cauda epididymis of the males did not differ significantly (data not shown).

The use of a different promoter to direct expression of Lis1 to all stages of spermatogenesis could be considered. Another possibility could be the generation of double transgenic mice (hEFE-1α and PGK2 line), which would express a LIS1 fusion protein in most germ cells. These mice could then be used for a ‘rescue’ experiment. Moreover, the use of Lis1 5’-UTR and 3’-UTR in transgenic constructs could help to get transgene expression that is similar to the endogenous Lis1 expression and thus is more likely to rescue the infertility of the gene trap line. In addition, it could be of interest to overexpress the testis-specific form of Lis1 in different germ cell stages to further characterize the function of this Lis1 transcript.

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