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

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Key Words

Genetic rescue · Infertility · Spermatogenesis · Transgenic mice

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 abnormal-

ities. 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.

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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 em-

bryonic lethality of *Lis1*-null mutants. The gene trap mouse line *Lis1*^{GT/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 male 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 *Lis1*^{GT/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 *Lis1*^{GT/GT}.

Materials and Methods

Generation of Transgenic Animals and Backcross of the Gene Trap Line *Lis1*^{GT/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-1α*. 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 *Bam*HI and *Xba*I restriction sites (fig. 3A). The final construct was released by *Sph*I digestion. From 2 transgenic founders, 2 mouse lines were established and analyzed. Genotyping was done with primers Lispi-F and Lispi-R.

For PGK2 (phosphoglycerate kinase-2)-controlled *Lis1* overexpression, a 1.4-kb fragment containing the PGK2 promoter was cloned into *Xho*I and *Eco*RI restriction sites of pBluescript II SK(-) vector (Stratagene, Amsterdam, The Netherlands) [Tascou et al., 2001]. A 1,377-bp *Lis1* cDNA fragment (containing an *Eco*RI restriction site 136 bp 5'-upstream of the *Lis1* start codon and a *Bam*HI restriction site that replaced the *Lis1* stop codon) was cloned into *Eco*RI and *Bam*HI restriction sites of the plasmid. Then a 410-bp fragment containing 6 c-Myc tags followed by 2 stop codons and the SV40 poly(A) was amplified. pCS2-3' mt plasmid (Hammerschmidt Plasmid Stocks, Freiburg, Germany) was used as a template (the reverse primer consists of a template binding region and a tail comprising the SV40 poly(A) sequence) and cloned into *Bam*HI and *Not*I restriction sites of the plasmid downstream of *Lis1* cDNA integration (fig. 3A). The final construct was released by *Not*I and *Bgl*II digestion. From 3 transgenic founders, 3 transgenic lines were established and analyzed. Primers used for genotypings were RT-PGK2-Prom-F99 and RT-PGK2-Prom-R99.

For hEF-1 α (human elongation factor-1 α)-controlled *Lis1* overexpression, a 1.2-kb *Hind*III-*Eco*RI fragment containing *hEF-1 α* promoter was derived from pEF-BOS (BCCM/LMBP Plasmid collection, Ghent, Belgium) and cloned into pEGFP-C1 vector (Clontech, Saint-Germain-en-Laye, France). The 410-bp (c-Myc tag)₆-SV40poly(A) fragment mentioned above was cloned into *Bam*HI and *Not*I restriction sites of the plasmid, thereby replacing the EGFP gene. A 1,377-bp *Lis1* cDNA fragment was cloned into *Eco*RI and *Bam*HI restriction sites upstream of the (c-Myc tag)₆-SV40poly(A) (fig. 3A). The final construct was released by *Not*I and *Bcl*VI digestion. From 5 transgenic founders, 5 transgenic lines were established and analyzed. Primers used for genotyping were RT-hEF-Prom-F99 and RT-hEF-Prom-R99.

To generate homozygous transgenic males, heterozygous F₁ animals were mated and positive male offspring (either heterozygous or homozygous) were mated with wild-type females. Embryos from 13.5 dpc were genotyped. All embryos of a homozygous should be heterozygous (positive for the transgenic construct), while only about 50% of the embryos of a heterozygous male should be positive. Two to four males (F₂ generation) of each line were testbred and 1 homozygous male per line analyzed.

Generation of the gene trap line *Lis1*^{GT/GT} was described previously [Nayernia et al., 2003]. *Lis1*^{GT/GT} mutant mice (on NMRI background) were backcrossed to C57BL/6J, FVB/N, CD-1 and 129/Sv/Ola mouse strains (Charles River Laboratories, Wilmington, Mass., USA) for 7 generations. Primers used for genotyping were *Lis1* 20 sense, *Lis1* 21 antisense and 2A53 antisense.

All experiments with animals were performed at the Animal Facility of Institute of Human Genetics (Goettingen, Germany).

Experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Goettingen.

Histology and Analysis of Impaired Spermatogenesis

Testes 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 μ m) were deparaffinized, rehydrated, and stained with hematoxylin and eosin (H&E).

To analyze putative spermatogenic defects in 'rescued' mice and *Lis1*^{GT/GT} mice testis cross-sections of NMRI wild type, *Lis1*^{GT/GT}, *Lis1*^{GT/-}/PGK2/L3^{Tpos}, *Lis1*^{GT/GT}/PGK2/L3^{Tpos} and *Lis1*^{GT/GT}/hEF-1 α /L15^{Tpos} animals were analyzed. Testes of three 46-day-old animals were fixed in Bouin's solution for each genotype, cut in half and embedded in paraffin. Five 3- μ m sections were transferred onto 1 glass slide with the following 7 sections being 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 $\times$ 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 (Peqlab, 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 μ l 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-phosphoribosyl-transferase) 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 [Kurrsch et al., 2004]. Each 10- μ l reaction was performed in 384-well format using SYBRgreen PCR Master Mix (Qiagen, Hilden, Germany) and 9 μ M 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

were considered statistically significant. Levels of mRNA expression were normalized to those of the mouse housekeeping gene *Sdha* (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 tissues were 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 (5% BSA, 1% Triton X-100 or Tween-20 in DPBS) for 1 h at room temperature. Testis sections were incubated with 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. Spermatozoa were allowed to swim out of caudae epididymidis for 1 h at 37°C , 5% CO_2 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 unspecific 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 Lis1^{GT/GT} Males

The gene trap integration in *Lis1^{GT/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 *Lis1^{GT/GT}* males [Nayernia et al., 2003]. To analyze *Lis1* expression pattern in testes of homozygous *Lis1^{GT/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) *Lis1^{GT/GT}*, *Lis1^{GT/-}* 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 Lis1^{GT/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 *Lis1^{GT/GT}*/NMRI mice, incipient congenic strains were generated. Heterozygous *Lis1^{GT/-}* 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-

Fig. 1. Relative expression levels of *Lis1* 'Ex2/3' and *Lis1* 'Ex2a/3' splicing forms detected by quantitative RT-PCR in testes of *Lis1*^{GT/GT}/NMRI, *Lis1*^{GT/-}/NMRI and NMRI wild-type mice of different ages. Expression levels are normalized to the expression of endogenous control (*Sdha*). NMRI wild-type testes were used as calibrator in each independent experiment. Three biological replicates were used for each stage and genotype. Non-parametric Mann-Whitney U test was used to determine p values. Asterisk depicts statistical significance (* p < 0.05 and ** p < 0.005) in comparison with NMRI wild-type male (*Lis1* 'Ex2/3' transcript). Expression in testes of 10- (A), 15- (B), 20- (C), 25- (D) and 36-day- (E) and 2-month- (F) old mice was analyzed. The exon-specific primers are indicated in the schematic gene drawing (G).

mozygous males in all backgrounds was markedly reduced in comparison to heterozygous siblings (data not shown). To confirm the extensive germ cell degeneration observed in seminiferous tubules of *Lis1*^{GT/GT}/NMRI mice, sections of testes and epididymides of adult *Lis1*^{GT/-} and *Lis1*^{GT/GT} mice on CD-1, C57/BL/6J, FVB/N and 129/Sv/Ola backgrounds were stained with hematoxylin and eosin. Sections of testis of homozygous males on different backgrounds revealed the same extensive degeneration of germ cells in a large fraction of seminiferous tubules as seen in *Lis1*^{GT/GT}/NMRI males (fig. 2). Even though mutant testes exhibit early germ cells as well as Sertoli and Leydig cells, the number of late meiotic (i.e. late pachytene and diplotene spermatocytes) and postmeiotic (i.e. spermatids and spermatozoa) germ cells is markedly reduced. Moreover, the tubular structure is severely disturbed. In contrast to this, heterozygous males exhibit full spermatogenesis with no obvious differences from wild-type males. Tubules of epididymides of heterozygous males are packed with spermatozoa, while most tubules of homozygous males are devoid of them and some are filled with few prematurely released germ cells. Western blotting of testicular protein extracts showed a reduction of LIS1 protein in testis of homozygous animals on different genetic backgrounds in comparison to hetero-

zygous and wild-type animals (data not shown). Taken together, these results demonstrate that the infertility phenotype of *Lis1*^{GT/GT} is not attributable to a genetic background effect, i.e. the phenotype of *Lis1*^{GT/GT} mice is not altered by genetic background.

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 pachytene 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 pachytene spermatocytes and following stages, a construct was gener-

Fig. 2. H&E staining of testis and epididymis sections of adult *Lis1*^{GT/GT} and *Lis1*^{GT/-} mice on different genetic backgrounds. Sections through testis of homozygous mice on FVB/N (A), CD-1 (B) and C57/BL/6J (C) genetic background revealed extensive degeneration of a large fraction of seminiferous tubules, whereas testis sections of heterozygous males (D, on 129/Sv/Ola background) demonstrated robust spermatogenesis. Epididymides of homozygous males on 129/Sv/Ola background (E) were devoid of spermatozoa and only some prematurely released germ cells were present. Epididymides of heterozygous males showed normal histology with numerous spermatozoa inside (F) (original magnification A, C–F $\times 200$; B $\times 600$).

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-1 α gene followed by *Lis1* cDNA and 6 c-Myc tags followed by SV40 poly(A) (from now on referred to as hEF-1 α construct). Five lines from different founders were established and analyzed. The 2 lines discussed here were named *hEF-1 α /L15* and *hEF-1 α /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

Fig. 3. Transgenic constructs, expression analysis and morphology of testis of transgenic mice. **A** Schematic illustration of transgenic constructs. The black arrows correspond to primers used for genotyping and for RT-PCR analysis. **B** Expected expression pattern of transgenic transcripts in spermatogenesis. **C** Expression of LIS1-c-Myc tag fusion transcript in transgenic mice (on FVB/N background) was detected with 2 different primer pairs (**C-1**, **C-2**). Integrity of the RNA used for RT-PCR was proven by amplification of the mHPRT transcript (**C-3**).

display a specific staining pattern around the nucleus. LIS1-c-Myc tag protein in *PGK2/L3* (fig. 4B) is more widely distributed, with most meiotic and postmeiotic germ cells being positively stained. The expression in *hEF-1α/L15* (fig. 4D) and *hEF-1α/L19* (fig. 4E) is restricted to premeiotic germ cells. Taken together, these data show that a germ cell-specific overexpression of LIS1 protein has no influence on spermatogenesis of transgenic animals.

Overexpression of *Lis1* in Germ Cells of Infertile *Lis1^{GT/GT}* Males Has No Effect on Spermatogenesis
As *Lis1^{GT/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 *Lis1^{GT/GT}* males. For this genetic rescue approach, *Lis1^{GT/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-

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 *Lis1*^{GT/GT} 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.

Fig. 4. Determination of LIS1 protein in transgenic animals on FVB/N background. **B–G** Expression pattern was determined by immunohistochemistry using a c-Myc tag antibody (red). Nuclei were counterstained with DAPI (blue). The expression in *PGK2/L9* (**C**) is restricted to some meiotic and postmeiotic cells, with a particularly strong staining in haploid spermatids, which display a specific staining pattern around the nucleus. LIS1-c-Myc tag protein in *PGK2/L3* (**B**) is more widely distributed, with most mei-

otic and postmeiotic germ cells being positively stained. The expression in *hEF-1α/L15* (**D**) and *hEF-1α/L19* (**E**) in the seminiferous epithelium is restricted to premeiotic germ cells. As a negative control, testis sections were immunostained with secondary antibody (anti-mouse-Cy3-conjugated antibody) only. No staining in germ cells could be detected (**F**). Brain sections of *PGK2/L9* mice did not display specific staining for c-Myc tag antibody (**G**). All pictures are dual image overlays of Cy3 and DAPI fluorescence.

zygous F_1 animals were mated to produce *Lis1*^{GT/GT}/transgenic^{Tpos} 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 *Lis1*^{GT/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 *Lis1*^{GT/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 *Lis1*^{GT/-}/*PGK2/L3*^{Tpos} mice, while LIS1 antibody detects LIS1 protein in all testicular cells. Moreover, the over-expression of LIS1 in ‘rescued’ males compared to *Lis1*^{GT/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^{GT/GT}/PGK2/L3^{Tpos}* (**B**) mice revealed normal spermatogenesis, while testis sections of 'rescued' *Lis1^{GT/GT}/PGK2/L3^{Tpos}* (**A**), *Lis1^{GT/GT}/hEF-1α/L15^{Tpos}* (**C**) and *Lis1^{GT/GT}/TNP2/L7^{Tpos}* (**D**) mice demonstrated severe impairment of spermatogenesis in most tubules. Sections through epididymides revealed a high number of spermatozoa in heterozygous males of *Lis1^{GT/GT}/hEF-1α/L15^{Tpos}* (**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** $\times 100$; **C, D** $\times 200$; **E, F** $\times 600$). **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^{GT/GT}/PGK2/L3^{Tpos}* 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^{GT/GT}/PGK2/L3^{Tpos}* 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^{GT/GT}/hEF-1α/L15* and *Lis1^{GT/GT}* mice confirmed an increase in LIS1 protein level in transgenic mice compared to *Lis1^{GT/GT}* animals.

Fig. 6. Comparison of spermatogenesis defects in *Lis1*^{GT/-}/*PGK2*/*L3*^{Tpos}, *Lis1*^{GT/GT}/*PGK2*/*L3*^{Tpos}, *Lis1*^{GT/GT}/*hEF-1α*/*L15*^{Tpos} (all on mixed FVB/N/NMRI background), NMRI wild-type and *Lis1*^{GT/GT}/NMRI mice. Testis sections of 3 animals per genotype were analyzed. No significant difference was found in NMRI wild-type and *Lis1*^{GT/-}/*PGK2*/*L3*^{Tpos} or *Lis1*^{GT/GT}, *Lis1*^{GT/GT}/*PGK2*/*L3*^{Tpos} and *Lis1*^{GT/GT}/*hEF-1α*/*L15*^{Tpos} mice.

To compare spermatogenesis defects in ‘rescued’ *Lis1*^{GT/GT}/*PGK2*/*L3*^{Tpos}, *Lis1*^{GT/GT}/*hEF-1α*/*L15*^{Tpos} and *Lis1*^{GT/GT} in a more quantitative manner, testis cross-sections of three 46-day-old animals per genotype (*Lis1*^{GT/GT}, *Lis1*^{GT/GT}/*PGK2*/*L3*^{Tpos}, *Lis1*^{GT/-}/*PGK2*/*L3*^{Tpos}, *Lis1*^{GT/GT}/*hEF-1α*/*L15*^{Tpos}, *Lis1*^{GT/-}/*hEF-1α*/*L15*^{Tpos} 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 *Lis1*^{GT/-}/*PGK2*/*L3*^{Tpos} was found. ‘Rescued’ mice showed severe spermatogenesis defects as seen in *Lis1*^{GT/GT} males. Some tubules of ‘rescued’ mice showed mild hypospermatogenesis, while in *Lis1*^{GT/GT} all tubules were more severely affected, but these differences were not statistically significant.

As single sperm cells were found in epididymal sections of *Lis1*^{GT/GT}/*hEF-1α*/*L15*^{Tpos} males (fig. 5E), we counted sperm cells in caudae epididymides of *Lis1*^{GT/GT}/NMRI, *Lis1*^{GT/GT}/FVB/N, *Lis1*^{GT/GT}/129/Sv/Ola and ‘rescued’ animals *Lis1*^{GT/GT}/*PGK2*/*L3*^{Tpos}, *Lis1*^{GT/GT}/*hEF-1α*/*L15*^{Tpos} and *Lis1*^{GT/GT}/*hEF-1α*/*L19*^{Tpos}. 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 *Lis1*^{GT/GT} mice. Taken together, the overexpression of *Lis1* in meiotic and post-meiotic cells in testes of the gene trap line *Lis1*^{GT/GT} had no influence on spermatogenesis of these animals.

Discussion

Analysis of the Gene Trap Line *Lis1*^{GT/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 [Peterfy 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 *Lis1*^{GT/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* ‘Ex2/3’ 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 *Piwil-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

[Gold et al., 1983; McCarrey and Thomas, 1987]. 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-1 α -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-1 α -Lis1/L15* and *hEF-1 α -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-1 α* 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 cop-

ies 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 *PAFAH1B1* haploinsufficiency, which causes lissencephaly, submicroscopic duplication in 17p13.3 including *PAFAH1B1* 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 $\alpha 1$ and $\alpha 2$ of PAFAH1b. Both catalytic subunits are expressed in testis, and mutants lacking $\alpha 2$ or $\alpha 1$ and $\alpha 2$ subunits are infertile with no other apparent phenotype. According to Koizumi et al. [2003], $\alpha 1$ subunit-deficient mice are indistinguishable from wild-type mice, whereas $\alpha 2$ subunit-deficient mice show a reduction in testis size, but are fertile. However, $\alpha 1^{-/-}/\alpha 2^{-/-}$ 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 $\alpha 1$ subunit-deficient mice have normal fertility, while $\alpha 2$ -deficient mice are infertile. Spermatogenesis is disrupted at mid- or late pachytene stages of meiosis or early spermiogenesis. $\alpha 1^{-/-}/\alpha 2^{-/-}$ 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 $\alpha 2^{-/-}$ and $\alpha 1^{-/-}/\alpha 2^{-/-}$ males (suggested to be due to post-transcriptional control, as mRNA levels of *Lis1* are unaltered in $\alpha 2^{-/-}$ 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 $\alpha 1^{-/-}/\alpha 2^{-/-}$ mutants. Interestingly, the infertility phenotype of the double mutant mice ($\alpha 1^{-/-}/\alpha 2^{-/-}$) could be 'rescued' by generating triple mutant mice ($\alpha 1^{-/-}/\alpha 2^{-/-}/Lis1^{+/+}$). 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 *Lis1*^{GT/GT}, where heterozygous males (*Lis1*^{GT/+}) 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 $\alpha 2$ subunit in cultured mammalian CHO cells was found to induce centrosomal amplification and microtubule disorganization, while overexpression of the $\alpha 1$ 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 $\alpha 2$ subunit that cannot bind LIS1, suggesting that binding of the $\alpha 2$ subunit and LIS1 is a prerequisite for structural cell abnormality and that the quantitative balance of $\alpha 2$ and LIS1 are 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 (*Lis1*^{GT/GT}/PGK2/L3, *Lis1*^{GT/GT}/PGK2/L9, *Lis1*^{GT/GT}/hEF-1 α /L15 and *Lis1*^{GT/GT}/hEF-1 α /L19) remained infertile. 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 $\alpha 1^{-/-}/\alpha 2^{-/-}$ (catalytic subunits of PAFAH1b) could be rescued by generating triple mutant mice ($\alpha 1^{-/-}/\alpha 2^{-/-}/Lis1^{+/+}$), 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 caudae epididymidis 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 (*hEF-1 α* 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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References

- Alexander GM, Erwin KL, Byers N, Deitch JS, Augelli BJ, et al: Effect of transgene copy number on survival in the G93A SOD1 transgenic mouse model of ALS. *Brain Res Mol Brain Res* 130:7–15 (2004).
- Bi W, Sapir T, Shchelochkov OA, Zhang F, Withers MA, et al: Increased LIS1 expression affects human and mouse brain development. *Nat Genet* 41:168–177 (2009).
- Cahana A, Jin XL, Reiner O, Wynshaw-Boris A, O'Neill C: A study of the nature of embryonic lethality in *Lis1*^{-/-} mice. *Mol Reprod Dev* 66:134–142 (2003).
- Faulkner NE, Dujardin DL, Tai CY, Vaughan KT, O'Connell CB, et al: A role for the lissencephaly gene *LIS1* in mitosis and cytoplasmic dynein function. *Nat Cell Biol* 11:784–791 (2000).

- Friede RL: Developmental Neuropathology, 2nd ed (Springer Verlag, Berlin 1989).
- Furuchi T, Masuko K, Nishimune Y, Obinata M, Matsui Y: Inhibition of testicular germ cell apoptosis and differentiation in mice misexpressing Bcl-2 in spermatogonia. *Development* 122:1703–1709 (1996).
- Gambello MJ, Darling DL, Yingling J, Tanaka T, Gleeson JG, Wynshaw-Boris A: Multiple dose-dependent effects of Lis1 on cerebral cortical development. *J Neurosci* 23:1719–1729 (2003).
- Gold B, Fujimoto H, Kramer JM, Erickson RP, Hecht NB: Haploid accumulation and translational control of phosphoglycerate kinase-2 messenger RNA during mouse spermatogenesis. *Dev Biol* 98:392–399 (1983).
- Hattori M, Adachi H, Aoki J, Tsujimoto M, Arai H, Inoue K: Cloning and expression of a cDNA encoding the beta-subunit (30-kDa subunit) of bovine brain platelet activating factor acetylhydrolase. *J Biol Chem* 270:31345–31352 (1995).
- Hirotsune S, Fleck MW, Gambello MJ, Bix GJ, Chen A, et al: Graded reduction of Pafah1b1 (Lis1) activity results in neuronal migration defects and early embryonic lethality. *Nat Genet* 19:333–339 (1998).
- Hogan B, Constantini F, Lacy E: Manipulating the Mouse Embryo: A Laboratory Manual. (Cold Spring Harbor Lab Press, New York 1986).
- Kato M, Dobyns WB: Lissencephaly and the molecular basis of neuronal migration. *Hum Mol Genet* 12 Spec No 1:R89–R96 (2003).
- Kerjan G, Gleeson JG: Genetic mechanisms underlying abnormal neuronal migration in classical lissencephaly. *Trends Genet* 23:623–630 (2007).
- Kleene KC, Distel RJ, Hecht NB: Translational regulation and deadenylation of a protamine mRNA during spermiogenesis in the mouse. *Dev Biol* 105:71–79 (1987).
- Koizumi H, Yamaguchi N, Hattori M, Ishikawa TO, Aoki J, et al: Targeted disruption of intracellular type I platelet activating factor-acetylhydrolase catalytic subunits causes severe impairment in spermatogenesis. *J Biol Chem* 278:12489–12494 (2003).
- Kramer JM, Erickson RP: Development program of PGK-1 and PGK-2 isozymes in spermatogenic cells of the mouse: specific activities and rates of synthesis. *Dev Biol* 87:37–45 (1981).
- Ksiazek I, Burkhardt C, Lin S, Seddik R, Maj M, et al: Synapse loss in cortex of agrin-deficient mice after genetic rescue of perinatal death. *J Neurosci* 27:7183–7195 (2007).
- Kumar TR, Low MJ, Matzuk MM: Genetic rescue of follicle-stimulating hormone beta-deficient mice. *Endocrinology* 139:3289–3295 (1998).
- Kurrasch DM, Huang J, Wilkie TM, Repa JJ: Quantitative real-time polymerase chain reaction measurement of regulators of G-protein signaling mRNA levels in mouse tissues. *Methods Enzymol* 389:3–15 (2004).
- Leventer RJ, Cardoso C, Ledbetter DH, Dobyns WB: LIS1: from cortical malformation to essential protein of cellular dynamics. *Trends Neurosci* 24:489–492 (2001).
- McCarrey JR, Thomas K: Human testis-specific PGK gene lacks introns and possesses characteristics of a processed gene. *Nature* 326:501–505 (1987).
- Meng X, Lindahl M, Hyvönen ME, Parvinen M, de Rooij DG, et al: Regulation of cell fate decision of undifferentiated spermatogonia by DNF. *Science* 287:1489–1493 (2000).
- Montaguelli X: Effect of the genetic background on the phenotype of mouse mutations. *J Am Soc Nephrol* 11:101–105 (2000).
- Nayernia K, Boehm D, Topaloglu O, Schlueter G, Engel W: Rat transition nuclear protein 2 regulatory region directs haploid expression of reporter gene in male germ cells of transgenic mice. *Mol Reprod Dev* 58:368–375 (2001).
- Nayernia K, Vauti F, Meinhardt A, Cadenas C, Schweyer S, et al: Inactivation of a testis-specific Lis1 transcript in mice prevents spermatid differentiation and causes male infertility. *J Biol Chem* 278:48377–48385 (2003).
- Peterfy M, Gyuris T, Basu R, Takacs L: Lissencephaly-1 is one of the most conserved proteins between mouse and human: a single amino-acid difference in 410 residues. *Gene* 150:415–416 (1994).
- Peterfy M, Gyuris T, Grosshans D, Cuaserna C, Takacs L: Cloning and characterization of cDNAs and the gene encoding the mouse platelet-activating factor acetylhydrolase Ib alpha subunit/lissencephaly-1 protein. *Genomics* 47:200–206 (1998).
- Robinson MO, McCarrey JR, Simon MI: Transcriptional regulatory regions of testis-specific PGK2 defined in transgenic mice. *Proc Natl Acad Sci USA* 86:8437–8441 (1989).
- Smith DS, Niethammer M, Ayala R, Zhou Y, Gambello MJ, et al: Regulation of cytoplasmic dynein behaviour and microtubule organization by mammalian Lis1. *Nat Cell Biol* 11:767–775 (2000).
- Tascou S, Nayernia K, Meinhardt A, Schweyer S, Engel W, et al: Targeted expression of SV40 large tumour antigen (TAg) induces a transient enhancement of spermatocyte proliferation and apoptosis. *Mol Hum Reprod* 7:1123–1131 (2001).
- Tinkle BT, Jay G: Molecular biology, analysis, and enabling technologies: Analysis of transgene integration, in Pinkert CA (ed): *Transgenic Animal Technology: A Laboratory Handbook*, pp 459–471 (Academic Press, New York 2002).
- Weis J, Fine SM, David C, Savarirayan S, Sanes JR: Integration site-dependent expression of a transgene reveals specialized features of cells associated with neuromuscular junctions. *J Cell Biol* 113:1385–1397 (1991).
- Woldeyesus MT, Britsch S, Riethmacher D, Xu L, Sonnenberg-Riethmacher E, et al: Peripheral nervous system defects in erbB2 mutants following genetic rescue of heart development. *Genes Dev* 13:2538–2548 (1999).
- Wynshaw-Boris A: Lissencephaly and LIS1: insights into the molecular mechanisms of neuronal migration and development. *Clin Genet* 72:296–304 (2007).
- Yamaguchi N, Koizumi H, Aoki J, Natori Y, Nishikawa K, et al: Type I platelet-activating factor acetylhydrolase catalytic subunits over-expression induces pleiomorphic nuclei and centrosome amplification. *Genes Cells* 12:1153–1161 (2007).
- Yan W, Assadi AH, Wynshaw-Boris A, Eichele G, Matzuk MM, Clark GD: Previously uncharacterized roles of platelet-activating factor acetylhydrolase 1b complex in mouse spermatogenesis. *Proc Natl Acad Sci USA* 100:7189–7194 (2003).