THE ROLE OF LISSENCEPHALY-1 PROTEIN

IN

MALE GERM CELL DIFFERENTIATION

Dissertation
zur Erlangung des Doktorgrades
der Mathematisch-Naturwissenschaftlichen Fakultäten
der Georg-August-Universität zu Göttingen

vorgelegt von
Nadja Drusenheimer
aus Remscheid

Göttingen 2009

D 7

Referent:	Prof. Dr. med Dr. h.c. W. Engel
Korreferentin:	Prof. Dr. S. Hoyer-Fender
Tag der mündlichen Prüfung:	

TABLE OF CONTENTS

Abbreviations

129/Sv	129/Sv/Ola mouse strain
ABI	Applied Biosystem Instrument
AP	Alkaline Phosphatase
ATP	Adenosinetriphosphate
BCIP	1-bromo-3-chloropropane
bp	base pair
BLAST	Basic Local Alignment Search Tool
BSA	Bovine Serum Albumin
°C	Degree Celsius
C57BL	C57BL/6J mouse strain
cDNA	complementary DNA
d	day/s
dATP	desoxyriboadenosintriphosphate
dH_20	destilled water
DAPI	Diamidino-2-phenylindole dihydrochloride
dCTP	Desoxyribocytosinetriphosphate
DMEM	Dulbecco's Modified Eagle Medium
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic acid
DNAse	Deoxyribonuclease
dNTP	deoxynucleotidetriphosphate
dpc	days post coitum
dT	deoxythymidinate
DTT	Dithiothreitol
EDTA	Ethylene Diamine Tetraacetic Acid
EGFP	Enhanced Green Fluorescence Protein
ES	Embryonic Stem
et al.	et alii (and others)
EtBr	Ethidium Bromide
Fig.	Figure
FCS	Fetal Calf Serum

FVB	FVB/N mouse strain
g	gravity
GT/GT	homozygous Gene Trap mouse
GT/-	heterozygous Gene Trap mouse
HBSS	Hanks' Balanced Salt Solution
HE	Heterozygote
HEPES	N-(-hydroxymethyl)piperazin,N'-3-propanesulfoneacid
HPRT	hypoxanthin-phosphoribosyl-transferase
hr(s)	hour(s)
HO	Homozygote
ICM	Inner Cell Mass
ICSI	Intracytoplasmic Sperm Injection
IPTG	Isopropyl-ß-thiogalactopyranoside
kb	kilobase
kDa	Kilodalton
mg	milligram
ml	milliliter
µl	microliter
µm	micrometer
min	minute
mM	millimolar
mRNA	Messenger-RNA
NaAc	Sodium Acetate
NBT	Nitro-blue Tetrazolium
NCBI	National Center for Biotechnology Information
Neo	Neomycin
ng	nanogram
nm	nanometer
nt	nucleotide
OD	Optical Density
ORF	Open Reading Frame
PBS	„Phosphate Buffered Saline“

PCR	„Polymerase Chain Reaction“
PFA	Paraformaldehyde
PMSF	Phenylmethanesulfonilfluoride
RNA	Ribonucleic Acid
RNase	Ribonuclease
RT-PCR	Reverse-Transcription-PCR
RT	Room Temperature
Sdha	Succinate dehydrogenase
SD	Standard Deviation
SDS	Sodium Dodecyl Sulfate
SV40	Simian Virus 40
Tris	Tris(hydroxymethyl)-aminomethane
U	Unit
UTR	„untranslated region“
Vol.	Volume
v/v	Volume/Volume
WT	Wild Type
X-Gal	5-brom-4-chlor-3-indolyl-ß-D-galactopyranoside

Symbols of nucleic acids

A	Adenosine
C	Cytidine
G	Guanosine
T	Thymidine
U	Uridine

Symbols of amino acids

A	Ala	Alanine
B	Asx	Asparagine or Aspartic acid
C	Cys	Cysteine
D	Asp	Aspartic acid
E	Glu	Glutamic acid
F	Phe	Phenylalanine

G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophan
Y	Tyr	Tyrosine
Z	Glx	Glutamine or Glutamic acid

1. Introduction

1.1 *Pafah1b1/Lis1* is an evolutionary conserved gene

PAFAH1B1 (*LIS1*) gene has been identified as a non-catalytic subunit of type I platelet-activating factor acetylhydrolase [PAF-AH (I)], a heterotrimeric enzyme, which inactivates the platelet-activating factor (PAF) (Reiner et al., 1993, Hattori et al., 1994). Homologs of *Lis1* are known to exist amongst others in the fungus *Aspergillus nidulans* (Xiang et al., 1995), in *Saccharomyces cerevisiae* (Geier et al., 1997), in *Caenorhabditis elegans* (Dawe et al., 2001), in *Drosophila melanogaster* (Liu et al., 2000) and in *Mus musculus* (Peterfy et al., 1995, Hirotsune et al., 1998). Comparison of the predicted amino acid and nucleotide sequences of *Lis1* from ten species reveals a high level of sequence conservation (Table 1.1). The protein sequence identity ranges from 58.6% between mouse and the fungi *Magnaporthe grisea* and 99.8% between mouse and human and mouse and chimpanzee (Table 1.1). There is only one single amino acid difference between mouse and human (Peterfy et al., 1994) and three amino acid differences between human and bovine (Hattori et al., 1995).

Table 1.1: Homologs of *Pafaha1b1/Lis1* gene in different eukaryotic species using NCBI HomoloGene software. Gene name (Symbol) for different species, gene ID, chromosomal location and the similarity (%) of the alignment of nucleotide and amino acid sequences for *Pafah1b1* gene between mouse and other species are given.

Symbol	Species	Gene ID	Chromosomal location	similarity protein (%)	similarity DNA (%)
Pafah1b1	*M. musculus*	18472	11 B3	-	-
PAFAH1B1	*H. sapiens*	5048	17p13.3	99.8	95.1
PAFAH1B1	*P. troglodytes*	454422	17	99.8	95.0
PAFAH1B1	*B. taurus*	282513	19	94.6	94.6
Pafah1b1	*R. norvegicus*	83572	10q24	98.3	98.3
PAFAH1B1	*G. gallus*	374224	19	88.5	88.5
pafaha1b1b	*D. rerio*	394247	21	82.1	82.1
Lis-1	*D. melanogster*	36791	2R52F4-52F5	65.8	65.8
Lis-1	*C. elegans*	176758	III	58.6	58.6
MGG_09369	*M. grisea*	2680413	IV	48.3	52.3

The LIS1 protein contains a so-called LisH motif at the N terminus, followed by a coiled-coil region and a seven WD-40 repeat forming b-propeller structure at the C terminus (Reiner et al., 1993, Cardoso et al., 2000) (Fig. 1.1). The LisH domain consists

of an alpha-helical motif and is found in over 100 eukaryotic intracellular proteins, from metazoa, fungi and plant that have a wide range of functions (Emes and Ponting, 2001). In LIS1 it is suggested to contribute to the regulation of microtubule dynamics, either by mediating dimerisation or by binding cytoplasmic heavy chain or microtubules directly (Kim et al., 2004, Mateja et al., 2006). The coiled coil is a ubiquitious protein motif that mediates protein-protein interaction. It is found in many different types of proteins, which are involved in multiple cellular processes. WD-40 repeats (also known as WD or beta-transducin repeats) are short ~40 amino acid motifs, often terminating in a Trp-Asp (W-D) dipeptide. WD-containing proteins have 4 to 16 repeating units, all of which are thought to form a circularised beta-propeller structure. WD-repeat proteins are a large family found in all eukaryotes and are implicated in a variety of functions ranging from signal transduction and transcription regulation to cell cycle control and apoptosis. The underlying common function of all WD-repeat proteins is coordinating multi-protein complex assemblies, where the repeating units serve as a rigid scaffold for protein interactions. The specificity of the proteins is determined by the sequences outside the repeats themselves (Neer et al., 1994, Li and Roberts, 2001).

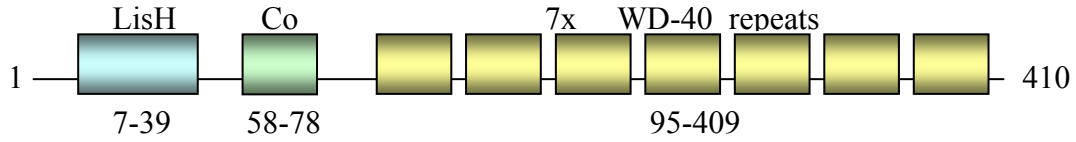

Figure 1.1: Schematic representation of LIS1 protein structure with its three conserved motifs. LisH domain (residues 7-39) is labelled in blue, Coiled-coil domain (Co) (residues 58-78) is labelled in green and seven WD-40 repeats (residues 95-409) are labelled in yellow.

1.2 Mutations in *LIS1* gene cause classical lissencephaly

Human lissencephaly (derived from the Greek “lissos” meaning smooth and “encephalos” meaning brain) is a developmental abnormality characterised by a smooth cerebral suface, 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 (Hirotsune 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 (Cahana et al., 2003).

Submicroscopic duplication within the subtelomeric region of chromosome 17p13.3, including PAFAH1B1, leads to neurobehavioral deficits and subtle brain abnormalities in several patients. Brains of transgenic mice with increased *Lis1* expression in the developing brain are significantly smaller than brains of control mice. Further analysis revealed reduced cellular polarity in the ventricular zone and delayed radial and tangential migration in *Lis1* overexpressing mice (Bi et al., 2009).

1.3 Expression and function of *Lis1*

The expression and function of *Lis1* has been elaborately studied in several species including *S. cerevisiae, C. elegans, D. melanogaster*, mouse and human. Expression analysis in mouse 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 (Fig. 1.2).

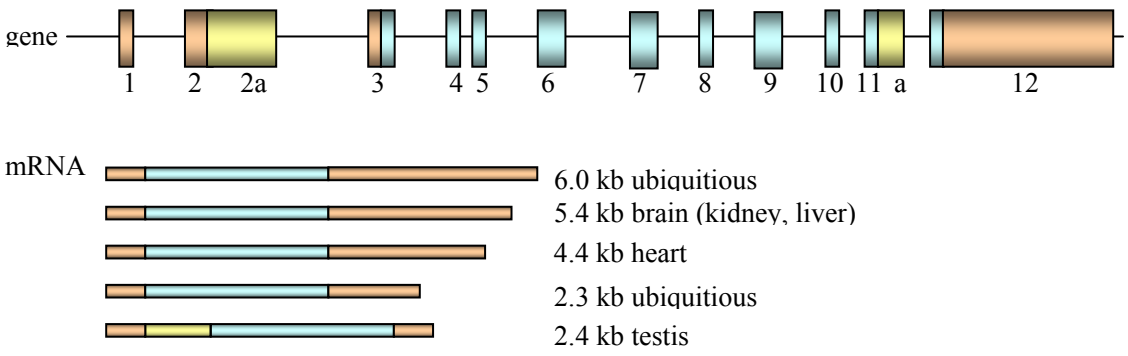

Figure 1.2: Schematic illustration of murine *Lis1* gene and its various transcripts. *Pafah1b1/Lis1* gene is located on chromosome 11, consists of 12 exons and spans more than 50kb of genomic DNA. The

first two exons are untranslated and the third exon contains the rest of 5`UTR and the ATG start codon. Exon 12 contains 75 bp of translated sequence, the TGA translational stop codon and 3714 bp of 3`UTR (coding exons are labelled in blue, non-coding exons in red and alternatively spliced exons 2a and 11a in yellow). Multiple *Lis1* transcripts arise by different polyadenylation and alternative splicing. The 6.0 kb and the 2.3 kb transcript is expressed ubiquitously, while the 5.4 kb transcript (exclusively expressed in brain and to a lower extent in kidney and liver), the 4.4 kb transcript (exclusively expressed in heart) and the 2.4 kb transcript (exclusively expressed in testis) are expressed in a tissue specific manner (Peterfy et al., 1998).

LIS1 is an important protein that is involved in numerous protein-protein interactions and cellular processes. Besides its involvement in regulation of 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).

PAF-AH (I) inactivates PAF, a potent signalling phospholipid that is involved in a variety of physiological events by removing the acetyl moiety at the sn-2 position of PAF (Arai et al. 2002). PAF is expressed in various tissues (Venable et al., 1993), including the central nervous system (Kornecki and Ehrlich, 1988, Marcheselli and Bazan, 1994, Maclennan et al., 1996) and reproductive organs (Minhas et al., 1996, Levine et al., 2002), where it plays a role in sperm motility (Roudebush, 2001) and acrosomal function (Angle et al., 1993).

In the yeast *Saccharomyces cerevisiae* the *Lis1* homolog *Pac1* is involved in dynein mediated nuclear migration. It targets dynein to microtubule tips, which is necessary for sliding of microtubules along the bud cortex during mitosis. Cells lacking *Pac1* fail to display microtubule sliding movement and nuclear segregation (Lee et al., 2003).

In the filamentous fungus *Aspergillus nidulans* the *Lis1* homolog *nudF* is again required for nuclear migration. Nuclei of mutants can divide, but fail to migrate, leading to a cluster of nuclei in the spore end of the germ tube (Xiang et al., 1995). The nuclear distribution pathway in *A. nidulans* involves several proteins, including NudF, NudE, cytoplasmic dynein and dynactin, and is highly conserved in eukaryotes (Osmani et al., 1990).

In the nematode *Caenorhabditis elegans* null alleles of *lis-1* results in defects identical to those observed after inactivation of dynein heavy chain, including defects in centrosome separation and spindle assembly (Cockell et al., 2004). Abrogation of *lis-1*

results in embryonic lethality, sterility, altered vulval morphology, uncoordinated movement and nuclear positioning defects during early embryonic cell divisions (Dawe et al., 2001).

In *Drosophila melanogaster* the *Lis1* homolog *DLis1* is essential for normal embryonic development. Homozygous *DLis1* mutant embryos hatch normally, but soon experience growth retardation and die 5 to 6 days later. Moreover analysis of ovarian mutants of *DLis1* revealed that *DLis1* is required for germline cell division, fusome integrity and oocyte differentiation (Liu et al., 1999). A genetic interaction between DLIS1 and cytoplasmic dynein heavy chain (CDHC) in neuroblast proliferation, dendritic growth and axonemal transport was shown in somatic clones of the *Drosophila* nervous system (Liu et al., 2000).

The regulation of dynein motor function by LIS1 and microtubule organisation 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 Wynshaw-Boris, 2007, Kerjan and Gleeson, 2007). LIS1 interacts directly with NDE1 (a mammalian NudE homolog), which interacts with the cytoplasmic dynein light chain (CDLC) to regulate protein localisation and function. LIS1 also directly interacts with NUDEL1 (another mammalian NudE homolog), and LIS1 and NUDEL1 directly interact with the cytoplamic dynein heavy chain (CDHC) to regulate centrosomal protein localisation and function as well as microtubule dynamics. These interactions are critical for nuclear movement and neuronal migration. Point mutations in *Lis1* that cause human lissencephaly severely affect the interaction of LIS1 with itself, with NUDEL and NDE1 (Feng et al., 2000, Sasaki et al., 2000), confirming the functional importance of these interactions.

LIS1 protein is accumulated in regions of high microtubule density in neurons and fibroblasts, especially at the centrosome and microtubule-organising 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).

1.4 Generation of the gene trap line L39$^{GT/GT}$

Gene trapping is a high-throughput approach that is used to introduce insertional mutations across the genome in mouse embryonic stem (ES) cells (Stanford et al., 2001, Hansen et al., 2003). The gene-trapping principle is based on the random integration of a reporter/selector cassette into a genome in order to simultaneously select for, mutate and identify the trapped locus. Gene trap mutagenesis of mouse ES cells generates random loss-of-function mutations, which can easily be identified and can often report the endogenous expression of the mutated gene (Wurst et al., 1995).

In a gene trap approach performed by Christina Cadenas (University of Braunschweig) in cooperation with Prof. Dr. P. Gruss (Max-Planck-Institute for Biophysical Chemistry, Göttingen), using an exon trap vector, the embryonic stem cell (ES) clone 2A-53 was isolated and a stable mouse line, line 39 (L39), was generated. The trapped gene was most abundantly expressed in heart, neural tube, brain and dorsal root ganglia during embryogenesis. To identify the integration site of the vector a DNA library from L39 mice was generated, cloned into λDASH-II phage vector and packed. After hybridisation with a Neo probe, two positive clones were isolated and analysed. Sequencing results showed that the exon trap vector had integrated in the second intron of *Lis1* gene. 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 ribosome 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. Figure 1.3 shows a schematical drawing of the integration of the vector in the second intron of *Lis1* gene.

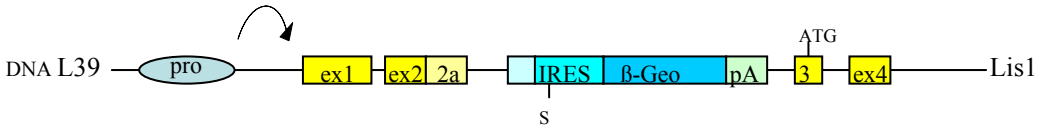

Figure 1.3: Schematical drawing of the integration site of the gene trap vector into intron 2 of *Lis1* gene. The gene trap vector contains an IRES sequence (internal ribosome entry site; for cap-independent mRNA translation), followed by a β-Geo (β-galactosidase /neomycin-resistance fusion gene) and a poly(A) signal (pA) from SV40. Abbrevations are: pro: *Lis1* promoter region, which activates expression (illustrated by the arrow), ex: exon, S: cryptic splice acceptor site present within the IRES sequence, ATG: translation start codon present within exon 3 of *Lis1* gene.

Analysis of heterozygous and homozygous gene trap mice revealed no apparent pathological phenotype. In particular, Nissl staining of brains failed to show any signs of lissencephaly, suggesting that the gene trap insertion had not generally disrupted *Lis1* gene. However, subsequent breedings indicated that homozygous mutant males were consistently infertile, whereas mutant females reproduced normally.

1.5 Analysis of the gene trap line L39$^{GT/GT}$

(Nayernia et al., 2003)

In the following section a brief summary of the analysis of the gene trap line L39$^{GT/GT}$ is given (Nayernia et al. 2003) and the experiments which were performed as part of this thesis to confirm the published data are mentioned.

Integration of a gene trap vector in intron 2 of *Lis1* gene resulted in male infertiliy of homozygous mutant mice (L39$^{GT/GT}$ mice), while females reproduced normally. Testes of these males are about 50% smaller than testes of wild type males and histological analysis revealed that epididymes contained essentially no spermatozoa (Nayernia et al. 2003). To further prove the lack of spermatozoa, epididymial suspensions of gene trap males were analysed and sperm motility was quantified using the computer assisted semen analyis (CASA) in this thesis.

To determine the onset of the phenotype in mutant mice testis sections of 15 d, 25 d, 35 d, 45 d and 90 d old L39$^{GT/GT}$ males were histologically analysed. Sections of mutant testes up to d 45 exhibited intact seminiferous tubules of normal diameter and germ cells as well as Sertoli and Leydig cells present at the appropriate location. At d 90, however, round and elongating spermatids were released prematurely from the epithelium and located inside the lumen of the tubules. Moreover, only few spermatozoa were present in the testis and the tubular structure had collapsed, lacking the epithelial architecture and a clearly visible lumen (Nayernia et al. 2003). These results suggested that the gene trap mutation affects 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. Investigation of the expression of spermatocyte- and spermatid- specific markers of 45 d old mice by Northern blot analysis revealed no difference in wild type and gene trap males (Nayernia et al. 2003), suggesting that all germ cell progenitors are formed and that the

gene trap mutation causes a blockade of late spermatid differentiation resulting in a severe reduction of mature sperm cells.

As histological analysis of 45 d old $L39^{GT/GT}$ mice revealed no abnormalities in spermatogenesis, the unaltered expression levels of spermatocyte- and spermatid-specific markers of 45 d old homozygous males was not surprising. To support these results, Northern blot analysis of spermatocyte- and spermatid-specific markers in 1 month (30 d) and 2 months (60 d) old animals were performed in this thesis. Moreover, H&E staining of testis sections of 45 d old and 2 months old animals, and of 25 d, 35 d, 45 d and 2 months old gene trap males of different gemetic backgrounds were performed to further determine the onset of the phenotype.

A testis specific *Lis1* transcript (including exon 2a as part of the 5` untranslated region) 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 (Nayernia et al. 2003). These results indicated that the integration of the gene trap vector has resulted in the selective abolition of a testis specific transcript without affecting *Lis1* expression in brain. In recent years quantitative RT-PCR became a well-established method. It is widely used to validate cDNA arrays (Rajeevan et al., 2001) and to quantify expression levels of target genes (Wang and Brown, 1998). To investigate the expression of *Lis1* transcripts in testes of gene trap and wild type mice in a more quantitative manner Northern blotting and RT-PCR analysis were repeated and a detailed qRT-PCR during testicular development was performed to quantify the downregulation of the testis specific transcript.

Western blotting experiments demonstrated slightly increasing levels of LIS1 protein in testes of mice between 10 d, 15 d, 20 d and 25 d after birth and adulthood. A reduction of LIS1 protein in testes of gene trap males was confirmed, while protein levels in brain were unaltered. Immunohistochemical analysis of wild type testis sections showed that the protein was exclusively detected in myoid stroma cells and in spermatids but not in earlier germ cells or in Leydig and Sertoli cells. In mutant males LIS1 protein was present only in myoid stroma cells but not in spermatids (Nayernia et al. 2003). These results showed the accumulation of LIS1 protein in spermatids and suggested that the gene trap mouse $L39^{GT/GT}$ constitutes an effective LIS1 null mutant in the male germ line.

Due to the clear expression of LIS1 protein in testes of young animals, which lacked spermatids, and the data of immunohistochemical analysis performed by another group (Koizumi et al., 2003) which demonstrated LIS1 immunoreactivity in all seminiferous tubule cell types with an intense staining in meiotically dividing spermatocytes and elongating spermatids, expression of LIS1 protein was analysed again by Western blotting and immunohistochemistry to detail the testicular expression pattern of LIS1.

1.6 Objectives of this study

The aim of this study was the detailed characterisation of the mutant line L39, the genetic rescue of $L39^{GT/GT}$ males and the expression and functional analysis of *Lis1* gene in testis. Scientific approaches undertaken in this study are as follows:

1. Detailed analysis of the infertile gene trap line $L39^{GT/GT}$.

2. Expression analysis of *Lis1* on transcriptional and translational level during germ cell differentiation.

3. Analysis of germ cell specific regulation.

4. Determination of the role of LIS1 in spermatogenesis in *in vivo* "gain of function" transgenic models.

5. Transgenic rescue of the infertile gene trap line $L39^{GT/GT}$ with different transgenic lines, which overexpress *Lis1* under control of male germ cell specific promoters.

2. Materials and Methods

2.1 Materials

2.1.1 Chemicals

Acrylamide/Bisacrylamide	Roth, Karlsruhe
Acetic acid	Merck, Darmstadt
Agar	Roth, Karlsruhe
Agarose	Peqlab, Erlangen
Ammonium acetate	Fluka, Neu Ulm
Ampicillin	Sigma, Deisenhofen
Ampuwa	Fresenius, Bad Homburg
Bacto-Peptone	Roth, Karlsruhe
Bacto-Yeast-Extract	Roth, Karlsruhe
BCIP	Applichem, Darmstadt
Blocking powder	Roth, Karlsruhe
Boric acid	Roth, Karlsruhe
BSA	Biomol, Hamburg
Coomasie G-250	Sigma, Deisenhofen
Chloroform	J.T.Baker, Deventer, Niederlande
Vectashield (DAPI)	Vector, Burlingame
Diethyl pyrocarbonate (DEPC)	Sigma, Deisenhofen
Dulbecco's Modified Eagle Medium (DMEM)	PAN, Aidenbach
Dimethyl sulfoxid (DMSO)	Sigma, Deisenhofen
Dithiothreitol	Applichem, Darmstadt
DNA ladder (1kb)	Invitrogen, Karlsruhe
dNTPs (100 mM)	Invitrogen, Karlsruhe
EDTA	Sigma, Deisenhofen
Effectene	Qiagen, Hilden
Ethanol	J.T.Baker, Deventer, Niederlande
Ethidium bromide	Roth, Karlsruhe

FCS	PAN, Aidenbach
Formaldehyde	Merck, Darmstadt
Formamide	Sigma, Deisenhofen
Glutaraldehyde	Fluka, Neu Ulm
Glycerol	Invitrogen, Karlsruhe
Glycine	Biomol, Hamburg
HBSS medium	Sigma, Deisenhofen
Horse serum	Sigma, Deisenhofen
HCl	Roth, Karlsruhe
H_2O_2	Merck, Darmstadt
HEPES	Merck, Darmstadt
Ionophore A23187	Calbiochem, Bad Soden
IPTG	Biomol, Hamburg
Isopropanol	J.T.Baker, Deventer, Niederlande
KCl	Merck, Darmstadt
Lipofectamine^{TM}2000	Invitrogen, Karlsruhe
M2 medium	Sigma, Deisenhofen
Methanol	J.T.Baker, Deventer, Niederlande
$MgCl_2$	Merck, Darmstadt
MOPS	Applichem, Darmstadt
β-Mercaptoethanol	Serva, Heidelberg
Na acetate	Merck, Darmstadt
Na citrate	Merck, Darmstadt
Na deoxycholate	Merck, Darmstadt
NaCl	Applichem, Darmstadt
Na_2HPO_4	Merck, Darmstadt
NaH_2PO_4	Merck, Darmstadt
NaOH	Merck, Darmstadt
NBT	Applichem, Darmstadt
Neomycin(G-418)	PAN, Aidenbach
NuPage® LDS sample buffer (4x)	Invitrogen, Karlsruhe
NuPage® Mops SDS running buffer	Invitrogen, Karlsruhe
Orange G	Sigma, Deisenhofen
OPTI-MEM I	Invitrogen, Karlsruhe

Peptone	Roth, Karlsruhe
Paraformaldehyde	Applichem, Darmstadt
Penicillin/Streptomycin	PAN, Aidenbach
PBS	Invitrogen, Karlsruhe
peqGOLDTriFast	Peqlab, Erlangen
Phosphoric acid	Merck, Darmstadt
Picric acid	Fluka, Neu Ulm
Phenol	Biomol, Hamburg
Proteinase K	Applichem, Darmstadt
Protein marker	Invitrogen, Karlsruhe
Radioactive substances:	
[α32P]-dCTP	Amersham, Braunschweig
Rapid-hybridization Puffer	Amersham, Freiburg
Ready primeTM II (DNA Labelling Kit)	Amersham, Freiburg
RNase Inhibitor	Boehringer, Mannheim
RNA length standard	Invitrogen. Karlsruhe
RNAse –Exitus Plus	Applichem, Darmstadt
Saccharose	Roth, Karlsruhe
Salmon sperms DNA	Sigma, Deisenhofen
SeeBlue® plus2 Pre-Stained Standard	Invitrogen, Karlsruhe
SDS	Serva, Heidelberg
S.O.C Medium	Invitrogen, Karlsruhe
Tris	Sigma, Deisenhofen
Triton X-100	Serva, Heidelberg
Tween-20	Promega, Mannheim
Viagen DirectPCR-Tail	Peqlab, Erlangen
X-Gal	Biomol, Hamburg
Xylol	Merck, Darmstadt

All those chemicals, which are not mentioned above, were ordered from Merck, Darmstadt, or Roth, Karlsruhe.

2.1.2 Solutions, buffers and media

All standard buffers and solutions were prepared according to Sambrook *et al.* (1989).

AP buffer	100 mM Tris-HCl (pH 9.5) 100 mM NaCl 50 mM $MgCl_2$
Bouin's solution	15 volumes of Picric acid (in H_2O) 5 volumes Formaldehyde (37%) 1 volume Acetic acid
BCIP-Solution	50 mg/ml BCIP 70% Dimethyl formamide
Blocking buffer (10 x)	40.913 g NaCl 6.057 g Tris in 500 ml dH_20 adjust pH to 7.5
Blocking buffer B1 (1 x)	50 ml 10 x blocking puffer 500 µl Tween-20 450 ml dH_20
Blocking solution (immunostaining)	5% BSA 1% Tween-20 in dPBS
Denaturation solution	1.5 M NaCl 0.5 M NaOH
DEPC-H_2O	0.1% (v/v) Diethylpyrocarbonate
Depurination solution	250 mM HCl

E-buffer (10x)	300 mM NaH_2PO_4 50 mM EDTA
Fix A	1.35 ml formaldehyde (37%) 0.4 ml glutaraldehyde (25%) 0.1 ml NP-40 (10%) 5 ml 10 x PBS up to 50 ml with dH_2O
Fix B:	1.35 ml formaldehyde (37%) 0.4 ml glutaraldehyde (25%) 1 ml NP-40 (10%) 5 ml 10x PBS 5 ml 1% NaDOC (Sodium desoxycholate)
Formalin Fixative Solution	4% Paraformaldehyde in dPBS
Glycerol loading buffer	10 mM Tris/HCl (pH 7.5) 10 mM EDTA (pH 8.0) 0.025% Orange G 30% Glycerol
Ligation buffer (10x)	600 mM Tris/HCl (pH 7.5) 80 mM $MgCl_2$ 100 mM DTT
Lysis buffer (DNA)	100 mM Tris-HCl (pH8) 5 mM EDTA 0.2% SDS 200 mM NaCl 100 µg/ml Proteinase K
Lysis buffer (protein)	10 mM Tris-HCl (pH 8.0)

	1 mM EDTA 2.5% SDS 1 mM PMSF Protease Inhibitor Cocktail Tablet
MOPS Buffer (10x)	41.8 g MOPS 16.6 ml 3 M Sodium acetate 20 ml 0.5 M EDTA in 1 liter of DEPC water adjust pH to 6.75
Neutralisation solution	1.5 M NaCl 1 M Tris/HCl (pH 7.0)
PBS buffer (10 x)	80 g NaCl 2 g KCl 26.8 g Na_2HPO_4-$7H_2O$ 2.4 g NaH_2HPO_4 adjust pH to 7.4
PBT buffer	0.1% Tween-20 in PBS (1 x) SSC (20x) 3 M NaCl 0.3 M Na_3 citrate (pH 7.0)
SSC (20 x)	3 M NaCl 0.3 M Na_3 citrate (pH 7.0)
5 x TBE buffer	450 mM Tris 450 mM Boric acid 20 mM EDTA (pH 8.0)
TE-buffer	5 mM Tris/HCl (pH 7.4) 1 mM EDTA

Washing solution I	2 x SSC 0.1% SDS
Washing solution II	0.2 x SSC 0.1% SDS
X-Gal staining solution:	2.5 ml X-Gal (20 mg/ml) 1.0 ml $K_3Fe(CN)_6$ (250 mM) 1.0 ml $K_4Fe(CN)_6$ (250 mM) 1.0 ml $MgCl_2$ (100 mM) up to 50 ml with dPBS

2.1.3 Laboratory materials

The laboratory materials, which are not listed here, were bought from Schütt and Krannich (Göttingen).

Cell culture flask	Greiner, Nürtingen
Culture slides	BD Falcon, Heidelberg
Disposable filter Minisart NMI	Sartorius, Göttingen
Hybond C	Amersham, Braunschweig
Hybond N	Amersham, Braunschweig
HPTLC Aluminum folio	Merck, Darmstadt
Microcentrifuge tubes	Eppendorf, Hamburg
Petri dishes	Greiner, Nürtingen
Pipette tips	Eppendorf, Hamburg
RotiPlast paraffin	Roth, Karlsruhe
Transfection flasks	Lab-Tek/Nalge, Nunc, IL, USA
Superfrost slides	Menzel, Btaunschweig
Whatman blotting paper	Schleicher and Schüll, Dassel
X-ray films	Amersham, Braunschweig

2.1.4 Sterilisation of solutions and equipments

All solutions that are not heat sensitive were sterilised at 121°C, 10^5 Pa for 20 min in an autoclave (Webeco, Bad Schwartau). Heat sensitive solutions were filtered through a disposable sterile filter (0.2 to 0.45 µm pore size). Plastic wares were autoclaved as above. Glassware were sterilised overnight in an oven at 220°C.

2.1.5 Media, antibiotics and agar-plates

2.1.5.1 Media for bacteria

LB Medium (pH 7.5):	1% Bacto-Peptone 0.5% Yeast extracts 1% NaCl
LB-Agar:	1% Bacto-Peptone 0.5% Yeast extracts 1% NaCl 1.5% Agar

The LB medium was prepared with distilled water, autoclaved and stored at 4°C.

2.1.5.2 Media for cell culture

NIH 3T3 and fibroblasts (MEFs):	DMEM supplemented with sodium pyruvate (1 mM) 10% fetal calf serum (FCS), heat inactivated 1% glutamine (200mM), 1% penicillin (50units/ml)/ streptomycin (50 µg/ml)
GC-1:	DMEM supplemented with sodium pyruvate (1mM)

	10% FCS, heat inactivated 1% penicillin (50 units/ml)/ streptomycin (50 µg/ml)
HeLa cells:	DMEM supplemented with sodium pyruvate (1 mM) 10% FCS, heat inactivated 2% penicillin (50 units/ml)/ streptomycin (50 µg/ml)
SSC/129/Sv and ES:	DMEM 20% FCS, heat inactivated 1 mM Non essential amino acids 1 mM Sodium pyruvate 10 µM ß-Mercaptoethanol 2 mM L-Glutamine 2% penicillin (50 units/ml)/ streptomycin (50 µg/ml) 1000 U/ml Recombinant leukaemia inhibitory factor (LIF)
SSC/129/Sv differentiation:	DMEM 20% FCS, heat inactivated 1 mM Non essential amino acids 1 mM Sodium pyruvate 10 µM ß-Mercaptoethanol 2 mM L-Glutamine 2% penicillin (50 units/ml)/ streptomycin (50 µg/ml) 1x 10^{-6}M retinoic acid

For long time storage of the cells in liquid nitrogen, the following freezing medium was used:

30% culture media

20% DMSO
50% FCS

2.1.5.3 Antibiotics

Stock solutions were prepared for the antibiotics. The stock solutions were then filtered through sterile disposable filters and stored at –20°C. When antibiotic was needed, it was added after the autoclaved medium has cooled down to a temperature lower than 55°C.

Antibiotic	**Master solution**	**Solvent**	**Final concentration**
Ampicillin	50 mg/ml	H_2O	50 µg/ml
Chloramphenicol	25 mg/ml	EtOH	25 µg/ml
Kanamycin	50 mg/ml	H_2O	50 µg/ml
Mitomycin C	1 mg/ml	dPBS	10 µg/ml

2.1.5.4 IPTG/X-Gal plates

LB-agar with 50 µg/ml ampicillin, 100 µM IPTG and 0.4% X-Gal was poured into Petri dishes. The dishes were stored at 4°C.

2.1.6 Bacterial strain

E. coli	DH5α	Invitrogen, Karlsruhe

2.1.7 Plasmids

pBluescript-PGK2-Promoter (clone 442)	Human Genetics, Göttingen
pCMV-ßGal	G.C. Sheveler, Moskau
pCS2-3`mt	Hammersmith, Freiburg
pEGFP-N1	Clontech, Saint-Germain-en-Laye France
pEGFP-hEF-1α (clone 1031)	Human Genetics, Göttingen
FPCA-V1	Prof. Dr. S. Hoyer-Fender, Göttingen
FPCA-V2	Prof. Dr. S. Hoyer-Fender, Göttingen

pGEM-T Easy	Promega, Mannheim
pGL3-Basic	Promega, Mannheim
pGL3-Control	Promega, Mannheim
pGL3-Promoter	Promega, Mannheim
pRL-SV40	A. Craig, Freiburg
pSDK-LacZ	Human Genetics, Göttingen

2.1.8 Synthetic oligonucleotides

The synthetic oligonucleotide primers used in this study were obtained from OPERON and dissolved in dH_2O (Ampuwa) to a final concentration of 100 µM.

Primers used for **genotyping**:

2A53 3AS:	5`AGTGAGGCTTCTAGGACAAGAGG3`
GTpR4:	5`TGCCAGTTTGAGGGGACGACGACAG3´
Lis1 21 AS:	5`CACACCAGCATTCTAAACAGTGCAAT3`
Lis1 20 sense:	5`AAGGTGGTCAAGATTGAGGTGATGAG3`
Lis1 Intron2F:	5`CACTGTGGCTATCTTCAGACG3`
Lis1 Intron2R:	5`GAAGAACTAGCCCCACCTGAC3`
Lispi-F:	5`TTCTCTGTCACTACCCTCAGC3`
Lispi-R:	5`CGGAATATGCCTCTTCATAGC3`
RT-hEF-Prom-F99:	5`AGCCTCAGACAGTGGTTCAAAG3`
RT-hEF-Prom-R99:	5`GCACCATTTTGGCTCTAATGTC3`
RT-PGK2-Prom-F99:	5`CAGCATTAAGATCCAGGTGTCAG3`
RT-PGK2-Prom-R99:	5`GCACCATTTTGGCTGTAATGTC3`

Primers used for **"Genome Walk"**:

GSP1-BS:	5`CTCCAACCTCCGCAAACTCCTATTTCT3`
GSP2-BS:	5`CATACTTTCCGTTCCTCTTCCCATGAA3`
GSP1-BS-gen:	5`CACACCAGCATTCTAAACAGTGCAATG3`
GSP2-BS-gen:	5`CAGTGGTAAAGAGCACCGACTGCTCTT3`
GSP1-BS-gen-5`:	5`AAGGTGGTCAAGATTGAGGTGATGAGC3`
GSP2-BS-gen-5`:	5`CAGTGTGAGTGATACTTCACCTAGTAG3`
GSP1-Gö:	5`TGCCAGTTTGAGGGGACGACGACAGTA3`

GSP2-Gö:	5`TCTTCGCTATTACGCCAGCTGGCGAAA3`
GSP2-Gö-gen:	5`ATAGAAGAACTAGCCCCACCTGACTCT`3`
GSP1-Gö-gen:	5`GTGATCCTGTTCTTTGAGACACTAACC3`
GSP1-Gö-gen-5`:	5`GTGGGCAGTGGTAGTATATGCCTTTAA3`
GSP2-Gö-gen-5`:	5`AGGTAAGTGTAAGGAAGAGGCCTAATG3`

Primers used for **qPCR**:

Lis1-Ex2-F2:	5`GGGAGTGAAGGACGGAAGAG3`
Lis1-Ex3-R1-Iris:	5`GGGACAGCACCATTTTGGCTG3`
Lis1-RT-Ex2a-F9:	5`CATCCCATCTCTCCACTCCATTAGG3`
Lis1-RT-Ex3-R9:	5`GTGGCTTCCACAGAGGGAAAAATG3`
qPCR-Acr-F:	5`ACGTAGTGACGGAGGGAAATG3`
qPCR-Acr-R:	5`TAGCAGGTGTGGGGTATTTGG3`
qPCR-Pelo-F:	5`TGAGCCCAGACTGTCGTGAC3`
qPCR-Pelo-R	5`TCTGCACCTTAGCGTGAAGCC3`
qPCR-Sdha-F:	5`GCTTGCGAGCTGCATTTGG3`
qPCR-Sdha-R:	5`CATCTCCAGTTGTCCTCTTCCA3`

Primers used for the amplification and sequencing of **transgenic constructs**:

442-XbaI-R1:	5`AGTTCTAGAGGCTCGAGAGGG3`
442-BamHI-F1:	5`GTGGATCCGGTGGAATCGATGATACTAGTGT AGCA3`
1031-5`Seq-F1:	5`TACTAGCGCTACCGGACTCAG3`
1031-3`Seq-NotI-R1:	5`GCTGATTATGATCTAGAGTCG3`
cMyc-F1:	5`GTCGGAGCAAGCTTGATTTAGGTGACA3`
cMyc-PolyA-NotI-R1:	5`CGCCGGCGCAGTGAAAAAAATGCTTTA TTTGTGAAATTTGTGATGCTATTGCTTTAT TTGGATCTACGTAATACGACTCACTATAGTTC3`
hEF-Prom-F1:	5`GAAGTTAGGCCAGCTTGGCAC3`
Lis-442-Seq-F1:	5`GTCCACCTGAGAAATACGCAT3`
Lis-442-Seq-R1	5`CGTGCATGGTTCTGATGCATT3`
Lis1-cDNA-BamHI-R1:	5`TGACTGGATCCACGGCACTCCCA3`
Lis1-cDNA- EcoRI-F1:	5`GACTGGTTGCTGATTGAATTC3`
PGK2-Promoter-F1:	5`TTCCACTACATGACCCTCTGC3`

PGK2-Promoter-R1:	5`CCTCAATGTTGAACATCGAGTC3`
SV40PolyA-NotI-R2	5`GCGGCCGCCAGTGAAAAAAATGCTTTATTTGT3`
SV40PolyA-NotI-R3:	5`AGTGATGCGGCCGCGCAGTGAAAA3`

Primers used for **Enhancer- and Promoteranalysis**:

Enh-BS-BamHI-F1:	5`GGATCCTCTGATAGAACAGGTAGTGG3`
Enh-BS-SalI-R1:	5`GTCGACACTGAGTCAGGTAGAGACATC3`
Enh-Gö-BamHI-F1:	5`GGATCCGCAGTCAGTGCTCTTAGCACT3`
Enh-Gö-SalI-R1:	5`GTCGACTGTATATGGGTTCTGGGCATC3`
pGL3-Promoter-Seq-R:	5`GACGATAGTCATGCCCCGCG3`
pGL3-Promoter-Seq-R1:	5`GTAAAACCTCTACAAATGTGG3`
pGL3-Promoter-Seq-R2:	5`TCAGGGGGAGGTGTGGGAGGT3`
pGL3-Prom-Seq-F9:	5`CATCTCAATTAGTCAGCAACC3`
pGL3-Prom-Seq-R9:	5`CCTGTCCTACGAGTTGCATG3`
Pro4-f1:	5`AATGCCAGTGCACCGGGTAC3`
Pro4-r1:	5`CCTGGGAGCTCAGTGTTAGC3`
Pro6-f1:	5`GGCTTACTCGAGTTAAGGCTA3`
Pro6-r1:	5`CTTGGGAAGCTTCCCTTTTG3`
Prom-Lis1-KpnI-F2:	5`AAATGCCAGTGCACCGGGTAC3`
Prom-Lis1-HindIII-R2:	5`CCTGATCCCTTGGGAAGCTTC3`
SV40-Enh-NheI-F:	5`GCTAGCTCGATAAGGATCTGAACGATG3`
SV40-Enh-NheI-R:	5`GCTAGCGCTGTGGGAATGTGTGTCAGTTAG3`

Primers used to create probes for **Northern blotting**:

Lis1-C-F1:	5`GGCGTCGGGTTCTCCGCTTGTCCTTA3`
Lis1-D-R1	5`TGGATTTGAATTAAATATGCCACTATGTGGCT CCACAG3`
mbeta-actin-F1:	5`GTGGGAATGGGTCAGCAGAAGGAC3`
mbeta-actin-R1:	5`GACTCATCGTACTCCTGCTTG3`
mOdf1-N:	5`GAGCTCAAGCTTTGGCCGCACTGAGTTGTC3`
mOdf1-C:	5`CCGCGGTACCCAAGATCATCTTCCTACA3`
mPGK2-F:	5`TCTCATGAGTCACCTCGGTCG3`
mPGK2-R:	5`AACTGTGAGCCCGATGTGCAG3`
mSYCP3-F:	5`AGTGGAAGATTTTTGTTCCTGGTT3`

mSYCP3-R:	5`TTGACACAATCGTGGAGAGAA3`
mTP2-F1:	5`CGGCCTCAAAGTCACACCAGT3`
mTP2-R1:	5`AGTCCGTTTCCGCCTCCTGAC3`

Primers used for **RT-PCR**:

mHPRT-F:	5`CGTCGTGATTAGCGATGATG3`
mHPRT-R:	5`TATGTCCCCCGTTGACTGAT3`
LIS-Ex1-F1:	5`CGTCAGGCAGTTTAGAGCAAA3`
LIS-Ex1-F2:	5`TACAGCCGCCAGCTCACC3`
LIS-Ex2-f2:	5`GGGAGTGAAGGACGGAAGAG3`
Lis1-Ex2-R1:	5`CTCTTCCGTCCTTCACTCCC3`
LIS-Ex2a-f2:	5`AAAGATCTGCAGCATCCACC3`
LIS-Ex2a-R1:	5`AGAACCTGGGAGCTCAGTGTT3`
LIS-Ex3-r2:	5`CTCTGGGACGACACCATTTT3`

Primers used for **BiFC-Assay**:

BRAP-XhoI-F1:	5`CTCGAGCAGCGCCTGTCCTATGAGT3`
BRAP-XmaI-R1:	5`AGAACATGACATCCCGGGGCT3`
BRAP-Seq-F1:	5`CCTGTCTCTGCACAGAGAAGT3`
BRAP-Seq-R1:	5`CGATTCCAGCTGGCTTGTCAA3`
LopAct-SacI-F1:	5`CACTTCATCCGGAGCTCTACA3`
LopAct-ApaI-R1:	5`GAGCAAGGGCCCAGCAGATTTC3`
Nudel-XhoI-F1:	5`GCTTTCTCGAGCATGGATGGT3`
Nudel-SacII-R1:	5`AGCATCCGCGGTGCTGACGAT3`
Nudel-Seq-F1:	5`GAGCATCAGTATGCACAGAGC3`
Nudel-Seq-R1	5`GTGCTGATATCCTAGCAGAAG3`
pACT2-F:	5`GCTTACCCATACGATGTTCC3`
pACT2-R:	5`TTGAGATGGTGCACGATGCA3`
V2-KpnI-R1:	5`CCCTATGGTACCTCTAGACAAC3`
V2-SmaI-F1:	5`GAACTAACCCGGGCTATAGCAGAT3`

Primers used for **other applications**:

GT-LacZ-F1:	5`GAGAATCCGACGGGTTGTTACTC3`
GT-LacZ-R1:	5`AATTCGCGTCTGGCCTTCCTGTAG3`

GT-LacZ-R2:	5`ACGGCGGATTGACCGTAATGGGAT3`
GT-Test-F1:	5`GACTAGGAGTGTGACTCAGT3`
GT-Test-R1:	5`CGATTAAGTTGGGTAACGCC3`
Lis1 Intron2R2.2:	5`TGAGCCACCTTGATGACTCCA3`
LIS-Ex2-F1:	5`TGGTGGATGGGAGTGAAGGA3`
LIS-Ex2a-F1:	5`TGTCCTTAGGTTGAGGGTGG3`
LIS-Ex3a-R1:	5`TATGTGGCTTCCACAGAGGG3`
RT-IRES-R1:	5`TCGATCCCCACTGGAAAGACC3`
RT-LacZ-F4:	5`ACTGATGGAAACCAGCCATC3`
RT-LacZ-R3:	5`CTTCATCCACCACATACAGG3`
RT-NEO-R1:	5`CATTGCATCAGCCATGATGG3`
SP6:	5`ATTTAGGTGACACTATAGAAT3`
T7:	5`TAATACGACTCACTATAGGG3`

2.1.9 cDNA probes for Northern blotting

ß-actin	generated by Dr. J. Nolte
c-myc Tag	generated in present study
Lis1cDNA	generated in present study
Lis1 Exon2a	generated in present study
Odf1	generated in present study
PGK2	generated by Dr. J. Nolte
SCP3	generated by Dr. J. Nolte
TP2	generated by Dr. J. Nolte

2.1.10 Eukaryotic cell lines

GC-1	Hofmann, USA
HeLa	ATCC, Rockville, USA
NIH 3T3	S.A. Aaronson, Bethesda, U.S.A.
SSC/129/Sv	Human Genetics, Göttingen
ES 2A-53	K. Chowdhury, Göttingen

2.1.11 Mouse strains

Mouse strains C57BL/6N, 129/Sv/Ola, CD-1, NMRI and FVB/N were initially ordered from Charles River Laboratories, Wilmington, USA, and further bred in Animal Facility of Institute of Human Genetics, Göttingen.

2.1.12 Antibodies

Primary Antibodies:

Mouse anti mouse α-tubulin Antibody	Sigma, Deisenhofen
Monoclonal Anti-LIS1, Clone LIS1-338	Sigma, Deisenhofen
Mouse monoclonal IgG Anti-LIS1 (G-3)	Santa Cruz Biotechnology, Heidelberg
Goat polyclonal IgG Anti-LIS1 (N-19)	Santa Cruz Biotechnology, Heidelberg
Goat polyclonal IgG Anti-LIS1 (K-16)	Santa Cruz Biotechnology, Heidelberg
Mouse Monoclonal IgG Anti-c-Myc Tag	Milipore, Schwalbach/Ts.

Secondary Antibodies:

Anti-Goat IgG AP conjugated Antibody	Sigma, Deisenhofen
Anti-Goat IgG Cy3 conjugated Antibody	Sigma, Deisenhofen
Anti-Goat IgG FITC conjugated Antibody	Sigma, Deisenhofen
Anti-Goat IgG Peroxidase conjugated Antibody	Sigma, Deisenhofen
Anti-Mouse IgG Cy3 conjugated Antibody	Sigma, Deisenhofen
Anti-Mouse IgG AP conjugated Antibody	Sigma, Deisenhofen
Anti-Mouse IgG FITC conjugated Antibody	Sigma, Deisenhofen
Anti-Mouse IgG Peroxidase conjugated Antibody	Sigma, Deisenhofen

2.1.13 Enzymes

Platinum Taq polymerase	Invitrogen, Karlsruhe
Proteinase K	Sigma, Deisenhofen
Restriction enzymes (with supplied buffers)	Invitrogen, Karlsruhe
	NEB, Frankfurt
DNAse I , Amplification Grade	Sigma, Deisenhofen
Immolase DNA Polymerase	Bioline, Luckenwalde

LA Taq	Takara, Potsdam
RNase inhibitors	Invitrogen, Karlsruhe
Superscript-II	Invitrogen, Karlsruhe
T4 DNA ligase	Promega, Mannheim

2.1.14 Kits

Dual-Luciferase Reporter Assay System	Promega, Mannheim
Dye Terminator Cycle Sequencing-Kit	Applied Biosystems, Darmstadt
DYEnamic ET-Terminator mix	Amersham Pharmacia, Braunschweig
Endo Free Plasmid Maxi Kit	Qiagen, Hilden
Galacto-Light™ System	Applied Biosystems, Darmstadt
GenomeWalker™ Universal Kit	Clontech, Saint-Germain-en-Laye France
ProbeQuant G-50 Micro Colums	Amersham Pharmacia, Braunschweig
QIAquick Gel Extraction Kit	Qiagen, Hilden
Rediprime II DNA Labeling System	Amersham Pharmacia, Braunschweig

2.1.15 Instruments

ABI Prism 7900 HT	Applied Biosystem, Darmstadt
Autoclave	Webeco, Bad Schwartau
Biophotometer	Eppendorf, Hamburg
Centrifuge 5415D	Eppendorf, Hamburg
Centrifuge 5417R	Eppendorf, Hamburg
Cryostat (Modell CM 1900-1-1)	Leica Microsysteme Vertrieb GmbH, Bensheim
DNA Sequencer MegaBACE 1000	Amersham, Freiburg
GeneAmp PCR System 9600	Perkin Elmer, Berlin
Histocentre 2 embedding machine	Shandon, Frankfurt a M.
Microscope BX60	Olympus, München
Microscope IX81	Olympus, München
Neubauer cell chamber	Schütt Labortechnik, Göttingen
Power supply	Gibco BRL, Karlsruhe

Refrig. Superspeed Centrifuge RC-5B	Sorvall, Langenselbold
Semi-Dry-Blot Fast Blot	Biometra, Göttingen
Spectrophotometer Ultraspec 3000	Amersham, Freiburg
SpeedVac concentrator SVC 100H	Schütt, Göttingen
Thermomixer 5436	Eppendorf, Hamburg
TurboblotterTM	Schleicher & Schüll, Dassel
X-Ray Automatic Processor Curix 60	Agfa, München

2.2 Methods

2.2.1 Isolation of nucleic acids

2.2.1.1 Isolation of plasmid DNA

(Sambrook et al., 1989)

2.2.1.1.1 Small-scale isolation of plasmid DNA

A single *E.coli* colony was inoculated in 5 ml of LB medium with the appropriate antibiotic and incubated in a shaker for 16 hrs at 37°C with a vigorous shaking. After the incubation the medium was centrifuged at 2000 x g for 10 min. The pellet was resuspended in 100 µl of solution P1. The bacterial cells were lysed with 200 µl of P2 solution and then neutralised with 150 µl of P3 solution. The precipitated solution was centrifuged at 10000 x g at RT. The supernatant was transferred into a new tube and centrifugation was done again. The supernatant was transferred into a new tube and 1 ml of 100% ethanol was added to precipitate the DNA. The solution was then stored on ice for 15 min, centrifuged at full speed for 20 min, and finally the pellet was washed with 70% ethanol and after air-drying was dissolved in 30-50 µl of Ampuwa.

P1:	50 mM Tris-HCl, pH 8.0
	10 mM EDTA
	100 µg/ ml RNase A
P2:	200 mM NaOH
	1% SDS

P3: 3.0 M Potassium acetate, pH 5.5

2.2.1.1.2 Large-scale isolation of plasmid DNA

A single clone was inoculated in 2 ml LB medium with appropriate antibiotic as a pre-culture for 8 hrs in 37°C shaker. This pre-culture was added in a dilution of 1/50 fold in 100 ml LB medium with appropriate antibiotics and incubated overnight at 37°C with shaking. The saturated culture was centrifuged at 6000 x g for 15 min. The pellet was resuspended in 5 ml of solution PI and cells were lysed with P2 and P3 as described above. The precipitated solution was centrifuged at 20000 x g for 30 min at 4°C. Meanwhile, the column (Qiagen-tip) that was provided with the midi preparation kit was equilibrated with 10 ml of QBT solution. After centrifugation, the lysate was poured into this equilibrated column to allow the DNA to bind with the resin present in the bed of the column. The column was then washed twice with 10 ml of solution QC. Finally, the DNA was eluted with 5 ml of QF solution. To precipitate the DNA, 3.5 ml of isopropanol was added and mixed thoroughly and centrifuged at 14000 x g for 30 min at 4°C. The DNA pellet was washed with 70% ethanol and dissolved in 100 µl of TE buffer.

QBT:	750 mM	Sodium chloride
	50 mM	MOPS pH 7.0
	15%	Ethanol
	0.5 %	Triton X-100
QC:	1 mM	Sodium chloride
	50 mM	MOPS pH 7.0
	15%	Ethanol (absolute)
QF:	1.25 M	Sodium chloride
	50 mM	Tris/HCl (pH 8.5)

2.2.1.1.3 Endotoxin free preparation of plasmid DNA

Endotoxins, also known as lipopolysaccharides or LPS, are cell membrane components of Gram-negative bacteria (e.g., *E. coli*). During lysis of bacterial cells, endotoxin molecules are released from the outer membrane into the lysate. Endotoxins strongly influence the transfection efficiency of cultured cells like embryonic stem (ES) cells. Increased endotoxin levels lead to sharply reduced transfection efficiencies. Endofree plasmid preparation kit integrates endotoxin removal into standard plasmid preparation procedure. The neutralised bacterial lysate was filtered through a QIA filter cartridge (provided in kit) and incubated on ice with a specific Endotoxin Removal buffer (patented by Qiagen). The endotoxin removal buffer prevents LPS molecules from binding to the resin in the columns (QIAGEN-tips), thus allowing purification of DNA containing less than 0.1 EU endotoxin per µg plasmid DNA.

2.2.1.2 Isolation of genomic DNA from murine tail biopsies

(Laird et al., 1991)

Lysis buffer I: 100 mM Tris/HCl (pH 8.0)
100 mM EDTA
0.5% SDS

Up to 1 cm of the tail from a mouse was incubated in 700 µl of lysis buffer containing 35 µ1 proteinase K (10 µg/µl) at 55°C overnight in Thermomixer 5436. To the tissue lysate an equal volume of phenol was added, mixed by inverting several times and centrifuged at 8000 x g for 5 min at RT. After transferring the upper aqueous layer into a new tube, the same procedure was repeated, first with 1:1 ratio of phenol and chloroform and then with chloroform only. Finally, the DNA was precipitated with 0.7 volume of isopropanol, washed with 70% ethanol, dissolved in 50-100 µl of Ampuwa and incubated at 68°C for 10 min.

2.2.1.3 Isolation of total RNA from tissue samples and cultured cells

Total RNA isolation reagent is an improved version of the single-step method for total RNA isolation. The composition of reagent includes phenol and guanidine thiocyanate

in a mono-phase solution. 100-200 mg of tissue sample was homogenised in 1-2 ml of peqGOLD TriFast by using a plastic homogeniser. The sample volume should not exceed 10% of the volume of reagent used for the homogenisation. The homogenate was incubated at RT for 5 min to permit the complete dissociation of nucleoprotein complexes. Then, 0.2 ml of chloroform was added, mixed vigorously and stored at 4°C for 10 min. After centrifugation at 12000 x g for 15 min at 4°C, the colourless upper aqueous phase was transferred into a new tube. The RNA was precipitated by adding 0.5 ml of isopropanol. Finally, the pellet was washed twice with 70% ethanol and dissolved in 30-50 µl of DEPC-H_2O.

2.2.2 Determination of nucleic acid concentration

The concentration of nucleic acids was determined spectrophotometrically by measuring absorption of the samples at 260 nm. The quality of nucleic acids i.e. contamination with salt and protein was checked by the measurements at 280 nm and 320 nm. The concentration was calculated according to the formula (Lambert-Beer Law):

$$C = (E\,260 - E\,320)fc$$

C= concentration of sample (µg/µl)
E 260= ratio of extinction at 260 nm
E 320= ratio of extinction at 320 nm
f = dilution factor
c= concentration (standard) / absorption (standard)
for double stranded DNA : c = 0.05 µg/µl
for RNA : c = 0.04 µg/µl
for single stranded DNA : c = 0.03 µg/µl

2.2.3 Gel electrophoresis

Gel electrophoresis is the technique by which mixtures of charged macromolecules, especially nucleic acids and proteins, are separated in an electrical field according to their mobility which is directly proportional to macromolecule's charge to mass ratio.

2.2.3.1 Agarose gel electrophoresis of DNA

Agarose gels are used to electrophorese nucleic acid molecules from as small as 50 bp to more than 50 kb, depending on the concentration of the agarose and the precise nature of the applied electrical field (constant or pulse). Usually, 1 g- 1.5 g of agarose was added to 100 ml of 0.5 x TBE buffer and boiled in the microwave to dissolve the agarose, then cooled down to about 60°C before adding one drop of ethidium bromide (0.5 %). These 1-1.5% agarose gels were poured into a horizontal gel chamber.

2.2.3.2 Agarose gel electrophoresis of RNA

(Hodge, 1994)

Single-stranded RNA molecules often have complementary regions that can form secondary structures. Therefore, RNA was run on a denaturing agarose gel that contained formaldehyde, and before loading, the RNA was pre-treated with formaldehyde and formamide to denature the secondary structure of RNA. 1 g of agarose was added to 10 ml of l0 x MOPS Buffer and 74 ml DEPC treated H_20 and dissolved by heating in a microwave. After cooling down to about 50°C, 16.6 ml of formaldehyde (37%) were added, stirred and poured into a horizontal gel chamber.
RNA samples were treated as follows:
10 µg RNA (1 µg/µl) was mixed with sample buffer (3 µl 10x MOPS Buffer, 5 µl Formaldehyde and 12 µl Formamide (40%)), denaturated at 65°C for 10 min and chilled on ice. Then 8 µl Stopmix (containing 0.1% ethidium bromide) was added before loading onto the gel. The gel was run at 25 V at 4°C for about 16-20 hrs. To determine the size of the nucleic acid fragments on agarose gels, molecular weight ladder (0.5 - 10 kb RNA ladder) was loaded with samples in parallel slots.

2.2.3.3 SDS-PAGE for separation of proteins

(Laemmli, 1970)

The NuPAGE Pre-Cast Gel System (Invitrogen) is a polyacrylamide gel system for high performance gel electrophoresis and is based on SDS-PAGE gel chemistry. It consists of NuPAGE Bis-Tris Pre-Cast Gels and specially optimized buffers which have an operating pH of 7.0. The NuPAGE System is based upon a Bis-Tris-HCl buffered (pH

6.4) polyacrylamide gel, with a separating gel that operates at pH 7.0. While NuPAGE Bis-Tris Gels do not contain SDS, they are formulated for denaturing gel electrophoresis applications only.
7.5 µl of NuPAGE LDS Sample Preparation Buffer 4 x (pH 8.4) were mixed with 2 µl 0.1 M DTT, 10 to 20 µg of whole protein extract and Ampuwa to a total volume of 30 µl. The samples were denaturated by heating in 70ºC for 10 min and cooled on ice for 5 min. Samples were then centrifuged at 10000 x g for 10 min and loaded onto NuPAGE Bis-Tris Gels of acrylamide concentration of 10% and run in 1 x NuPAGE MOPS Buffer at 60 V for 3 to 5 hrs at RT producing an expected separation range. To determine the molecular weight of the proteins on the gel, 8 µl of a pre-stained molecular weight standard (See Blue Plus2, Invitrogen) was also loaded.

2.2.4 Isolation of DNA fragments after gel electrophoresis

The QIAquick Gel Extraction method is designed to extract and purify DNA of 70 bp to 10 kb in length from agarose gels. Up to 400 µg agarose can be processed per spin column. The principle of this method depends on selective binding of DNA to uniquely designed silica-gel membrane. To the excised DNA fragment from agarose, 3 volumes of QG buffer were added and incubated at 50°C for 10 min. After the gel slice was dissolved completely, it was applied to a QIAquick column and centrifuged for 1 min. The flow through was discarded and the column was washed with 0.75 ml of PE buffer. The column was then placed into a fresh microcentrifuge tube. To elute the DNA, 25 to 50 µl of Ampuwa was applied to the centre of the QIAquick membrane and centrifuged for 1 min.

2.2.5 Enzymatic modifications of DNA

2.2.5.1 Digestion of DNA using restriction enzymes

Restriction enzyme digestions were performed by incubating double-stranded DNA with an appropriate amount of restriction enzyme in its respective buffer as recommended by the supplier, and at the optimal temperature for the specific enzyme. Standard digestions included 2-10 U enzyme per microgram of DNA. These reactions were usually incubated for 1-3 hrs to ensure complete digestion at the optimal

temperature for enzyme activity, which was typically 37°C. For genomic DNA digestion, the reaction solution was incubated overnight at 37°C.

2.2.5.2 Ligation of DNA fragments

Ligation of an insert DNA into a vector (digested with appropriate restriction enzyme) was carried out in the following reaction mix:

30 µg vector DNA (digested)
50-100 µg insert DNA (1:3, vector: insert ratio)
1 µl ligation buffer (10x)
1 µl T4 DNA ligase (5U/µl)
in a total volume of 10 µl

Ligations were carried out at RT for 2-4 hrs or 16°C overnight.

2.2.5.3 Phenol-chloroform extraction and ethanol precipitation

Protein impurities were removed by vigorous shaking of nucleic acid solution with an equal volume of phenol/chloroform mixture (1:1). The emulsion was then centrifuged for 5 min, 10000 x g, at RT, and the upper aqueous phase was collected, mixed with an equal volume of chloroform and centrifuged (5 min, RT, 10000 x g). Finally, the upper aqueous phase was collected for precipitation. Nucleic acids were precipitated by addition of NaAc (final conc. 0.3 M) and 2.5 volumes of absolute ethanol. The mixture was then vortexed and centrifuged (5 min, 4°C, 10000 x g). The pellet was washed with 70% ethanol and centrifuged (5 min, RT, 10000 x g). After washing, the supernatant was aspirated and the pellet was air dried. The dried pellet was redissolved in Ampuwa.

2.2.5.4 TA-Cloning

(Clark, 1988; Hu, 1993)

Taq polymerase and other DNA polymerases have a terminal transferase activity that results in the non-template addition of a single nucleotide to the 3` ends of PCR

products. In the presence of all 4 dNTPs, dATP is preferentially added. This terminal transferase activity is the basis of the TA- cloning strategy. For cloning of PCR products, the pGEM-T Easy vector system that has 5' T overhangs was used. The mixture was as follows:

50 µg pGEM-T Easy Vector
PCR product (1:3, vector to insert ratio)
1 µl of T4 DNA Ligase l0x buffer
1 µl of T4 DNA Ligase
in a total volume of 10 µl

The content was mixed by pipetting and the reaction was incubated overnight at 16°C. For transformation of the ligation reaction, DH5α competent cells were used (Invitrogen).

2.2.5.5 Filling-up reaction

(Costa and Weiner, 1994)

0.1-4 µg of digested DNA was mixed with 0.05 mM dNTPs and 1-5 U of Klenow fragment with reaction buffer in a total volume of 50 µl. The reaction was incubated at 37°C for 15 min, and then stopped by heating at 75°C for 10 min.

2.2.6 Transformation of competent *E.coli* bacteria

(Ausubel et al., 1994)

Transformation of competent DH5α (Hanahan, 1983) purchased by Invitrogen was done by gently mixing one aliquot of the competent bacteria (50 µl) with 10 µl of ligation reaction. After incubation for 30 min on ice, bacteria were heat shocked for 45 sec at 42°C, and cooled down for 5 min on ice. After adding 900 µl of SOC medium (Invitrogen), bacteria were incubated at 37°C, 200 rpm for l hr, to allow recovery of heat shocked bacteria and were plated out on LB-agar plates containing appropriate antibiotic and whenever required 1 mM IPTG and 40 mg/ml X-Gal for "Blue-White" selection.

2.2.7 Polymerase Chain Reaction (PCR)

(Saiki et al., 1988)

The polymerase chain reaction (PCR) represents the most important technique in the field of molecular biology. It is a very sensitive and powerful technique (Saiki et al., 1988) that is widely used for the exponential amplification of specific DNA sequences *in vitro* by using sequence specific synthetic oligonucleotides (primers). The general principle of PCR starts from a pair of oligonucleotide primers that are designed so that a forward or sense primer directs the synthesis of DNA towards a reverse or antisense primer, and vice versa. During the PCR, the Taq DNA polymerase (a heat stable polymerase) (Chien et al., 1976) catalyses the synthesis of a new DNA strand that is complementary to a template DNA from the 5' to 3' direction by a primer extension reaction, resulting in the production of the DNA region flanked by the two primers. It allows the rapid and unlimited amplification of specific nucleic acid sequences that may be present at very low concentrations in very complex mixtures.

2.2.7.1 PCR amplifications of DNA fragments

The amplification cycles were performed in an automatic thermocycler. The PCR reaction contains in general the following substances:

1.0 µl DNA
l.0 µl forward primer (l0 mM)
l.0 µl reverse primer (10 mM)
0.5 µl l0 mM dNTPs
2.5 µl l0x PCR buffer
1.0 µl 50mM $MgCl_2$
0.2 µl Platinum Taq DNA Polymerase (5 U/µl)
Up to 25 µl Ampuwa

The reaction mixture was placed in a 200 µl reaction tube in a thermocycler. A standard PCR program is shown here:

Initial denaturation	95°C	5 min	
Denaturation	94°C	30 sec	28-35x
Annealing	55-60°C	30 sec	
Elongation	72°C	1 min	
Final extension	72°C	10 min	
Storage	8°C		

2.2.7.2 Reverse transcription PCR (RT-PCR)

Reverse Transcription PCR (RT-PCR) is a technique, which generates cDNA fragments from RNA templates, and thereafter amplifies them by PCR. It is a very useful technique to determine the expression of genes in specific tissues or in different developmental stages. 5 µg of total RNA was mixed with 1 µl of oligo (dT) primer (10 µM) in a total volume of 12 µl. To avoid the possible secondary structure of the RNA, which might interfere with the synthesis, the mixture was heated to 65°C for 5 min, and then chilled on ice. After a brief centrifugation, the followings were added to the mixture:

4 µl 5x First strand buffer
2 µl 0.1M DTT
1 µl 10 mM dNTPs

The content of the tube was mixed gently and incubated at 42°C for 2 min. Then, 1 µl of reverse transcriptase enzyme (Superscript II, Invitrogen) was added and further incubated at 42°C for 50 min for the first strand cDNA synthesis. Next, the reaction was inactivated by heating at 70°C for 15 min. 0.5 µl of the first strand reaction was used for the PCR reaction (as described above).

2.2.7.3 Quantitative Real-Time PCR

In contrast to regular PCR, Real-Time PCR or quantitative PCR (qPCR) allows accurate quantification of starting amounts of DNA targets. In Real-Time PCR using SYBR Green I, the increase of fluorescence as the dye binds to the increasing amount of DNA in the reaction tube is measured. SYBR Green I binds to all double-stranded

DNA molecules, emitting a fluorescent signal of a defined wavelength on binding. The excitation and emission maxima of SYBR Green I are at 494 nm and 521 nm, respectively, and are compatible for use with any Real-Time cycler. Detection takes place in the extension step of Real-Time PCR. Signal intensity increases with increasing cycle number due to the accumulation of PCR product.

DNA was isolated from transgenic mice tails by the method described above (2.2.1.2) followed by an ethanol precipitation (2.2.5.3). Serial dilutions of sample and standard DNA's were made. Standard DNA (a mixture of Founder DNA and two F1 males) was serially diluted to 20, 10, 5, 2.5, 1.25 and 0.625 ng/µl for the generation of standard curve, while each sample DNA (transgenic mice) was diluted to a concentration of 10 ng/µl. Primers were designed to generate amplicons less than 200 bp, thus enhancing the efficiency of PCR amplification. Real-Time quantitative PCR was performed using QuantiTect SYBR Green PCR Master mix (Quiagen) in an ABI Prism 7900HT sequence detection system. Each reaction was run in triplicate and the melting curves were analysed to ensure that only a single product was amplified. *Pelota* gene (GenBank, NM_134058) primers (qPCR-Pelo-F and qPCR-Pelo-R (2.1.8)) were used for the normalisation of each DNA sample and *Acrosin* gene (GenBank, NM_013455) primers (qPCR-Acr-F and qPCR-Acr-R) were used for calibration of normalised samples measured. Quantitative real-time PCR reactions of DNA specimens and standards were conducted in a total volume of 10 µl with 5 µl of 2 x QuantiTect SYBR-Green PCR-Master-Mix, 1 µl of each forward and reverse primer in a final concentration of 9 µM and 2.5 µl of DNA. The following cycling parameters were used:

2 min 50°C
15 min 95°C
15 sec 95°C
30 sec 60°C } 40x
30 sec 72°C
15 sec 95°C
15 sec 60°C } melting curve
15 sec 95°C

Standard curves of the threshold cycle number versus the log number of copies of genes were generated for transgenic integration sites and were used to extrapolate the number of integration sites of transgene. Quantitative Real-Time PCR results were reported as the number of transgenic allele in comparison to homozygous *Acrosin* allele.

2.2.7.4 Quantitative Real-Time RT-PCR

In general Quantitative Real-Time RT-PCR for mRNAs was performed as for DNA (2.2.7.3). cDNA synthesis was done according to section 2.2.7.2 with preceding DNAse treatment. 5 µl 2x QuantiTect SYBR-Green PCR-Master-Mix, 1 µl Forward Primer (9µM), 1 µl Reverse Primer (9µM), 0.3µl $MgCl_2$ (50mM) and 1µl of cDNA (in a 1/20 dilution) were mixed with RNase free water to a total volume of 10 µl. The following PCR program was used:

2 min 50°C
15 min 95°C
15 sec 95°C
30 sec 60°C } 40x
30 sec 72°C
15 sec 95°C
15 sec 60°C } melting curve
15 sec 95°C

Primer sequences are provided in section 2.1.8. *Sdha* was used as endogenous reference. For standard curves, a mixture of NMRI testes cDNA and GT/GT testes cDNA was used. Selection of the appropriate sample for the standard curve was based on preliminary experiments testing detection of expression of each gene by RT-PCR (2.2.7.2). Specificity of the PCR products was confirmed with subsequent cloning and sequencing while reliability of Real-time PCR data was also assessed in connection with the respective dissociation curves.

2.2.7.5 PCR-based "Genome-Walking"

GenomeWalker DNA walking is a simple method for finding unknown genomic DNA sequences adjacent to a known sequence such as a cDNA (Siebert et al., 1995). The GenomeWalker™ Universal Kit (Clontech) was used to verify the integration sites of the gene trap vector into the genome of L39 mice. A schematic overview of the methodical approach is shown in figure 2.1 (modified from GenomeWalker™ Universal Kit).

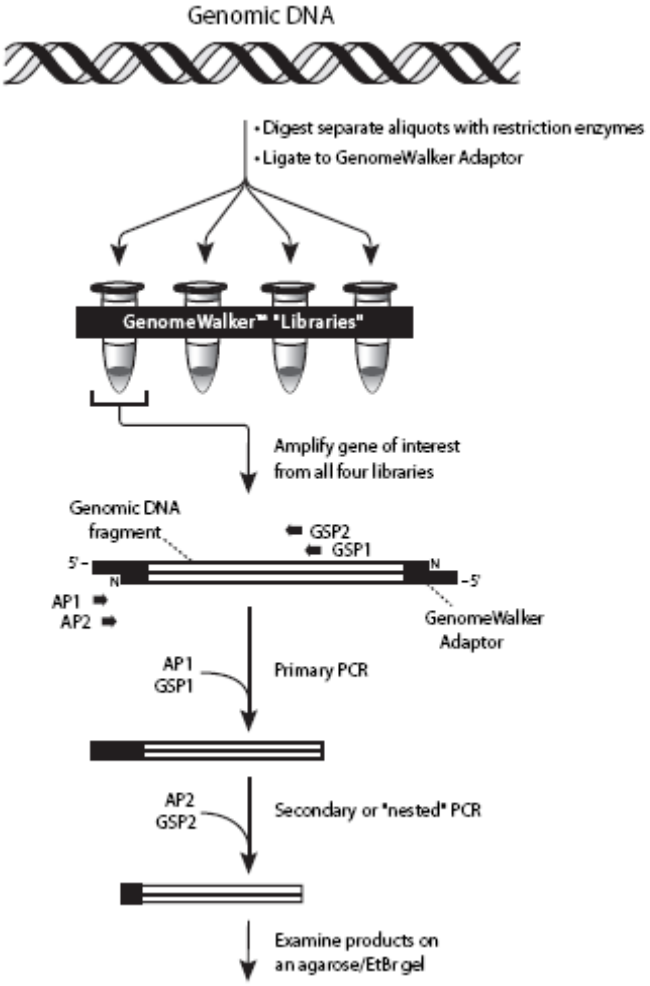

Figure 2.1: Flow chart of the GenomeWalker™ protocol. Genomic DNA is digested by four different "blunt-end" restriction enzymes and ligated to GenomeWalker Adaptors. These DNA pools are then subjected to two rounds of PCR, with Adaptor primer (AP1) and Gene-specific primer (GSP1) and AP2 and GSP2, respectively. Amplified PCR products are examined on agarose gel.

Genome Walk was performed according to GenomeWalker^{Tm} Universal Kit User Manual (Clontech). Briefly, DNA was isolated from different L39$^{GT/GT}$ mice organs by the method described above (2.2.1.2), followed by an ethanol precipitation (2.2.5.3).

DNA was then digested with four different restriction enzymes (supplied by Clontech) and ligated to GenomeWalker Adaptors. After construction of the libraries, a primary PCR using adaptor primer (AP1) provided in the kit and an outer, gene-specific primer (GSP1) located in *Lis1* Intron 2 or the gene trap vector was run. The primary PCR mixture was then diluted and used as a template for a secondary or "nested" PCR with the nested adaptor primer (AP2) and a nested gene-specific primer (GSP2) located 5`downstream of the corresponding GSP1. Each PCR product was then cloned into pGEM-T Easy and sequenced.

2.2.8. Protein and biochemical methods

2.2.8.1 Isolation of total proteins

Proteins were extracted from ~30 mg frozen mouse tissues (disrupted before by mortar and pestle) by homogenization in 500 µl – 1 ml protein lysis buffer. Lysates were maintained on ice for 60 min and then sonicated on ice and centrifuged at 12000 x g for 20 min at 4°C. Supernatant was distributed in several microcentrifuge tubes, quantified and stored at -80°C or used immediately for Western blotting.

2.2.8.2 Determination of protein concentration

(Bradford, 1976)

To determine the protein concentration Bio-Rad protein assay was employed, which is a dye-binding assay based on the differential colour change of a dye in response to various concentrations of protein. The assay is based on the observation that the absorbance maximum for an acidic solution of Coomasie Blue G-250 shifts from 494 to 595 nm when the binding to protein occurs. The BSA stock solution of 1 µg/ml was diluted in order to obtain standard dilutions in range of 10 µg/ml to 100 µg/ml. The Bio-Rad's color reagent was diluted 1:5 with H_2O. In a 96-well microtiter plate 20 µl of each standard dilution and the samples to be measured were pipetted with 280 µl of the color reagent. The absorption of the colour reaction was measured at 595 nm in a microplate reader (Microplate Reader 450, Bio-Rad).

2.2.9 Blotting techniques

2.2.9.1 Southern blotting of DNA onto nitrocellulose filter

(Southern, 1975)

In Southern blotting, the transfer of denatured DNA from agarose gels to nitrocellulose membrane is achieved by capillary flow. 20 x SSC buffer, in which nucleic acids are highly soluble, is drawn up through the gel into the nitrocellulose membrane, taking with it the single-stranded DNA that becomes immobilised in the membrane matrix. After electrophoresis of DNA, the gel was treated with 0.25 M HCl for depurination. It was followed by incubation in denaturation solution for 30 min and 45 min in neutralization solution. The transfer of the DNA to the nitrocellulose membrane was done in a Turbo-Blot-Apparatus (Schleiher & Schuell, Dassel). About 20 Whatman filter papers (GB 003) were layered on a Stack Tray followed by four Whatman filter papers (GB 002) and one Whatman filter paper GB 002 soaked with 2 x SSC. The equilibrated nitrocellulose filter that was also soaked with 2 x SSC was laid on the top. The agarose gel, which was treated as described above, was placed on the filter and was covered with three Whatman filter papers GB 002 soaked with 2x SSC. The buffer tray was placed and filled with 20 x SSC. Finally a wick, which was soaked with 20 x SSC, and the wick cover were put on the top of the blot. The transfer was carried out overnight. Finally, after disassembling of the blot, the filter was washed briefly in 2 x SSC and the DNA was fixed onto the filter by baking it at 60°C for 2 hrs.

2.2.9.2 Northern blotting of RNA onto nitrocellulose filter

For the transfer of RNA onto a nitrocellulose filter, the same procedure as described above (2.2.9.1) was performed. In this case, however, the gel does not need to be denatured, but was transferred directly onto the filter.

2.2.9.3 Western blotting of protein onto PVDF membrane

(Gershoni and Palade, 1982)

Semi-dry transfer buffer (1x): 25 mM Tris pH 8.3
150 mM Glycin
10 % Methanol

After the electrophoresis of proteins on a SDS-PAGE, the gel and the PVDF membrane, which were cut of the size of the gel, were first moistened with methanol and then equilibrated in semidry transfer buffer. Six pieces of GB004 Whatman filter paper were also cut of the size of the gel. First, three papers soaked with transfer buffer were placed on semi dry transfer machine's lower plate and then the equilibrated membrane was placed over them. Next, the gel was placed avoiding any air bubbles. Another three Whatman papers soaked with transfer buffer were placed over to complete the sandwich model. The upper plate was placed over this sandwich and the transfer was carried out at 10 W (150 – 250 mA, 39 V) for 1 hr. The membrane was air-dried and used for incubation with antibodies. To confirm transfer efficiency of proteins onto nitrocellulose membranes, the gel was incubated for 30 min in Coomassie blue solution at RT and then destained in water overnight.

The membrane was first incubated in blocking buffer I with 5% non-fat dry milk for 1 hr at 4°C in order to block unspecific binding sites, followed by incubation with 2% milk in B1 buffer for 5 min. Membrane was then incubated with a primary antibody at the recommended antibody dilution in buffer B1 with 2% non-fat dry milk overnight at 4°C. Then, the membrane was washed 3 times in B1 buffer with 2% dry milk for 60 min and then incubated with the HRP conjugated secondary antibody in B1 buffer with 2% non-fat dry milk for 1 hr at 4°C. After this step, the membrane was washed three times for 20 min in B1 buffer with 2% dry milk and once in PBS for 5 min at 4°C. Finally, protein bands were visualized using enhanced chemiluminescence as described by the manufacturer (Santa Cruz Biotech.).

2.2.10 "Random Prime" method for generation of ^{32}P-labeled DNA

(Denhardt, 1966; Feinberg and Vogelstein, 1989)

Rediprime™ II Random Prime Labeling System (Amersham Pharmacia) was used for labelling of DNA probes. The method depends on the random priming principle developed by Feinberg and Vogelstein (1989). The reaction mix contained dATP, dGTP, dTTP, Klenow fragment (4-8 U) and random oligodeoxyribonucleotides. Firstly, 25-50 ng of DNA were denatured in a total volume of 46 µl (in TE buffer) in boiling water for 10 min and quickly chilled on ice for 5 min. After pipetting the denatured probe in a Rediprime II Random Prime Labeling System cup, 4 µl of [γ-^{32}P] dCTP (3000 Ci/mmol) was added to the reaction mixture. The labelling reaction was carried out at 37°C for 45 min. The labelled probe was purified from nonincorporated [γ-^{32}P] dCTP by using microspin columns (Amersham Pharmacia).

2.2.11 Hybridisation of nucleic acids

(Denhardt, 1966)

The membrane to be hybridised was equilibrated in 2 x SSC and transferred to a hybridisation tube. After adding 10 ml of hybridisation solution and 500µl sheared salmon DNA, the membrane was incubated for at least 4 hrs in the hybridization oven at 65°C. Then, the labelled probe was denatured at 95°C for 10 min, quickly chilled, and added to the hybridisation solution. The hybridisation was carried out overnight in the oven. The next day, the filter was washed for 10 min with 2 x SSC at RT. Finally, it was washed with 0.2 x SSC containing 0.1% SDS at the hybridisation temperature. After drying the filter, it was sealed in Saran wrap and exposed to autoradiography for an appropriate time at -80°C. The film was developed in X-Ray Automatic Processor Curix 60. For quantification of detected bands, the program Quantity One (Bio-Rad) was used.

2.2.12 Non-radioactive dye terminator cycle sequencing

(Sanger, 1977)

Non-radioactive sequencing was performed with the Dye Terminator Cycle Sequencing-Kit (Applied Biosystems). The reaction products were analysed with

automatic sequencing equipment, namely MegaBASE 1000 (Amersham). For the sequencing reaction, four different dye labelled dideoxy nucleotides were used (Sanger et al., 1977), which, when exposed to an argon laser, emit fluorescent light which can be detected and interpreted. The reaction was carried out in a total volume of 10 µl containing 300-800 ng plasmid DNA or 100-200 ng purified PCR products, 10 pmol primer and 4 µl reaction mix (contains dNTPs, dideoxy dye terminators and Taq DNA polymerase). Elongation and chain termination take place during the following program in a thermocycler:
5 min denaturation followed by 25 cycles at 95°C, 30 sec; 55°C, 15 sec; 60°C, 2 min. After the sequencing reaction, the DNA was precipitated with 1/10 volume 3 M NaAc and 2.5 volume 100% ethanol and washed in 70% ethanol. The pellet was dissolved in 4 µl of loading buffer, denatured at 95°C for 3 min, and finally loaded onto the sequence gel.

2.2.13 Histological techniques

2.2.13.1 Tissue preparation for paraffin-embedding

The freshly prepared tissues were fixed in Bouin's solution or 4% PFA for 24 hrs to prevent alterations in the cellular structure. The tissue to be embedded in paraffin should be free of water. The dehydration process was accomplished by passing the tissue through a series of increasing alcohol concentrations. For this purpose, the tissue was incubated in 70%, 80%, 90%, 96% and 100% ethanol for at least 1 hr at RT. Later, the ethanol was removed from the tissue by incubating it in isopropanol overnight. Tissue was then incubated in different mixtures of isopropanol/xylol in ratios 3:1, 1:1 and 1:3 for 3 hrs at RT. Then tissue was incubated in 100% xylol overnight. Further, tissue was incubated in paraplast at 60°C overnight. Before embedding, paraplast was changed at least three times. Finally, the tissue was placed in embedding mould and melted paraffin was poured into the mould to form a block. The block was allowed to cool and was then ready for sectioning or was stored at 4°C or RT.

2.2.13.2 Sections of the paraffin block

The paraffin blocks were pre-cut to the optimal size and clamped into the microtom. The cut-thickness of the paraffin embedded tissues was 3-5 µm. The sections were floated on 40°C prewarmed water to allow actual spread and subsequently put onto Superfrost slides. A fine brush was used to transfer the sections to slides. Slides were then dried at 40°C and incubated in 60°C for about 15 min to get rid off excess of paraffin. Slides were then stored at RT for further analysis.

2.2.13.3 Tissue preparation for cryopreservation

For cryopreservation of tissues, animals were perfused prior to dissection. Animals were anesthetized with Rompun (Bayer) or Ketavet (Bayer), the thoracic cavity was opened, the heart exposed and an 18-gauge needle was inserted through the left ventricle into ascending aorta and then the right atrium was snipped. The animal was perfused slowly with about 20 ml of dPBS, then with 20 ml of 4% PFA. After perfusion was completed, the animal limbs were cut and the mouse stored overnight in 4% PFA. The next day, the appropriate tissue was removed and post-fixed in PBS with 30% sucrose for 16-30 hrs. Then the tissue was embedded in Tissue freezing medium (Leica Microsystems) and stored at -80°C.

2.2.13.4 Cryosectioning

The cryostat (Leica Microsystems) was cooled down to approximately -20°C. The embedded tissues were cut into 5-6µm thick slices and transferred to Superfrost slides. The slides were used immediately for LacZ staining or immunohistochemistry.

2.2.13.5 Hematoxylin & Eosin staining of histological sections

Slides with paraffin sections were first incubated three times in Xylol for 5 min, followed by incubation in 100%, 96%, 80%, 70% and 50% ethanol for 3 min each. Slides were then washed 1 min in dH_2O and stained for 5 min in hematoxylin. Staining was followed by washing in running tap water for 10 min. Thereafter slides were destained in acidic ethanol (70%) according to the staining intensity required,

then stained with eosin (0.1% + 2% acetic acid) for 2-10 sec, then washed in dH_2O for 1 min and incubated in 50%, 70%, 80%, 90%, 96% and 100% ethanol for 3 min each. Finally they were incubated twice in Xylol for 3 min and closed with cover slides.

2.2.13.6 LacZ staining of tissue sections

Freshly cut cryosections of testis or brain of different gene trap mice were fixed in Fix A solution for 30 min, washed in dPBS for 10 min and then fixed in Fix B for another 30 min at RT. Incubation with X-Gal staining solution was performed overnight, followed by another washing step with dPBS. Slides were mounted with Eukitt.

2.2.13.7 Tissue preparation for electron microscopy

For conventional electron microscopy, mouse testes were fixed with 1% paraformaldehyde and 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 8-12 hrs. Fixed testes were 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% OsO4 in cacodylate buffer for 2 hrs, 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.

2.2.14 Indirect immunohistochemistry

Fixation and subsequent treatment of mouse testes were performed as described in 2.2.13. Tissue cross sections (3-7 µm) were deparaffinized with Xylol and rehydrated by descending ethanol concentrations. For immunostaining, sections were washed 3 times in PBS and were then incubated with a 5% blocking solution (5% BSA, 1% Triton X-100 or Tween-20 in PBS) for 1 hr at RT. Testis sections were incubated with primary antibody (1:50-1:200) in a 1.5% blocking solution (1.5% BSA, 1% Triton X-100 or Tween-20 in PBS) overnight at 4°C. Sections were then rinsed three times in PBS and subsequently incubated with secondary antibody (1:500 in 1.5% blocking solution) for 1 hr at RT. After incubation with secondary antibody, sections were washed again in PBS and the nuclei were counterstained with VectaShield, a DAPI-containing mounting medium (Linearis). Immunostaining of the sections was

examined using the fluorescence microscope BX60 (Olympus) or the Laser Scanning microscope IX81 (Olympus).

2.2.15 Generation of transgenic mice

(Hogan et al., 1986)

Generation of transgenic mice was performed by "Transgenic Service" of Max Planck Institute for Experimental Medicine in Göttingen by pronuclear microinjection of DNA. Method for transgenic animal production is based on Hogan et al. (1986).

2.2.15.1 Preparation of DNA for pronuclear microinjection

Transgenic constructs were released from cloning vector by restriction digestion. Digested fragments were separated by agarose gel electrophoresis (without EtBr) in the way that 25 µg of digested plasmid was loaded to slots of the 0.8% agarose gel. After separation, outer lanes were cut out and stained with EtBr. After staining, the gel was reconstructed and appropriate gel slices were cut out from the rest of the gel. DNA was then eluted from gel with QIAquick extraction kit (Qiagen).
Concentration of DNA was estimated by EtBr electrophoresis of DNA aliquot in comparison with Smart ladder marker (defined DNA amounts in each band). For microinjection, DNA was diluted to 30 µg/ml in microinjection buffer (10mM Tris, pH 7.5, 0.1 mM EDTA, pH 8.0).

2.2.16 Determination of sperm parameters

2.2.16.1 Sperm count in epididymis, uterus and oviduct

Caudae epididymes of mice were dissected under aseptic conditions and put into 0.4 ml of in vitro fertilization (IVF) medium. Spermatozoa were allowed to swim out of caudae epididymes for 1 hr at 37°C, 5% CO_2. Sperm suspension was diluted 40-100 times with PBS before counting, when necessary. 5 µl of this suspension was put into a Neubauer counting chamber and sperms were counted in 4 independent fields (each having an area of 0.0025 mm^2) under the microscope BX60 with x200 magnification. Total sperm numbers were calculated by following formula:

Total Sperm = average no. of sperm x 10 x 400 x dilution

For determination of sperm number in the uterus and the oviduct males were mated with wild type females. The uteri and oviducts of those females which were positive for vaginal plug were dissected in M2 medium, sperms were flushed out and counted in a Neubauer counting chamber as described above.

2.2.16.2 Sperm motility

13 µl of sperm suspension was put on a dual sided sperm analysis chamber. Sperm motility was quantified using the computer assisted semen analysis (CASA) system (CEROS version 10, Hamilton Thorne Research). 5000-10000 spermatozoa from 3 mice of mutant line and one wild type mouse were analysed using the following parameters: path velocity (VAP), progressive velocity (VSL), track speed (VCL), lateral displacement (ALH), beat cross frequency (BCF) and straightness (STR). Frequencies of these six sperm motility parameters were examined by probability plots categorised by mouse type (wild type/mutant) and by time of observation (1.5, 3.5 and 5.5 hrs after preparation) for statistical analysis.

2.2.17 Eukaryotic cell culture methods

2.2.17.1 Cell culture conditions

All cells were grown in their respective growth media (2.1.5.2). All the cells were cultured at 37°C in a humidified incubator with 5% CO_2 and grown to 80% confluency. ES-2A53 and SSC/129/Sv cells were grown on mytomycin C-treated MEFs (mouse embryonic fibroblasts).

2.2.17.2 Preparation of MEFs feeder layers

A frozen vial of MEFs (mouse embryo fibroblasts) was quickly thawed at 37°C and transferred to 10 ml MEFs medium. After centrifugation at 270 x g for 5 min, the cell pellet was gently resuspended in 10 ml MEFs medium and plated on a 50 mm culture flask. Cells were incubated at 37°C in 5% CO_2. When the cells formed a confluent monolayer, they were either trypsinised, transferred to five 150 mm dishes and grown until they formed confluent monolayer, or directly treated with mitomycin C. To treat

MEFs with mitomycin C, the medium was removed and 10 ml fresh medium containing 100 µl of mitomycin C (1 mg/ml) was added. After 2-3 hrs of incubation, cells were washed twice with 10 ml PBS, trypsinized, centrifuged, resuspended in MEFs medium and plated onto dishes, which were treated with 0.1% gelatine for at least 2 hrs. The feeder cells were allowed to attach by incubation overnight at 37°C, 5% CO_2. Before adding ES or SSC/129/Sv cells on feeder layer, the medium was changed to ES cell medium.

2.2.17.3 Trypsinization of eukaryotic cells

Cells were washed twice with sterile DPBS and incubated in minimal amount of trypsin- EDTA (0.5 g/l trypsin, 0.2 g/l EDTA) (PAN) at 37°C until they had detached from the dish. The process was controlled under an inverted microscope. Trypsin activity was inhibited by addition of growth medium in which the cells were subsequently resuspended. Cell counting was performed, when necessary, using an improved Neubauer chamber, and the cells were plated out or harvested for cryopreservation.

2.2.17.4 Cryopreservation and thawing of eukaryotic cells

Resupended cells were spun down (800 x g for 5 min at 4°C) in 4 ml growth medium. The supernatant was aspirated and the cells were resuspended (1 - 5 x 10^7 cells/ml) in ice cold medium (DMEM or RPMI, 20% FKS, 10% DMSO). Cells were kept for 7 days at -80°C and then stored in liquid nitrogen. For revitalization, frozen cells were quickly thawed at 37°C water bath, gently transferred to disposable Falcon (BD Falcon, USA) tubes containing 4 ml growth medium and spun down as described above. Supernatant was discarded by aspiration and cells were plated out after being resuspended in a suitable amount of prewarmed growth medium.

2.2.17.5 Transfection of eukaryotic cells with plasmids

Approximately 4 x 10^5 of the cells were plated on 12 well plates and cultured overnight in 2 ml growth medium. Transfection was done using Lipofectamin

transfection reagent (Invitrogen) or Effectene transfection reagent (Qiagen) according to supplier`s protocol. The cells were then washed with prewarmed PBS. About 2 ml of fresh growth medium was applied and cells were incubated at 37°C and 5% CO_2. After 48 hrs, cells were lysed and processed for Luciferase activity measurement.

2.2.18 Assay of Luciferase Activity

The Luciferase Assay provides a rapid, sensitive, and quantitative measurement of the activity of the reporter enzyme firefly (*Photinus pyralis*) Luciferase in cultured mammalian cells. Luciferase catalyses the following chemiluminescent oxidation–reduction reaction:

Luciferase substrate (Luciferin) + ATP + O2 →

Oxyluciferin + light (560 nm) + AMP + PPi + CO2

The Luciferase assay was performed by adding 200 µl of Luciferase-assay-buffer to 30 µl of cell lysate. Luciferase in the cell lysate catalyzes the chemiluminescent reaction, which emits light. The emitted light was measured with a luminometer (Typ LB 953, Berthold).

2.2.19 β-Galactosidase measurement

(Jain et al., 1991)

Measuring of β-Galactosidase- activity was done with 10 µl of the same cell lysate that was used for the analysis of Luciferase activity. The Galacto-Light-Kit was used according to suppliers (Tropix) protocol. β-Galactosidase activity was used to normalize the Luciferase activity by dividing the RLUs (relative light units) of the Luciferase measurement by the RLUs of the β-Galactosidase measurement.

2.2.20 Software and Computer tools

For the analysis of the nucleotide sequences, programs like BLAST, MEGABLAST and other programs from National Center for Biotechnology Information (NCBI) were used (www.ncbi.nlm.nih.gov). For restriction analysis of DNA, NEBcutter V2.0 programm was used (http://tools.neb.com/NEBcutter2/index.php). Information about mouse alleles, phenotypes and strains were obtained from the Jackson Laboratory web

page (www.informatics.jax.org). Mouse genome sequence and other analysis on mouse genes, transcripts and putative proteins were downloaded from Celera discovery system (www.celera.com). For statistical analysis of data, the STATISTICA software was used.

3. Results

3.1 Analysis of the gene trap line L39

3.1.1 Histological analysis of L39 mice

To confirm the observed extensive germ cell degeneration of seminiferous tubules in $L39^{GT/GT}$ mice, testes of 46 d and 2 months old $L39^{GT/-}$ and $L39^{GT/GT}$ mice were fixed in Bouins solution, sliced into 5 µm thick sections and stained with hematoxilin and eosin (H&E) (Fig. 3.1).

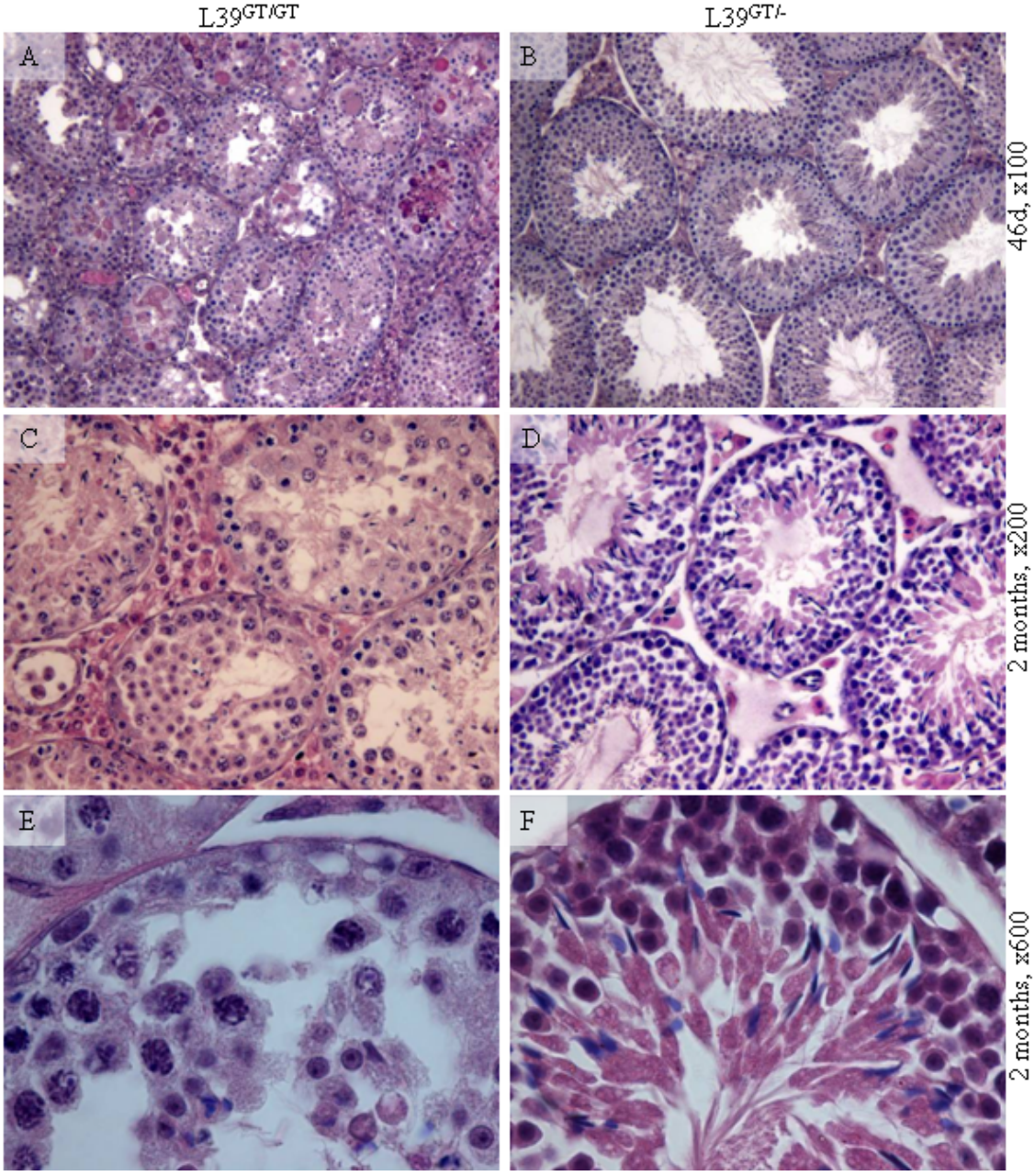

Figure 3.1: H&E staining of testis sections of 46d (A, B) and 2 months old (C-F) $L39^{GT/GT}$ and $L39^{GT/-}$ mice. Sections through testes of homozygous gene trap mice revealed extensive degeneration of a large fraction of seminiferous tubules (A, C, E), whereas testis sections of heterozygous animals demonstrated robust spermatogenesis (B, D, F) (original magnification: A, B x100; C, D x200; E, F: x600).

The histological analysis of testes of $L39^{GT/GT}$ mice revealed extensive degeneration of germ cells in a large fraction of seminiferous tubules. Even though mutant testes exhibited 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 was markedly reduced. This phenotype was visible in the younger males (46 d) as well as in the adult males (2 months old) with an increasing amount of collapsed tubules in the adult males. In marked contrast to this were heterozygous males, which exhibited full spermatogenesis with no obvious differences from wild type males.

3.1.2 Expression analysis of *Lis1* in L39 mice

3.1.2.1 Expression analysis of *Lis1* gene in L39 mice by RT-PCR

To analyse the testis specific and the ubiquitously expressed *Lis1* transcript in L39 and wild type mice, total RNA was isolated (2.2.1.3) from different 36 d old mouse tissues including brain, heart and testes. By RT-PCR analysis (Fig. 3.2) all transcripts could be detected in all analysed tissues. The testis specific *Lis1 Ex2a/3* transcript was clearly detectable in testes and to a lower amount in brain and heart. A reduction of the *Lis1 Ex 2a-specific* transcript in testes of homozygous gene trap males was not clearly visible by RT-PCR. In contrast to this was the downregulation of *Lis1 Ex2/3* transcript in testes of the $L39^{GT/GT}$ male. This transcript was very low expressed in brain, and hardly expressed in heart. Eventually, the *Lis1 Ex5/6* transcript was expressed at a high level in all tissues analysed. Integrity of the RNA used for RT-PCR was proven by amplification of *HPRT* transcript.

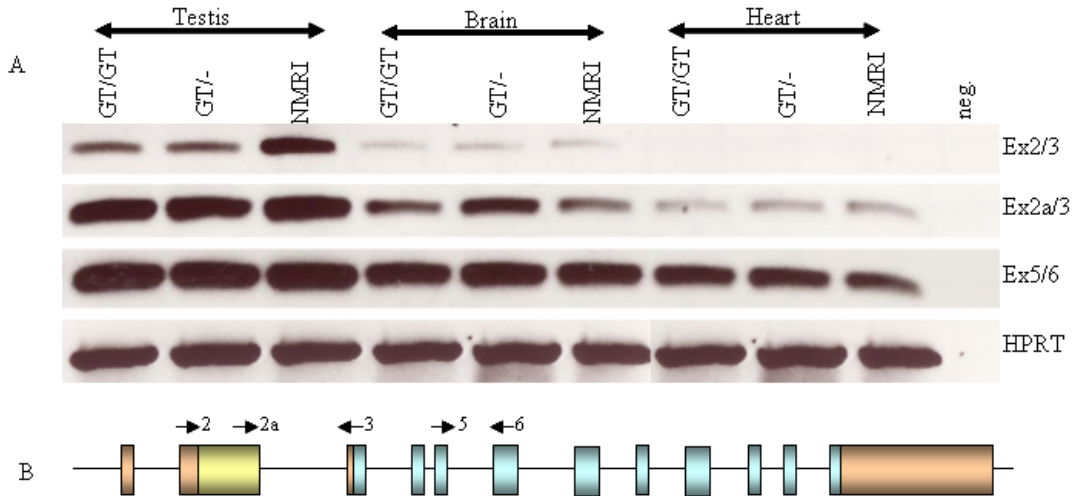

Figure 3.2: RT-PCR expression analysis of different *Lis1* transcripts in testis, brain and heart of 36 d old L39 and NMRI males (A). The reduction of the testis specific *Lis1 "2a"* transcript is not clearly visible in testes of $L39^{GT/GT}$ animals. The exon specific primers are indicated in the schematic gene drawing (B).

3.1.2.2 Analysis of *Lis1 "2a"*-transcripts in L39 mice by Northern blot

To further determine expression of the testis specific *Lis1 Ex2a/3* transcript in mutant testes of L39 mice, Northern blot analysis was performed using a ^{32}P-labelled *Lis1* "2a" specific probe. The primers used to generate the probe (Lis1-C-F1 and Lis1-D-F1; 2.1.8) were the same that Peterfy et al. (1998) used to show testis specific expression of *Lis1 Ex2a/3* transcript. The probe detected the expected 2.4kb fragment specifically in testis. Rehybridisation with a β-actin probe showed unequal amounts of loaded RNA. Again, downregulation of the testis-specific *Lis1 Ex2a/3* transcript can only be estimated by the higher amount of loaded RNA in comparison to the lower amount of loaded RNA of NMRI testes (Fig. 3.3).

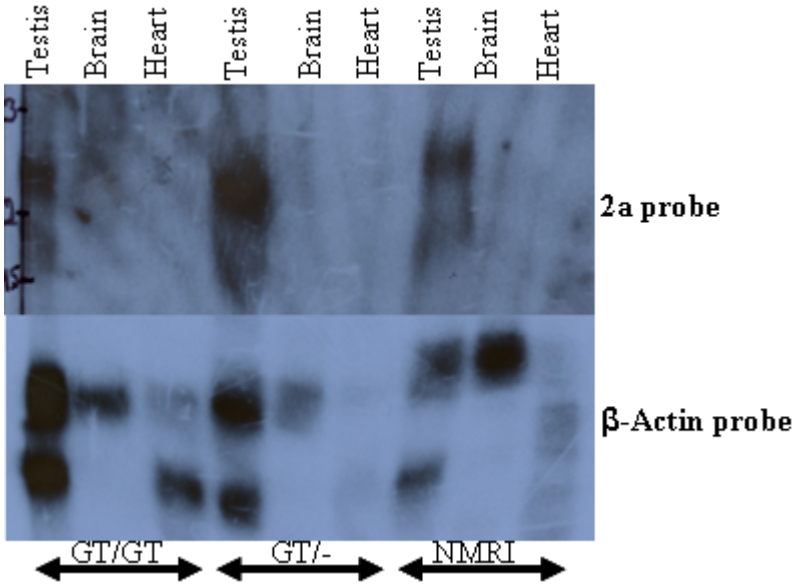

Figure 3.3: Northern blot analysis for *Lis1 Ex2a/3* specific transcript. Expression of the *Lis1 Ex2a/3* specific transcript is restricted to testis, as no expression in heart or brain was detected in 36 d old mice. NMRI wild type males as well as L39 males showed clear expression of the transcript at the predicted size of 2.4 kb. Integrity of RNA was checked by rehybridisation of the membrane with a β-actin probe that detects a 2.1 and a 1.5 kb isoform.

3.1.2.3 Expression analysis of *Lis1* gene in L39 mice by quantitative RT-PCR

To analyse *Lis1* expression pattern in testes of homozygous L39 males during testicular development, quantitative RT-PCR analysis was performed. Total RNA was isolated from testes of 10 d, 15 d, 20 d, 25 d, 36 d old and adult (2 months old) $L39^{GT/GT}$, $L39^{GT/-}$ and NMRI wild type males, followed by cDNA synthesis of 5 µg DNAse treated RNA. Real-Time RT-PCR was performed as described in 2.2.7.4, with *Sdha* (succinate dehydrogenase complex, subunit A, flavoprotein (Fp)) as endogenous reference. Three animals per age and genotype were investigated in independent experiments. Two *Lis1* splicing variants were analysed, the testis-specific *Lis1 "2a"* transcript (with primer pair Lis1-RT-Ex2a-F9 and Lis1-RT-Ex3-R9 located in exon 2a and exon 3, respectively) and the ubiquitously expressed *Lis1 "2"* transcript (with primer pair Lis1-Ex2-F2 and Lis1-Ex3-R1-Iris located in exon 2 and exon 3, respectively). The normalised values were calibrated to the mean value for NMRI wild type testes in each experiment. Three biological replicates were evaluated in independent experiments and statistically analysed using Statistica software. As obtained data showed non-normal

distribution, the non-parametric "Mann-Whitney-U-Test" was used to calculate p-values.

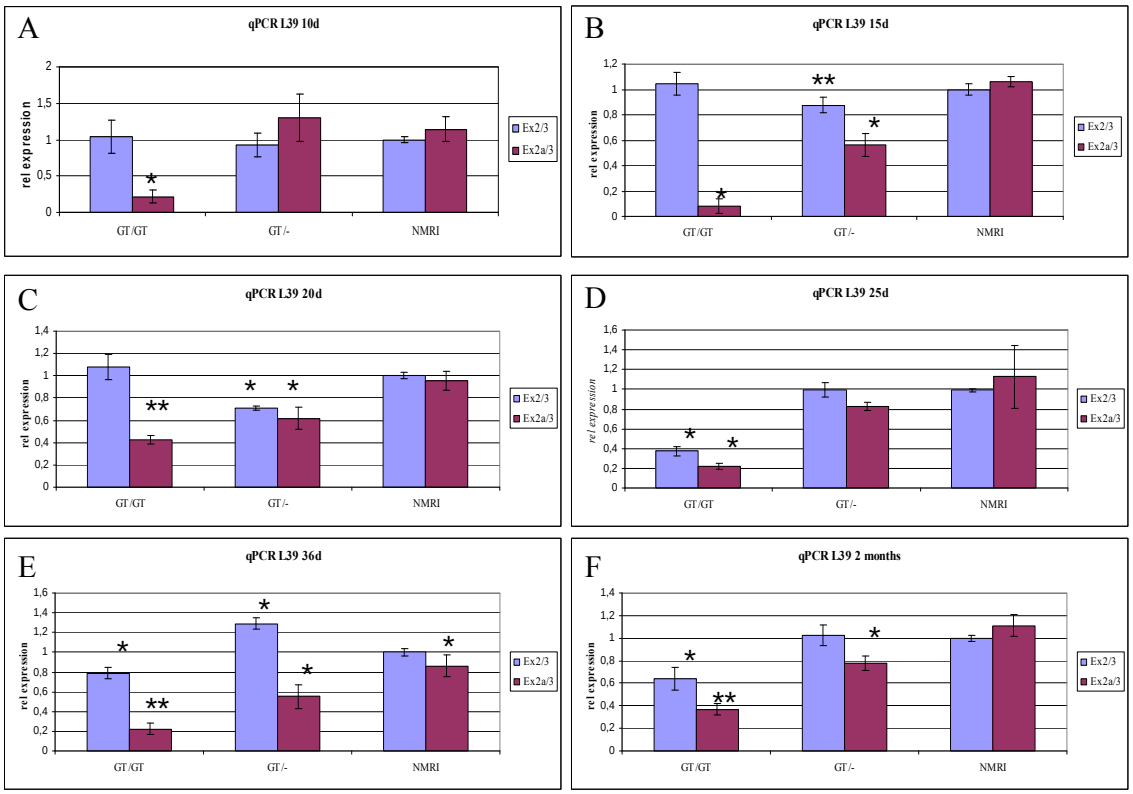

Figure 3.4: Relative expression levels of *Lis1 "2"* (Ex2/3) and *Lis1 "2a"* (Ex2a/3) splicing forms detected by quantitative RT-RCR in testes of $L39^{GT/GT}$, $L39^{GT/-}$ and NMRI wild type mice of different ages. Expression levels are normalised to the expression of endogenous control (*Sdha*). NMRI 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 "2"* transcript). Expression in testes of 10 d (A), 15 d (B), 20 d (C), 25 d (D), 36 d (E) and 2 months (F) old mice was analysed.

The expression of *Lis1 "2a"* transcript is significantly reduced ($p<0.05$) in 10 d old homozygous mutants, while no difference in expression level of *Lis1 "2"* transcript in analysed genotypes could be found. The expression levels of both splicing forms are similar in NMRI wild type (Fig. 3.4 A). The same expression pattern was found in 15 d old testes, with a significant reduction of the *Lis1 "2a"* transcript in heterozygous animals ($p<0.05$) and a further reduction in homozygous mice ($p<0.05$). Again, both transcripts share similar expression intensities in the NMRI wild type mice (Fig. 3.4 B). The observed expression pattern continues in 20 d old mice (Fig. 3.4 C). Reduction of

the relative expression of *Lis1 "2a"* transcript in homozygous mice is not as strong as the reduction of the splicing form in 15 d old mice, but highly significant ($p<0.005$). In 25 d old mice, the expression pattern changes (Fig. 3.4 D). Both transcripts are significantly downregulated ($p<0.05$) in homozygous mice. This pattern continues in 36 d old mice (Fig. 3.4 E), where the significant reduction of the *Lis1 "2"* transcript ($p<0.05$) is not as drastic as the reduction of the *Lis1 "2a"* transcript ($p<0.05$). The relative expression level of *Lis1 "2a"* transcript in NMRI wild type mice is slightly reduced in comparison to the *Lis1 "2"* transcript. In adult homozygous mice both splicing forms are reduced. *Lis1 "2a"* transcript is strongly downregulated ($p<0.005$), while the *Lis1 "2"* transcript is downregulated to about 60% of the amount of the wild type level ($p<0.05$) (Fig. 3.4 F). There is no strong difference in expression level of both splicing forms in NMRI mice in all stages analysed. Downregulation of relative expression of *Lis1 "2a"* transcript in $L39^{GT/GT}$ males is very prominent in all stages, while downregulation of *Lis1 "2"* transcript occurred in later stages (from 25 d on), but not as prominent as the reduction of *Lis1 "2a"* transcript.

3.1.2.4 Expression of LIS1 protein in L39 mice by Western blot analysis

To evaluate the expression of LIS1 protein in tissues of L39 mice, total protein extracts from testes and brain of 4 months old mutant animals and wild type controls were analysed by Western blot. The 45 kDa LIS1 protein was found in lysates of all tissues and genotypes studied (Fig. 3.5).

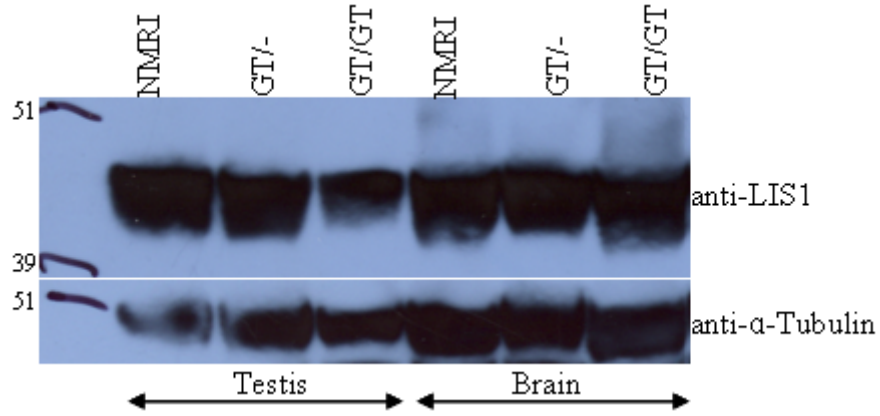

Figure 3.5: Expression of LIS1 protein in testes and brain of adult mutant and wild type (NMRI) mice. Monoclonal anti-LIS1 (Sigma) recognises the 45 kDa protein in lysates of testes and brain of all animals studied. 50 kDa α-tubulin protein band is shown as a control.

In testes of homozygous L39$^{GT/GT}$ mice LIS1 protein amount is reduced as compared to LIS1 protein in NMRI mice. α-tubulin loading control indicates a lower amount of testicular NMRI protein, which strengthens the observed reduction of LIS1 protein in testes of L39$^{GT/GT}$ mice. In contrast to this, LIS1 protein is equally expressed in brain lysates of all genotypes.

3.1.3 Expression of spermatogenic markers in L39 mice by Northern blot analysis

To determine the distribution of germ cells at different stages of spermatogenesis, the expression of the spermatocyte-specific marker transcripts *Pgk2* (Boer, 1987) and *Scp3* (Yuan, 2000), as well as the spermatid-specific markers *Tp2* (Adham, 2001) and *Odf-1* (Burfeind, 1996) in testes of NMRI wild type, heterozygous and homozygous gene trap mice of different ages was investigated (Fig. 3.6).

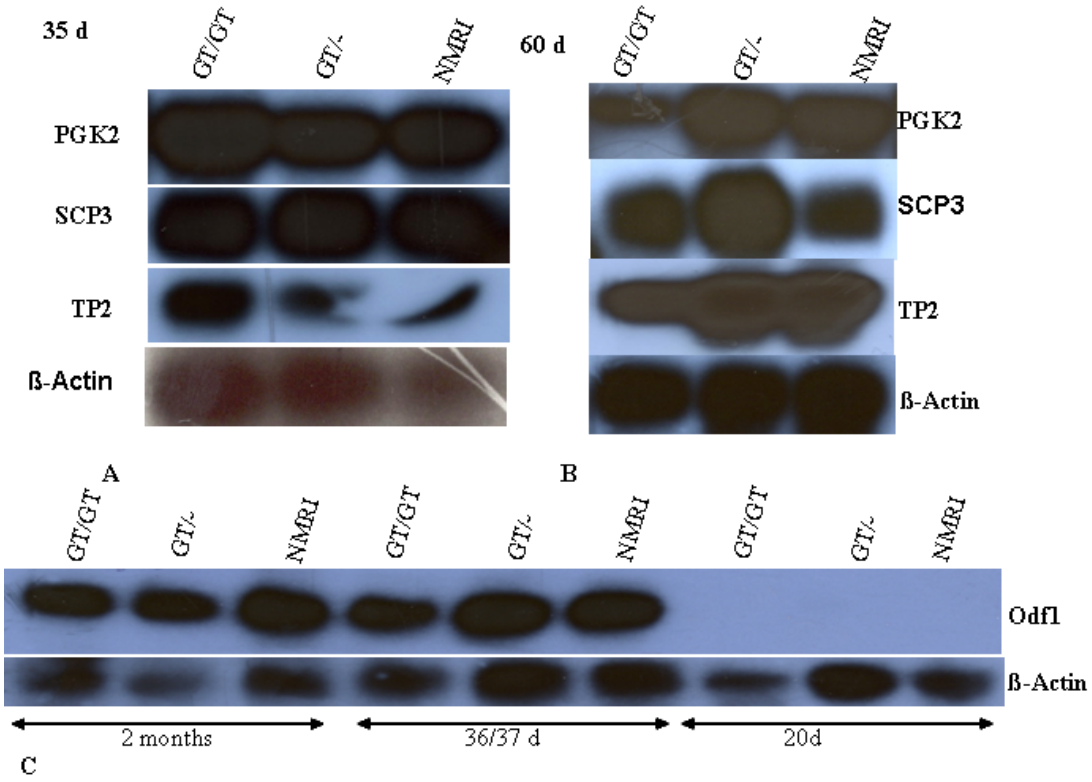

Figure 3.6: Northern blot analysis of spermatogenic markers in NMRI wild type and heterozygous (GT/-) and homozygous (GT/GT) L39 mice. Total RNA (10µg) was extracted from testes of mice of different ages and subjected to Northern blot hybridisation for several spermatogenic markers used as probes. Expression of *Pgk2*, *Scp3* and *Tp2* in testes of 35 d old mice (A), in testes of 60 d old mice (B) and expression of *Odf1* in testes of different ages (C) was analysed. Integrity of the RNA was checked by hybridisation with ß-actin probe.

All transcripts were expressed at similar levels in NMRI wild type and mutant mice. As expected *Odf1* transcripts were not detectable in 20 d old NMRI wild type and L39 testes lacking spermatids. Integrity of RNA was checked by rehybridization of ß-actin probe.

3.1.4 Immunohistochemistry of testes of L39 mice

(performed by B. Jung)

To investigate the distribution of LIS1 protein in testis, paraffin embedded 36 d old wild type mouse testes were cross-sectioned to a thickness of 5-7 µm. These testis sections were then immunostained with monoclonal anti-LIS1 antibody (Sigma) in a 1:200 dilution and superimposed by DAPI staining. Specific immunostaining was observed in all testicular cells (Fig. 3.7).

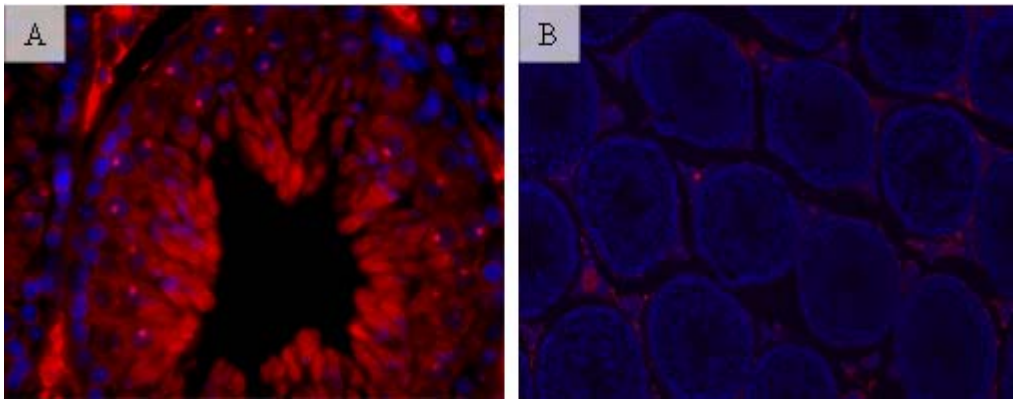

Figure 3.7: Expression analysis of LIS1 in testes of 36 d old wild type mice using immunohistochemical analysis. Staining was observed in all testicular cells (A). Negative control with secondary antibody only is depicted in B. Pictures are dual image overlays of Cy3 and DAPI fluorescences. Experiment was performed by B. Jung.

The expression of LIS1 was restricted to the cytoplasm of testicular cells, with an intense staining of LIS1 in meiotically dividing spermatocytes and elongating spermatids. These data confirms the observations obtained by Koizumi et al. (2003).
To investigate the distribution of LIS1 protein in testes of gene trap males immunohistochemical analysis were repeated for heterozygous and homozygous L39 males.

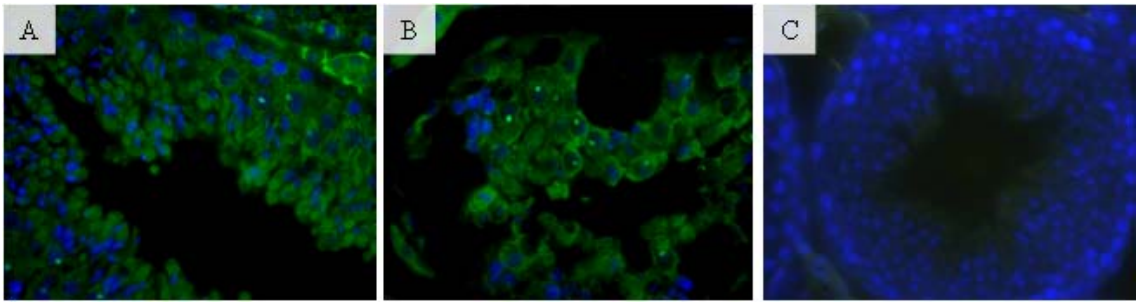

Figure 3.8: Expression analysis of LIS1 in testes of 6 months old $L39^{GT/-}$ (A) and $L39^{GT/GT}$ (B) mice using immunohistochemical analysis. Staining was observed in all testicular cells. A negative control with secondary antibody only is depicted in C. Pictures are dual image overlays of Cy2 and DAPI fluorescences. Experiment was performed by B. Jung.

Again, specific immunostaining was observed in cytoplasm of all testicular cells (Fig. 3.8). Immunohistochemical analysis was performed by Bomi Jung (TU Braunschweig).

3.1.5 Detection of fusion transcripts in L39 mice

To determine expression of fusion transcripts between *Lis1 Exon2* and the gene trap vector and *Lis1 Exon2a* and the gene trap vector, RT-PCR analysis was performed. Total RNA was isolated from testes of homozygous gene trap mice and wild type mice as negative control. As expected fusion transcripts of *Lis1 Exon2* with gene trap vector (Fig. 3.9) and *Lis1 Exon2a* with gene trap vector (Fig. 3.10) were detected in homozygous gene trap mice only. The RT-PCR products were extracted from agarose gel, cloned into pGEM-T Easy vector und subsequently sequenced.

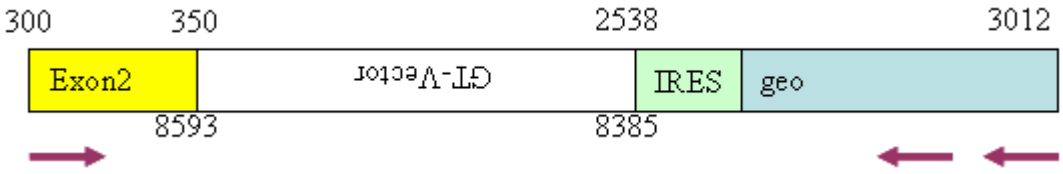

Figure 3.9: Sequenced part of the fusion transcript *Lis1 Exon2* and the gene trap vector. The primers used for the RT-PCR are Lis1-Ex2-F2, GT-Test-R1 and GT-LacZ-R1, respectively. The sequence was blasted against *Lis1* cDNA (Ensembl, ENSMUST00000021091) and the gene trap vector pUHachi (GenBank, AB242616), respectively.

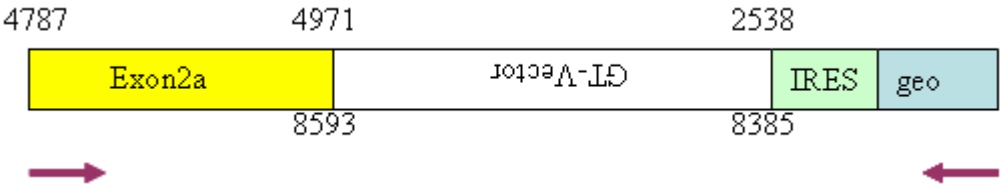

Figure 3.10: Sequenced part of the fusion transcript *Lis1 Exon2a* and the gene trap vector. The primers used for RT-PCR are Lis1-Ex2-F2 and GT-Test-R1, respectively. The sequence was blasted against *Lis1* cDNA (Ensembl, ENSMUST00000102520) and the gene trap vector pUHachi (GenBank, AB242616), respectively.

The splicing of the fusion transcript occurs as expected in the gene trap vector, which was fragmented during integration in the genome, and is spliced to the 3` end of *Lis1* exon 2 or *Lis1* exon 2a, respectively. Parts of the IRES-Sequence and the geo sequence were detected in the fusion transcript in 3` to 5` direction.

3.1.6 LacZ staining in L39 mice

Expression of the *ß-Galactosidase* reporter gene of the gene trap vector pKC421, used to generate the mutant L39 line, can be visualised by LacZ staining (2.2.13.6.). To show activity of the *Lis1* promoter in the mutant line, testes of L39 mice at different ages were fixed, embedded in Tissue freezing medium (Leica Microsystems) and cryosectioned. Thereafter sections were stained by LacZ staining. This experiment was performed in collaboration with Prof. Meinhardt (University of Giessen).

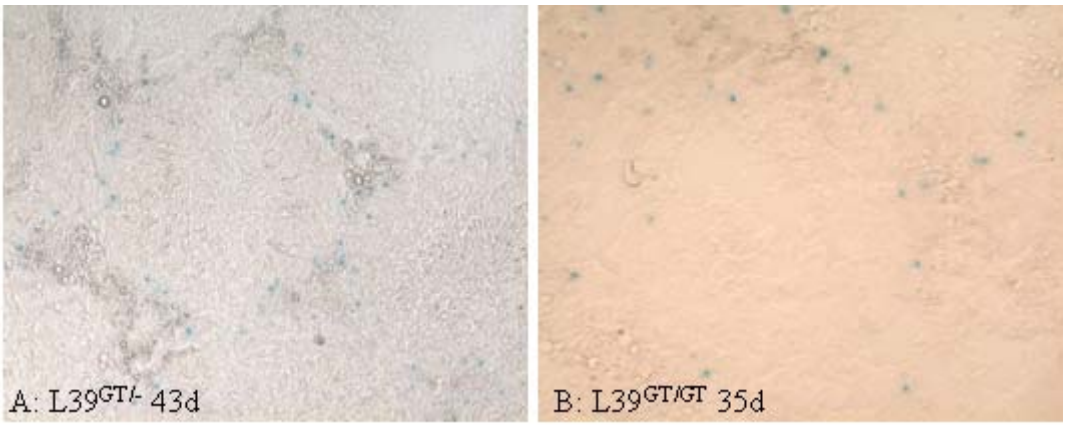

Figure 3.11: LacZ staining of testes of L39 males. Cryosections of 43d old heterozygous mutants (A) and 35 d old homozygous mutants (B) were stained for expression of *ß-Galactosidase*. Some spermatogonia and some early primary spermatocytes are stained blue.

Some spermatogonia and few early primary spermatocytes were positive for the staining (Fig. 3.11). Even though the morphology of the cryosection is of low quality, positively stained, blue cells are always localised near the basal lamina of seminiferous tubules, which leads to the presumption that these cells are spermatogonia. Not all spermatogonia are stained. Few cells are blue, which are located closer to the lumen of the seminiferous tubules. These are presumably early primary spermatocytes.

3.1.7 Sperm analysis of L39$^{GT/GT}$ mice

To confirm infertility of L39$^{GT/GT}$ males a couple of homozygous males were tested by mating them with homozygous gene trap or wild type females. All matings were performed for several weeks, but no litters were obtained.

Then, total sperm count in caudae epididymes of L39 homozygous and heterozygous control males was determined. Three homozygous (2, 3 and 6.5 months old) and one wild type male (3.5 months old) were used for analysis. 2.35 x 10^7 sperms were found in the wild type control male, while a few sperms were found in all L39$^{GT/GT}$ males via counting in a Neubauer counting chamber. To evaluate the morphology of sperm cells, 20 µl of epididymal cell suspension was transferred to slides, fixed in formaldehyde and covered with DAPI-containing mounting medium. Slides were then analysed for sperms under the fluorescence microscope BX60 (Olympus).

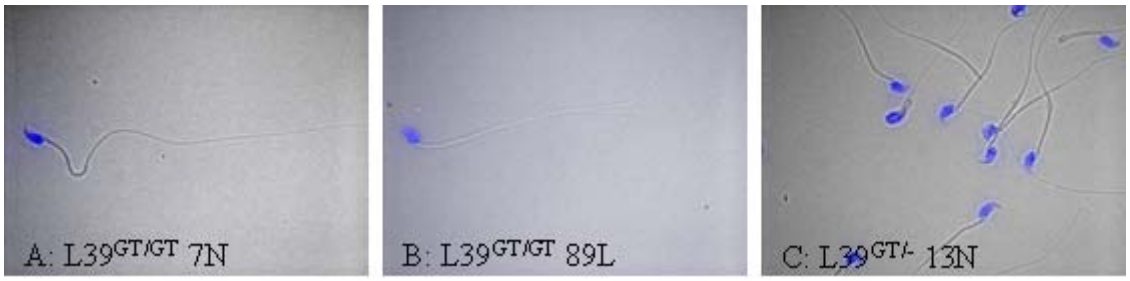

Figure 3.12: DAPI staining of sperms isolated from caudae epididymes of L39 mice. Epididymes of homozygous (A, B) and heterozygous (C) control mice were dissected and smear was fixed and stained with DAPI. A low number of sperm was found in homozygous males in contrast to a high number of sperms in heterozygous animals. Sperms of all genotypes looked morphologically normal (original magnification x600).

With this analysis the existence of spermatozoa in caudae epididymes of homozygous L39 males was shown. In comparison to the heterozygous control the number of sperms

is significantly reduced, as expected. Nevertheless, the morphology of all sperms found looked fairly normal (Fig.3.12).

3.1.8 Genomic localisation of integrated gene trap construct in L39 mice

Genotyping of L39 mice was established separately in the Institute of Human Genetics in Göttingen and the Institute for Biochemistry and Biotechnology in Braunschweig. In the course of this work genotyping primers were exchanged and genomic localisation was checked. It turned out that two different regions located in intron 2 of *Lis1* gene were amplified by genotyping primers (Fig. 3.13).

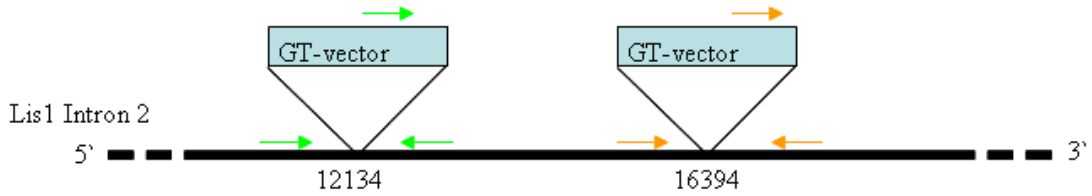

Figure 3.13: Schematic drawing of the putative integration sites of the gene trap vector in L39 mice. The black line presents intron 2 of *Lis1* gene. Numbers indicate the sites of integration of the vector (GT-vector). Green arrows show location of primerset I ("Goe"-primers) and orange arrows location of primerset II ("BS"-primers) used for genotyping.

Primerset I ("Gö"-primers) amplified a DNA region of *Lis1 intron 2* that is separated by roughly 4kb from the DNA region amplified by primerset II ("BS"-primers). All PCR-products were cloned into pGEM-T Easy vector and subsequently sequenced to exclude unspecific binding. To confirm that the gene trap vector was integrated in both regions a "Genome walk" approach was used.

3.1.8.1 "Genome Walk" approach

GenomeWalker™ DNA walking (Clontech) is a simple PCR-based method for walking upstream or downstream in genomic DNA from a known sequence, thus finding unknown genomic DNA sequences adjacent to a known sequence such as a cDNA (Siebert et al., 1995).

Separate aliquots of $L39^{GT/GT}$ DNA were completely digested with different restriction enzymes that leave blunt ends. Each batch of digested genomic $L39^{GT/GT}$ DNA was then

ligated separately to the GenomeWalker Adaptor. After the libraries had been constructed, primary PCR that uses the outer adaptor primer (AP1) provided in the kit and outer, gene-specific primers (GSP1) was run. The primary PCR mixture was then diluted and used as a template for a secondary or "nested" PCR with the nested adaptor primer (AP2) and nested gene-specific primers (GSP2). Each of the DNA fragments amplified was then cloned into pGEM-T Easy and sequenced. A schematical drawing of the location of GSP1 and GSP2 primers is shown in figure 3.14.

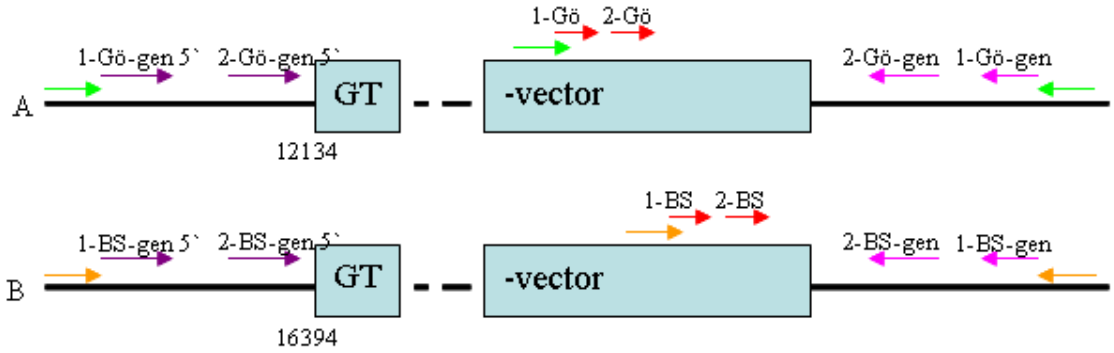

Figure 3.14: Schematical drawing of the primers used for "Genome Walk" to confirm the putative integration sites of the gene trap vector in L39 mice. Location of the genotyping primers are illustrated with green arrows (primerset Gö, A) and orange arrows (primerset BS, B). GSP1 primers (abbreviated with 1) are located 3`downstream of the corresponding genotyping primer, and GSP2 primers (abbreviated with 2) are located 3`downstream of the corresponding GSP1 primer. Altogether six experimental approaches were used to determine the integration sites, three for each gene trap vector. Primerset 1-Gö and 2-Gö (A) as well as primerset 1-BS and 2-BS (B) (illustrated by red arrows) are located in the 3`region of the gene trap vector. Primerset 1-Gö-gen 5` and 2-Gö-gen 5` (A) as well as primerset 1-BS-gen 5`and 2-BS-Gen 5` (B) (illustrated by violet arrows) are located 5`of the putative integration site. Primerset 1-Gö-gen and 2-Gö-gen (A) as well as primerset 1-BS-gen and 2-BS-gen (B) (illustrated by pink arrows) are located 3`of the gene trap vector.

Altogether six approaches using different primers were used to confirm two integration sites of the gene trap vector. For each integration three different primersets (located downstream of the corresponding genotyping primer) were designed. One primerset is localised at the 3` end of the vector, thus being able to sequence from the vector into *Lis1 intron 2*. One primerset is localised 5` upstream of the gene trap vector, thus being able to sequence into the 5`part of the gene trap vector, and one primerset is localised 3` downstream of the integration, thus being able to sequence into the 3`end of the gene trap vector. With all approaches the exact integration point in *Lis1 intron 2* was determined.

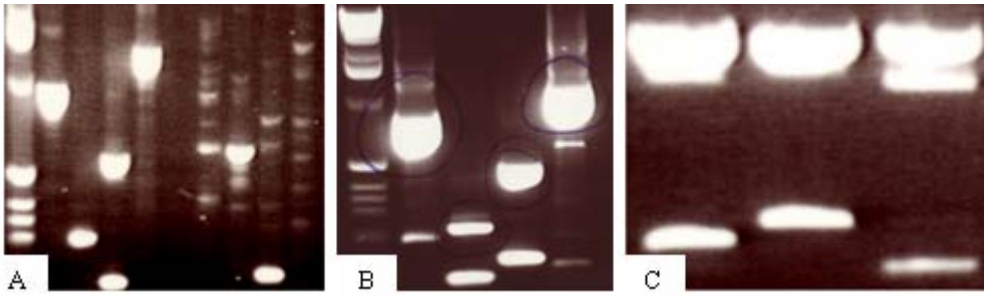

Figure 3.15: Examples of agarose gels of primary (A) and secondary (B) PCR-products of L39$^{GT/GT}$ digested DNA and *EcoRI* digested pGEM-T Easy vectors with cloned PCR-products from secondary PCR (C).

Secondary PCR amplified DNA fragments in at least two of the four batches of digested genomic L39$^{GT/GT}$ DNA in each experimental approach (Fig. 3.15). After gel extraction and cloning of DNA fragments into pGEM-T Easy vector, all fragments were sequenced and subjected to BLAST search (www.ncbi.nlm.gov/BLAST). As a reference *Lis1* (Ensembl, ENSMUSG00000020745) and the gene trap vector pUHachi (GenBank, AB242616) were used. Both integration sites could be confirmed (Fig 3.16).

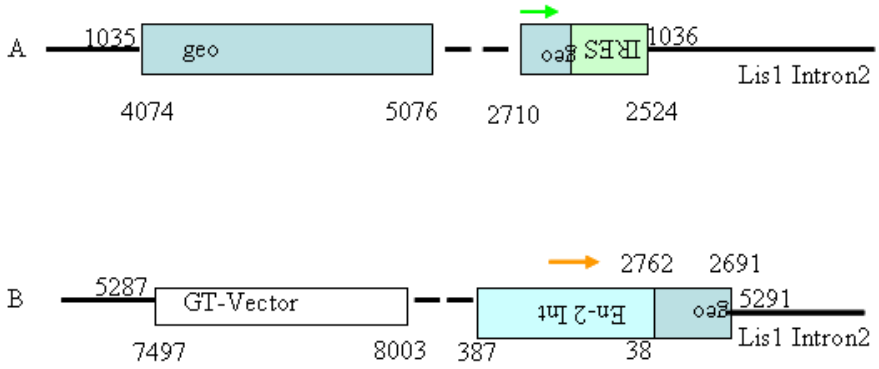

Figure 3.16: Schematical drawing of integrated gene trap vector into Intron2 of *Lis1* gene. (A) displays the integration amplified by primerset "Gö" while (B) displays the integration amplified by primerset "BS". Location of the genotyping primers are illustrated by arrows. Numbers on the black line refer to integration point in intron 2 of *Lis1* gene (with 1 being nucleotide 74410112 on chromosome 11), while the other numbers refer to nucleotide position in the pUHachi vector. Note, that parts of the vector were detected in 3` to 5` direction.

Integration of the gene trap vector at position 5287 (Fig. 3.16 B) in intron 2 led to a deletion of 3 nucleotides, while the integratin of the vector at position 1034 in intron 2 (Fig. 3.16 A) did not lead to any mutation in the genome of L39 mice.

3.1.8.2 Confirmation of the gene trap integration in ES 2A-53 cells

To confirm that both integration sites were present in L39 from the beginning, the ES cell clone from which the L39 mice was generated, namely 2A-53 (kindly provided by K. Chowdhury, Max-Planck-Institute for Biophysical Chemistry, Göttingen), was cultivated and DNA was isolated. Genotyping PCRs with both primersets were performed (Fig. 3.17).

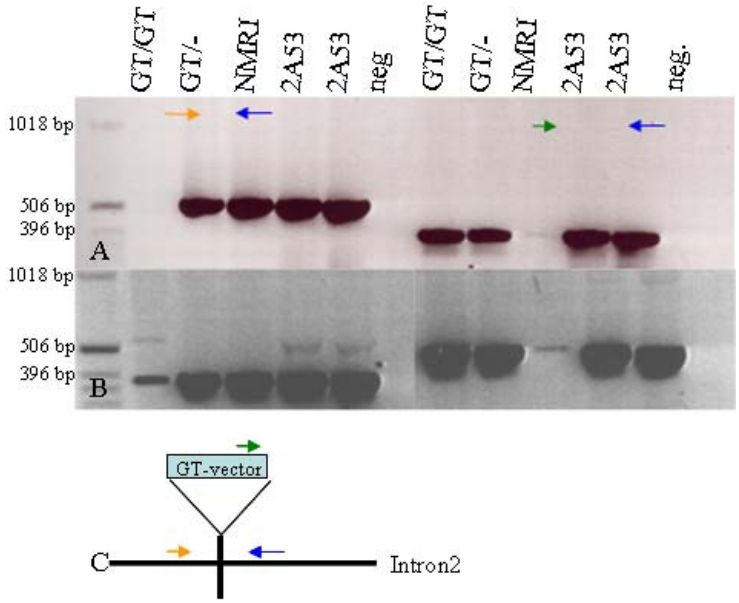

Figure 3.17: Agarosegel of genotyping PCR of ES-cell clone 2A-53. In (A) primerset "Gö" was used. Lis1-Intron2 and Lis1-Intron2-R amplified a 525 bp wild type fragment while GTpR4 and Lis1-Intron2-R amplified a 351 bp mutant fragment. In (B) primerset "BS" was used. Lis1-20-sense and Lis1-21-AS amplified a 375 bp wild type fragment while 2A53AS and Lis1-21-AS amplified a 480 bp mutant fragment. Schematic overview of primer positions is visualized in (C). Note that only one integration is displayed. Colored arrows indicate genotyping primers, with yellow indicating Lis1-Intron2-F and Lis1-20-sense, green indicating GTpR4 and 2A53AS, and blue indicating Lis1-Intron2-R and Lis1-21-AS, respectively. DNA of homozygous (GT/GT), heterozygous (GT/-) and wild type (NMRI) mice was used as control. 2A-53 ES cells are heterozygous for the gene trap integration.

ES cell clone 2A-53 contains both integrations in a heterozygous manner. Homozygous, heterozygous and NMRI DNA was used as control and displayed the expected pattern.

3.1.9 Phenotypical analysis of L39 mice on different genetic backgrounds

An increasing number of scientific articles report 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 Montagutelli, 2000). To analyse the effects of genetic background on phenotype of L39 mice incipient congenic strains were generated. To generate full transgenic animals, with the aim of transferring a mutation from one inbred genetic background to another, mice heterozygous for the mutation with inbred background A are crossed with mice of strain B. Among the F1 animals, mice heterozygous for the mutation are selected by genotyping. These F1 animals are crossed with strain B to produce N2 progeny, among which only mice heterozygous for the mutation are used for the next generation. The same cycle is repeated until N10 mice are obtained. Heterozygous N10 mice are crossed to produce mice homozygous for the mutation, with a genetic background very close to strain B.
The gene trap line L39 is of NMRI background. Heterozygous L39 males on NMRI outbred were crossed with females of CD-1, C57BL, 129/Sv or FVB genetic background. Backcrossing was done for seven generations before heterozygous animals were crossed to produce homozygous incipient congenic strains.

3.1.9.1 Fertility test of L39 males on different genetic backgrounds

Several homozygous adult $L39^{GT/GT}$ males were mated with wild type females. The uteri and oviducts of those mice which were positive for vaginal plug were dissected in IVF medium, flushed out and sperm number was determined. No sperms were found in uterus and oviduct of females mated with homozygous males. The experiment was repeated two times for each male. Heterozygous L39GT/- males of the same age were mated with wild type females as controls. Sperm count revealed the expected number of sperm in uterus and oviduct of females mated with heterozygous control mice. Table 3.1 shows the number of animals tested for each background, the age of the animals and the number of sperms found in uterus and oviduct. The testes sizes of homozygous males in all backgrounds were markedly reduced in comparison with heterozygous siblings. Figure 3.18 despicts testes of L39 mice on CD-1 background.

Table 3.1: Sperm count in uterus and oviduct of wild type females mated with L39 males of different backgrounds. The background and genotype, the number of animals tested, the age of the males, and the number of sperms found in uterus and oviduct are diplayed.

Background & genotype	Animals	Age (mo)	No of sperms in uterus	No of sperms in oviduct
CD-1 GT/GT	6	2-3	0	0
CD-1 GT/-	3	2-3	$7.6 \pm 2.9 \times 10^6$	$4.2 \pm 2.5 \times 10^4$
C57BL GT/GT	4	2-3	0	0
C57BL GT/-	2	2-3	$4.5 \pm 2.5 \times 10^6$	0
FVB GT/GT	4	2-3	0	0
FVB GT/-	2	2-3	$6.4 \pm 3.9 \times 10^6$	0
129/Sv GT/GT	4	2-4	0	0
129/Sv GT/-	2	2-4	$4.9 \pm 3.7 \times 10^6$	$5.8 \pm 5.3 \times 10^3$

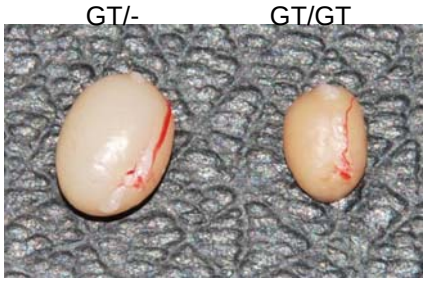

Figure 3.18: Testes of adult (7 months old) homozygous (GT/GT) and heterozygous (GT/-) L39 siblings on CD-1 background.

3.1.9.2 Histological analysis of testis sections of L39 males on different genetic backgrounds

To confirm the observed extensive germ cell degeneration in seminiferous tubules of L39/NMRI$^{GT/GT}$ mice (Fig. 3.1), testes and epididymes of 25 d, 35 d, 45 d old and adult L39$^{GT/-}$ and L39$^{GT/GT}$ mice on CD-1, C57BL, FVB and 129/Sv backgrounds were fixed in Bouins solution, sliced into 5 µm thick sections and stained with hematoxilin and eosin (H&E) (Fig. 3.19 to Fig. 3.22).

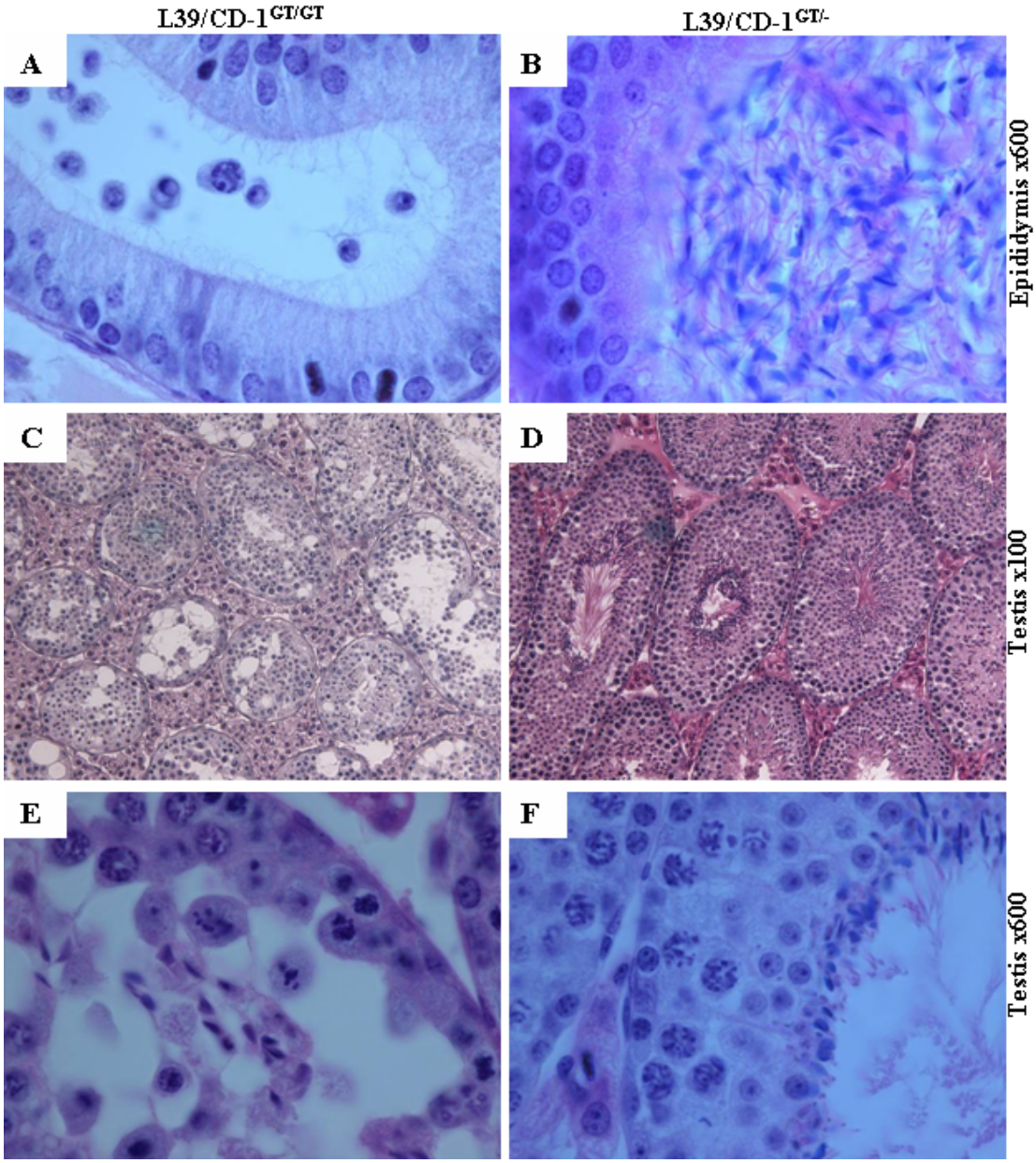

Figure 3.19: H&E staining of epididymes and testis sections of 45 d old and adult L39/CD-1$^{GT/GT}$ and L39/CD-1$^{GT/-}$ mice. Sections through epididymes revealed a high number of sperms in heterozygous 45 d old males (B), whereas epididymes of 45 d old homozygous males were found to be filled with some premature released germ cells, but no sperms (A). Sections through testes of adult (C) and 45 d old (E) homozygous gene trap mice revealed extensive degeneration of a large fraction of seminiferous tubules, whereas testis sections of adult (D) and 45 d old (F) heterozygous animals demonstrated robust spermatogenesis (original magnification A, B, E, F x600; C, D x100).

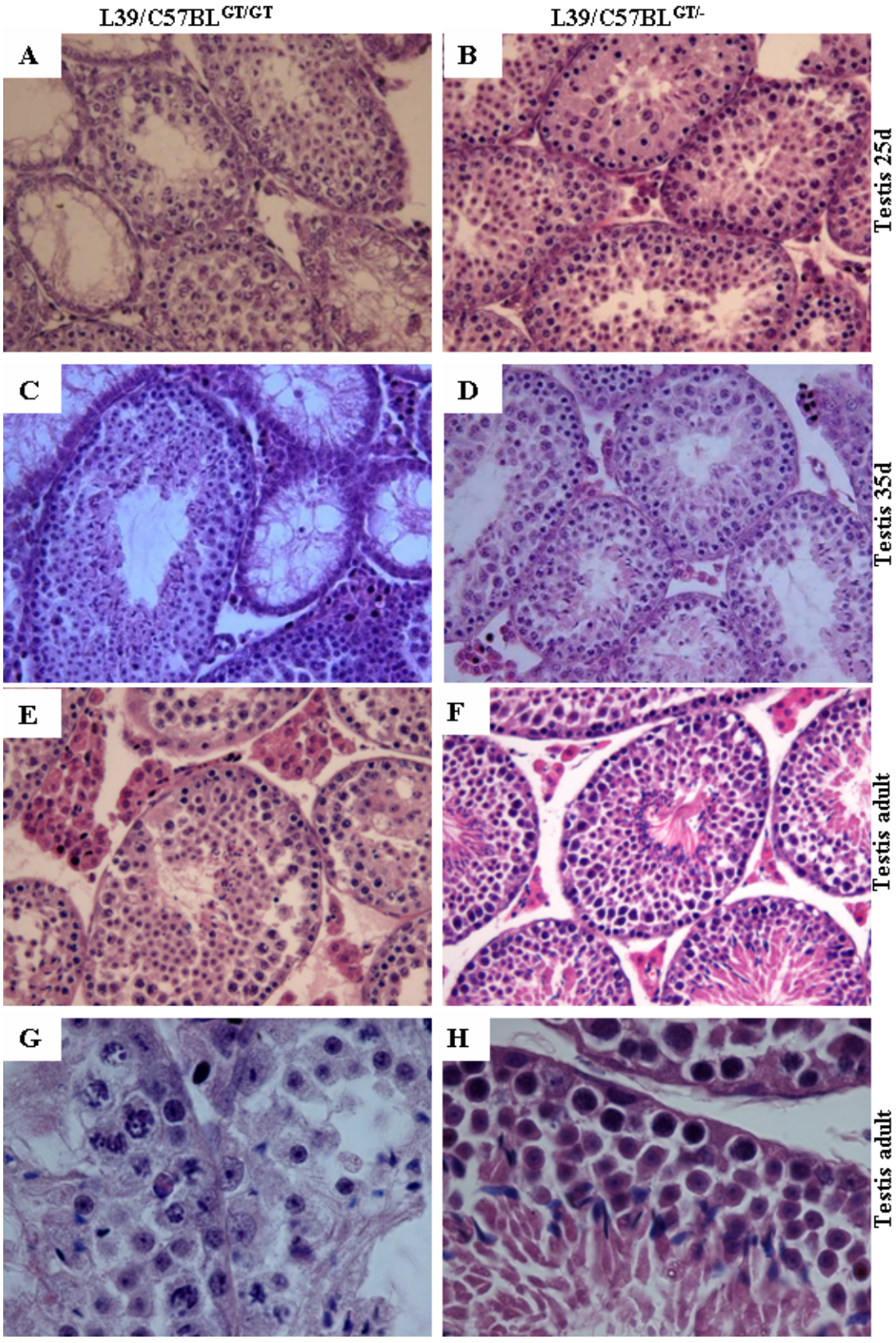

Figure 3.20: H&E staining of testis sections of 25 d, 35 d old and adult L39/C57BL$^{GT/GT}$ and L39/C57BL$^{GT/-}$ mice. Sections through testes of 25 d (A), 35 d old (C) and adult (E, G higher magnification) homozygous gene trap mice revealed extensive degeneration of a large fraction of

seminiferous tubules, whereas testis sections of 25 d (B), 35 d old (D) and adult (F, H higher magnification) heterozygous animals demonstrated robust spermatogenesis (original magnification A- F x200; G, H x600).

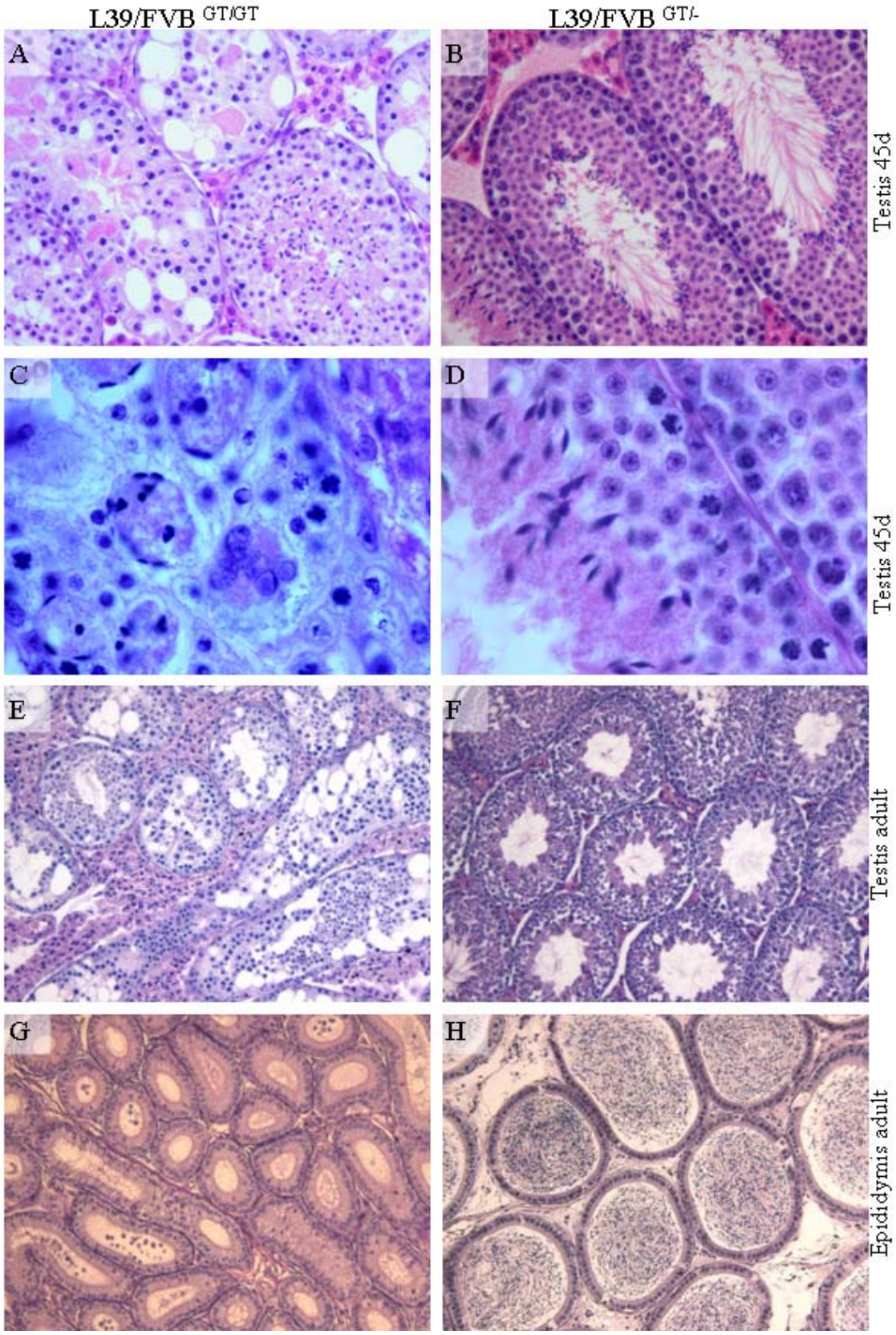

Figure 3.21: H&E staining of epididymis and testis sections of 45 d old and adult L39/FVB$^{GT/GT}$ and L39/FVB$^{GT/-}$ mice. Sections through epididymis revealed a high number of sperms in heterozygous adult males (H), whereas most tubules of epididymis of adult homozygous males are empty and few were found to be filled with some premature released germ cells, but no sperms (G). Sections through testes of 45 d old (A, C) and adult (E) homozygous gene trap mice revealed extensive degeneration of a large fraction of seminiferous tubules, whereas testis sections of 45 d old (B, D) and adult (F) heterozygous animals demonstrated robust spermatogenesis (original magnification A, B x200; C, D x600; E-H x100).

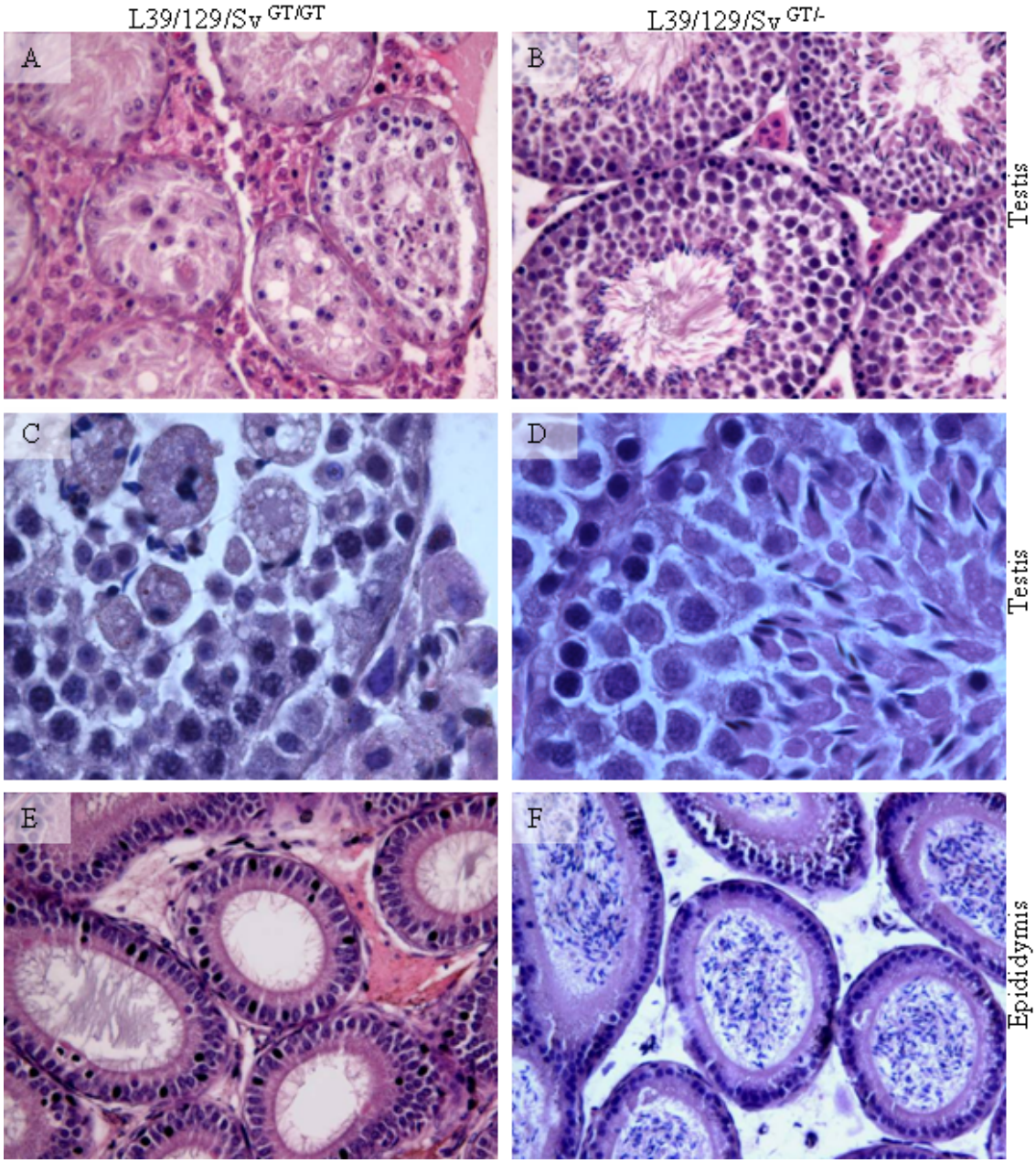

Figure 3.22: H&E staining of epididymis and testis sections of adult L39/129/Sv$^{GT/GT}$ and L39/129/Sv$^{GT/-}$ mice. Sections through epididymes revealed a high number of sperms in heterozygous adult males (F), whereas most tubules of epididymes of adult homozygous males are empty (E). Sections through testes of adult (A, C) homozygous gene trap mice revealed extensive degeneration of a large fraction of

seminiferous tubules, whereas testis sections of adult (B, D) heterozygous animals demonstrated robust spermatogenesis (original magnification A, B, E, F x200; C, D x600).

The histological analysis of testes of L39$^{GT/GT}$ mice on different backgrounds revealed the same extensive degeneration of germ cells in a large fraction of seminiferous tubules as observed in L39/NMRI$^{GT/GT}$ males (Fig. 3.1). 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. This phenotype was visible in 45 d old males (Fig. 3.19 E) as well as in adult male on CD-1 background (Fig. 3.19 C) with an increasing amount of collapsed tubules in the adult male. In marked contrast to this are heterozygous males, which exhibit full spermatogenesis with no obvious differences from wild type males (Fig. 3.19 D, F). This phenotype was also detected in 35 d old mice, where homozygous males exhibit a high number of degenerated tubules, with a reduced number of late spermatogenic cells, giant germ cells and vacuolisation of tubules (data not shown). Tubules of epididymes of heterozygous males were packed with sperms (Fig. 3.19 B), while most tubules of homozygous males were empty and some (Fig. 3.19 A) were filled with few premature released germ cells. No sperm cells could be detected.

On C57BL background the phenotype was already visible in 25 d old males (Fig. 3.20 A). Spermatogenesis in some tubules was completely abolished. The number of affected tubules increased with age (Fig. 3.20 C, E, G). In marked contrast to this were heterozygous males, which exhibited full spermatogenesis with no obvious differences from wild type males (Fig. 3.20 B, D, F, H). Sections of epididymes of heterozygous males at different ages were packed with sperms while tubules of homozygous males were mainly empty and few were filled with single premature released germ cells. No sperm cells could be detected (data not shown).

Histological analysis of sections of 45d and 2 months old gene trap males of FVB (Fig. 3.21) and 129/Sv (Fig. 3.22) background showed the same extensive degeneration of germ cells in seminiferous tubules and a lack of sperm cells in epididymes as observed in L39$^{GT/GT}$ males on the other backgrounds. Again, heterozygous animals displayed full spermatogenesis and epidiymes of males were found to be filled with sperms.

3.1.9.3 Expression analysis of LIS1 protein in L39 mice on different genetic backgrounds

3.1.9.3.1 Expression analysis of LIS1 in L39/C57BL mice

To evaluate expression of LIS1 protein in testis of L39/C57BL mice, total protein extracts from testes of 25 d, 35 d, 45 d old and adult mutant animals were analysed by Western blot. The 45 kDA LIS1 protein was found in lysates of all testes and genotypes studied (Fig. 3.23).

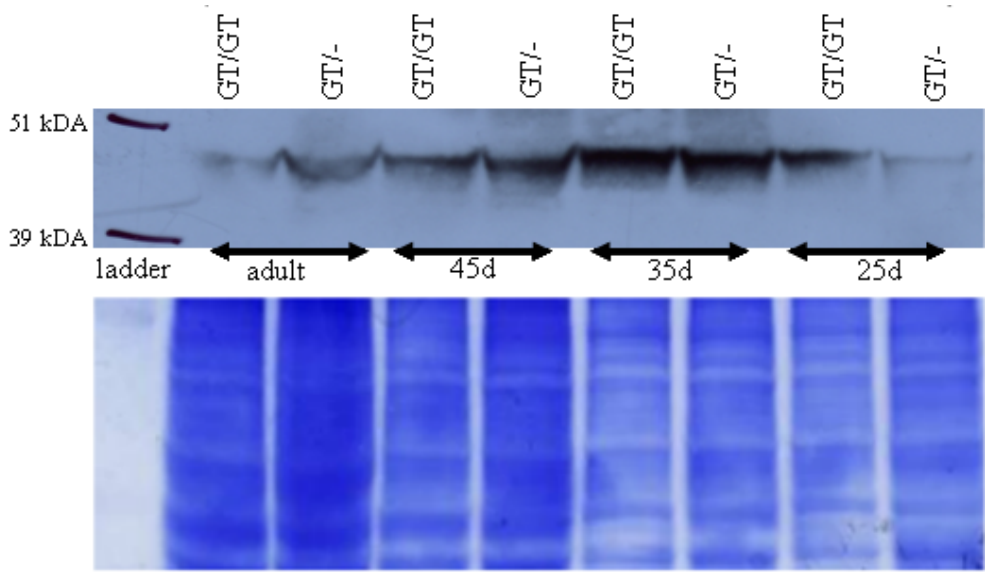

Figure 3.23: Expression of LIS1 protein in testes of 25 d, 35 d and 45 d old and adult L39/C57BL mutant mice. Monoclonal anti-LIS1 (Sigma) recognises the 45 kDa protein in testicular lysates of all animals studied. Part of the coomassie stained gel is shown as a control for protein loading.

A reduction of LIS1 protein in testis of homozygous L39/C57BL$^{GT/GT}$ mice is detected in adult mice only in comparison to heterozygous control mice. In younger mice the amout of LIS1 protein does not seem to differ between heterozygous and homozygous siblings. The Coomassie stained gel indicates comparable amounts of protein loaded for each age stage investigated.

3.1.9.3.2 Expression analysis of LIS1 in L39/FVB and L39/129/Sv mice

To evaluate expression of LIS1 protein in testis of L39/FVB and L39/129/Sv mice, total protein extracts from testes of adult (2 months old) mutant animals were analysed by

Western blot. The 45 kDA LIS1 protein was found in testes of all animals studied (Fig. 3.24).

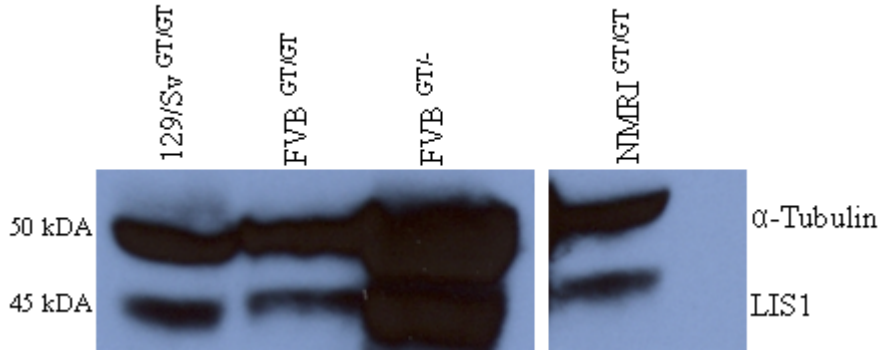

Figure 3.24: Expression of LIS1 protein in testes of old adult L39 mice (3 to 4 months old) of different genetic backgrounds. Monoclonal anti-LIS1 (Sigma) recognises the 45 kDa protein in testicular lysates of all animals studied. 50 kDa α-tubulin protein is shown as a control for protein loading.

A reduction of LIS1 protein in testes of homozygous L39/FVB$^{GT/GT}$ mice, which was detected in L39/NMRI (Fig. 3.5) and L39/C57BL (Fig 3. 23) could not be confirmed in L39/FVB males and L39/129/Sv males in this Western blot. The amount of testicular protein loaded differed between the heterozygous and the homozygous mice, with a higher amount in the heterozygous mutant. Thus it is not clear, whether the amount of LIS1 is reduced in L39/FVB$^{GT/GT}$ males.

3.1.9.4 Expression analysis of *Odf1* in L39/C57BL mice

The spermatocyte-specific marker transcripts, *Pgk2* (Boer, 1987) and *Scp3* (Yuan, 2000), as well as the spermatid-specific markers *Tp2* (Adham, 2001) and *Odf-1* (Burfeind, 1996), were found to be expressed at similar levels in testes of NMRI wild type, heterozygous and homozygous gene trap mice L39/NMRI of different ages (Fig. 3.6). To confirm this data for L39/C57BL mice, Northern blot analysis of *Odf1* expression of adult, 45 d, 35 d and 25 d old L39/C57BL$^{GT/GT}$ and L39/C57BL$^{GT/-}$ mice was performed (Fig. 3.25).

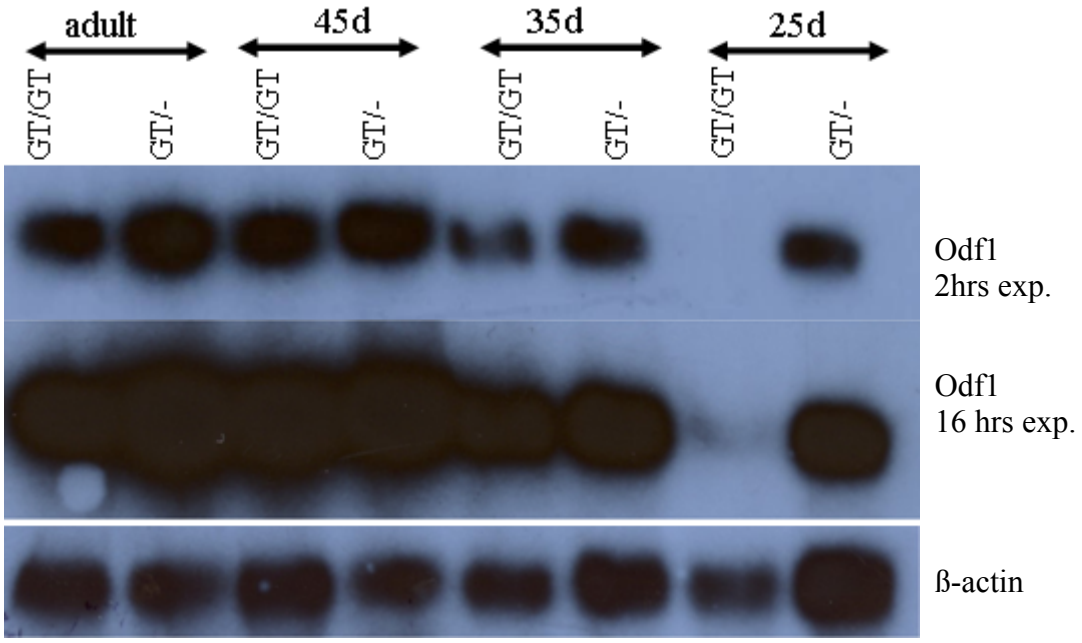

Figure 3.25: Northern blot analysis of *Odf1* expression in heterozygous (GT/-) and homozygous (GT/GT) L39/C57BL mice. Total RNA (10µg) was extracted from testes of mice of different ages and subjected to Northern blot hybridization of Odf1 probe. Integrity of the RNA was checked by hybridization with ß-actin probe.

Odf1 was expressed at similar levels in adult, 45 d and 35 d old heterozygous and homozygous L39/C57BL mice. The expression of *Odf1* was low in 25 d old heterozygous and not detectable in 25 d old homozygous males after 2 hrs of exposure. As *Odf1* expression starts in round spermatids (Burfeind, 1996), one expects *Odf1* transcript to be present in testis of 25 d old mice, as seen in heterozygous animals. Comparison of RNA amount loaded (checked by rehybridization with ß-actin probe) revealed a rather big difference in 25 d old heterozygous and homozygous males, which could explain the absence of *Odf1* in the L39/C57BL$^{GT/GT}$ male. Hence, after 16 hrs of exposure a slight band could be detected in the homozygous 25 d old L39/C57BL male.

3.1.9.5 Sperm analysis of L39 mice on different genetic backgrounds

To confirm infertility of L39/FVB$^{GT/GT}$ males on sperm level, total sperm count in caudae epididymes of L39/FVB homozygous (2.5 months and 4.5 months old) and heterozygous (4.5 months old) control males was determined. Three homozygous and one heterozygous male were used for analysis. 3.34 x 10^7 sperms were found in heterozygous control mice, while only single sperms were found in L39/FVB$^{GT/GT}$ males

via counting in a Neubauer counting chamber. To evaluate the sperm morphology, 20 µl of epididymal cell suspension was transferred to slides, fixed in formaldehyde and covered with DAPI-containing mounting medium. Slides were then analysed for sperms under the fluorescence microscope BX60 (Olympus).

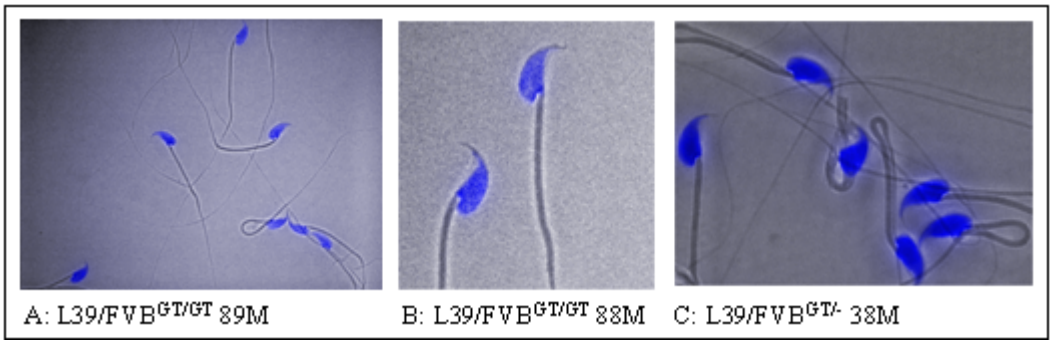

Figure 3.26: DAPI staining of sperms isolated from caudae epididymes of L39/FVB mice. Epididymes of homozygous mice (A, B) and heterozygous (C) control mice were dissected and smear was fixed and stained with DAPI. A low number of sperm was found in homozygous males in contrast to a high number of sperms in heterozygous animals. Sperms of all genotypes looked morphologically normal (original magnification x600).

Spermatozoa were found in caudae epididymes of homozygous L39/FVB males (Fig. 3.26). In comparison to the heterozygous control, as expected, the number of sperms was significantly reduced. Nevertheless, the morphology of all sperms found looked normal, as can be seen on the high magnification image of L39/FVB$^{GT/GT}$ male no 88M (Fig. 3.26 B).

To confirm infertility of L39/129/Sv$^{GT/GT}$ males on sperm level, total sperm count in caudae epididymes of 2.5 months old L39/129/Sv homozygous (three animals) and heterozygous (one animal) males was determined. 1.52 x 10^7 sperms were found in heterozygous control mice, while in two out of three L39/129/Sv$^{GT/GT}$ males a low number of sperms were found (7.15 x 10^4 and 1.65 x 10^4, respectively) via counting in a Neubauer counting chamber. 13 µl of sperm suspension was put on a dual sided sperm analysis chamber. Sperm motility was quantified using the computer assisted semen analysis (CASA) system. In the heterozygous control mouse 64% of counted sperms were motile and 42% were progressive. In contrast to this in one out of three homozygous males 4% of sperms were motile and 2% were progressive. The total number of sperms in homozygous males was low, with 233 total sperm cells counted and 1577 sperm cells in the control. L39/129/Sv line is the only line where sperms

could be analysed via CASA. To evaluate the sperm morphology, 20 µl of epididymal cell suspension was transferred to slides, fixed in formaldehyde and covered with DAPI-containing mounting medium. Slides were then analysed for sperms under the fluorescence microscope BX60 (Olympus).

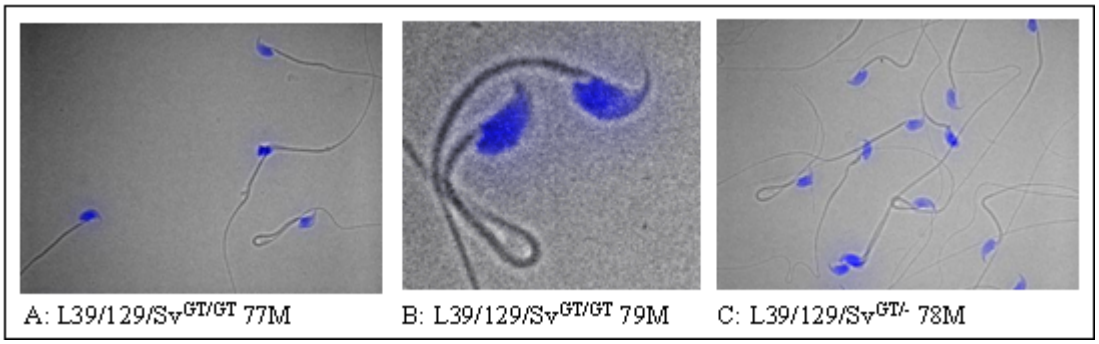

Figure 3.27: Sperms isolated from caudae epididymes of L39/129/Sv mice. Epididymes of homozygous (A, B) and heterozygous (C) control mice were dissected and smear was fixed and stained with DAPI. A low number of sperms was found in homozygous males in contrast to a high number of sperms in heterozygous animals. Sperms of all genotypes looked morphologically normal (original magnification x600).

The existence of spermatozoa in caudae epididymes of homozygous L39/129/Sv males was shown (Fig. 3.27). In comparison to the heterozygous control the number of sperms was significantly reduced, as expected. Nevertheless the morphology of all sperms found looked normal, as can be seen on the high magnification image of L39/129/Sv$^{GT/GT}$ male no 79M (Fig. 3.27 B).

3.2 Colocalisation of LIS1 with putative interaction partners

3.2.1 Yeast two hybrid Assay

(performed by B. Jung)

The yeast two-hybrid system developed by Stanley Fields and co-workers (Fields, 1989) is a well-established highly sensitive *in vivo* assay system for the detection of protein/protein interaction. In order to find new interaction partners for LIS1 in testis, a yeast-two-hybrid assay was performed by Bomi Jung (TU Braunschweig), with *Lis1* cDNA used as bait and a mouse testis cDNA library as prey. Four putative new interaction partners were identified, namely Nude-like protein (Nudel), BRCA1-associated protein (BRAP), Ran binding protein 9 and Lim-only protein ACT (ACT).

3.2.2 BiFC-Assay

The BiFC (Bimolecular Fluorescence Complementation) assay is based on the observation that N- and C-terminal subfragments of GFP (or derivatives thereof, e.g. YFP) do not spontaneously reconstitute a functional fluorophore. However, if fused to interacting proteins, the two non-functional halves of the fluorophore are brought into tight contact, refold together and generate *de novo* fluorescence. Thus, by BiFC, the interaction status of two proteins of interest can be easily monitored via fluorescence emission upon excitation with a suitable wavelength. A schematic image of the principle of the BiFC assay is depicted in figure 3.28.

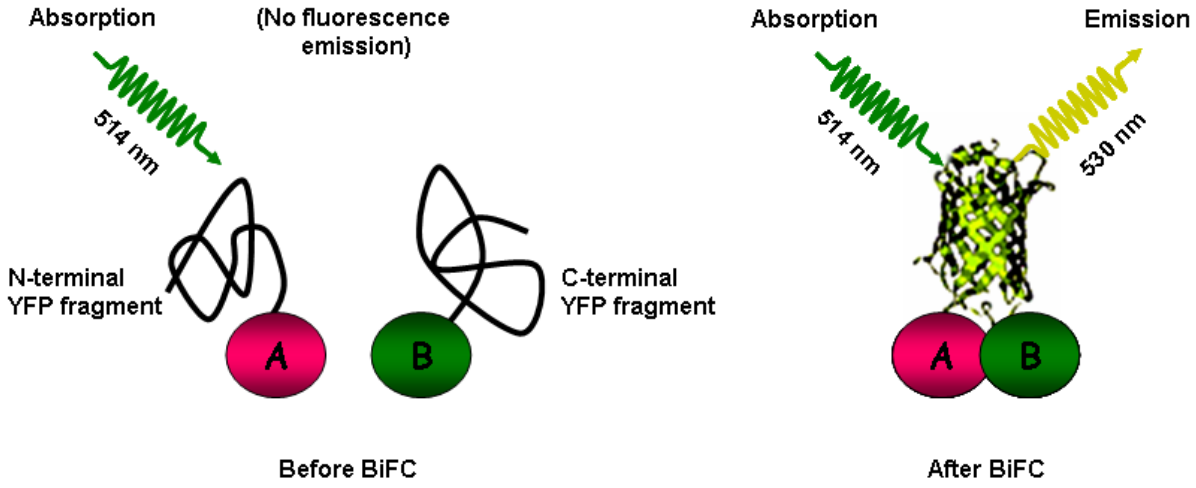

Figure 3.28: Principle of the BiFC assay. The scheme depicts the principle of the BiFC assay, exemplified by a split YFP fluorophore. Proteins A and B are fused to N- and C-terminal fragments of YFP, respectively. In the absence of an interaction between A and B, the fluorophore halves remain non-functional. Following interaction between A and B, a functional fluorophore is reconstituted which exhibits emission of fluorescence upon excitation with an appropriate wavelength (figure taken from Bhat, 2006**).**

The BiFC-Assay used in this study was established by Prof. Dr. S. Hoyer-Fender (Department of Developmental Biology, University of Göttingen). The vectors FPCA-V1 and FPCA-V2 were generated and kindly provided by Prof. Dr. S. Hoyer-Fender. The modified vector FPCA-V1 contains the constitutive CMV promoter, followed by the ORF of the putative LIS1 interacting proteins (ACT, BRAP and NUDEL, respectively), a short spacer region and the C-terminal end of EGFP (amino acids 158 to 239). The modified vector FPCA-V2 contains the constitutive CMV promoter, followed by E2 tag fused to the N-terminal end of EGFP (amino acids 1 to 157), a short spacer region and the ORF of *Lis1* (Fig. 3.29).

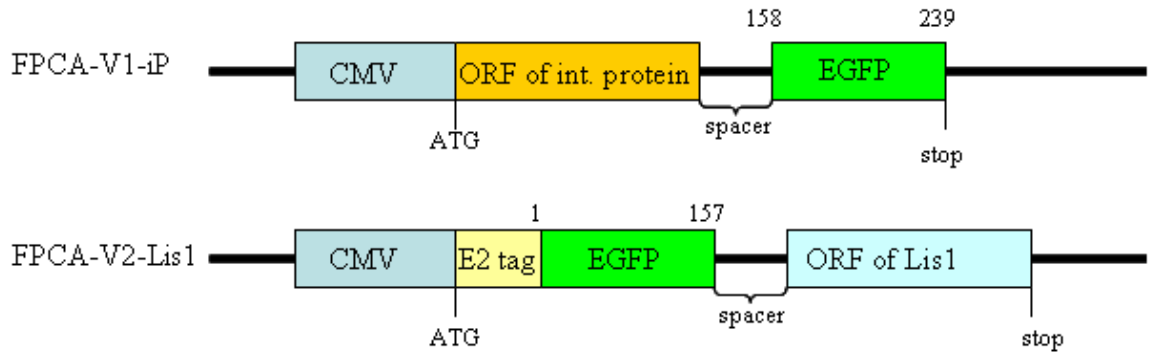

Figure 3.29: Schematic drawing of the vectors used for BiFC-Assay (provided by Prof. Dr. S. Hoyer-Fender). In FPCA-V1-iP ORFs of the putative interaction partners (iP) of LIS1 were cloned 5`upstream

of the C-terminal part of EGFP (amino acids 158-239). ORF of *Lis1* was cloned 3`downstream of the N-terminal part of EGFP (amino acids 1-157) in FPCA-V2-Lis1.

The ORFs for all constructs were amplified with primers described in 2.1.8, cloned into pGEM-T Easy, sequenced and subcloned into FPCA-V1 and FPCA-V2, respectively.

3.2.2.1 Interaction of LIS1 and LIM-only-protein-ACT

NIH-3T3 cells and HeLa cells were transiently transfected with FPCA-V1-LIM-only-protein-ACT and FPCA-V2-Lis1. After 48 hrs cells were fixed, counterstained with DAPI and examined under the confocal microscope IX81 (Olympus). EGFP fluorescence could be detected in cytoplasm of NIH-3T3 cells (Fig. 3.30 A, B) as well as in cytoplasm of HeLa cells (Fig. 30, C). As a positive control, cells were transfected with FPCA-V1-Pelota and FPCA-V2-CDK2-AP1 (Fig. 3.30 D). Interaction of these proteins has been shown by O. Burnicka-Turek (unpublished data). To check the specificity of the vectors, NIH-3T3 cells were cotransfected with FPCA-V1-LIM-only-protein-ACT and FPCA-V2-CDK2-AP1 (Fig. 3.30 E), and FPCA-V1-Pelota and FPCA-V2-Lis1, respectively (Fig. 3.30 F). No EGFP fluorescence could be detected in these negative controls.

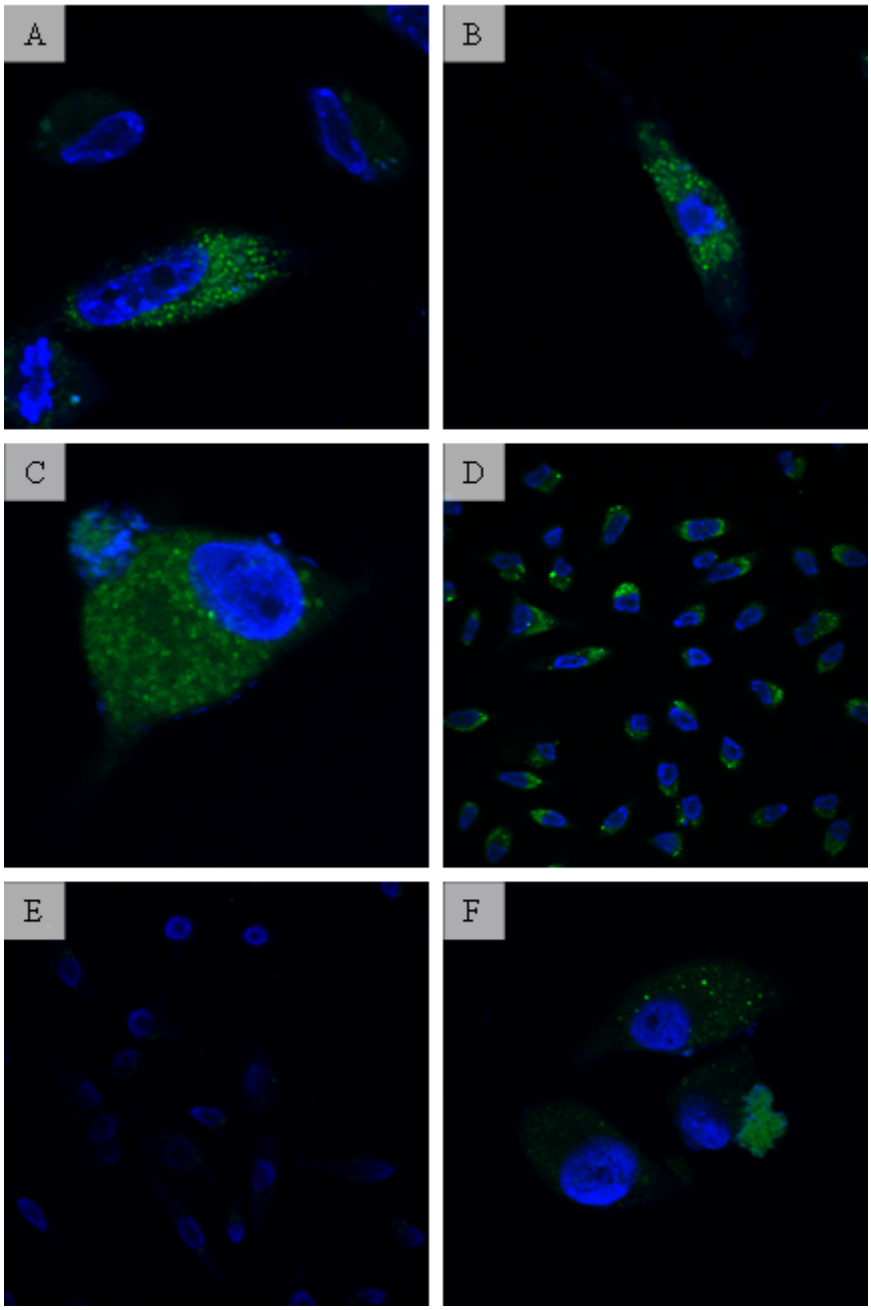

Figure 3.30: Confocal images of specific interaction of LIS1 and Lim-only protein-Act detected by BiFC-Assay. NIH-3T3 (A, B) and HeLa (C-F) cells were transiently transfected with FPCA-vectors, fixed 48h after transfection and counterstained with DAPI. Interaction of LIS1 and Lim-only-protein-Act is clearly visible in NIH-3T3 cells (A,B) and in HeLa cells (C). As a positive control the interaction of Pelota and CDK2-AP1 is shown in D. Specificity of the vectors was analysed by transfection of cells with FPCA-V1-Lim-only-protein-Act and FPCA-V2-CDK2-AP1 (E) and FPCA-V1-Pelota and FPCA-V2-Lis1 (F), respectively. No EGFP fluorescence was detected in these negative controls. All pictures are dual image overlays of EGFP and DAPI fluorescences.

3.2.2.2 Interaction of LIS1 and BRCA1-Associated-Protein

HeLa cells were transiently transfected with FPCA-V1-BRAP and FPCA-V2-Lis1. After 48 h, cells were fixed, counterstained with DAPI and examined under the confocal microscope IX81 (Olympus). EGFP fluorescence could be detected in cytoplasm of HeLa cells (Fig. 3.31 A, B). To check the specificity of the vectors, NIH-3T3 cells were cotransfected with FPCA-V1-BRAP and FPCA-V2-CDK2-AP1 (Fig. 3.31 C), and FPCA-V1-Pelota and FPCA-V2-Lis1, respectively (Fig. 3.31 D). No EGFP fluorescence could be detected in these negative controls. The experiment was repeated twice to confirm the interaction between LIS1 and BRAP.

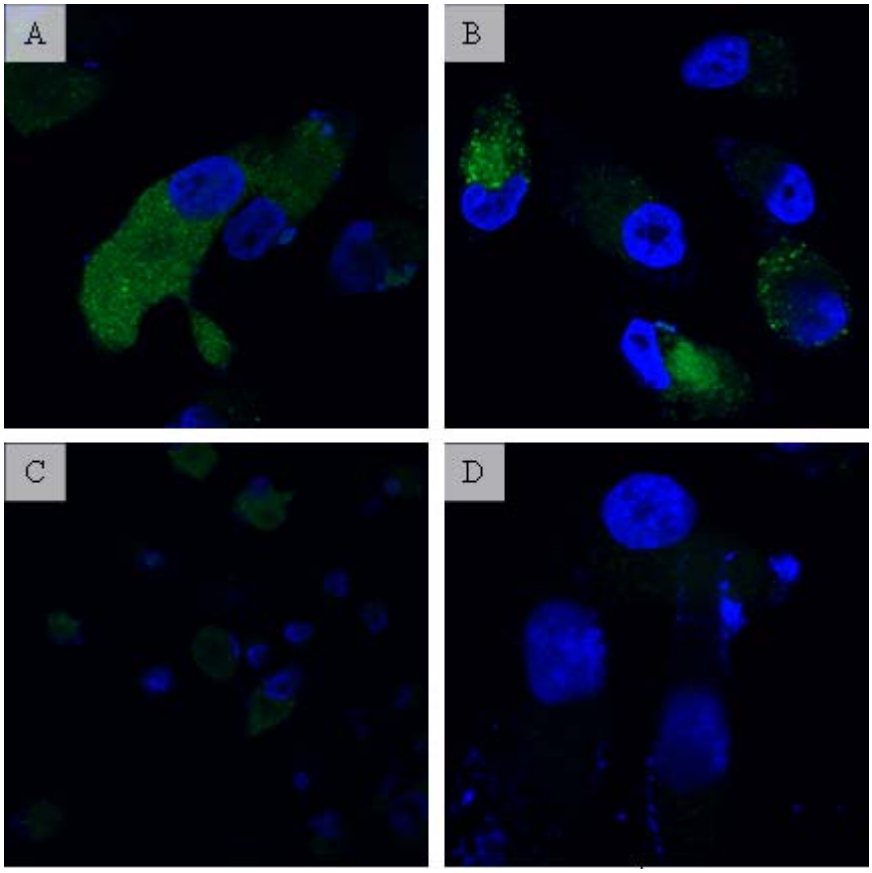

Figure 3.31: Confocal images of specific interaction of LIS1 and BRAP detected by BiFC-Assay. HeLa cells were transiently transfected with FPCA-vectors, fixed 48h after transfection and counterstained with DAPI. Interaction of LIS1 and BRAP is clearly visible in cytoplasm of HeLa cells (A, B). No EGFP fluorescence was detected in negative controls (C, D). HeLa cells were transiently transfected with FPCA-V1-BRAP and FPCA-V2-CDK2-AP1 (C) and FPCA-V1-Pelota and FPCA-V2-Lis1 (D), respectively. All pictures are dual image overlays of EGFP and DAPI fluorescences.

3.2.2.3 Interaction of LIS1 and NUDEL

HeLa cells were transiently transfected with FPCA-V1-Nudel and FPCA-V2-Lis1. After 48 hrs cells were fixed, counterstained with DAPI and examined under the confocal microscope IX81 (Olympus). EGFP fluorescence could be detected in cytoplasm of HeLa cells (Fig. 3.32 A, B). Nudel (Nuclear distribution gene E-like) is involved in neuronal migration during brain development. To check the specificity of the vectors, NIH-3T3 cells were cotransfected with FPCA-V1-Nudel and FPCA-V2-CDK2-AP1 (Fig. 3.32 D). EGFP fluorescence could be detected in the negative control, while in untransfected HeLa cells no EGFP fluorescence was detected (Fig. 3.32 C). The experiment was repeated twice to confirm the interaction between LIS1 and NUDEL.

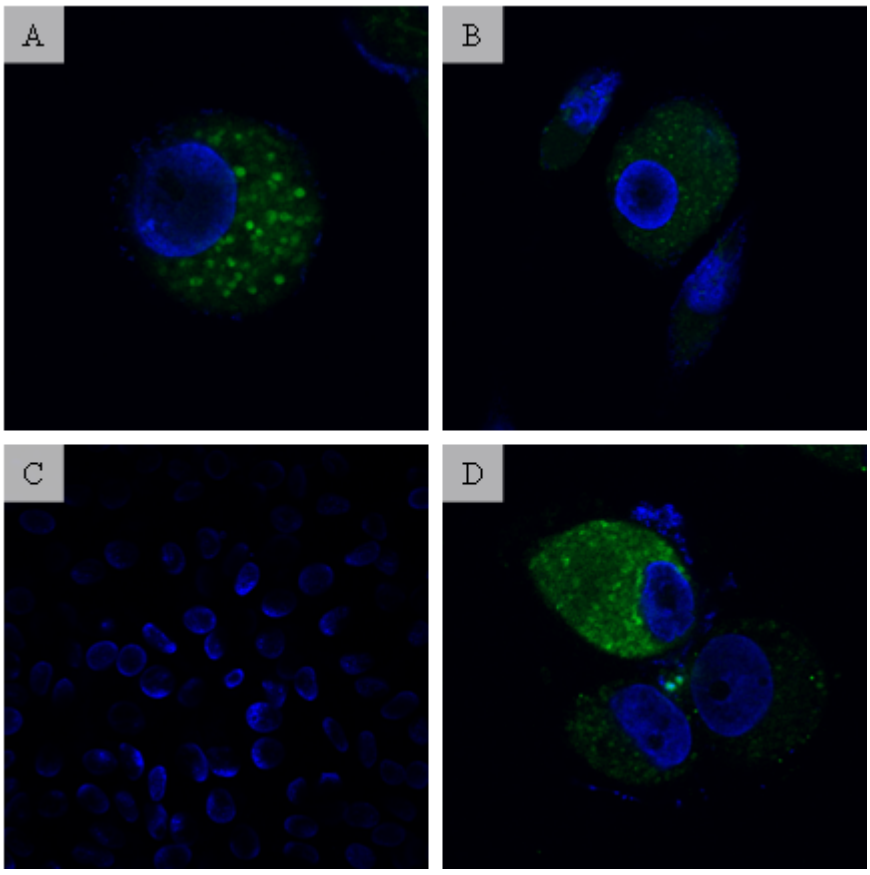

Figure 3.32: Confocal images of specific interaction of LIS1 and NUDEL detected by BiFC-Assay. HeLa cells were transiently transfected with FPCA-vectors, fixed 48h after transfection and counterstained with DAPI. Interaction of LIS1 and NUDEL is clearly visible in cytoplasm of HeLa cells (A, B). No EGFP fluorescence was detected in untransfected HeLa cells (C), but transiently transfected cells with FPCA-V1-NudeL and FPCA-V2-CDK2-AP1 show a clear EGFP signal (D). All pictures are dual image overlays of EGFP and DAPI fluorescences.

3.3 Analysis of germ cell specific regulation

3.3.1 *Lis1* promoter analysis with pGL3 vectors

To determine *Lis1* promoter region, *Lis1* genomic sequence was analysed with ECR (Evolutionary conserved Regions) Browser (http://ecrbrowser.dcode.org). This tool enables users to find ECRs in genomes of 13 species. *Lis1* genomic sequence including 5`-flanking region was compared to Human, Chimpanzee, Rhesus macaque, Chicken, Dog, Rat, Zebrafish and Frog genomes. A high number of conserved nucleotides was found within 2 kb of *Lis1* 5`-flanking region. This was confirmed by a blast search (blastn) where human and mouse 5`-flanking regions are compared. Moreover, ElDorado, the Genomatix genome annotation and analysis project (www.genomatix.de), was used to identify promoter regions of murine *Lis1* gene. Again, Gene2Promoter and PromoterInspector program revealed putative promoter regions within 2 kb of *Lis1* 5`-flanking region.

To see whether the *Lis1* 5`-flanking sequence contains a *Lis1* specific promoter, a reporter plasmid was constructed by cloning *Lis1* 5`-flanking region into pGL3-Basic vector. pGL3 vectors contain a modified coding region for firefly (Photinus pyralis) Luciferase that has been optimized for monitoring transcriptional activity in transfected eukaryotic cells.

3.3.1.1 Construction of plasmid

A 1846 bp PCR fragment of *Lis1* 5`-flanking region was amplified using primers Pro6-f1 (containing a *XhoI* restriction site) and Pro6-r1 (containing a *HindIII* restriction site), cloned into pGEM-T Easy and sequenced. After digestion with *HindIII* and *XhoI*, the PCR fragment was subcloned into the digested pGL3-Basic vector and sequenced again. The resulting vector, pGL3-Pro6, is shown in figure 3.33.

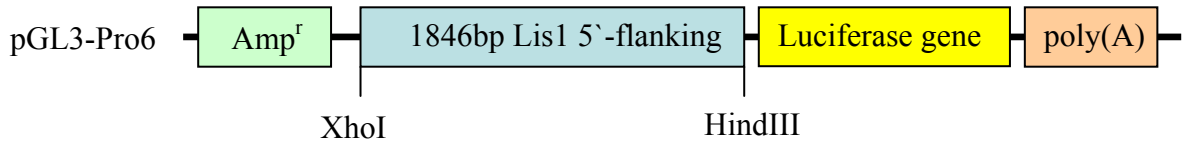

Figure 3.33: Schematic drawing of pGL3-Pro6 vector. 1846 bp *Lis1* 5`-flanking region was cloned into *XhoI* and *HindIII* sites of pGL3-Basic vector 5`upstream of *Luciferase* gene. The vector contains a

ß-lactamase gene (Amp^r), which confers resistance to ampicillin and the SV40 late poly(A) signal for the *Luciferase* reporter gene.

As a positive control pGL3-Control vector was used, which contains 240 bp of SV40 core promoter and 236 bp of SV40 enhancer sequence followed by the SV40 poly(A) signal. Transfection with this vector leads to high expression of the *Luciferase* reporter gene in eukaryotic cells. As a negative control pGL3-Basic vector was used, that contains no promoter or enhancer sequence. This vector was used to show background expression of the *Luciferase* reporter gene.

3.3.1.2 Analysis of Luciferase activity in transient transfected cells

Luciferase activity was measured in three different eukaryotic cell lines, namely GC-1 spg, NIH 3T3 and NS20Y. GC-1 spg is a spermatogonial cell line, isolated from testis of 10 d old BALB/c mice. The type B spermatogonia were immortalised by transfection with pSV3-neo (a plasmid containing coding sequence for the SV40 large T antigen and neomycin resistance) (Hofmann, 1992). NIH 3T3 is a murine embryonic fibroblast line, which was isolated from NIH/Swiss mouse (Jainchill, 1969). NS20Y is a mouse neuroblastoma line, established from A/Jax mouse strain with neuroblastoma (Amano, 1972).

Luciferase expression was determined 48 hrs after cotransfection of cells with pGL3 vector and pCMV-ßGal vector. Cells were lysed and enzymatic activity was measured according to 2.2.18 and 2.2.19. To normalise for transfection efficiency, values (RLUs (relative light units) of Luciferase activity) were divided by ß-Galactosidase values of the same sample. Mean value of normalised pGL3-Control vector was used as a calibrator. Each cell line was transfected at least two times, with three technical replicates each. Figure 3.34 shows Luciferase activity measurement results of all tested cell lines.

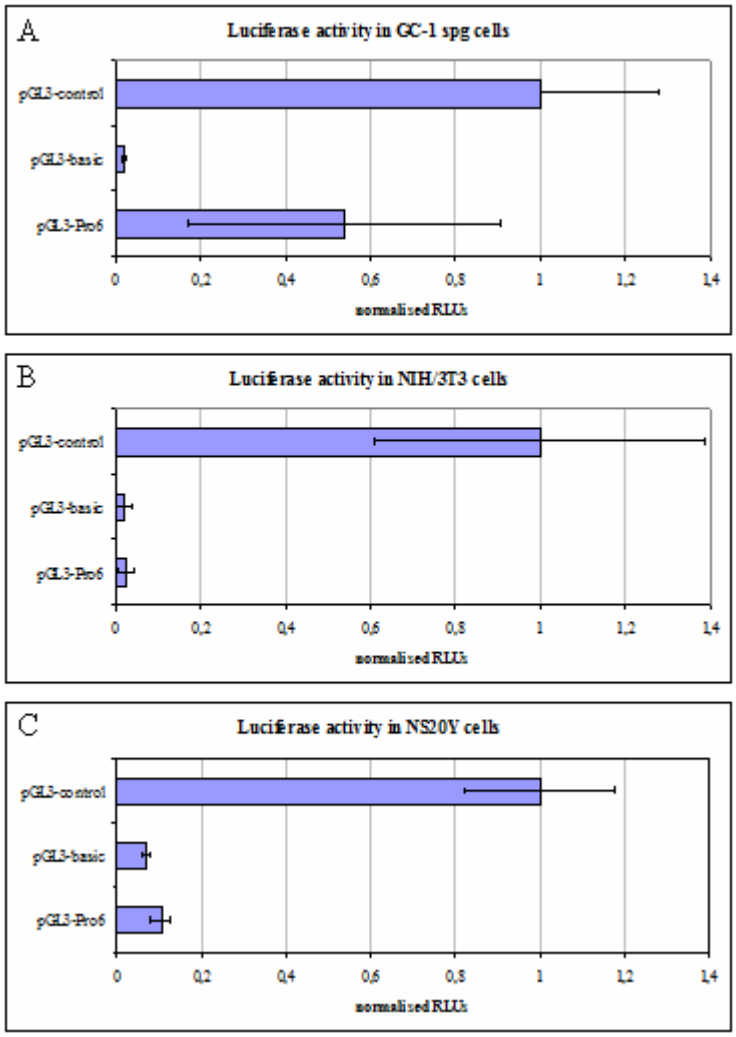

Figure 3.34: Luciferase activity in different cell lines transfected with pGL3-Pro6 vector. As positive control cells were transfected with pGL3-Control vector, as negative control cells were transfected with pGL3-basic vector. Cells were lysed 48 hrs after cotransfection with pGL3 and pCMV-ßGal vectors and assayed for Luciferase activity. The graphs depict the mean (± SD) of two independent experiments of transfected GC-1 spg (A), NIH 3T3 (B) and NS20Y (C) cells, respectively.

Luciferase activity in cells transfected with pGL3-Pro6 vectors was not significantly increased in comparison to Luciferase activity of cells transfected with pGL3 basic vector. Although transfected GC-1 spg cells showed an increased enzyme activity (Fig. 3.34 A), results were not significant as compared to pGL3-Basic vector (Mann-Whitney-U-Test; p=0.28). NIH 3T3 (Fig. 3.34 B) and NS20Y (Fig. 3.34 C) cells did not differ in enzyme activity after transfection of pGL3-Pro6 or pGL3-Basic vector. The 1.8 kb 5`-flanking region of *Lis1* gene had no effect on Luciferase expression in the analysed cells.

3.3.2 *Lis1* Enhancer analysis with pGL3 vectors

An enhancer is a cis-regulatory DNA sequence that can greatly increase the transcription rate of genes even though they may be far upstream or downstream from the promoter they stimulate. In the gene trap line L39 a testis specific transcript (*Lis1 "2a"* transcript) is downregulated by the gene trap integration. One could explain this downregulation by an integration of the gene trap vector into a testis specific enhancer sequence that destroyed DNA binding of testis specific transcription factors and thus caused low expression of *Lis1 "2a"* transcript. To verify this hypothesis, putative enhancer sequences were cloned into pGL3-Promoter vector and SSC/129/Sv cells were transfected with these constructs.

3.3.2.1 Construction of plasmids

A 819 bp PCR fragment of *Lis1 intron 2* region, surrounding the first integration site of the gene trap vector (amplified with primerset "Gö") and a 1287 bp PCR fragment, surrounding the second integration site of the gene trap vector (amplified by primerset "BS") were amplified using primers Enh-Gö-BamHI-F1 (containing a *BamHI* restriction site) and Enh-Gö-SalI-R1 (containing a *SalI* restriction site) and Enh-BS-BamHI-F1 (containing a *BamHI* restriction site) and Enh-BS-SalI-R1 (containing a *SalI* restriction site), respectively, were cloned into pGEM-T Easy vector and sequenced. After digestion with *BamHI* and *SalI*, the PCR fragment was subcloned into the digested pGL3-Promoter vector and sequenced again. Moreover, a 1780 bp putative promoter fragment was amplified with primer Prom-Lis1-KpnI-F2 (including a *KpnI* restriction site) and Prom-Lis1-HindIII-R2 (including a *HindIII* restriction site), cloned into pGEM-T Easy and sequenced. After digestion with *KpnI* and *HindIII*, the PCR fragment was subcloned into the digested pGL3-Control vector (the SV40 core promoter region was cut out with the used enzymes) and sequenced again. The resulting vectors, pGL3-Pro-Gö, pGL3-Pro-BS and pGL3-Con-Pro4 are shown in figure 3.35.

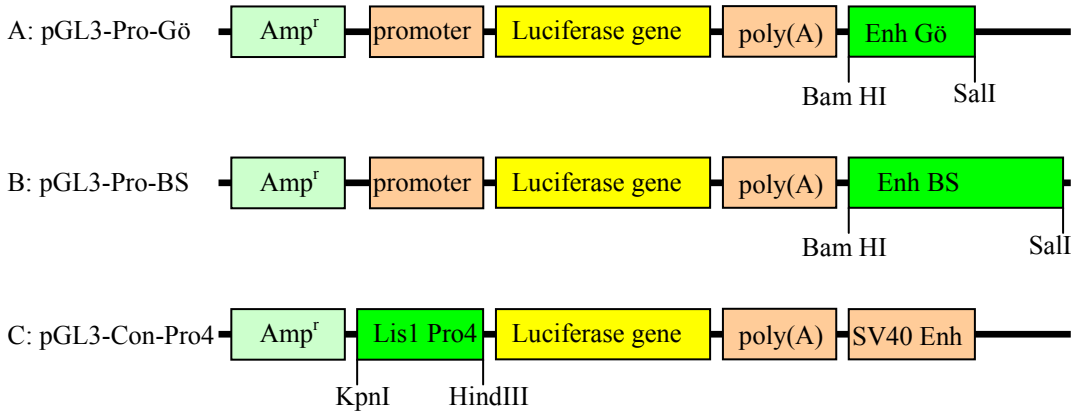

Figure 3.35: Schematical drawing of pGL3-Pro-Gö (A), pGL3-Pro-BS (B) and pGL3-Con-Pro4 (C) vectors. In (A) a 819 bp fragment of *Lis1 intron 2* region (containing the integration site of the gene trap vector amplified by "Gö" primerset) was cloned into *BamHI* and *SalI* sites of pGL3-Promoter vector 5`upstream of *Luciferase* gene. In (B) a 1287 bp fragment of *Lis1 intron 2* region (containing the integration site of the gene trap vector amplified by "BS" primerset) was cloned into *BamHI* and *SalI* sites of pGL3-Promoter vector 5`upstream of *Luciferase* gene. In (C) a 1780 bp fragment of 5`-flanking region of *Lis1* was cloned into *KpnI* and *HindIII* sites of pGL3-Control vector, thereby replacing the SV40 promoter fragment. Vectors contain a *ß-lactamase* gene (Amp^r), which confers resistance to ampicillin and the SV40 late poly(A) signal for the *Luciferase* reporter gene.

As a positive control pGL3-Control vector was used, which contains 240 bp of SV40 core promoter and 236 bp of SV40 enhancer sequence followed by the SV40 poly(A) signal. Transfection with this vector leads to high expression of the *Luciferase* reporter gene in eukaryotic cells. As a negative control pGL3-Basic vector was used, that contains no promoter or enhancer sequence. This vector was used to show background expression of the *Luciferase* reporter gene.

3.3.2.2 Analysis of Luciferase activity in transient transfected SSC/129/Sv cells

SSC/129/Sv (Spermatogonial Stem Cell) line was transfected with pGL3 vectors to detect *Lis1* enhancer activity. In our group SSC/129/Sv, a murine spermatogonial cell line, was isolated from testes of adult 129/Sv wild type mice and transfected with Stra8/EGFP construct (Guan et al., 2006). FACS (fluorescence activated cell sorting) analysis of EGFP expressing cells was performed two times to obtain a pure SSC/129/Sv population. Differentiation of SSC lines can be achieved by deprivation of leukaemia inhibitory factor (LIF) and by addition of retinoic acid (RA). One day before transfection, SSC/129/Sv cells (cultured on MEFs) were split on two gelatine-coated 24

well plates, on one plate cells were cultured with SSC/129/Sv medium while on the other plate cells were cultured with the SSC/129/Sv differentiation medium.
Endogenous activity of *Lis1* promoter was shown by Western blot analysis of SSC/129/Sv cell lysates (Fig. 3.36).

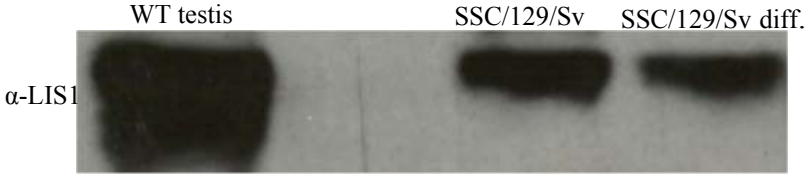

Figure 3.36: Expression of LIS1 protein in SSC/129/Sv and differentiated SSC/129/Sv (SSC/129/Sv diff.). Monoclonal anti-LIS1 (Santa Cruz) detects the 45 kDa protein in lysates of SSC/129/Sv cells and testicular lysate of wild type testis (WT testis), used as a positive control.

Luciferase expression was determined 48 hrs after cotransfection of cells with pGL3 vector and pCMV-ßGal vector (or pRL-SV40 vector). Cells were lysed and enzymatic activity was measured according to 2.2.18 and 2.2.19. To normalise for transfection efficiency, values (RLUs of Luciferase activity) were divided by ß-Galactosidase values of the same sample (or by renilla Luciferase values of the same sample, when transfected with pRL-SV40 vector). Mean value of normalised pGL3-Control vector (in undifferentiated SSC/129/Sv cells) was used as a calibrator. SSC/129/Sv cells were transfected three times, with three technical replicates each. Figure 3.37 shows Luciferase activity measurement results.

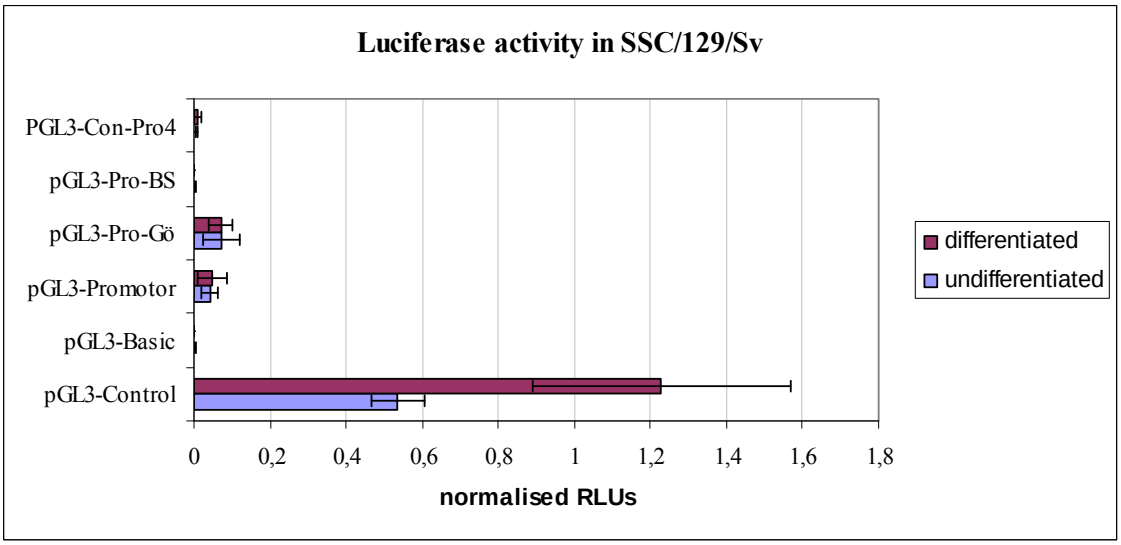

Figure 3.37: Luciferase activity in SSC/129/Sv transfected with pGL3 vector constructs. As positive control cells were transfected with pGL3-Control vector, as negative control cells were transfected with

pGL3-Basic vector. Cells were lysed 48 hrs after cotransfection with pGL3 and pCMV-ßGal (or pRL-SV40) vectors and assayed for Luciferase activity. The graphs depict the mean (± SD) of three independent experiments. Differentiated SSC/129/Sv were cultured in differentiation medium for 72 hrs, while undifferentiated SSC/129/Sv were cultured in usual SSC/129/Sv medium.

Luciferase activity in cells transfected with pGL3-Pro-Gö and pGL3-Pro-BS vectors was not significantly increased in comparison to Luciferase activity of cells transfected with pGL3 basic vector. Small differences in the enzyme activity in undifferentiated versus differentiated cells were not significant. Expression of the reporter gene in differentiated cells transfected with pGL3-Control vector was not significantly higher ($p<0.1$) than in undifferentiated cells. The cloned putative enhancer regions of *Lis1* gene and the putative promoter region had no effect on Luciferase expression in the analysed cell line.

3.3.3 Generation of a transgenic construct with putative *Lis1* enhancer sequence

To check if the DNA region of *Lis1 intron 2* that surrounds both integration sites has an enhancer activity *in vivo*, a transgenic construct was generated that expresses LacZ under control of the SV40 core promoter and a 6 kb *Lis1 intron 2* fragment. A schematic drawing of the transgenic enhancer construct is shown in figure 3.38.

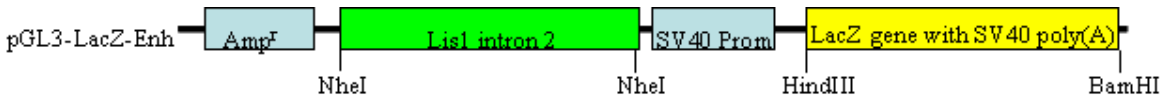

Figure 3.38: Enhancer construct for *in vitro* analysis of *Lis1 intron 2* region. The 3.3 kb *LacZ* gene with adjacent SV40 poly(A) signal was cloned into *HindIII* and *BamHI* restriction sites of pGL3-Promoter vector, thereby replacing *Luciferase* gene and SV40 poly(A) signal of the vector. Next, a 6 kb fragment of *Lis1 intron 2* was cut out of a *Lis1* containing BAC clone (bMQ-108I8, kindly provided by Dr. A. Holz, Braunschweig) and cloned 5` upstream of the SV40 core promoter region (SV40 Prom). The Amp^r gene confers resistance to ampicillin.

pSDKLacZ vector was digested with *HindIII* and *BamHI* to release a 3.34 kb fragment including the *LacZ* gene with the SV40 poly(A) signal. This fragment was then cloned into *HindIII* and *BamHI* restriction site of pGL3-Promoter vector, thereby replacing the *Luciferase* gene and the SV40 poly(A) signal of the vector. Next, a 6 kb fragment of the *Lis1* gene containing BAC clone bMQ-108I8 (BACPAC Resources, California, USA; kindly provided by Dr. A. Holz, Braunschweig) (including the *Lis1 intron 2* region,

where the gene trap vector integrated in L39 mice) was cloned into *NheI* restriction site of the vector 5` upstream of the SV40 core promoter region.

As a negative control, the pGL3-LacZ vector was used, which does not contain the putative enhancer region of *Lis1*. As a positive control the SV40 enhancer region was amplified (with primers SV40-Enh-NheI-F and SV40-Enh-NheI-R), and cloned into *NheI* restriction site of the vector 5` upstream of the SV40 core promoter region.

The pGL3-LacZ-Enh construct has not been finished during course of this work.

3.4 Genetic rescue of the infertile mice $L39^{GT/GT}$

To confirm that the reproductive defect in $L39^{GT/GT}$ males is due to the gene trap integration in *Lis1* gene locus, to determine whether germ-cell-specific expression of *Lis1* is the cause of infertility and to gain further insights into the role and requirement of LIS1 in spermatogenesis, transgenic rescue approaches were used.
For this, three transgenic mice were generated, which overexpress *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.

3.4.1 Analysis of TNP2-Lis1 (Lispi) transgenic mice

Transition nuclear protein 2 (TNP2) is expressed postmeiotically in male germ cells and is involved in chromatin condensation during spermiogenesis in the mouse (Kleene, 1987). To generate transgenic mice, which overexpress *Lis1* gene under control of the TNP2 promoter, a construct was generated (from now on referred to as Lispi construct) that includes 525 bp of 5`-flanking region of rat *TNP2* gene, followed by 1.5 kb of *Lis1* cDNA and 920 bp of 3`-flanking region of rat *TNP2* gene. This construct was produced by Yeroolt Tangad.

3.4.1.1 Generation and breeding of transgenic Lispi mice

The Lispi construct was prepared according to 2.2.15.1 followed by pronuclear microinjection, which was performed by “Transgenic Service” of Max-Planck-Institute for Experimental Medicine, Göttingen. Two mice of F0 offspring were genotyped positive for the transgenic integration. These founders (#2 and #7) were further bred with FVB wild type mice and F1 offsprings were genotyped and used for expression analysis of the *Lis1* transgene. These experiments were performed by Khaliun Tseden.

3.4.1.2 Expression analysis of transgenic Lispi mice

(performed by K. Tseden)

To further determine testis specific expression of the transgenic *Lispi* transcript in mutant animals of Lispi mice, Northern blot analysis was performed using a ^{32}P-labelled *Lispi* specific probe. The expression was restricted to testes of mice of both Lispi lines (#7 and #2), while brain, kidney, liver, lung and spleen in these lines showed no expression (Fig. 3.39).

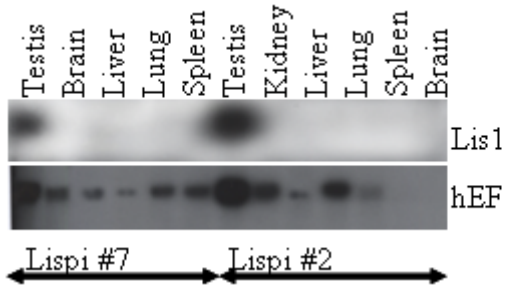

Figure 3.39: Northern blot analysis of *Lispi* specific transcript. Expression was restricted to testes of Lispi line #7 and #2. Integrity of RNA was checked by rehybridisation of the membrane with a hEF probe.

This Northern blot analysis was performed by Khaliun Tseden.

3.4.1.3 Histological analysis of testis sections of Lispi^{Tpos} males

Histological analysis of testes of adult Lispi^{Tpos} males revealed no obvious phenotype. Males exhibit full spermatogenesis with no obvious differences from wild type males (data not shown). All experiments were performed by Khaliun Tseden.

3.4.2 Analysis of "rescued" L39$^{GT/GT}$/Lispi^{Tpos} males

3.4.2.1 Breeding strategy to generate L39$^{GT/GT}$/Lispi^{Tpos} males

To generate males, that are homozygous for the gene trap vector integration and positive for the transgene Lispi, the following breeding strategy was used:

(i) F0: ♀ L39$^{GT/GT}$ x ♂ Lispi^{Tpos}
(ii) F1: ♀ L39$^{GT/-}$/Lispi^{Tpos} x ♂ L39$^{GT/-}$/Lispi^{Tpos}
(iii) F2: ♂ L39$^{GT/GT}$/Lispi^{Tpos}

Homozygous gene trap females are fertile and could be used for breeding with Lispi^{Tpos} males. All resulting offspring were heterozygous for the gene trap integration (L39$^{GT/-}$), and about 50% of the animals were Lispi^{Tpos}. L39$^{GT/-}$/Lispi^{Tpos} siblings of the F1 generation were bred to generate L39$^{GT/GT}$/Lispi^{Tpos} males. According to Mendelian ratio the genotype of 3/16 of offspring was L39$^{GT/GT}$/Lispi^{Tpos} (1/4 GT/GT x 3/4 Lispi positive). About 50% of offspring were male, thus about 10% (3/32) of F2 generation were L39$^{GT/GT}$/Lispi^{Tpos} males. From now on, these mice are referred to as Lispi "rescued" males.

3.4.2.2 Fertility test of L39$^{GT/GT}$/Lispi^{Tpos} males

Fertility of three Lispi "rescued" males was tested by mating them with homozygous gene trap or wild type females. All matings were performed for several weeks, but no litter was obtained.

Eight adult homozygous Lispi "rescued" males (between 2 and 6 months old) were then mated with wild type females. The uteri and oviducts of those mice which were positive for vaginal plug were dissected in IVF medium, flushed out and sperm number was determined. No sperms were found in uterus and oviduct of females mated with homozygous males. The experiment was repeated two times for each male. L39$^{GT/-}$ /Lispi^{Tpos} animals were mated with wild type females as controls. Sperm count revealed the expected number of sperms in uterus ($10.9x10^6 \pm 2.6x10^6$, N=5) of females mated with L39$^{GT/-}$/Lispi^{Tpos} males. As observed in L39$^{GT/GT}$, the testes sizes of Lispi

"rescued" males were markedly reduced in comparison with heterozygous siblings (data not shown).

For the determination of sperm motility 13 µl of sperm suspension from caudae epididymes was put on a dual sided sperm analysis chamber. Sperm motility was quantified using the computer assisted semen analysis (CASA) system. Number of motile sperm cells in "rescued" mice was too low to be detected by CASA, while number of sperms in heterozygous animals was sufficient to be analysed (data not shown).

3.4.2.3 Histological analysis of testis sections of L39$^{GT/GT}$/Lispi^{Tpos} males

To see whether the overexpression of *Lis1* in haploid germ cells has an effect on testis morphology in Lispi "rescued" mice, testes of adult Lispi "rescued" males, L39$^{GT/GT}$ and FVB wild type mice were fixed in Bouins solution, sliced into 5 µm thick sections and stained with hematoxilin and eosin (H&E) (Fig. 3.40).

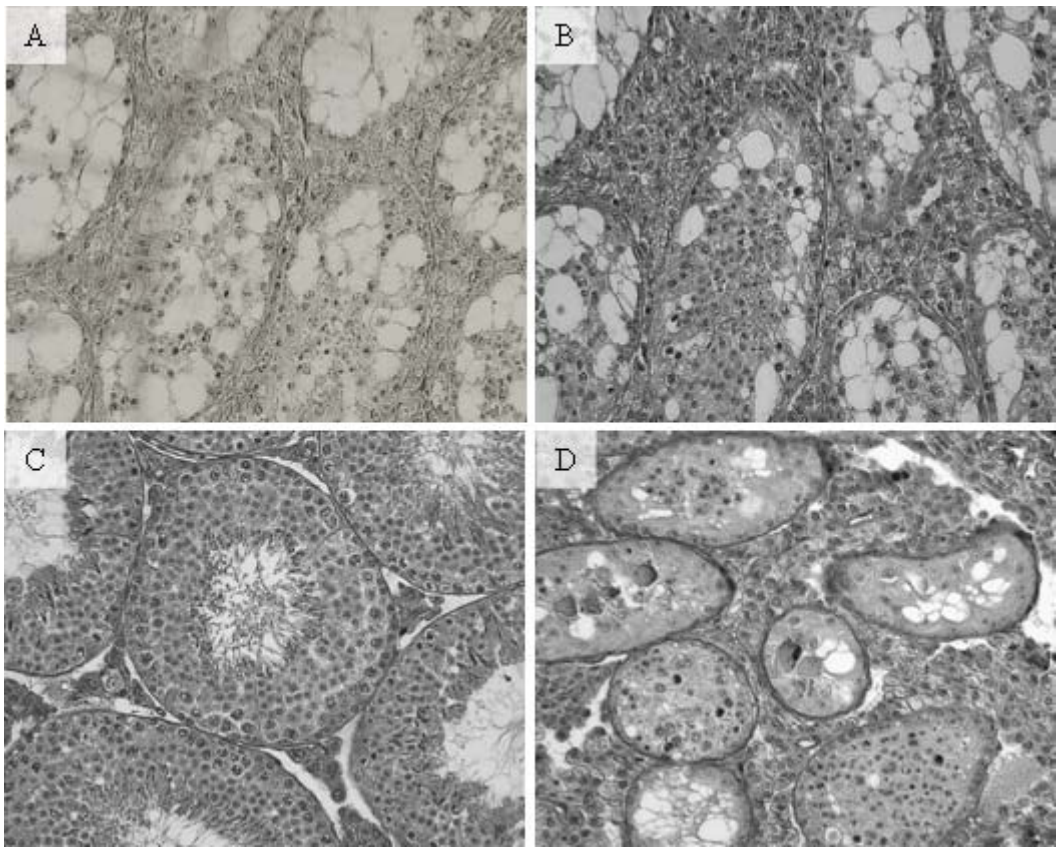

Figure 3.40: H&E staining of testis sections of adult Lispi "rescued" males, L39$^{GT/GT}$ and FVB wild type mice. Sections through testes of adult Lispi "rescued" mice (A, no 12A; B, no 13A) revealed similar extensive degeneration of a large fraction of seminiferous tubules as observed in testes of homozygous

gene trap mice (D), whereas testis sections of adult FVB wild type animals (C) demonstrated robust spermatogenesis. original magnification of depicted images is 200x and 100x for (D), respectively.

The histological analysis of testes of Lispi "rescued" mice revealed the same extensive degeneration of a large fraction of seminiferous tubules as observed in $L39^{GT/GT}$ males of all genetic backgrounds (Fig. 3.1, 3.19 to 3.22 and 3.40 D). Like in the gene trap line, the mutant testis exhibits early germ cells as well as Sertoli and Leydig cells, but the number of late meiotic (i.e. late pachytene and diplotene spermatocytes) and postmeiotic (i.e. spermatids and spermatozoa) germ cells was markedly reduced. This phenotype was visible in both Lispi "rescued" mice.
The overexpression of *Lis1* in postmeiotic germ cells has no visible influence on testis morphology of homozygous gene trap mice.

3.4.2.4 Electron microscopy of testis sections of $L39^{GT/GT}$/$Lispi^{Tpos}$ males

Electron microscopy of testis sections from early postnatal stages of $L39^{GT/GT}$ mice revealed no ultrastructural alterations of spermatogenic precursor cells, including pachytene spermatocytes but severely affected spermatids at later postnatal stages (Nayernia, 2003). To confirm these data for Lispi "rescue" males, the ultrastructure of testes of Lispi "rescue" mice at the age of 28 d and 4 months was examined by electron microscopy in cooperation with Prof. Meinhardt (Institute for Anatomy and Cell Biology, Giessen).
An overview of defects found in $L39^{GT/GT}$/$Lispi^{Tpos}$ males is shown in figure 3.41. In 28 d old mice few vacuoles were found, but the nuclear structure of all germ cells looked similar. No differentiation of germ cells was found, thus no acrosome or tail formation or condensation of nuclei were detected. This phenotype became more severe in 4 months old mice, were a complete failure of spermatogenesis was observed, with many vacuoles and inclusion bodies. Some cells were found, which looked similar to round and elongating spermatids, but without tail formation. The overexpression of *Lis1* in haploid cells did not improve the phenotype of $L39^{GT/GT}$ mice.

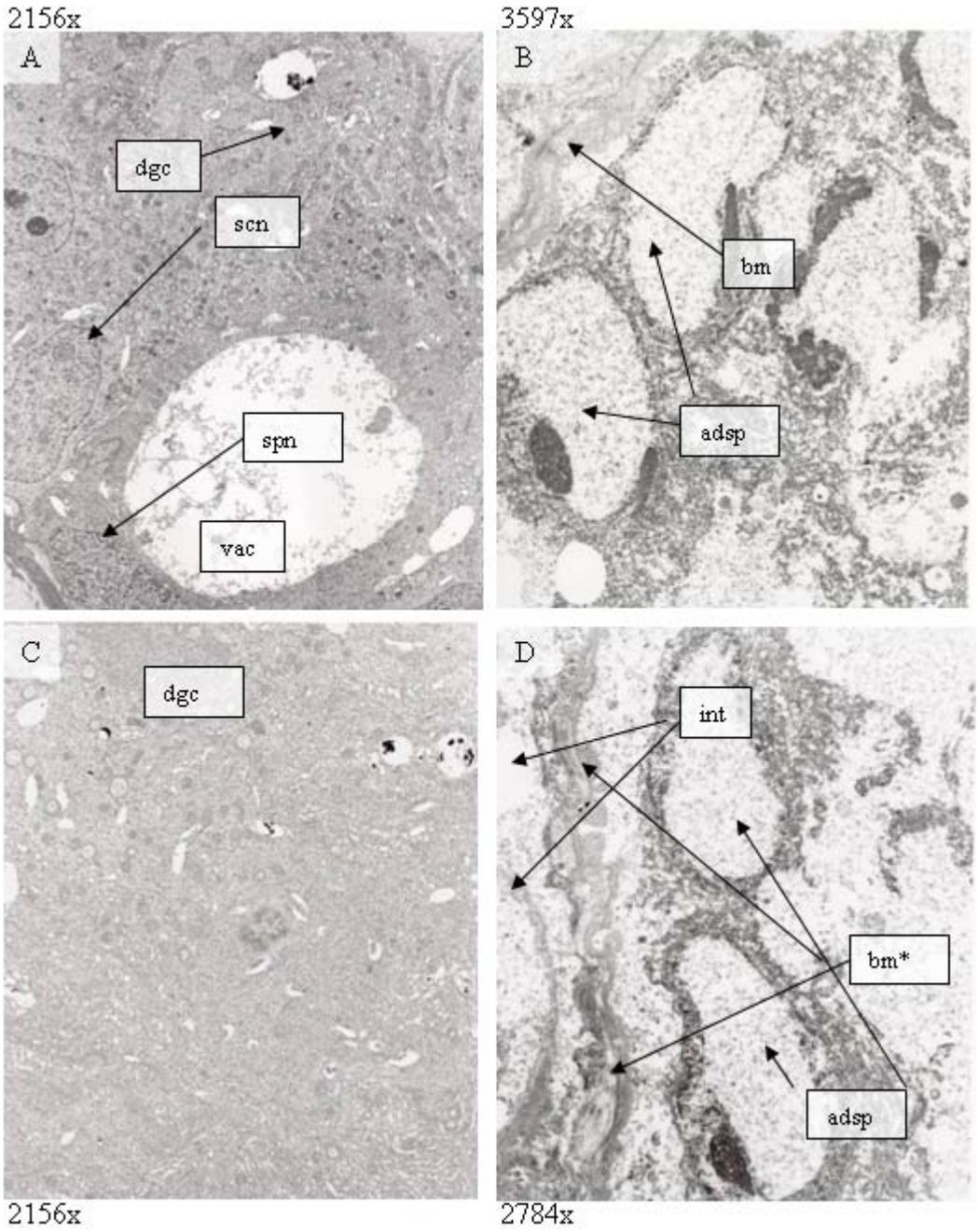

Figure 3.41: Electron microphotographs of testes of 28 d old (C, D) and 4 months old (A, B) Lispi "rescued" mice. Abbrevations are: **adsp**, atypical, degenerating spermatogonia; **bm**, basal membrane; **bm***, basal membrane with peritubular cell extension; **dgc**, degenerating germ cells; **int**, interstitium; **scn**, Sertoli cell nucleus; **spn**, Spermatogonia nucleus and **vac**, vacuole.

3.4.3 Analysis of transgenic PGK2-Lis1-c-myc Tag mice

Phosphoglycerate Kinase-2 (*PGK2*) is an autosomal gene expressed in a testis-specific manner exclusively in the late stages of spermatogenesis (Kramer, 1981). To generate transgenic mice, which overexpress *Lis1* gene under control of the PGK2 promoter, a construct was generated (from now on referred to as PGK2 construct) that includes 1.4 kb of 5`-flanking region of human *PGK2* gene, followed by 1377 bp of *Lis1* cDNA and a 410 bp fragment of (c-myc Tag)$_6$-SV40 poly(A). A schematic drawing of the PGK2 construct is depicted in figure 3.42.

Figure 3.42: Schematic drawing of the PGK2 construct used to generate transgenic mice which overexpress *Lis1* gene under control of human PGK2 promoter. A 1377 bp fragment of *Lis1* cDNA (containing ATG codon) was cloned into *EcoRI* and *BamHI* restriction sites of vector clone 442 (pBluescript II SK (-) with 1.4 kb PGK2 promoter). Next, a 410 bp fragment of (c-mycTag)$_6$ (containing two stop codons) followed by SV40 poly(A) signal was cloned into *BamHI* and *NotI* restriction sites. The black arrows correspond to primers used for genotyping and for RT-PCR analysis.

The detailed cloning strategy was as follows:

(i) The plasmid clone 442 (containing a 1.4 kb fragment of PGK2 promoter cloned into pBluescript II SK (-) vector (Stratagene)) was sequenced and restriction sites and promoter sequences were confirmed.

(ii) A 1377 bp *Lis1* cDNA fragment was amplified by PCR (using primer Lis1-cDNA-EcoRI and Lis1-cDNA-BamHI), cloned into pGEM-T Easy and sequenced. The PCR product contains an *EcoRI* restriction site 136 bp 5` upstream of *Lis1* start codon and a *BamHI* restriction site that replaced the *Lis1* stop codon.

(iii) Sequenced *Lis1* cDNA fragment was cloned into *EcoRI* and *BamHI* restriction sites of plasmid clone 442.

(iv) A 410 bp PCR fragment was amplified (using primer c-myc-F1 and c-Myc-PolyA-NotI-R1), cloned into pGEM-T Easy and sequenced. pCS2-3`mt plasmid (Hammerschmidt Plasmid Stocks, Freiburg), that contains six c-myc Tags followed by two stop codons, was used as a template in the PCR reaction. The reverse primer

consists of a template binding region and a tail comprising the SV40 poly(A) sequence with an artificial *NotI* restriction site.
(v) The 410 bp fragment (iv) contained the *NotI* restriction site in 3` to 5` direction. So a new PCR product was amplified (using primer cMyc-F1 and SV40PolyA-NotI-R2), cloned into pGEM-T Easy and sequenced.
(vi) Cloning strategy was changed. Two glycine residues were introduced upstream of the c-myc Tags as a spacer to ensure proper peptide folding. A PCR product was amplified using 442-BamHI-F1 and SV40PolyA-NotI-R2 primer, cloned into pGEM-T Easy and sequenced.
(vii) The sequenced PCR fragment was cloned into *BamHI* and *NotI* restriction sites of plasmid 442-Lis1 (iii) and sequenced again.

3.4.3.1 Generation and breeding of transgenic PGK2-Lis1-c-myc Tag mice

The PGK2 construct was prepared according to 2.2.15.1 followed by two pronuclear microinjections, which were performed by "Transgenic Service" of Max-Planck-Institute for Experimental Medicine, Göttingen. Tail biopsies of F0 offspring were genotyped with the construct specific primers RT-PGK2-Prom-F99 and RT-PGK2-Prom-R99 (depicted in figure 3.42, left primer pair). To exclude contamination in the genotyping process, a freshly cut tail biopsy of wild type mice was processed in all genotypings of transgenic lines. Three mice of F0 offspring were genotyped positive for the transgenic integration. These founders (#3, #5 and #9, all females) were further bred with FVB wild type males and F1 offsprings were genotyped and used for expression analysis of the *Lis1* transgene. All founders were transmitting the transgenic construct to their offspring.

3.4.3.2 Expression analysis of transgenic PGK2-Lis1-c-myc Tag mice

3.4.3.2.1 Expression of the fusion transcript in PGK2-Lis1-c-myc Tag mice by RT-PCR

To analyse the expression of *Lis1* fusion transcript in transgenic F1 animals of PGK2-Lis1-c-myc Tag lines at the RNA level, total RNA was isolated from testes of 6 weeks old transgenic mice and FVB and NMRI wild type controls, followed by DNAse treatment. By RT-PCR analysis (Fig. 3.43), the fusion transcript (amplified with two

different primer pairs; depicted in figure 3.42) could be detected in testes of all transgenic lines, while in testes of wild type mice no transcript was found. Integrity of the RNA used for RT-PCR was proven by amplification of the *HPRT* transcript.

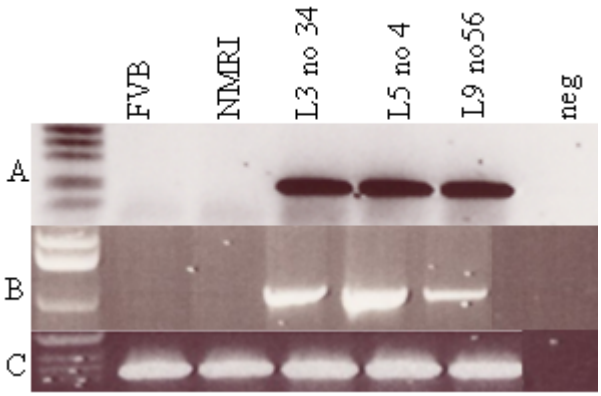

Figure 3.43: RT-PCR expression analysis of *Lis1* fusion transcript in testes of PGK2-Lis1-c-myc Tag lines (L3, L5 and L9) and FVB and NMRI wild type mice. Expression of the fusion transcript is restricted to testes of transgenic lines. In (A) primers RT-PGK2-Prom-F99 and RT-PGK2-Prom-R99 were used, and in (B) primers Lis1-Ex7-F1 and 442-XbaI-R1. Integrity of the RNA used for RT-PCR was proven by amplification of the *HPRT* transcript (C).

3.4.3.2.2 Expression of the fusion transcript in PGK2-Lis1-c-myc Tag mice by Northern blot analysis

To validate the expression of the *Lis1-c-myc Tag* fusion transcripts in transgenic testis and brain of PGK2-Lis1-c-myc Tag lines, Northern blot analysis was performed using a ^{32}P-labelled c-myc Tag specific probe. For the generation of the probe the PGK2 construct was digested with *BamHI* and *XhoI* to release a 307 bp fragment, which contained the six c-myc Tags. The probe detected the expected 1.8 kb fragment. The expression was restricted to testis of the transgenic lines, while testis of wild type mice and brain of transgenic mice showed no expression (Fig. 3.44 A). Clearly evident was the stronger expression in the L5 male compared to the L3 male. Integrity of RNA was checked by rehybridisation of the membrane with a β-actin probe (Fig. 3.44 B).

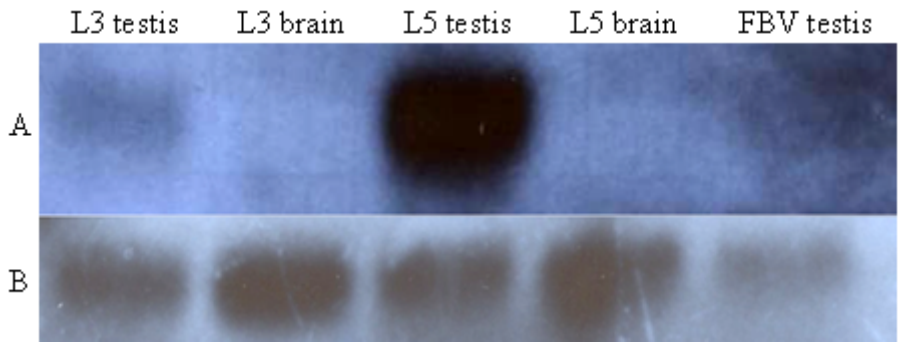

Figure 3.44: Northern blot analysis of *Lis1 c-myc Tag* specific transcript. Expression of the *Lis1 c-myc Tag* transcript is restricted to testes of transgenic lines, as no expression in testes of wild type control and brain of transgenic animals was detected in 6 weeks old mice. Testis of PGK2-Lis1-c-myc- Tag males showed clear expression of the transcript at the predicted size of 1.8 kb (A). Integrity of RNA was checked by rehybridisation of the membrane with a β-actin probe (B).

3.4.3.2.3 Expression of LIS1 in PGK2-Lis1-c-myc Tag mice by Western blot analysis

To evaluate the expression of LIS1-c-myc Tag fusion protein in tissues of transgenic mice, total protein extracts from testes of wild type and $L39^{GT/GT}$ controls and tissues of mutant animals were analysed by Western blot (Fig. 3.45). Monoclonal LIS1 antibody (Sigma) could detect the 45 kDa protein in all testicular lysates analysed (Fig. 3.45 A), while the LIS1-c-myc Tag fusion protein was detectable only in testes of L3 animal. Monoclonal c-myc Tag antibody (Milipore) detected only the 59 kDA LIS1-c-myc Tag fusion protein in testicular lysate of L3. As the monoclonal c-myc Tag antibody was working well in Western blot, it was used to further analyse tissue-specificity of the fusion protein in the different transgenic lines. In L3 (Fig. 3.45 B), L5 (Fig. 3.45 C) and L9 (Fig. 3.45 D) the fusion protein was detectable only in testes. As a positive control testicular protein of L3 animal (which was used in Western blot analysis in figure 3.45 A) was used, while protein of wild type testes served as a negative control. 50 kDa α-tubulin protein was used as a control for protein loading (Fig. 3.45 B-D). Transgenic mice of all three lines overexpress LIS1 protein in a testis-specific manner.

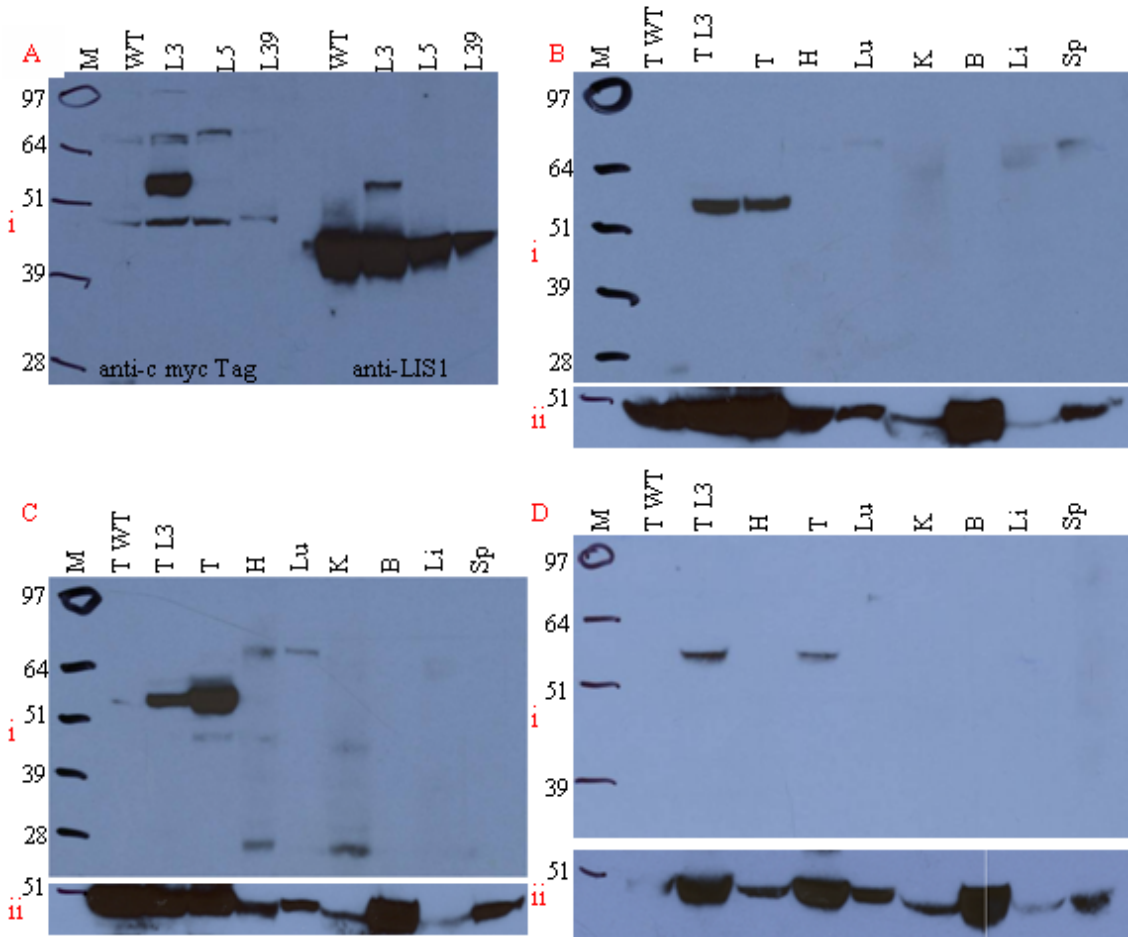

Figure 3.45: Expression of LIS1-c-myc-Tag fusion protein in tissues of adult (36 d old) PGK2-Lis1-c-myc-Tag mice and in testes of wild type (WT) and L39$^{GT/GT}$ (L39) mice. Monoclonal anti-LIS1 (Sigma) recognises the 45 kDa protein in testicular lysates of all animals studied, while the 59 kDa fusion protein is only detectable in testes of L3 (A i, right side). Monoclonal anti-c-myc Tag antibody (Milipore) verified this result (A i, left side). Testis specific expression of the fusion protein was shown for L3 (B i), for L5 (C i) and for L9 (D i) with monoclonal c-myc Tag antibody. The tissues analysed were testis (T), heart (H), lung (Lu), kidney (K), brain (B), liver (Li) and spleen (Sp). 50 kDa α-tubulin protein is shown as a control for protein loading in ii (B, C and D) (M: Prestained protein marker, Invitrogen).

3.4.3.3 Immunohistochemistry of testis sections of PGK2-Lis1-c-myc Tag males

3.4.3.3.1 LIS1 staining

(performed by B. Jung)

To investigate the distribution of LIS1 protein in testes of transgenic animals, paraffin embedded testes of a 4 months old L3 male were cross-sectioned to a thickness of 5-7 µm and then immunostained with monoclonal anti-LIS1 antibody (Sigma) in a 1:50

dilution and superimposed by DAPI staining. This experiment was performed by Bomi Jung (TU Braunschweig). Specific immunostaining was observed in all testicular cells (Fig. 3.46).

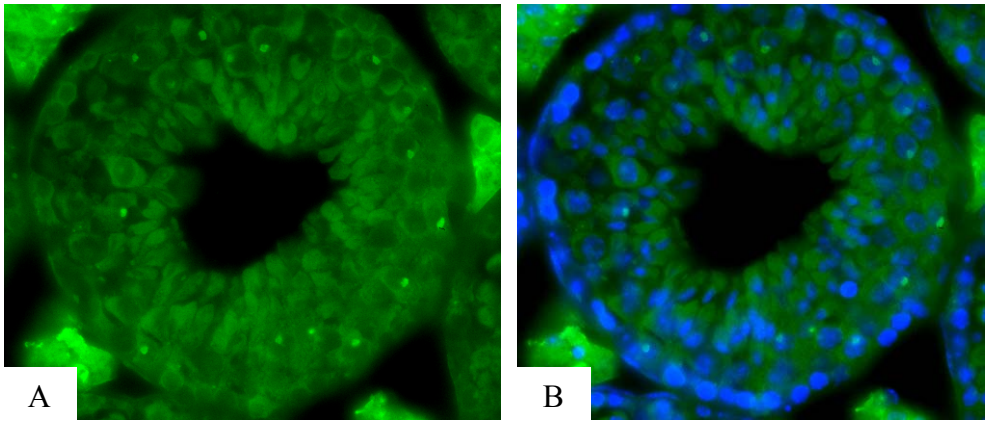

Figure 3.46: Expression of LIS1 in testes of transgenic L9 mice using immunohistochemistry. Specific expression of LIS1 protein was detected in all testicular cells. Cy-2 fluorescence is shown in (A) and an image overlay of DAPI and Cy-2 fluorescences is shown in (B). This experiment was performed by B. Jung (Braunschweig).

3.4.3.3.2 c-myc Tag staining

To analyse the expression pattern of the LIS1-c-myc Tag fusion protein in tubuli seminiferi of transgenic animals, adult mice of L3 and L9 were perfused with PFA according to 2.2.13.3 and 5-6 µm thick cryosections of testes were immunostained with monoclonal c-myc Tag antibody in a 1:200 dilution. Specific immunostaining was observed in meiotic and postmeiotic cells of L3 and L9 males (Fig. 3.47). The staining intensity in testes of L9 mice (Fig. 3.47 A, B) was stronger than in testes of L3 mice (Fig. 3.47 C, D). In particular haploid elongated spermatids displayed strong staining around the nucleus in tubules of L9 mice (Fig. 3.47 B). This expression pattern differed in L3 males, were staining was less intense and distributed to meiotic and postmeiotic germ cells (Fig. 3.47 C, D). The PGK2 construct directs testis specific overexpression of LIS1 in meiotic and postmeiotic cells.

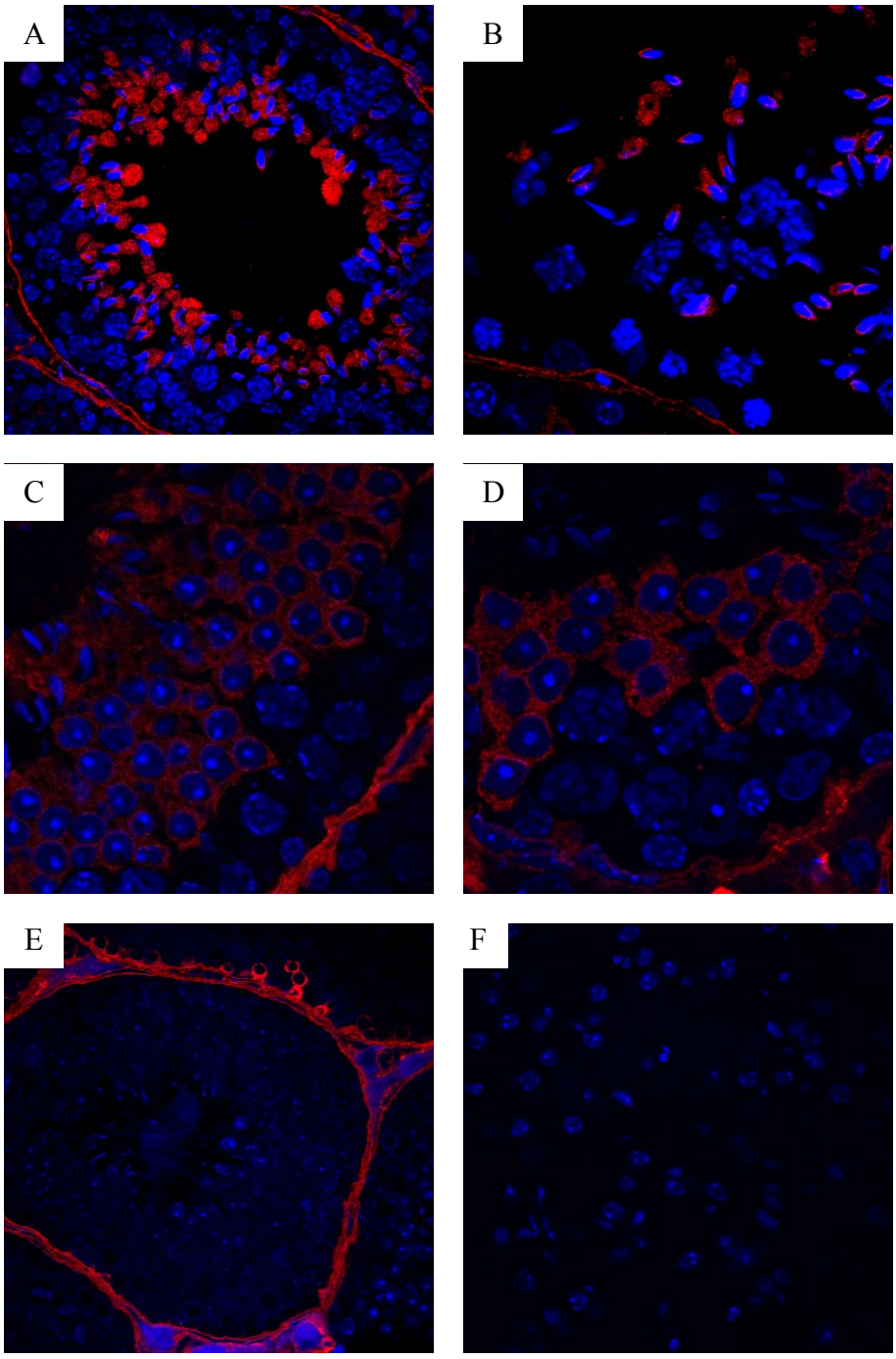

Figure 3.47: Expression of LIS1-c-myc Tag fusion protein in testis sections of transgenic PGK2-Lis1-c-myc Tag mice by using immunohistochemistry. The fusion protein was detected by a monoclonal c-myc Tag antibody (1:200). The expression in L9 (A, B) is restricted to some meiotic and postmeiotic cells, with a particular strong staining in haploid spermatids, which display a specific staining pattern around the nucleus (B). LIS1-c-myc-Tag protein in L3 (C, D) is more widely distributed, with most meiotic and postmeiotic germ cells being positively stained. 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 (E). Brain sections of L9 mice did not display specific staining for c-myc Tag antibody (F). All pictures are dual image overlays of Cy3 and DAPI fluorescences.

3.4.3.4 Histological analysis of testis sections of PGK2-Lis1-c-myc Tag mice

To evaluate the influence of meiotic and postmeiotic overexpression of *Lis1* on testis morphology, adult PGK2-Lis1-c-myc Tag mice of line 3 (L3) and line 5 (L5) were fixed in PFA, sliced into 5 µm thick sections and stained with hematoxilin and eosin (H&E) (Fig. 3.48).

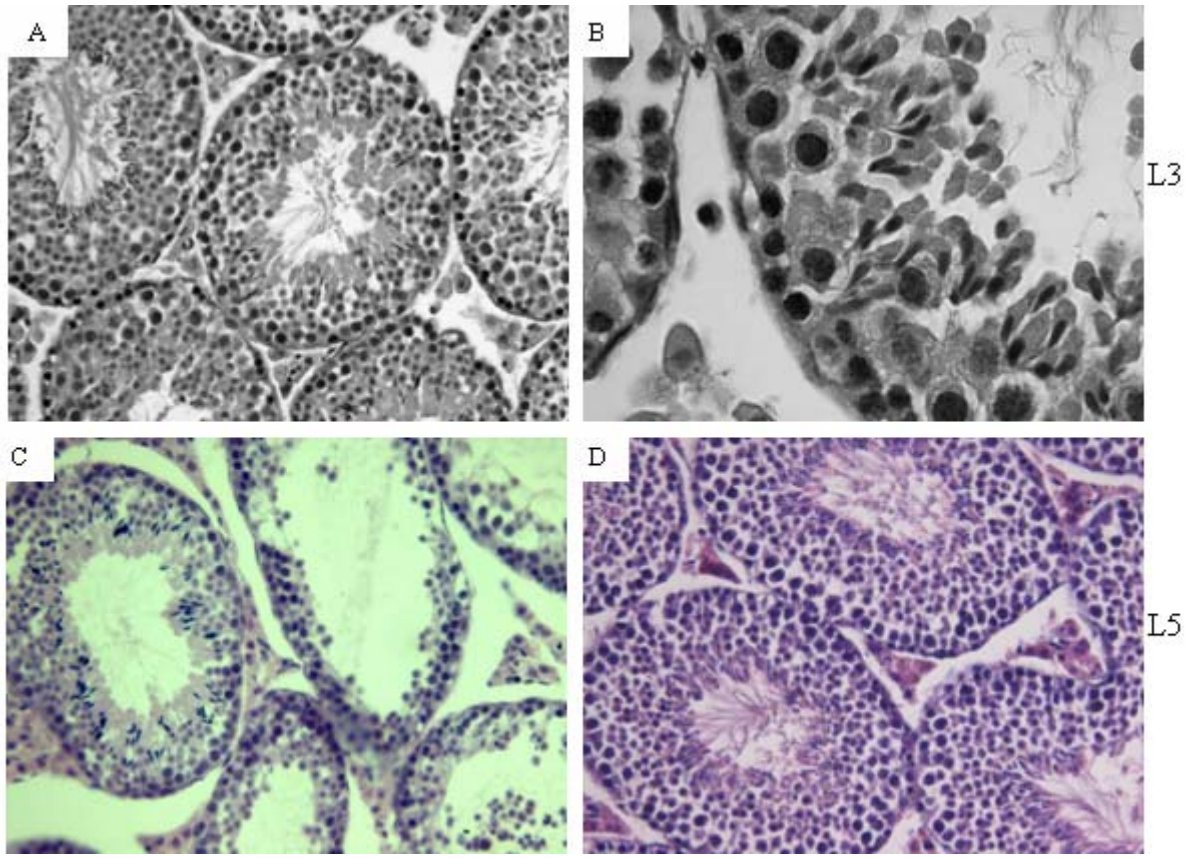

Figure 3.48: H&E staining of testis sections of adult PGK2-Lis1-c-myc Tag mice. Sections through testes of L3 no 33 (A, B) and L5 no 54A (D) mice revealed robust spermatogenesis, while testis sections of L5 no 4 mice demonstrated severe depletion of spermatogenesis in most tubules (C) (original magnification A, C, D x200; B, x600).

Testis sections of PGK2-Lis1-c-myc Tag mice of L3 (Fig. 3.48 A, B) and L5 (no 54A, Fig. 3.48 D) displayed no morphological abnormalities. Full spermatogenesis could be observed in these males. In contrast to this is the severe depletion of spermatogenesis in most tubules of L5 no 4 mice (Fig. 3.48 C). About 80% of tubuli seminiferi displayed no haploid cells and only few meiotic cells. Testicular protein extract of this mouse was used in Western blot analysis to detect expression of Lis-c-myc Tag fusion protein (Fig. 3.45 A). No fusion protein was detected, which correlates with the observed lack of

meiotic and postmeiotic cells in this animal. To further analyse fertility of L5 animals, two positive F1 males were crossed with wild type females. Both females became pregnant and about half of the offspring was transgenic. Moreover, expression analysis of a second L5 animal revealed testis specific expression of the fusion protein (Fig. 3.45 C) and the morphological analysis of a third male displayed no abnormalities in spermatogenesis (Fig. 3.48 D). Thus, the observed phenotype of L5 no 4 male is probably not on account of the expression of the transgenic construct. All males used for further matings were fertile with normal litter size. Histological analysis of L9 males revealed no abnormalities in spermatogenesis as well as analysis of epididymes of transgenic lines (data not shown).

3.4.3.5 Generation of homozygous transgenic PGK2-Lis1-c-myc Tag males

To analyse a dose-dependent effect on *Lis1* overexpression on spermatogenesis of homozygous transgenic mice, PGK2-Lis1c-myc Tag^{Tpos} mice of F1 generation were mated and offspring were genotyped. Positive males (either heterozygous or homozygous) were mated with wild type females and embryos (13.5 dpc) were genotyped. All offspring of a homozygous male were heterozygous (positive for the transgenic construct), while only about 50% of the offspring of a heterozygous male were positive. Two to four males (F2 generation) of each line were testbred and one homozygous male per line analysed.
Homozygous males of all lines were fertile (as all wild type mice got pregnant). Histological analysis of testes and epididymes sections displayed no abnormalities (data not shown).

3.4.3.6 Determination of integration sites in transgenic PGK2-Lis1-c-myc Tag lines

In transgenic animals created by pronuclear microinjection, the site of integration of the transgene within the genome is a random event. Thus, when multiple embryos have been injected with the same construct, the integration site will be different in each founder animal. In the case of pronuclear injection, there is typically one insertion site, although multiple transgene copies are often found in a tandem array at that integration site (Lo, 1987).

To identify the number of integration sites in the transgenic lines the transgenic ratio of offspring was determined for all lines. In case of one integration site, the transmission of the transgene should be in Mendelian fashion, which means, that about 50% of offspring (of F1 generation) are heterozygous (positive) for the transgene. Figure 3.49 summarizes the genotype and sex distribution in F1 offspring of all transgenic PGK-Lis-c-myc Tag lines. Crossings of founders with wild type males did not show deviation from Mendelian ratio, as it was shown by chi^2 test. Moreover, no differences was seen in sex distribution between the two groups (Chi^2-test, L3 p= 0.92, L5 p=0.75 and L9 p= 0.0843).

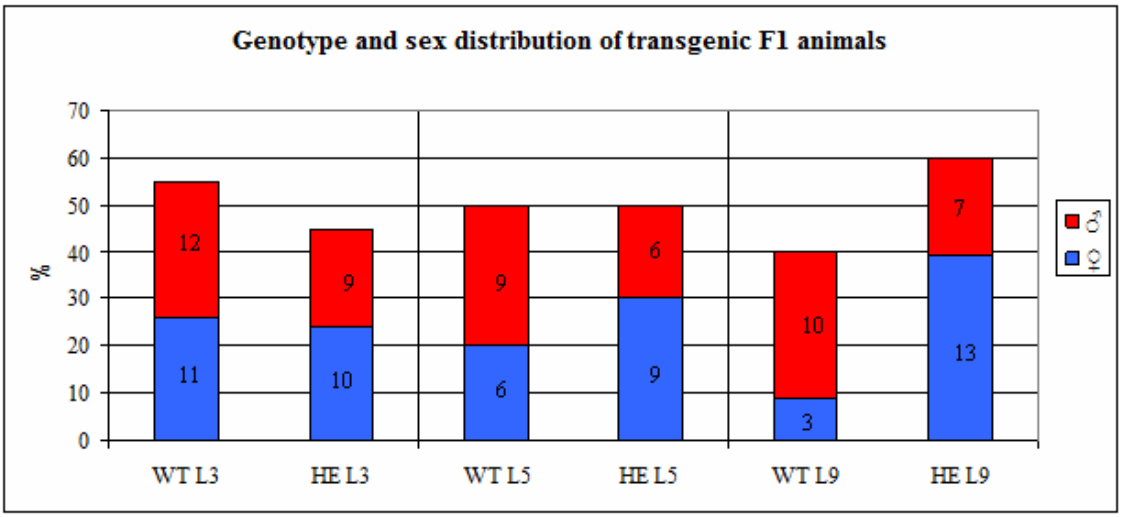

Figure 3.49: Genotype and sex distribution of transgenic mice in founder x FVB wild type breedings. Breeding of founder 3, founder 5 and founder 9 with FVB wild type males did not show deviation from Mendelian ratio. 42 animals from 4 litters were genotyped for L3, 30 animals from 3 litters were tested for L5 and 33 animals from 3 litters were genotyped for L9. Abbreviations are: HE, heterozygous; WT, wild type. Numbers in columns display number of animals for each genotype and sex.

To further confirm this data, integration site number was determined by qPCR for L3 and L5. *Pelota* gene was used for the normalisation of each DNA sample and the single copy gene *acrosin* was used for calibration of the normalised samples. The results are shown in figure 3.50.

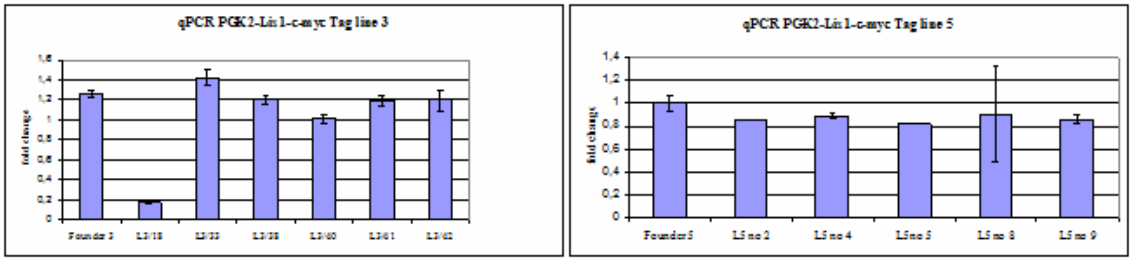

Figure 3.50: Determination of integration sites in PGK2-Lis-c-myc Tag mice of line 3 and line 5. *Pelota* gene was used for the normalisation of each DNA sample and the single copy gene *acrosin* was used for calibration of the normalised samples. Founder and F1 offspring had comparable fold change values (relative to *acrosin* gene) in both lines. This result confirms the presence of just one integration site of the transgenic construct.

Founder and F1 offspring of both analysed lines displayed similar fold change values relative to *acrosin* gene. In case of one integration site in the founder animal, all heterozygous offspring inherit the same amount of transgenic DNA, while in case of multiple integration sites, segregation of the transgene occurs and heterozygous offspring display different fold changes. PGK2-Lis1-c-myc-Tag mice of line 3 and line 5 have just one integration site of the transgene.

3.4.4 Analysis of "rescued" L39$^{GT/GT}$/PGK2-Lis1-c-myc Tag^{Tpos} males

3.4.4.1 Breeding strategy to generate L39$^{GT/GT}$/PGK2-Lis1-c-myc Tag^{Tpos} males

To generate males, that are homozygous for the gene trap vector integration and positive (heterozygous or homozygous) for the transgene PGK2-Lis1-c-myc Tag (PGK2-Lis1), the following breeding strategy was used:

(iv) F0: ♀ L39$^{GT/GT}$ x ♂ PGK2-Lis1$^{T/-}$
(v) F1: ♀ L39$^{GT/-}$/ PGK2-Lis1$^{T/-}$ x ♂ L39$^{GT/-}$/ PGK2-Lis1$^{T/-}$
(vi) F2: ♂ L39$^{GT/GT}$/PGK2-Lis1$^{T/-}$ & ♂ L39$^{GT/GT}$/PGK2-Lis1$^{T/T}$

Homozygous gene trap females are fertile and could be used for breeding with PGK2-Lis1$^{T/-}$ males. All resulting offspring were heterozygous for the gene trap integration (L39$^{GT/-}$), and about 50% of the animals were PGK2-Lis1$^{T/-}$. L39$^{GT/GT}$/PGK2-Lis1$^{T/-}$ siblings of F1 generation were bred to generate L39$^{GT/GT}$/PGK2^{Tpos} (heterozygous or homozygous) males. According to Medelian ratio the genotype of 3/16 of offspring was L39$^{GT/GT}$/PGK2-Lis1^{Tpos} (1/4 GT/GT x 3/4 PGK2-Lis1 positive (HE or HO)). About 50% of offspring were males, thus about 10% (3/32) of F2 generation were L39$^{GT/GT}$/PGK2-Lis1^{Tpos} males. PGK2-Lis1-c-myc-Tag mice of line3 (L3) and line 9 (L9) were used for the generation of L39$^{GT/GT}$/ PGK2-Lis1-c-myc Tag^{Tpos} males. These animals are from now on referred to as L39/L3 and L39/L9 animals, respectively.

3.4.4.2 Fertility test of L39$^{GT/GT}$/PGK2-Lis1-c-myc Tag^{Tpos} males

Fertility of two L39$^{GT/GT}$/L3^{Tpos} males was tested by mating them with wild type females. All matings were performed for several weeks, but no litter was obtained.
Eight L39$^{GT/GT}$/L3^{Tpos} males were then mated with wild type females. The uteri of those mice which were positive for vaginal plug were dissected in IVF medium, flushed out and sperm number was determined. No sperms were found in uterus of females mated with "rescued" males. The experiment was repeated two times for each male. L39$^{GT/-}$/L3^{Tpos} animals were mated with wild type females as controls. Sperm count revealed the expected number of sperm in uterus ($5.8 \pm 2.1 \times 10^6$, N=3) of females mated with L39$^{GT/-}$/L3^{Tpos} males. As observed in L39$^{GT/GT}$, the testes sizes of "rescued"

$L39^{GT/GT}/L3^{Tpos}$ males were markedly reduced in comparison with heterozygous siblings (data not shown).
One $L39^{GT/GT}/L9^{Tpos}$ male was mated with wild type females, but no litter was obtained. Uteri of females which were mated with $L39^{GT/GT}/L9^{Tpos}$ male contained no sperms, whereas in uterus of a female which was mated with $L39^{GT/GT}/L9^{Tpos}$ male $3.2x10^7$ sperms were found.

3.4.4.3 Expression analysis of $L39^{GT/GT}$/PGK2-Lis1-c-myc Tag^{Tpos} mice

To validate the expression of LIS1-c-myc Tag fusion protein in testes of "rescued" mice, total protein extracts from testes of L39/L3 and wild type and $L39^{GT/GT}$ control animals were analysed by Western blot (Fig. 3.51). Monoclonal LIS1 antibody (Sigma) could detect the 45 kDa protein in all testicular lysates analysed, while the LIS1-c-myc Tag fusion protein was detectable only in testes of L3 animals and L39/L3 no 13 ($L39^{GT/GT}/L3^{Tpos}$). Monoclonal c-myc Tag antibody (Milipore) detects the 59 kDA LIS1-c-myc Tag fusion protein in testicular lysate of L3 and all L39/L3 mice. The expression of fusion protein in L3 mice was stronger than in L39/L3 mice. No difference in expression level was detected in homozygous (no 4A) and heterozygous (no 7A) L3 animals. As expected, the fusion protein was not expressed in wild type and L39/CD-1 controls.

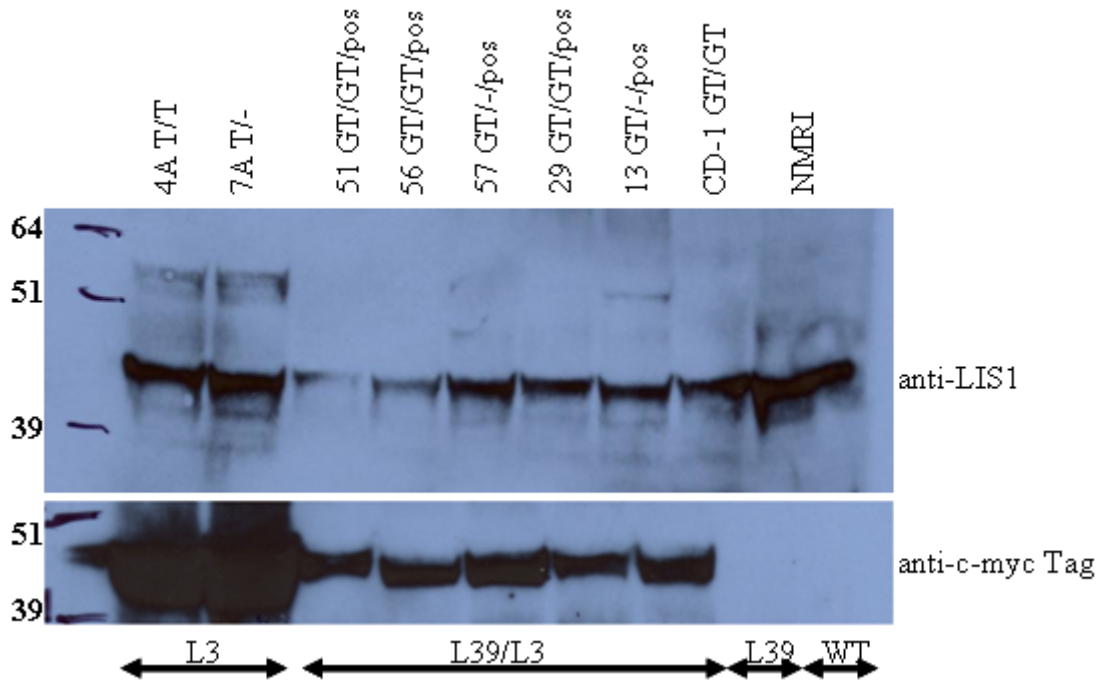

Figure 3.51: Expression of LIS1-c-myc-Tag fusion protein in testes of adult L39/L3, L39/CD-1$^{GT/GT}$ and wild type (NMRI) mice. Monoclonal anti-LIS1 (Sigma) recognises the 45 kDa protein in testicular lysates of all animals studied, while the 59 kDa fusion protein is only detectable in testes of L3 (no 4A$^{T/T}$ and no 7A$^{T/-}$) and L39/L3 no 13 (L39$^{GT/GT}$/L3^{Tpos}) animals. Monoclonal anti-c-myc Tag antibody (Milipore) detects the fusion protein in testes of L3 and all L39/L3 mice, while no expression could be detected in wild type and L39/CD-1$^{GT/GT}$ animals.

Western blot analysis could confirm the overexpression of LIS1 in testes of L39$^{GT/GT}$/PGK2-Lis-c-myc Tag mice. The expression level of LIS1-c-myc-Tag fusion protein is lower than in transgenic PGK2-Lis1-c-myc Tag mice.

3.4.4.4 Histological analysis of testis sections of L39$^{GT/GT}$/PGK2-Lis1-c-myc Tag^{Tpos} males

To evaluate the influence of meiotic and postmeiotic overexpression of *Lis1* on testis morphology of gene trap mice testes and epididymes of L39$^{GT/GT}$/L3^{Tpos} and heterozygous L39$^{GT/-}$/L3^{Tpos} controls were fixed in Bouin solution, sliced into 5 µm thick sections and stained with hematoxilin and eosin (H&E) (Fig. 3.52).

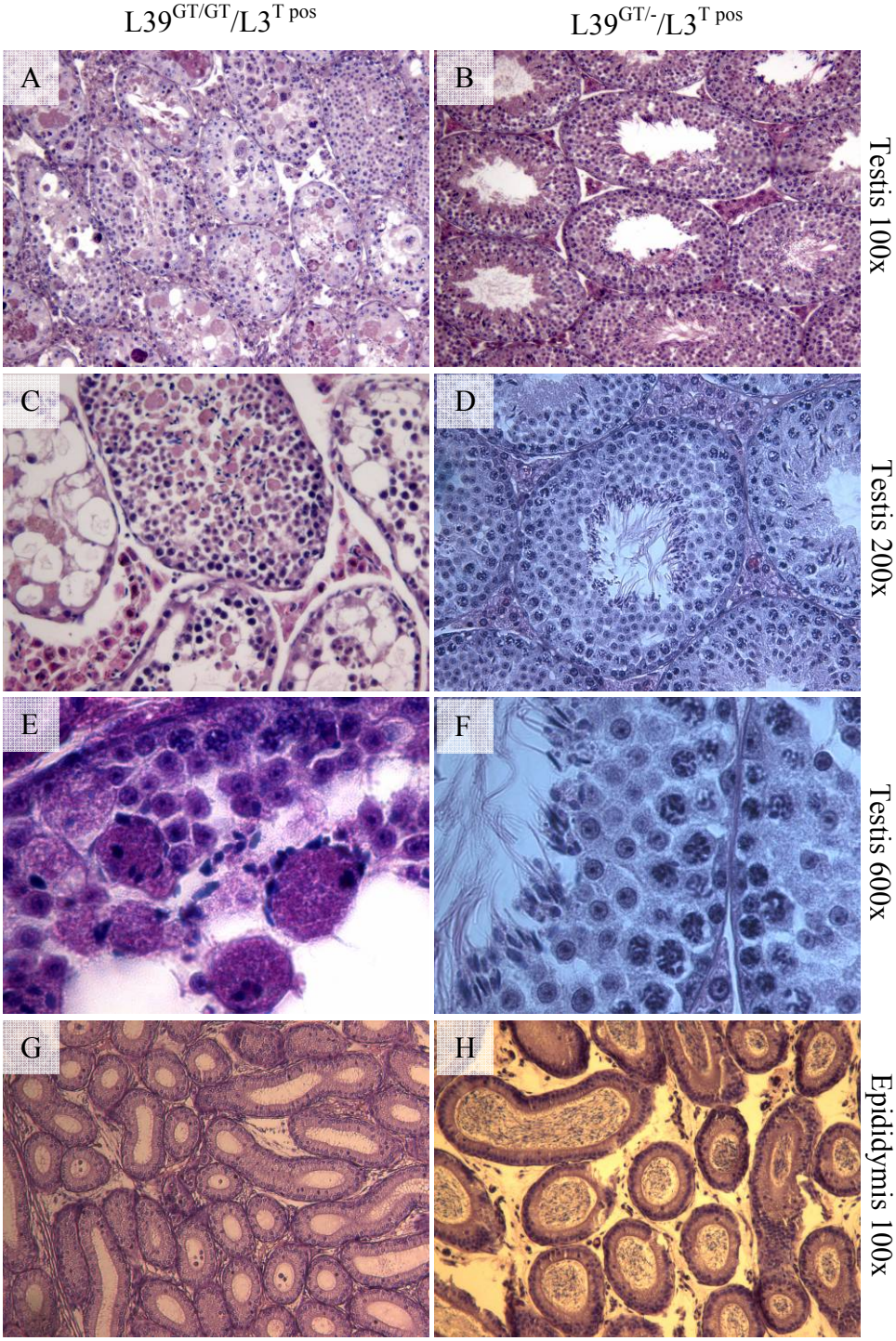

Figure 3.52: H&E staining of testes and epididymes sections of adult L39/PGK2-Lis1-c-myc Tag mice. Sections through testes of heterozygous L39/L3 mice revealed robust spermatogenesis (B, D, F), while testis sections of "rescued" mice demonstrated severe depletion of spermatogenesis in most tubules (A, C, D). Sections through epididymes revealed a high number of sperms in heterozygous males (H), whereas most tubules of epididymes of "rescued" males were empty and some were filled with some premature released germ cells, but no sperms (G) (original magnification A, B, G, H x100; C, D x200; E, F x600).

The histological analysis of testes of "rescued" L39/L3 mice revealed the same extensive degeneration of a large fraction of seminiferous tubules as observed in $L39^{GT/GT}$ males of all genetic backgrounds (Fig. 3.1; 3.19 to 3.22 and 3.40 D). Like in the gene trap line, mutant testes exhibit early germ cells as well as Sertoli and Leydig cells, but the number of late meiotic (i.e. late pachytene and diplotene spermatocytes) and postmeiotic (i.e. spermatids and spermatozoa) germ cells is markedly reduced. This phenotype was confirmed in 35 d and 45 d old $L39^{GT/GT}/L3^{Tpos}$ mice (data not shown). In marked contrast to this are heterozygous males ($L39^{GT/-}/L3^{Tpos}$), which exhibit full spermatogenesis with no obvious morphological differences from wild type males. Epididymal sections of "rescued" mice were mainly empty, except for few premature released germ cells in some tubules (Fig. 3.52 G), while epididymal sections of heterozygous control mice were packed with sperms (Fig. 3.52 H).
The overexpression of *Lis1* in meiotic and postmeiotic germ cells has no visible influence on testis morphology of homozygous gene trap mice.

3.4.4.5 Immunohistochemistry of testis sections of $L39^{GT/GT}$/PGK2-Lis1-c-myc Tag^{Tpos} males

3.4.4.5.1 LIS1 staining
(performed by B. Jung)

To investigate the distribution of LIS1 protein in testis of L39/L3 mice, adult paraffin embedded heterozygous and homozygous L39/L3 testes were cross-sectioned to a thickness of 5-7 µm. These testis sections were then immunostained with monoclonal anti-LIS1 antibody (Sigma) in a 1:50 dilution and superimposed by DAPI staining. This experiment was performed by Bomi Jung (TU Braunschweig). Specific immunostaining was observed in cytoplasm of all testicular cells (Fig. 3.53).

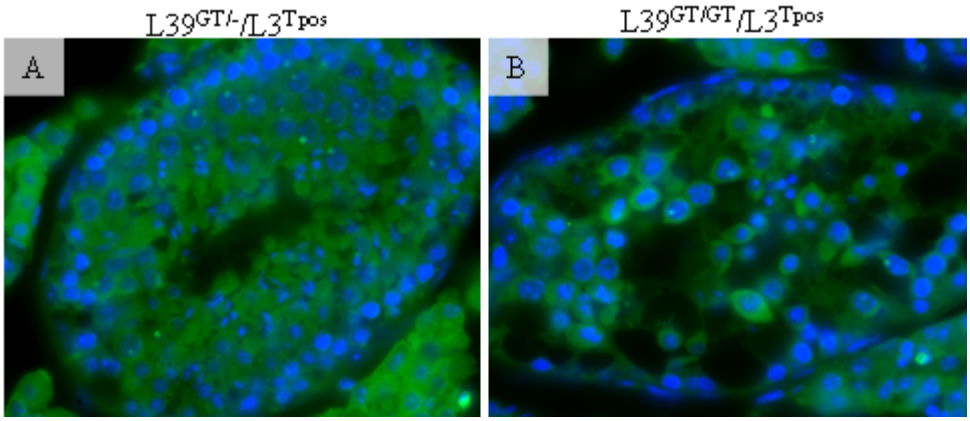

Figure 3.53: Expression analysis of LIS1 in testis of adult L39/L3 mice using immunohistochemical analysis. Staining was observed in all testicular cells. Tubules of heterozygous mice ($L39^{GT/-}$ /$L3^{Tpos}$, A) displayed full spermatogenesis, while tubules of "rescued" mice ($L39^{GT/GT}$ /$L3^{Tpos}$, B) were degenerated. All pictures are dual image overlays of Cy2 and DAPI fluorescences. This experiment was performed by B. Jung (TU Braunschweig).

3.4.4.5.2 c-myc Tag staining

To analyse the expression pattern of the LIS1-c-myc Tag fusion protein in tubuli seminiferi of L39/L3 animals, adult mice of L39/L3 were perfused with PFA according to 2.2.13.3 and 5-7 µm thick cryosections of testis were immunostained with monoclonal c-myc Tag antibody in a 1:200 dilution. Specific immunostaining was observed in meiotic and postmeiotic cells of L39/L3 males (Fig. 3.54). Staining was not as intense as in PGK2-Lis1-c-myc Tag L3 mice (Fig. 3.47 C, D), but demonstrated the same pattern (Fig. 3.54 A, B). In "rescued" L39/L3 mice, staining is visible mostly in haploid cells with clear cytoplasmic staining (Fig. 3.54 C). In the negative control no testicular cells were stained (Fig. 3.54 D). Brain sections of immunostained L39/L3 mice did not show any specific signal (data not shown).
Immunohistochemistry analysis could confirm the meiotic and postmeiotic overexpression of LIS1 in testes of L39/L3 mice.

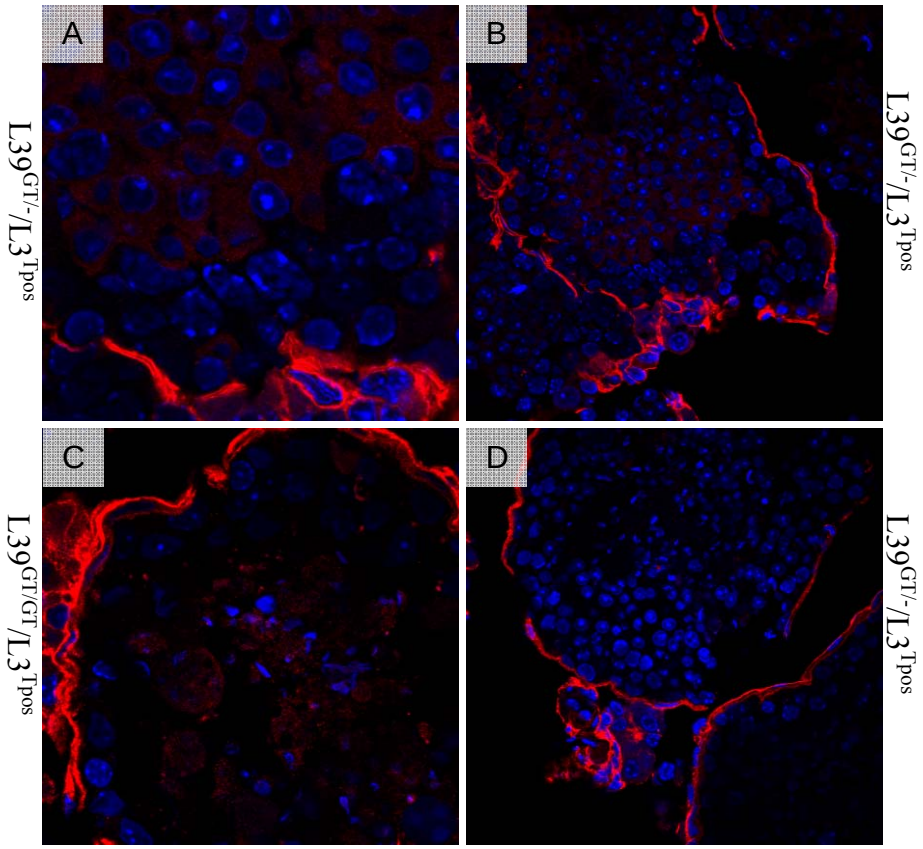

Figure 3.54: Expression of LIS1-c-myc Tag fusion protein in testis sections of L39/L3 mice by using immunohistochemistry. The fusion protein was detected by monoclonal c-myc Tag antibody (1:200). The expression in $L39^{GT/-}/L3^{Tpos}$ mice (A, B) is restricted to meiotic and postmeiotic cells. LIS1-c-myc Tag protein in $L39^{GT/GT}/L3^{Tpos}$ mice (C) was detected mainly in haploid cells with a clear cytoplasmic location. 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 (D). All pictures are dual image overlays of Cy3 and DAPI fluorescences.

3.4.4.6 Sperm analysis of $L39^{GT/GT}$/PGK2-Lis1-c-myc Tag^{Tpos} males

To confirm infertility of "rescued" $L39^{GT/GT}$/PGK2-Lis1-c-myc Tag^{Tpos} males on sperm level, total sperm count in caudae epididymes of 2.5 months old $L39^{GT/GT}$/PGK2-Lis1-c-myc Tag^{Tpos} and heterozygous $L39^{GT/-}$/PGK2-Lis1-c-myc Tag^{Tpos} control males was determined. Three homozygous and one heterozygous male were used for analysis. 5.03×10^7 sperms were found in heterozygous control mice, while 1.2×10^4 sperms were found in one "rescued" male and few sperms were found in the other two "rescued" males via counting in a Neubauer counting chamber. To evaluate sperm morphology, 20 µl of epidiymal solution was transferred to slides, fixed in

formaldehyde and covered with DAPI-containing mounting medium. Slides were then analysed for sperms under the fluorescence microscope BX60 (Olympus).

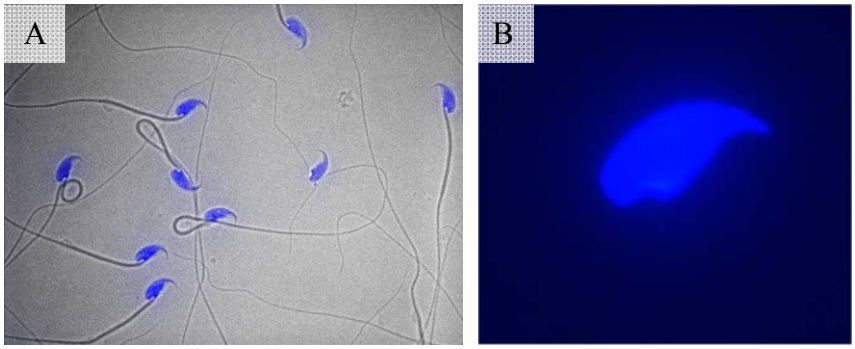

Figure 3.55: DAPI staining of sperms isolated from caudae epididymes of L39/L3 mice. Epididymes of "rescued" mice (B) and heterozygous control mice (A) were dissected and smear was fixed and stained with DAPI. A low number of spermatozoa was found in $L39^{GT/GT}$/PGK2-$Lis1^{Tpos}$ males in contrast to a high number of sperm cells in the control animal. Spermatozoa of all genotypes looked morphological normal (original magnification x600).

The existence of spermatozoa in caudae epididymes of "rescued" L39/L3 males was shown (Fig. 3.55). In comparison to the heterozygous control the number of sperms was significantly reduced, as expected. Nevertheless the morphology of all sperms found looked normal, as can be seen on the high magnification image of $L39^{GT/GT}/L3^{Tpos}$ male no 97 (Fig. 3.55 B).

3.4.4.7 Detailed comparison of spermatogenesis defects in $L39^{GT/GT}$ and $L39^{GT/GT}$/PGK2-Lis1-c-myc Tag^{Tpos} males

To compare spermatogenesis defects in "rescued" L39/L3 mice and $L39^{GT/GT}$ mice in a more quantitative manner, testis cross-sections of NMRI wild type, $L39^{GT/GT}$, $L39^{GT/-}/L3^{Tpos}$ and $L39^{GT/GT}/L3^{Tpos}$ animals were analysed. Testes of three 46 d old animals per genotype were fixed in Bouins solution, cut in half and embedded in paraffin. Five 3 µm cuttings were transferred to one slide, 7 cuttings were discarded, the following 5 cuttings were transferred to a slide and so on. 10 slides were prepared for each genotype. Three slides (no 1, no 6 and no 10) per animal were stained with H&E and analysed. Two cross-sections per slide were viewed with a fluorescence microscope and two images per cross-section were collected at 100x 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 analysed. First, total numbers of tubules were determined and then tubules were assigned 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 vacuolisation
- few haploid cells
- premature release of immature germ cells

An example for each of the four classes is shown in figure 3.56.

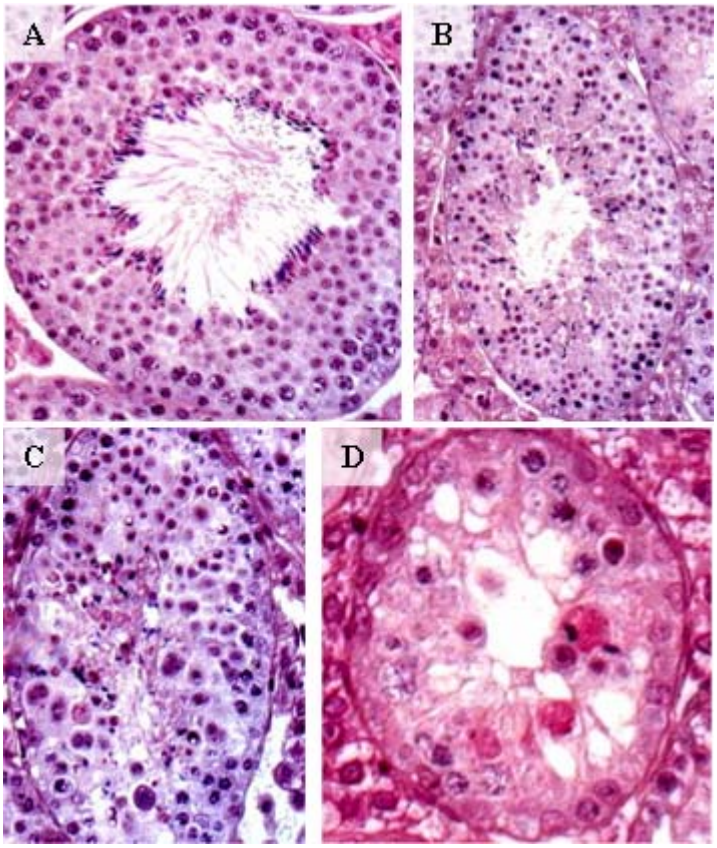

Figure 3.56: H&E staining of testis sections of transgenic animals. **A** displays a tubule with **normal spermatogenesis (class 1)**, **B** displays a tubule with **mild hypospermatogenesis (class 2)**, **C** displays a tubule with **medium hypospermatogenesis (class 3)** and **D** displays a tubule with **severe hypospermatogenesis (class 4)** (original magnification x200).

Table 3.2 gives an overview of the distribution of spermatogenesis defects in the analysed genotypes.

Table 3.2: Distribution of spermatogenesis defects in NMRI wild type, $L39^{GT/-}/L3^{Tpos}$, $L39^{GT/GT}$ and $L39^{GT/GT}/L3^{Tpos}$ animals. Three animals per genotype were analysed. Overall number of counted tubules is stated (∑) and tubules of each class are expressed as percentage (±SD).

	∑	class 1	class 2	class 3	class 4
NMRI	314	86.4(±3.8)	13.2(±3.9)	0.4(±0.6)	
$L39^{GT/-}/L3^{Tpos}$	325	88.8(±3.0)	10.3(±1.7)	0.8(±1.4)	
$L39^{GT/GT}$	549			7.4(±8.2)	92.6(±8.2)
$L39^{GT/GT}/L3^{Tpos}$	473		2.8(±4.8)	14.1(±15.7)	83.1(±20.5)

Even though the percentage of class 4 tubules (severe hypospermatogenesis) in $L39^{GT/GT}$ gene trap mice was higher than in "rescued" $L39^{GT/GT}/L3^{Tpos}$ mice and

$L39^{GT/GT}$ males displayed no class 2 tubules (mild hypospermatogenesis), while 2.8% of $L39^{GT/GT}/L3^{Tpos}$ tubules displayed mild hypospermatogenesis, no statistically significant differences (Mann-Whitney-U-Test, p=0.27 for class 3 and 4 and p=0.31 for class 2) in spermatogenesis defects were found in these two lines (Fig. 3.57).

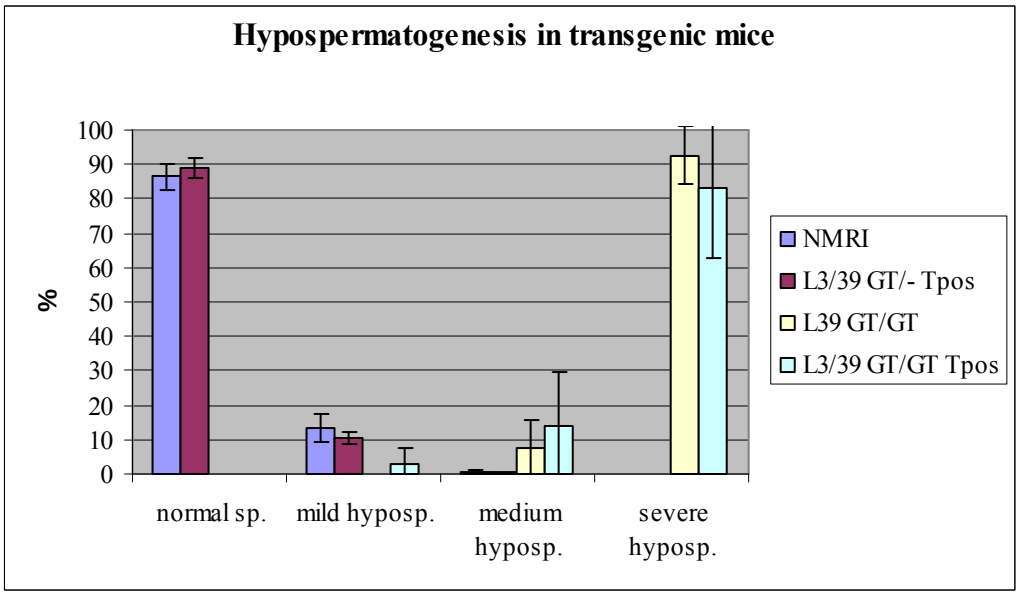

Figure 3.57: Analysis of spermatogenesis defects in NMRI wild type, $L39^{GT/GT}$, $L39^{GT/GT}/L3^{Tpos}$ and $L39^{GT/-}/L3^{Tpos}$ mice. Testis sections of three animals per genotype were analysed. No significant difference was found in NMRI and $L39^{GT/-}/L3^{Tpos}$ mice or $L39^{GT/GT}$ and $L39^{GT/GT}/L3^{Tpos}$ mice.

As expected no significant difference in spermatogenesis defects in NMRI wild type and $L39^{GT/-}/L3^{Tpos}$ was found.

The overexpression of *Lis1* in meiotic and postmeiotic cells in testes of gene trap line had no influence on spermatogenesis of these animals.

3.4.5 Analysis of transgenic hEF-1α-Lis1-c-myc Tag mice

It was previously shown, that a 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). To generate transgenic mice, which overexpress *Lis1* gene under control of the hEF-1α promoter, a construct was generated (from now on referred to as hEF-1α construct) that includes 1.2 kb of 5`-flanking region of *human elongation factor-1α* gene, followed by 1377 bp of *Lis1* cDNA and a 410 bp fragment of (c-myc Tag)$_6$-SV40 poly(A). A schematic drawing of the hEF-1α construct is depicted in figure 3.58.

Figure 3.58: Schematic drawing of the hEF-1α construct used to generate transgenic mice which overexpress *Lis1* gene under control of human elongation factor-1α promoter. A 410 bp fragment of (c-myc Tag)$_6$ (containing two stop codons) followed by SV40 poly(A) signal was cloned into *BamHI* and *NotI* restriction sites of vector clone 1031 (pEGFP-1 with 1.2 kb hEF-1α promoter), thereby replacing the *EGFP* gene. Next a 1377 bp fragment of *Lis1* cDNA (containing ATG codon) was cloned into *EcoRI* and *BamHI* restriction sites. The black arrows correspond to primers used for genotyping and RT-PCR analysis.

The detailed cloning strategy was as follows:

(i) The plasmid clone 1031 (containing a 1.2 kb fragment of hEF-1α promoter cloned into pEGFP vector (Clontech)) was sequenced and restriction sites and promoter sequences were confirmed.

(ii) The sequenced 410 bp fragment of (c-myc Tag)$_6$ (containing two stop codons) followed by SV40 poly(A) signal (amplification is described in 3.5.3.1 in detail) was cloned into *BamHI* and *NotI* restriction sites of vector clone 1031, thereby replacing the EGFP gene.

(iii) The sequenced 1377 bp *Lis1* cDNA fragment (amplification is described in 3.5.3.1 in detail) was cloned into *EcoRI* and *BamHI* restriction sites of plasmid 1031-c-myc Tag-SV40poly(A) (ii).

(iv) The resulting hEF-1α construct was sequenced again.

3.4.5.1 Generation and breeding of transgenic hEF-1α-Lis1-c-myc Tag mice

The hEF-1α construct was prepared according to 2.2.15.1 followed by two pronuclear microinjections, which were performed by "Transgenic Service" of Max-Planck-Institute for Experimental Medicine, Göttingen. Tail biopsies of F0 offspring were genotyped with the construct specific primers RT-hEF-Prom-F99 and RT-hEF-Prom-R99 (depicted in figure 3.58, left primer pair). To exclude contamination in the genotyping process, a freshly cut tail biopsy of wild type mice was processed in all genotypings of transgenic lines. Five mice of F0 offspring were genotyped positive for the transgenic integration. These founders (#1 (♂), #13 (♀), #15 (♂,) #19 (♀) and #27 (♂)) were further bred with FVB wild type animals and F1 offspring were genotyped and used for expression analysis of the *Lis1* transgene. All founders transmitted the transgenic construct to their offspring.

3.4.5.2 Expression analysis of transgenic hEF-1α-Lis1-c-myc Tag mice

3.4.5.2.1 Expression of the fusion transcript in hEF-1α-Lis1-c-myc Tag mice by RT-PCR

To analyse the expression of *Lis1* fusion transcript in transgenic F1 animals of PGK2-Lis1-c-myc Tag lines at the RNA level total RNA was isolated from tissues of 6 weeks old transgenic mice and FVB and NMRI wild type controls, followed by DNAse treatment. By RT-PCR analysis (Fig. 3.59), the fusion transcript (amplified with two different primer pairs; depicted in figure 3.58) could be detected in testis of all transgenic lines, while in testis of wild type mice no transcript was found. Different tissues of L13 males were analysed, the fusion transcript was detectable only in testis. Integrity of the RNA used for RT-PCR was proven by amplification of the *HPRT* transcript. Moreover, for line 13 a minusRT control of testis sample was performed to exclude DNA contamination.

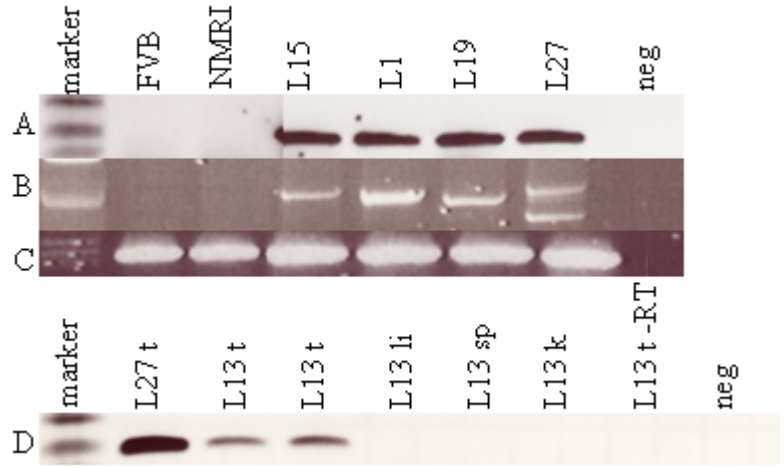

Figure 3.59: RT-PCR expression analysis of *Lis1 fusion* transcript in testes of hEF-1α-Lis1-c-myc Tag lines (L1, L13, L15, L19 and L27) and FVB and NMRI wild type mice. Expression of the fusion transcript is restricted to testes of transgenic lines. In (A) primers RT-hEF-Prom-F99 and RT-hEF-Prom-R99 were used, and in (B) primers Lis1-Ex7-F1 and 442-XbaI-R1. Integrity of the RNA used for RT-PCR was proven by amplification of the *HPRT* transcript (C). In (D) different tissues of L13 mice were analysed. Again, expression of the fusion transcript (amplified with primers RT-hEF-Prom-F99 and RT-hEF-Prom-R99) was restricted to testes. As a control for DNA contamination a minus-RT-control of testes RNA was performed. Abbreviations are: t, testis; li, liver; sp, spleen and k, kidney.

3.4.5.2.2 Expression of the fusion transcript in hEF-1α-Lis1-c-myc-Tag mice by Northern blot analysis

To validate the expression of the *Lis1-c-myc Tag* fusion transcripts in transgenic testis of hEF-1α-Lis1-c-myc Tag lines, Northern blot analysis was performed using a ^{32}P-labelled c-myc Tag specific probe. For the generation of the probe the hEF-1α construct was digested with *BamHI* and *XhoI* to release a 307 bp fragment, which contained the six c-myc Tags. The probe detected the expected 1.8 kb fragment. The expression was restricted to testis of the transgenic lines, while brain of L5 (PGK2-Lis1-c-myc Tag) mice showed no expression (Fig. 3.60 A). Integrity of RNA was checked by rehybridisation of the membrane with a β-actin probe (Fig. 3.60 B).

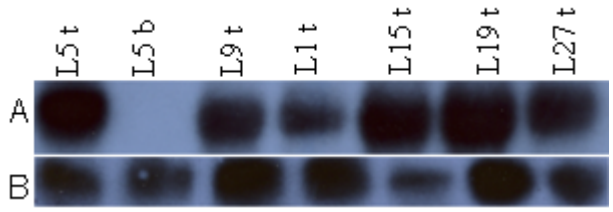

Figure 3.60: Northern blot analysis of *Lis1 c-myc Tag* specific transcript. Expression of the *Lis1 c-myc Tag* transcript is restricted to testis of transgenic lines. No expression in brain of L5 male was detected. Testes of hEF-1α-Lis1-c-myc Tag males showed clear expression of the transcript at the predicted size of 1.8 kb (A). Integrity of RNA was checked by rehybridisation of the membrane with a β-actin probe (B).

3.4.5.2.3 Expression of LIS1 in hEF-1α-Lis1-c-myc Tag mice by Western blot analysis

To evaluate the expression of LIS1-c-myc-Tag fusion protein in tissues of transgenic mice, total protein extracts from testes of L3 (PGK2-Lis1-c-myc Tag) and $L39^{GT/GT}$ controls and tissues of mutant animals were analysed by Western blot (Fig. 3.61). Monoclonal c-myc Tag antibody (Milipore) detects the 59 kDA LIS1-c-myc Tag fusion protein in testicular lysate of all analysed lines, but not in $L39^{GT/GT}$ and the tissues tested. Unspecific staining was detected in most tissues at about 50 kDa. In line 1 (Fig. 3.61 A i) and line 27 (Fig. 3.61 C i) an additional band was detected at about 64 kDa. As a positive control testicular protein of L3 was used, while protein of gene trap testes served as a negative control. 50 kDa α-tubulin protein was used as a control for protein loading (Fig. 3.61 A ii- D ii).

Transgenic mice of all five lines overexpress LIS1 in a testis-specific manner.

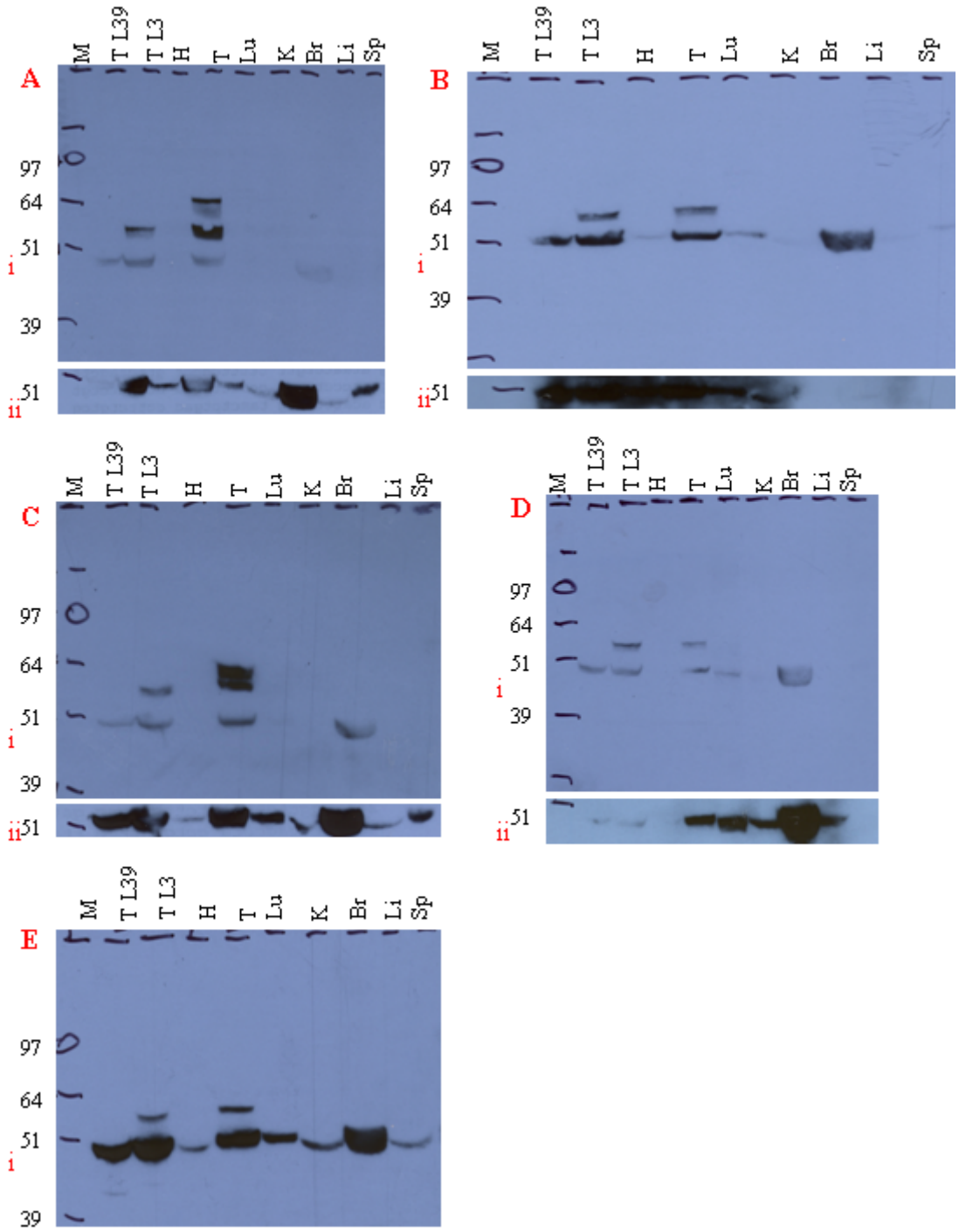

Figure 3.61: Expression of LIS1-c-myc Tag fusion protein in tissues of adult (36 d old) hEF-1α-Lis1-c-myc Tag mice and in testes of homozygous gene trap (L39) and L3 (PGK2-Lis1-c-myc Tag) mice. Monoclonal anti-c-myc Tag antibody (Milipore) recognises the 59 kDa LIS1-c-myc-Tag fusion protein in testicular lysates of all lines studied, while all other analysed tissues and testes of L39 are negative. In (A) protein lysates of line 1 was analysed, in (B) protein lysate of L19, in (C) protein lysate of L27, in (D) protein lysate of L13 and in (E) protein lysate of L15. The tissues analysed are testis (T), heart (H), lung (Lu), kidney (K), brain (Br), liver (Li) and spleen (Sp). 50 kDa α-tubulin protein is shown as a control for protein loading in ii (A-D), in E i membrane was hybridised with anti-c-myc Tag and anti-LIS1 antibody simultaneously.

3.4.5.3 Immunohistochemistry of testis sections of hEF-1α-Lis1-c-myc Tag males

To analyse the expression pattern of the LIS1-c-myc Tag fusion protein in tubuli seminiferi of transgenic animals, adult mice of line 15 and line 19 were perfused with PFA according to 2.2.13.3 and 5-6 µm thick cryosections of testis were immunostained with monoclonal c-myc Tag antibody in a 1:200 dilution. Specific immunostaining was observed in premeiotic cells of L15 and L19 males (Fig. 3.62). The staining intensity in testes of L19 mice (Fig. 3.62 C, D) was stronger than in testes of L15 mice (Fig. 3.62 A, B). No germ cells were stained in the negative control (secondary antibody only; Fig. 3.62 E). Immunostaining of brain sections of line 19 males revealed no specific staining (Fig. 3.62. F). The hEF-1α construct directs testis specific overexpression of LIS1 in premeiotic cells.

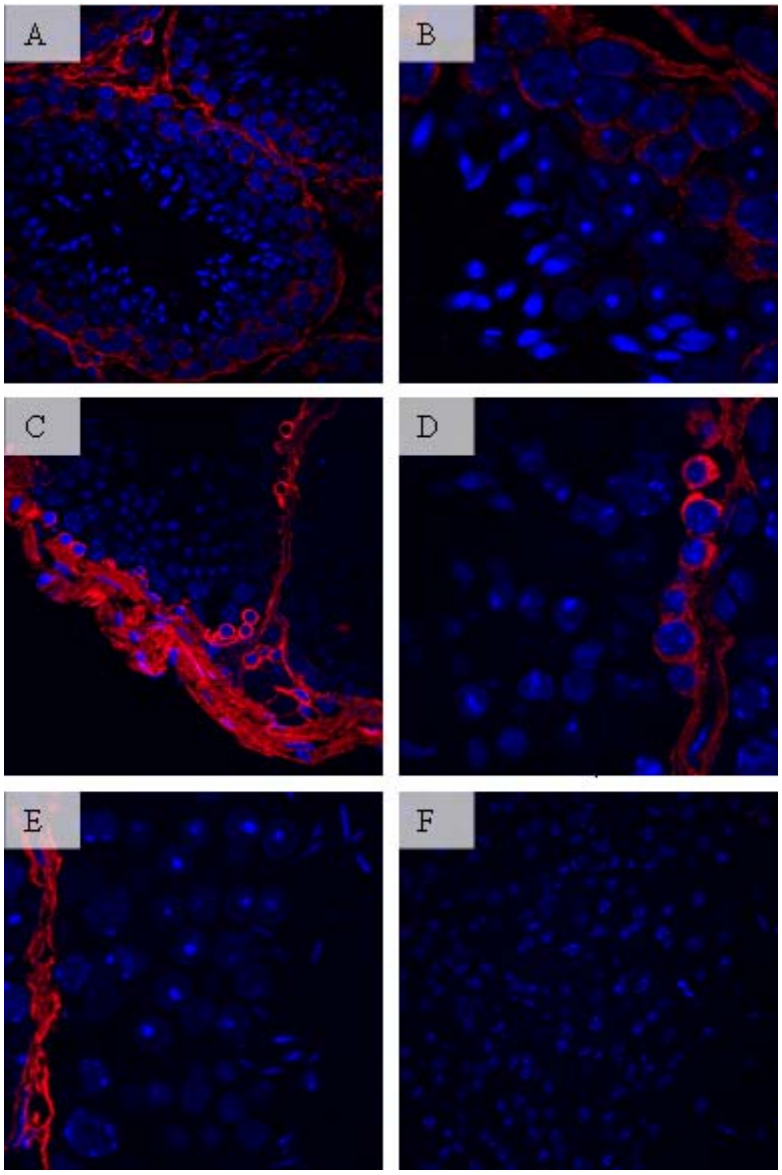

Figure 3.62: Expression of LIS1-c-myc Tag fusion protein in testis sections of transgenic hEF-1α-Lis1-c-myc Tag mice by using immunohistochemistry. The fusion protein was detected by a monoclonal c-myc Tag antibody (1:200). The expression in L15 (A, B) and L19 (C, D) is restricted to premeiotic germ 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 (E). Brain sections of L9 mice did not display specific staining for c-myc Tag antibody (F). All pictures are dual image overlays of Cy3 and DAPI fluorescences.

3.4.5.4 Histological analysis of testis sections of hEF-1α-Lis1-c-myc Tag mice

To evaluate the influence of premeiotic overexpression of *Lis1* on testis morphology, adult hEF-1α-Lis1-c-myc Tag mice of line 1, line 15 and line 19 were fixed in PFA,

sliced into 5 µm thick sections and stained with hematoxilin and eosin (H&E) (Fig. 3.63).

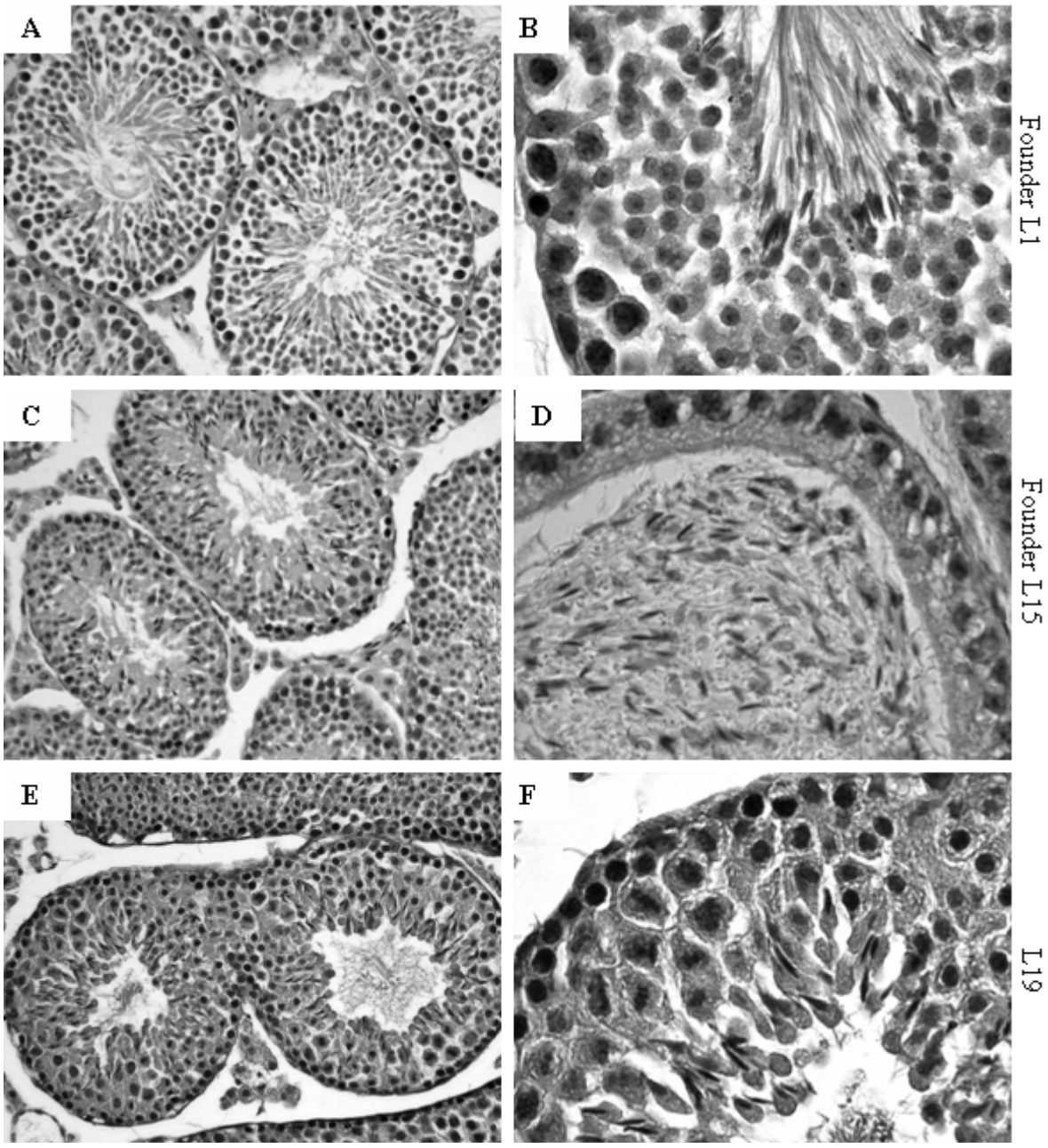

Figure 3.63: H&E staining of testis and epididymis sections of adult hEF-1α-Lis1-c-myc Tag mice. Sections through testes of L1 founder (A, B), L15 founder (C) and L19 no 14A (E, F) mice revealed robust spermatogenesis. Sections through epididymes of L15 founder revealed a high number of sperms (D) (original magnification A-E, x200; F, x600).

Testis sections of hEF-1α-Lis1-c-myc Tag mice of L1 (Fig. 3.63 A, B), L15 (Fig. 3.63 C) and L19 (Fig. 3.63 E, F) displayed no morphological abnormalities. Full spermatogenesis could be observed in these males. Tubules of epididymes of L15

founder were packed with sperm cells (Fig. 3.63 D). Histological analysis of L13 and L27 males revealed no abnormalities in spermatogenesis (with the exception of one L13 male, which showed severe disruption of tubule architecture) and caudae epididymes were filled with sperms (data not shown). Overexpression of *Lis1* in premeiotic cells had no visible influence on spermatogenesis of transgenic animals.

3.4.5.5 Generation of homozygous transgenic hEF-1α-Lis1-c-myc Tag males

To analyse a dose-dependent effect on *Lis1* overexpression on spermatogenesis of homozygous transgenic mice, hEF-1α-Lis1-c-myc Tag^{Tpos} mice of F1 generation (line 15 and line 19) were mated and offspring was genotyped. Positive males (either heterozygous or homozygous) were mated with wild type females and embryos (13.5 dpc) were genotyped. All offspring of a homozygous males are heterozygous (positive for the transgenic construct), while only about 50% of the offspring of a heterozygous male are positive. Two to four males (F2 generation) of each line were testbred and one homozygous male per line analysed.
Homozygous males of both lines were fertile (as all wild type females got pregnant). Histological analysis of testis and epididymis sections displayed no abnormalities (data not shown).

3.4.5.6 Determination of integration sites in transgenic hEF-1α-Lis1-c-myc Tag lines

To identify the number of integration sites in the transgenic lines, the transgenic ratio of offspring was determined for all lines. In case of one integration site, the transmission of the transgene should be in Mendelian fashion, which means, that about 50% of offspring (of F1 generation) are heterozygous (positive) for the transgene. Figure 3.64 summarizes the genotype and sex distribution in F1 offspring of all transgenic hEF-1α-Lis-c-myc Tag lines. Crossings of founders with wild type males did not show deviation from Mendelian ratio, with the exception of L1 ($p< 0.0001$), as it was shown by chi^2 test. Moreover, no differences were seen in sex distribution between the two groups in all lines but line 1 (Chi^2-test, L13 p=0.55, L15 p=0.29, L19 p=0.62 and L27 p= 0.99).

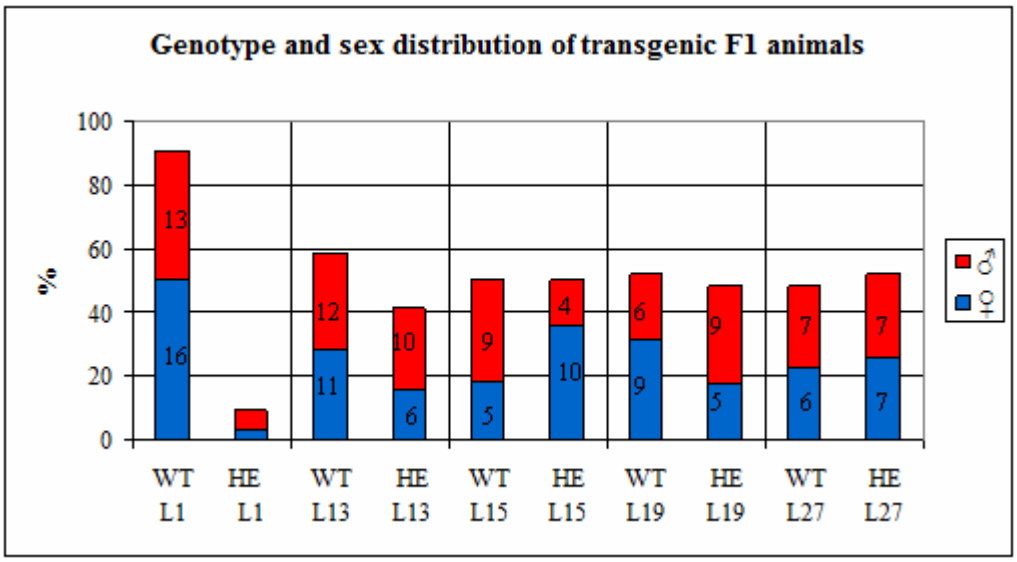

Figure 3.64: Genotype and sex distribution of transgenic mice in Founder x FVB wild type breedings. Breeding of founder 1, founder 13, founder 15, founder 19 and founder 27 with FVB wild type animals did not show deviation from Mendelian ratio, with the exception of line 1 ($p<0,0001$). 32 animals from 4 litters were genotyped for L1, 39 animals from 4 litters for L13, 28 animals from 2 litters for L15, 29 animals from 4 litters for L19 and 27 animals from 2 litters for L27. Abbreviations are: HE, heterozygous; WT, wild type. Numbers in columns display number of animals for each genotype and sex.

These data suggests that all transgenic lines, with the exception of line 1, have one transgenic integration site. Founder 1 might be a mosaic mouse.

To further confirm this data integration site number was determined by qPCR for L15 and L19. Single copy gene *pelota* was used for the normalisation of each DNA sample. The results are shown in figure 3.65.

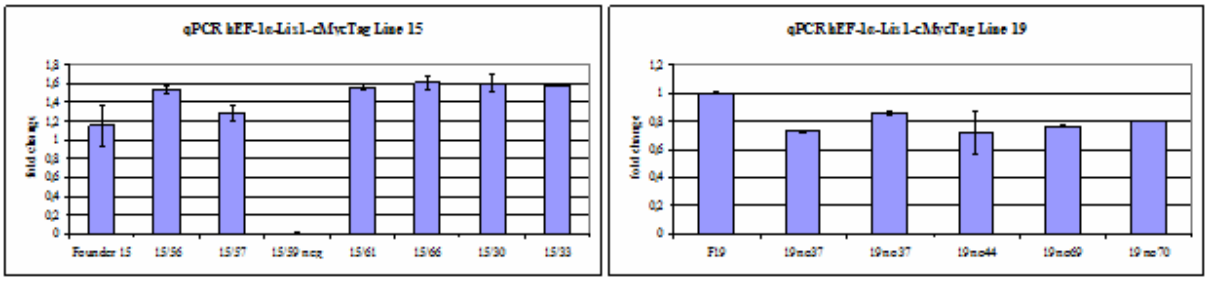

Figure 3.65: Determination of integration sites in hEF-1α-Lis-c-myc Tag mice of line 15 and line 19. Single copy gene *pelota* was used for the normalisation of each DNA sample. F1 offspring had comparable fold change values in both lines. This result confirms the presence of just one integration site of the transgenic construct in line 15 and line 19 mice.

Fold change values of positive F1 animals did not differ significantly, therewith confirming the existence of just one integration site of the transgenic construct in line 15 and line 19.

3.4.6 Analysis of "rescued" L39$^{GT/GT}$/hEF-1α-Lis1-c-myc Tag^{Tpos} males

3.4.6.1 Breeding strategy to generate L39$^{GT/GT}$/hEF-1α-Lis1-c-myc Tag^{Tpos} males

To generate males, that are homozygous for the gene trap vector integration and positive (heterozygous or homozygous) for the transgene hEF-1α-Lis1-c-myc Tag (hEF-1α-Lis1), the following breeding strategy was used:

(vii) F0: ♀ L39$^{GT/GT}$ x ♂ hEF-1α-Lis1$^{T/-}$

(viii) F1: ♀ L39$^{GT/-}$/ hEF-1α-Lis1$^{T/-}$ x ♂ L39$^{GT/-}$/ hEF-1α-Lis1$^{T/-}$

(ix) F2: ♂ L39$^{GT/GT}$/hEF-1α-Lis1$^{T/-}$ & ♂ L39$^{GT/GT}$/hEF-1α-Lis1$^{T/T}$

Homozygous gene trap females are fertile and could be used for breeding with hEF-1α-Lis1$^{T/-}$ males. All resulting offspring were heterozygous for the gene trap integration (L39$^{GT/-}$), and about 50% of the animals were hEF-1α-Lis1$^{T/-}$. L39$^{GT/GT}$/hEF-1α-Lis1$^{T/-}$ siblings of F1 generation were bred to generate L39$^{GT/GT}$/hEF-1α-Lis1^{Tpos} (heterozygous or homozygous) males. According to Mendelian ratio the genotype of 3/16 of offspring was L39$^{GT/GT}$/hEF-1α-Lis1^{Tpos} (1/4 GT/GT x 3/4 hEF-1α-Lis1 positive (HE or HO)). About 50% of offspring were males, thus about 10% (3/32) of F2 generation were L39$^{GT/GT}$/hEF-1α-Lis1^{Tpos} males. hEF-1α-Lis1 c-myc Tag mice of line 15 (L15) and line 19 (L19) were used for the generation of L39$^{GT/GT}$/hEF-1α-Lis1-c-myc Tag^{Tpos} males. These animals are from now on referred to as L39/L15 and L39/L19 animals, respectively.

3.4.6.2 Fertility test of L39$^{GT/GT}$/hEF-1α-Lis1-c-myc Tag^{Tpos} males

Fertility of two L39$^{GT/GT}$/L15^{Tpos} males (2 to 4 months old) was tested by mating them with wild type females. All matings were performed for several weeks, but no litter was obtained.

Five L39$^{GT/GT}$/L15^{Tpos} and three L39$^{GT/GT}$/L19^{Tpos} males were then mated with wild type females. The uteri and oviducts of those mice, which were positive for vaginal plug, were dissected in IVF medium, flushed out and sperm number was determined. No sperms were found in uterus and oviduct of females mated with "rescued" males of both lines. The experiment was repeated two times for each male. L39$^{GT/-}$ /L15^{Tpos} animals

were mated with wild type females as controls. Sperm count revealed the expected number of sperm in uterus (11.6 ± 10.3x10^6, N=3) and oviduct (3.2 ± 4.6x10^3, N=3) of females mated with L39$^{GT/-}$/L15^{Tpos} males. As observed in L39$^{GT/GT}$, the testis sizes of "rescued" L39$^{GT/GT}$/L15 and L39$^{GT/GT}$/L19 males were markedly reduced in comparison with heterozygous siblings (data not shown).

3.4.6.3 Expression analysis of L39$^{GT/GT}$/hEF-1α-Lis1-c-myc Tag^{Tpos} mice

To validate the expression of LIS1-c-myc Tag fusion protein in testis of "rescued" mice, total protein extracts from testes of L39/L15, L39/L19 and L39$^{GT/GT}$ control animals were analysed by Western blot (Fig. 3.66). Monoclonal LIS1 antibody (Sigma) could detect the 45 kDa protein in all testicular lysates analysed, while the LIS1-c-myc Tag fusion protein was detectable only in testes of transgenic animals, with the exception of L39$^{GT/GT}$/L19^{Tpos} animal (where the protein concentration was too low to detect the fusion protein). Monoclonal c-myc Tag antibody (Milipore) detected the 59 kDA LIS1-c-myc Tag fusion protein in testicular lysate of L39/L15 and L39/L19 mice, but not in L39$^{GT/GT}$ mice.

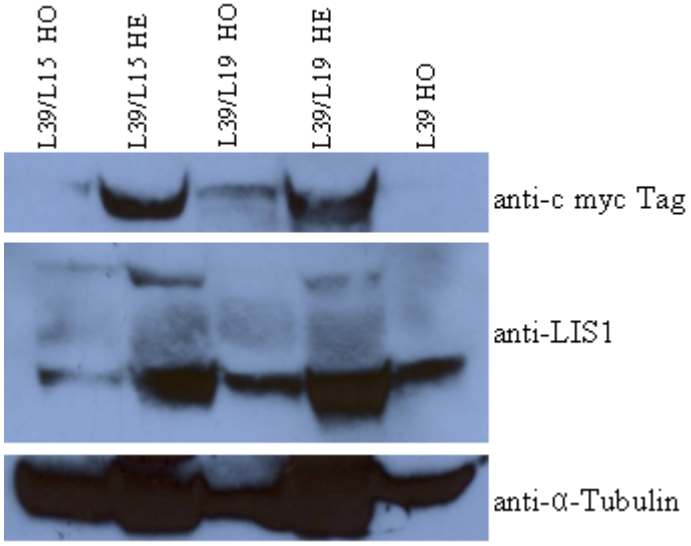

Figure 3.66: Expression of LIS1-c-myc Tag fusion protein in testes of adult L39/L15, L39/L19 and L39/$^{GT/GT}$ mice. Monoclonal anti-LIS1 (Sigma) recognises the 45 kDa protein in testicular lysates of all animals studied, while the 59 kDa fusion protein is only detectable in testes of L39/L15 and L39$^{GT/-}$/L19^{Tpos} animals. Monoclonal anti-c-myc Tag antibody (Milipore) detects the fusion protein in testes of all L39/L15 and L39/L19 mice, while no expression could be detected in L39$^{GT/GT}$ animal. 50 kDa α-tubulin protein is shown as a control for protein loading. Abbrevations are: HE: heterozygous mice (L39$^{GT/-}$/Lx^{Tpos}) and HO: homozygous mice (L39$^{GT/GT}$/Lx^{Tpos}).

Western blot analysis could confirm the overexpression of LIS1 in testis of $L39^{GT/GT}$/hEF-1α-Lis-c-myc Tag mice of both lines.

3.4.6.4 Histological analysis of testis sections of $L39^{GT/GT}$/hEF-1α-Lis1-c-myc Tag ^{Tpos} males

To evaluate the influence of premeiotic overexpression of *Lis1* on testis morphology of gene trap mice, testes and epididymes of adult $L39^{GT/GT}/L15^{Tpos}$, $L39^{GT/GT}/L19^{Tpos}$ "rescued" mice and heterozygous $L39^{GT/-}/L15^{Tpos}$ and $L39^{GT/-}/L19^{Tpos}$ controls were fixed in Bouin solution, sliced into 5 µm thick sections and stained with hematoxilin and eosin (H&E) (Fig. 3.67).

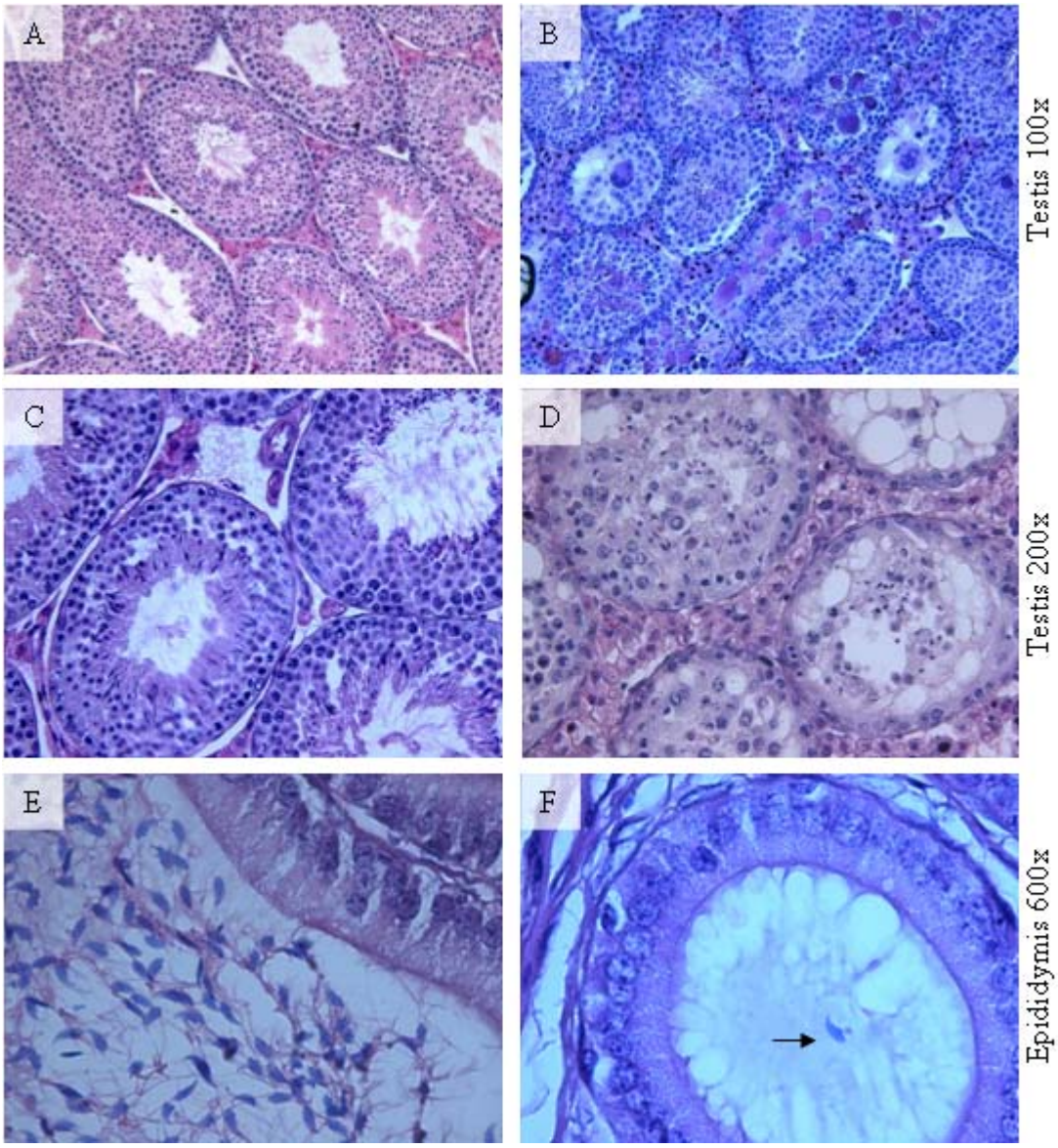

Figure 3.67: H&E staining of testis and epididymis sections of adult L39/hEF-1α-Lis1-c-myc Tag mice. Sections through testes of heterozygous L39/L15 (A) and L39/L19 (C) mice revealed robust spermatogenesis, while testis sections of "rescued" L39/L15 (B) and L39/L19 (D) mice demonstrated severe depletion of spermatogenesis in most tubules. Sections through epididymes revealed a high number of sperms in heterozygous males of L39/L15 (E), whereas most tubules of epididymes of "rescued" males were empty, but some were filled with few premature released germ cells or single sperm cells (marked with an arrow) (F) (original magnification A, B x100; C, D x200; E, F x600).

The histological analysis of testes of "rescued" L39/L15 and L39/L19 mice revealed the same extensive degeneration of a large fraction of seminiferous tubules as observed in $L39^{GT/GT}$ males of all genetic backgrounds (Fig. 3.1, 3.19 to 3.22 and 3.40 D). Like in the gene trap line mutant testes exhibit early germ cells as well as Sertoli and Leydig cells, but the number of late meiotic (i.e. late pachytene and diplotene spermatocytes)

and postmeiotic (i.e. spermatids and spermatozoa) germ cells is reduced. This phenotype was confirmed in 45 d old $L39^{GT/GT}/L15^{Tpos}$ mice (data not shown). In marked contrast to this are heterozygous males ($L39^{GT/-}/L15^{Tpos}$ and $L39^{GT/-}/L19^{Tpos}$), which exhibit full spermatogenesis with no obvious morphological differences from wild type males. Epididymal sections of "rescued" mice were mainly empty, except for few premature released germ cells in some tubules and even some with single sperm cells (Fig. 3.67 F), while epididymal sections of heterozygous control mice were packed with sperms (Fig. 3.67 E).

The overexpression of *Lis1* in premeiotic germ cells has no significant influence on testis morphology of homozygous gene trap mice.

3.4.6.5 Sperm analysis of $L39^{GT/GT}$/hEF-1α-Lis1-c-myc Tag^{Tpos} males

To evalutate sperm parameters of "rescued" $L39^{GT/GT}$/ hEF-1α-Lis1-c-myc Tag^{Tpos} males on sperm level, total sperm count in caudae epididymes of 2 months old $L39^{GT/GT}$/hEF-1α-Lis1-c-myc Tag^{Tpos} and heterozygous $L39^{GT/-}$/hEF-1α-Lis1-c-myc Tag^{Tpos} control males was determined.

Two homozygous and four heterozygous males of L39/L15 were used for analysis. $1.6 \times 10^7 \pm 1.4 \times 10^7$ sperms were found in heterozygous control mice, while $2.3 \times 10^4 \pm 0.9 \times 10^4$ sperms were found in "rescued" males via counting in a Neubauer counting chamber.

Two homozygous and one heterozygous male of L39/L19 were used for analysis. 3.7×10^6 sperms were found in heterozygous control mice, while 3.4×10^4 sperms were found in one out of two "rescued" males via counting in a Neubauer counting chamber.

13 µl of sperm suspension of L39/L19 mice was put on a dual sided sperm analysis chamber. Sperm motility was quantified using the computer assisted semen analysis (CASA) system. In the heterozygous control mouse 39% of counted sperms were motile and 27% were progressive. In contrast to this, in one out of two homozygous males 6% of sperms were motile and 1% progressive. The total number of sperms in the homozygous male was low, with 119 total sperm cells counted and 1517 sperms in the control.

To evaluate sperm morphology, 20 µl of epididymal cell suspension was transferred to slides, fixed in formaldehyde and covered with DAPI-containing mounting medium.

Slides were then analysed for sperms under the fluorescence microscope BX60 (Olympus).

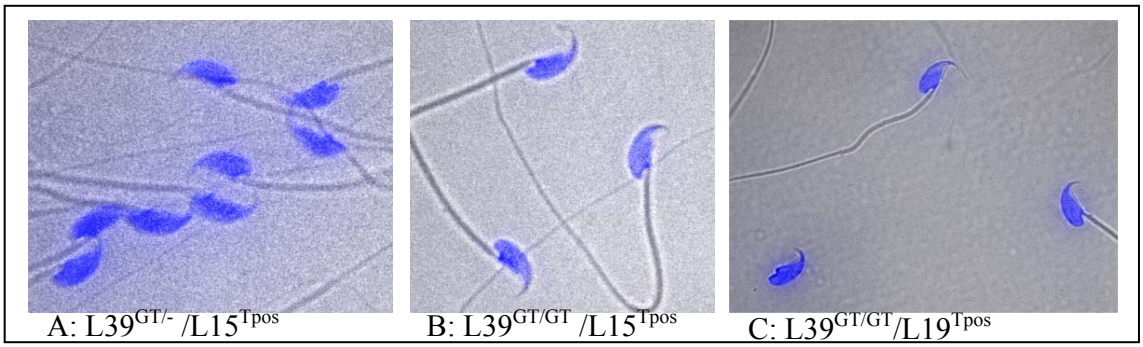

Figure 3.68: DAPI staining of sperms isolated from caudae epididymes of L39/L15 and L39/L19 mice. Epididymis of "rescued" L39/L15 (B) and L39/L19 (C) mice and heterozygous control mice (A) were dissected and smear was fixed and stained with DAPI. A low number of sperms was found in $L39^{GT/GT}$/hEF-1α-Lis1-c-myc Tag^{Tpos} males in contrast to a high number of sperms in the control animal. Sperms of all genotypes looked morphological normal (original magnification x600).

The existence of spermatozoa in caudae epididymes of "rescued" L39/L15 and L39/L19 males was confirmed (Fig. 3.68). In comparison to the heterozygous control the number of sperms was significantly reduced, as expected. Nevertheless the morphology of all sperm cells found looked normal.

3.4.6.6 Comparison of spermatogenesis defects in $L39^{GT/GT}$ and $L39^{GT/GT}$/hEF-1α-Lis1-c-myc Tag^{Tpos} males

To compare spermatogenesis defects in "rescued" L39/L15 mice and $L39^{GT/GT}$ mice in a more quantitative manner, testis cross-sections of NMRI wild type, $L39^{GT/GT}$, and $L39^{GT/GT}/L15^{Tpos}$ animals were analysed as in 3.4.4.7 (an example for each of the four classes is shown in figure 3.56). Table 3.3 gives an overview of the distribution of spermatogenesis defects in the analysed genotypes.

Table 3.3: Distribution of spermatogenesis defects in 46 d old NMRI wild type, $L39^{GT/GT}$ and $L39^{GT/GT}/L15^{Tpos}$ animals. Three animals per genotype were analysed. Overall number of counted tubules is stated (∑) and tubules of each class are expressed as percentage (±SD).

	∑	class 1	class 2	class 3	class 4
NMRI	314	86.4(±3.8)	13.2(±3.9)	0.4(±0.6)	
$L39^{GT/GT}$	549			6.38(±8.2)	93.62(±8.2)
$L39^{GT/GT}/L3^{Tpos}$	383		0.26(±0.5)	10.97(±0.58)	88.8(±0.2)

Even though the percentage of class 4 tubules (severe hypospermatogenesis) in $L39^{GT/GT}$ gene trap mice was higher than in "rescued" $L39^{GT/GT}/L3^{Tpos}$ mice and $L39^{GT/GT}$ males displayed no class 2 tubules (mild hypospermatogenesis), while a low number of $L39^{GT/GT}/L3^{Tpos}$ tubules (0.26%) displayed mild hypospermatogenesis, no statistically significant differences (Mann-Whitney-U-Test, p=0.51 for class 3 and 4 and p=0.37 for class 2) in spermatogenesis defects were found in these two lines (Fig. 3.69).

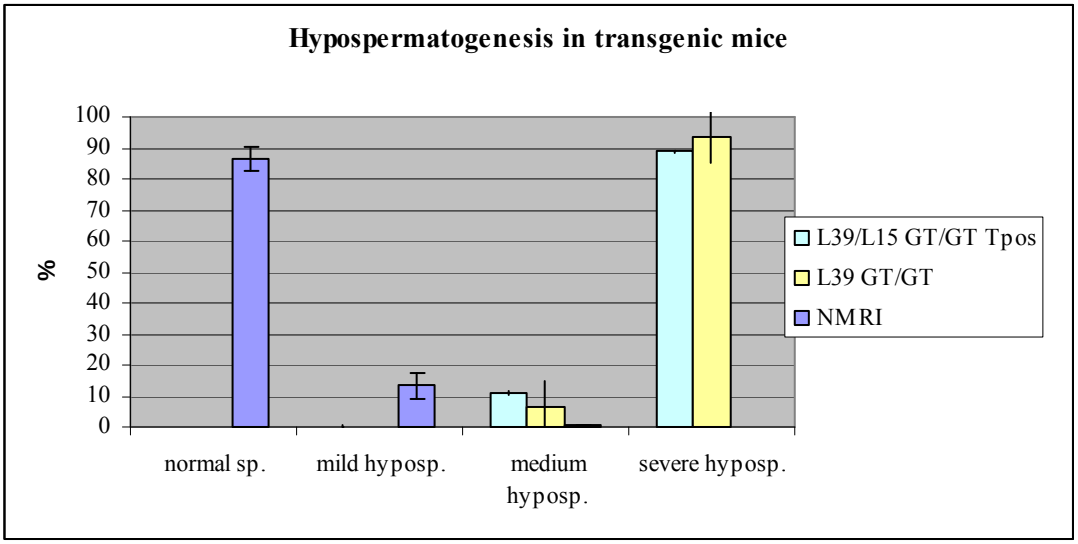

Figure 3.69: Analysis of spermatogenesis defects in NMRI wild type, $L39^{GT/GT}$ and $L39^{GT/-}/L15^{Tpos}$ mice. Testis sections of three animals per genotype were analysed. No significant difference was found in $L39^{GT/GT}$ and $L39^{GT/GT}/L15^{Tpos}$ mice.

The overexpression of *Lis1* in premeiotic germ cells in testes of gene trap line had no measureable influence on spermatogenesis of these animals.

4. Discussion

4.1 Brief overview of results

The present work discusses the role of Lissencephaly-1 protein in male germ cell differentiation. The following results were obtained:

1 Characterisation of the gene trap line L39$^{GT/GT}$:

● H&E staining of testis sections revealed an extensive germ cell degeneration in tubuli seminiferi of 46 d and 2 months old homozygous gene trap males with a markedly reduced number of late meiotic and postmeiotic germ cells. Heterozygous males exhibit full spermatogenesis.

● Expression analysis of *Lis1* transcripts by qRT-PCR could show a significant reduction of *Lis1 "Ex2a/3"* transcript in homozygous gene trap mice of all analysed ages (from 10 d to 2 months). The *Lis1 "Ex2/3"* transcript was reduced in homozygous males from d 25 on, but the reduction was not as prominent as the reduction of the *Lis1 "Ex2a/3"* transcript.

● Northern blot analysis of spermatocyte- and spermatid-specific markers in mutant mice showed similar expression levels for all genotypes in 35 d and 60 d old mice.

● Western blot analysis of 4 months old mice revealed a reduction of LIS1 protein in testes of L39$^{GT/GT}$ males in comparison to heterozygous and wild type males, while the amount of LIS1 protein in brain did not differ. Immunohistochemical analysis revealed that the expression of LIS1 protein is restricted to the cytoplasm of all testicular cells, with an intense staining in meiotically dividing spermatocytes and elongating spermatids.

● Presence of a fusion transcript of *Lis1* with the gene trap vector was confirmed. Spermatogonial cells and some early primary spermatocytes were positive for LacZ staining.

● Caudae epididymes of L39$^{GT/GT}$ mice contained a low number of morphologically normal looking spermatozoa.

● Two integration sites of the gene trap vector in intron 2 of *Lis1* gene could be shown by a "Genome Walk" approach. The existence of both integration sites in the ES cell clone "2A53" was confirmed.

● The gene trap mutation was analysed on different genetic backgrounds, namely CD-1, FVB, C57BL and 129/Sv. All genetic backgrounds exhibit the same phenotype as the original gene trap line L39/NMRI. All homozygous males were infertile, with caudae epidiymes containing a low number of morphologically normal looking spermatozoa. H&E staining of testis sections showed an early onset of the degeneration of germ cells, with a defect of spermatogenesis visible in 25 d old L39/C57BL$^{GT/GT}$ mice.

2 Colocalisation of LIS1 with putative interaction partners

● Four putative new interaction partners of LIS1 were identified by a yeast two-hybrid assay (performed by B. Jung, TU Braunschweig), namely Nude-like protein (Nudel), BRCA1-associated protein (BRAP), Ran binding protein 9 and Lim-only protein ACT (ACT).

● An interaction of LIS1 with Nude-like protein, BRCA1-associated protein and Lim-only protein ACT could be confirmed by Bimolecular Fluorescence Complementation (BiFC) assay.

3 Analysis of germ cell specific regulation

● Luciferase activity in different cell lines (NIH 3T3, NS20Y and GC-1 spg) transfected with pGL3-Basic vector containing a putative *Lis1* promoter region (1846bp of *Lis1* 5`flanking region) was not increased in comparison to controls.

● Luciferase activity in differentiated and undifferentiated SSC/129/Sv cells transfected with pGL3-Promoter vectors containing putative *Lis1* enhancer regions was not increased in comparison to controls.

● Generation of a transgenic construct containing putative *Lis1* enhancer sequences was not finished during course of this work.

4 Genetic rescue of the infertile mice L39GT/GT

● The transgenic line TNP2-Lis1 (Lispi) was generated that overexpresses *Lis1* in postmeiotic germ cells under control of the *Transition Nuclear Protein 2* (TNP2) promoter. Expression of the transgene was confirmed by Northern blotting analysis. Transgenic animals are fertile and testis sections of transgenic males displayed no abnormalities in comparison with sections of wild type mice.

● Double transgenic L39$^{GT/GT}$/Lispi^{Tpos} males were generated to rescue the infertility phenotype of the gene trap line. Analysed males remained infertile and histological

analysis of testis sections revealed no differences between $L39^{GT/GT}$ mice and rescued males. Electron microscopy confirmed the degeneration of germ cells in testes of "rescued" mice.

● The transgenic line PGK2-Lis1-c-myc Tag was generated that overexpresses *Lis1* from pachytene spermatocytes on under control of the *Phosphoglycerate Kinase-2* (PGK2) promoter. Three transgenic lines were analysed. Testis specific expression of the transgenic fusion transcript was proven by RT-PCR, Northern blotting and Western blotting. Immunohistochemical analysis could confirm the expression of the transgene in pachytene spermatocytes and following stages. All transgenic animals were fertile and testis sections of heterozygous and homozygous transgenic males displayed no abnormalities in comparison with wild type sections.

● Two lines of double transgenic $L39^{GT/GT}$/PGK2-Lis1-c-myc Tag^{Tpos} males were generated to rescue the infertility phenotype of $L39^{GT/GT}$. Transgenic expression was confirmed by Western blotting and immunohistochemical analysis. Males remained infertile and number of sperms in caudae epididymes was as low as in $L39^{GT/GT}$ males. Detailed comparison of defects in spermatogenesis of $L39^{GT/GT}$ and "rescued" males revealed no significant differences.

● The transgenic line hEF-1α-Lis1-c-myc Tag was generated that overexpresses *Lis1* in premeiotic germ cells under control of the *human Elongation Factor 1α* (hEF-1α) promoter. Five transgenic lines were analysed. Testis specific expression of the transgenic fusion transcript was proven by RT-PCR, Northern blotting and Western blotting. Immunohistochemical analysis confirmed restricted expression of the transgene in premeiotic spermatogonial cells. All transgenic animals were fertile and testis sections of heterozygous and homozygous transgenic males displayed no abnormalities in comparison with wild type sections.

● Two lines of double transgenic $L39^{GT/GT}$/hEF-1α-Lis1-c-myc Tag^{Tpos} mice were generated to rescue the infertility phenotype of gene trap males. Transgenic expression was confirmed by Western blotting. Males remained infertile and number of sperms in caudae epididymes was not increased in comparison to $L39^{GT/GT}$ males. Testis sections revealed the same severe germ cell degeneration as observed in the gene trap line.

4.2 Characterisation of L39 and comparison to published data

The main characteristic of the gene trap line L39 is the infertility of its homozygous males, while females reproduce normally. This prominent phenotype is sustained for about 10 years now, with more than 1400 mice being produced. The first analyses of L39, concerning the infertility, were performed in the Institute of Human Genetics in Göttingen by K. Nayernia and coworkers and published in "The Journal of Biological Chemistry" in 2003 (Nayernia et al., 2003). Continuous improvement in the field of molecular biology and technical innovations in recent years led to new methodical applications which could be used in this work for a more detailed and quantitative investigation of L39 mice. The infertility of homozygous mice was confirmed periodically by mating them with wild type or L39 females and the reduction in testis size was confirmed also in comparison with heterozygous and wild type siblings. To prove the lack of sperms in epididymal sections of $L39^{GT/GT}$ males cell suspension from caudae epididymes was fixed on slides, covered with DAPI-containing medium and analysed for sperms under the fluorescence microscope. With this experiment several morphologically normal looking sperms were found in homozygous males (Fig. 3.12). Even though the number was markedly reduced in comparison with heterozygous siblings, it is clear that spermatozoa are produced and stored in epididymes of L39 males, even at the age of 6 months. Table 4.1 lists the experiments performed in the first analysis (JBC) and in this work (revision) to determine the phenotype of mutant mice.

Table 4.1: Experiments performed to determine the phenotype of $L39^{GT/GT}$ males. Results obtained in the first analysis (Nayernia et al., 2003) (JBC)and in this work (Revision) are listed (ND, not determined).

Phenotype $L39^{GT/GT}$ males	JBC	Revision
fertility test	infertility	infertility
testis size	50% of wild type	50% of $L39^{GT/-}$ males
epididymal sperm analysis	ND	several spermatozoa morphologically normal

To determine the onset of the phenotype in mutant mouse testes, sections were histologically evaluated. Sections of mutant testis initially (up to postnatal d 45) exhibit intact seminiferous tubules of normal diameter and germ cells as well as Sertoli and Leydig cells present at appropriate locations. Phenotypical changes were first observed

at d 90 when round and elongating spermatids were released prematurely from the epithelium and located inside the lumen of tubules. Also the tubular structure seemed to have collapsed in the mutant, lacking the epithelial architecture and a clearly visible lumen. Only very few spermatozoa are present in mutant testes and the residual ones appeared in small clusters (Nayernia et al., 2003). This published phenotype was confirmed in this study. But a clear difference was found in the age of onset. Investigation of testis sections of 46 d old homozygous mutants clearly showed degeneration of germ cells in a large fraction of seminiferous tubules, with reduced numbers of late meiotic and postmeiotic germ cells as well as a significant vacuolisation (Fig. 3.1). Younger stages of the original gene trap line (on NMRI background) were not investigated, but 25 d and 35 d old homozygous gene trap males on CD-1 (Fig. 3.19) and C57/BL (Fig. 3.20) background. Animals displayed phenotypical changes at 25 d (analysed on C57/BL background only) and 35 d. Even though the number of affected tubules was lower than in older mice, the onset of the phenotype occurs earlier than demonstrated in the first analysis (Nayernia et al., 2003). To determine the distribution of sperm progenitor cells Nayernia and colleagues investigated the expression of spermatocyte- and spermatid-specific markers in 45 d old mice by Northern blotting. All markers were expressed at similar levels in wild type and mutant animals. In this work this experiment was repeated in younger animals (35 d old) to analyse the first wave of spermatogenesis and in older animals (60 d old) to investigate whether the expression levels changes with increasing numbers of affected tubules. In both analysed stages the former result was confirmed (Fig. 3.6), as the expression levels in all genotypes were similar. This data leads to the assumption that spermatogonia, spermatocytes and spermatids are formed in seminiferous tubules and the gene trap mutation affects the terminal differentiation of spermatids, resulting in a severe reduction of spermatozoa. To revise the expression levels of different spermatogenic markers in a quantitative manner, qRT-PCR analysis could be performed. Table 4.2 lists the experiments performed in the first analysis (JBC) and in this work (revision) to determine the onset of the phenotype in mutant mice.

Table 4.2: Experiments performed to determine the onset of the phenotype of $L39^{GT/GT}$ males. Results obtained in the first analysis (Nayernia et al., 2003) (JBC) and in this work (Revision) are listed. Abbreviations are: ND, not determined; x mo, x months old; xd, x days old.

Onset of phenotype in $L39^{GTGT}$ males	Age	JBC	Revision
Histological analysis	15 d	no anomalies	ND
	25 d	no anomalies	ND for NMRI background collapsed tubules on CD-1 background
	35 d	no anomalies	ND for NMRI background collapsed tubules, reduced number of meiotic and postmeiotic germ cells, vacuolisation on CD-1 and C57/BL background
	45/46 d	no anomalies	tubule structure collapsed reduced number of meiotic and postmeiotic germ cells vacuolisation
	2 mo	ND	as in 45/46d old mice
	3 mo	tubule structure collapsed very few spermatozoa	ND
expression of spermatocyte -specific markers	35 d	ND	Pgk-2 & Scp3: similar expression levels in wild type and mutants
	45 d	Pgk-2 & ACR: similar expression levels in wild type and mutants	ND
	60 d	ND	Pgk-2 & Scp3: similar expression levels in wild type and mutants
expression of spermatid -specific markers	35 d	ND	Tnp2 & Odf-1 (36/37d) similar expression levels in wild type and mutants
	45 d	Tnp2, Prm-2 & Hook1: similar expression levels in	ND

		wild type and mutants	
	60 d	ND	Tnp2 & Odf-1 similar expression levels in wild type and mutants

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 "2a"* transcript in homozygous gene trap mice. The expression of this transcript was first detected in 20 d old 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 d 10 to 60 (Fig. 3.4). A strong, significant downregulation of *Lis1 "2a"* splicing form from 10 d on could be detected in $L39^{GT/GT}$ males. With this result the insight on *Lis1* expression pattern in testis was extended. The downregulation of *Lis1 "2a"* transcript could be confirmed, but the expression of this splicing form was found to start earlier than published. 10 d old mice display already a significant downregulation of the *Lis1 "2a"* transcript. This result raises the possibility that *Lis1* is expressed in earlier stages of spermatogenesis. Both splicing forms are more or less equally expressed in all analysed stages, making it very unlikely that the "2a" transcript is expressed solely in later stages and the "2" transcript in earlier stages of spermatogenesis. Moreover a significant downregulation of *Lis1 "2"* splicing form was detected from 25 d on in homozygous mice which was not shown before. It is not clear whether this is due to the integration of the gene trap vector or the downregulation of the "2a" transcript, which might have an influence on the expression of the other splicing form. Moreover, fusion transcripts of the gene trap vector spliced to *Lis1 exon 2* (Fig. 3.9) and to *Lis1 exon 2a* (Fig. 3.10) could be amplified, thus it is not surprising that both splicing forms are affected by the gene trap integration.

With a "Genome Walk" experiment two integration sites of the gene trap vector within intron 2 of *Lis1* gene were determined (Fig. 3.16). Table 4.3 gives an overview about the experiments that were done before and in this work to characterise the genotype of $L39^{GT/GT}$ males and to evaluate the expression of *Lis1* transcripts in testis.

Table 4.3: Experiments performed to characterise the genotype of L39 males and to evaluate the expression of *Lis1* in testis. Results obtained in the first analysis (Nayernia et al., 2003) (JBC) and in this work (Revision) are listed. Abbreviations are: ND, not determined; ad., adult; xd, x days old.

Genotype & *Lis1* expression	Age	JBC	Revision
Northern blotting	5 d-ad.	reduction of a 2.3kb transcript in testis of adult $L39^{GT/GT}$ males; detection with a "2a specific" probe from 20 d on in wild type testis; no expression in $L39^{GT/GT}$ males and W/W^{v} mutants	reduction of "2a" transcript in testis of 36 d old $L39^{GT/GT}$ males
RT-PCR	5 d-ad.	low expression of "2a" transcript from 10 d on, high expression from 25 d on; *Lis1* expression in mutant testis is reduced compared with wild type, expression in brain is unaffected	no clear downregulation of *Lis1* transcripts in 36 d old mutant mice; expression of fusion transcripts of exon 2 and the gene trap vector and exon 2a and the gene trap vector
qRT-PCR	10 d-ad.	ND	prominent downregulation of "2a" transcript in $L39^{GT/GT}$ males from 10 d on; "2" transcript is significantly downregulated from 25 d on
"Genome Walk"		ND	two integration sites of the gene trap vector in intron 2 of *Lis1* gene

Expression analysis of LIS1 on protein level was performed by Western blotting and immunohistochemstry. Protein extracts of mice between 10 d after birth and adulthood revealed similar levels of LIS1 protein on Western blots of wild type mice. A clear reduction of LIS1 protein in testes of homozygous mutant mice was shown, while brain lysates of mutant mice and wild type controls exhibited similar levels of LIS1 expression. Immunostaining of testis sections from wild type and mutant mice showed that LIS1 protein is present in myoid stroma cells and in spermatids, but not in other germ cells. In the homozygous mutant the expression is restricted to myoid stroma cells, while LIS1 protein in spermatids was entirely abolished (Nayernia et al., 2003).

Western blotting of adult mice was repeated in this work and a reduction of LIS1 protein in homozygous L39 males was confirmed (Fig. 3.5). In contrast, the distribution of LIS1 protein in testes analysed by immunohistochemistry revealed a different pattern. LIS1 protein is expressed in all testicular cells in wild type and mutant mice (Fig. 3.7 & 3.8). This result is in line with immunohistochemical staining of adult mouse testes performed by Koizumi and coworkers (2003). They showed LIS1 immunoreactivity in cytoplasm of all seminiferous tubule cell types including Sertoli cells, with intense staining of LIS1 in meiotically dividing spermatocytes and elongating spermatids. Moreover, LIS1 was also localised at meiotic spindles of spermatocytes and manchettes of elongating spermatids (localisation with perinuclear microtubules in the spermatid manchette was also confirmed by electron microscopy by Nayernia et al. (2003)). These results and the clear expression of LIS1 in testes of 10 d old mice by Western blotting leads to the assumption that LIS1 is expressed in all stages of spermatogenesis. Moreover it was shown by Western blotting that SSC/129/Sv cells (a spermatogonial cell line) and differentiated SSC/129/Sv cells express LIS1 protein (Fig. 3.36), thus showing that the expression of LIS1 in 10 d old animals rather comes from spermatogonial cell than entirely from myoid stroma cells as suggested by Nayernia et al. (2003). Moreover, Western blot data by Yamaguchi et al. (2004) further confirms expression of LIS1 in testes of young males (from d 8 on with a slight increase in older males). Table 4.4 gives an overview about the experiments that were done before and in this work to evaluate the expression of LIS1 on protein level.

Table 4.4: Experiments performed to evaluate the expression of LIS1 on protein level. Results obtained in the first analysis (Nayernia et al., 2003) (JBC) and in this work (Revision) are listed. Abbreviations are: ND, not determined; ad, adult; 6mo, 6 months old; xd, x days old.

Expression of LIS1 protein	Age	JBC	Revision
Western blotting (testes of wild type mice)	10 d	expressed	ND
	15 d	expressed	ND
	25 d	expressed	expressed (C57/BL)
	ad.	expressed	expressed

Western blotting (testes of L39 and wild type mice)	ad.	decreased expression in testes of GT/- and GT/GT mutants, unchanged expression in brain	decreased expression in testes of GT/- and GT/GT mutants, unchanged expression in brain (4 months)
Immunohistochemistry (on wild type testes)	ad.	expression restricted to spermatids and myoid stroma cells	expression in cytoplasm of all testicular cells (36 d old mouse)
Immunohistochemistry (on $L39^{GT/-}$ testes)	6 mo	ND	expression in cytoplasm of all testicular cells
Immunohistochemistry (on $L39^{GT/GT}$ testes)	ad.	expression restricted to myoid stroma cells	expression in cytoplasm of all testicular cells (6 months old mouse)

Taken together, the results of this thesis validated that homozygous L39 males are infertile with severe defects in spermatogenesis. Reduction of late meiotic and postmeiotic cells, especially spermatozoa, collapsed tubular structure and vacuolisation could be confirmed. Expression of both *Lis1* transcripts in young animals, analysed by qRT-PCR, together with immunohistochemical analysis and Western blotting revealed expression of *Lis1* in all germ cells. In line with the publication the testis specific *Lis1 "2a"* transcript is highly downregulated in testes of $L39^{GT/GT}$ males.

4.3 L39 on different genetic backgrounds

An increasing number of scientific articles report 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. For example, the *ob* and *db* mutations (which affect the leptin and leptin receptor genes, respectively) are responsible for hyperglycemia and obesity in the C57BL/6 genetic background, but induce overt diabetes in the related C57BL/Ks inbred strain (Hummel, 1972; Coleman, 1973). C57BL/6 mice that are heterozygous for the *ApcMin* (multiple intestinal neoplasia) mutation, which is a germline mutation in the *Apc* (adenomatous polyposis coli) gene, develop an average of approximately 30 tumors throughout their intestines, whereas their heterozygous F1 progeny (C57BL/6 x AKR/J hybrids) exhibits only an average of approximately 6

polyps (Moser et al., 1992). For knock-out mouse models similar reports have been published.

EGFR (Epidermal Growth Factor Receptor Locus) deficiency on a CF-1 background resulted in peri-implantation death due to degeneration of the inner cell mass. On a 129/Sv background, homozygous mutants died at mid-gestation due to placental defects while on a CD-1 background, the mutants lived for up to 3 weeks and showed abnormalities in skin, kidney, brain, liver, and gastrointestinal tract (Threadgill et al., 1995). Another example is the *Cftr* (Cystic fibrosis transmembrane conductance regulator)-deficient mouse which demonstrated prolonged survival among backcross and intercross progeny with different inbred strains (Rozmahel et al., 1996).

The genetic background effect is attributable to so-called modifier genes, which act in combination with the causative gene (Montaguelli, 2000). Modifiers can affect penetrance, dominance modification, expressivity and pleiotropy. Depending on the nature of the phenotypic effect, modifiers might cause enhanced phenotypes, reduced phenotypes, novel phenotypes or wild-type phenotypes (reviewed by Nadeau, 2001).

In this work the gene trap line L39 (on NMRI outbred) was backcrossed to the inbred strains 129/Sv, FVB, C57BL and the outbred strain CD-1, respectively. After backcrossing for seven generations heterozygous littermates were crossed to produce homozygous incipient congenic strains. Fully backcrossed congenic strains are defined as 10 generations of backcrossing. Therefore the incipient congenic strains might still contain genes segregating from NMRI background.

The infertility phenotype of the L39 mice is not altered on different genetic backgrounds. All homozygous gene trap males remained infertile. Also immunohistological analysis revealed the same severe phenotype as previously shown on NMRI background (Fig. 3.19 to 3.22). LIS1 protein level in testes of homozygous males was reduced compared with heterozygous siblings. The spermatid specific marker *Odf-1* was expressed at a similar level in all genotypes on C57BL background (Fig. 3.25). In homozygous gene trap males on 129/Sv (Fig. 3.27) and FVB (Fig. 3.26) background a low number of morphologically normal looking spermatozoa was found. Changing of the background does not seem to have a prominent effect on spermatogenesis of homozygous gene trap mice. There are several examples where the genetic background influences fertility in different mouse models. One example is the *Cyclin A1* gene (*Ccna1*) deficient mouse. *Ccna1* is expressed during meiosis and is required for spermatogenesis. Homozgous knockout mice on a mixed genetic

background (129S6/SvEv x MF) are sterile due to spermatogenic arrest prior to the first meiotic division, while heterozygous males are subfertile. On the inbred strain (129S6/SvEv) heterozygous males are sterile, with a severe reduction in total sperm number (van der Meer et al., 2004). Another example is the *Nxf2* (Nuclear RNA export factor 2) deficient mouse. *Nxf2*-deficient males exhibit fertility defects that differ between mouse strains. One third of *Nxf2*-deficient males on a mixed (C57BL/6x129) genetic background were sterile, whereas the remaining males were fertile. On inbred (C57BL/6J) genetic background males were subfertile (Pan et al., 2009).

4.4 LIS1 and its interaction partners in testis

A lot is known about the function of LIS1 especially in neuronal migration during brain development. In testis the function of LIS1 as part of the PAF-AH (I) complex was investigated in detail (Koizumi et al., 2003, Yan et al., 2003) and is discussed in 4.5. To find new interaction partners of LIS1 in murine testis a yeast two hybrid assay was performed by Bomi Jung (TU Braunschweig), with *Lis1* cDNA used as bait and a mouse testis cDNA library as prey. With this approach one might be able to deduce new functions of LIS1 in testis. A detailed analysis and discussion of LIS1 and its putative interaction partners is done by Bomi Jung in the course of her PhD thesis (unpublished data). In this work the interaction of LIS1 with three putative new interaction partners, namely Nude-like protein (NUDEL), Lim-only protein ACT (ACT) and BRCA1-associated protein (BRAP) was analysed by a BiFC-Assay.

The interaction of LIS1 with NUDEL (Nuclear distribution gene E-like product) is well analysed. In the filamentous fungus *Aspergillus nidulans* the LIS1 homolog NUDF interacts with the coiled coil domain of the NUDEL homolog NUDE (Efimow and Morris, 2000). In mammals interaction of LIS1 and NUDEL was first identified in a yeast two-hybrid screen of a human third trimester brain cDNA library using full length *Lis1* gene (Niethammer et al., 2000). They could show colocalisation of LIS1 and NUDEL in centrosome-like structures near the nucleus in Cos7 cells. NUDEL expression was also detected in centrosomal structures in cultured hippocampal neurons and in growth cones as was reported for LIS1 (Smith et al., 2000). Moreover, NUDEL, like LIS1, interacts with cytoplasmic dynein in GST pulldown experiments from brain extracts (Niethammer et al., 2000). Interaction of LIS1 and NUDEL in transiently

transfected HeLa cells was confirmed by BiFC assay in this study (Fig. 3.32). The subcellular localisation of EGFP fluorescence was restricted to cytoplasm in HeLa cells in punctuate staining patterns. Staining in centrosome like structures like in Cos7 cells and hippocampal neurons could not be confirmed. Further experiments like BiFC assay followed by antibody staining with e.g. centrosomal markers would be helpful to analyse subcellular localisation in more detail. In testis, the expression of *Nudel* was analysed by Yamaguchi et al. (2004). By Western Blotting analysis Nudel became detectable in testes of wild type mice on P12, correlating with the appearance of zygotene spermatocytes, and the expression increased five fold on P27, correlating with the appearance of elongating spermatids. This data could be confirmed by immunohistochemical analysis, where expression of *Nudel* was low in spermatogonia and spermatocytes but clearly detectable in cytoplasm of round spermatids. High expression was detected in manchettes of elongating spermatids and in centrosomal region of maturing spermatids. This data suggests an involvement of *Nudel* in spermatid differentiation, most probably in manchette formation and function. The expression pattern of LIS1 and NUDEL differs somewhat, as LIS1 immunoreactivity is present in essentially all seminiferous tubule cell types (Koizumi et al., 2003), but overlap in meiotic spindles of spermatocytes and manchettes of elongating spermatids. Both structures are microtubule specific, which is in line with LIS1`s and NUDEL`s known microtubule associated functions.

The ACT (Lim-only-protein Activator of CREM in testis) protein was first identified by a yeast two-hybrid screen as a putative interaction partner of CREM (cAMP-responsive element modulator) (Fimia et al., 1999). The transcriptional activator CREM is highly expressed in male germ cells and controls transcription of postmeiotic genes like protamines and transition proteins (reviewed by Sassone-Corsi, 1998). CREM deficient mice display a block in germ cell development at early spermatid stage (Nantel et al., 1996, Blendy et al., 1996) confirming its crucial role in postmeiotic germ cell differentiation. ACT contains four complete copies and one amino-terminal half of the LIM motif, which are constituted by double zinc finger structures that mediates protein-protein interaction (Dawid et al, 1998, Curtiss and Heilig, 1998). The third LIM domain seems to be critical for interaction between CREM and ACT (Fimia et al., 1999). *ACT* expression is highly specific to testis, where it is expressed in nucleus of round and elongating spermatids, but not in mature sperm. The protein colocalises in purified spermatids with CREM (Fimia et al., 1999). The nuclear expression pattern of ACT is

surprising, as LIS1 is described as a cytoplasmic protein. Macho et al. (2002) analysed the expression of *ACT* in more detail and found that the subcelluar localisation of ACT is regulated by the kinesin motor protein KIF17b, which is expressed in spermatids only. KIF17b colocalises with ACT in haploid spermatids and mediates the transport of ACT from the nucleus to the cytoplasm during spermatid elongation. This relocalisation of ACT to the cytoplasm temporally correlates with the cessation of transcription of CREM- regulated genes. As expected from cytoplasmic expression pattern of LIS1 in BiFC assay the EGFP signal was detectable only in cytoplasm of NIH-3T3 and HeLa cells, but not in nuclei of these cells (Fig. 3.30). From these results one can speculate that the interaction of LIS1 and ACT is restriced to elongating spermatids. Nevertheless, the function of this interaction is unknown and needs to be studied in detail. Interestingly, *Act*-deficient mice are fertile and testis sections of adult $Act^{-/-}$ mice exhibit no morphological abnormalities compared to wild type mice, leading to the assumption that *Act* is not essential for spermatogenesis (Kotaja et al., 2004). Further analysis of caudae epididymes of mutant mice revealed a dramatically lowered sperm number at a level of only 35% of WT mice. A large majority of spermatozoa of *Act*-null mice has folded tails and abnormally shaped heads (Kotaja et al., 2004).

BRAP (BRCA1-associated protein) was identified in a yeast two hybrid assay, where a fragment of BRCA1 containing both nuclear localization signals (NLS) was used as a bait. BRAP was shown to interact specifically with the NLS motifs of BRCA1 (Chen et al., 1996). Li et al. (1998) could show expression of *Brap* in breast epithelial cells and adult mouse tissues. Interestingly, *Brap* is most abundantly expressed in testis while the expression in other tissues is low. The expression is restricted to cytoplasm in the breast epithelial cell line HBL100 (Li et al., 1998). The precise expression or function of *Brap* in testis has yet to be explored, while its function in MAP kinase signal transduction cascade in different cells lines is well analysed. BRAP (IMP) modulates sensitivity of the MAP kinase cascade by negative regulation of MAP kinase activation by limiting the formation of Raf/MEK complexes probably by inactivation of the KSR1 scaffold protein. It also acts as a Ras responsive E3 ubiquitin ligase that, on activation of Ras, is modified by auto-polyubiquitination resulting in the release of inhibition of Raf/MEK complex formation (Matheny et al., 2004). Moreover, BRAP functions as a cytoplasmic retention protein for p21 during monocyte differentiation. Binding of p21 and Brap is required for the cytoplasmic localisation of p21 (Asada et al. 2004). Because nothing is known about the localisation or function of BRAP in murine testis it is hard to speculate

about its interaction with LIS1 and the possible impact on LIS1 function. The expression pattern of *Brap* in testis should be analysed in detail to deduce possible functions. In BiFC assay the interaction of LIS1 and BRAP was confirmed (Fig. 3.31). The EGFP signal was detectable in cytoplasm of transiently transfected HeLa cells.
Table 4.5 summarizes the expression patterns of LIS1 and its three analysed interaction partners in testis.

Table 4.5: Expression pattern, subcellular localisation and function in testis as well as putative interaction partners and domains of LIS1, ACT, BRAP and NUDEL are listed. Abbreviation: ND, not determined.

	LIS1	**ACT**	**BRAP**	**NUDEL**
testicular expression	all testicular cell types	round and elongating spermatids only	expressed in testis	spermatocytes and following stages; high expression in elongating spermatids; no expression in spermatozoa
subcellular localisation in testis	cytoplasmic; meiotic spindles of spermatocytes; manchettes of elongating spermatids	nucleic in round spermatids, mainly cytoplasmic in elongating spermatids	ND	cytoplasmic; manchettes of elongating spermatids
function in testis	microtubule associated functions; role in spermatid differentiation	stimulates CREM transcriptional activity; involved in regulation of postmeiotic genes	ND	role in spermatid differentiation
putative interaction partners in testis	PAFAH (I) subunit α1 & PAFAH (I) subunit α2; α-TUBULIN; DYNEIN; NUDEL	CREM; KIF17b	ND	LIS1; DYNEIN
domains	LisH domain; coiled-coil; 7x WD40 repeats	four and a half LIM domains	coiled-coil Zinc-finger	coiled-coil

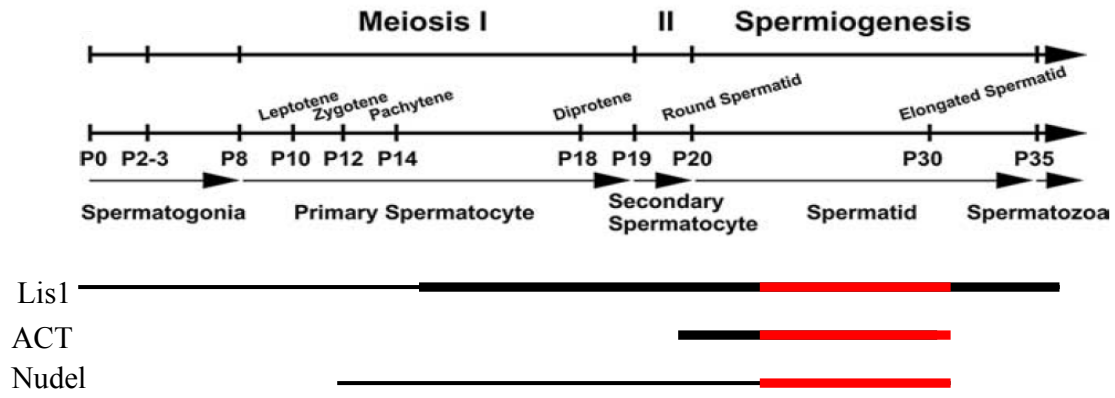

Figure 4.1: Putative expression pattern of LIS1, ACT and NUDEL in murine spermatogenesis. LIS1 is expressed in cytoplasm of all germ cells, including mature spermatozoa. ACT is expressed in nucleus of haploid round spermatids, where it interacts with CREM, and is translocated to the cytoplams in elongating spermatids by the kinase KIF17b, where its expression overlaps with the expression of LIS1. The expression of NUDEL starts in zygotene spermatocytes and increases markedly at P27, where it is mainly localised, like LIS1, in manchettes of elongating spermatids. The testicular expression pattern of BRAP is unknown. The red line indicates overlapping subcellular expression of LIS1 with ACT and NUDEL, respectively.

As visualised in figure 4.1 expression of ACT and NUDEL overlaps with LIS1`s expression in elongating spermatids. A possible interaction of ACT and NUDEL and a detailed analysis of the interaction of LIS1 and ACT and its subcellular localisation could help to rule out the function in elongating spermatids. As nothing is known about the function of BRAP in testis, it is highly interesting to analyse the expression pattern of *Brap* in testis in detail and to find out in which testicular cells LIS1 and BRAP are interacting and in what possible pathways they are involved.

The expression of LIS1 in all testicular cells leads to the assumptions that LIS1 could be involved in a variety of processes during spermatogenesis in addition to meiosis and manchette formation and function, although precise functions are unclear so far.

4.5 Testicular overexpression of *Lis1* and genetic rescue of L39 mice

To confirm that the reproductive defect in $L39^{GT/GT}$ males is due to the gene trap integration in *Lis1* gene locus and to gain further insights into the role and requirement of LIS1 in spermatogenesis, a transgenic rescue approach was used.

For this, three transgenic mice lines were generated, which overexpress *Lis1* under the control of testis specific promoters. The promoters used to overexpress *Lis1* in a testis specific manner were:

(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.

(i) *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, 1996; Meng, 2000). In our group this 1.2 kb *HindIII-EcoRI* fragment containing *human EF-1α* promoter (derived from pEF-BOS (Mizushima, 1990)) was used to overexpress *Piwil-2* specifically in spermatogonia (Lee, 2006).

(ii) *Pgk2* (Phosphoglycerate kinase-2) is an autosomal gene expressed in a testis-specific manner exclusively in the late stages of spermatogenesis (Kramer, 1981). *Pgk2* transcription is first seen in pachytene spermatocytes with message levels increasing during later stages of spermatogenesis (Gold, 1983; McCarrey, 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, 1989). The same promoter sequence was also used in our institute to examine the susceptibility of spermatocytes to transformation by targeted expression of SV40 TAg in spermatocytes of transgenic mice (Tascou et al., 2001).

(iii) Transition Nuclear Protein 2 (TNP2) is involved in chromatin condensation during spermiogenesis in the mouse (Kleene, 1987). It is expressed postmeiotically in male germ cells. Trancription of the *TNP2* mRNA starts in round spermatids and is then stored for about 6 d 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 d later in elongating spermatids, which is an evidence for posttranscriptional regulation of transgene expression (Nayernia et al., 2001).

Figure 4.2 gives an overview of the putative expression pattern of the transgenic *Lis1*-c-*myc Tag* fusion transcript in spermatogenesis for each line.

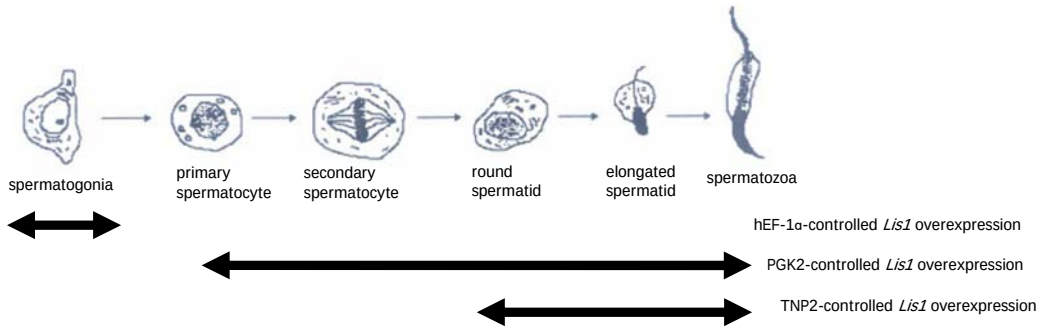

Figure 4.2: Expected expression pattern of transgenic *Lis1-c-myc Tag* fusion transcripts in spermatogenesis.

With these three different transgenic lines all stages of spermatogenesis are covered. 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 in mice from different founder lines to determine whether the phenotype is linked to the transgene integration site or the construct itself. Three PGK2-Lis1-c-myc Tag transgenic lines, five hEF-1α-Lis1-c-myc Tag lines and two TNP2-Lis1 lines were analysed. All analysed lines were transmitting and no phenotypical abnormalities were found (with the exception of line 1, which was discarded). Western blot analysis confirmed testis specificity of the fusion transcript (Fig. 3.45 & 3.61). Immunohistochemical stainings of tesis sections of transgenic animals revealed the expected staining pattern for hEF-1α-Lis1-c-myc Tag line 19 and line 15 in spermatogonial cells (Fig. 3.62) and for PGK2-Lis1-c-myc Tag line 9 and line 3 in meiotic and postmeiotic cells (Fig. 3.47). These results indicate that expression of the LIS1-c-myc Tag fusion protein is under control of the designed promoters (hEF-1α and PGK2, respectively). Nevertheless, slight differences were seen in PGK2-Lis1-c-myc Tag lines, where line 9 displayed strong staining in haploid, elongating spermatids while in line 3 staining was more widely distributed in meiotic and postmeiotic cells. The integration sites might have an additional effect on the expression due to regulatory

elements specific for the integration site (for an example see Weis et al., 1991). Transgenic constructs usually integrate in multiple copies into the genome in a head-to-tail orientation (Tinkle and Jay, 2002). This copy number can affect the resulting phenotype. Well studied examples are transgenic mouse models for amyotrophic lateral sclerosis, where the onset of the neurodegeneration correlates well with transgene copy number (Alexander et al., 2004). All transgenic lines generated in this study have a single integration site (see 3.4.3.6 & 3.4.5.6), 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 analysed, but no effect was found (see 3.4.3.5 & 3.4.5.5). 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 cause milder brain structural abnormalities, moderate to severe developmental delay and failure to thrive. It would be of interest to analyse, whether patients with this milder phenotype have any problems with fertility. Moreover, a transgenic mouse model was analysed, which overexpresses *Lis1* in the developing brain. Animals had a decreased brain size, an increase in apoptotic cells and a distorted cellular organisation in the ventricular zone (Bi et al., 2009). Again, this phenotype is milder than in mice with decreased levels of LIS1. In cultured mammalian cells the overexpression of *Lis1* leads to mitotic progression and spindle misorientation, while downregulation of LIS1 interferes with attachment of chromosomes to the metaphase plate and leads to chromosome loss (Faulkner et al., 2000).

LIS1 forms complexes with the two catalytic subunits α1 and α2 of Pafah1b. Both catalytic subunits are expressed in testis and mutants lacking *α2* or *α1* and *α2* subunits are infertile with no other apparent phenotype. According to Koizumi et al. (2003) *α1* subunit deficient mice are indistinguishable from wild-type mice, whereas *α2* subunit deficient mice show a reduction in testis size, but are fertile. However, $\alpha1^{-/-}/\alpha2^{-/-}$ 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 is the observation by Yan et al. (2003). *α-1* subunit deficient mice have normal

fertility, while *α-2* deficient mice are infertile. Spermatogenesis is disrupted at mid- or late pachytene stages of meiosis or early spermiogenesis. $\alpha1^{-/-}/\alpha2^{-/-}$ 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 $\alpha2^{-/-}$ and $\alpha1^{-/-}/\alpha2^{-/-}$ males (suggested to be due to post-transcriptional control, as mRNA levels of *Lis1* are unaltered in $\alpha2^{-/-}$ mice), Yan et al. (2003) found an increase in LIS1 protein expression. This discrepancy could be explained by the age of the analysed animals. While Koizumi and coworkers analysed adult males, Yan and coworkers analysed 12 d 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 d old animals only pachytene spermatocytes and earlier germ cells are present which are not affected in $\alpha1^{-/-}/\alpha2^{-/-}$ mutants. Interestingly, the infertility phenotype of the double mutant mice ($\alpha1^{-/-}/\alpha2^{-/-}$) could be "rescued" by generating triple mutant mice ($\alpha1^{-/-}/\alpha2^{-/-}/Lis1^{+/-}$). These mice exhibit full spermatogenesis although some degenerating germ cells and multinucleated giant cells were observed. These results suggest that *Lis1* expression 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 L39, where heterozygous males with a reduced expression of *Lis1* transcripts are fertile (Fig. 3.4). Only a further reduction of *Lis1* expression in homozygous mice leads to infertility of the males.

Overexpression of the *α2* subunit in cultured mammalian CHO cells induced centrosomal amplification and microtubule disorganisation, while overexpression of the *α1* subunit leads to a less prominent phenotype (Yamaguchi et al, 2007). Interestingly, these phenotypical changes were not found in cells overexpressing a mutant form of the *α2* subunit that can not bind LIS1, suggesting that binding of the α2 subunit and LIS1 is a prerequisite for structural cell abnormality and that the quantitative balance of α2 and LIS1 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. Table 4.6 gives an overview on above mentioned mutant mouse models, which have an influence on *Lis1* expression level. The upregulation of LIS1 protein in testis of transgenic mice should be analysed further in a quantitative

manner, to study the amount of upregulation and to be able to compare different LIS1 protein levels.

Table 4.6: Mouse models that effect *Lis1* expression. Abbreviation: ND, not determined.

mouse model	**reference**	**effect on brain**	**effect on testis**	***Lis1* expression in testis**
L39GT/-	Nayernia et al., 2003	no effect	no effect	downregulation of *Lis1 "2a"* transcript (80%) (this work)
L39GT/GT	Nayernia et al., 2003	no effect	infertile; reduced testis weight; blockade of late spermatid differentiation	downregulation of *Lis1 "2"* transcript (60%) and *Lis1 "2a"* transcript (40%) (this work)
Lis1$^{+/-}$	Hirotsune et al., 1998 Yan et al., 2003	cortical, hippocampal and olfactory bulb disorganization (Hirotsune et al., 1998)	fertile; reduced testis weight and tubule diameter; degenerating germ cells (Yan et al., 2003)	reduction of LIS1 protein (50%) (Yan et al.; 2003)
Lis1$^{-/-}$	Hirotsune et al., 1998	embryonic lethal after embryo implantation	-	-
α1$^{-/-}$/α2$^{-/-}$	Koizumi et al., 2003	no effect	infertile; spermatogenesis disrupted at spermatocyte stage	reduction of LIS1 protein (20%) in adult mice
α1$^{-/-}$/α2$^{-/-}$	Yan et al., 2003	no effect	infertile; spermatogenesis disrupted at spermatocyte stage	strong upregulation of LIS1 protein in 12 d old mice
α1$^{-/-}$/α2$^{-/-}$ /Lis1$^{+/-}$	Yan et al., 2003	ND	fertile; reduced testis weight; some degenerating germ cells	slight up- regulation of LIS1 protein
testis specific *Lis1* overexpression	this work	no effect	no effect; full spermatogenesis	expression of LIS1-c-myc Tag fusion protein and endogenous LIS1 protein
brain specific *Lis1* overexpression	Bi et al., 2009	reduced brain size; disorganisation in the ventricular zone	ND	ND

The 5'-UTR and 3'-UTR of eukaryotic mRNAs contain sequence motifs crucial for many aspects of gene regulation and expression, like mRNA stability, mRNA localisation and translational efficiency (Kuersten and Goodwin, 2003). In the testis, posttranscriptional mechanisms play prominent roles in controlling the timing of protein synthesis. Many proteins that are essential for sperm assembly and function are synthesized in late-stage male germ cells and are dependent upon the translation of

stored mRNAs (Kierszenbaum and Tres, 1978, Braun, 1998). The function of many gene specific 5`-UTRs and 3`UTRs have been analysed. For example function of the 3`UTR of the *nanos2* gene in mice has been analysed. Tsuda et al. (2006) generated lacZ knock-in mice with and without a native *nanos2* 3`UTR. A clear difference in expression of the reporter gene was found. In oocytes *lacZ* mRNA was expressed in both lines, but ß-Gal expression was restricted to the line without *nanos2* 3`UTR. In male germ cell development the reverse phenomenon was observed with ß-Gal reporter activity being detectable over a longer period than in mice lacking *nanos2* 3`UTR. To further understand the significance of the *nanos2* 3`UTR *in vivo*, they generated the mouse line nanos2$^{pA/pA}$, which lacks the endogenous *nanos2* 3`UTR. Nanos2$^{pA/pA}$ mice were fertile but revealed defects in early spermatogenesis. Other well studied examples are the *mouse Protamine 1* (*mP1*) and the *Transition Nuclear Protein 2* (*TNP2*) genes. Transcription of *mP1* starts in round spermatids, its mRNA is stored and translated about one week later in elongating spermatids (Balhorn et al., 1984, Kleene et al., 1984). Braun et al (1989) demonstrated that the 3`UTR of *mP1* gene is sufficient for proper temporal expression. They generated two different transgenic lines. In both lines the *human growth hormone* (*hGH*) gene was expressed under control of the *mP1* promoter. In one line the 3`UTR of *hGH* was used and in the other 156 bp of *mP1* 3`UTR. In both lines *hGH* mRNA was expressed in round spermatids as expected. However, the translation of *hGH* mRNA was repressed (until elongating spermatid stage) only in the line that carried the construct with the 3`UTR of *mP1* gene, while in the other line immediate *mP1* translation was observed. *TNP2* gene is expressed postmeiotically in male germ cells. Transcription of the *TNP2* mRNA starts in round spermatids and is then stored for about 6 d 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 (Nayernia et al., 2001) and was therefore used for the generation of Lispi mice in this study. A transgenic line that carries a *TNP2* transgene with the 3`UTR of *hGH* showed simultaneous transcription and translation of *TNP2* in round spermatids. This premature translation caused abnormal head morphology, reduced sperm motility and male infertility (Tseden et al., 2007).

In the transgenic constructs used in this study the SV40 polyadenylation signal (or the 3`UTR of *TNP2* gene in Lispi lines) was used instead of the 3`UTR of *Lis1* gene, which

consists of 3714 bp with several alternative polyadenylation sites (Peterfy et al., 1998). To use *Lis1* 3`UTR in transgenic constructs could be useful to generate mice in which transgene expression is under control of posttranscriptional regulation of *Lis1* gene. In other words, the correct temporal and stage specific expression of *Lis1* might be a prerequisite for a successful transgenic rescue approach. Moreover, also the 5` UTR of *Lis1* gene could be used for transgene expression, especially the testis specific alternatively spliced exon 2a might have additional regulatory functions.

Several successful transgenic rescue experiments have been reported. One example are *agrin*-deficient mice (Lin et al., 2001). *Agrin*$^{-/-}$ mice die at birth because of aberrant development of the neuromuscular junctions. Ksiazek et al. (2007) generated transgenic mice (Tg) that express the chick *agrin* gene under control of the motor neuron-specific homeobox factor Hb9 and bred this line with agrin knock-out mice. In the resulting line (*Tg*/*agrin*$^{-/-}$) perinatal death was prevented. Another example for a successful transgenic rescue experiment are *ErbB2* deficient mice (Lee et al., 1995). *ErbB2*$^{-/-}$ mice die at midgestation because of heart malformation. Woldeyesus et al. (1999) report a genetic rescue of heart development by expression of *erbB2* cDNA under control of the *Nkx2.5* gene, which is expressed specifically in the myocardium. “Rescued” animals survived to birth. Moreover, reproductive defects in *FSH* (Follicle-Stimulating Hormone) - deficient mice could be rescued by expression of *FSHß* transgene in two different transgenic lines (Kumar et al., 1998). The infertility phenotype of the double mutant mice *α1*$^{-/-}$/*α2*$^{-/-}$ (catalytic subunits of Pafah1b) could be rescued by generating triple mutant mice (*α1*$^{-/-}$/*α2*$^{-/-}$/*Lis1*$^{+/-}$), which had a decreased LIS1 level (Yen et al., 2003, discussed above).

“Rescued” males of all analysed lines in this study remained infertile. A detailed analysis of spermatogenesis defects in “rescued” males and gene trap males revealed no significant differences (Fig 3.57 for L39/L3 mice & 3.69 for L39/L15 mice). Also sperm number in caudae epididymes of the males did not differ significantly (Table 4.7).

Table 4.7: Sperm numbers in caudae epididymes of homozygous L39 males and "rescued" males. The age (in months) and the number of sperms in caudae epididymes are listed. Few refer to a low number of sperms, which could not be calculated with a Neubauer counting chamber. Moreover, spermatozoa of all animals looked morphologically normal. No significant differences were found between the gene trap line (analysed on different backgrounds) and the "rescued" males.

Line	Age [mo]	No of sperms [x 10^4]	Line	Age [mo]	No of sperms [x 10^4]
L39 (NMRI)	2	few	L39/L3	2.5	1.2
	3	few		2.5	few
	6.5	few		2.5	few
L39 (FVB)	2.5	few	L39/L15	2	3.2
	2.5	few		2	1.4
	4.5	few	L39/L19	2	3.4
L39 (129/Sv)	2.5	7.2		2	few
	2.5	1.7			
	2.5	few			

A possible reason that the rescue approach did not work in the different mouse models is the LIS1 protein level. The overexpression of *Lis1* might not be strong enough to reach a LIS1 protein level that is sufficient to overcome the lack of protein. For this reason animals that are homozygous for the transgene should be tested. In the performed breedings one third of the "rescued" males should be homozygous for the transgene. With eight $L39^{GT/GT}/L3^{Tpos}$, eight $L39^{GT/GT}/Lispi^{Tpos}$ and eight $L39^{GT/GT}$ /hEF-1α-Lis1-c-myc Tag^{Tpos} males tested, the probability that no male is homozygous is significantly low (Chi^2-test; $0.05>p>0.01$), therefore it can be assumed that within these eight males some homozygous were present. Still, expression of *Lis1* under control of the PGK2 promoter, the TNP2 promoter or the hEF-1α promoter in testis of homozygous "rescued" males was not sufficient to improve fertility.

4.6 Future perspectives

The main goal of this work was to determine whether the integration of the gene trap vector into the second intron of *Lis1* gene in the gene trap line L39 is the reason for the infertility of homozygous males. With the transgenic rescue approach used in this study, the infertility phenotype could not be improved. 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-Lis1-c-myc Tag and PGK2-Lis1-c-myc Tag), that would express LIS1 fusion protein in most germ cells. These mice could then be used for a "rescue" experiment. Moreover, the above mentioned 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.

It was shown in this work that *Lis1* transcripts are downregulated in L39 mice. Nevertheless, integration of the gene trap vector into the genome led to a fragmentation of the vector, so it can not be excluded that parts of the gene trap vector integrated into other parts of the genome and intensify or influence the phenotype. To analyse this, a knock-in mouse could be generated, which carries the *LacZ* gene at the same locus as the gene trap line. These knock-in mice would display the same testicular phenotype as L39 mice, if no other locus is affected in L39 mice.

Interaction of LIS1 with its three putative interaction partners, namely ACT, BRAP and NUDEL could be confirmed by a BiFC assay in this study. A detailed analysis of this interaction partners is currently being done by Bomi Jung (TU Braunschweig) in the course of her PhD work and will help to elucidate the functions of LIS1 in testis. Especially experiments that can identify the stage specific interaction and subcellular localisation of LIS1 and interaction partners could help to figure out new functions of LIS1 in testis.

To study germ cell specific regulation of *Lis1* gene promoter and enhancer analysis were performed using Luciferase vectors. A major disadvantage of these studies is the lack of meiotic and postmeiotic germ cell lines. Even though induced SSC/129/Sv cells differentiate to meiotic and postmeiotic stages, the percentage of these cells is low and probably not sufficient to detect stage specific enhancers. For this reason a transgenic

approach could be used to analyse the effects of putative enhancer sequences in testis of transgenic animals. A potential construct is described in 3.3.3.

5. Summary

The subject of this study was the function of Lissencephaly-1 protein in male germ cell differentiation.

A gene trap mouse model (L39) in which a gene trap vector had integrated in intron 2 of *Lis1* gene resulted in male infertility of homozygous $L39^{GT/GT}$ mice (Nayernia et al., 2003) and was further analysed in this study. The extensive germ cell degeneration in tubuli seminiferi of homozygous gene trap males with markedly reduced number of late meiotic and postmeiotic germ cells could be confirmed. To analyse the reported downregulation of a testis specific *Lis1 "2a"* transcript in a quantitative manner qRT-PCR experiments were performed and a significant reduction of *Lis1 "2a"* transcript was confirmed in homozygous males of all analysed ages (from 10 d to 2 months). Moreover, ubiquitiously expressed *Lis1 "2"* transcript was downregulated in homozygous males from d 25 on. The reduction in *Lis1* transcripts led to a reduced LIS1 protein level in testis but not in brain, which could be demonstrated on Western blot analysis. The expression of LIS1 protein could be detected in cytoplasm of all testicular cells with an intense staining in meiotically dividing spermatocytes and elongating spermatids. To study the gene trap mutation on different genetic backgrounds, namely CD-1, FVB, C57BL and 129/Sv, incipient congenic animals were generated. No differences between homozygous animals of different genetic backgrounds were found, therefore a genetic background effect could be excluded. Analysis of epidiymal cell suspension of gene trap males revealed that a low number of morphologically normal looking sperms could be found in all homozygous animals on all backgrounds.

To find new interaction partners of LIS1 in testis a yeast two-hybrid assay was performed by B. Jung (TU Braunschweig) and four putative new interaction partners were identified. The detailed analysis of this interaction partners is currently being done by B. Jung in the course of her PhD work and might help to elucidate new function of LIS1 in testis. In this work the interaction of LIS1 with Nude-like protein (NUDEL), BRCA-1 associated protein (BRAP) and Lim-only protein ACT (ACT) was confirmed by BiFC assay.

As a testis specific *Lis1* transcript is downregulated in L39 males the gene trap vector integration might have disrupted a germ cell specific enhancer region. To study this

hypothesis putative *Lis1* promoter and enhancer regions were analysed in a cell culture system using Luciferase vectors. No Luciferase activity was detected, which could be due to a lack of meiotic and postmeiotic germ cells. Further studies in *in vivo* models could help to investigate germ cell specific regulation of *Lis1* gene.

To confirm that the infertility of $L39^{GT/GT}$ males is due to the gene trap integration in *Lis1* gene locus and to gain further insights into the role and requirement of LIS1 in spermatogenesis, a transgenic rescue approach was used. Three transgenic lines were generated, which overexpress *Lis1* under the control of testis specific promoters. The promoters used were (i) hEF-1α promoter, which is exclusively active in spermatogonial cells, (ii) PGK2 promoter, which is active in pachytene spermatocytes and following stages of spermatogenesis, and (iii) TNP2 promoter, which is active in round spermatids and following stages of spermatogenesis. Five hEF-1α-Lis1-c-myc Tag lines, three PGK2-Lis1-c-myc Tag lines and two TNP2-Lis1 (Lispi) lines were analysed. The expression of the transgene was confirmed by Northern blotting, Western blotting and immunohistochemical analysis. All transgenic lines were fertile and testis sections displayed no abnormalities in comparison with wild type animals. Also homozygous transgenic males (PGK2-Lis1-c-myc Tag and hEF-1α-Lis1-c-myc Tag) were fertile with no abnormalities in testis morphology. The overexpression of *Lis1* in different germ cells had no influence on fertility of transgenic animals.

Transgenic animals were mated with L39 mice to generate “rescued” $L39^{GT/GT}/Lis1^{Tpos}$ males. Two lines of PGK2-Lis1-c-myc Tag mice (L3 and L9), two lines of hEF-1α-Lis1-c-myc Tag mice (L15 and L19) and one line of Lispi mice were used for the rescue experiment. The expression of LIS1 fusion protein was confirmed by Western blotting and immunohistochemical analysis. All “rescued” animals remained infertile. A detailed analysis of spermatogenesis defects in two ”rescued” lines (L39/L3 and L39/L15) in comparison to $L39^{GT/GT}$ males revealed no significant differences. Also the number of sperm cells in caudae epididymes of “rescued” males was as low as in $L39^{GT/GT}$ mice. The overexpression of *Lis1* could not rescue the infertility phenotype of homozygous gene trap males.

6. References

Adham IM, Nayernia K, Burkhardt-Göttges E, Topaloglu Ö, Dixkens C, Holstein AF and Engel W, 2001. "Teratozoospermia in mice lacking the transition protein 2 (Tnp2)." *Mol. Hum. Reprod*. **7**:513-20.

Alexander GM, Erwin KL, Byers N, Deitch JS, Augelli BJ, Blankenhorn EP and Heiman-Patterson TD, 2004. "Effect of transgene copy number on survival in the G93A SOD1 transgenic mouse model of ALS." *Brain Res Mol Brain Res.* **130**(1-2):7-15.

Amano T, Richelson E and Nirenberg M, 1969. "Neurotransmitter Synthesis by Neuroblastoma Clones." *Proc Natl Acad Sci U S A.* **69**(1):258–63.

Angle MJ, Tom R, Jarvi K and McClure RD, 1993. "Effect of platelet-activating factor (PAF) on human spermatozoa-oocyte interactions." *J Reprod Fertil.* **98**(2):541-8.

Arai H, Koizumi H, Aoki J and Inouef K, 2002. "Platelet-Activating Factor Acetylhydrolase (PAF-AH)." *J. Biochem.***131**:635-40.

Asada M, **Ohmi K**, **Delia D**, **Enosawa S**, **Suzuki S**, **Yuo A**, **Suzuki H and Mizutani S**, **2004. "**Brap2 functions as a cytoplasmic retention protein for p21 during monocyte differentiation**"** *Mol Cell Biol.* **24**(18):8236-43.

Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA and Struhl K, 1994. "Current protocols in molecular biology." John Wiley & Sons Inc., USA.

Balhorn R, Weston S, Thomas, C and Wyrobek AJ, 1984. "DNA packaging in mouse spermatids. Synthesis of protamine variants and four transition proteins." *Exp. Ceil. Res.* **150**:298-308.

Bhat RA, Lahaye T and Panstruga R, 2006. "The visible touch: in planta visualization of protein-protein interactions by fluorophore-based methods." *Plant Methods.* **2**:12.

Bi W, Sapir T, Shchelochkov OA, Zhang F, Withers MA, Hunter JV, Levy T, Shinder V, Peiffer DA, Gunderson KL, Nezarati MM, Shotts VA, Amato SS, Savage SK, Harris DJ, Day-Salvatore DL, Horner M, Lu XY, Sahoo T, Yanagawa Y, Beaudet AL, Cheung SW, Martinez S, Lupski JR and Reiner O, 2009. "Increased LIS1 expression affects human and mouse brain development." *Nat Genet.* **41**(2):168-77.

Blendy JA, Kaestner KH, Weinbauer GF, Nieschlag E and Schütz G, 1996. "Severe impairment of spermatogenesis in mice lacking the CREM gene." *Nature.* **380**(6570):162-5.

Boer PH, Adra CN, Lau YF and McBurney MW, 1987. "The testis-specific phosphoglycerate kinase gene pgk-2 is a recruited retroposon." *Mol. Cell. Biol*. **7**:3107-12.

Bradford MM, 1976. "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem.* **72**:248-54.

Braun RE, 1998. "Post-transcriptional control of gene expression during spermatogenesis." *Semin Cell Dev Biol.* **9**:483–9.

Braun RE, Peschon JJ, Behringer RR, Brinster RL and Palmiter RD, 1989. "Protamine 3'-untranslated sequences regulate temporal translational control and subcellular localization of growth hormone in spermatids of transgenic mice." *Genes Dev.* **3**(6):793-802.

Burfeind P and Hoyer-Fender S, 1996. "Transcription and translation of the outer dense fiber gene Odf1 during spermiogenesis in the rat: a study by in situ analyses and polysome fractionation." *Mol. Reprod. Dev.* **45**:10–20.

Cahana A, Jin XL, Reiner O, Wynshaw-Boris A and O'Neill C, 2003. "A study of the nature of embryonic lethality in LIS1-/- mice." *Mol Reprod Dev.* **66**:134–42.

Cardoso C, Leventer RJ, Matsumoto N, Kuc JA, Ramocki MB, Mewborn SK, Dudlicek LL, May LF, Mills PL, Das S, Pilz DT, Dobyns WB and Ledbetter DH,

2000. "The location and type of mutation predict malformation severity in isolated lissencephaly caused by abnormalities within the LIS1 gene." *Hum Mol Genet.* **9**:3019-28.

Chen CF, Li S, Chen Y, Chen PL, Sharp ZD and Lee WH, 1996. "The nuclear localization sequences of the BRCA1 protein interact with the importin-alpha subunit of the nuclear transport signal receptor." *J Biol Chem.* **271**(51):32863-8.

Chien A, Edgar DB and Trela JM, 1976. "Deoxyribonucleic acid polymerase from the extreme thermophile Thermus aquaticus." *J Bacteriol.* **127**:1550-1557.

Clark JM, 1988. "Novel non-templated nucleotide addition reactions catalyzed by procaryotic and eucaryotic DNA polymerases." *Nucleic Acids Res.* **16**:9677-86.

Cockell MM, Baumer K and Gönczy P, 2004. "lis-1 is required for dynein-dependent cell division processes in C. elegans embryos." *J Cell Sci.* **117**:4571-82.

Coleman DL and Hummel KP, 1973. "The influence of genetic background on the expression of the obese (*ob*) gene in the mouse." *Diabetologia.* **9**:287-93.

Costa GL and Weiner MP, 1994. "Polishing with T4 or *Pfu* polymerase increases the efficiency of cloning of PCR fragments." *Nucleic Acids Res.* **22**:2423.

Curtiss J and Heilig JS, 1998. "DeLIMiting development." *Bioessays.* **20**(1):58-69.

Dawe AL, Caldwell KA, Harris PM, Morris NR and Caldwell GA, 2001. "Evolutionary conserved nuclear migration genes required for early embryonic development in Caenorhabditis elegans." *Dev Genes Evol.* **211**:434-41.

Dawid IB, Breen JJ and Toyama R, 1998. "LIM domains: multiple roles as adapters and functional modifiers in protein interactions."*Trends Genet.* **14**(4):156-62.

Denhardt DT, 1966. "A membrane-filter technique for the detection of complementary DNA." *Biochem Biophys Res Commun.* **23**:641-46.

Emes RD and Ponting CP, 2001. "A new sequence motif linking lissencephaly, Treacher Collins and oral-facial-digital type 1 syndromes, microtubule dynamics and cell migration." *Hum Mol Genet.* **10**(24):2813-20.

Efimov VP and Morris NR, 2000. "The LIS1-related NUDF protein of Aspergillus nidulans interacts with the coiled-coil domain of the NUDE/RO11 protein." *J Cell Biol.* **150**(3):681-8.

Faulkner NE, Dujardin DL, Tai CY, Vaughan KT, O'Connell CB, Wang Y and Vallee RB, 2000. "A role for the lissencephaly gene LIS1 in mitosis and cytoplasmic dynein function." *Nat Cell Biol.* **11**:784-91.

Feinberg AP and Vogelstein B, 1989. "A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity." *Anal Biochem.* **123**:6-13.

Feng Y, Olson EC, Stukenberg PT, Flanagan LA, Kirschner MW and Walsh CA, 2000. "LIS1 regulates CNS lamination by interacting with mNudE, a central component of the centrosome." *Neuron.* **3**:665-79.

Fields S and Song O, 1989. "A novel genetic system to detect protein-protein interactions." *Nature.* **340**(6230):245-6.

Fimia GM, De Cesare D and Sassone-Corsi P, 1999. "CBP-independent activation of CREM and CREB by the LIM-only protein ACT." *Nature.* **398**(6723):165-9.

Friede RL, 1989. "Developmental Neuropathology." 2nd edn. Springer, Berlin.

Furuchi T, Masuko K, Nishimune Y, Obinata M and Matsui Y, 1996. "Inhibition of testicular germ cell apoptosis and differentiation in mice misexpressing Bcl-2 in spermatogonia." *Development.* **122**(6):1703-9.

Gambello MJ, Darling DL, Yingling J, Tanaka T, Gleeson JG and Wynshaw-Boris A, 2003. "Multiple dose-dependent effects of Lis1 on cerebral cortical development." *J Neurosci.* **23**:1719–29.

Geiser JR, Schott EJ, Kingsbury TJ, Cole NB, Totis LJ, Bhattacharyya G, He L and Hoyt MA, 1997. "Saccharomyces cerevisiae genes required in the absence of the

CIN8-encoded spindle motor act in functionally diverse mitotic pathways." *Mol Biol Cell.* **8**:1035-50.

Gershoni JM and Palade GE, 1982. "Electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to a positively charged membrane filter." *Anal Biochem.* **124**:396-405.

Gold B, Fujimoto H., Kramer JM, Erickson RP and Hecht NB, 1983. "Haploid accumulation and translational control of phosphoglycerate kinase-2 messenger RNA during mouse spermatogenesis." *Devl. Biol.* **98**:392−99.

Guan K, Nayernia K, Maier LS, Wagner S, Dressel R, Lee JH, Nolte J, Wolf F, Li M, Engel W and Hasenfuss G, 2006. "Pluripotency of spermatogonial stem cells from adult mouse testis." *Nature*. **440**:1199–203.

Hanahan D, 1983. "Studies on transformation of Escherichia coli with plasmids." *J Mol Biol.* **166**(4):557-80.

Hansen J, Floss T, Van Sloun P, Fuchtbauer EM, Vauti F, Arnold HH, Schnutgen F, Wurst W, von Melchner H and Ruiz P, 2003. "A large-scale, gene-driven mutagenesis approach for the functional analysis of the mouse genome." *Proc Natl Acad Sci USA*. **100**:9918-22.

Hattori M, Adachi H, Aoki J, Tsujimoto M, Arai H and Inoue K, 1995. "Cloning and expression of a cDNA encoding the beta-subunit (30-kDa subunit) of bovine brain platelet activating factor acetylhydrolase." *J Biol Chem.* **270**:31345-52.

Hattori M, Adachi H, Tsujimoto M, Arai H and Inoue K, 1994. "Miller-Dieker Lissencephaly gene encodes a subunit of brain platelet-activating factor acetylhydrolase [corrected]." *Nature.* **370**:216-8.

Hirotsune S, Fleck MW, Gambello MJ, Bix GJ, Chen A, Clark GD, Ledbetter DH, McBain CJ and Wynshaw-Boris A, 1998. "Graded reduction of Pafah1b1 (Lis1) activity results in neuronal migration defects and early embryonic lethality." *Nat Genet.* **19**:333-9.

Hodge R, 1994. "Preparation of RNA gel blots." *Methods Mol Biol.* **28**:49-54.

Hofmann MC, Narisawa S, Hess RA and Millán JL, 1992. "Immortalization of germ cells and somatic testicular cells using the SV40 large T antigen." *Exp. Cell Res.* **201**:417-35.

Hogan B, Costantini F and Lacy E, 1986. "Manipulating the Mouse Embryo. A Laboratory Manual." Cold Spring Harbor: Cold Spring Harbor Labortory Press.

Hu G, 1993. "DNA polymerase-catalyzed addition of nontemplated extra nucleotides to the 3' end of a DNA fragment." *DNA Cell Biol.* **12**:763-70.

Hummel KP, Coleman DL and Lane P, 1972. "The influence of genetic background on expression of mutations at the diabetes locus in the mouse C57BL/KsJ and C57BL/6J strains." *Biochem Genet.* **7**:1-13.

Jain VK and Magrath IT, 1991. "A chemiluminescent assay for quantitation of betagalactosidase in the femtogram range: application to quantitation of beta-galactosidase in lacZ-transfected cells." *Anal Biochem* **199**(1):119-24.

Jainchill JL, Aaronson SA and Todaro GJ, 1969. "Murine sarcoma and leukemia viruses: assay using clonal lines of contact-inhibited mouse cells." *J Virol.* **4**(5):549–53.

Kato M, **Dobyns WB, 2003.** "Lissencephaly and the molecular basis of neuronal migration." *Hum Mol Genet.* **12** Spec No 1:R89-96.

Kerjan G and Gleeson JG., 2007. "Genetic mechanisms underlying abnormal neuronal migration in classical lissencephaly." *Trends Genet.* **23**(12):623-30.

Kierszenbaum AL and Tres LL, 1978. "RNA transcription and chromatin structure during meiotic and postmeiotic stages of spermatogenesis." *Fed Proc.* **37**:2512–16.

Kim MH, Cooper DR, Oleksy A, Devedjiev Y, Derewenda U, Reiner O, Otlewski J, and Derewenda ZS, 2004. "The structure of the N-terminal domain of the product of the lissencephaly gene Lis1 and its functional implications." *Structure.* **12**(6):987-98.

Kleene K.C, Distel RJ and Hecht NB, 1987. „Translational regulation and deadenylation of a protamine mRNA during spermiogenesis in the mouse. *Dev. Biol.* **105**:71-9.

Kleene KC and Flynn JF, 1987. "Characterization of a cDNA clone encoding a basic protein, TP2, involved in chromatin condensation during spermiogenesis in the mouse." *J. Biol. Chem.* **262**:17272-7.

Koizumi H, Yamaguchi N, Hattori M, Ishikawa TO, Aoki J, Taketo MM, Inoue K and Arai H, 2003. "Targeted disruption of intracellular type I platelet activating factor-acetylhydrolase catalytic subunits causes severe impairment in spermatogenesis." *J. Biol. Chem.* **278**:12489–94.

Kornecki E and Ehrlich YH, 1988. " Neuroregulatory and neuropathological actions of the ether-phospholipid platelet-activating factor." *Science.* **240**(4860):1792-4.

Kotaja N, De Cesare D, Macho B, Monaco L, Brancorsini S, Goossens E, Tournaye H, Gansmuller A and Sassone-Corsi P, 2004. "Abnormal sperm in mice with targeted deletion of the act (activator of cAMP-responsive element modulator in testis) gene." *Proc Natl Acad Sci U S A.* **101**(29):10620-5.

Kramer JM and Erickson RP, 1981. "Development program of PGK-1 and PGK-2 isozymes in spermatogenic cells of the mouse: specific activities and rates of synthesis." *Devl Biol.* **87**:37−45.

Ksiazek I, Burkhardt C, Lin S, Seddik R, Maj M, Bezakova G, Jucker M, Arber S, Caroni P, Sanes JR, Bettler B and Ruegg MA, 2007. "Synapse loss in cortex of agrin-deficient mice after genetic rescue of perinatal death." *J Neurosci.* **27**(27):7183-95.

Kuersten S and Goodwin EB, 2003. "The power of the 3' UTR: translational control and development." *Nat Rev Genet.* **4**:626–37.

Kumar TR, Low MJ and Matzuk MM, 1998. "Genetic rescue of follicle-stimulating hormone beta-deficient mice." *Endocrinology.* **139**(7):3289-95.

Laemmli UK, 1970. "Cleavage of structural proteins during the assembly of the head of the bacteriophage T4." *Nature.* **227**:680-685.

Laird PW, Zijderveld A, Linders K, Rudnicki MA, Jaenisch R and Berns A, 1991. "Simplified mammalian DNA isolation procedure." *Nucleic Acids Res.* **19**:4293.

Lee JH, 2006. "Analysis of the Role of Piwil2 gene in Tumorigenesis and Germline Stem Cell Metabolisms." PhD thesis.

Lee KF, Simon H, Chen H, Bates B, Hung MC and Hauser C, 1995. "Requirement for neuregulin receptor erbB2 in neural and cardiac development." *Nature.* **378**:394–98.

Lee WL, Oberle JR and Cooper JA, 2003. "The role of the lissencephaly protein Pac1 during nuclear migration in budding yeast." *J. Cell Biol.* **160**:355-64.

Leventer RJ, Cardoso C, Ledbetter DH and Dobyns WB, 2001. "LIS1: from cortical malformation to essential protein of cellular dynamics." *Trends Neurosci.* **24**(9):489-92.

Levine AS, Kort HI, Toledo AA and Roudebush WE, 2002. "A review of the effect of platelet-activating factor on male reproduction and sperm function." *J Androl.* **23**(4):471-6.

Li D and Roberts R, 2001. "WD-repeat proteins: structure characteristics, biological function, and their involvement in human diseases." *Cell Mol Life Sci.* **58**(14):2085-97.

Li S, Ku CY, Farmer AA, Cong YS, Chen CF and Lee WH, 1998. "Identification of a novel cytoplasmic protein that specifically binds to nuclear localization signal motifs." *J Biol Chem.* **273**(11):6183-9.

Lin W, Burgess RW, Dominguez B, Pfaff SL, Sanes JR and Lee KF, 2001. "Distinct roles of nerve and muscle in postsynaptic differentiation of the neuromuscular synapse." *Nature.* **410**:1057–64.

Liu Z, Steward R and Luo L, 2000. "Drosophila Lis1 is required for neuroblast proliferation, dendritic elaboration, and axonal transport." *Nat Cell Biol.* **2**:776-83.

Liu Z, Xie T and Ruth Steward R, 1999. "Lis1, the Drosophila homolog of a human lissencephaly disease gene, is required for germline cell division and oocyte differentiation." *Development.* **126**:4477-88.

Lo CW, Coulling M and Kirby C, 1987. "Tracking of mouse cell lineage using microinjected DNA sequences: analyses using genomic Southern blotting and tissue-section in situ hybridizations." *Differentiation.* **35**:37-44.

Macho B, Brancorsini S, Fimia GM, Setou M, Hirokawa N and Sassone-Corsi P, 2002. "CREM-dependent transcription in male germ cells controlled by a kinesin." *Science.* **298**(5602):2388-90.

Maclennan KM, Smith PF and Darlington CL, 1996. "Platelet-activating factor in the CNS." *Prog Neurobiol.* **50**(5-6):585-96.

Marcheselli VL and Bazan NG, 1994. "Platelet-activating factor is a messenger in the electroconvulsive shock-induced transcriptional activation of c-fos and zif-268 in hippocampus." *J Neurosci Res*. **37**(1):54-61.

Mateja A, Cierpicki T, Paduch M, Derewenda ZS and Otlewski J, 2006. "The dimerization mechanism of LIS1 and its implication for proteins containing the LisH motif." *J Mol Biol*. **357**(2):621-31.

Matheny SA, Chen C, Kortum RL, Razidlo GL, Lewis RE and White MA, 2004. "Ras regulates assembly of mitogenic signalling complexes through the effector protein IMP." *Nature.* **427**(6971):256-60.

Matsuo K, Shintani S, Tsuji T, Nagata E, Lerman M, McBride J, Nakahara Y, Ohyama H, Todd R and Wong DT, 2000. "p12(DOC-1), a growth suppressor, associates with DNA polymerase alpha/primase." *FASEB J.* **14**(10):1318-24.

McCarrey JR and Thomas K, 1987. "Human testis-specific PGK gene lacks introns and possesses characteristics of a processed gene." *Nature.* **326**:501–5.

Meng X, Lindahl M, Hyvönen ME, Parvinen M, de Rooij DG, Hess MW, Raatikainen-Ahokas A, Sainio K, Rauvala H, Lakso M, Pichel JG, Westphal H, Saarma M and Sariola H, 2000. "Regulation of cell fate decision of undifferentiated spermatogonia by GDNF." *Science.* **287**(5457):1489-93.

Minhas BS, Ripps BA, Zhu YP, Kim HN, Burwinkel TH and Gleicher N, 1996. "Platelet activating factor and conception. *Am J Reprod Immunol*. **35**(3):267-71.

Mizushima S and Nagata S, 1990. "pEF-BOS, a powerful mammalian expression vector." *Nucleic Acids Res.* **18**(17):5322.

Montaguelli, X, 2000. "Effect of the genetic background on the phenotype of mouse mutations." *J Am Soc Nephrol*. **11**:101–5.

Moser AR, Dove WF, Roth KA and Gordon JI, 1992. "The Min (multiple intestinal neoplasia) mutation: Its effect on gut epithelial cell differentiation and interaction with a modifier system." *J Cell Biol.* **116:**1517–26.

Nadeau JH, 2001. "Modifier genes in mice and humans." *Nat Rev Genet.* **2**(3):165-74.

Nantel F, Monaco L, Foulkes NS, Masquilier D, LeMeur M, Henriksén K, Dierich A, Parvinen M and Sassone-Corsi P, 1996. "Spermiogenesis deficiency and germ-cell apoptosis in CREM-mutant mice." *Nature.* **380**(6570):159-62.

Nayernia K, Boehm D, Topaloglu O, Schlueter G and Engel W, 2001. "Rat transition nuclear protein 2 regulatory region directs haploid expression of reporter gene in male germ cells of transgenic mice." *Mol Reprod Dev.* **58**:368-75.

Nayernia K, Vauti F, Meinhardt A, Cadenas C, Schweyer S, Meyer BI, Schwandt I, Chowdhury K, Engel W and Arnold HH, 2003. "Inactivation of a testis-specific Lis1 transcript in mice prevents spermatid differentiation and causes male infertility." *J Biol Chem.* **278**(48):48377-85.

Neer EJ, Schmidt CJ, Nambudripad R and Smith TF, 1994. "The ancient regulatory-protein family of WD-repeat proteins." *Nature.* **371**:297-300.

Niethammer M, Smith DS, Ayala R, Peng J, Ko J, Lee MS, Morabito M and Tsai LH, 2000. "NUDEL is a novel Cdk5 substrate that associates with LIS1 and cytoplasmic dynein." *Neuron.* **28**(3):697-711.

Osmani AH, Osmani SA and Morris NR, 1990. "The role of the lissencephaly protein Pac1 during nuclear migration in budding yeast." *J. Cell Biol.* **111**:543-51.

Pan J, Eckardt S, Leu NA, Buffone MG, Zhou J, Gerton GL, McLaughlin KJ and Wang PJ, 2009. "Inactivation of Nxf2 causes defects in male meiosis and age-dependent depletion of spermatogonia." *Dev Biol.* Epub ahead of print doi:10.1016/j.ydbio.2009.03.022

Peterfy M, Gyuris T, Basu R and Takacs L, 1994. "Lissencephaly-1 is one of the most conserved proteins between mouse and human: a single amino-acid difference in 410 residues." *Gene.* **150**:415-6.

Peterfy M, Gyuris T, Grosshans D, Cuaresma C and Takacs L, 1998. "Cloning and characterization of cDNAs and the gene encoding the mouse platelet-activating factor acetylhydrolase Ib a subunit/lissencephaly-1 protein." *Genomics.* **47**:200-6.

Peterfy M, Hozier JC, Hall B, Gyuris T, Peterfy K and Takecs L, 1995. "Localization of the mouse lissencephaly-1 gene to mouse chromosome 11B3, in close proximity to D11Mit65." *Somat Cell Mol Genet.* **21**:345-9.

Rajeevan MS, Ranamukhaarachchi DG, Vernon SD and Unger ER, 2001."Use of real-time quantitative PCR to validate the results of cDNA array and differential display PCR technologies." *Methods.* **25**(4):443-51.

Reiner O, Carrozzo R, Shen Y, Wehnert M, Faustinella F, Dobyns WB, Caskey CT and Ledbetter DH, 1993. "Isolation of a Miller-Dieker lissencephaly gene containing G protein beta-subunit-like repeats." *Nature.* **364**:717-21.

Robinson MO, McCarrey JR and Simon MI, 1989. "Transcriptional regulatory regions of testis-specific PGK2 defined in transgenic mice." *Proc. Natl. Acad. Sci. USA.* **86**:8437-41.

Roudebush WE, 2001. "Role of platelet-activating factor in reproduction: sperm function." *Asian J Androl.* **3**(2):81-5.

Rozmahel R, Wilschanski M, Matin A, Plyte S, Oliver M, Auerbach W, Moore A, Forstner J, Durie P, Nadeau J, Bear C and Tsui LP, 1996. "Modulation of disease severity in cystic fibrosis transmembrane conductance regulator deficient mice by a secondary genetic factor." *Nat Genet.* **12**:280–7.

Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, Mullis KB and Erlich HA, 1988. "Primer directed enzymatic amplification of DNA with a thermostable DNA polymerase." *Science.* **239**:487-91.

Sambroock J, Fritsch EF and Maniatis T, 1989. "Molecular cloning: a laboratory manual (2nd edition)." Cold Spring Harbour, New York, USA.

Sanger F, Nicklen S and Coulson AR, 1977. "DNA sequencing with the chain terminating inhibitors." *Proc Natl Acad Sci U S A.* **74**:5463-7.

Sapir T, Cahana A, Seger R, Nekhai S and Reiner O, 1999. "LIS1 is a microtubule-associated phosphoprotein." *Eur J Biochem.* **265**(1):181-8.

Sasaki S, Shionoya A, Ishida M, Gambello MJ, Yingling J, Wynshaw-Boris A and Hirotsune S, 2000. "A LIS1/NUDEL/cytoplasmic dynein heavy chain complex in the developing and adult nervous system." *S Neuron.* **28**(3):681-96.

Sassone-Corsi P, 1998. "CREM: a master-switch governing male germ cells differentiation and apoptosis." *Semin Cell Dev Biol.* **9**(4):475-82.

Siebert PD, Chenchik A, Kellogg DE, Lukyanov KA and Lukyanov SA, 1995. "An improved PCR method for walking in uncloned genomic DNA." *Nucleic Acids Res.* **23**:1087-8.

Smith DS, Niethammer M, Ayala R, Zhou Y, Gambello MJ, Wynshaw-Boris A and Tsai LH, 2000. "Regulation of cytoplasmic dynein behaviour and microtubule organization by mammalian Lis1." *Nat Cell Biol.* **11**:767-75.

Southern EM, 1975. "Detection of specific sequences among DNA fragments separated by gel electrophoresis." *J Mol Biol.* **98**:503-17.

Stanford WL, Cohn JB and Cordes SP, 2001. "Gene-Tap mutagenesis: past, present and beyond." *Nat. Rev. Genet*. **2**:756–68.

Tascou S, Nayernia K, Meinhardt A, Schweyer S, Engel W, Trappe R and Burfeind P, 2001. "Targeted expression of SV40 large tumour antigen (Tag) induces a transient enhancement of spermatocyte proliferation and apoptosis." *Mol Hum Reprod.* **7**(12):1123-31.

Threadgill DW, Dlugosz AA, Hansen LA, Tennenbaum T, Lichti U, Yee D, LaMantia C, Mourton T, Herrup K, Harris RC, Barnard JA, Yuspa SH, Coffey

RJ and Magnuson T, 1995: "Targeted disruption of mouse EGF receptor: Effect of genetic background on mutant phenotype." *Science*. **269**:230–4.

Tinkle BT and Jay G, 2002. "Molecular biology, analysis, and enabling technologies: Analysis of transgene integration." In: Pinkert CA, ed. Transgenic Animal Technology: A Laboratory Handbook. New York: *Academic Press*. 459-71.

Tseden K, Topaloglu O, Meinhardt A, Dev A, Adham I, Müller C, Wolf S, Böhm D, Schlüter G, Engel W and Nayernia K, 2007. "Premature translation of transition protein 2 mRNA causes sperm abnormalities and male infertility." *Mol Reprod Dev*. **74**(3):273-9.

Tsuda M, Kiso M and Saga Y, 2006. "Implication of nanos2-3'UTR in the expression and function of nanos2." *Mech Dev.* **123**(6):440-9.

Van der Meer T, Chan WY, Palazon LS, Nieduszynski C, Murphy M, Sobczak-Thépot J, Carrington M and Colledge WH, 2004. "Cyclin A1 protein shows haplo-insufficiency for normal fertility in male mice." *Reproduction.* **127**(4):503-11.

Venable ME, Zimmerman GA, McIntyre TM and Prescott SM, 1993. "Platelet-activating factor: a phospholipid autacoid with diverse actions." *J Lipid Res.* **34**(5):691-702.

Wang T and Brown MJ, 1998. "mRNA quantification by real time TaqMan polymerase chain reaction: validation and comparison with RNase protection." *Anal Biochem.* **269**(1):198-201.

Weis J, Fine SM, David C, Savarirayan S and Sanes JR, 1991. "Integration site-dependent expression of a transgene reveals specialized features of cells associated with neuromuscular junctions." *J Cell Biol.* **113**(6):1385-97.

Woldeyesus MT, Britsch S, Riethmacher D, Xu L, Sonnenberg-Riethmacher E, Abou-Rebyeh F, Harvey R, Caroni P and Birchmeier C, 1999. "Peripheral nervous system defects in erbB2 mutants following genetic rescue of heart development." *Genes Dev.* **13**(19):2538-48.

Wurst W, Rossant J, Prideaux V, Kownacka M, Joyner A, Hill D.P. Guillemot F, Gasca S, Cado D, Auerbach A and Ang SL, 1995. "A large-scale gene-trap screen for insertional mutations in developmentally regulated genes in mice." *Genetics* **139:**889-99.

Wynshaw-Boris A, 2007. "Lissencephaly and LIS1: insights into the molecular mechanisms of neuronal migration and development." *Clin Genet*. **72**:296–304.

Xiang X, Osmani AH, Osmani SA, Xin M and Morris NR, 1995. "NudF, a nuclear migration gene in Aspergillus nidulans, is similar to the human LIS-1 gene required for neuronal migration." *Mol Biol Cell.* **6**:297-310.

Yan W, Assadi AH, Wynshaw-Boris A, Eichele G, Matzuk MM and Clark GD, 2003. "Previously uncharacterized roles of platelet-activating factor acetylhydrolase 1b complex in mouse spermatogenesis." *Proc Natl Acad Sci U S A.* **100**(12):7189-94.

Yamaguchi N, Koizumi H, Aoki J, Natori Y, Nishikawa K, Natori Y, Takanezawa Y and Arai H., 2007. "Type I platelet-activating factor acetylhydrolase catalytic subunits over-expression induces pleiomorphic nuclei and centrosome amplification." *Genes Cells.* **12**(10):1153-61.

Yamaguchi N, Takanezawa Y, Koizumi H, Umezu-Goto M, Aoki J and Arai H, 2004. "Expression of NUDEL in manchette and its implication in spermatogenesis." *FEBS Lett.* **566**(1-3):71-6.

Yuan L, Liu L, Zhao L, Brundell E, Daneholt B and Höög C, 2000. "The murine SCP3 gene is required for synaptonemal complex assembly, chromosome synapsis, and male fertility." *Molecular Cell*. **5**(1):73-83.

Curriculum vitae

Personal details:

Name: **Nadja Drusenheimer**
Address: Düstere-Eichen-Weg-30
37073 Göttingen
Telephone: 0551 4885930
E-mail: Nadjadrusenheimer@hotmail.com
Date of Birth: 26.07.1979
Place of Birth: Remscheid
Nationality: German

Educational background:

since Aug 2005: **PhD study**, Institute of Human Genetics, Georg-August University, Goettingen
Title of thesis: "The Role Of Lissencephaly-1 Protein In Male Germ Cell Differentiation"

Aug 2004-Jul 2005: **Diploma thesis**, Institute of Human Genetics, Georg-August University, Göttingen
Title of thesis: "Zur Differenzierung von Stammzellen des Knochenmarks und von Monozyten zu männlichen Keimzellen"

Okt 2000- Juli 2004: Study of Biology (Diplomstudium) at the Georg-August University, Göttingen

Aug 1990- Jul 1999: Secondary School (Leibniz Gymnasium, Remscheid)

Aug 1986- Jul 1990: Primary School (Adolf-Clarenbach Grundschule, Remscheid)

Work Experience:

Aug 1999- Aug 2000: European Voluntary Service (EVS) at the Lord Mayor Treloar

College, Alton, United Kingdom

Publications:

Nayernia K, Lee JH, **Drusenheimer N**, Nolte J, Wulf G, Dressel R, Gromoll J and Engel W. **"Derivation of male germ cells from bone marrow stem cells."** *Lab Invest.* 2006 Jul;86(7):654-63.

Nayernia K, Nolte J, Michelmann HW, Lee JH, Rathsack K, **Drusenheimer N**, Dev A, Wulf G, Ehrmann IE, Elliott DJ, Okpanyi V, Zechner U, Haaf T, Meinhardt A and Engel W. **"In vitro-differentiated embryonic stem cells give rise to male gametes that can generate offspring mice."** *Dev Cell.* 2006 Jul;11(1):125-32.

Nayernia K, Lee JH, Engel W, Nolte J, **Drusenheimer N**, Rathsack K, Dev A, Wulf G, Ehrmann IE, Elliott D, Zechner U, Haaf T, Meinhardt A, Michelmann HW, Hasenfuss G and Guan K. **"From stem cells to germ cells and from germ cells to stem cells."** *Iranian Journal of Reproductive Medicine.* Spring 2007; 5(2):41-4.

Drusenheimer N, Wulf G, Nolte J, Lee JH, Dev A, Dressel R, Gromoll J, Schmidtke J, Engel W and Nayernia K. **"Putative human male germ cells from bone marrow stem cells."** *Soc Reprod Fertil Suppl*. 2007;63:69-76.

Zovoilis A, Nolte J, **Drusenheimer N**, Zechner U, Hada H, Guan K, Hasenfuss G, Nayernia K and Engel W. **"Multipotent adult germline stem cells and embryonic stem cells have similar microRNA profiles."** *Mol Hum Reprod.* 2008 Sep;14(9):521-9.

Acknowledgements

I would like to express my gratitude to Prof. Dr. med. Dr. h.c. W. Engel for his support, encouragement, excellent scientific supervision and financial support for my Ph.D study. It has been a pleasure working in such an inspiring and friendly atmosphere as he has created at the Institute of Human Genetics.

I wish to express my appreciation to Prof. Dr. K. Nayernia, who initiated this project and provided me with excellent scientific guidance, instructive ideas and theoretical discussion over the period of my PhD study.

I sincerely thank PD Dr. S. Hoyer-Fender for being my co-referee. I also extend my sincere thank you to Prof Dr. S. Poeggeler and Prof Dr. R. Heinrich for being my dissertation examiners.

I would like to thank Prof. Dr. HH. Arnold, Dr. A. Holz and especially Bomi Jung for good cooperation and discussions.

I would like to thank Prof. A. Meinhardt and E. Schneider for their help in LacZ staining and evaluation of spermatogenesis defects.

I would like to thank Pawel Grzmil for scientific discussions and instructive ideas during my work.

I would like to thank Sandra for proofreading of this work and Janine for technical help.

I have to thank all members of the animal house, especially Jutta, for all their help with mice.

I would like to thank all my institute colleagues and friends for their help, their numerous advices, constant support and fantastic work atmosphere.

I would like to appreciate the current and former members of our group: Angeliki, Amal, Arvind, Birgit, Byambaa, Christian, Cornelia, Haliuna, Harald, Heike, Ilona, Jae Ho, Janine, Jessica, Karina, Katy, Krishna, Kristin, Kristina, Linda, Lukasz, Maiada, Moneef, Ogi, Ola, Sandra, Sandra, Stefan and Thanasis. Thanks for spending so much time with me in and outside the lab. I really enjoyed it!

Mein herzlichster Dank gilt meiner Familie, besonders meinen Eltern für ihre uneingeschränkte Unterstützung. Danke!!!