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Inactivation of a Testis-specific Lis1 Transcript in Mice Prevents Spermatid Differentiation and Causes Male Infertility*

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Karim Nayernia‡, Franz Vauti§, Andreas Meinhardt¶, Christina Cadenas§, Stephan Schweyer‖, Barbara I. Meyer, Iris Schwandt‡, Kamal Chowdhury**, Wolfgang Engel‡, and Hans-Henning Arnold§‡‡**

*From the ‡Institute for Human Genetics and ‖Department of Pathology, University of Göttingen, Göttingen 37073, the §Department of Cell and Molecular Biology, Technical University Braunschweig, Spielmannstrasse7, Braunschweig 38106, the ¶Department of Anatomy and Cell Biology, University of Giessen, Giessen 35378, and the **Max-Planck-Institute for Biophysical Chemistry, Göttingen 37077, Germany*

Lis1 protein is the non-catalytic component of platelet-activating factor acetylhydrolase 1b (PAF-AH 1B) and associated with microtubular structures. Hemizygous mutations of the *LIS1* gene cause type I lissencephaly, a brain abnormality with developmental defects of neuronal migration. Lis1 is also expressed in testis, but its function there has not been determined. We have generated a mouse mutant (*LIS1*$^{GT/GT}$) by gene trap integration leading to selective disruption of a Lis1 splicing variant in testis. Homozygous mutant males are infertile with no other apparent phenotype. We demonstrate that Lis1 is predominantly expressed in spermatids, and spermiogenesis is blocked when Lis1 is absent. Mutant spermatids fail to form correct acrosomes and nuclei appear distorted in size and shape. The tissue architecture in mutant testis appears severely disturbed displaying collapsed seminiferous tubules, mislocated germ cells, and increased apoptosis. These results provide evidence for an essential and hitherto uncharacterized role of the Lis1 protein in spermatogenesis, particularly in the differentiation of spermatids into spermatozoa.

Type 1 lissencephaly is an autosomal dominant congenital disorder in humans, characterized by a smooth surface of the brain due to abnormal neuronal migration during early development (1). Humans afflicted by isolated lissencephaly carry hemizygous mutations of the lissencephaly 1 gene (*LIS1*), suggesting that haplo-insufficiency of *LIS1* causes the disease (2–4). Mice with one inactive *LIS1* allele also display disorganization of the brain cortex, hippocampus, and the olfactory bulb, whereas homozygous Lis1 null mice die soon after implantation during early embryogenesis (5).

The *LIS1* gene encodes a protein (Lis1) with seven WD-40 repeats at the N terminus (2). The protein has been identified biochemically as the non-catalytic subunit of the trimeric type I platelet-activating factor acetylhydrolase (PAF-AH 1B)[1] that inactivates platelet-activating factor (PAF) (3). PAF is a potent signaling phospholipid in various tissues (6, 7), including the central nervous system (8–12) and the reproductive organs (13, 14). PAF has been implicated in sperm motility (15, 16) and acrosomal function (17) but not in spermatogenesis. It has also been suggested that PAF plays a role in the pathogenesis of testicular ischemia (18). The intracellular type I PAF-AH is a G protein-like complex with two catalytic subunits (alpha1 and alpha 2) and the regulatory β-subunit, Lis1 (19). PAF-AH 1B is expressed in developing brain structures (20, 21) and in testis (22), supporting a role in these organs. Lis1 protein also associates with tubulin (23), cytoplasmic dynein (24, 25), and NudE (26–28) and localizes to the cell cortex and to mitotic kinetochores suggesting microtubule-associated functions, such as cell division, chromosome segregation, nuclear migration, and vesicular transport. Overexpression of Lis1 in cultured cells interferes with orientation of spindles and progression into mitosis, whereas blocking of Lis1 perturbs the metaphase plate (24). Whether the apparently different biochemical interactions of Lis1 are functionally related remains to be seen.

Proteins homologous to vertebrate Lis1 have been identified in several organisms, including *Saccharomyces cerevisiae* (29), *Aspergillus nidulans* (30), *Caenorhabditis elegans* (31), and *Drosophila* (32). In yeast and *A. nidulans* Lis1 homologues play a role in dynein-mediated nuclear migration (30, 33–35). In the mouse, Lis1 also interacts with the nuclear migration proteins NUDC (36) and mNudE-L (37) suggesting a similar role. Abrogation of lis-1 in *C. elegans* results in embryonic lethality, sterility, altered vulval morphology, uncoordinated movement, and nuclear positioning defects during early embryonic cell divisions (31). Lis1 in *Drosophila* is also essential for normal development (32). Ovarian mutant clones of Lis1 in the fly indicated that it is required for germ line cell division and oocyte differentiation, supporting the notion that Lis1 interacts with the dynein complex to regulate the function of the membrane skeleton, necessary for nuclear and neuronal migration. Lis1 also functions in dendritic elaboration and axonal transport in *Drosophila* and in cultured neurons (38). Collectively, these data argue that Lis1 has been highly conserved during evolution and may exert similar cellular functions in various developmental processes.

Murine Lis1 mRNA is widely expressed in many cell types, but certain splicing and polyadenylation variants are differentially found in adult brain, heart, and testis (39). Particularly in testis, an alternatively spliced transcript exists that contains

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‡‡ To whom correspondence should be addressed. Tel.: 49-531-391-5735; Fax: 49-531-391-8178; E-mail: h.arnold@tu-bs.de.

[1] The abbreviations used are: PAF-AH 1B, platelet-activating factor acetylhydrolase type I; Mops, 4-morpholinepropanesulfonic acid; RT, reverse transcription; PBS, phosphate-buffered saline; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; TBS, Tris-buffered saline; FCS, fetal calf serum; P, postnatal days; E, embryonic days; EM, electron microscopy.

the additional exon 2a as part of the 5′-untranslated leader sequence. The functional role of Lis1 in organs other than brain has not been determined, partly due to early embryonic lethality of Lis1-null mutants. We have taken advantage of a gene trap mouse line that carries a mutagenic insertion within the *LIS1* gene causing selective disruption of a testis-specific Lis1 transcript. This permitted us to explore the role of Lis1 in the male germ line. Here we report that Lis1-deficient male mice are infertile, whereas females show normal fertility. The defect in spermatogenesis leads to a blockade of spermatid differentiation and severely distorted tissue architecture of the seminiferous tubules. Mutant spermatids fail to form correct acrosomes and frequently do not undergo appropriate nuclear condensation. The mutant phenotype also presents significantly increased apoptosis of germ cells in adult testis. Thus, our results reveal a unique and novel function of Lis1 in spermatogenesis.

EXPERIMENTAL PROCEDURES

Generation of the LIS1 Gene Trap Mouse—The exon gene trap vector pKC421 was constructed from the plasmid pGT1.8 IRESβgeo (52) by deletion of the engrailed 2 (En-2) splice acceptor site with BamHI/BglII digestion and by removal of an SalI site downstream of theSV40 poly (A) addition signal. The ES cell line 2A-53 containing an insertion of the gene trap vector within the *LIS1* gene was generated as described previously (53). The 129/Sv-derived ES cell clone was used in morula aggregations according to published procedures to obtain chimeric male founders that were mated with outbred *NMRI* females (53). Heterozygous progeny was mated to maintain the allele. For genotyping by Southern blot analysis, DNA was extracted from tail biopsies and digested with SacI restriction enzyme. Blots on Hybond N membranes (Amersham Biosciences, Freiburg, Germany) were hybridized in 6× SSC, 5× Denhardt's, 0.1% SDS, 100 μg/ml denatured salmon sperm DNA at 65 °C overnight using an SacI/fragment of the second intron of the *LIS1* gene as probe (see Fig. 1). Hybridized filters were washed twice at 65 °C with 0.2× SSC containing 0.1% SDS. Wild type and mutant alleles are represented by 4.3- and 8-kb SacI fragments, respectively.

Construction of Genomic Phage Library—To clone the site of vector integration, a genomic phage library of a heterozygous mouse was generated in the λDASH-II vector (Stratagene) according to standard procedures. Two independent recombinant phage clones carrying inserts of 21 and 17.2 kb were isolated with LacZ- and neo-specific hybridization probes. Regions flanking the integrated vector were sequenced and searched using Blast against mouse genome databases.

Northern Blot Analysis—Total RNA was isolated from testis and brain of wild type and mutant mice at various postnatal stages using the Total RNA Isolation Reagent (Biomol) according to the manufacturer's instructions. RNA samples (30 μg) were denatured at 65 °C for 10 min in loading buffer (50% formamide, 13 Mops buffer, 6.5% formaldehyde) and run on 0.8% agarose gels containing 0.12% formaldehyde. Electrophoresis and RNA blotting was performed according to standard procedures (54). Blots were hybridized with radioactively labeled testis-specific Lis1 cDNA. The human elongation factor 2 cDNA probe was used as loading control (55).

RT-PCR Analysis—Total RNA (1 μg) was reverse-transcribed in a final volume of 20 μl containing 200 units of Superscript reverse transcriptase (Invitrogen, Karlsruhe, Germany), 40 units of RNasin (Roche Applied Science, Mannheim, Germany), and oligo-dT primer. PCR was carried out with 2 μl of cDNA, 10 pmol each of forward and reverse primers, and 3 units of *Taq* polymerase. Cycling condition were 94 °C, 58 °C, and 68 °C for 30 s each. Glyceraldehyde-3-phosphate dehydrogenase was used as internal control. The glyceraldehyde-3-phosphate dehydrogenase 5′ primer was 5′-ACC ACA GTC CAT GCC ATC AC-3′; the glyceraldehyde-3-phosphate dehydrogenase 3′ primer was 5′-TCC ACC ACC CTG TTG CTG TA-3′.

Western Blot Analysis—Protein extracts from testis and brain were prepared in SE buffer containing 0.32 mol/liter sucrose, 1 mmol of EDTA, and 0.1% β-mercaptoethanol. For electrophoresis samples (50 μg/lane) were boiled for 10 min, briefly spun down at 500 rpm, and loaded onto an 8% polyacrylamide gel. Proteins were blotted onto a polyvinylidene difluoride membrane (Roche Applied Science) and probed with goat polyclonal anti-Lis-1 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) and rabbit polyclonal α-tubulin antibody (Sigma-Aldrich) as internal reference. Antibody reactions were visualized on filters with a 1:10,000 dilution of alkaline phosphatase-conjugated anti-goat or anti-rabbit immunoglobulin (Sigma-Aldrich) and incubated in 0.35 mg/ml nitro blue tetrazolium and 0.18 mg/ml 5-bromo-4-chloro-indolylphosphate substrate.

Analysis of Fertility—Reproductive capacity of Lis-$1^{GT/GT}$ and Lis-$1^{+/GT}$ males was determined by breeding with wild type and mutant females. Ten males of each genotype were mated with females for 6 months. Females were checked for vaginal plugs every day and separated for 21 days when positive. The total number of vaginal plugs and born offspring was counted for each male during the entire investigation period.

Histology—Testis and epididymis from mice of different postnatal age were fixed in Bouin's fixative for 48 h at room temperature. The fixative was removed with 70% ethanol for 2–3 days, and tissues were embedded in paraffin. Mounted sections (4–6 mm) were deparaffinized, rehydrated, and stained with hematoxylin-eosin, or used for immunohistochemistry. Spermatozoa were prepared and resuspended in PBS. Air-dried smears were fixed in methanol/acetone (1:1) for 5 min and used for immunohistochemistry.

Immunohistochemistry—Spermatozoa smears and sections of testis were incubated for 16–18 h at 4 °C with one of the following antibodies in a 1:200 dilution: goat anti-Lis-1 antibody (Santa Cruz Biotechnology), rabbit anti-OAM antibody (outer acrosomal membrane), rabbit anti-acrosin antibody, rabbit anti-Tnp-2 antibody, goat anti-HSP90α antibody (Santa Cruz Biotechnology). For co-localization studies, sperm smears were incubated with a 1:200 dilution of anti-Lis-1 antibody and a 1:300 dilution of rabbit anti-OAM or rabbit anti-acrosin antibodies. Slides were then washed three times with PBS and incubated for several hours at room temperature with 1:100 dilution of fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Sigma-Aldrich Chemie, Deisenhofen, Germany). Double immunostainings were performed with a mixture of 1:100 fluorescein isothiocyanate-conjugated goat anti-mouse IgG and Cy3-conjugated anti-rabbit IgG (Sigma-Aldrich). After three further washes with PBS, slides were mounted in 4′,6-diamidino-2-phenylindole mounting solution (Vector Laboratories Inc., Burlingame, CA).

Electron Microscopy—For conventional electron microscopy, mouse testis was fixed with 1% paraformaldehyde and 3.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 8–12 h. Fixed testes were cut into small pieces and thoroughly washed over 3–4 days at 4 °C in 0.1 M cacodylate buffer containing 0.1 M saccharose. Tissue fragments were then treated with 1% OsO_4 in cacodylate buffer for 2 h, washed three times, dehydrated, and embedded in epoxy resin. Ultrathin sections were contrasted using uranyl acetate and lead citrate and examined with a Leo 906 electron microscope. For immunoelectron microscopy wild type testes fragments were fixed in 4% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M phosphate buffer for 4 h. Specimens were washed in 0.1 M phosphate buffer supplemented with 7% sucrose, subsequently dehydrated in increasing concentrations of ethanol, and embedded in LR White resin. Ultrathin sections were blocked in 2% bovine serum albumin and 0.04 M glycine in phosphate-buffered saline (PBS, pH 7.4) for 1 h at room temperature. The anti-Lis-1 antibody was incubated at room temperature for 1 h at a 1:200 dilution in 2% bovine serum albumin in PBS. After thorough washing, specimens were decorated with the rabbit anti-goat secondary antibody coated on 15-nm colloidal gold particles (dilution 1:20; Biocell) for 1 h. Finally, the sections were contrasted with 10% uranyl acetate for 20 min in the dark. Omission of the primary antibody served as a negative control.

Nuclear DNA Fragmentation Labeling (TUNEL)—Testes were removed from 90-day-old wild type and Lis-$1^{GT/GT}$ mice, fixed in formalin solution, embedded in paraffin, and cut into 5-μm sections on which hematoxylin-eosin stainings and TUNEL assays were performed. To determine apoptotic cells sections were dewaxed prior to digestion with 0.7 unit/ml proteinase K (Sigma, Deisenhofen/Germany) in Tris-buffered saline (50 mM Tris-HCl, 150 mM NaCl, pH 7.5, TBS), supplemented with 2 mM $CaCl_2$. Sections were then incubated in TBS containing 10% fetal calf serum (FCS) and 0.3% H_2O_2 to block endogenous peroxidase activity. Subsequently, slides were rinsed with TBS and incubated for 60 min at 37 °C in reaction buffer for terminal transferase (Roche Applied Science, Germany), containing 50 μl of labeling buffer (250 units/ml terminal transferase, 20 μl/ml 10× digoxigenin-DNA labeling mix, and 1 mM $CoCl_2$). After labeling, sections were washed in TBS, blocked with 10% FCS (Roche Applied Science) for 15 min, and then incubated with a rabbit horseradish peroxidase-conjugated $F(ab)_2$ fragment against digoxigenin (Dako/Hamburg, Germany) for 60 min. The horseradish peroxidase-conjugated $F(ab)_2$ fragment was applied in a 1:200 dilution with TBS containing 10% FCS. Nuclear signals were visualized using 3,3′-diaminobenzidine. Negative controls were performed without terminal transferase. Lymph nodes with reactive follic-

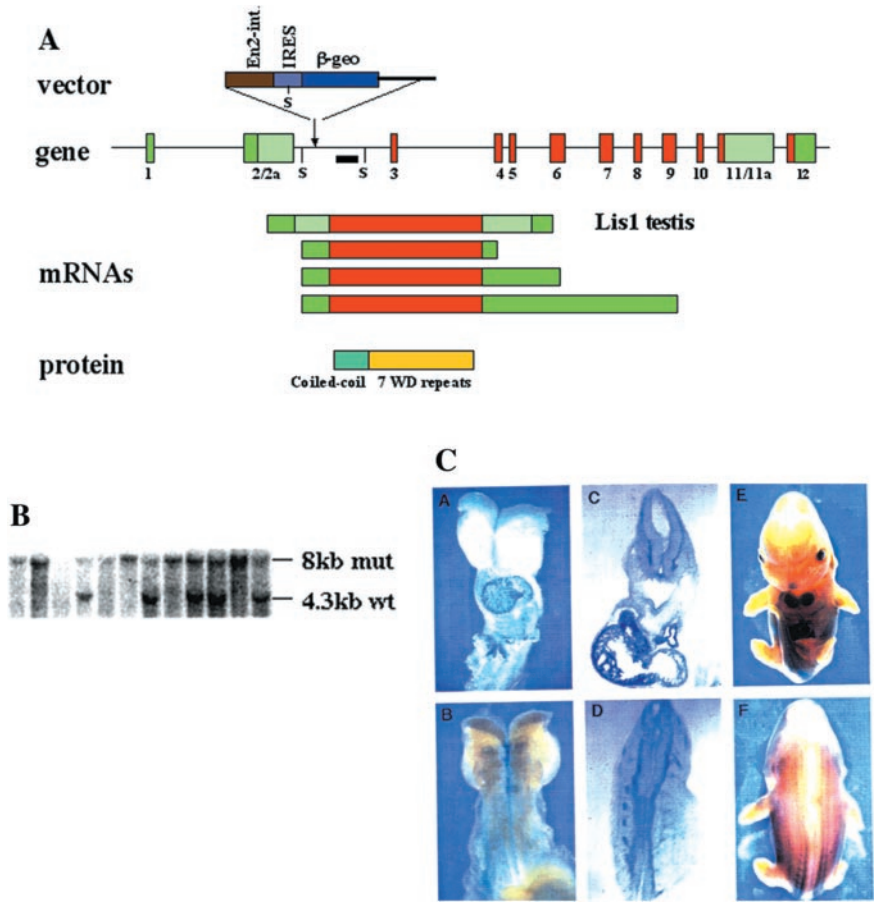

FIG. 1. **Scheme illustrating the organization of the *LIS1* gene, its various transcripts, and the domain structure of the protein.** *A*, the gene trap vector and its site of integration in the second intron are shown. Coding exons are labeled in *red*, non-coding exons in *dark green*, and alternatively spliced exons in *light green*. The coiled-coil and seven WD-40 repeat domains in Lis1 protein are indicated. *B*, Southern blot containing DNA isolated from postnatal pups of heterozygous parents. DNA was digested with SacI and hybridized with the probe shown in *A* (*black bar*). Note that $Lis1^{+/GT}$ and $Lis1^{GT/GT}$ animals were born alive. *C*, LacZ staining of $Lis1^{+/GT}$ mouse embryos at E8.5 (*panels A* and *B*) and E12.5 (*panels E* and *F*) on whole-mounts, and sections of E10.5 embryos (*panels C* and *D*). Strong expression is observed in heart, neural tube, and dorsal root ganglia.

ular hyperplasia were used as positive control. Statistical significance of the obtained data was determined using the Man Whitney *U* test with $p < 0.05$ considered to be significant.

RESULTS

Integration of a Gene Trap Vector in the LIS1 Locus Results in Male Infertility—In a gene trap approach using an exon trap vector, we isolated the embryonic stem (ES) cell clone 2A-53 from which a stable mouse line was generated. The trapped gene was most abundantly expressed in heart, neural tube, brain, and dorsal root ganglia during embryogenesis (Fig. 1*C*). 5′ Rapid amplification of cDNA ends from known vector sequences failed to identify a fusion transcript in RNA from heart and brain. Therefore, the gene trap vector, including flanking genomic sequences, was cloned. Sequence analysis of two independently isolated phage clones showed that the exon trap vector had unintentionally integrated in the second intron of the mouse *LIS1* gene (Fig. 1*A*). RT-PCR on RNA from the ES cell clone 2A-53 identified a transcript containing the second exon of the *LIS1* gene spliced to a cryptic splice acceptor site present within the internal ribosomal entry site sequence of the vector. This aberrant splicing event generates the β-Geo mRNA and explains why the trapped ES cell clone could be obtained under Geneticin selection.

We next generated mice homozygous for the gene trap integration ($Lis1^{GT/GT}$). Offspring of heterozygous parents displayed normal Mendelian distribution of genotypes and no apparent pathological phenotype (Fig. 1*B*). In particular, Nissl staining of brains from adult mutants failed to show any signs of lissencephaly and compound heterozygotes with a Lis1-defective allele had no augmentation of the brain phenotype (data not shown). These observations suggested that the gene trap insertion had not generally disrupted the *LIS1* gene. However, subsequent breeding indicated that homozygous mutant males were consistently infertile, whereas mutant females reproduced normally (Table I). Mating with homozygous males yielded vaginal plugs, but sperm could not be recovered from the uterus and no pregnancies were recorded. In line with this result, we found that testes of homozygous males were considerably smaller (~50%) and the epididymis contained essentially no spermatozoa (Fig. 2*A*). To determine the onset of the phenotype in mutant mice, we histologically evaluated testes from postnatal days 15–90 (P15–P90). Sections of mutant testis initially (P14–P45) exhibited intact seminiferous tubules of normal diameter and germ cells as well as Sertoli and Leydig cells present at the appropriate locations (Fig. 2*B*). Notably, spermatogonia at the lamina propria, spermatocytes of the post-pachytene stage, and round spermatids at the luminal side of seminiferous tubules were observed until P45 when elongating spermatids begin to accumulate in the inner lining of the seminiferous epithelium. At postnatal P90, however, round and elongating spermatids were released prematurely from the epithelium and located inside the lumen of tubules, in contrast, to wild type spermatids assembling correctly at the luminal surface of seminiferous tubules. At this postnatal stage also the tubular structure seemed to have collapsed in the mutant, lacking the epithelial architecture and a clearly visible lumen. Moreover, only very few spermatozoa were present in mutant testis as compared with wild type (Fig. 2*B*) and the residual ones appeared in small clusters and frequently abnormal (data not shown). These results then suggested that the gene trap mutation affected the maintenance phase of spermatogenesis, including terminal differentiation of spermatids, but apparently did not interfere with the initiation phase up to the generation of spermatids. To determine the distribution of sperm progenitor cells in a more quantitative fashion, we investigated the expression of the spermatocyte-specific marker

TABLE I
Fertility of wild type, LIS1$^{+/GT}$, and LIS1$^{GT/GT}$ mutant mice

Mating of genotypes			
Male[a]	Female[a]	Mice born	Litter size[b]
LIS$^{+/+}$	*LIS*$^{+/+}$	168	7.3
LIS$^{+/+}$	*LIS1*$^{+/GT}$	176	6.6
LIS$^{+/+}$	*LIS1*$^{GT/GT}$	191	7.6
LIS1$^{+/GT}$	*LIS*$^{+/+}$	159	7.1
LIS1$^{GT/GT}$	*LIS*$^{+/+}$	0	0

[a] Age-matched 3- to 9-month-old animals were used for crossing.
[b] Mean of more than 20 litters.

transcripts, testis-specific phosphoglycerate kinase (Pgk-2) (40), and proacrosin (ACR) (41), as well as the spermatid-specific markers, transition protein 2 (Tnp2) (42), protamin 2 (Prm-2) (43), and Hook1 (44) in testis of wild type, heterozygous, and homozygous mice at 45 days of age. As shown in Fig. 2*C*, all transcripts were expressed at similar levels in wild type and mutant animals confirming that germ cell progenitors, including spermatids, were normally formed. From these results we concluded that the gene trap mutation causes a blockade of late spermatid differentiation resulting in a severe reduction of mature gametes. However, Lis1 is not essential for testis development, because testis histology during early postnatal stages appeared normal in the mutant and spermatogonia, spermatocytes, and round spermatids were formed in seminiferous tubules, whereas the late transition to spermatozoa essentially failed to occur.

To evaluate the disruption of tissue architecture and dislocation of spermatids from seminiferous tubules in the mutant, immunohistological stainings were performed on testis sections of adult mice using the anti-Tnp-2 and anti-Hsp90α antibodies that specifically recognize spermatids and spermatogonia, respectively. Significantly, spermatogonia that are normally found at the base of the seminiferous epithelium in close association with the lamina propria in wild type were misplaced toward the lumen of tubules in mutant testis (Fig. 3*A*). Likewise, the normal localization of spermatids in the apical region of the tubules was markedly changed in the mutant and spermatids appeared mostly round rather than elongated (Fig. 3*A*). Because the general histology of adult mutant testis argued for an overall impairment of spermatogenesis, possibly as a consequence of the primary defect in spermatid differentiation, we wondered whether apoptosis might be increased in mutant testis. Using the TUNEL assay, significantly elevated numbers of apoptotic cells were observed in mutant testis as compared with wild type (Fig. 3*B*). The programmed cell death was not limited to spermatids but also affected other germ cells. These observations were consistent with the interpretation that blocking differentiation of spermatids leads to disruption of seminiferous tubules and results in augmented apoptosis of spermatogenic cells.

The Gene Trap Integration Abrogates Expression of a Testis-specific Transcript—The murine *LIS1* gene consists of 12 exons with the translational start codon located in exon 3 (see Fig. 1). It was shown previously that in mouse multiple Lis1 mRNAs arise by different polyadenylation and alternative splicing (39). In testis, a 2.3-kb transcript is present that utilizes an alternative splice donor site within the second intron to include exon 2a sequence in mRNA (Fig. 1*A*). To evaluate whether Lis1 transcripts were affected by the gene trap integration, Northern blot analysis was performed with testis RNA from wild type and mutant animals, and from various mouse mutant strains that are arrested at discrete stages of spermatogenesis (45). Using Lis1 cDNA as hybridization probe, the 2.3-kb transcript was markedly reduced in testis of homozygous mutants as compared with wild type (Fig. 4*A*). The same transcript was

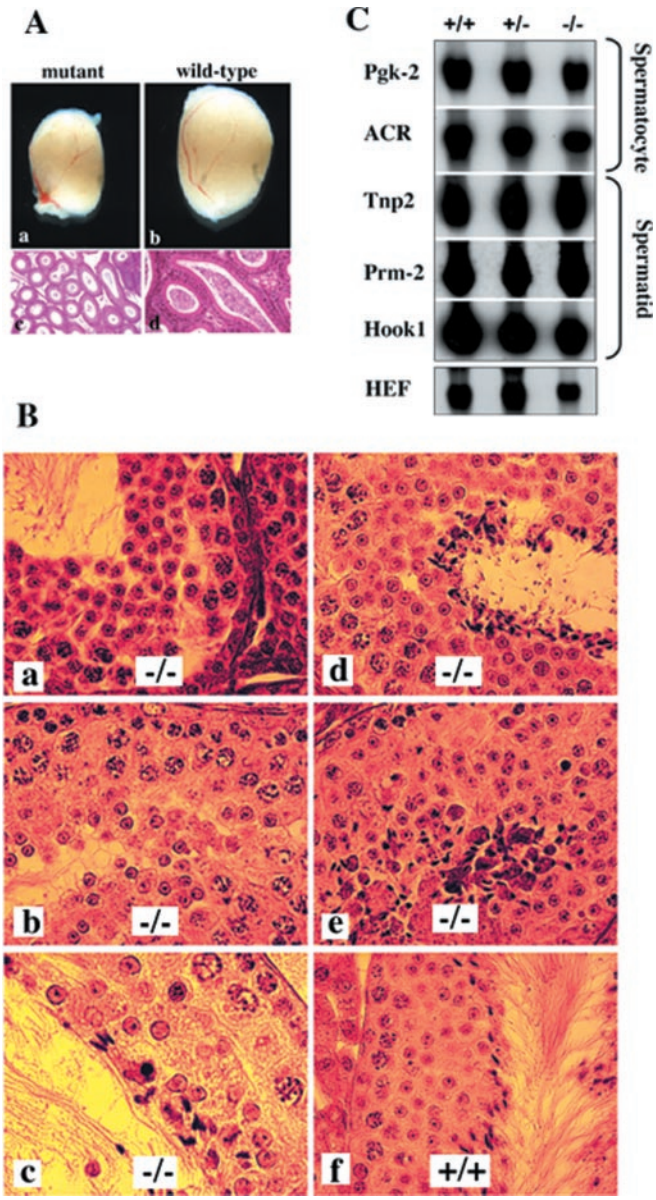

FIG. 2. **Macroscopic and histological examination reveals phenotypic alterations of testis from Lis1$^{GT/GT}$ mutant animals.** *A*, testis size is severely reduced (50% of wild type) in the homozygous mutant (*panels a* and *b*). Sections through epididymis demonstrate tubules of smaller diameter that are devoid of spermatozoa in the mutant (*panel c*) in contrast to wild type males (*panel d*). *B*, histological sections through testes from homozygous mutant animals at postnatal days 15 (*a*), 25 (*b*), 35 (*c*), 45 (*d*), and 90 (*e*). Control section of wild type testis at postnatal day 90 is shown for comparison (*f*). Note the fairly normal appearance of germ cells in the mutant until day 45 after birth with spermatocytes and spermatids present in seminiferous tubules (*a–d*). However, at day 90 the tissue architecture appears grossly disturbed in the mutant, and spermatids are clustered instead of lining the luminal epithelium of the seminiferous tubules (*e*). In contrast to wild type testis (*f*), no spermatozoa are present in the mutant. *C*, Northern blot analysis of testis RNA from P45 wild type, heterozygous, and homozygous mutant animals hybridized with probes for marker transcripts in spermatocytes and spermatids. Human elongation factor (HEF) was used to control RNA loading of the gel. Based on these molecular markers, levels of sperm progenitor cells are unaltered by the mutation.

present in the *qk/qk* strain and at lower level in *Tfm* and *Insl3*-deficient mice, which still generate some elongated spermatids at a reduced level. In contrast, *W/W^v* mutants lacking germ cells failed to express the 2.3-kb Lis1 transcript. Taken together these observations suggest that the 2.3-kb Lis1 transcript is expressed in germ cells, most likely in spermatids.

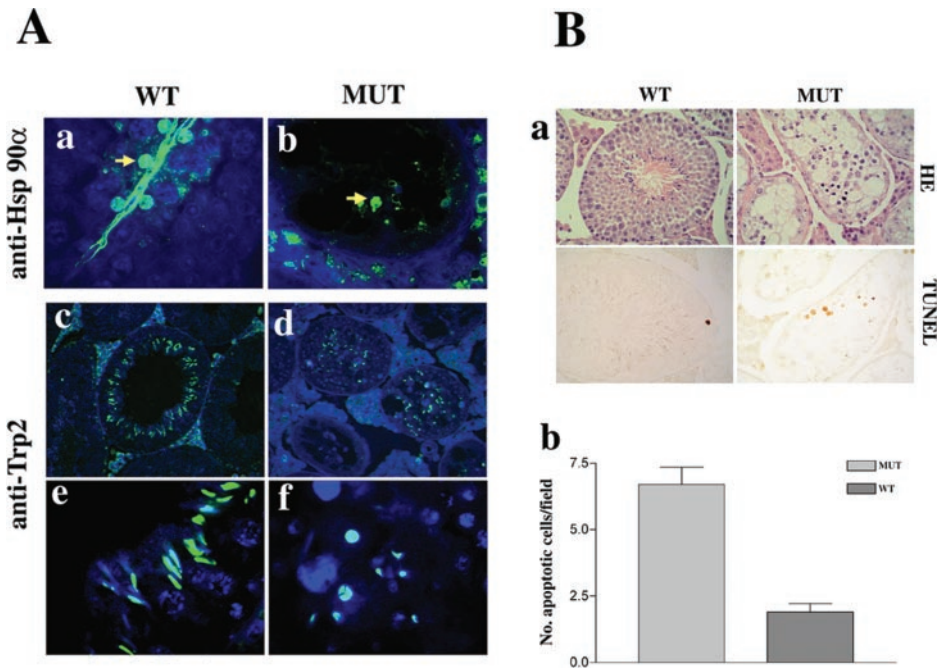

FIG. 3. **Germ cells in mutant testis are misplaced and undergo apoptosis.** *A*, immunohistochemical identification of spermatogonia and spermatids using Hsp90α- and Trp2-specific antibodies, respectively, illustrates the aberrant location of these cells in testis of adult $Lis1^{GT/GT}$ mutant mice (*panels b* and *d*) as compared with wild type (*panels a* and *c*). The higher power magnification shows the elongated shape of wild type spermatids (*e*) and mostly round spermatids in mutant testis (*f*). *B*, hematoxylin/eosin (*HE*) staining and Tunel assay on sections of adult wild type and mutant testis (*a*). Note the disrupted tubular structure, dilated vacuoles, and drastic reduction of spermatogenic cells in the mutant. A typical example of TUNEL labeling (*brown cells*) illustrates increased apoptosis of presumably spermatocytes in mutant testis. Statistical evaluation of apoptotic cells was performed by counting TUNEL-positive cells in at least 10 independent microscopic fields for each wild type and mutant (*b*). The mean value indicates significantly elevated numbers of apoptotic cells in the mutant.

Support for this notion came from a time-course experiment using an exon 2a-specific probe. As illustrated in Fig. 4*B*, the 2.3-kb Lis1 mRNA containing exon 2a began to accumulate to appreciable concentrations at postnatal day 20 in wild type testis, concomitant with the onset of spermatid generation. Significantly, this Lis1 transcript was almost absent in the homozygous mouse mutant and barely found in testis of *Tfm* and *olt/olt*, or W/W^v, mutant mice that predominantly contain spermatocytes or no germ cells, respectively. Lis1 transcripts were also absent from Leydig cells, representing an interstitial space cell type. These results were confirmed by RT-PCR, using primers from exons 2 and 3, although low concentrations of Lis1 transcripts were detected at earlier developmental stages of testis maturation compared with Northern blot analysis, probably due to higher sensitivity of the method (Fig. 4*D*). Thus, a low level of Lis1 transcription may occur in spermatocytes. We also compared Lis1 expression in testis and brain of wild type and mutant mice by RT-PCR using different primers that together covered 11 exons of the Lis1 mRNA. With all primers used, Lis1 expression in mutant testis was always markedly reduced compared with wild type, in contrast to Lis1 expression in brain that was largely unaffected by the mutation (Fig. 4, *C* and *E*). Interestingly, only the testis-specific Lis1 splice variant containing exon 2a was diminished in the mutant, whereas transcripts lacking exon 2a appeared unaltered in both, mutant brain, and testis (Fig. 4*C*). Taken together these results indicated that fortuitous integration of the gene trap vector into the second intron of the mouse *LIS1* gene had resulted in the selective abolition of a testis-specific transcript without affecting Lis1 expression in brain. This mutation offered the opportunity to investigate the role of Lis1 in spermatogenesis that had been precluded from previous studies due to embryonic lethality of conventionally targeted *LIS1* mouse mutants (5). Consistent with the expression pattern of Lis1 transcripts, mutant males did not show the lissencephaly brain phenotype but exhibited drastically reduced testis size and infertility.

Lis1 Protein Is Absent in Spermatids But Present in Myoid Stroma Cells of Mutant Testis—To investigate the distribution of Lis1 protein in testis, we performed Western blot and immunohistochemical analyses on sections with a Lis1-specific antibody. Protein extracts from testis of mice between 10 days after birth and adulthood revealed slightly increasing levels of Lis1 protein on Western blots (Fig. 5*A*, *panel a*). Sections through seminiferous tubules showed that the protein was exclusively detected in spermatids and not in earlier germ cell progenitors nor in Leydig and Sertoli cells, in good agreement with the RNA analysis (Fig. 5*A*, *panel b*). Comparative immunostaining of testis sections from wild type and mutant animals showed for the first time that Lis1 protein was also present in myoid stroma cells, but this expression was not affected by the mutation (Fig. 5*B*). In contrast, Lis1 protein in spermatids was entirely abolished in testis of mutant males. Western blot analysis confirmed the genotype-dependent reduction of Lis1 protein in testis, whereas protein levels in brain were unaltered (Fig. 5*C*). Thus, we have demonstrated that Lis1 protein accumulates in spermatids and the gene trap mouse $Lis1^{GT/GT}$ constitutes an effective Lis1 null mutant in the male germ line. These results argue that Lis1 is absolutely required in a cell autonomous fashion for differentiation of spermatids into spermatozoa.

Subcellular Localization of Lis1 Protein in Spermatids and Sperm Cells—The localization of Lis1 protein within spermatids and spermatozoa was determined by immunohistochemical staining of sections from adult mouse testis and on sperm preparations. In keeping with the notion that Lis1 interacts with a number of microtubule-associated proteins, including tubulin (23), cytoplasmic dynein (24, 25, 46), and NUDE (26–28, 47), in various organisms, we also analyzed the distribution of tubulin and dynein in spermatids by double immunofluorescence together with Lis1. As illustrated in Fig. 6*A*, dynein was found throughout the cytoplasm of early spermatids, partially co-localizing with Lis1 protein in a domain around the nucleus that might reflect the Golgi apparatus. Tubulin accumulated in a cap-like domain on the nucleus, and this area almost completely coincided with Lis1 suggesting that both proteins may interact directly or indirectly (Fig. 6*B*). These data were consistent with the view that Lis1 in spermatids associates with microtubular structures, possibly via microtubule-associated proteins, such as dynein, similar to what has been shown in other cell types. Immunogold labeling of Lis1 protein on electron micrographs of testis sections confirmed the association of Lis1 with microtubules, particularly in the temporary microtubular manchette that forms around the nucleus in spermatids

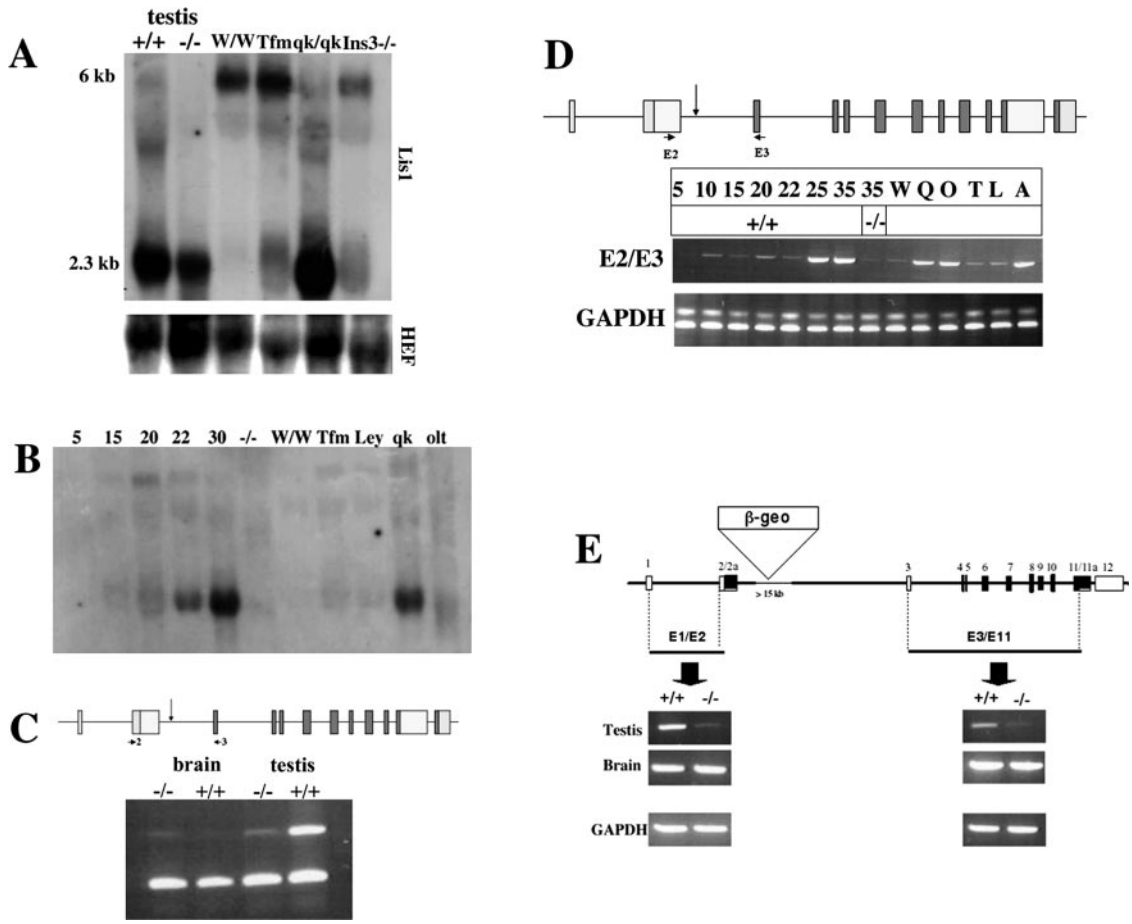

FIG. 4. **Expression analysis reveals reduction of Lis1 transcripts in testis of Lis1$^{GT/GT}$ mutant mice.** Northern blots were loaded with testis RNA from adult wild type and mutant mice (*A*), from adolescent mice between 5 and 30 days after birth (*B*), and from several mutant mouse strains that are arrested at different stages of spermatogenesis (*A* and *B*). Total Lis1 cDNA was used as a hybridization probe in *A*, and an exon 2a-specific probe was used in *B*. Hybridization with an human elongation factor (HEF)2 probe served as RNA loading control. Note that a 2.3-kb mRNA containing exon 2a accumulates in testis with the first wave of spermatogenesis, and this mRNA isoform was almost completely abolished in mutant testis, whereas another 2.3-kb variant lacking exon 2a remained unaffected (*A* and *B*). RT-PCR reactions were performed on RNA isolated from brain and testis of adult wild type and mutant animals (*C* and *E*), from testis of the indicated postnatal days, and from various mouse mutants (*D*). The exon-specific PCR primers are indicated in the schematic gene drawings shown at the *top* of each gel. The data confirm the dramatic reduction of the testis-specific Lis1 mRNA (containing exon 2a) in homozygous mutants. Glyceraldehyde-3-phosphate dehydrogenase-specific primers were used to calibrate the RNA input.

(Fig. 6*D*, *panels A′* and *C′*). Lis1-specific immunogold staining was also observed in the cytoplasm where it was partly associated with electron-dense structures, not however in microtubules of the tail (Fig. 6*D*, *panel B′*). These observations are in line with a microtubule-associated function of Lis1 in spermatids. We also found that Lis1 protein is still present in mouse sperm cells where it appears to co-localize with the acrosome (Fig. 6C).

Impairment of Acrosome and Tail Formation, and Nuclear Condensation in Lis1-deficient Spermatids—Electron microscopy of testis sections from early postnatal stages revealed no ultrastructural alterations of spermatogenic precursor cells, including pachytene spermatocytes (Fig. 7*B*, *panels a* and *d*) but severely affected spermatids at later postnatal stages (see Fig. 7*B*). The complex transition of initially round to elongated spermatids is accompanied by extensive condensation of the nucleus, establishment of the acrosome, and formation of the tail. To obtain information on possible Lis1 functions in these processes, we immunostained spermatids with anti-OAM antibody to visualize the acrosome. We observed multiple acrosomal vesicles that were loosely arranged around the nucleus in Lis1-deficient spermatids, in contrast to the single acrosomal vesicle in wild type cells (Fig. 7*A*). Consistent with a defect in the formation of the acrosome, electron microscopy of mutant testis frequently revealed vastly dilated acrosomes, which were not seen in wild type (Fig. 7*B*, *panels b*, *e*, and *f*). Moreover, mutant spermatids often contained two distinct vesicles with proacrosomal granules that already made contact with the nucleus, in contrast to normal spermatids in which the two vesicles typically fuse to a single, rounded proacrosomal vesicle prior to contacting the nuclear envelop (Fig. 7*B*, *panel c*). Theses observations indicated that Lis1 provides a crucial function for generating the acrosome concerning either the proper formation or fusion of vesicles from the Golgi cisternae or the correct transport and targeting of preacrosomal vesicles to the nuclear membrane, or both. As vesicular transport usually occurs on microtubules, we also stained spermatids for α-tubulin. Occasionally more than one tail-like protrusion was beginning to form in several locations of Lis1$^{GT/GT}$ spermatids, whereas only a single tail was generated in wild type cells (Fig. 7*A*). Moreover, EM images illustrated that tail formation was obviously impaired in mutant spermatids, because many of them contained the basal tail cuff at the point of insertion but failed to add axonemes to elongate the tail structure (Fig. 7*B*, *panels g* and *h*). To test for nuclear condensation we followed the distribution of Tnp-2 with a specific antibody. This protein accumulated normally in the condensed and hook-shaped nucleus of wild type spermatids but appeared dispersed in mul-

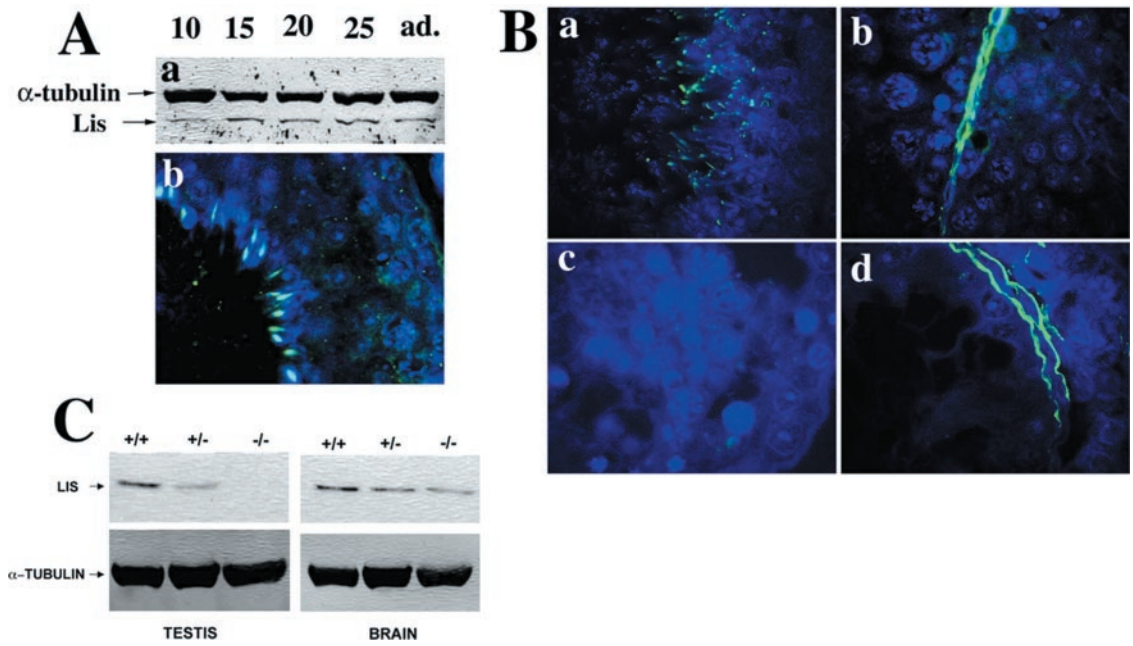

FIG. 5. **Determination of Lis1 protein in testis of wild type (*A*) and mutant (*B* and *C*) mice.** Western blots of protein extracts from testis of early postnatal stages (10–25 days) illustrate the accumulation of Lis1 protein in comparison to α-tubulin (*A*, *panel a*). Immunohistochemical staining of adult testis from a wild type mouse reveals Lis1 expression in spermatids located at the luminal side of a seminiferous tubule (*A*, *panel b*). Western blot of proteins extracted from brain and testis of adult mice shows decreased levels of Lis1 protein in testis of heterozygous and homozygous mutant mice (*C*). In contrast, levels of Lis1 protein are virtually unchanged in brain of the same animals. An anti-α-tubulin antibody was always used as internal reference. Comparison of wild type and mutant testis by immunohistochemistry (*B*) illustrates Lis1 protein present in spermatids of wild type (*a*) that is entirely lacking in the mutant (*c*). However, myoid stroma cells express comparable levels of Lis1 protein in both, wild type and mutant testis (*b* and *d*).

tiple patches throughout the still round nucleus of mutant cells reflecting that nuclear condensation and possibly condensation of chromatin had not properly taken place in the absence of Lis1 protein (Fig. 7*A*). This observation was confirmed by EM analysis of mutant spermatids revealing nuclei with poorly condensed chromatin (data not shown) and of grossly abnormal shape with numerous invaginations and kinks that were never seen in wild type cells (Fig. 7*B*). Taken together these data strongly argue that Lis1 fulfils a unique and essential role for several cellular aspects of spermatid differentiation, most likely mediated by microtubular functions.

DISCUSSION

Lis1 is required for germ cell division in the *Drosophila* (32), which is certainly not the case in the Lis1 mouse mutant. Clearly, germ cell progenitors undergo mitotic and meiotic cell divisions in mutant testis and appropriately generate haploid spermatids. It also seems unlikely that Lis1 is generally required for proper migration of germ cells, as suggested for neurons in the brain cortex of Lis1 mutants. Spermatocytes in the mutant testis move correctly from the periphery toward the center of seminiferous tubules and only later lose their precise allocation, probably as a result of the late differentiation defect in Lis1-deficient spermatids. The disruption of seminiferous tubules in mature testis of mutant males may also contribute to mislocation of germ cell progenitors. What causes the general loss of tissue organization in Lis1-deficient testis is unclear, but it seems secondary to the cell autonomous defect in spermatogenesis. Likewise, massive apoptosis of spermatogenic cells appears to be mediated by one or more unknown signals as a secondary result of the primary defect in mutant spermatids.

In the absence of Lis1, spermatids frequently retain large nuclei of irregular shape and fail to develop the typical cap-like acrosome on top of the nucleus. Moreover, mutant spermatids tend to develop several tail-like structures around the nucleus instead of a single one that is normally located opposite to the acrosome. Whether both aspects of the phenotype are related is currently unclear. Consistent with a function in acrosome formation, Lis1 co-localizes with Golgi vesicles. Thus, one might hypothesize that Lis1 is required for transport of these vesicles within the cytoplasm, possibly by a microtubule-dependent mechanism. The co-localization of Lis1 with tubulin and dynein in mouse spermatids supports this idea. Alternatively or in addition to being part of a motor protein complex, Lis1 might also function as an adaptor protein that provides target specificity to proacrosomal vesicles or might be involved in vesicle fusion. Significantly, vesicles formed in Lis1-deficient spermatids appear frequently dilated and display abnormal distribution of granular material. In addition, many of the preacrosomal granules contact the nucleus prior to fusion suggesting that they may be unable to merge into a functional acrosome. Interestingly, Lis1 protein remains associated with the acrosome in mouse spermatozoa and may therefore be necessary to maintain the intact structure. Further biochemical and cell biological studies will be required to determine specific interaction partners of Lis1 protein in spermatids and examine their functional significance.

Condensation of the nucleus in spermatids occurs primarily by extrusion of liquid through nuclear pores resulting in 10-fold reduced volume of karyoplasm and the hook-like shape of the nucleus in mouse. Signals and molecular mechanisms underlying these changes in nuclear structure are unknown, although the temporary accumulation of microtubules in a basket-like fashion around the nucleus of spermatids may be related to this process. Based on the observed association of Lis1 protein with these perinuclear microtubules and the phenotype of spermatids in the Lis1-deficient mouse mutant, we propose that Lis1 plays a role in nuclear condensation, although it is yet impossible to pinpoint its precise functional involvement. As a working hypothesis for the process of nuclear condensation one could envisage a squeezing mechanism that

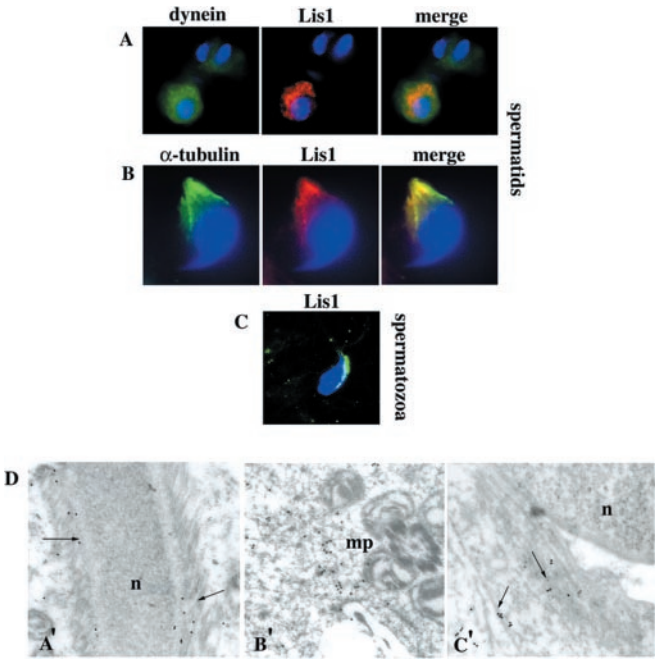

FIG. 6. **Lis1 protein co-localizes with dynein and tubulin in mouse spermatids.** Double immunofluorescence was performed on testis sections of mouse using specific antibodies for dynein (*green* in *A*), tubulin (*green* in *B*), and Lis1 (*red* in *A* and *B*) as described under "Experimental Procedures." Although Lis1 only partially overlaps with a subdomain of dynein next to the nucleus (*A*), it coincides almost entirely with tubulin (*B*). In mouse spermatozoa Lis1 protein (*green*) appears to be associated with the acrosome (*C*). Electron micrographs of immunogold-labeled Lis1 protein in spermatids indicate that Lis1 protein preferentially localizes to perinuclear microtubules in the spermatid manchette (*arrows* in *A′* and *C′*). Gold particles labeling Lis1 are also found in the cytoplasm, frequently associated with electron-dense material but not in tail structures (*B′*). *n*, nucleus; *mp*, midpiece of tail.

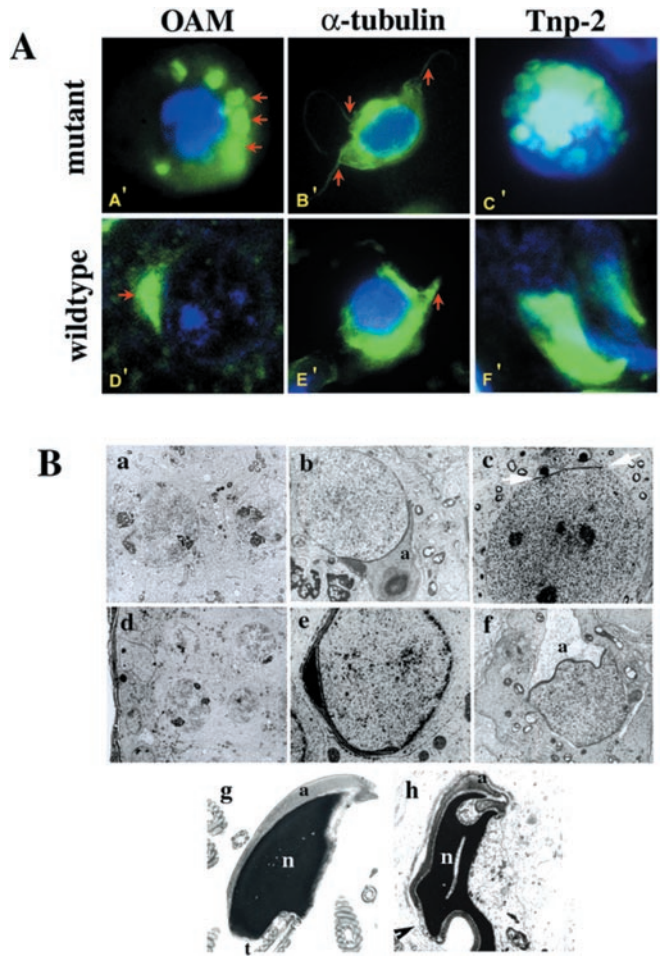

FIG. 7. **Lack of Lis1 protein in spermatids affects formation of acrosome and tail as well as nuclear condensation.** *A*, immunohistochemistry on mutant (*A′*, *B′*, and *C′*) and wild type (*D′*, *E′*, and *F′*) testis reveals severe defects in mutant spermatids. *Red arrows* in *A′* indicate the numerous acrosomal vesicles (outer acrosomal membrane is stained *green*) in mutant as compared with the single acrosome in wild type (*D′*). Also note the beginning tail structures (tubulin staining in *green*) at several positions in mutant spermatids (*B′*, *red arrows*) but only at one position in wild type (*E′*, *red arrow*). The distribution of transition protein 2 (*Tnp-2*) illustrates the decondensed nucleus in mutant spermatids (*C′*), whereas it outlines the hook-shaped, condensed nucleus in wild type cells (*F′*). Nuclei were always counterstained with 4′,6-diamidino-2-phenylindole (*blue*). *B*, electron micrographs of testis sections from wild type (*panels d*, *e*, and *g*) and mutant (*panels a*, *b*, *c*, *f*, and *h*) mice at P45 Note the fairly normal appearance of spermatocyte nuclei in mutant (*a*) compared with wild type (*d*). Typical acrosome of wild type spermatids (*e*) and dilated acrosome in the mutant (*b*) that sometimes lacks acrosomal granules (*f*). In mutant spermatids distinctly flattened vesicles with proacrosomal granules frequently appear in close contact with the nuclear membrane suggesting that they fail to fuse to the typical proacrosomal vesicle prior to nuclear attachment (*arrows* in *c*). *Panel g* shows a normal wild type spermatid with acrosome (*a*), hook-shaped nucleus (*n*), and tail (*t*). In contrast, the mutant spermatid exhibits a grossly misshaped nucleus, abnormal acrosome, and only the basal cuff of the tail (*arrowhead* in *f*).

leads to the extrusion of karyoplasm by the action of motor protein complexes associated with Lis1 in the spermatid manchette.

Besides interacting with microtubular structures Lis1 also forms complexes with two catalytic subunits α1 and α2 of the trimeric phospholipase, intracellular type I platelet-activating factor acetylhydrolase (PAF-AH 1B), in which it constitutes the non-catalytic subunit. Two recent studies (22, 48) demonstrated that both catalytic subunits of PAF-AH 1B are expressed in testis and mutants lacking the α2 or α2 and α1 subunits exert male infertility and marked loss of germ cells at an early stage of spermatogenesis. These findings suggest that Lis1 may mediate its function in testis as part of the PAF-AH complex. According to Koizumi *et al.* (22), the catalytic α1 subunit is only expressed in spermatogonia and null mutants display no overt phenotype. In contrast, α2 is highly expressed in all cells of seminiferous tubules and the male mutant shows significant reduction of testis size indicating that PAF-AH activity is functionally relevant during spermatogenesis. Interestingly, both mentioned studies associate the testis phenotype with altered expression levels of Lis1 in α2 single and α1/α2 double mutant mice, although they profoundly disagree on the direction of changes. Although Koizumi *et al.* (22) report drastically reduced levels (20% of wild type) of Lis1 protein, supposedly by post-translational control, Yan *et al.* (48) show increased Lis1 expression. Moreover, the latter study demonstrates that reducing Lis1 levels by heterozygosity rescues the mutant phenotype. These observations then complicate the interpretation, because either loss of catalytic subunits or altered Lis1 levels or both may cause the complex defects in testis. In any case, it is unlikely that the testicular phenotype elicited by the Lis1 mutation is solely mediated via the catalytic activity of PAF-AH 1B, because the various mutants generate different phenotypes. Although the Lis1 mutation primarily affects late steps of spermatid differentiation, the α1/α2 double mutant exhibits early degeneration of primary spermatocytes as well as defects in spermatogenic cells of later developmental stages. The apparently overlapping but distinct phenotypes of Lis1 and PAF-AH mutants therefore argue for distinct roles and requirements of Lis1 in several processes of spermatogenesis, at least partly independent of the catalytic activity. Although it is known that PAF-AH and its substrate

PAF are present in mammalian sperm and may modulate motility and fertility (13), cellular functions of the enzyme activity during spermatogenesis and even the physiological substrate in germ cells are entirely unclear. The structure of PAF-AH 1B closely resembles that of trimeric G proteins. Lis1 constitutes the homologue of the β subunit, and the tertiary fold of the α1 subunit is very reminiscent of a small GTPase, like p21 ras (49). Thus, in addition to the potential of regulating the signaling molecule PAF, Lis1 may also be involved in intracellular signal transduction as part of a GTP switch protein complex, as suggested previously (3). Clearly a plethora of extra- and intracellular signaling molecules is important in reproductive physiology (50). Of note in this context, overexpression of the very low density lipoprotein receptor in mouse results in a similar testicular phenotype as described here (51).

In summary, Lis1 $^{GT/GT}$ mutant mice provide a model system for severe oligozoospermia and present conclusive evidence that Lis1 protein is absolutely required for the differentiation of spermatids. The subcellular localization with tubulin and dynein argues for microtubule-mediated processes that critically depend on Lis1 protein. Determination of interaction partners for Lis1 in germ cells will clarify the molecular mechanism(s) by which Lis1 exerts its function in spermiogenesis.

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Molecular Basis of Cell and Developmental Biology:

Inactivation of a Testis-specific Lis1 Transcript in Mice Prevents Spermatid Differentiation and Causes Male Infertility

Karim Nayernia, Franz Vauti, Andreas Meinhardt, Christina Cadenas, Stephan Schweyer, Barbara I. Meyer, Iris Schwandt, Kamal Chowdhury, Wolfgang Engel and Hans-Henning Arnold

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