Expression and functional analysis of *Tex18* and *Stra8* genes in male germ cells

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D7

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ABBREVIATIONS

ABI	Applied Biosystem Instrument
AP	Alkaline Phosphate
ATP	Adenosintriphosphate
BCIP	1-bromo-3-chloropropane
bp	base pair
BSA	Bovine serum albumin
°C	Degree Celsius
CASA	Computer Assisted Semen Analysis
cDNA	complementary DNA
dATP	Desoxyriboadenosintriphosphate
dH_20	distilled water
DAPI	Diamidino-2-phenylindole dihydrochloride
dCTP	Desoxyribocytosintriphosphate
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic acid
DNAse	Deoxyribonuclease
dNTP	deoxynucleotidetriphosphate
dpc	day post coitum
dT	deoxythymidinate
DTT	Dithiothreitol
EDTA	Ethylene diamine tetraacetic acid
EGFP	Enhanced green fluorescence protein
ES	Embryonic stem
EtBr	Ethidium bromid
FACS	Fluorescence activated cell sorting
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
GST	Glutathione S-transferase
g	gravity

HBSS	Hanks' balanced salt solution
HE	heterozygote
HEPES	N-(-hydroxymethyl) piperazin, N'-3-propansulfoneacid
hr(s)	hour(s)
HO	homozygote
ICM	Inner cell mass
ICSI	Intracytoplasmic sperm injection
IPTG	Isopropyl-ß-thiogalactopyranoside
IVF	In vitro fertilization
kb	kilobase
kDa	kiloDalton
LB	Luria-Bertrani
LIF	Recombinant leukaemia inhibitory factor
LPS	lipopolysaccharides
M	molarity
Mb	Mega base pair
MOPS	3-[N-Morpholino]-Propanesulfate
mPHGPx	mitochondrial phospholipid hydroperoxide glutathione peroxidase
mRNA	messenger Ribonucleic acid
mg	milligram
ml	milliliter
µl	microliter
µm	micrometer
min	minute
NaAc	Sodium acetate
NBT	Nitro-blue tetrazolium
NCBI	National Center for Biotechnology Information
Neo	Neomycin
ng	nanogram
nm	nanometer
OAM	Outer acrosome membrane
OD	Optimal density
ORF	Open Reading Frame

PAGE	Polyacrylamide Gel Electrophoresis
PCR	Polymerase chain reaction
PGC(s)	Primordial germ cell(s)
pH	Prepondirance of hydrogen ions
PBS	Phosphatebuffersaline
PBT	Phosphatebuffersaline + Tween
PMSF	Phenylmethylsulfonyl fluoride
RNA	Ribonucleic acid
Rnase	Ribonuclease
RT	Room temperature
RT-PCR	Reverse transcriptase-PCR
RZPD	the Resource Center and Primary Database
SDS	Sodium Dodecylsulfate
SDS-PAGE	SDS-Polyacrylamide Gel Electrophoresis
sec	second
SSC(s)	Spermatogonial stem cell(s)
Stra	Stimulated by retinoic acid
SV 40	Simian Virus 40
Ta	Thermus aquaticus
TBE	Tris-Borate-EDTA-Electrophoresis buffer
TCP	Total cell protein
TE	Tris-EDTA buffer
Tex	Testis expressed
Tris	Trihydroxymethylaminomethane
U	Unit
UTR	untranslated region
UV	Ultra violet
V	Voltage
WT	wild type
X-Gal	5-bromo-4-chloro-3-indolyl-ß-galactosidase
ZP	Zona Pellucida

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

Symbols of nucleic acids

A	Adenosine
C	Cytidine
G	Guanosine
T	Thymidine
U	Uridine

1 INTRODUCTION

1.1 Spermatogenesis – short review

Infertility affects 13-18% of couples and growing evidence from clinical and epidemiological studies suggests an increasing incidence of male reproductive problems. Male factor is involved in up to half of infertile couples. Male reproductive success depends notably on the ability of the testis to produce sufficient number of normal sperm at a consistent rate during spermatogenesis. Spermatogenesis is a complex process that requires cooperation of germ cells and testicular somatic cells. Continuous spermatogenesis depends on a pool of spermatogonial stem cells (SSCs) and their ability to self renew and to differentiate. Disruption or incorrect activity of genes responsible for the regulation of this process might lead to impaired spermatogenesis, abnormal sperm function and male infertility.

Spermatogonial stem cells originate from the primordial germ cells (PGCs), which are derived from embryonal ectoderm of epiblast. In mice PGCs are recognized at 7.5 days post coitum (dpc) (Matsui, 1998; Lawson and Pederson, 1992). The PGCs proliferate and migrate from the site of origin (allantois) along the hindgut to the genital ridges, where they associate with somatic gonadal precursor cells to form gonad (Matsui, 1998). During this migration the number of PGCs increases from the initial 100 to10000 cells (Tam and Snow, 1981). Primordial germ cells can be recognized by their expression of alkaline phosphatase (Ginsburg et al., 1990). Once within the gonad, PGCs differentiate in a sex specific manner, including a distinct program of proliferation and quiescence. In male genital ridges they become enclosed by somatic supporting cells, the precursor Sertoli cells. Together they form solid strands of cells called seminiferous cords. In later development, seminiferous cords form a lumen and become seminiferous tubules. Germ cells enclosed within seminiferous cords differ morphologically from PGCs, therefore they are called gonocytes (Clermont and Perey, 1957). Gonocytes proliferate for a few days and become arrested in the G_0/G_1 phase of the cell cycle at 16 dpc (Clermont and Perey, 1957). In rats and mice they resume proliferation shortly after birth to give rise to spermatogonial stem cells (Vergouwen et al., 1991) (Fig.1.1).

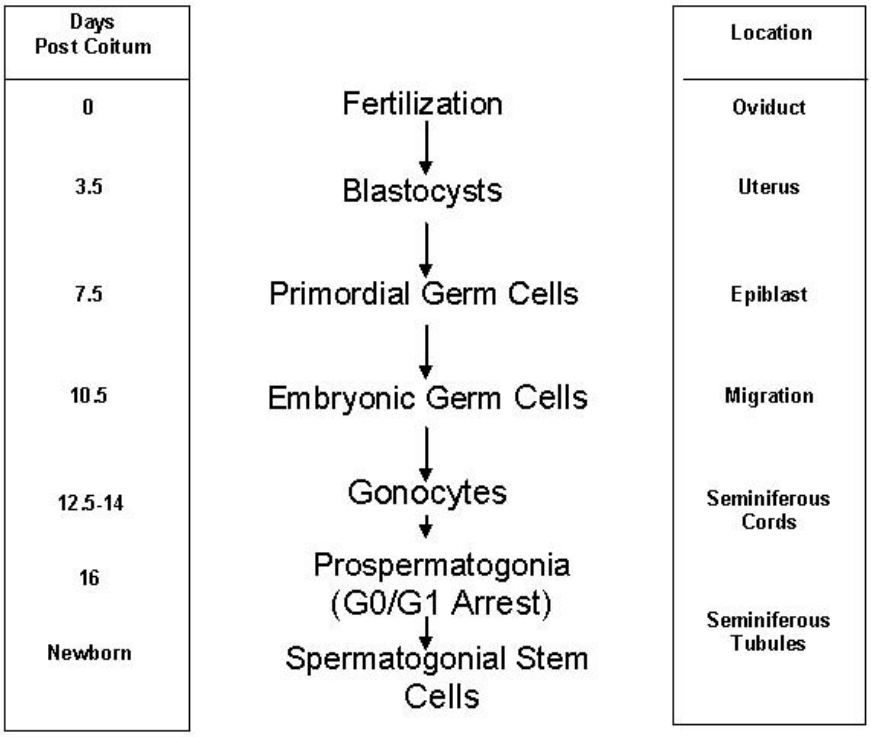


Figure 1.1 Origin and specification of spermatogonial stem cells in the mouse: The male germ line proceeds through several developmental steps prior to establishment and initiation of spermatogonial stem cell division in the testis. Temporal appearance and location of different cell types are shown in this figure (from Nayernia et al., 2004b).

According to the model proposed by Huckins (1971) and Oakberg (1971) A_s (A single) spermatogonia are believed to be stem cells of spermatogenesis. In mice there are about 35000 stem cells in one testis (Tegelenbosch and de Rooij, 1993) Upon division of the A_s spermatogonia, the daughter cells can either separate from each other and become two new stem cells, or they can remain connected by intercellular bridges and become A_{pr} (A paired) spermatogonia. Subsequently, A_{pr} cells divide into chains of 4, 8 and 16 A_{al} (A aligned) spermatogonia (de Rooij, 1998). In the normal situation, about half of the A_s cell population will self renew and half will differentiate into A_{al} spermatogonia. In such a way, the stem cell number is maintained. A_s, A_{pr} and A_{al} are sometimes called undifferentiated spermatogonia. A_{al} cells are able to differentiate into A1 spermatogonia, the first type of differentiated spermatogonia. A1 spermatogonia divide then mitotically into A2, A3, A4, In (Intermediate) and finally B spermatogonia, which divide into primary spermatocytes (de Rooij, 1998) (Fig 1.2).

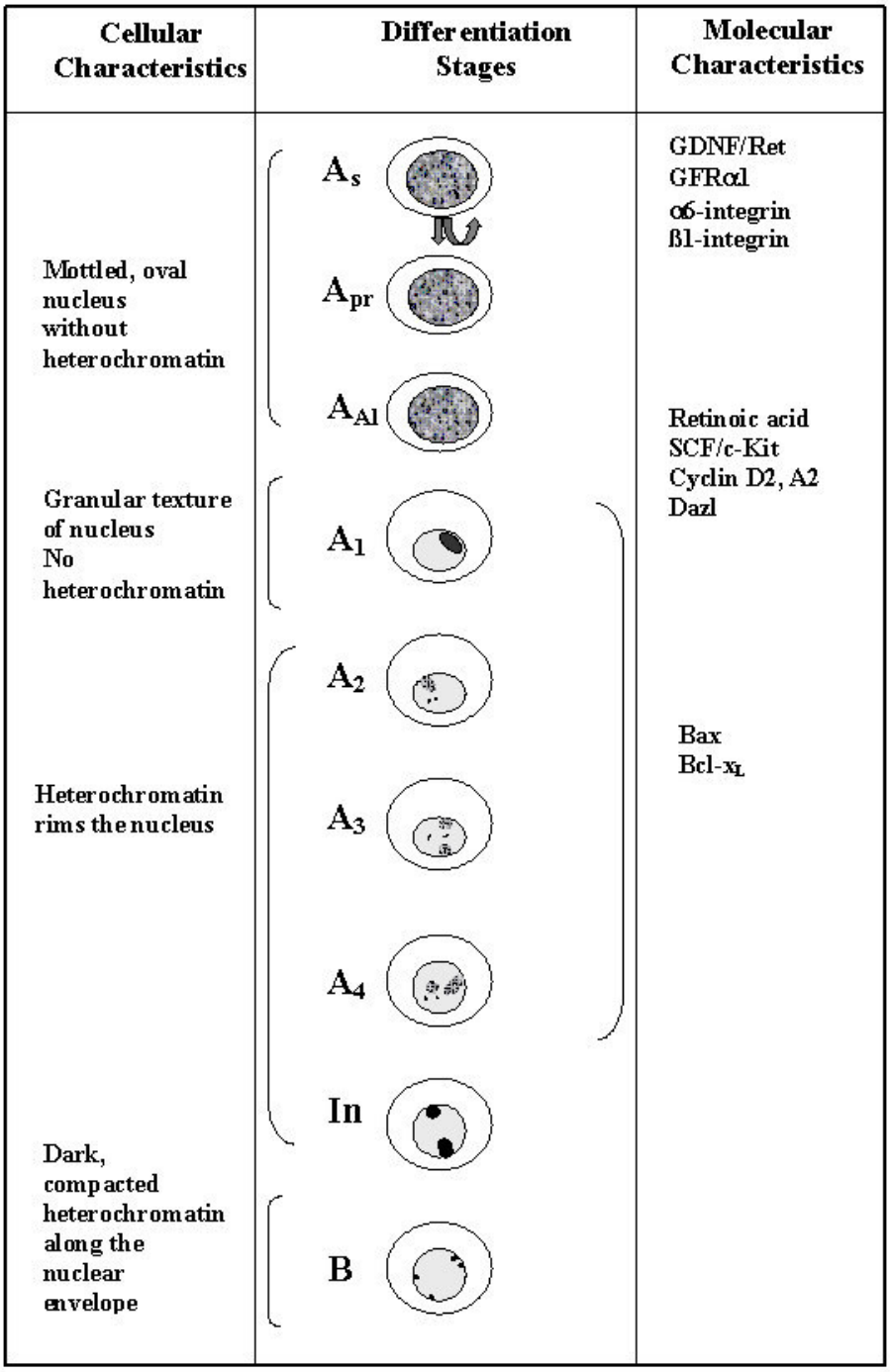


Figure 1.2 Different stages of spermatogonial stem cell differentiation and molecules, which are known to be involved in proliferation and differentiation of spermatogonial stem cells (SSC) (from Nayernia et al., 2004b).

Types of spermatogonia are described morphologically based on the appearance of heterochromatin in the cell. Undifferentiated spermatogonia show mottling of heterochromatin throughout the nucleus in the absence of heterochromatin lining the nuclear envelope. The A1 cells display finely granular chromatin throughout the

nucleus and virtually no flakes of heterochromatin along the nuclear membrane. The A2 to A4 spermatogonia contain progressively more heterochromatin rimming the nucleus. Intermediate-type spermatogonia display flaky or shallow heterochromatin that completely rims the nucleus, while type B spermatogonia show rounded heterochromatin periodically around the nuclear envelope (Oakberg, 1971; Chiarini-Garcia and Russel, 2001). Primary spermatocytes divide meiotically into round spermatids, which develop into spermatozoa in 16 steps (review Russel et. al., 1990). The interval of time between the formation of subsequent cohorts of new A1 spermatogonia is always similar in particular species and is called the duration of the epithelial cycle. In mice, the first twelve steps of spermatid development are used to divide the epithelial cycle into twelve stages. Different spermatogonia, spermatocytes and spermatid types are present at the same time at every stage of epithelial cycle. Spermatogenesis in mammals occurs in seminiferous epithelium, in seminiferous tubules composing testis. Germ cells move radially inward as spermatogenesis proceeds until spermatozoa are released into the lumen of the tubules. Spermatogenesis happens in successive waves along the wave of the tubule, each wave contains a discrete cohort of germ cells stages. The scheme of mammalian spermatogenesis is shown in Figure 1.3.

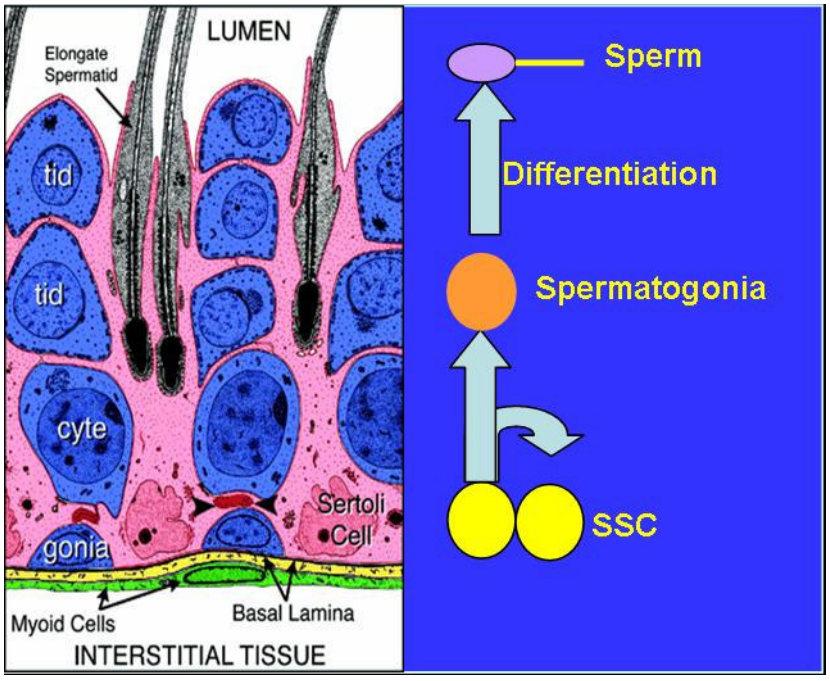


Figure 1.3 The scheme showing spermatogenesis in mammalian seminiferous tubule. Spermatogonial stem cells, localised close to the basal lamina of the seminiferous tubule can either renew themselves, or differentiate into spermatogonia. Spermatogonia move towards the lumen of the seminiferous tubule as they differentiate into spermatocytes, spermatids and finally into mature spermatozoa, which are shed into the lumen. Abbreviations are: gonia: spermatogonia, cyte: spermatocyte, tid: elongated spermatid.

1.2 Regulation of spermatogenesis

There are many genes involved in the mechanisms that regulate the number and determine the proliferation and differentiation fate of SSCs as well as of differentiating germ cells. In normal seminiferous tubules, the ratio between self-renewing and differentiating SSCs is 1.0. When self-renewing predominates, seminiferous epithelium would be reduced to stem cells only and a tumour might form. If differentiation prevails, the population of stem cells will be depleted, leading to seminiferous tubules with Sertoli cells only. Overexpression of glial cell-line derived neurotrophic factor (GDNF) produced by Sertoli cells was found to favour the self-renewal of SSCs, while low level promotes differentiation. GDNF receptors: ret and GFRα1 are expressed by spermatogonia, therefore the number of spermatogonial stem cells can be regulated by Sertoli cells (Meng et al., 2000). Differentiation of A_{al} spermatogonia is a crucial step and different molecules have been reported to be involved in this process. Mutations in the stem cell factor (SCF, expressed by Sertoli cells) and c-kit (expressed by differentiating spermatogonia) genes encoding SCF/c-kit system have a variable effect on spermatogonia. For example, mutant Sl17H/Sl17H appears to arrest at the differentiation step from A_{al} to A1 spermatogonia (de Rooij et al., 1999). Another example are mice deficient in RNA-binding protein encoded by *Dazl* (deleted in azoospermia-like) which is normally expressed in spermatogonia. In these mice differentiation of A_{al} into A1 spermatogonia does not take place. This indicates to the essential role of *Dazl* in spermatogenesis (Schrans-Stassen et al., 2001). There are other factors that have an influence on the differentiation of A_{al} to A1 spermatogonia. Spermatogenesis could be arrested at this step, like in the case of cryptorchid or juvenile spermatogonial depletion (jsd) mutant mice (de Rooij et al., 1999). Also retinoic acid is involved in the differentiation of A_{al} into A1 spermatogonia, because its deficiency

prevents A_{al} spermatogonia from differentiation (van Pelt and de Rooij, 1990a and b). The number of the spermatogonia is regulated at the stage of differentiated spermatogonia. When too much A1 spermatogonia are formed in some areas of the seminiferous tubules (which is the normal situation), an even germ cell density is achieved by apoptosis of the surplus A2, A3 and A4 spermatogonia. The Bcl-2 family plays a role in this process with some members promoting cell survival (like Bcl-2, Bcl-x_L or Bcl-w) and others promoting cell death (for example Bax, Bad, Bak and Bim) (Adams and Cory, 1998). For example, overexpression of Bcl-2 and Bcl-x_L and a deficiency of Bax cause accumulation of spermatogonia in the testis and finally apoptosis of all cells soon after the start of the meiotic prophase (Knudson et al., 1995; Furuchi et al., 1996). There are more known molecular markers for SSCs, like α6 and β1 integrins (Shinohara et al., 1999) or CD9 (Kanatsu-Shinohara, 2004) and for differentiating spermatogonia, like Notch-1 (von Schoenfeldt et al., 2004).

Over 100 genes have been shown to be involved in spermatogenesis (Escalier, 2001). Invalid function of these genes could lead, for example, to reduced number, motility and morphology of sperm. Mouse models for azoospermia (Kuo et al., 2005), asthenozoospermia (Pilder et al., 1997) or teratozoospermia (Mendoza-Lujambio et al., 2002) are widely described in literature. Generation of knock – out mice is a powerful tool to study the function of the gene which could be involved in failure of spermatogenesis. For example, *Zfp145*, encoding the transcriptional repressor Plzf, whose expression is restricted to gonocytes and undifferentiated spermatogonia, is required to regulate self-renewal and maintenance of the stem cell pool. Mice lacking the gene underwent a progressive loss of spermatogonia coming with age, increase in apoptosis and subsequent loss of tubule structure (Costoya et al., 2004).

1.3 *Tex18* and *Stra8* as novel, spermatogonia expressed genes

Many new spermatogonia expressed genes have been discovered in recent years. The functions of most of them are still unclear. *Tex18* and *Stra8* genes are examples of such testis expressed genes of unknown function; therefore they were chosen as the subject of this study.

Tex18 gene (**T**estis **ex**pressed gene **18**) was discovered by the Page group (Wang et al., 2001) in a systematic search for genes expressed in mouse spermatogonia, but not in somatic tissues. Genes expressed specifically in germ cells were identified through the "cDNA subtraction" method, where a pool of transcripts present in one cell type ("tracer") was depleted of transcripts shared with other cell types ("driver"). Tracer cDNA was generated from purified mouse spermatogonia while driver cDNA was generated from a combination of 11 somatic tissues (heart, brain, lung, liver, skeletal muscle, kidney, spleen, stomach, thymus, skin). None of the recovered known genes were specific to meiotic or post-meiotic germ cells. Authors concluded then, that spermatogonial cDNA subtraction would provide an efficient and sensitive route to the identification of germ-cell–specific genes expressed before meiosis. *Tex18* gene is 1191 bp long, consists of only one exon and contains a relatively long 5' untranslated region. It is localised on mouse chromosome 10 and its putative protein is 80 aminoacids long

Stra8 gene (**St**imulated by **r**etinoic **a**cid gene **8**) was identified by the Chambon group (Bouillet et al. 1995a) by a subtractive hybridization cloning strategy that was used to identify retinoic acid-responsive genes in P19 embryonal carcinoma cells. *Stra8* was one of the 50 genes identified in this screen, from which 40 were novel. It is 1455 bp long, contains 9 exons of different lengths, codes for a 45 kDa protein of 393 aminoacids and is localised on mouse chromosome 6. It was shown, that the *Stra8* expression is restricted to the early premeiotic spermatogonia, and the protein encoded by the gene is cytoplasmic (Oulad-Abdelghani et al. 1996).

Because both genes were described as expressed in the testis during premeiotic spermatogenesis, we hypothesised that they could take part in the regulation of the fate of SSCs. It can not be excluded, that they are involved in other ways in the process of spermatogenesis, and therefore are important for male fertility.

1.4 Aims of the study

The aims of this study were the expression and functional analysis of both *Tex18* and *Stra8* genes. Scientific approaches undertaken in this study were summarized and categorized as follows:

1. Determination of genomic structure of both genes.
2. An expression study of *Tex18* and *Stra8* using RT-PCR.
3. Generation and analysis of transgenic models, in which EGFP is expressed under the promoters of each gene.
4. Subcellular localisation of *Tex18* in the cell.
5. Efforts towards generation of antibodies against both genes and analysis of *Stra8* gene expression.
6. Functional analysis of *Tex18* and *Stra8* genes in knock – out mouse models.

2 MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals

Acrylamide/Bisacrylamide	Roth, Karlsruhe
Acetic acid	Merck, Darmstadt
Agar	Roth, Karlsruhe
Agarose	Invitrogen, Karlsruhe
Ammonium acetate	Fluka, Neu Ulm
Ampicillin	Sigma, Deisenhofen
Ampuwa	Fresenius, Bad Homburg
Bacto-tryptone	Roth, Karlsruhe
Bacto-Yeast-Extract	Roth, Karlsruhe
BCIP	Applichem, Darmstadt
Blocking powder	Roth, Karlsruhe
Boric acid	Scharlau Chemie, Barcelona
BSA	Biomol, Hamburg
Cell culture media	Invitrogen, Karlsruhe
Coomasie G-250	Sigma, Deisenhofen
Chloroform	Merck, Darmstadt
Vectashield (DAPI)	Vector, Burlingame
Diethyl pyrocarbonate (DEPC)	Sigma, Deisenhofen
Dimethyl sulfoxid (DMSO)	Merck, Darmstadt
Dithiothreitol	Sigma, Deisenhofen
DNA Markers	Invitrogen, Karlsruhe
dNTPs (100 mM)	Invitrogen, Karlsruhe
Dye Terminator Mix	Applied Biosystems
EDTA	Sigma, Deisenhofen

Ethanol	Baker, Deventer, NL
Ethidium bromide	Roth, Karlsruhe
Ficoll 400	Applichem, Darmstadt
FCS	Invitrogen, Karlsruhe
Formaldehyd	Merck, Darmstadt
Formamide	Sigma, Deisenhofen
Glutaraldehyde	Serva, Heidelberg
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	Merck, Darmstadt
IVF Media	Medicult, Berlin
1 kb DNA Ladder	Gibco BRL, Karlsruhe
0.24-9.5 RNA Ladder	Gibco BRL, Karlsruhe
KCl	Merck, Darmstadt
M16 medium	Sigma, Deisenhofen
Methanol	Merck, Darmstadt
$MgCl_2$	Merck, Darmstadt
MOPS	Applichem, Darmstadt
β-Mercaptoethanol	Serva, Heidelberg
Mineral oil	Sigma, Deisenhofen
Na azide	Sigma, Deisenhofen
Na acetate	Merck, Darmstadt
Na citrate	Merck, Darmstadt
Na deoxycholate	Merck, Darmstadt
NaCl	Merck, Darmstadt

Na_2HPO_4	Merck, Darmstadt
NaH_2PO_4	Merck, Darmstadt
NaN_3	Merck, Darmstadt
NaOH	Merck, Darmstadt
NBT	Applichem, Darmstadt
NuPAGE LDS sample buffer (4x)	Invitrogen, Karlsruhe
NuPAGE MOPS SDS running buffer	Invitrogen, Karlsruhe
Orange G	Sigma, Deisenhofen
Penicillin/Streptomycin	PAN, Aidenbach
PBS	Invitrogen, Karlsruhe
Phosphoric acid	Merck, Darmstadt
Picric acid	Fluka, Neu Ulm
Phenol	Biomol, Hamburg
Proteinase K	Applichem, Darmstadt
Protein marker	Invitrogen, Karlsruhe
Radioactive substances:	
[γ^{32}P]-ATP [α^{32}P]-dCTP	Amersham, Braunschweig
Rediprime™ II	Amersham, Freiburg
RNase Inhibitor	Boehringer, Mannheim
RNA length standard	Invitrogen, Eggenstein
RNA reagent	Biomol, Hamburg
RNase away	Biomol, Hamburg
Saccharose	Roth, Karlsruhe
Salmon sperm DNA	Sigma, Deisenhofen
SDS	Serva, Heidelberg
S.O.C Medium	Invitrogen, Karlsruhe
Triton X-100	Serva, Heidelberg
Tris	Sigma, Deisenhofen
Tween-20	Promega, Mannheim
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

2.1.2.1 Agarose gel electrophoresis

5X TBE buffe	450 mM Trisbase 450 mM Boric acid 20 mM EDTA (pH 8.0)
Glycerol loading buffer –I	10 mM Tris/HCl (pH 7.5) 10 mM EDTA (pH 8.0) 0.025% Orange G 30% Glycerol

2.1.2.2 SDS-PAGE

40% Acrylamide stock solution	Acrylamide 29.2% (w/w) Bis-acrylamide 0.8% (w/w)

10% Ammonium persulfate solution in H_20

NuPAGE LDS sample buffer (4x)

Running buffer (5x)	25 mM Tris/HCl (pH 8.3) 192 mM Glycine 0.1% SDS
Stacking gel buffer (4x)	0.5 M Tris/HCl (pH 6.8) 0.4% SDS

Separating gel buffer (4x)	1.5 M Tris/HCl (pH 8.3) 0.4% SDS

2.1.2.3 Frequently used buffers and solutions

AP buffer	100 mM Tris-HCl (pH 9.5) 100 mM NaCl 50 mM $MgCl_2$
BCIP-Solution	50 mg/ml BCIP 70% Dimethyl formamide
Blocking solution (immunostaining)	60 µl of horse serum, 150 µl of 10%Triton X-100 2790 µl of PBS
Bouin's solution	5 volume of picric acid (in H_2O) 5 volume 37% formaldehyde 1 volume acetic acid
Carrier DNA	sonicated salmon sperm DNA, 5 mg/ml
Denaturation solution	1.5 M NaCl 0.5 M NaOH
Depurination solution	0.25 M HCl
E-buffer (10x)	300 mM NaH_2PO_4 50 mM EDTA

Elution buffer1	1.5 M NaCl 20 mM Tris/HCl (pH 7.5) 1 mM EDTA
Ligation buffer (10x)	600 mM Tris/HCl (pH 7.5) 80 mM $MgCl_2$ 100 mM DTT
Lysis buffer I	100 mM Tris/HCl (pH 8.0) 100 mM NaCl 100 mM EDTA 0.5% SDS
Lysis-buffer II	100 mM Tris/HCl (pH 8.0) 5 mM EDTA 200 mM NaCl 0.2% SDS 100 µg/ml proteinase K
10 X MOPS buffer	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
NBT- Solution	75 mg/ml NBT 70% Dimethyl formamide
Neutralisation solution	1.5 M NaCl 1 M Tris/HCl (pH 7.0)

PBS buffer	130 mM NaCl 7 mM Na_2HPO_4 4 mM NaH_2HPO_4
Protein lysis buffer	150 mM NaCl 10 mM EDTA 50 mM Tris/HCl pH7.6 1% Triton X-100 1% sodium deoxycholate
Semidry transfer buffer (1x)	25 mM Tris pH 8.3 150 mM Glycin 10 % Methanol
SSC (20x)	3 M NaCl 0.3 M Na_3 citrate (pH 7.0)
Stop-Mix	15% Ficoll 400 200 mM EDTA 0.1% Orange G
TE-buffer	10 mM Tris/HCl (pH 8.0) 1 mM EDTA
Washing solution I	2x SSC 0.1% SDS
Washing solution II	0.2x SSC

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
Dialysis hoses	Serva, Heidelberg
Disposable filter Minisart	NMI Sartorius, Göttingen
Filter paper 0858	Schleicher and Schüll, Dassel
Hybond – C	Amersham, Braunschweig
Hybond - N	Amersham, Braunschweig
Microcentrifuge tube	Eppendorf, Hamburg
Petri dishes	Greiner, Nürtingen
Pipette tips	Eppendorf, Hamburg
Roti-plast paraffin	Roth, Karlsruhe
Superfrost slides	Menzel, Gläser
Transfection flask	Lab-Tek/Nalge, Nunc, IL, USA
Whatman blotting paper (GB 002, GB 003 and GB 004)	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 60 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-tryptone
	0.5% Yeast extracts
	1% NaCl
LB-Agar	1% Bacto-trypton
	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

ES-cell medium:

DULBECCO´s MEM (DMEM)

0.1 mM	Non essential amino acids
1 Mm	Sodium pyruvate
10 µM	ß-Mercaptoethanol
2 mM	L-Glutamine
20%	Fetal calf serum (FCS)
1000 U/ml	Recombinant leukaemia inhibitory factor (LIF)

Fibroblast cell medium (EmFi)

DULBECCO´s MEM (DMEM)

2 mM	L-Glutamine
10%	FCS

Tera 1 cells medium:
Mc Coy's 5a medium (Gibco)
+ L-Glutamin
10% FCS
1% penicillin/streptomycin

For long time storage of the cells in liquid nitrogen, the following freezing media were used:

ES cell – freezing medium:	30%	ES cell medium
	50%	FCS
	20%	DMSO
EmFi cells – freezing medium:	30%	EmFi cell medium
	50%	FCS
	20%	DMSO

2.1.5.3 Antibiotics

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

	Master solution	Solvent	Final concentration
Ampicillin	50 mg/ml	H_2O	50 µg/ml
Kanamycin	25 mg/ml	H_2O	50 µg/ml
G 418	40mg/ml	PBS	400 µg/ml
Gancyclovir	100 mM	PBS	2 µM
Mitomycin C	1 mg/ml	PBS	10 µg/ml

2.1.5.4 IPTG / X-Gal plate

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 strains

E. coli	DH5α	Invitrogen
E. coli	BL21 (DE3)	Novagen

2.1.7 Plasmids

pBluescript SK (+/-)	Stratagene
pBluescript KS (+/-)	Stratagene
pGEM-T Easy	Promega
pTK-Neo	Prof. N. Brose, MPI für Experimentelle Medizin, Göttingen
pPNT-M1	Prof. R. Mulligan, Children's Hospital, Boston, USA; modified by Prof . H. Hahn, Institut für Humangenetik, Göttingen
pZERO-2	Invitrogen
pEGFP-1	Clontech
pEGFP-N1	Clontech
pET 41a+	Novagen
Lawrist7	RZPD, Berlin

2.1.8 Synthetic oligonucleotide primers

The synthetic oligonucleotide primers used in this study were obtained either from Eurogentec (Köln, Germany) or Roth (Karlsruhe, Germany) and dissolved in water to a final concentration of 100 pmol/µl.

Tex18F:	5’ GAT CAT TGC TTC AGG CTA CCA 3’
Tex18R:	5’ CTT CAC TTA AAA GGA GGC AAA 3’
Stra8F:	5’ TCA CAG CCT CAA AGT GGC AGG 3’
Stra8R:	5’ GCA ACA GAG TGG AGG AGG AGT 3’
GapdhF:	5’ ACC ACA GTC CAT GCC ATC AC 3’
GapdhR:	5’ TCC ACC ACC CTG TTG CTG TA 3’
hGapdhF:	5’ CCA GCA AGA GCA CAA GAG GAA GAG 3’
hGapdhR:	5’ AGC ACA GGG ATA CTT TAT TAG ATG 3’
SryF:	5’ TCA TGA GAC TGC CAA CCA CAG 3’
SryR:	5’ CAT GAC CAC CAC CAC CAC CAA 3’
Text F2:	5’ TAG GCA GAG CTG TTT CCG CTC TGT GAT 3’
Text R2:	5’ GTT CCC CTA GCC TTC TAC CTT CTG AAC 3’
EGFPF2:	5’ CTG AAG TTC ATC TGC TGC ACC AAA 3’
EGFPR2 :	5’ TTG AAG TCG ATG CCC TTC AGC 3’
Tex18SalI :	5’ TAT TAG TCG ACA ACC CAC CTC TTA CTC TGA GC3’
Tex18ClaI :	5’ ATA GCA TCG ATG TCC TCT ATT TTC CCT GTC CC 3’
SalI 1:	5’ GGC ATT TCC CCA ACT GAA GCT CCT TTC 3’
SalI 2:	5’ CCA GGT ACT CTG CCA AGT ATC AAC CCC 3’
SalI 3:	5’ ACC CCA TCC CAC TTC GGC TCT GTT ATA 3’
SalI 4:	5’ CAT ACA GGC CTG GCT CCA ACC TGA TCT 3’
ClaI 1:	5’ CTT CCT AGT TTC CCT CTG GCC AAA CCA 3’
ClaI 2:	5’ AGA CGC CAC ATA AAA CCA GAC GTA GCC 3’
ClaI 3:	5’ AGG GGT TGA TAC TTG GCA GAG TAC CTG 3’
ClaI 4:	5’ AAG GAG CTT CAG TTG GGG AAA TGC CTC 3’

Text F2:	5’ GCA AAC GAA ATG GTT GGG AGA TGG GGG 3’
Text R2:	5’ GAA AGT CCT GTT GGT CTC TTC AGG CAG 3’
TexPCRF3:	5’ CCA TTG AAG ACA GTC TTC GGG 3’
TexPCRR3:	5’ CTC TTA CCG TAC ATC GGC TAC 3’
TexEGFPN1F:	5’ CGC GAA TTC CCT CTG TCA GGA GAA TG 3’
TexEGFPN1R:	5’ GCG GGA TCC GCA TTC ACA GTA AAC AC 3’
hStra8F2:	5’ CAG ACG ATG GAC CTT CTG ACT 3’
hStra8R2:	5’ GCT TGC CAC ATC AAA GGC ATC 3’
fpTex18F:	5’ CGG AAT TCC TGC TTT GGA ATC ATT GCC C 3’
fpTex18R:	5’ CGG AGC TCA CAA GTT CTC AGA ATT GAC G 3’
fpStra8F:	5’ TGG AAT TCA CCC CTG GAG AAG GCA ACC A 3’
hStra8FN:	5’ CAG CGC TCT TCA ACA ACC TCA GGA 3’
hStra8RN:	5’ ACC AAG GGG AGG AAC CAT TCT GAG 3’
fpStra8R:	5’ ACG AGC TCA AAG GTC TCC AGG CAC TTC A 3’
pTKNf:	5’ ATT GTC TGA GTA GGT G 3‘
pTKNr:	5’ GCG CGA ATT CGA TGA TCC TGA ACG GC 3‘
pTKR:	5’ AAC AGC TAT GAC CAT GAT TAC G 3‘
pPNTF1:	5’ GAA CAA AAG CTG GAG CTC CAC 3’
pPNTR1:	5’ CTA CCC GGT AGA ATT GAC CTG 3’
pPNTF2 :	5’ GCA GCC TCT GTT CCA CAT ACA 3’
pPNTR2:	5’ CTA AAG CGC ATG CTC CAG ACT 3’
pET41aF:	5’ GTA CTG CAA TTG GTA TGA AAG AAA CCG CTG 3’
pET41aR:	5’ GTT TAG AGG CCC CAA GGG GTT ATG 3’
StrextF:	5’ TCT CAG TGC AGA GGC AGA CAT CTC 3’
StrextR:	5’ GCC TAT GTA AAG CTC TCA GAG GCG 3’
StraPCRF:	5’ GAG AAA GGG GTC AAA GGA CAC 3’
StraPCRR:	5’ GAA AGC TCT CAC TGT AGC TGG 3’
NeoRStra2:	5’ GTT GGC TAC CCG TGA TAT TGC 3’
NeoRI:	5’ AGG AGC AAG GTG AGA TGA CAG 3’
pEGFPN1F:	5’ CGG TGG GAG GTC TAT ATA AGC 3’
pEGFPN1R:	5’ CTG AAC TTG TGG CCG TTT ACG 3’
T7:	5’ TAA TAC GAC TCA CTA TAG GG 3’

T3:	5’ ATT AAC CCT TCA CTA AAG 3’
SP6:	5’AGG TGA CAC TAT AGA ATA C 3’
ST F1:	5’GAT CTT TTA TAC ACA AGT CAT AGC 3’
ST R1:	5’ GTG GTA CAG AAC TTA GGT GTT TAA TTG 3’
Stra8tr F:	5’ AGT TGA GCT CTG GAA ACC CAC AAC GAA AGG C 3’
Stra8tr R:	5’ CAT TGT CGA CGA TGC ACA GAT CCT CTA GGA G 3’
Tex18tr F:	5’ AGT TGA GCT CTG CTG ACT TTG GTA CTC TCC T 3’
Tex18tr R:	5’ CAT TGG TAC CTA CCC TTA GGA AGG TGA GCT T 3’

2.1.9 cDNA probes

β-actin cDNA	Clontech
Neo probe	generated in present study
Tex18 probe	generated in present study
Stra8 3’ cDNA probe	generated in present study
Stra8 5’ cDNA probe	generated in present study
Tex18 external probe	generated in present study
Stra8 external probe	generated in present study

2.1.10 Eukaryotic cell lines

RI mouse embryonic stem cell line (Passage 11)	Dr. A. Nagi, Toronto, Canada
NIH 3T3	Institut für Humangenetik, Goettingen
Tera1	ATCC, Rockville, USA

2.1.11 Mouse strains

Mouse strains C57BL/6J, 129/Sv, and FVB 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

Rabbit anti mouse Stra8 peptid	Eurogentec
Rat 1D4B anti α-lamp1	Developmental Studies Hybridoma Bank
Rabbit polyclonal anti-GST	Novagen
Rabbit anti OAM	Sigma
Mouse anti mPHGPx	Sigma
Mouse anti α-tubulin	Sigma
Goat anti-rabbit FITC and Cy3-conjugated	Sigma
Sheep anti-mouse Cy3-conjugated	Sigma
Goat anti-rabbit alkaline phosphatase conjugated	Sigma

2.1.13 Enzymes

Alkaline phosphatase	New England Biolabs
Collagenase	Sigma
DNAse I Amplification Grade	Invitrogen
Klenow Fragment	Invitrogen
Platinum Taq polymerase	Invitrogen
Pfx Platinum polymerase	Invitrogen
Proteinase K	Sigma
Restriction enzymes (with supplied buffers)	Invitrogen, NEB
RNase A	Qiagen

Rnase H	Invitrogen
Rnase inhibitors	Invitrogen
Superscript-II	Invitrogen
Taq Polymerase	Invitrogen
T4 DNA ligase	Promega
T4 RNA ligase	Invitrogen
Trypsin	Invitrogen

2.1.14 Kits

Bug Buster GST-bind purification kit	Novagen
CLONfectin	Clontech
Dye Terminator Cycle Sequencing-Kit	Applied Biosystem
DYEnamic ET-Terminator mix	Amersham Pharmacia
Endo Free Plasmid Maxi Kit	Qiagen
Invisorb Forensic Kit I	Invitek
Megaprime DNA Labeling Kit	Amersham Pharmacia
Maxi Plasmid Kit	Qiagen
Mega Plasmid Kit	Qiagen
Mini Plasmid Kit	Qiagen
QIAEX II	Qiagen
QIAquick Gel Extraction Kit	Qiagen
QIA shredder kit	Qiagen
Rediprime™ II Random Prime Labeling System	Amersham Pharmacia
RNeasy Minikit	Qiagen
pET GST Fusion Systems 41	Novagen
SulfoLink Kit	Pierce

2.1.15 Instruments

ABI 3100 Genetic Analyzer	Applied Biosystem
Autoclave	Webeco
Centrifuge 5415 D	Eppendorf
Centrifuge 5417 R	Eppendorf
Biophotometer	Eppendorf
Biofuge 13	Heraeus
FACStar Plus	Becton Dickinson
FACScan	Becton Dickinson
GeneAmp PCR System 9700	Perkin Elmer
Histocentre 2 embedding machine	Shandon
Megabace 1000 Sequencer	Amersham
Megafuge 1.0 R	Heraeus
Microscope BX60	Olympus
Microtom Hn 40 Ing.	Nut hole
Microplate-Reader, Model 450	BioRad
Neubauer cell chamber	Schütt Labortechnik
Power supply	Gibco BRL
Refrigerated Superspeed Centrifuge RC-5B	Sorvall
Semi-Dry-Blot Fast Blot	Biometra
Spectrophotometer Ultraspec 3000	Amersham Pharmacia
SpeedVac concentrator SVC 100H	Schütt Labortechnik
Thermomixer 5436	Eppendorf
Turboblotter™	Schleicher & Schüll
UV Stratalinker™ 1800	Leica
X-Ray Automatic Processor Curix 60	Agfa

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 (mini – prep)

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. 0.5 ml of this culture was used for making glycerol stock (0.5 ml of culture and 0.5 ml of glycerol) and rest 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 4°C. The supernatant was transferred into a new tube and centrifugation was done again. The supernatant was transferred again into a new tube and 1 ml of 100% ethanol was added to precipitate the DNA. It was then stored in 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 µl of sterile water.

P1: 50 mM Tris-Cl, 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 preparation of plasmid DNA (midi - prep)

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 1:100 fold into 100 ml LB medium with appropriate antibiotic and incubated overnight at 37°C with shaking. The culture was centrifuged then at 6000 x g for 15 min. The pellet was resuspended in 4 ml of solution P1 and cells were then lysed with 4 ml of P2 and incubated on ice for 5 min. 4 ml of P3 buffer was added, mixed and incubated on ice for 15 min. 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 thus allowing 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, 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
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 (LPS), are cell membrane components of Gram-negative bacteria (e.g. *E.coli*). During lysis of bacterial cells for plasmid preparations, endotoxin molecules are released from the outer membrane into the lysate. Endotoxins strongly influence transfection of DNA into primary cells and 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 QIAfilter 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 endotoxin unit per µg plasmid DNA.

2.2.1.2 Isolation of genomic DNA from tissue samples

(Laird et al., 1991)

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

The method was performed according to Laird et al. (1991). 1 to 2 cm of the tail from a mouse was incubated in 700 µl of lysis buffer I containing 30 µl Proteinase K (10 µg/µl) at 55°C for overnight in Thermomixer 5436. To the tissue lysate, equal volume of phenol was added, mixed by inverting several times, and centrifuged at 10000 x g at RT for 5 min. 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 alone. Finally, the DNA was precipitated with 700 µl of isopropanol, washed with 500 µl of 70%

ethanol, dissolved in 100-200 µl of sterile water and incubated at 60°C for 10 - 20 min. DNA was then stored at 4°C.

2.2.1.3 Isolation of genomic DNA from sperm

Invisorb Forensic Kit I was used for the isolation of DNA from sperm. Mouse sperm was transferred to 2 ml centrifuge tube and 1 ml of lysis buffer was added. Probe was incubated at RT for at least 2 hrs. Then probe was centrifuged at 10000 x g for 30 sec and supernatant was transferred into new tube. 15 µl of carrier suspension was added, tube was vortexed briefly and incubated at RT for 5 min. After 1 sec centrifugation and removing of supernatant, pellet was washed twice with wash buffer at 6000 x g. Dried pellet was resuspended in elution buffer and incubated in 60°C for 5 min. After 2 min centrifugation at 10000 x g DNA containing supernatant was transferred into a new tube.

2.2.1.4 Isolation of genomic DNA from ES cells

Lysis buffer II:	100 mM Tris/HCl (pH 8.0)
	5 mM EDTA
	200 mM NaCl
	100 µg/ml Proteinase K
	0.2% SDS

To isolate the DNA from ES cells, cells in a 24 well plate were washed with PBS and incubated overnight in 500 µl lysis buffer II at 55°C. Equal volume of isopropanol was added and mixed for 15 min to precipitate the DNA. After washing with 70% ethanol, the DNA was transferred into a microcentrifuge cup containing 80 µl sterile water and incubated at 60°C for 10 –20 min.

2.2.1.5 Isolation of total RNA from tissue samples and cultured cells

(Chomczynski and Sacchi, 1987).

Total RNA isolation reagent is an improved version of the single-step method for total RNA isolation described first by Chomczynski and Sacchi (1987). The composition of reagent includes phenol and guanidine thiocyanate in a monophase solution. In order to avoid any RNase activity, homogeniser used for RNA isolation was previously treated with RNase away and DEPC water and special RNase free Eppendorf cups were used during the procedure. 100 mg tissue sample was homogenised in 1 ml of RNA reagent by using a glass-teflon homogeniser. The sample volume should not exceed 10% of the volume of reagent used for the homogenisation. The homogenate was vortexed and incubated on ice for 5 min to permit the complete dissociation of nucleoprotein complexes. Then, 0.2 ml of chloroform was added, vortexed and incubated on ice for 5 min. After centrifuging at 800 x g for 15 min at 4°C, the colourless upper aqueous phase was transferred into a new tube. 500 µl of isopropanol was added, solution was mixed by vortexing and RNA was precipitated by centrifugation at 10000 x g for 1 min. Finally, the pellet was washed with 75% ethanol, and dissolved in 50-100 µl DEPC-H_2O. The RNA was stored at –80°C. To isolate total RNA from cultured cells, 350 µl of reagent was added to the 6 cm diameter petri dish. Cells were collected with a rubber stick and the lysate was transferred into a QIA shredder in 2 ml cup. Probe was then centrifuged for 2 min at 10000 x g in order to homogenise. 350 µl of 70% ethanol was added and mixed. Mixture was put in RNeasy mini spin column, centrifuged for 15 sec at 6000 x g and washed with 700 µl of RW1 buffer. Filter was put into new cup, 500 µl of RPE was added and centrifuged for 2 min. at 6000 x g. After removing of supernatant, RPE washing was repeated. Finally, filter was put into new 1.5 ml cup, 30- 50 µl of DEPC treated H_2O was added and centrifuged for 1 min at 6000 x g.

2.2.2 Determination of the 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 230, 280, and 320 nm. The concentration was calculated according to the formula:

$C = (E\ 260 – E\ 320)\ fc$

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 mixture 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 base pairs to more than 50 kilobases, depending on the concentration of the agarose and the precise nature of the applied electrical field (constant or pulse). Usually, 1 g of agarose was added in 100 ml 0.5x TBE buffer, boiled in the microwave to dissolve the agarose, then cooled down to about 60°C before adding 3 µl ethidium bromide (10 mg/ml). This 1% agarose gel was poured into a horizontal gel chamber. 0.5x TBE buffer was used also as electrophoresis buffer. Before loading the samples, about 0.1 volume of loading buffer was added and mixed. The samples were then loaded into the wells of the gel and electrophoresis was carried out at a steady voltage (50 – 100 V). Size of the DNA fragments on agarose gels was determined using 1 kb DNA ladder, which was loaded with samples in parallel slots. DNA fragments were observed and photographed under UV light.

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. 2 g of agarose was added to 20 ml of 10x MOPS buffer and 148 ml of DEPC water and dissolved by heating in microwave oven. After cooling it to about 50°C, 33.2 ml of formaldehyde (37%) was added, stirred and poured into a horizontal gel chamber. RNA samples were treated as follows:

10 – 20 µg RNA
2 µl 10 x MOPS Buffer
3 µl Formaldehyde
7 µl Formamide (40%)
1 µl Ethidium bromide
5 µl Loading buffer

Samples were denatured at 65°C for 10 min and chilled on ice before loading into the gel. The gel was run at 30 V at 4°C overnight. To determine the size of the nucleic acid fragments on agarose gels, molecular weight ladder (0.24 – 9.5 RNA ladder) was loaded with samples in parallel slots.

2.2.3.3. SDS-PAGE for the separation of proteins

(Laemmli, 1970)

SDS-Page (Sodium Dodecylsulfate-Polyacrylamide Gel Electrophoresis) gel electrophoresis can be used for separating proteins for analysis and molecular weight determination. The proteins are denatured and rendered monomeric by boiling in the presence of reducing agents (β-mercaptoethanol or dithiothreitol) and negatively charged

detergent (SDS). The proteins, which normally differ according to their charges, are all coated with the SDS molecules, which are negatively charged. Hence, all the proteins in the sample become negatively charged and achieve constant charge to mass ratio. In this way, the separation is according to the size of the proteins. A SDS-PAGE consists of two gels; firstly, a 10-12 % separating gel was poured. In order to achieve a smooth boundary between separating and stacking gel, the separating gel was covered with a layer of water. After polymerisation of the separating gel, a 4 % stacking gel was poured over it. The samples were heated in 70°C in NuPage LDS sample buffer for 10 min before loading into the gel. The gel was run in NuPage MOPS SDS running buffer at 15 mA for 1 hr, then at a constant current of 30 mA.

2.2.4 Isolation of DNA fragments from agarose gel

2.2.4.1 Glass silica method

(Vogelstein and Gillespie, 1979)

For the isolation of DNA fragments of 300-4000 base pairs (bp) in length from agarose gels, the QIAEX II Gel Extraction System kit from Qiagen was used. The principle of this method depends on the binding capacity of DNA to silica in high salt concentrations and elution in low salt solutions. After separation of DNA on an agarose gel, the DNA fragment to be isolated was excised with a razor blade and weighed. DNA isolation was performed according to protocol in QIAEXII handbook supplied with the kit.

2.2.4.2 QIAquick gel extraction method

This method is designed to extract and purify DNA of 70 bp to 10 kilobase pairs (kb) in length from agarose gels. Up to 400 mg agarose can be processed per spin column. The principle of this method depends on selective binding of DNA to uniquely designed silica-gel membrane. Excised DNA fragment in agarose was isolated as described in QIAquick Spin Handbook supplied by producer (Qiagen).

2.2.5 Enzymatic modifications of DNA

2.2.5.1 Digestion of DNA using restriction enzymes

Restriction enzymes are class of bacterial enzymes that cut DNA at specific sites. In bacteria their function is to destroy foreign DNA, such as that of bacteriophages. This attribute of restriction endonucleases are widely utilized in molecular biology. 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 that specific enzyme. Standard digestions include 2-10 U enzyme per microgram of DNA. Reactions were usually incubated for 1-3 hrs to ensure complete digestion at the optimal temperature for enzyme activity, which was typically 37°C. However, for genomic DNA digestion the reaction solution was incubated overnight at 37°C.

2.2.5.2 Ligation of DNA fragments

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

30 ng vector DNA (digested)
50-100 ng 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
Blunt-end ligations were carried out at 16°C for overnight, whereas overhang-end ligations were carried out at 4°C overnight.

2.2.5.3 Dephosphorylation of 5’ ends of DNA

To prevent the recircularization of plasmids without insertion of DNA, in case when only one restriction enzyme was used, alkaline phosphatase treatment was performed. Alkaline phosphatase catalyses the hydrolysis of 5'-phosphate residues from DNA. The following components were mixed: 1-5 µg vector DNA, 5 µl 10 x reaction buffer, 1 µl alkaline phosphatase (1 U) in a total volume of 50 µl and incubated at 37°C for 30 min. Subsequently the reaction was stopped by heating at 85°C for 15 min. The dephosphorylated DNA was purified by phenol/ chloroform extraction and ethanol precipitation.

2.2.5.4 TA-Cloning

(Clark, 1988; Hu, 1993)

Taq and other 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, pGEM-T Easy vector systems that has 5’ T overhangs were used. The followings were mixed:

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

The content was mixed by pipetting and the reaction was incubated for 1 hr at RT. For transformation of the ligation reaction, DH5α competent cells were used (Invitrogen).

2.2.5.5 Filling-up reaction

(Costa and Weiner, 1994)

To make blunt-end from overhang-end 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 bacteria

(Ausubel et al., 1994)

Transformation of the bacteria was done by gently mixing one aliquot of competent bacteria (50 µl) with 10 µl of ligation reaction. After incubation for 35 min on ice, bacteria were heat shocked for 20- 60 sec at 42°C and cooled down for 2 min on ice. After adding 600 µl of S.O.C. medium, bacteria were incubated at 37°C with shaking for 1 hr to allow recovery of heat shocked bacteria. They were then plated out on LB-agar plates containing appropriate antibiotic (50µg/ml), and whenever required, 1 mM IPTG and X-Gal 40 mg/ml were added for "Blue-White" selection.

2.2.7 Polymerase Chain Reaction (PCR)

The polymerase chain reaction (PCR) is one of 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 amplification of DNA fragments

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

1 µl	DNA
1 µl	forward primer (10 pmol)
1 µl	reverse primer (10 pmol)
1 µl	10 mM dNTPs
5 µl	10x PCR buffer
1.5 µl	50 mM $MgCl_2$
1 µl	*Taq* DNA Polymerase (5U/µl)
Up to 50 µl	H_2O

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

Initial denaturation	95°C	5 min	
Elongation	95°C	30 sec (denaturation)	30-35 cycles
	55°C - 65°C	30-45 sec (annealing)	
	72°C	1-2 min (extension)	
Final extension	72°C	10 min	

2.2.7.2 Reverse transcription PCR (RT-PCR)

RT-PCR is a technique which generates cDNA fragments from RNA templates and thereafter amplify it by PCR. It is very useful to determine the expression of genes in specific tissues or in different development stages. 1-5 µg of total RNA was mixed with 1 µl of oligo $(dT)_{18}$ primer (10 pmol/µl) and sterile water was added to 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 70°C for 10 min and then quickly chilled on ice. After a brief centrifugation, the followings were added to the mixture:

4 µl	5x First strand buffer
2 µl	0.1 M 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) was added and further incubated at 42°C for 50 min for the first strand cDNA synthesis. Then, the reaction was inactivated by heating at 70°C for 15 min. One µl of the first strand reaction was used for the PCR reaction (as described above).

2.2.8 Generation of constructs for recombinant fusion proteins

A number of systems exist for overexpressing specific polypeptides in bacterial cells. Fusion vectors facilitate the purification of the required protein. The sequence of interest is cloned behind the gene for a protein such as glutathione-S-transferase (GST), and introduced into bacteria. Commercial vectors are available for the production of fusion constructs. Expression is usually under the control of an inducible promoter. Production of the fusion protein is induced by the addition of IPTG, and purification from bacterial protein is aided by a property of the fusion partner. GST will bind to glutathione-sepharose

beads. The fusion pair is eluted from the immobilized matrix by the addition of reduced glutathione.

2.2.8.1 Production of GST-*T*ex18 and GST-Stra8 fusion protein construct.

2.2.8.1.1 Amplification of *Tex18* and *Stra8* cDNA.

Tex18 and *Stra8* cDNA fragments (195 and 1167 bp, respectively) were amplified using primers with introduced convenient restriction sites for subsequent cloning (fpTex18F, fpTex18R and fpStra8F, fpStra8R). Fragments were designed to be cloned into open reading frames (ORF) of GST gene. PCR was performed using *Platinum Pfx* DNA polymerase with proof-reading activity to avoid mismatches in amplification. PCR conditions:

94°C	5 min	
95°C	30 sec	3 cycles
61°C	30 sec	
72°C	1 min	
95°C	30 sec	3 cycles
59°C	30 sec	
72°C	1 min	
94°C	30 sec	30 cycles
56°C	30 sec	
72°C	1 min	
72°C	10 min	

2.2.8.1.2 Sub-cloning and sequencing of PCR products.

PCR products were digested with *Eco*RI and *SSt*I enzymes (restriction sites for these emzymes were introduced in primers sequences) and then purified from a 1% agarose gel by QiaQuick method (2.2.4.2). The purified fragments were ligated between the *Eco*RI and *SSt*I sites of pET 41a+ expression vector and constructs were transformed into competent *E.coli* DH5α cells. Plasmid DNA from few colonies was sequenced with vector specific primers (pET41aF and pET 41aR) to confirm correct ORF and to check for mismatches. One GST-Tex18 clone, which showed no mismatches comparing to *Tex18* cDNA, was chosen for further work. Though checking of dozens of GST-*Stra8* clones, no clone without mismatches was found. Therefore production of GST-*Stra8* fusion protein was not continued.

2.2.9 Expression of recombinant proteins.

2.2.9.1 Preparation for induction of expression

Plasmid with fusion protein construct GST-*Tex18* was isolated from DH5α cells and transfected into expression host bacterial strain *E.coli* BL21 (DE3). Single colony of bacteria containing vector with fusion construct was picked from a freshly streaked plate and inoculated in 50 ml LB culture with kanamycin / ampicillin.

2.2.9.2 Sample induction protocol

Bacterial cell culture was incubated with shaking at 37°C until OD600 reached 0.4–1. Uninduced sample was removed for control (see total cell protein sample protocol). To the remainder, IPTG from a 100 mM stock was added to a final concentration of 0.4 mM and incubation was continued for 2–3 hrs. Induced sample was removed (see total cell protein sample protocol), flasks were placed on ice for 5 min and then cells were harvested by

centrifugation at 5000 x g for 5 min at 4°C. They were then resuspended in 0.25 culture volume of cold 20 mM Tris-HCl pH 8.0, and centrifuged as above. Finally supernatant was removed and cells were stored as a frozen pellet at –70°C or used directly for purification according to BugBuster method (see below).

2.2.9.3 Analysis of protein from bacterial cell cultures.

2.2.9.3.1 Total cell protein (TCP) sample

The expression of target genes was assessed by analysis of total cell protein on a SDS-polyacrylamide gel followed by Coomassie blue staining. Prior to harvesting the cells, 1 ml sample of well-mixed culture was taken and centrifuged at 10000 x g for 1 min. Pellet was resuspended by mixing in 150 µl of 1x phosphate-buffered saline (PBS). 50 µl of 4x sample buffer and fresh DTT was added and sample was sonicated for 20 sec. Proteins were heated for 10 min at 70°C to denature and then stored at –20°C until SDS-PAGE analysis. Detection of the expressed protein was achieved by Western blotting.

2.2.9.3.2 Preparation of cell soluble fraction extracts with BugBuster™ protein extraction reagent.

Bacteria were harvested from liquid culture by centrifugation at 6500 x g for 5 min. Pellet was drained to remove as much liquid as possible. Cell pellet was resuspended in the BugBuster at room temperature, using 2 ml reagent for cells from a 50 ml culture. Cell suspension was subsequently incubated on a shaking platform at a slow setting for 10 min at RT. Insoluble cell debris was removed by centrifugation at 16000 x g for 20 min at 4°C. Supernatant (soluble extract) was transferred to a fresh tube and sample was subjected to SDS-PAGE electrophoresis.

2.2.10 Protein methods

2.2.10.1 Isolation of total protein

Proteins were extracted from fresh or frozen mouse tissues by homogenization in protein lysis buffer (150mM NaCl, 10mM EDTA, 50mM Tris/HCl, pH7.6, 1% Triton X-100 and 1% sodium deoxycholate) containing protease inhibitors (1μg/μl leupeptin, 3μg/μl aprotinin, 1μg/μl pepstatin). Lysates were sonicated on ice (about 20 impulses) and centrifuged at 1200 x g for 10 min at 4°C. Supernatant, containing membranes, organelles and cytosolic proteins was collected and stored at –80°C, or used immediately for Western blot.

2.2.10.2 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 bovine serum albumin (BSA) stock solution of 1 mg/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 and filtered through 0.45 µm filters. 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 colour reagent. The absorption of the colour reaction was measured at 595 nm in a microplate reader (Microplate Reader 450, Bio-Rad).

2.2.11 Blotting techniques

2.2.11.1 Southern blotting of DNA to nitrocellulose filters

(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 for 20 min with 0.25 M HCl for depurination.

It was followed by denaturation solution for 45 min and 1 hr in neutralization solution. The transfer of the DNA to the nitrocellulose membrane was done in a Turbo-Blot-apparatus (Schleicher & Schüll, Dassel). About 20 Whatman filter papers (GB 003) were layered on a Stack Tray , then 4 Whatman filter papers (GB 002) on it and finally 1 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 covered with 3 Whatman filter papers GB 002 soaked with 2 x 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 for overnight. Finally, after disassembling of the blot, the filter was washed briefly in 2 x SSC, air-dried and the DNA was fixed onto the filter by either baking it at 80°C for 2 hrs or by UV-crosslinking in UV Stratalinker 1800.

2.2.11.2 Dot blot of DNA to nitrocellulose filters (colony hybridization)

Filter with colonies of bacteria was taken from agarose plate and put on the Whatman paper soaked with 10% SDS for 3 min (with colonies on the external side). Subsequently, filter was put on the Whatman papers soaked with denaturation solution (0.5 M NaOH, 1.5 M NaCl) and neutralizing solution (1.5 M NaCl, 0.5 M Tris-HCl, pH 7.4) for 5 min. Finally, filter was washed in 2 x SSC for 1 min, dried on air for 30 min and baked in 80°C for at least 2 hrs.

2.2.11.3 Northern blotting of RNA onto nitrocellulose filters

For the transfer of RNA onto a nitrocellulose filter, the same procedure as described above (2.2.11.1) was performed. In this case, however, the gel does not need to be denatured, but was transferred directly onto the nitrocellulose filter, as described in section 2.2.11.1.

2.2.11.4 Western blotting of protein onto PVDF membrane

(Gershoni and Palade, 1982)

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

After electrophoresis of proteins on a SDS-PAGE, the gel and the PVDF membrane, which was cut at the size of the gel, was first moistened with methanol and then equilibrated in semidry transfer buffer. Six pieces of GB004 Whatman filter paper were also cut at 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. For protein dot blot, specified amounts of protein solution in different concentrations were poured onto PVDF membrane. Membrane was air-dried and used for incubation with antibodies.To assess transfer efficiency of proteins onto nitrocellulose membranes, the gel was incubated for 30 min in Coomassie blue solution at RT.

2.2.11.5 Incubation of protein-bound membranes with antibodies

The membrane was first incubated in P1 buffer with 5 % non-fat dry milk for 1 hr at RT in order to block unspecific binding sites, followed by incubation with 2% milk in P1 buffer for 5 min. Membrane was then incubated with a primary antibody at the recommended antibody dilution in P1 buffer with 2 % non-fat dry milk for overnight at 4°C. Then, the membrane was washed 4 times in P1 buffer with 2% dry milk for 5 to 10 min and then incubated with the alkaline phoshpatase conjugated secondary antibody in P1 buffer with 2% non-fat dry milk for 1 hr at RT. After this step the membrane was washed 4 times in P1 with 2% dry milk, one time in P1 without dry milk and one time in AP buffer

for 5 min at RT. Finally, the proteins on the membrane were visualized by an incubation step in the dark with 10 ml of staining solution (alkaline phosphatase substrate solution) for 15 min and rinsed with water to stop the reaction.

P1 buffer: 150 µl 5M NaCl
100µl 1M Tris/HCl pH 7.5

AP buffer: 100 mM Tris-HCl (pH 9.5)
100 mM NaCl
50 mM $MgCl_2$

Staining Solution: 66 µl NBT
33 µl BCIP
in 5 ml of AP buffer

2.2.12 "Random Prime" method for generation of ^{32}P labelled 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, dCTP, Klenow fragment (4-8 U) and random oligodeoxyribonucleotides. Firstly, 10 - 25 ng of DNA were denatured in a total volume of 46 µl at boiling water for 10 min and quick chilled in ice for 5 min. First, denatured probe was added to Rediprime™ II Random Prime Labeling System cup, and then 4 µl of [α^{32}P] dCTP (3000 Ci/mmol) was added to the reaction mixture. The labelling reaction was carried out at 37°C for 0.5-1 hr. The labelled probe was purified from unincorporated [α-^{32}P] dCTP by using microspin columns (Amersham Pharmacia).

2.2.13 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 8 ml of hybridisation solution and 150 µl of sheared salmon DNA, the membrane was incubated for 2 hrs in the hybridization oven at an appropriate temperature, which was usually 65°C. Then, the labelled probe was denatured at 95°C for 10 min, quick chilled on ice and added to the hybridisation solution, together with 150 µl of sheared salmon DNA. The hybridisation was carried out overnight in the oven. 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 and then with 0.02 x SSC at the hybridisation temperature. After drying the filter, it was sealed in plastic foil and exposed to autoradiography overnight to few days (depending on the value of radioactive signal) at -80°C. The film was developed in X-ray automatic processor Curix 60. If membrane has to be used again, it was stripped in 0.2 x SSC at 80°C, until no radioactive signal was detected.

2.2.14 Non-radioactive dye terminator cycle sequencing

Non-radioactive sequencing was performed with the Dye Terminator Cycle Sequencing-Kit (Applied Biosystem). The reaction products were analysed with automatic sequencer, model Megabace 1000 (Amersham, Freiburg). 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 1 µg plasmid DNA or 100-200 ng purified PCR product, 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: 4 min denaturation followed by 25 cycles at 95°C, 30 sec, denaturation; 55°C, 15 sec, annealing; 60°C, 1 min, elongation. After the sequencing reaction, the DNA was precipitated with 1/10 volume 3 M sodium acetate 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 into the sequence gel.

2.2.15 Generation of polyclonal antibody

2.2.15.1 Peptide analysis

Different computational tools were applied to select potential antigenic peptides. Before synthesis of the peptide, a hydrophilicity/hydrophobicity profile analysis was carried out and for further confirmation antigenicity prediction was performed. In next step, predictions of secondary structure such as β- turns and α-helices in combination with the surface probability of the protein region were the parameters which enabled us to select the best peptides. In the last step, we compared primary sequence of our protein with international data bank to select unique sequence for antibody generation. Two peptides for generation of antibody against Stra8 protein were selected and synthesised. The sequences of peptides are as follows:

Peptide EP034149: H_2N-ATP GEG NQP SDD GAP C-$COONH_2$

Peptide EP034150: H_2N –CVN TPL NQE PEP PDD D –$COONH_2$

2.2.15.2 Immunisation of rabbit

Eurogentec Company did immunisation under DOUBLE X program. Two peptides were selected and synthesised. Using modern algorithms for peptide selection, the success rate for peptide immunization can be as high as 75 %. This still means a 25 % chance of failure. Under DOUBLE X program the success rate is increased to 93.75 %. Two rabbits were immunised with 100 µg of antigen mixed with Freund's complete adjuvant in 1:1 ratio. Before injection, pre- immune sera were collected from the animals. After 14 days, first booster immunisation was performed with 1:1 ratio of antigen with Freund's incomplete adjuvant. Second booster was given after 28 days and a third booster after 56 days from

first immunisation. Final bleeding was done after 86 days from first immunisation. The antiserum was aliquoted and stored at -80°C.

2.2.15.3 Affinity purification of polyclonal antibody against peptide

For antibody purification, SulfoLink® Coupling Gel (PIERCE) was used. The gel consists of immobilized iodoacetyl on a crosslinked agarose support. SulfoLink® support binds specifically to sulfhydryls. The 12-atom spacer arm deduces steric and makes binding more efficient. This longer arm is designed for conjugating small peptides to the support.

2.2.15.3.1 Immobilization

Sample preparation buffer: 0.1 M sodium phosphate
5 mM EDTA-Na, pH6.0

Coupling buffer: 50 mM Tris
5 mM EDTA-Na, pH 8.5

The peptide (10mg) was dissolved in 1 ml of sample preparation buffer. The solution was added to vial containing 6 mg of reductant: 2-mercaptoethylamine HCl (2-MEA) and incubated at 37°C for 1.5 hrs. The mixture was cooled to room temperature and applied to the 5 ml desalting column (equilibrated with 20 ml of coupling buffer) to remove excess 2-MEA from the reduced sample. Reduced peptide was eluted from the column by applying 3 ml of coupling buffer.

2.2.15.3.2 Coupling to gel and blocking non-specific binding sites on gel

Reduced protein mixture (3 ml) was added to SulfoLink® Coupling Gel column after equilibrating with 8 ml of coupling buffer. The column was mixed at RT for 15 min and then incubated for 30 min without mixing. After that, the column was washed with 6 ml of coupling buffer. 2 ml of 0.05 M cysteine in coupling buffer was applied to the column to

block non-specific sites. The column was mixed for 15 min at RT, and then incubated for 30 min without mixing.

2.2.15.3.3 Washing and deactivation

Wash solution: 1.0 M NaCl
0.05% NaN_3

Column was washed with at least 12 ml of wash solution, then with 4 ml of 0.05% degassed sodium azide in PBS, finally, after replacing of bottom cap, 2 ml of 0.05% sodium azide in PBS was added. Column was stored at 4°C.

2.2.15.3.4 Purification

The column was equilibrated with 6 ml of PBS. The antiserum (1.5 ml sample) was applied onto the column. The column was incubated at room temperature for 1 hr. This procedure, using always 1.5 ml sample, was repeated, until whole antiserum (about 12 ml) was used. The column was washed with 12 ml of PBS. Elution was done with 8 ml of ImmunoPure®IgG Elution Buffer (Pierce). Fractions of 1 ml were collected and monitored by A280. Fractions of interest were pooled and exchange into PBS containing 0.05% sodium azide by dialysis. Purified antiserum was stored at -20°C and the column was re-equilibrated with 10 ml of PBS containing 0.05% sodium azide.

2.2.16 Histological techniques

2.2.16.1 Tissue preparation for paraffin-embedding

The freshly prepared tissues were fixed in Bouin's solution for 24 - 72 hrs to prevent the 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 let 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 (histoclear) in ratios 3:1, 1:1 and 1:3 for 30 min-1 hr 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 stored at 4°C.

2.2.16.2 Sections of the paraffin block

The paraffin blocks were pre-cut to the optimal size and clamped into the microtom (Hn 40 Ing., Nut hole, Germany). The cut-thickness of the paraffin embedded was for 3- 7 µm. The sections were floated on 40°C 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 80°C for about 15 min, to get rid off excess of paraffin. Slides were then stored at RT for further analysis.

2.2.16.3 Tissue preparation for electron microscopy

Freshly isolated testes were treated with fixation solution for 8-12 hrs in 4°C. Tissues were then washed in washing buffer for few hours and used for electron microscopical analysis.

Fixation solution: 1% paraformaldehyde
3% glutaraldehyde
in 0.1 M Cacodylat buffer, pH 7.4

Washing solution 3.4 % Saccharose
in 0.1 M Cacodylat buffer, pH 7.4

2.2.16.4 Staining of the histological sections (Hematoxylin-Eosin staining)

The stored slides with the paraffin sections were first incubated three times in histoclear (Xylol) for 3 min, followed by incubation in 100%, 96%, 80%, 70% and 50% ethanol for 2 min in each. Slides were then washed 1 min in H_2O and stained for 15 min in hematoxylin. Staining was followed by washing in running tap water for 10 min. Thereafter slides were stained with eosin (0.1% + 2% acetic acid) for 1 min, then in dH_2O for 1 min and incubated in 50%, 70%, 80%, 90%, 96% and 100% ethanol for 2 min in each. Finally they were incubated two times in histoclear (Xylol) for 3 min and closed with cover slides.

2.2.16.5 Staining of the histological sections for stage specific analysis (PAS - Hematoxylin staining)

For the stage specific histological analysis of testis sections, PAS (Periodic Acid Schiff) – Hematoxylin staining was applied. This method is used for detection of glycogens in tissues - they are stained purple while the nuclei are stained blue. Slides with the paraffin sections were deparaffinized and hydrated as described above, then they were oxidized in 0.5% periodic acid solution (0.5 g of periodic acid in 100 ml of distilled water) for 5 min, rinsed in distilled water and placed in Schiff reagent for 15 min. Subsequently they were washed in tap water for 5 minutes and counterstained with hematoxylin for 1 min**.** Finally, they were washed in tap water for 5 min, dehydrated and closed with cover slides.

2.2.17 Indirect immunohistochemistry

Fixation and subsequent treatment of mouse testis was performed as described in 2.2.16. Tissue cross sections (3-7 µm) were deparaffinized with roticlear solution (Roth) and rehydrated by descending ethanol concentrations. For immunostaining, sections were washed 3 times in PBS and were then incubated with a blocking solution (60 µl of horse serum, 150 µl of 10%Triton X-100 and 2790 µl o PBS) for 1 hr at RT. Testis sections were incubated with primary antibody (1:50 - 1:100) overnight at 4°C. Sections were then rinsed three times in PBS and subsequently incubated with secondary antibody (e.g. FITC-conjugated Ig or Cy3-conjugated Ig, 1:500; Sigma) for 1 hr at RT. After incubation with

secondary antibody sections were washed again in PBS and the nuclei were counterstained with DAPI (Vector). Immunostaining of the sections was examined using a fluorescence-equiped microscope (BX60; Olympus).

For immunostaining of sperm, suspensions were spread onto Superfrost slides, air-dried and fixed in 4% PFA for 10 min at RT, next washed twice in PBS and immunostained as described above. NIH 3T3 cells were also fixed with 4% PFA, washed in PBS and immunostained using the same method.

2.2.18 Transfection of NIH 3T3 cells with the Tex18 - EGFP construct

Approximately 4 x 10^5 fibroblast cells (NIH 3T3) were plated on a cell culture slide (Falcon) and cultured overnight in 1 ml DMEM medium containing 10% FCS and penicillin/streptomycin at 37°C and 5% CO_2. On the day of the transfection, fresh liposome solution was prepared. 90 ml of HEPES-NaCl buffer warmed to 45–55°C was added to a 100-mg aliquot of CLONfectin stock (CLONTECH laboratories. Inc) to make a final concentration of 1 mg/ml CLONfectin. After gently vortexing, mixture was placed on ice. Two solutions, A and B were prepared in sterile tubes:

Solution A:

2–4 mg Tex18-EGFP construct

100 ml Serum-free medium

Solution B:

2–8 mg CLONfectin (1 mg/ml in HEPES-Buffered Saline [HBS])

100 ml Serum-free medium

Solutions A and B were combined into one tube, mixed gently and incubated at room temperature for 10–30 min. Then 1.8 ml of serum-free medium was added to tube containing the CLONfectin/DNA solution and mixed gently. Old medium was removed from the culture and CLONfectin/DNA/media solution was applied. Cell culture slides were gently moved back and forth to distribute transfection solution evenly. Slides were incubated at 37°C for 4 hrs in a CO_2 incubator, after this time CLONfectin/DNA-containing medium was removed and cells were washed with PBS prewarmed to 37°C.

About 2 ml of fresh complete growth medium was applied and cells were incubated at 37°C. After 24 hrs cell culture slides were fixed and immunostaining was applied.

2.2.19 Techniques for production of targeted mutant mice

(Joyner, 2000)

The discovery that cloned DNA introduced into cultured mouse embryonic stem cells can undergo homologous recombination at specific loci has revolutionized our ability to study gene function in vitro and in vivo. This technique allows us to generate any type of mutation in any cloned gene. Over twenty years ago, pluripotent mouse embryonic stem (ES) cells derived from inner cell mass cells of mouse blastocysts were isolated and cultured (Martin, 1981; Evans and Kaufman, 1981). Using stringent culture conditions, these cells can maintain their pluripotent developmental potential even after many passages and following genetic manipulations. Genetic alterations introduced into ES cells in this way can be transmitted into the germ line by producing mouse chimeras. Therefore, applying gene targeting technology to ES cells in culture gives the opportunity to alter and modify endogenous genes and study their functions in vivo.

2.2.19.1 Production of targeted embryonic stem cell clones

2.2.19.1.1 Preparation of EmFi feeder layers

A frozen vial of EmFi cell was quickly thawed at 37°C and transferred to 10 ml EmFi medium. After centrifugation at 270 x g for 5 min, the cell pellet was gently resuspended in 10 ml EmFi medium and plated on a 50 mm culture flask. Cells were incubated at 37°C, 5% CO_2. When the cells formed a confluent monolayer (three days), they were either trypsinized, transferred to five 150 mm dishes and grown until they formed confluent monolayer, or directly treated with mitomycin C. To treat the EmFi with mitomycin C, the medium was removed and 10 ml fresh medium containing 100 µl mitomycin C (1mg/ml) was added. After 2-3 hrs of incubation, the monolayer of cells was washed twice with 10

ml PBS. The cells were then resuspended with 10 ml medium, and gentle pipetting dissolved any cell aggregates. The cells were centrifuged, resuspended in EmFi medium and plated onto dishes, which were treated with 0.1% gelatine for 30 min. The feeder cells were allowed to attach by incubation overnight at 37°C, 5% CO_2 or used after 2 hrs of incubation. Before adding ES cells on the feeder layer, the medium was changed to ES cell medium.

2.2.19.1.2 Growth of ES cells on feeder layer

One vial of frozen ES cells was quickly thawed and cells were transferred to a 12 ml tube containing 6 ml ES cell medium. After centrifugation, the cell pellet was resuspended in 5 ml ES cell medium and plated on 60 mm dishes containing EmFi cells at 37°C, 5% CO_2. Next day the medium was changed. The second day, cells were washed with PBS, treated with 2 ml trypsin/EDTA at 37°C, 5% CO_2 for 5 min. The cells were gently pipetted up and down to dissolve cell clumps, resuspended with 5 ml ES medium and centrifuged. The cell pellet was resuspended in 10 ml ES cell medium and distributed either to 5 or 6 dishes (60 mm) or to 2 dishes (100 mm) containing feeder layers. The cells were passaged every second day as described above.

2.2.19.1.3 Electroporation of ES cells

ES cells, which have grown for two days on 100 mm dishes, were trypsinized. The cell pellet was resuspended in 20 ml PBS and centrifuged .The cell pellet was then resuspended in 1 ml PBS. Cell suspension (0.8 ml) was mixed with 40 µg of linearized DNA-construct and transferred into an electroporation cuvette. The electroporation was performed at 240 V, 500 µF with the BIO RAD gene pulser™. After electroporation, the cuvette was placed on ice for 20 min. The cell suspension was transferred from cuvette into 20 ml of ES cell medium and plated onto two 100 mm dishes containing feeder layers. The medium was changed every two days. Two days after the electroporation, the drugs for the selection were added (active G418 at 150-250 µg/ml and gancyclovir at 2 µM). The medium was changed every day. After about eight days of selection, drug resistant colonies have appeared and were ready for screening by Southern blot analysis.

2.2.19.1.4 Analysis of recombinant ES cells

The drug resistant colonies that were formed after about eight days of selection were picked with a drawn-out Pasteur pipette under a dissecting microscope. Each colony was transferred into a 24 well plate containing feeders and ES cell medium. After 2 days, the ES cells were trypsinized for 5 min and resuspended in 500 µl ES cell medium. Half of the cell suspension in each well was transferred to a well on two different 24 well plates, one gelatinised plate, and the other containing feeder cells (master plate). The gelatinised plate was used for preparing DNA and the master plate was kept frozen. Prepared DNA was subjected for Southern blot analysis.

2.2.19.2 Production of chimeras by injection of ES cells into blastocyst.

The ability of mammalian embryos to incorporate foreign cells and develop as chimeras has been exploited for a variety of purposes including the perpetuation of mutations produced in embryonic stem (ES) cells by gene targeting and the subsequent analysis of these mutations. The standard procedure is to inject 10-20 ES cells, which are recombinant for a targeted locus into the blastocoel cavity of blastocysts that have been recovered by flushing the uteri of day 4 pregnant mice (C57BL/6J). After injection, embryos are cultured for a short period (2-3 hrs) to allow re-expansion of the blastocoel cavity, which collapses upon injection. Then the embryos were transferred to the uterine horns of day 3 CD1 pseudopregnant mice. Pseudopregnant females were obtained by mating 6-8 weeks old oestrous females with vasectomized males.

2.2.19.3 Detection of chimerism and mice breeding.

The most convenient and readily apparent genetic marker of chimerism is coat colour. Chimeric males (and sometimes females) are test bred to ascertain contribution of the ES cells to germ line. Once a germ line chimera has been identified, the first priority will be to obtain and maintain the targeted allele in living animals (inbred background). The chimeras

were bred with C57BL/6J and with 129/SvJ background mice to compare the phenotype in two different genetic backgrounds.

2.2.20 Generation of transgenic mice.

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 was based on Hogan et.al. (1986).

2.2.20.1 Preparation of DNA for pronuclear microinjection.

Transgenic constructs were released from cloning vector by restriction digestion. As it was described previously (Brinster et. al. 1985) linear form of DNA integrates more efficiently into the genome. Digested fragments were separated in agarose gel electrophoresis (without EtBr) in the way that 25 µg of digested plasmid was loaded to slots of the gel. After separation outer lanes were cut out and stained with EtBr. After staining, gel was reconstructed and appropriate gel slice was cut out from the rest of the gel under UV light. DNA was then eluted from gel with QIAquick extraction kit and filtered through 0.45 µm microfilter (Milipore). 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 4 ng/µl in microinjection buffer (10 mM Tris, pH 7.5, 0.1 mM EDTA, pH 8.0).

2.2.21 Fluorescence activated cell sorting (FACS)

Fluorescence activated cell sorting (FACS) is a method, which enables rapid separation of the cells in a suspension on the basis of size and the colour of their fluorescence. Cell suspension containing cells labeled with a fluorescent dye (or cells emitting fluorescence signal by itself, eg. expressing EGFP) is directed into a thin stream so that all the cells pass in single file. A laser beam is directed at the stream . As each labeled cell passes through the beam, its resulting fluorescence is detected by a photocell. If the signals from the detectors meet either of the criteria set for fluorescence and size, an electrical charge (+ or

-) is given to the cell. They retain this charge as they pass between a pair of charged metal plates, where they are sorted. FACS-positive and FACS-negative cells can be then analysed by PCR, immunochemistry and other methods.

2.2.21.1 Preparing of testicular cell suspension for FACS analysis

Freshly isolated testes were washed in PBS and put in dishes filled with collagenase in HBSS medium (without Ca and Mg). Tunica albuginea was removed and tubules were seperated. After 15-30 min of incubation in 37°C, tubules were washed 2-4 times in HBSS medium and centrifuged for 5 min at 150 x g each time. Cells were then incubated in 37°C in 1 M EDTA containing 0.25 % trypsin for 5 min. 10-20 % of total volume of FCS was added to stop the reaction. Cells were then filtrated in 70 µm pores Falcon filter. Filtrate was centrifuged for 5 min at 150 x g and pellet was resuspended in DMEM medium containing 10% FCS and antibiotics. Cells were then sorted on FACStar Plus (Becton Dickinson USA).

2.2.21.2 Determination of DNA content

For determination of the number of cells in different cell cycle stage (1N, 2N and 4N), EGFP positive cells and wild type cells (control) prepared as for FACS analysis were suspended in 100 µl of PBS. 1 ml of 98% ethanol was added, cells were vortexed and left at 4°C for 30 min. Cells were then centrifuged for 3 min at 350 x g, washed in PBS with 1% FCS, treated with 0.25% Triton X100 in PBS for 5 min, washed again 3 times with PBS + FCS and finally stained. DNA content was measured with propidium iodid (20 µg/ml) in PBS + FCS, with RNase A (100 µg/ml). DNA content was determined on FACScan device (Becton Dickinson, USA)

2.2.22 Determination of sperm parameters

2.2.22.1 Sperm count in epididymes, uterus and oviducts.

Epididymes of mice were dissected under aseptic condition and put in 0.5 ml of in vitro fertilization (IVF) medium. Spermatozoa were allowed to swim out of the epididymes for 1 hr at 37°C, 5 % CO_2. Sperm suspension was diluted 10 - 40 times with PBS before counting, when necessary. 5 µl of this suspension was put into Neubauer counting chamber and spermatozoa were counted in 8 independent fields (each having an area of 0.0025 mm^2) under the microscope (Olympus BX60) with 20x magnification. Total spermatozoa were calculated by following formula:

Total Sperm = average No. of sperm x 10 x 500 x B (B is the dillution)

For determination of sperm number in the uterus and the oviducts, *Tex18* deficient 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 and the spermatozoa were flushed out.

2.2.22.2 Determination of sperm abnormalities

For the determination of sperm abnormalities, sperm suspensions were spread onto Superfrost slides, air-dried and fixed in 4% PFA for 10 min at RT. Slides were then washed 1 min in H_2O and then stained 15 min in haematoxylin. Next they were washed in running tap water for approximately 10 min and finally stained with eosin (0.1% + 2% acetic acid) for 1 min and washed in H_2O for 1 min. 200 spermatozoa were counted and designed as normal or abnormal (normal or unusual sperm head shape). Percentage of abnormal sperm was determined.

2.2.22.3 Sperm motility

10 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). Then, 5000-10000 spermatozoa from 3 mice of mutant line and 2 of wild-type were analyzed using the following parameters: average path velocity (VAP), straight line velocity (VSL), curvilinear velocity (VCL),

lateral head amplitude (ALH), beat frequency (BCF) and straight forward movement (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 hr after preparation) for statistical analysis.

2.2.22.4 Acrosome reaction

Spermatozoa were isolated and capacitated by incubating for 1 hr at 37°C, 5 % CO_2. Sperms were transferred into two microcentrifuge tubes and centrifuged for 2 min at 3000 x g. The supernatant was aspirated, leaving only 50 µl for resuspension of sperms. 2.5 µl of Ionophore A23187 (final concentration 10 µm in DMSO) was added to sperm suspension, for negative control 2.5 µl of phosphoric acid (5 mM) was added and incubated at 37°C for 1 hr. The sperms were then fixed in 500 µl of 2 % formaldehyde (in PBS) for 30 min at 4°C. After completion of fixation, sperms were centrifuged at 4000 x g for 2 min. Sperms were further washed twice with 0.15 mM ammonium acetate. Finally they were resuspended in 100 µl of PBS and 30 µl of suspension was spread on superfrost slide and air-dried. The slides were stained with Coomasie G-250 in 3.5 % H_2O_2 for 2.5 min. Unbound dye was removed by washing several times with water. The slides were mounted with 30 % glycerol and observed under microscope. At least 200 sperms with and without blue head were counted. Here, blue head sperms mean those sperms which failed to undergo acrosome reaction. The acrosome reaction was calculated as follows:

$$\text{Acrosome reaction (in percentage)} = \frac{\text{Number of sperm without blue head}}{\text{Total number of sperm}} \times 100$$

2.2.23 Sperm egg binding assay

2.2.23.1 Oocyte isolation and zona pellucida removal

Mature oocytes were collected from 8-12-week-old superovulated female mice by intraperitoneal injections of 5 IU of pregnant mare serum gonadotrophin (PMSG) followed by 5 IU of human chorionic gonadotrophin. Oocytes were incubated with hyaluronidase (3 mg/ml) in embryo culture medium (M16) for 5-10 min at 37°C to dissociate cumulus cells, and washed through three 200-µl drops of fresh M16. To prepare zona-free eggs, oocytes were treated for 15 to 30 sec in acidic Tyrode's solution, pH 2.7.Oocytes were transferred into IVF medium, overlaid with light mineral oil and incubated for 1 hr at 37°C in a 5% CO_2 incubator before use.

2.2.23.2 Sperm-egg binding assay

Oocytes with or without zona pellucida were placed in 50-µl drops of IVF medium. Capacitated sperm were added to the drops at final concentration of 100000 sperm and incubated for 2.5 hrs. Eggs were washed two times in fresh medium to remove loosely attached sperm. All oocytes from one experiment were collected in 20µl of IVF medium; sperms were released by gentle pipetting and were counted as described in 2.2.21**.** Sperm number per oocyte was calculated.

2.2.24 Computer analysis

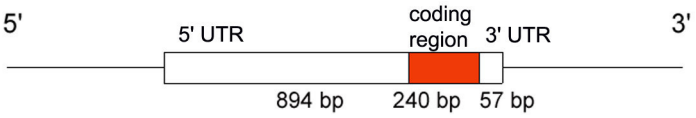
For the analysis of the nucleotide sequences, programs like BLAST, MEGABLAST and other programs from National Center for Biotechnology Information (NCBI) were used (www.ncbi.nlm.nih.gov). For restriction analysis of DNA NEBcutter V2.0 programm was used (http://tools.neb.com/NEBcutter2/index.php). Information about mouse alleles, phenotypes and strains were used from Jackson Laboratory (www.informatics.jax.org). For proteins studies ExPASy tools (www.expasy.ch) were used. Mouse genome sequence and other analysis on mouse genes, transcript and putative proteins were downloaded from Celera discovery system(www.celera.com). For statistical analysis Statistica software (Statsoft ®, http://www.statsoftinc.com) was used.

3 RESULTS

3.1 Introduction to result section of *Tex18*

Tex18 gene (**T**estis **ex**pressed gene **18** - access number NM 031385) was identified together with 24 other testis specific genes through cDNA subtraction method. (Wang et al., 2001). *Tex18* is a novel murine gene localised in mouse chromosome 10 and consisting of 1191 nucleotides, containing a long 5' UTR fragment of 894 bp and one exon of 240 bp, coding for a 80 aminoacids protein (Fig. 3.1 A). No specific domains are present in the sequence of Tex18 protein (Fig. 3.1 B).

A



B

```
   1 acaggtttcaggcttaggaagaaaggggtagtagtccaggaactctttcttcatggtagg

  61 aataacttaatagatgtgttacagttgggaatatcggatttcctctggcccaggtgtcca

 121 ggtgagcacttcaggcattactgaggaatctgtgttgctgtattactgttccgtgatgtc

 181 aaaccctgttttccacacagtataacgcaacagcatagtgtatagtattatagaccagac

 241 agcttgggcctggaattattgctcccccacacctatccctacccacacctcaggcaaaac

 301 aagcaaaaagccccaaatctacttttgagcaagagggtgtgctcaggaagaaggaacaca

 361 cagaggaagtatacttggtttttattccgaggaaggtggtaattgtatccttcccccttt

 421 ggctggggtctgaacctctgctagtctgagaattggtgcataagaaaatggaggaaggggg
                                 ________Tex18F_________

 481 aaaggagttattgctccaaagaggaaaggatcattgcttcaggctaccaaattcttagaa

 541 tagaaataggacctttgagtaaataaaagttttacttgaagtgggggggagggattaactc

 601 ctaaacacaagttttatagtatttgatagaaaaaaaatctcattatatatttcttataaa

 661 tccttcctctttttttcctttaaaacactcttttcttcaaactcattgtggttttcttt

 721 ttctgttctatcttggaataagaaactgccctggggagggagtagtacctgtttattaat

 781 agtcaactcaactatcctatgttatgttcctagaataaaaaattgttacatctatttcat

 841 taccatggtttaaggtaagctcaccttcctaagggtagttcctctgtcaggagaatgatc
                                                          M  I
                                                 _________Tex18R_______

 901 tatctgctttggaatcattgccctttgatgtgcaaaggagtttgcctccttttaagtgaa
      Y  L  L  W  N  H  C  P  L  M  C  K  G  V  C  L  L  L  S  E
     _

 961 gtatccagggtgtggggctccagaaagtattacagtgttttaaatgtagcatttgcttgt
      V  S  R  V  W  G  S  R  K  Y  Y  S  V  L  N  V  A  F  A  C


1021 aatatttgtaacatacatttaggcacttgtgaatcttctttgataattgggacacattcg
      N  I  C  N  I  H  L  G  T  C  E  S  S  L  I  I  G  T  H  S


1081 tcaattctgagaacttgttatttttttttctattttcatagtgtttactgtgaattaaaat
      S  I  L  R  T  C  Y  F  F  S  I  F  I  V  F  T  V  N


1141 ttgttatgccactgccaaaaaataaagcttactttggaacacaaaaaaaaa
```

Figure 3.1 (A) Schematic representation of *Tex18* gene. Lengths of the coding region, 5' UTR and 3' UTR are given. (B) Nucleotide sequence and deduced amino acid sequence of *Tex18*. ATG codon, indicating start of translation and TAA terminating codon are bold and coloured in blue. Positions of Tex18 F and Tex18 R primers used for expression analysis of *Tex18* are indicated and coloured in violet. Sequence from NCBI.

3.1.1. Expression analysis of *Tex18*

3.1.1.1 RT PCR analysis of *Tex18*

Expression of *Tex18* was found to be restricted to testis (Wang et al., 2001). This result was confirmed by RT-PCR, using RNA extracted from nine different tissues and primers Tex18F and Tex18R amplifying 453 bp fragment of *Tex18* gene (Fig 3.1 B) No PCR product was observed in other tissues. RT-PCR was done as well with RNA from testes of different mutants with spermatogenesis defects: W/W^V, Tfm/y, Leyl-/-, olt/olt and qk/qk. W/W^V mice are characterised by lack of any germ cell (review de Rooij and Boer, 2003), in Tfm/y and Leyl-/- mutants spermatogenesis is arrested at spermatocyte stage (Lyon and Hawkes, 1970; Zimmermann et al., 1999). In olt/olt at round spermatid stage and in qk/qk arrest at elongated spermatid stage is known (Bennett et al., 1971: Moutier, 1976). *Tex18* transcript was detectable in all of these mutants, except W/W^V – this indicates that expression starts as early as in spermatocyte stage. Because there is no expression in W/W^V mutant, it can be concluded that expression of the gene is restricted to germ cells. Expression studies were done also in postnatal and prenatal developmental stages (in prenatal stages whole embryos were used for RNA isolation). Transcript was present in all tested postnatal stages (P5 to P25) and in prenatal stages from day 15.5 dpc onward (however, earlier stages were not checked by RT-PCR). Interestingly, expression of *Tex18* was detected in ES cells, but not in early preimplantation stages (2, 4 and 8 cell stages). Taken together, these data indicate that *Tex18* gene expression starts at least in 15.5 dpc germ cells and in embryonic stem cells. (Fig. 3.2).

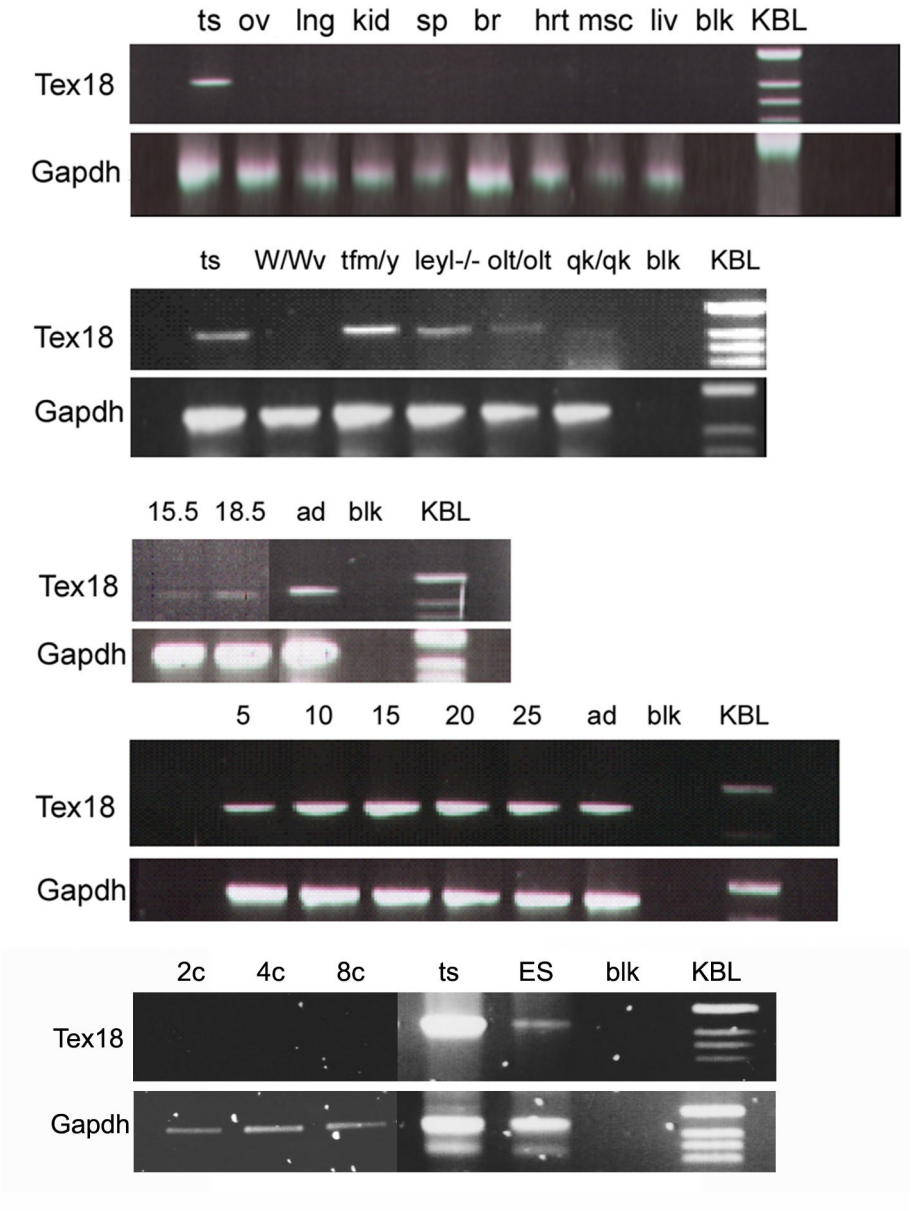


Figure 3.2 RT-PCR expression analysis of *Tex18* in different tissues; testes of mutants, pre- and postnatal developmental stages and ES cells using Tex18F and Tex18R specific primers. Expression is restricted to the testis. Expression of *Tex18* is observed in testes of all mutants except W/W^{V}, indicating that expression is restricted to germ cells. *Tex18* expression is present from 15.5 dpc onward and was observed in testes of all tested postnatal developmental stages. Expression of *Tex18* was detected also in ES cells. Gapdh served as a control. Abbreviations are: ts: testis, ov: ovary, lng: lungs, sp: spleen, br: brain, hrt: heart, msc: muscle, liv: liver, blk: blank = no - template control, ad: adult testis, 2c: 2 cell stage, 4c: 4 cell stage, 8c: 8 cell stage, ES – embryonic stem cells, KBL-standard molecular weight marker.

3.1.1.2 Translational analysis of *Tex18*

In order to analyse translation of *Tex18* RNA, efforts towards generation of antibodies against the protein were made. Tex18-GST fusion protein construct was made, as described in the section 2.2.8.1. First, pilot experiment was performed. For this purpose flask containing LB medium was inoculated with single colony of bacteria from *E.coli* BL21 (DE3) strain containing vector with fusion construct, and production of the fusion protein was induced by addition of IPTG. Total cell protein samples (see 2.2.9.3.1) of IPTG induced and not induced cultures were subjected to SDS-page electrophoresis, blotted on PVDF membrane and Western blot with antibodies against fusion tag (GST) was performed. Signal corresponding to the predicted size of fusion protein was obtained in the IPTG induced sample (Fig. 3.3).

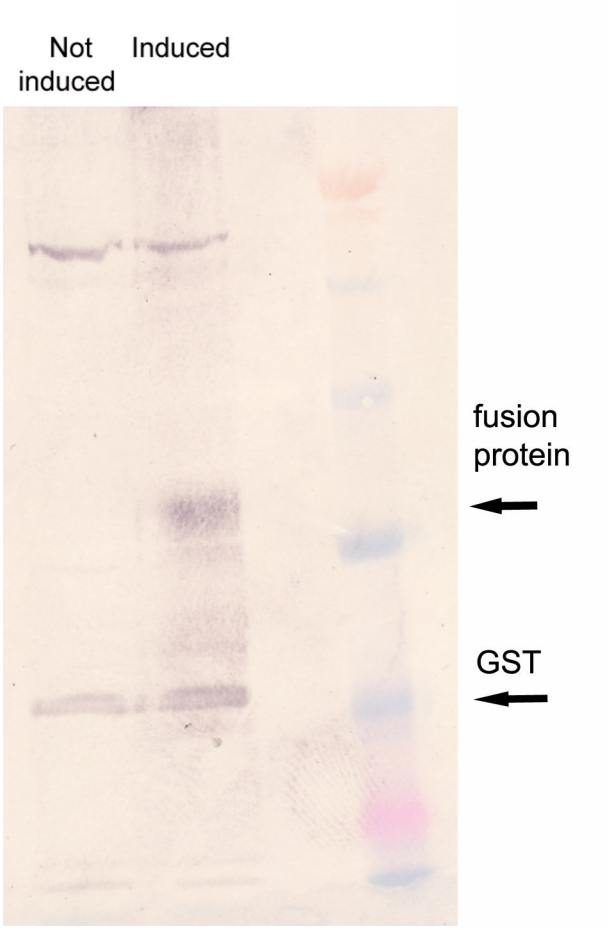


Figure 3.3 Western blot analysis of total protein sample from IPTG induced and not induced cultures of *E. coli* BL21 (DE3) strain. Anti-GST antibody recognizes fusion protein of expected size in the induced bacterial cells, while no signal was observed in uninduced probe. Lower bands in both probes indicate GST protein.

The next step was the production of Tex18-GST fusion protein in a bigger scale and purification of protein with Bugbuster™ method, as it was described in section 2.2.9. Fusion protein was produced for immunization of rabbits in order to obtain polyclonal antibody directed against Tex18 protein. Despite several trials of purification, fusion protein was always degradated after purification step, as it was shown by Western blot (Fig 3.4). Because fusion protein was not stable, it could not be used for the immunization of rabbit. Obtained antibody would show high affinity to GST, but not to Tex18-GST protein. Therefore production of *Tex18* antibody was not continued.

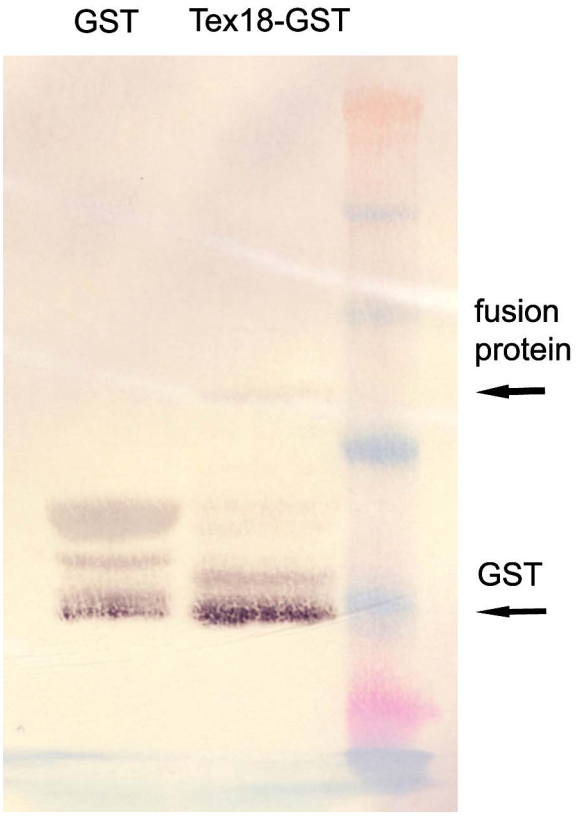


Figure 3.4 Western blot analysis of purified Tex18-GST fusion protein and GST protein (control) in large scale. Anti GST antibody recognizes GST protein in both probes, but very weak signal is visible in case of fusion protein.

3.1.1.3 Localisation of Tex18 protein in the cell.

Because generation of antibody against Tex18 protein was not successful, other method than immunochemistry had to be used for the subcellular localisation of the protein. For this purpose Tex18-EGFP fusion protein was generated. Coding region of *Tex18* gene was amplified with TexEGFPN1F and TexEGFPN1R primers and PCR product was checked for mismatches by sequencing. PCR product which showed no mutation was digested with *Eco* RI and *Bam* HI, as restriction sites for these enzymes were introduced in TexEGFPN1F and TexEGFPN1R primers sequences, respectively. Vector pEGFP N1 was digested with the same enzymes and cDNA was cloned. Tex18-EGFP fusion protein is expressed in this case under the control of CMV IE promoter (human cytomegalovirus immediate early promoter) (Fig 3.5).

Figure 3.5 Schematic representation of Tex18-EGFP fusion construct. Fusion protein is expressed under the control of CMV IE promoter.

Construct was transiently transfected into cultured NIH 3T3 fibroblast cells using Clonfectin kit, as it was described in section 2.2.18. After 24 hrs cells were fixed and observed under the microscope using UV light. Taking advantage of green signal emitted from EGFP, we were able to localise Tex18 protein, since Tex18 and EGFP build fusion protein. Tex18 is localised in the cytoplasm, however intensity of green is not the same in the whole cytoplasm and concentrates in some regions. They seemed to be lysosomes, therefore immunocytochemistry using anti-lysosomal antibody was applied. For this purpose, slides with fixed transfected cells were incubated with 1D4B antibody, directed against α-Lamp 1 – a 110 kDa lysosomal membrane glycoprotein (Chen et al., 1985). Microscopical observation proved colocalisation of Tex18-EGFP fusion protein and α-Lamp 1 protein (Fig. 3.6).

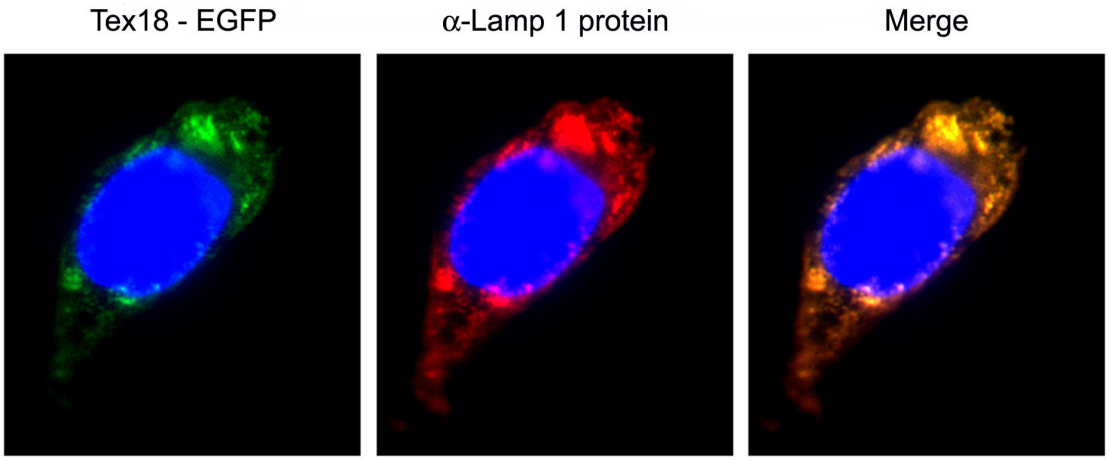


Figure 3.6 Subcellular localisation of Tex18 protein. NIH 3T3 cells were transiently transfected with Tex18 -EGFP construct and analysed under UV light (first picture). Immunocytochemistry of NIH 3T3 cells with 1D4B antibody directed against lysosomal protein α- Lamp 1 (second picture) showed colocalisation of Tex18-EGFP and α- Lamp 1 (third picture). Blue represents DAPI-stained cell nuclei.

3.1.2 Generation and analysis of *Tex18* transgenic mice

3.1.2.1 Generation of the transgenic construct and transgenic mice

For the more detailed expression studies of *Tex18*, transgenic mice were generated. For this purpose, fusion construct was designed (Fig. 3.7). A 1.6 kb promoter region of the *Tex18* gene amplified from genomic DNA with primers Tex18tr F and Tex18tr R was cloned in the pEGFP-1 vector. In this way, enhanced green fluorescent protein should be expressed under the *Tex18* promoter in transgenic mice in the same manner as endogenous *Tex18* gene, therefore promoter activity could be monitored. Green signal obtained from EGFP could be visible under UV light under microscopical observation of tissue sections. Expression of EGFP could be also detected by RT-PCR, Northern blot, Western blot and immunoassaying.

Figure 3.7 Schematic representation of Tex18-EGFP fusion gene showing 1.6 kb promoter region of mouse *Tex18* (p Tex18) linked to the coding region of EGFP.

Tex18-EGFP transgenic lines were generated by microinjection of Tex18-EGFP fragment (released from vector sequences by digestion with *Sac* I and purified after gel electrophoresis) into the pronuclei of fertilized mouse eggs. The injected embryos were transferred into FVB pseudopregnant hosts. Transgenic mice harbouring Tex18-EGFP construct were identified by Southern blot, using an EGFP cDNA probe (Fig. 3.8). Positive founder animals were bred with non- transgenic FVB mice and their transgenic progeny was crossed to produce homozygous animals. Two transgenic lines, namely Tex18/4 and Tex18/16, were generated.

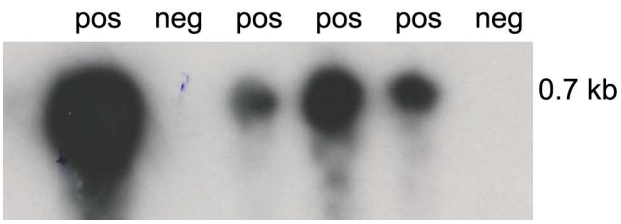


Figure 3.8 Southern blot screening of mice in order to obtain positive transgenic founders. pos: positive animal (transgene carrier), neg: negative animal (no integration has occurred).

3.1.2.2 Expression analysis of *Tex18* transgene

Northern blot hybridization performed on RNA isolated from different tissues of Tex18/4 transgenic line and using EGFP cDNA probe revealed that expression of EGFP under *Tex18* promoter is restricted to the testis, similarly like in case of endogenous gene (Fig 3.9). This result was confirmed also by RT-PCR performed on RNA isolated from both transgenic lines and using primers EGFPF2 and EGFPR2 (Fig 3.10).

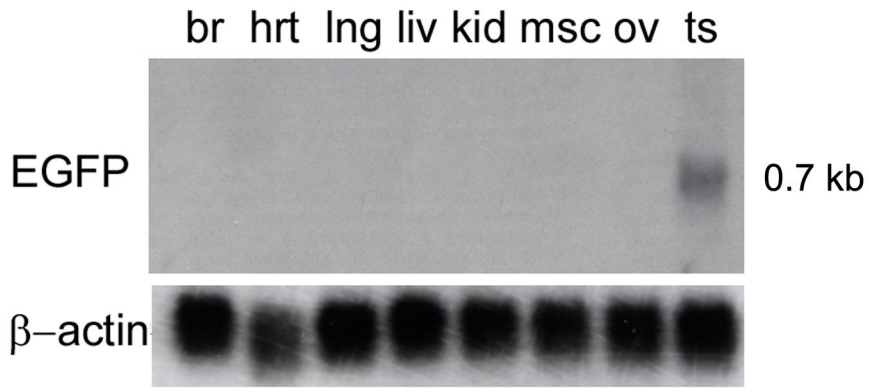


Figure 3.9 Northern blot analysis of different tissues of Tex18/4 transgenic line using EGFP probe. Abbreviations are: br: brain, hrt: heart, lng: lungs, liv: liver, kid: kidney, msc: muscle, ov: ovary, ts: testis. β-actin served as a positive control.

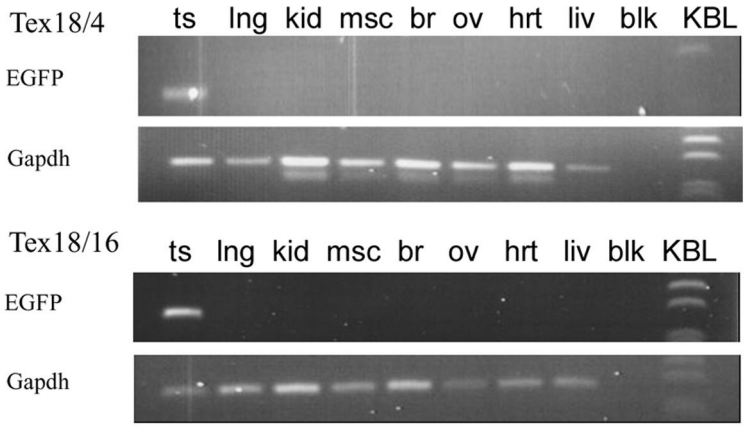


Figure 3.10 RT-PCR analysis of two independent transgenic lines Tex18/4 and Tex18/16 using EGFP primers in different tissues. Abbreviations are: ts: testis, lng: lung, kid: kidney, msc: muscle, br: brain, ov: ovary, hrt: heart, liv: liver, blk: blank = no-template probe. Gapdh served as a control.

Testes from transgenic line Tex18/4 were fixed in Bouin's solution; paraffin sections were prepared and stained with DAPI. Histological sections demonstrated green signal emitted by spermatids and sperm in the lumen of seminiferous tubules, which was not observed in the wild type control. It suggests that *Tex18* gene is expressed specifically

in testis and predominantly in postmeiotic stages of spermatogenesis (Fig. 3.11). Albeit, expression of EGFP is observed as early as in the blastocyst stage. Microscopical observations under UV light have shown that EGFP expression under *Tex18* promoter is recognizable in the 3.5 days old embryos of transgenic line Tex18/16 (Fig 3.12).

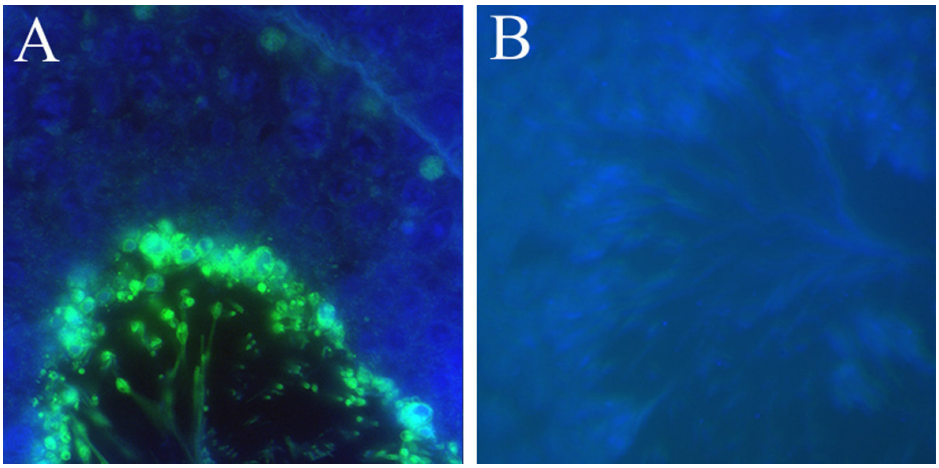


Figure 3.11 Histological analysis of testis from transgenic line Tex18/4. Green signal emitted from EGFP is visible in the late spermatids and in sperm of transgenic males (A), but is absent in the section of wild type testis (B). Blue colour comes from DAPI staining.

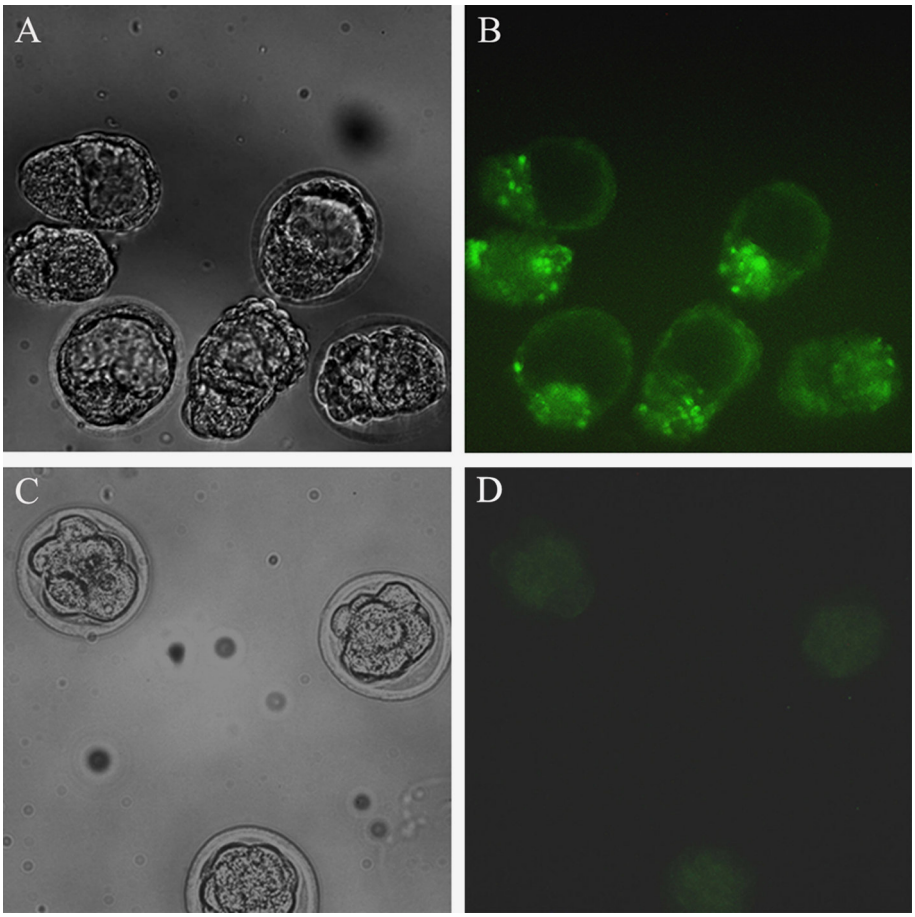


Figure 3.12 EGFP expression was observed in 3.5 days old blastocysts from transgenic line Tex18/16. Blastocysts are shown in normal light (A and C) and under UV light (B and D). Green signal is visible in some cells in the blastocysts of transgenic line (B), but is absent in the wild type control (D).

3.1.2.3 Fluorescence activated cell sorting (FACS) of EGFP positive cells

Expression of enhanced green fluorescent protein under the *Tex18* promoter provides an opportunity for isolation of EGFP-expressed cells by fluorescence-activated cell sorting (FACS). Whole testicular cell suspension from transgenic male was prepared as described in section 2.2.21.1. Subsequently, cells were applied for FACS analysis and a distinct population of EGFP positive cells was observed (Fig. 3.13). Interestingly, percentage of EGFP positive cells increases with the age of the male. While only 1-2 % of EGFP – positive cells were detectable in testes of 5 and 10 days old mice, in case of 15 days old males about 9 % and more than 20 % EGFP positive cells were detected in testes of adult males (Fig. 3.14).

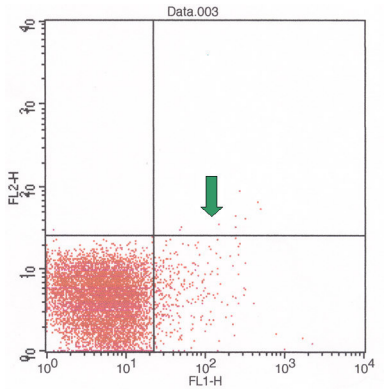


Figure 3.13 Fluorescence activated cell sorting (FACS) analysis of cell suspension from testis of 80 days old transgenic male from line Tex18/4. Arrow points at EGFP - positive cells (around 23% of cells were positive in this case).

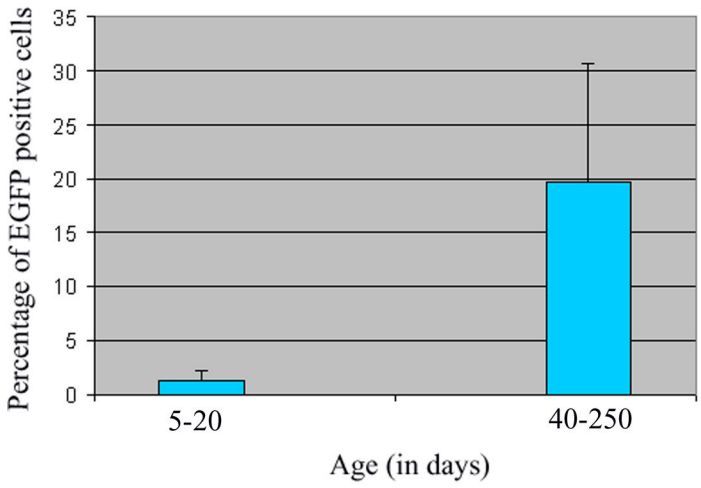


Figure 3.14 Diagram showing percentage of EGFP positive cells in testicular cell suspensions of transgenic males of different ages. Number of FACS- positive cells is smaller in the younger animals (in which postmeiotic germ cells are not present) than in older ones (which already have postmeiotic cells).

3.1.2.4 Determination of the DNA content of EGFP positive cells

Postmeiotic germ cells occur in the male gonad at about 20 dpc. Because an increase in the percentage of EGFP positive cells was observed at this time point, this observation supports the hypothesis that *Tex18* is expressed mainly during postmeiotic spermatogenesis. To examine this hypothesis, FACS positive cells were applied for DNA content measurement. Percentage of cells in haploid (1N), diploid (2N) and tetraploid (4N) stage was determined among EGFP-positive cells and total testis cell suspension from wild type control. This assay showed enrichment in number of haploid cells in the EGFP positive cells comparing to wild type control as the ratio of percentages of haploid cells to all cell types (1N/1N+2N+4N) makes 1.39 in EGFP positive cells and 0.53 in wild type control (Fig.3.15).

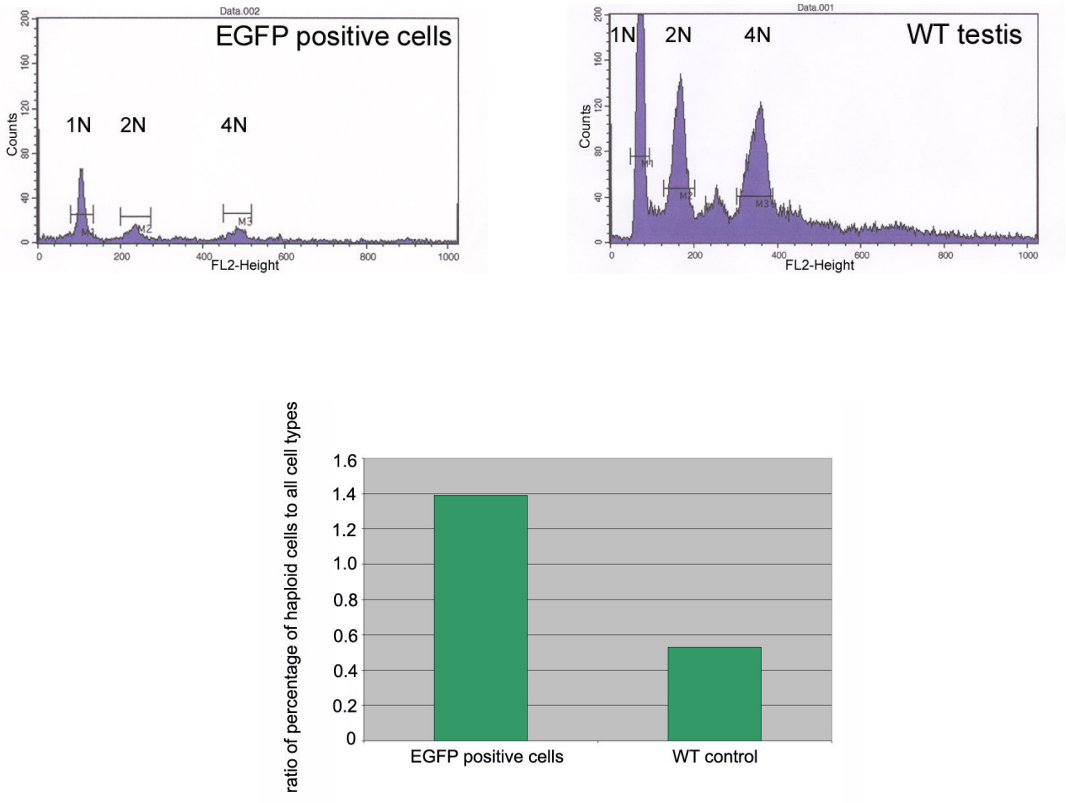


Figure 3.15 DNA content analysis showed that proportion of percentage of haploid cells to other cells (1N/1N+2N+4N) in testes of transgenic male is almost 3 times higher than in wild type control (1.39 in transgenic mouse as compared to 0.53 in wild type control). This indicates to an enrichment of haploid cells in the population of EGFP positive cells.

3.1.3 Targeted inactivation of mouse *Tex18* gene

One of the best ways to elucidate gene function is the generation of a knock - out animal model. For this purpose, *Tex18* knock – out mice were generated in this study. Analysis of the phenotype of mice with targeted disruption of *Tex18* gene could help in understanding the role of the gene in spermatogenesis and male fertility.

3.1.3.1 Isolation of cosmid clones from mouse genomic DNA library

The mouse RZPD (The Resource Center and Primary Database, Berlin) genomic library 129 ola was screened using 453 bp cDNA probe of *Tex18*, generated by PCR using primers Tex18F and Tex18R. The following cosmid clones were recognized as positive: 1 - MPM Gc 121P06227Q2, 2- MPM Gc 121P24194Q2, 3 -MPM Gc 121J03658Q2, 4 -MPM Gc 121F12439Q2, 5- MPM Gc 121H21642Q2, 6- MPM Gc 121B02744Q2 and 7 -MPM GC 121F07305Q2. Because *Tex18* is a relatively small gene, it was possible to design a targeting vector which disrupts the whole gene and replace it by *Neomycin* resistance gene. Mutant mice obtained from this construct will lack the entire *Tex18* gene, and therefore they will provide a convenient model for the study of *Tex18* function.

3.1.3.2 Restriction digestion analysis of cosmid clones

All of the clones which were designated as positive during cosmid library screening were digested with *Eco* RI and *Xba* I enzymes, blotted and hybridized radioactively with a 453 bp *Tex18* cDNA probe. Southern blot analysis and comparison of its results with known genomic structure revealed that only cosmid clones 1, 2, 4 and 6 were in fact positive (Fig. 3. 16 B)

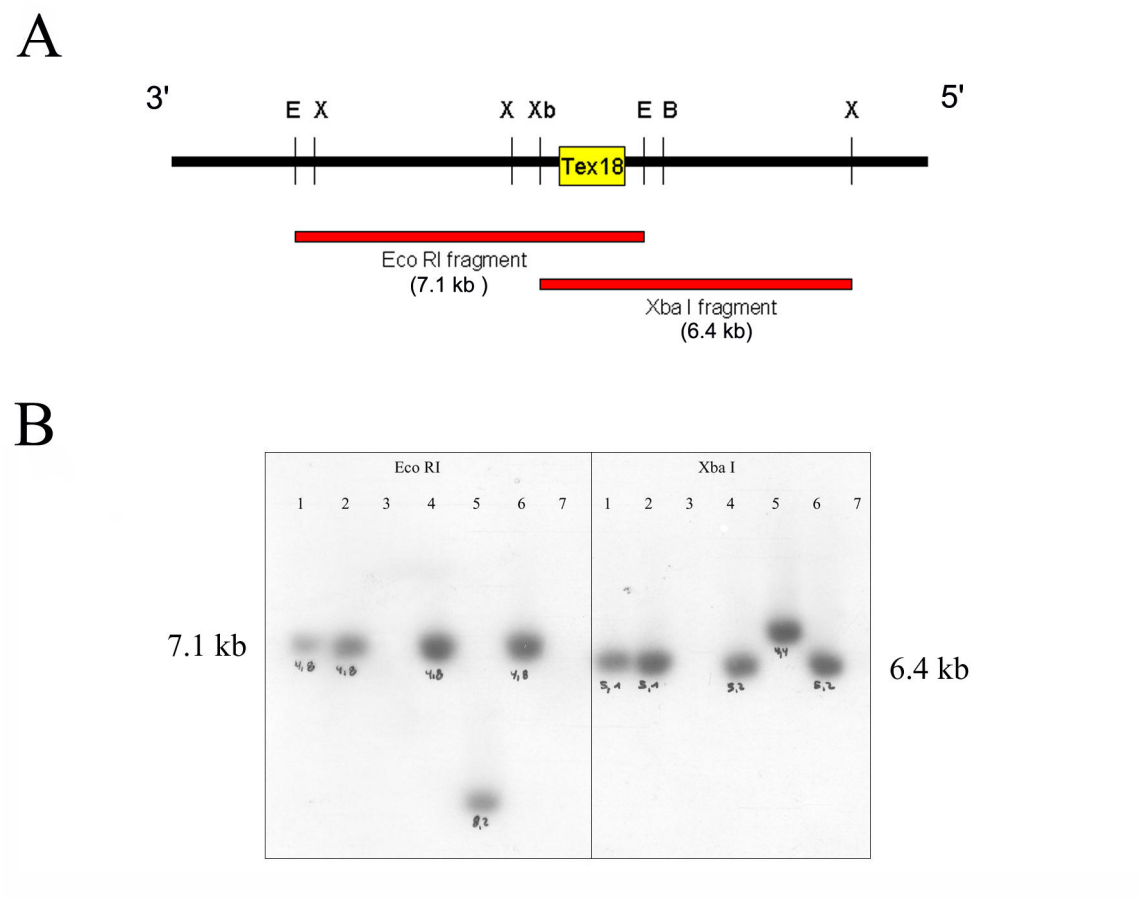


Figure 3.16. (A) Restriction digestion map of *Tex18* genomic DNA and fragments chosen for the targeting construct. Abbreviations are: E: *Eco* RI, X: *Xmn* I, Xb: *Xba* I, B: *Bam* HI. (B) Hybridization of cosmid clones digested with *Eco* RI and *Xba* I restriction enzymes with a 453 bp T*ex18* cDNA probe.

3.1.3.3 Generation of knock – out construct.

The *Xba* I fragment was selected for 5' wing of the construct. It was subcloned into the pBS II SK vector and then the 5 kb fragment was cut out with *Bam* HI and *Sst* II (from pBS II SK vector multicloning site) and cloned in pTK-Neo vector in the same sites. (Fig 3.16 A and 3.17). For the 3' wing, the following strategy was designed: *Eco* RI fragment was shortened with *Xmn* I enzyme, its ends filled up to create blunt ends and the fragment should be cloned into *Hinc* II site in the pBS II SK vector (blunt-end ligation). 3' wing should be then cut out from pBS vector with *Xho* I and *Cla* I and cloned into *Sal* I/*Cla* I (*Xho* I and *Sal* I have the same ends) sites of pTK-Neo vector. Unfortunately, blunt-end ligation was not successful. 2.5 kb 3' wing was finally

generated by PCR, using primers Tex18SalI and Tex18ClaI, with introduced sites for *Sal* I and *Cla* I enzymes. The whole PCR fragment was sequenced with primers: SalI 1, SalI 2, SalI 3, SalI 4, ClaI 1, ClaI 2, ClaI 3 and ClaI 4 to avoid any mismatch (Fig 3.16 A and 3.17). After homologous recombination, a 1.4 kb genomic fragment containing *Tex18* gene will be replaced by *neomycin* resistance gene. In the targeting construct *neomycin* is used as a marker for positive selection, while two copies of *thymidine kinase* (Tk) from Herpes virus are used as negative selection marker. In order to check positive orientation of both wings in the vector, pTKNf, pTKNr and pTKr primers, specific for vector, were used for sequencing.

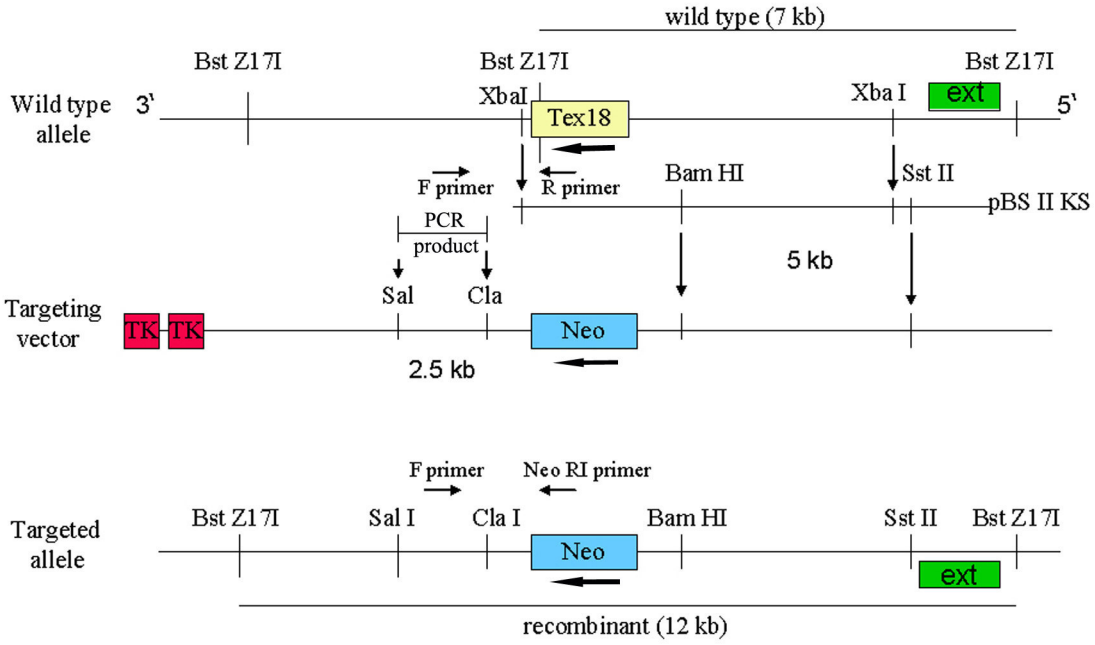


Figure 3.17 Schematic representation of targeted disruption of *Tex18* gene. The structures of the wild type allele, targeting vector and mutant allele are shown together with the relevant restriction sites. A 1.6 kb fragment containing whole *Tex18* gene was replaced by *Neo* selection cassette. *SalI/ClaI* fragment was amplified by PCR. Position of primers Tex18PCR F3 (described as F primer in the figure), Tex18 PCR R3 (R primer) and Neo RI used for genotyping of mice are indicated. Position of external probe used for screening of ES clones and lengths of fragments recognized by this probe by Southern blot in wild type and targeted allele are indicated too. TK stays for *thymidine kinase* cassette, while Neo for *neomycin* cassette.

3.1.3.4 Generation of the 5' external probe

The 5' probe for screening of ES cells was generated to distinguish between wild type and recombinant clones. For this purpose, a fragment of 0.8 kb was amplified by RT PCR, using primers Text F2 and Text R2. This external probe recognizes the following fragments in Southern blot hybridization after digestion of genomic DNA with *Bst* Z17I enzyme: 7 kb fragment in case of wild type and 12 kb in case of recombinant (Fig 3.17). The PCR fragment was cloned in pGEM T-Easy vector and then cut out with *Eco* RI.

3.1.3.5 Electroporation of the RI ES cells and screening of ES cells for recombinants

Tex18 targeting vector was linearized with *Sal* I enzyme and 50 µg of purified DNA was electroporated into RI embryonic stem cells, as it was described in section 2.2.19.1. Cells were plated on feeder layer and after 10 days of selection 57 clones resistant for neomycin were selected and cultured on 24 well plates. Genomic DNA was isolated from ES cells, as it was described in section 2.2.1.4, and used for Southern blot hybridization. DNA from each clone was digested with *Bst* Z17I enzyme, electrophoresed and blotted onto Hybond C membrane. Blots were then hybridized with radioactively labelled external probe. Two bands were recognized in case of homologous recombination: 7 kb wild type allele and 12 kb recombinant allele. When no recombination has occurred or non homologous recombination had taken place, only wild type band could be detected (Fig. 3.18). From 28 of 57 clones, DNA quality was not good enough to give clear results. In the remaining 27 clones no homologous recombination has occurred. Therefore second transfection was done and 68 clones were obtained. Again, in 24 of them DNA quality was too poor, in 17 of them no recombination was detected, but in 27 clones homologous recombination has taken place. These results were confirmed, using Neo probe for hybridization (Fig. 3.18). Clones nrs 14, 53 and 55 were rehybridized with external probe again and clone nr. 53 was chosen for blastocyst injection.

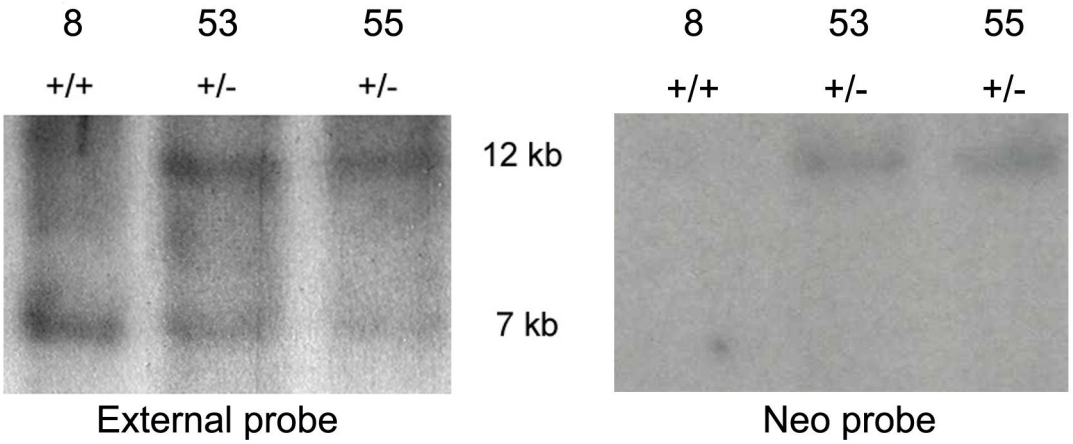


Figure 3.18 Southern blot analysis of ES clones. Genomic DNA extracted from ES clones was digested with *Bst* Z17I, separated electrophoreticaly and blotted onto Hybond C membrane. Blots were then hybridized with a 5' external probe. In case of wild type allele only one band of 7 kb was detected (+/+), in case of homologous recombination band of 12 kb was additionally seen (+/-). Rehybridization with Neo probe confirmed homologous recombination. The expected band of 12 kb was obtained. Clones' numbers are given.

3.1.3.6 Generation of chimeric mice

ES cells from the positive clone nr 53 were injected into 3.5 dpc blastocysts obtained from mice of C57 BL/6J background. Blastocysts were then implanted into uteri of pseudopregnant CD1 females in order to obtain chimeric mice. Four male chimeras were obtained. Their chimerism was estimated in percentage according to the coat colour: 95%, 50%, 15%, and 65%. All of them were bred with C57 BL /6J and 129/Sv females respectively, to obtain F1 generation on mixed background C57 BL/6J x 129/Sv and on pure background 129/Sv. Chimeras of 95% and 65 % chimerism were infertile and did not give any offspring, but males of 15% and 50% chimerism transmitted *Tex18* recombinant allele to the germline on both backgrounds. Transmission was checked by PCR genotyping, using primers TexPCRF3 (primer F), TexPCRR3 (primer R) and NeoRI (Fig 3.17) and genomic DNA isolated from tail biopsies of mice (Fig. 3.19).

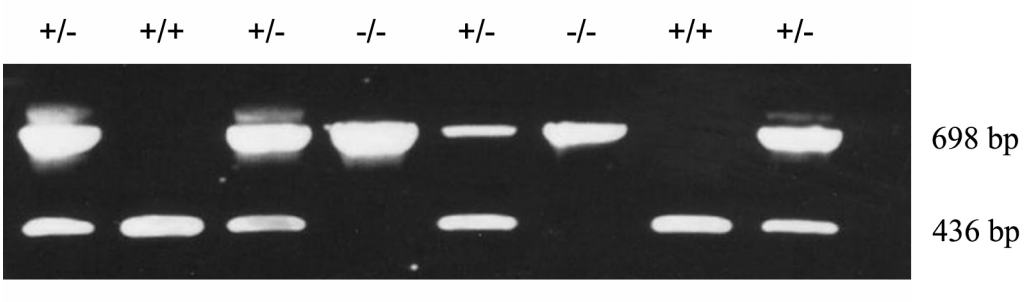


Figure 3.19 Genotyping results of Tex18 knock-out animals. Genomic DNA was extracted from mouse tails and PCR using primers Tex18PCR F3, Tex18PCR R3 and NEO RI was performed. Band of 436 bp was obtained in case of wild type animal (+/+), 698 bp band was amplified in case of homozygous (-/-) animals, while both bands were visible when DNA from heterozygous mice was used (+/-).

3.1.4 Generation and analysis of *Tex18* knock-out mice

F1 animals, heterozygous for *Tex18* gene, were used for further crossing in order to obtain F2 animals and establish knock-out lines on both C57 BL/6J x 129/Sv and 129/Sv backgrounds. F2 animals were genotyped as described above (Fig. 3.19).

3.1.4.1 *Tex18* gene expression in knock-out mice

Total RNA was isolated from testes of homozygous and heterozygous *Tex18* knock-out males. RNA was isolated also from testis of wild type control male. RT-PCR was performed using Tex18F and Tex18R primers (Fig 3.1 B). A 453 bp PCR product was obtained when RNA from wild type and heterozygous males was used, but not any product was visible in case of RT-PCR performed with RNA from homozygous mice. It proves that in homozygous *Tex18* knock-out mice the whole *Tex18* gene was disrupted (Fig 3.20).

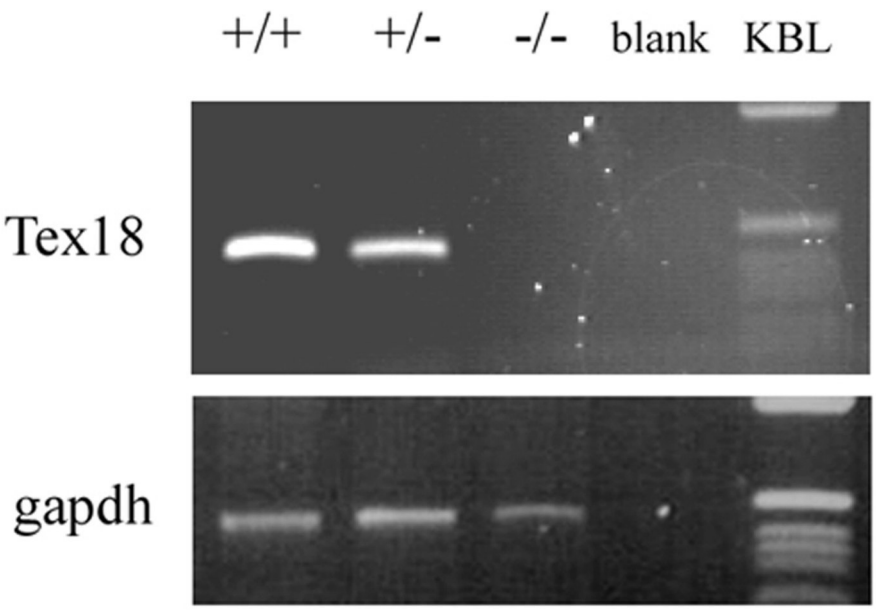


Figure 3.20 RT-PCR analysis of *Tex18* knock-out mice. RT-PCR using Tex18F and Tex18R primer was performed with RNA from homozygous (-/-), heterozygous (+/-) and wild type (+/+) animals. A 453 bp product of Tex18 gene was obtained from RNA of +/+ and +/- animals, but no band was visible in case of -/- mouse. Gapdh served as positive control.

3.1.4.2 Phenotypic analysis of *Tex18* knock-out mice.

3.1.4.2.1 Mode of inheritance

F1 heterozygous mice were intercrossed to obtain F2 generation. Mice were genotyped using DNA obtained from tail biopsies. Breeding of heterozygous mice with wild type mice were performed for a control. Mendelian inheritance pattern was not affected, as it was shown by chi^2 test ($p<0.05$), with two exceptions of breeding of heterozygous mice and breeding of heterozygous males with wild type females. Both situations respect 129/Sv background, where proportions of heterozygous and wild type animals were disrupted. Data are summarized in Table 3.1

A HE♀ x WT♂ on C57 BL/6J x 129/Sv background

	female	male
+/+	14 = 29%	16 = 33%
	30 =62%	
+/-	8 = 17%	10 = 21%
	18 =38 %	
	22 =46 %	26 = 54%

B HE x HE on C57 BL/6J x 129/Sv background

	female	male
+/+	19 = 16%	19 = 16%
	38= 32 %	
+/-	18 =15%	28 = 24%
	46 = 39%	
-/-	14 = 12 %	20 = 17%
	34= 29%	
	51= 43 %	67= 57%

C HE x WT on 129 SV background

	female	male
+/+	26 = 22%	40 = 34%
	66 = 56%	
+/-	23 = 19%	29 = 25%
	52 = 44 %	
	49= 41%	69 = 59%

D HE♂ x WT♀ on 129 SV background

	female	male
+/+	19 = 26%	26 = 36%
	45 = 62%	
+/-	14 =20%	13 = 18%
	27= 38 %	
	33= 46%	39= 54%

E HE♀ x WT♂ on 129 SV background

	female	male
+/+	6 = 13%	14 = 30%
	20 = 43%	
+/-	10 = 22%	16 = 35%
	26 = 57 %	
	16= 35%	30 = 65%

F HE x HE on 129 SV background

	female	male
+/+	27 = 19%	20 = 14%
	47 = 33 %	
+/-	38 = 26%	24 = 16%
	62 = 42 %	
-/-	14 = 10%	22= 15%
	36 = 25%	
	79=55 %	66 = 45%

Table 3.1 Statistical analysis of genotype distribution of *Tex18* in HE x WT and HE x HE breedings on C57 BL/6J x 129/Sv and 129/Sv backgrounds. (A) Breeding of heterozygous females with wild type males on mixed background did not show significant statistical differences, as it was shown by chi^2 test. 48 animals from 5 litters were genotyped (heterozygous males were not bred with wild type females in this case). (B) Crossing of heterozygous animals on mixed background also did not show deviation from Mendelian ratio. 118 animals and 14 litters were analyzed. (C) On 129/Sv background no deviations from expected numbers were observed when heterozygous animals were crossed with wild type ones. 118 animals in 17 litters were genotyped. (D) When only breeding of heterozygous males with wild type females was analyzed, statistical differences were observed by chi^2 test. They were 72 animals from 9 litters. (E) When wild type males were bred with heterozygous females, no disruption of Mendelian ratio was observed. They were 46 animals from 8 litters. (F) A statistically significant difference was observed by chi^2 test, when breeding of heterozygous animals on 129/Sv background was analyzed. 156 animals from 24 litters were genotyped. Abbreviations are: HE: heterozygote, WT: wild type.

3.1.4.2.2 Testing of fertility of *Tex18* deficient males

To evaluate consequences of *Tex18* disruption, fertility of homozygous males on both backgrounds were tested by mating them with homozygous and wild type females. All of the matings were performed for three months and usually after one month first litter was obtained. Number of born mice and mean litter size of these breedings together with results of breedings of heterozygous mice are summarized in Table 3.2. Average litter size of offspring of HO x HO mating on both backgrounds was significantly different from litter size of HE x WT and HO x WT matings on the mixed background and from HE x WT mating on 129/Sv background. These differences were shown by Mann – Whitney U Test ($p<0.05$), when average litter size on C57 Bl/6J is 7 and 5.9 on 129/Sv background (Silver, 1995). 8 males from C57 BL/6J x 129/Sv background and 12 males from 129/Sv background were used for the fertility test. All of the males from mixed background were fertile, but on 129/Sv background 8 of them gave usually not more than 2 offsprings, while other four were completely infertile. It means that approximately 33% of males from 129/Sv background are infertile and 67% subfertile.

Type of breeding	No. of mice born	No. of litters	Average litter size
C57BL/6J x 129Sv			
+/-♀ x +/+♂	48	5	9.6 ± 2.5
+/- x +/-	118	14	8.4 ± 2.9
-/- ♂ x +/+♀	114	16	10.4 ± 4.7
-/- x -/-	87	11	5.8 ± 2.7
129 Sv			
+/- x +/+	118	17	6.8 ± 3.5
+/-♂ x +/+♀	72	9	8.0 ± 3.4
+/-♀ x +/+♂	46	8	5.8 ± 3.4
+/- x +/-	156	24	6.5 ± 3.2
-/-♂ x +/+♀	45	11	5.6 ± 3.3
-/- x -/-	29	9	3.6 ± 2.1

Table 3.2 Fertility of *Tex18* +/- and *Tex18* -/- mice on genetic backgrounds C57BL/6J x 129/Sv and 129/Sv. Numbers of born mice, number of litters and mean litter sizes are given. Mean litter size from breeding of heterozygous animals is reduced, especially on 129/Sv background.

3.1.4.2.3 Sperm count and analysis of sperm abnormalities of *Tex18* deficient mice

Total sperm count in the cauda epididymes of *Tex18* homozygous, heterozygous and wild type males was determined. Eight homozygous, three heterozygous and three wild type males from both genetic backgrounds were used for sperm count. No statistically significant differences in sperm count were observed between knock-out and wild type animals. During counting, some abnormalities of sperm were observed, therefore sperm morphology was investigated in more details. Slides were prepared as it was described in section 2.2.22.2 and percentage of abnormal sperm was determined. High increase in percentage of sperm with unusual head shape was observed in C57 BL/6J x 129/Sv as well as in 1297Sv background. Statistically significant differences in percentages of abnormal spermatozoa between homozygous males and wild types were observed, as it was shown by Mann – Whitney U Test ($p<0.05$). It was approximately 47% of abnormal spermatozoa from cauda epididymes of males from mixed background and about 30% in case of males from 129/Sv background. There was no increase in sperm number with abnormal head shape in heterozygous males. These data are summarized in Table 3.3. Typical sperm head abnormalities are shown in Figure 3.21.

	Sperm in cauda epididymis	
Genotype	Number ($x10^6$)	Percentage of abnormal sperm
C 57BL/6J x 129SV		
+/+	16.43 ± 8.62	7.83 ± 3.51
-/-	17.78 ± 10.02	46.57 ± 17.73
129SV		
+/+	14.80 ± 3.87	3.83 ± 1.04
-/-	13.63 ± 10.66	29.68 ± 14.26

Table 3.3 Sperm count in the cauda epididymes of -/- and +/+ mice from both backgrounds. Statistically significant differences were observed in the percentage of abnormal sperm between *Tex18* deficient males and wild type in C 57BL/6J x 129SV and 129/Sv backgrounds.

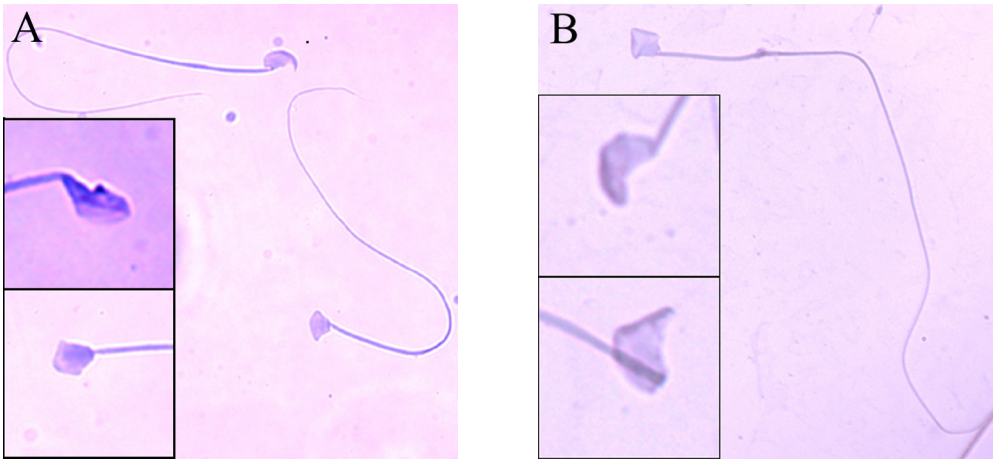
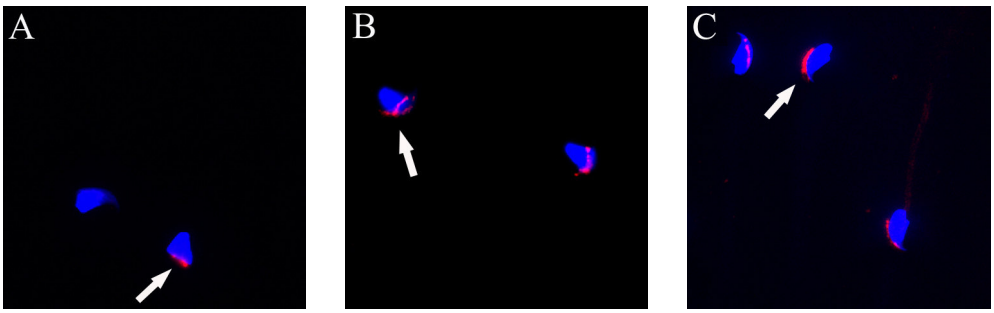


Figure 3.21 Typical sperm head abnormalities observed with sperm isolated from cauda epididymes of males from C 57BL/6J x 129/Sv (A) and 129/Sv (B) backgrounds. Higher magnifications are shown on the left side of the figures.

To investigate sperm abnormalities in more detail, immunostaining assays were applied. Three different antibodies (OAM –outer acrosomal membrane, directed against acrosome; mPHGPx - mitochondrial phospholipid hydroperoxide glutathione peroxidase, directed against sperm midpiece and antibody against α- tubulin) were applied as it was described in section 2.2.17. No differences in the structure of midpiece and tail between mutants and wild type control were observed. However, immunostaining with OAM antibody revealed abnormal shape and position of acrosomes in sperm with abnormal head (Fig.3.22). Abnormal acrosome shape was confirmed by electron microscopy (Fig. 3.23).



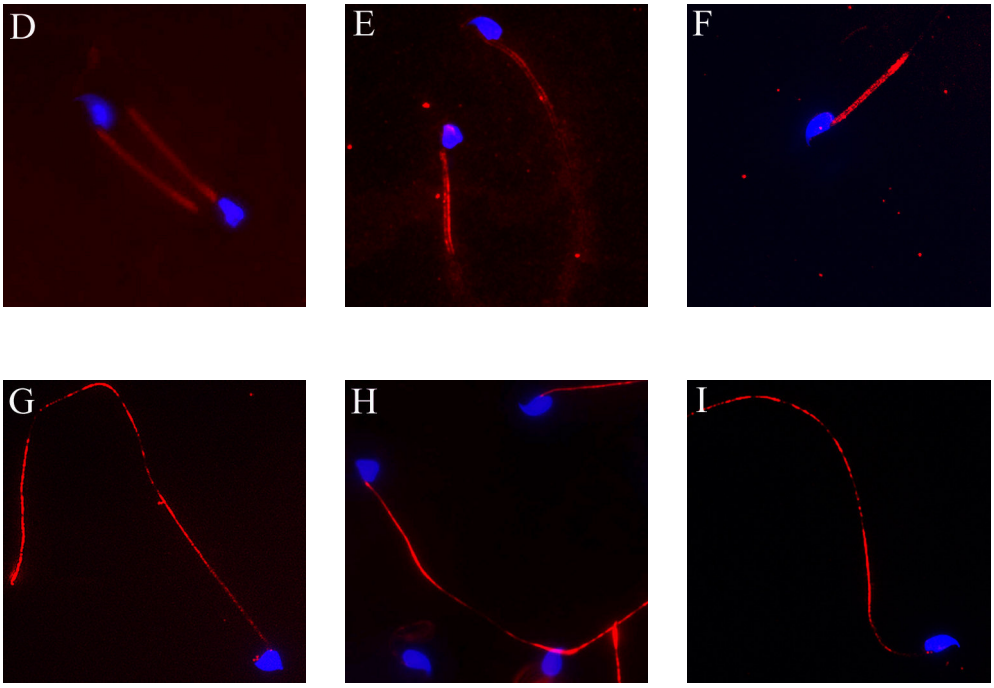


Figure 3.22 Results of immunostaining assays with anti OAM, anti mPHGPx and anti α- tubulin antibodies. Abnormal structure of acrosomes was shown on sperm from *Tex18* deficient males from C57 BL/6J x 129/Sv (A) and 129/Sv background (B), while normal acrosome structure was observed in wild type control (C), when OAM antibody was used. No abnormalities of midpiece of sperm of C57 BL/6J x 129/Sv (D) and 129/Sv (E) backgrounds and wild type (F) were detected, when mPHGPx antibody was applied, or when anti α-tubulin antibody was used for immunostaining of sperm from C57 BL/6J x 129/Sv (G), 129/Sv (H) backgrounds or from wild type (I).

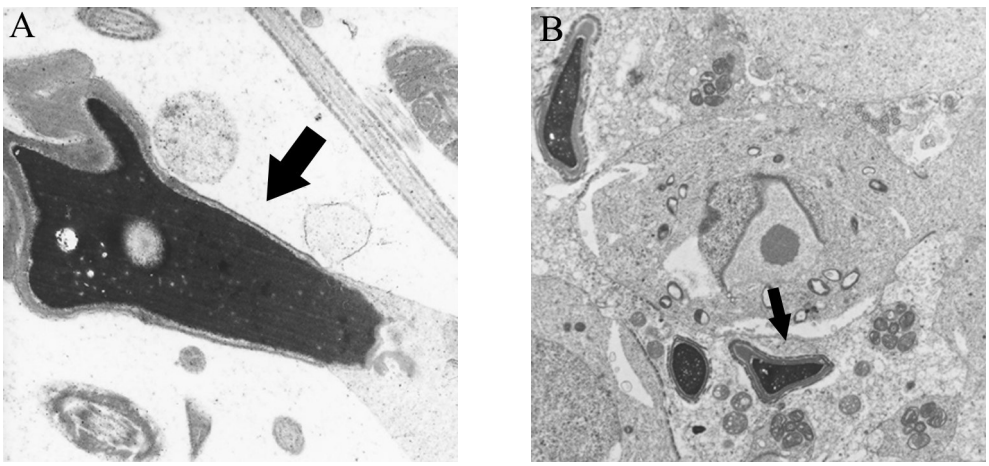


Figure 3.23 Electron microscopy of sperm in cauda epididymis (A) and sperm in testis (B) of *Tex18* deficient mice. Arrows point to abnormal head shape.

To answer the question if abnormal shape of acrosome has an effect on acrosome reaction, we examined the response of spermatozoa from mutant and wild-type mice to the calcium ionophore A23187 (method described in section 2.2.22.4). No significant difference in acrosome reaction between *Tex18* -/- and wild-type spermatozoa was observed. 91% of sperm of homozygous mice and 90% of sperm of wild type control from C57 BL/6J x 129/Sv background and 75.5% of -/- sperm and 82.5% of +/+ sperms from 129/Sv background underwent normal acrosome reaction. Because abnormal acrosomes can affect binding of spermatozoa to an egg, sperm – egg binding assay was applied, as it was described in section 2.2.23. No differences in sperm –egg interaction between mutant and wild type control spermatozoa were observed.

3.1.4.2.4 Analysis of motility and migration of sperm (VP test)

Taking into consideration the high number of abnormal sperm, migration of *Tex18*-deficient spermatozoa through the female genital tract was evaluated. For this purpose the number of sperm in uterus and oviducts was evaluated. *Tex18* -/- males from both backgrounds were mated several times with mature CD1 females. The next day, if vaginal plug was observed, uteri and oviducts from females were flushed with IVF medium and the sperm number was determined. Decrease in sperm number in uteri as well as in the oviducts was observed on both backgrounds. Average number of sperm in wild type control was approximately 0.4 x 10^6 in uterus and about 300 in oviducts (Table 3.4). These data support the hypothesis that sperm migration through the female genital tract is disrupted in homozygous knock-out mice.

A

Males	15A	16A	99	16A	15A	16A	35A	34A	99	72
Sperm in Uterus (10^6)	0	1.9875	0	0.005	0.0025	0.272	0.0006	0	0.468	0.456
Sperm in Oviducts	0	5775	0	0	0	110	0	0	0	0

B

Males	49C	49C	49C	49C	88C	88C	88C	88C
Sperm in Uterus(10^6)	0	0.13	2.17	0	0.003	0.59	0	0.01
Sperm in Oviducts	0	0	0	0	0	188	0	0

Males	82C	82C	82C	71C	71C	71C	71C	71C
Sperm in Uterus(10^6)	0	0	0.16	0	3.41	0	0	0.04
Sperm in Oviducts	0	0	0	0	150	0	0	0

Table 3.4 Sperm count in uterus and oviducts of vaginal plug positive females mated with *Tex18* -/- males from C57 BL/6J x 129/Sv (A) and 129/Sv (B) backgrounds. High decrease in sperm number in uteri and oviducts was observed on both backgrounds as compared to wild type mice.

To analyse whether disrupted migration of sperm in female genital tract is due to reduced motility, tests were applied. Motility of sperm from three *Tex18* -/- and two controls from both backgrounds were measured after 1.5, 3.5 and 5.5 hrs of incubation in vitro, as it was described in section 2.2.22.3. Essential differences in motility and progressive movement of sperm of *Tex18* deficient mice were observed, as compared to wild type. Proportions of motile and showing progressive movement spermatozoa of mutant mice were always 15 – 30 % lower than in control (like 52% of motile sperm of mutant vs. 79% of control at 1.5 hours or 32% of progressive moving sperm of *Tex18* males vs. 47.% of control at 3.5 hours on mixed background; 40% of motile mutant sperm vs. 60.5% of motile sperm from control at 3.5 hours or 19.6% of mutant sperm showing progressive movement vs. 38.5% in wild type at 3.5 hours on 129/Sv background). These data are summarized in Table 3.5.

A

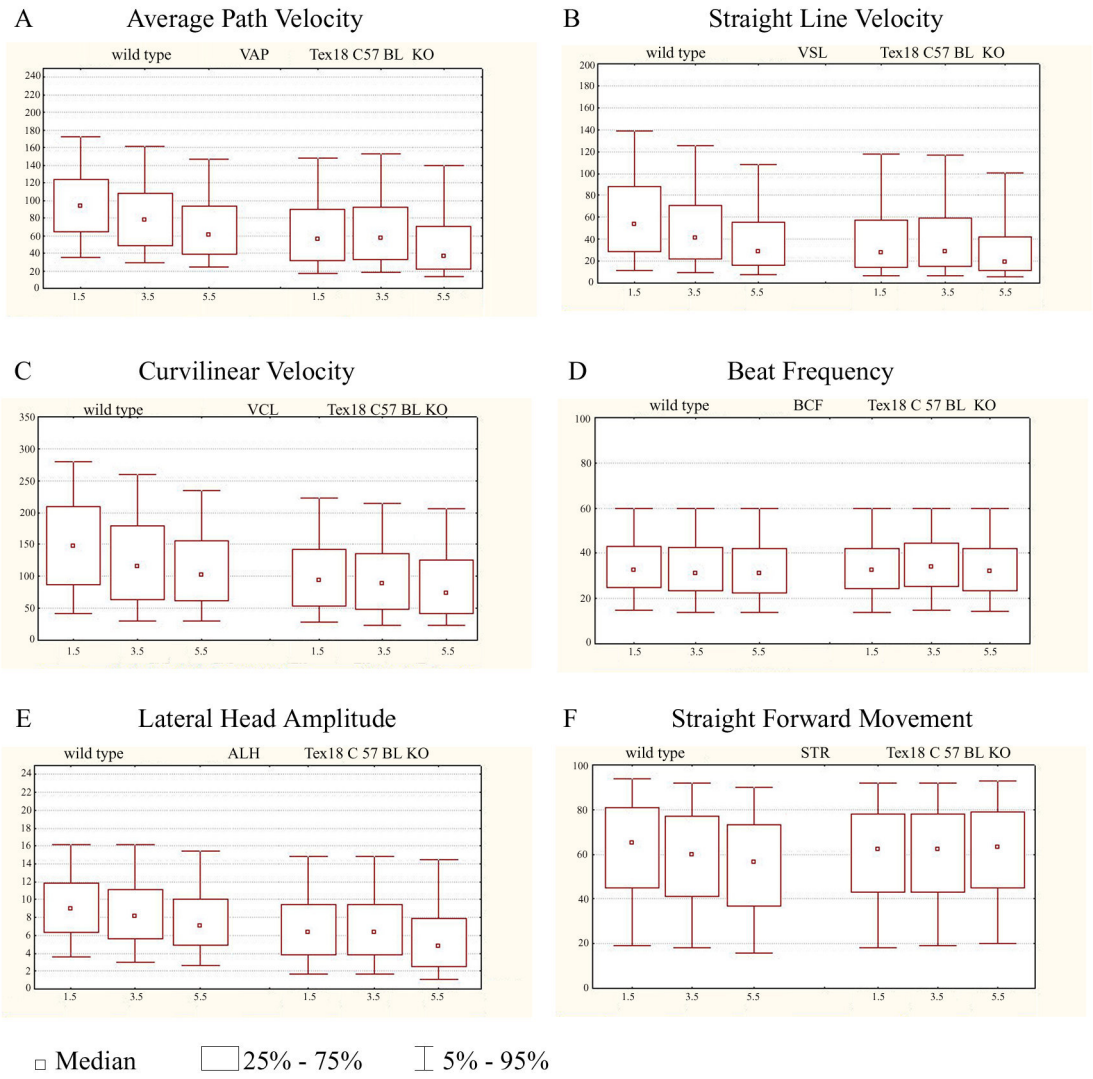
Genotype	Incubation time (h)	Percentage of motile spermatozoa	Percentage of spermatozoa with progressive movement
-/-	1.5	52.4	32.6
	3.5	51.0	32.0
	5.5	37.6	22.3
+/+	1.5	79.5	57.0
	3.5	69.5	47.5
	5.5	58.0	35.5

B

Genotype	Incubation time (h)	Percentage of motile spermatozoa	Percentage of spermatozoa with progressive movement
-/-	1.5	44.6	23.7
	3.5	40.0	19.6
	5.5	30.3	15.3
+/+	1.5	61.5	42.5
	3.5	60.5	38.5
	5.5	49.0	27.5

Table 3.5 Motility analysis of sperm from *Tex18* deficient males on C57 BL/6J x 129/Sv (A) and 129/Sv (B) backgrounds. Substantial differences in motility and progressive movement between mutant mice and wild type controls were observed.

For further investigation of sperm motility, following parameters were evaluated more in details: curvilinear velocity (VCL), average path velocity (VAP), straight line velocity (VSL), beat frequency (BCF), straight forward movement (STR) and lateral head amplitude (ALH) (Fig. 3.24). Mann-Whitney U-Test was done and statistically significant differences were observed for almost each parameter with $p<0.001$, with few exceptions. On C57 BL/6J x 129/Sv background significant differences were observed for BCF with $p=0.004$ at 1.5 and 3.5 hours and no differences were observed for this parameter at 5.5 hours ($p=0.26$), significant differences were found for STR at 3.5 hours with $p=0.004$. On 129/Sv background statistically significant differences were found for STR at 1.5 hours with $p = 0.0017$, BCF at 3.5 hours with $p= 0.006$ and STR at 5.5 hours with $p=0.024$. Differences were especially high for all velocities and lateral head amplitude. This finding indicates that motility of *Tex18* deficient mice sperm is greatly reduced.


A
Average Path Velocity
wild type
VAP
Tex18 C57 BL KO
B
Straight Line Velocity
wild type
VSL
Tex18 C57 BL KO
C
Curvilinear Velocity
wild type
VCL
Tex18 C57 BL KO
D
Beat Frequency
wild type
BCF
Tex18 C 57 BL KO
E
Lateral Head Amplitude
wild type
ALH
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F
Straight Forward Movement
wild type
STR
Tex18 C 57 BL KO
Median
25% - 75%
5% - 95%

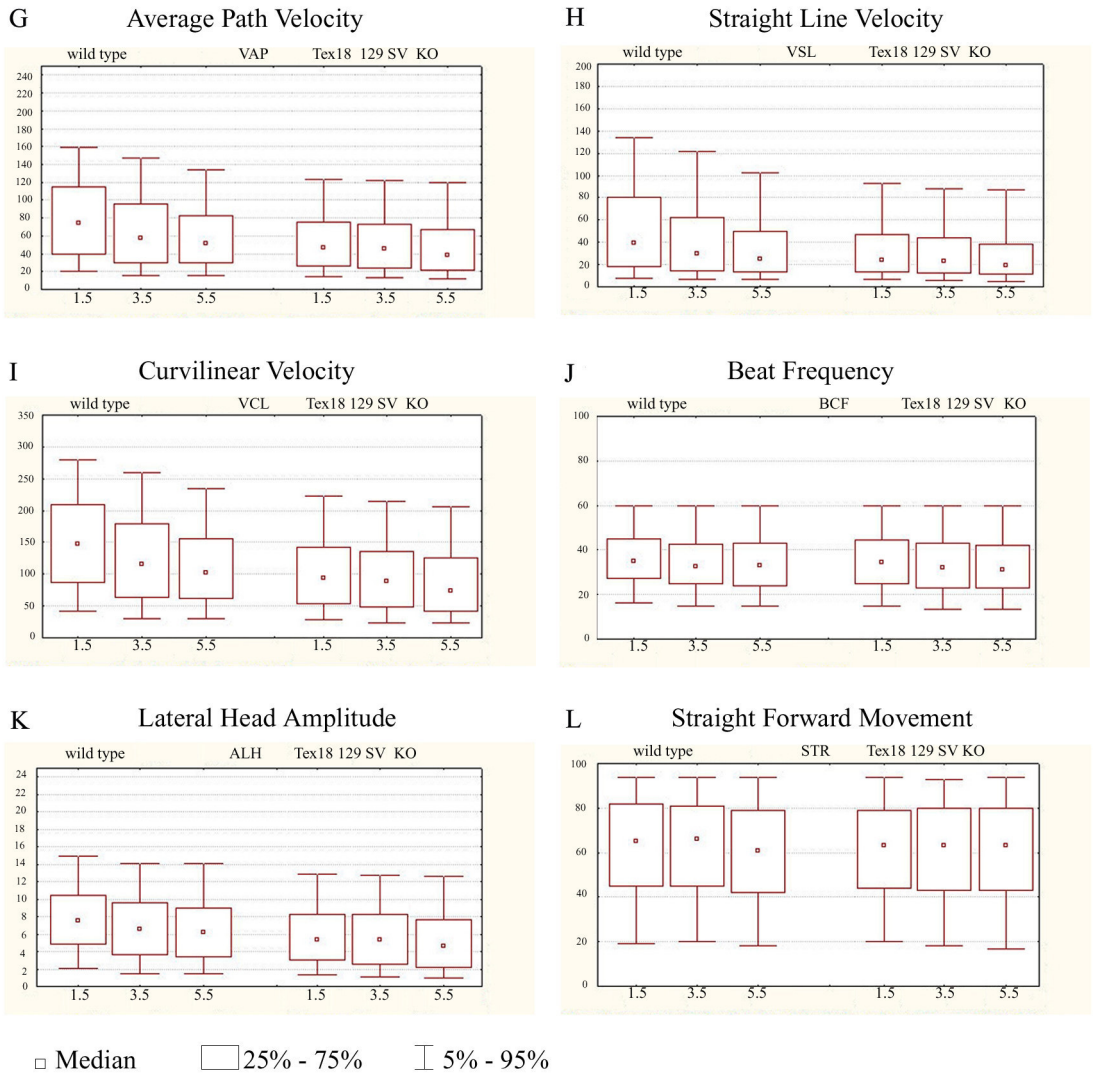
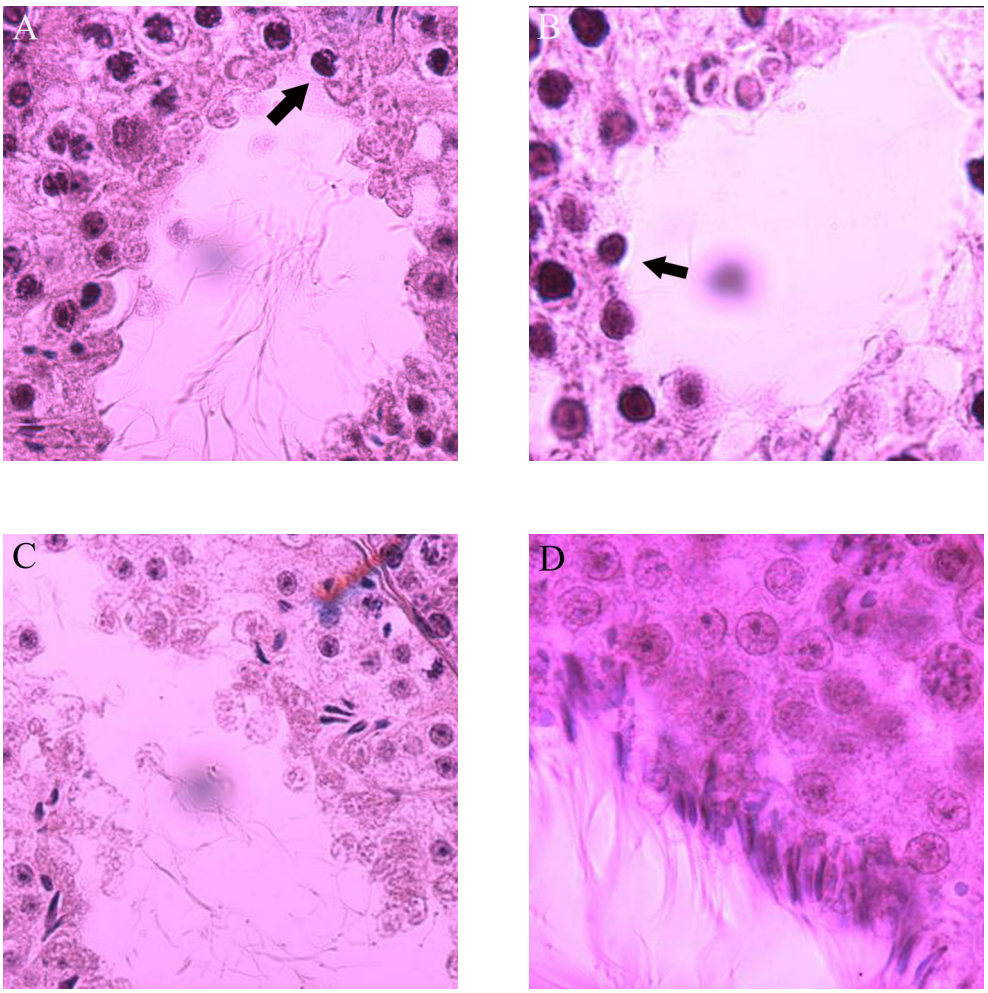


Figure 3.24 Computer-assisted analysis of sperm motility. The results of analyses of wild-type and *Tex18* knock-out spermatozoa on C57 BL/6J x 129/Sv (A, B, C, D, E and F) and 129/Sv (G, H, I, J, K and L) backgrounds are shown. Sperm velocities (micrometers/second), forward movement (percent), lateral amplitude of the sperm head (micrometers), and beat frequency (hertz) were measured after 1.5, 3.5, and 5.5 h. The means and appropriate standard deviations for each parameter are shown. The *Tex18*-deficient spermatozoa exhibit statistically significant reduction in all parameters (except BCF at 5.5 hours on C57 BL/6J x 129/Sv background), especially high in all velocities and lateral head amplitude, as compared to wild-type sperm, as it was shown by Mann – Whitney U Test (p< 0.001). (A and G): Average Path Velocity (VAP), (B and H): Straight Line Velocity (VSL), (C and I): Curvilinear Velocity (VCL), (D and J): Beat Frequency (BCF), (E and K): Lateral Head Amplitude (ALH), (F and L): Straight Forward Movement (STR).

3.1.4.2.5 Histological analysis of *Tex18* deficient males testes

Testes of *Tex18* -/- and *Tex18* +/- males were of normal size and weight. No abnormalities were noticed in their shape. Testes of few males were fixed in Bouin's solution; paraffin sections were prepared and stained with hematoxylin/eosin, as it was described in section 2.2.16. Histological sections of homozygous male testes revealed abnormalities in seminiferous tubule structure. Efficiency of spermatogenesis was disturbed – it was very often arrested at the stage of round spermatids. Therefore reduced number (or sometimes no any) of elongated spermatids and mature sperm was observed in the lumen of seminiferous tubules. Not full developed germ cells were observed in the lumen. Sometimes vacuoles were observed in round spermatids, between nucleus and cytoplasm and near the acrosome (Fig. 3.25).



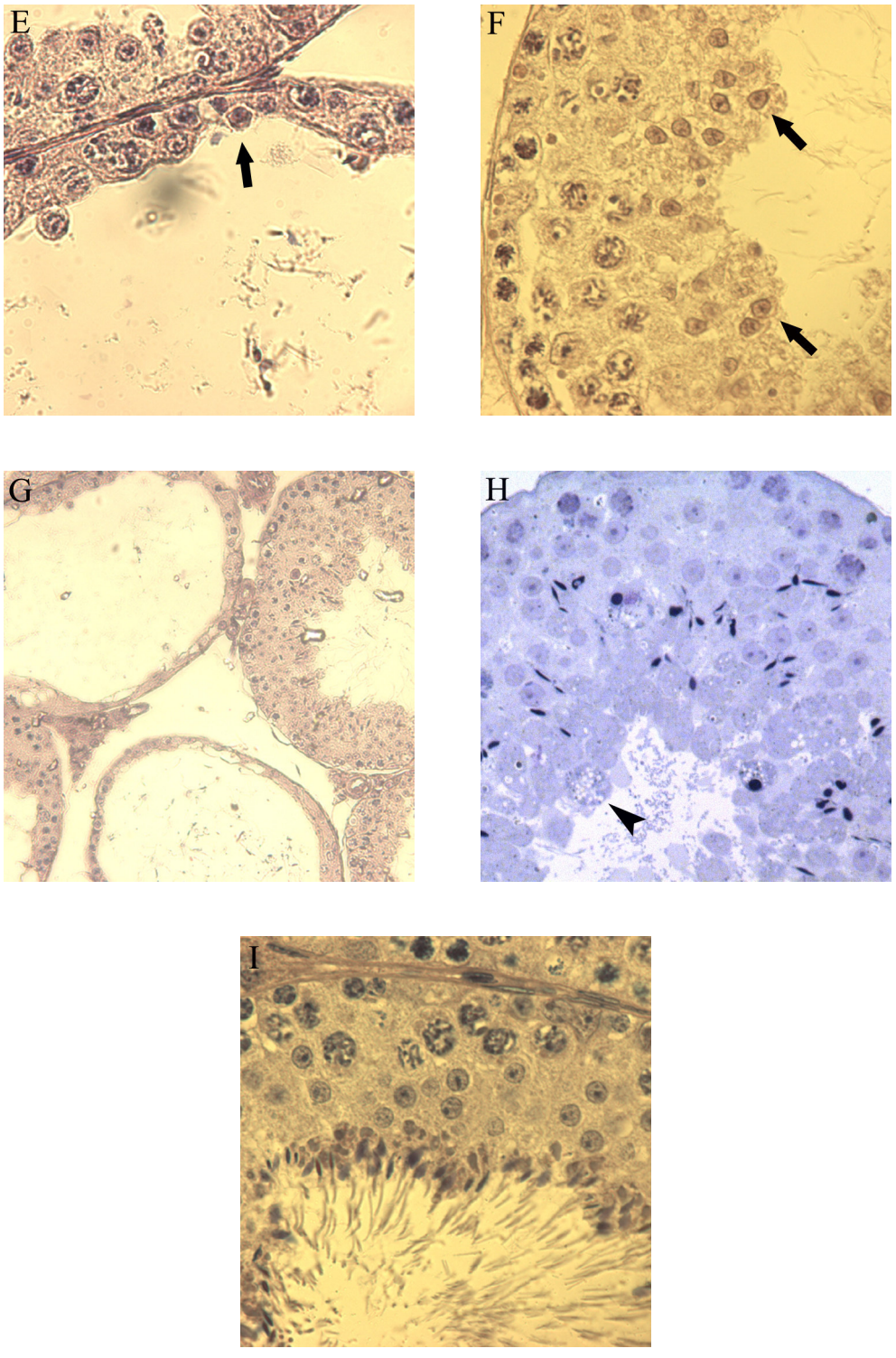


Figure 3.25 Histological sections of *Tex18* mutant males. Paraffin sections of testes of *Tex18* deficient mice reveal spermatogenesis abnormalities. Spermatogenesis was very often arrested at stage of round spermatids (arrows). Vacuoles were observed in spermatids (arrowhead). No spermatozoa were observed in the lumen of most of the seminiferous tubules. A, B and C: testis

section of *Tex18* deficient male from C 57 Bl/6J x 129/Sv background, D: wild type control from the same background. E, F, G and H: sections of testes from *Tex18* deficient males from 129/Sv background, I: wild type control from 129/Sv background.

Immunohistochemistry using mouse *Stra8* peptide antibody was applied on testis sections of *Tex18* -/- mice. *Stra8* is expressed in premeiotic male germ cells, therefore signal is observed in testis section in cells localised near the basal lamina of seminiferous tubules (Oulad – Abdelghani et al., 1996). Immunostaining of testis of *Tex18* deficient males revealed, that early spermatogenesis is not affected in the mutant mice, since *Stra8* signal in the *Tex18* -/- mice was the same as in wild type control (Fig. 3.26).

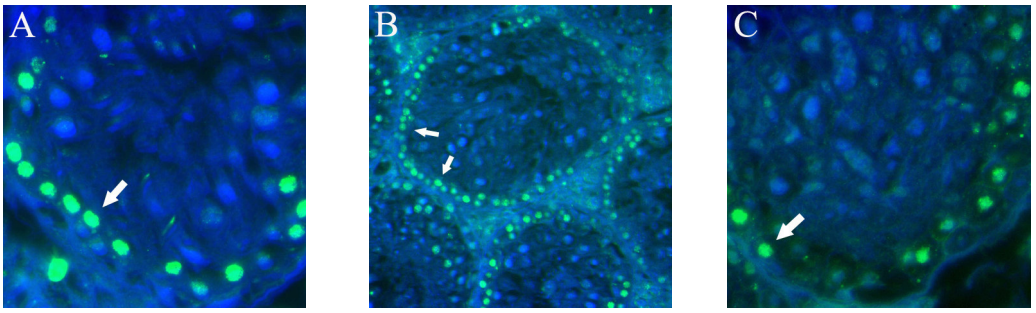


Figure 3.26 Immunohistochemistry of testis section from *Tex18* deficient males. No differences were observed between mutant mice (A and B) and wild type control (C)

3.1.4.2.6 Stage specific histological analysis of *Tex18* deficient males testes

Spermatogenesis is a cyclic process that could be divided into epithelial stages. Different numbers of stages occur in different species; in mouse spermatogenesis is divided into 12 stages (review Russel et al. 1990). Specified types of spermatogonia, spermatocytes and spermatids are observed in every epithelial stage. In *Tex18* knock – out mice deviations from this description were observed. Apoptotic spermatocytes were seen regularly in many seminiferous tubules (Fig. 27 A, B). This leads to tubules where the spermatocytes were largely missing. There were many diploid spermatids, indicating problems in the spermatocyte compartment as these cells failed to carry out the second meiotic division (Fig. 27 A, B, C). Subsequently, relatively often round spermatids did not start the elongation process or did it too slowly (Fig. 27 C, D, E).

Tubules, where most of the spermatids were elongated but some were still round, were also observed (Fig 27 F). However, apoptotic round spermatids and rarely morphologically abnormal elongated spermatids were also seen (Fig.27 A, D, E).

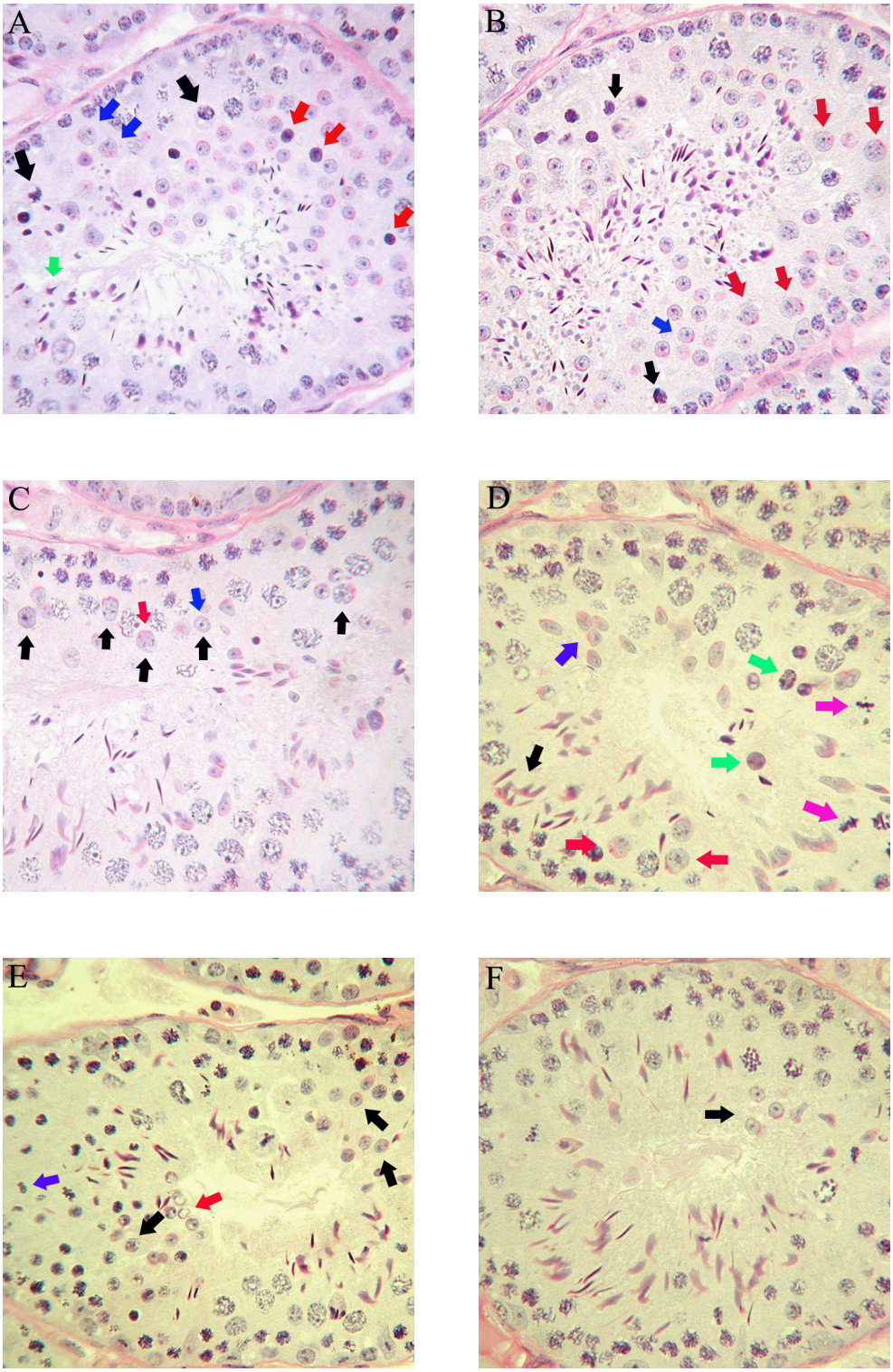


Figure 3.27 (A) Section of seminiferous tubule in epithelial stage VII. Black arrows point at apoptotic spermatocytes, while red arrows at apoptotic spermatids. Diploid spermatids are marked

with blue arrows and elongated spermatid showing abnormal morphology with green arrow. (B) Section of the tubule in epithelial stage VIII. Black arrows show apoptotic spermatocytes, while red ones show diploid spermatids, which skipped second meiotic division. Blue arrow indicates normal haploid spermatid. (C) Section of the tubule in epithelial stage XI. Arrows point at spermatids which are delayed in elongation process, because they are still in stage 8 –9. Haploid spermatid is marked with a blue arrow, diploid with red one. (D) Section of the seminiferous tubule in epithelial stage XI- XII. Although only elongated spermatids should be recognized at this stage (black arrow) spermatids from stages 7 – 8 (red arrows) and 9- 10 (blue arrow) are also visible. Spermatids starting apoptosis are marked with green arrows and normal meiotic divisions with pink ones. (E) Seminiferous tubule in epithelial stage XII. Spermatids from stage 7 which failed to start elongation process are visible (black arrows), round apoptotic spermatids are shown with red arrows. Meiotic divisions occur as well (blue arrow). (F) Seminiferous tubule in epithelial stage XII. Round spermatids which did not start elongation are present in this stage (black arrow).

3.2 Introduction to result section of *Stra8*

The *Stra8* gene (**St**imulated by **r**etinoic **a**cid gene **8** – access number Z75287) was identified in a screen performed to detect genes upregulated in P19 embryonal carcinoma cells treated with retinoic acid (Bouillet et al., 1995a). *Stra8* gene is localised in mouse chromosome 6 and contains 9 exons of 75 to 293 bp, separated by introns of different lengths. Translational start of the gene is localised in exon 2 and stop codon TAA in exon 9 (Fig. 3.28). It's 1455 bp mRNA encodes a 393 amino acid protein, containing a 51 aminoacid domain rich in glutamic acid (Fig 3.29). There is a human ortholog of *Stra8* gene localised in chromosome 7 and containing 9 exons (Fig. 3.30). Its 993 bp mRNA encodes a 330 aminoacid protein (Fig. 3.31). There is about 82% of homology between both genes on mRNA level and about 59% on protein level.

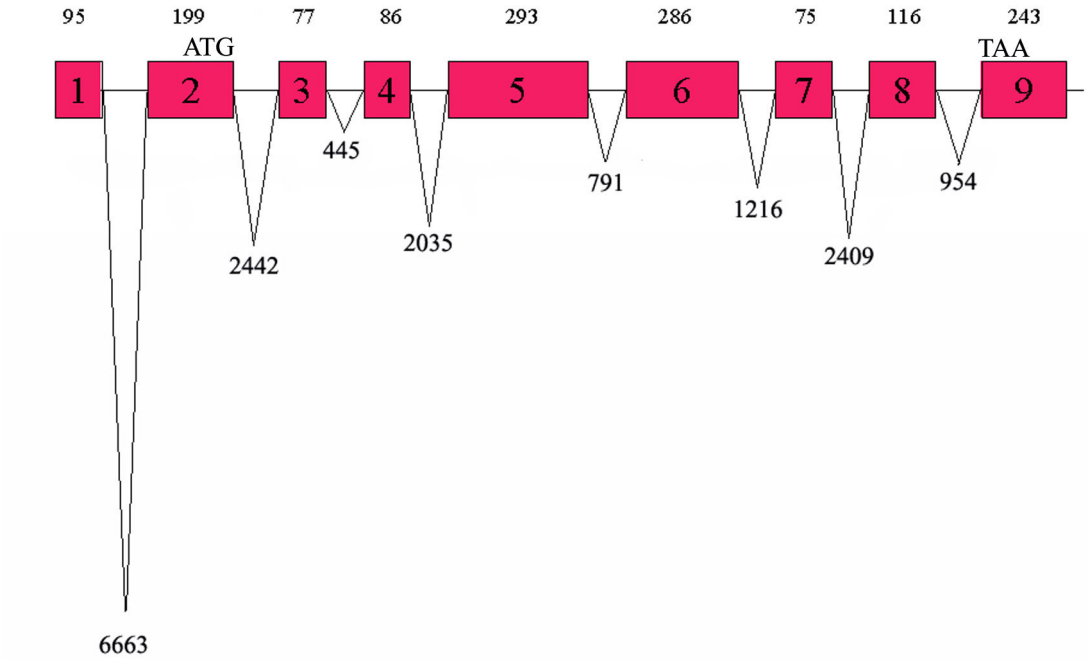


Figure 3.28 Exon – intron structure of the mouse *Stra8* gene. Red boxes represent exons. Lengths of exons and introns are given, ATG and stop codon TAA of the gene are marked.

```
   1 gcagtcgtgagtgactgactcgtcagggcttcgtgcagtttcctgcgtgttccacaagt

  62 gtcgaaggtgcatggttcaccgtggtggccttaaagattataatggccacccctggagaa
                                                 M  A  T  P  G  E

 122 ggcaaccaacccagtgatgatggggcaccccagccattggcgcagctgcagaagcttgag
      G  N  Q  P  S  D  D  G  A  P  Q  P  L  A  Q  L  Q  K  L  E
            EP034149 peptide
 182 cctcgggtggtccgcagacgcctgtcacaggcccgccatcgagccaccctggtagggctc
      P  R  V  V  R  R  R  L  S  Q  A  R  H  R  A  T  L  V  G  L
                                                 _______Stra8F_____
 242 ttcaacaacctaaggaaggcagtttactcccagtctgatatcacagcctcaaagtggcag
      F  N  N  L  R  K  A  V  Y  S  Q  S  D  I  T  A  S  K  W  Q
     _
 302 gtactgaataggacaaagattcatattcaggaacaggaagaaagcctggataagttgctg
      V  L  N  R  T  K  I  H  I  Q  E  Q  E  E  S  L  D  K  L  L

 362 aagctcaaagcatccttcaacctgcaagatgggaatcccaacagcttagaggaggtcaag
      K  L  K  A  S  F  N  L  Q  D  G  N  P  N  S  L  E  E  V  K

 422 gaagaatatgccagaatgtattccgagaatgacagtgtattcctaaacagttttcttcag
      E  E  Y  A  R  M  Y  S  E  N  D  S  V  F  L  N  S  F  L  Q

 482 gacagtccccctgagtggttcccctctgaggctgttggaccagatgctgaagaagaagga
      D  S  P  P  E  W  F  P  S  E  A  V  G  P  D  A  E  E  E  G

 542 gaagaagaaggagaagaagaaggagaagaaggagaagaagaagaggaaggagacgaagaa
      E  E  E  G  E  E  E  G  E  E  G  E  E  E  E  E  G  D  E  E

 602 ggagaagaagaagaagaaaacggtgaagagagagaggtagaggagtaccaggaagaggaa
      G  E  E  E  E  E  N  G  E  E  R  E  V  E  E  Y  Q  E  E  E
                                                 _______Stra8R_____
 662 gaagaagaagaggaggaggagaaaaaagtcgatctctcccactcctcctccactctgttg
      E  E  E  E  E  E  E  K  K  V  D  L  S  H  S  S  S  T  L  L
     _
 722 ccggacctcatggaatttgaacggtatctcaacttttacaagcagaccatggacctcctg
      P  D  L  M  E  F  E  R  Y  L  N  F  Y  K  Q  T  M  D  L  L

 782 accatgaacagcatcatctctgcacatgaagtgacacttcctattgtctctgccgccatc
      T  M  N  S  I  I  S  A  H  E  V  T  L  P  I  V  S  A  A  I

 842 tcccacctgtggcagactctctctgaggagaaaaaggccagactcctgcaggtgtgggaa
      S  H  L  W  Q  T  L  S  E  E  K  K  A  R  L  L  Q  V  W  E

 902 cagcagcacagcgccttcgcagacctcaccgaggcctgtctagagctggccggggtggag
      Q  Q  H  S  A  F  A  D  L  T  E  A  C  L  E  L  A  G  V  E

 962 ggcagcatgaaggacagcggcgtggacagccagggagcgagctgctcgctggagtccacc
      G  S  M  K  D  S  G  V  D  S  Q  G  A  S  C  S  L  E  S  T

1022 ccagaagagatccttttgaagatgcttttgacgtggcaagtttcctggacaagagtgag
      P  E  E  I  L  F  E  D  A  F  D  V  A  S  F  L  D  K  S  E

1082 gcccagcatatgtctaacatcagcgctatgtttgccacctgcaactcagaaaatccagag
      A  Q  H  M  S  N  I  S  A  M  F  A  T  C  N  S  E  N  P  E

1142 gagaaatttcagctctacatacagatcattgagttttcaaaagccttggctgtgttaac
      E  K  F  Q  L  Y  I  Q  I  I  E  F  F  K  S  L  G  C  V  N

1202 actccattaaaccaggaaccagagcccccagatgatgatgatgcaatgttgctgaagtgc
      T  P  L  N  Q  E  P  E  P  P  D  D  D  D  A  M  L  L  K  C
                  EP034150 peptide
```

```
1262 ctggagacctttgacgatctgtaaagcaggagggccggagaaggaggagattaaatgggg
      L   E   T   F   D   D   L

1322 gaggggcgggttcaagtttgaataccagtagctagagttatactgccttgtctttcaaga

1382 ctcattgagaaaggctgcttccaaaagccttcttgatgttcttagtttctgttactataa

1442 agttttaagagcaatg
```

Figure 3.29 Nucleotide sequence and deduced amino acid sequence of *Stra8* cDNA. ATG codon and TAA terminating codon are bold and coloured in blue. Glutamic acid rich domain is marked in green colour. The amino acid sequences of synthetic peptides used for generation of anti *Stra8* antibody are underlined. Positions of primers Stra8 F and Stra8 R are marked and coloured in violet. Sequence from NCBI.

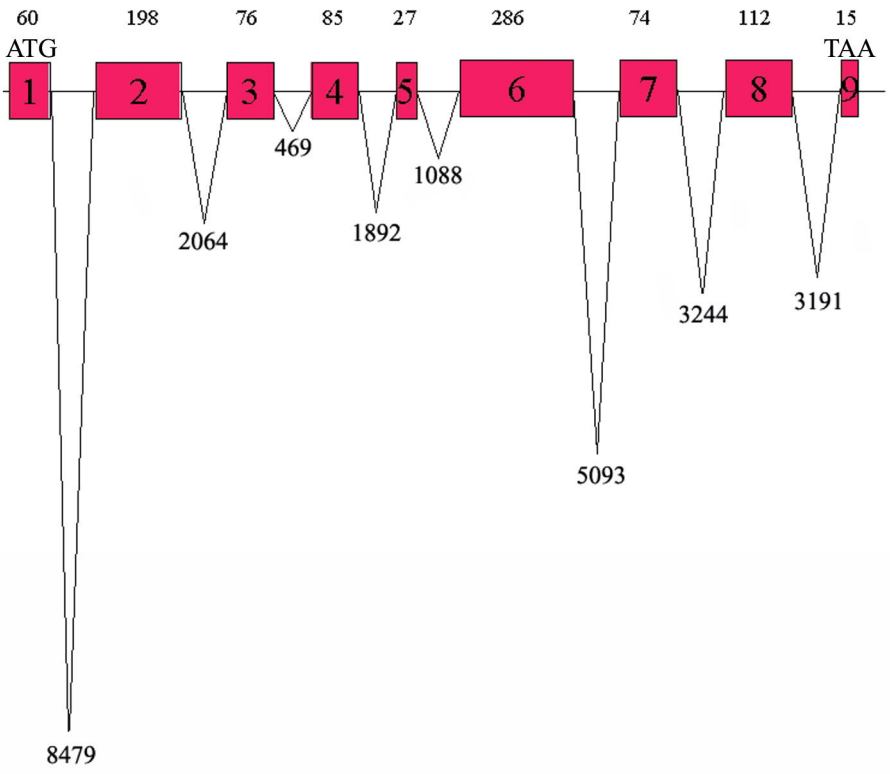


Figure 3.30 Exon – intron structure of the human *STRA8* gene. Red boxes represent exons. Lengths of exons and introns are given, ATG and stop codon TAA are marked.

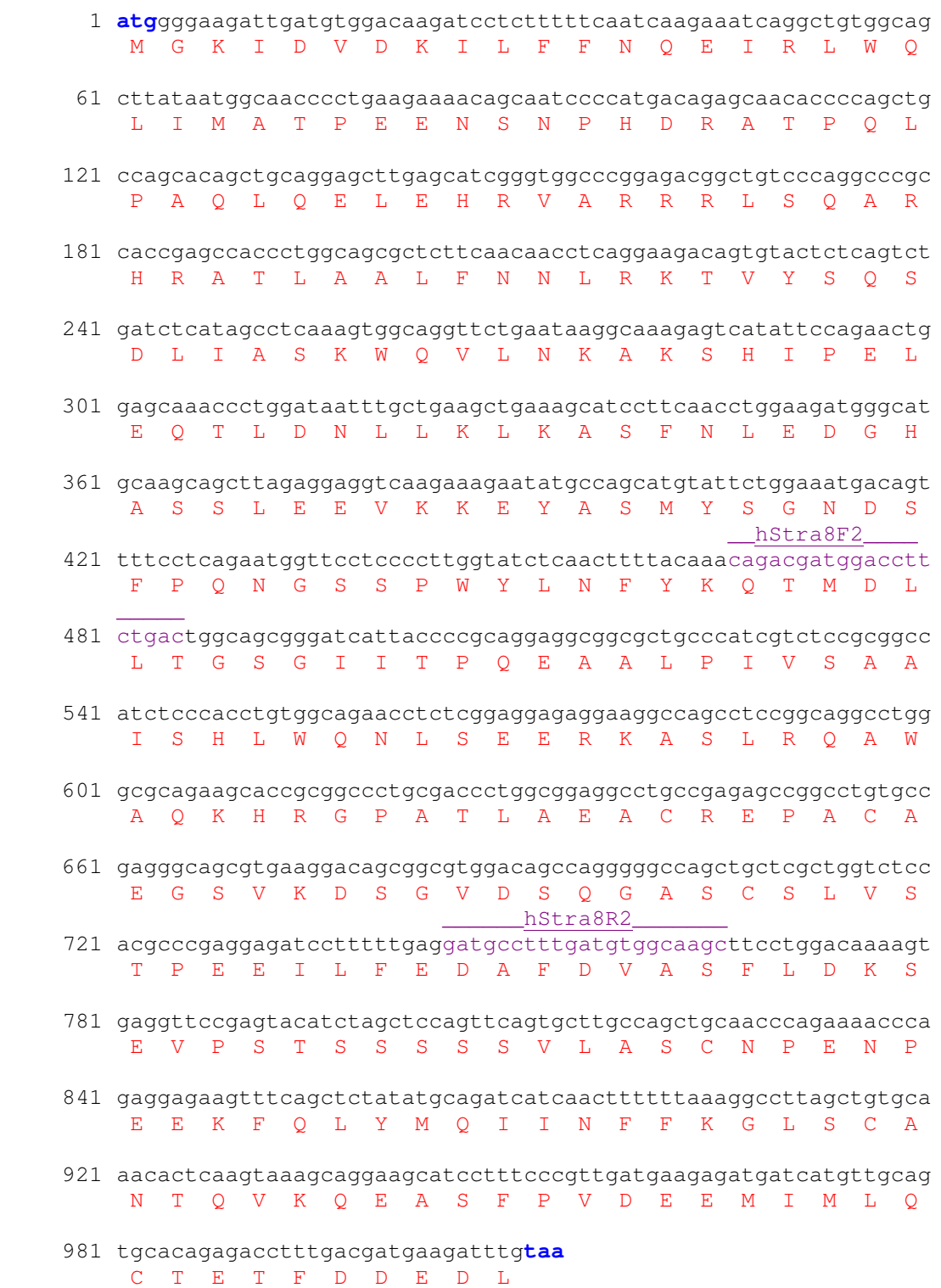


Figure 3.31 Nucleotide sequence and deduced amino acid sequence of human *STRA8 c*DNA. ATG codon and TAA terminating codon are bold and coloured in blue. Position of primers hStra8F2 and hStra8R2 used for expression analysis is marked and coloured in violet. Sequence from NCBI.

3.2.1 Expression analysis of *Stra8* gene

3.2.1.1 Transcriptional analysis

RT-PCR using primers Stra8F (localised in exons 2 and 3) and Stra8R (localised in exon 5, Fig. 3.29) amplifying a 440 bp fragment of *Stra8* gene was performed for expression analysis. First, RNA was isolated from different tissues and PCR product was observed exclusively in testis. RT-PCR with RNA from testes of different developmental mutants: W/W^V, Tfm/y, Leyl-/-, olt/olt and qk/qk revealed that the transcript is present in all mutants, except W/W^V. Expression of the gene is restricted to male germ cells. *Stra 8* transcript was present as well in all tested postnatal stages and in prenatal stages from day 15.5 onwards. Sex of embryos was determined by genotyping with SryF and SryR primers and male embryos were used for RNA isolation (Fig. 3.32). These results confirm the results obtained by other group (Oulad – Abdelghani et al., 1996).

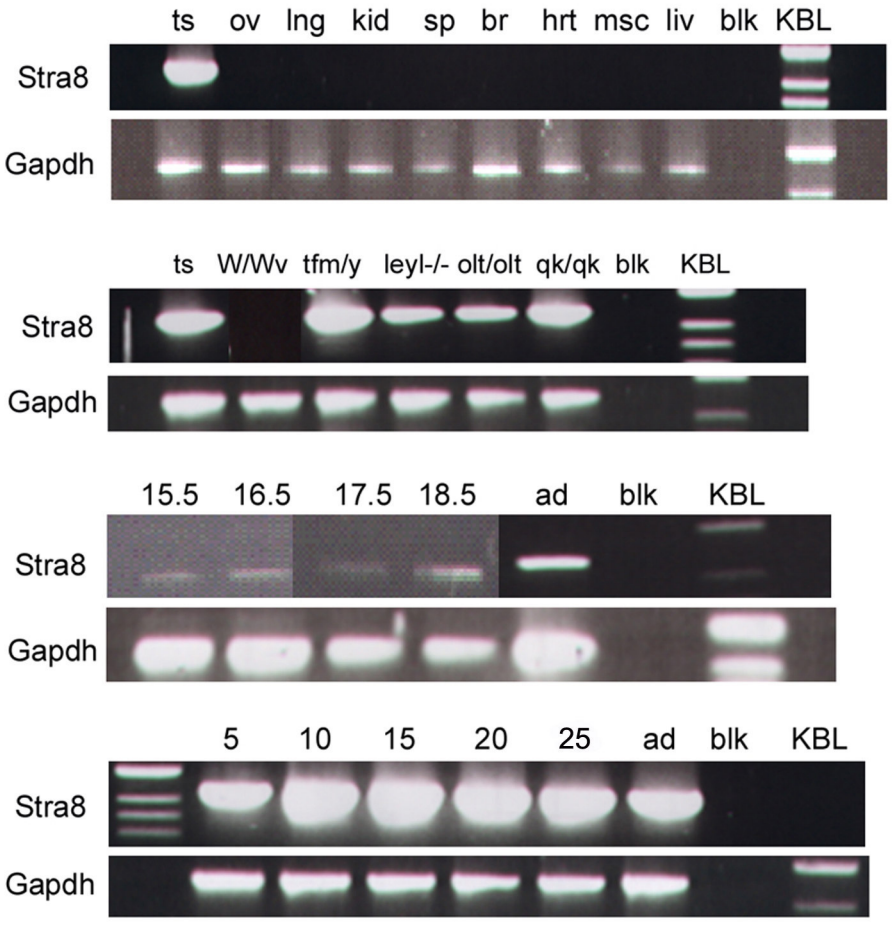


Figure 3.32 RT-PCR expression analysis of *Stra8* in different tissues and testes of mutants, pre- and postnatal developmental stages using Stra8F and Stra8R specific primers. Expression is restricted

to testis and germ cells only, since expression is not observed in W/W^{V} mutants. *Stra8* expression was determined from 15.5 dpc onwards and continues in postnatal development. Gapdh served as a control. Abbreviations are: ts: testis, ov: ovary, lng: lungs, sp: spleen, br: brain, hrt: heart, msc: muscle, liv: liver, blk: blank = no-template probe, ad: adult testis, KBL: standard molecular weight marker.

Expression of human *STRA8* gene was tested by RT-PCR using primers hStra8F2 and hStra8R2 amplifying a fragment of 309 nucleotides (Fig. 3.31) with RNA isolated from different human tissues. RT-PCR revealed that expression of *STRA8* is also restricted to the testis, as no band was detected in other tissues (Fig. 3.33).

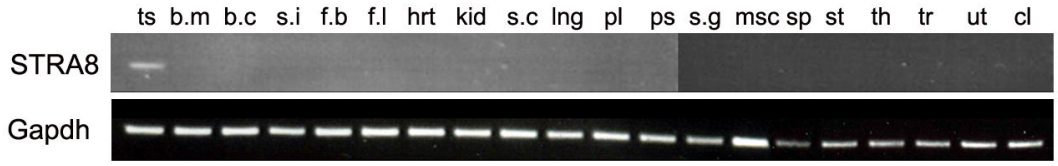


Figure 3.33 RT-PCR expression analysis of *STRA8* in different tissues: ts: testis, b.m.: bone marrow, b.c: brain cerebellum, s.i.: small intestine, f.b: fetal brain, f.l.: fetal liver, hrt: heart, kid: kidney, s.c.: spinal cord, lng: lung, pl: placenta, ps: prostate, s.g: salivary gland, msc: muscle, sp: spleen, st: stomach, th: thyroid, tr: trachea, ut: uterus, cl: colon.

RT-PCR analysis was also performed with RNA isolated from cultured human teratocarcinoma cells Tera 1. Higher expression of the gene was observed in cells incubated with retinoic acid (final concentration 10^{-6}M). It suggests that expression of human ortholog of murine *Stra8* is inducible by retinoic acid (Fig. 3.34).

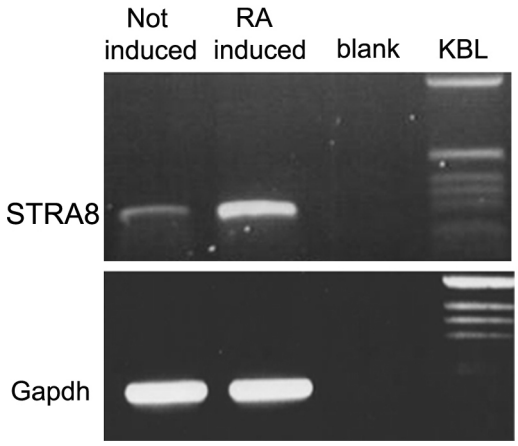


Figure 3.34. RT-PCR analysis of expression of *STRA8* in Tera 1 cells. Higher expression was observed in RA treated cells.

3.2.1.2 Translational analysis

Like in the case of *Tex18* gene, cDNA of *Stra8* gene was amplified in order to generate Stra8-GST fusion protein against which antibody could be developed. Despite of many trials of RT-PCR and using *Pfx Platinum* polymerase which has proof-reading activity, no product without mismatches was obtained. Therefore, polyclonal antibodies against two Stra8 peptides were developed, as it was described in section 2.2.15, by Eurogentec Company. The position and sequences of synthetic peptides are shown in Figure 3.29. Two purified antibodies, described as IgG vs. EP034149 and IgG vs. EP034150 were obtained. In order to check immunogenicity of both peptides, dot blot hybridization was performed applying both obtained antibodies. For this purpose, different amounts of peptides were blotted on Hybond-C membrane and were let to dry. Membranes were incubated, as in normal Western blot, in such a way, that each peptide was incubated with each antibody. Dot blot experiment revealed that only EP034150 was immunogenic since both antibodies recognized only this peptide and not the second one (Fig 3.35).

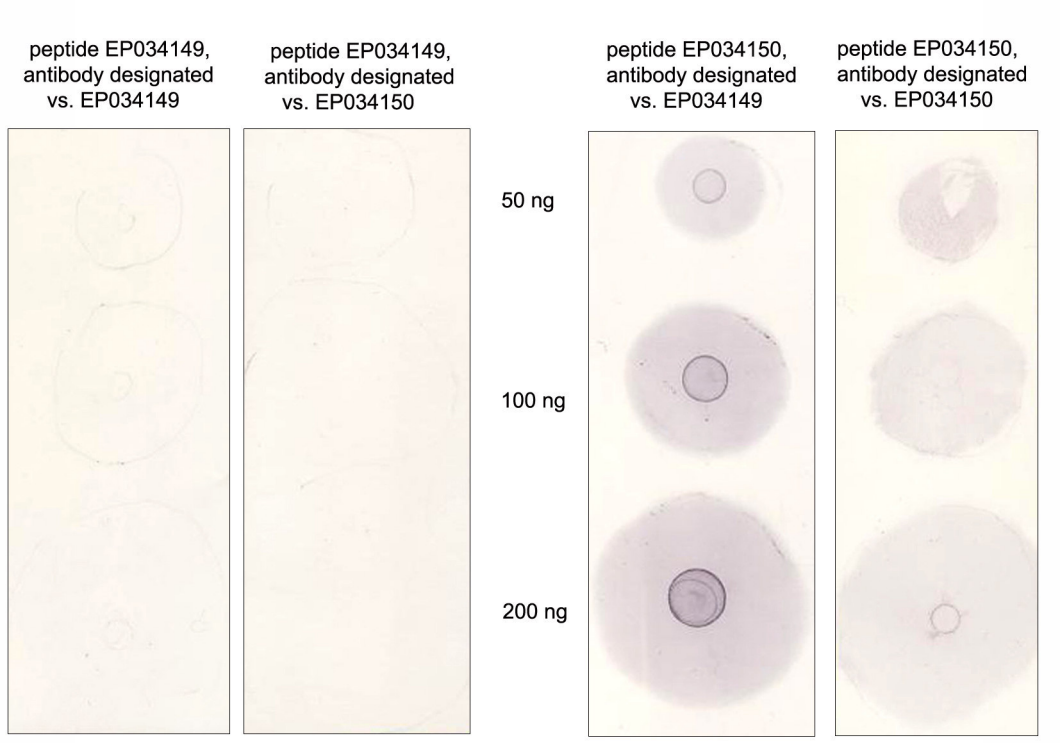


Figure 3.35 Dot blot hybridization of EP0341149 and EP034150 peptides with purified *Stra8* antibodies. Only second peptide was immunogenic and both antibodies recognize only EP034150.

To check affinity of both antibodies, Western blot analysis was performed. Total protein extracts from two tissues: testis and kidney (negative control) were separated on SDS-PAGE gel and transferred onto PVDV membrane. When antibody designated versus EP034149 were used, one specific band was obtained in total testicular cell extract (however the size was not as expected 45 kDa) and two weak signals in kidney cell extract. Many unspecific bands in both cell extracts were detected, when second antibody, defined as directed against EP034150 was applied (Fig. 3.36).

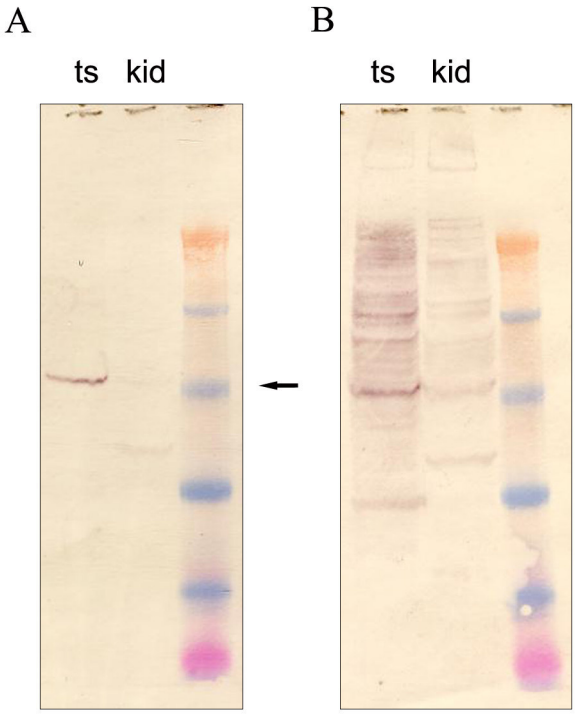


Figure 3.36 Western blot analyses with *Stra8* antibodies. When antibodies defined as directed against EP034149 were used, one specific band of not expected size was detected with total testis cell extract, however two weak bands in kidney extract were also observed (A). When antibody defined as directed against EP034150 was applied, many unspecific bands were detected in both extracts (B). Abbreviations are ts: testis, kid: kidney.

Because the second antibody gave to many unspecific signals, only the first antibody was used for further experiments. Despite of many Western blot trials, result shown in Figure 3.36A was not repeated. No band or different bands of not expected sizes were obtained. Assuming that these results could be due to not proper purification of antibody from serum, next purification was performed (2.2.15). An affinity column conjugated with EP034150 peptide was used for the purification. After binding of antiserum to the column, fractions of purified antibodies were eluted. Concentration of antibody in elutions was determined. Elutions which showed highest antibodies concentrations were used for further Western blots analyses. Unfortunately, purified antibody did not give expected result and no successful Western blot analysis was reached.

To check if *Stra8* antibody is able to recognize protein in histological sections and to determine *Stra8* expression pattern, immunostaining of paraffin embedded adult mouse

testis was performed. Slides were incubated overnight with *Stra8* antibody in a dilution of 1:100 followed by 1 hr incubation with FITC - conjugated antibody. A specific signal was only detected in premeiotic germ cells, localised close to the basal lamina of seminiferous tubules (Fig 3.37). These results are in accordance with the results of expression studies of EGFP signal in testes of *Stra8* transgenic mice and published data (Oulad – Abdelghani et al., 1996). In all cases the same expression pattern was observed.

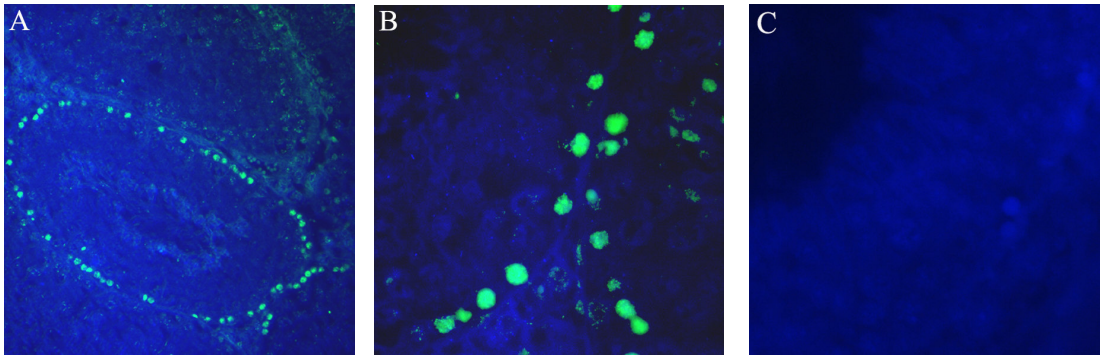


Figure 3.37 Immunostaining of testis of adult mice with *Stra8* antibody. Immunostaining using antibody against Stra8 protein followed by incubation with FITC conjugated secondary antibody revealed that the protein is expressed in early premeiotic spermatogonia localised near basal lamina of seminiferous tubules. General overview of testis seminiferous tubules (A) and higher magnification (B) are given. Incubation with only secondary antibody was performed as negative control (C).

3.2.2 Generation and analysis of *Stra8* transgenic mice

3.2.2.1 Generation of the transgenic construct

Like in the case of *Tex18* gene *Stra8* transgenic mice were generated. In this case a 1.4 kb promoter region of the *Stra8* gene was amplified from genomic DNA with primers Stra8tr F and Stra8tr R and cloned into pEGFP-1 vector (Fig.3.38). Expression of enhanced green fluorescent protein under the *Stra8* promoter in transgenic mice was expected.

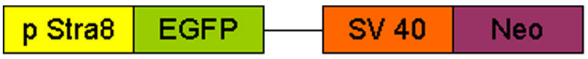


Figure 3.38 Schematic representation of Stra8-EGFP fusion gene showing 1.4 kb promoter region of mouse *Stra8* (p Stra8) linked to the coding region of EGFP.

Stra8-EGFP transgenic lines were generated by microinjection of Stra8-EGFP fragment (released from vector sequences by digestion with *Sac* I and purified after gel electrophoresis) into the pronuclei of fertilized 1-cell mouse embryos. The injected embryos were transferred into FVB pseudopregnant hosts. Transgenic mice harbouring Stra8-EGFP construct were identified by Southern blot, using a EGFP cDNA probe (Fig. 3.39). Positive founder animals were bred with non- transgenic FVB mice and their transgenic progeny was crossed to produce homozygous animals. Two independently established transgenic lines, namely Stra8/16 and Stra8/17 were generated.

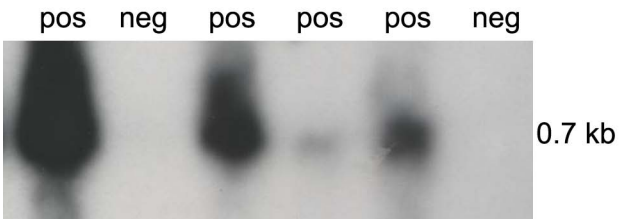


Figure 3.39 Southern blot screening of mice in order to obtain transgenic founders. pos: positive animal (transgene carrier), neg: negative animal (not transgenic).

3.2.2.2 Expression analysis in *Stra8* transgenic lines

Northern blot hybridization using RNA isolated from different tissues of Stra8/17 transgenic line and EGFP cDNA probe was performed and revealed that expression of EGFP under *Stra8* promoter is restricted to testis (Fig 3.40). This result was confirmed also by RT-PCR performed on RNA isolated from both transgenic lines and using primers EGFPF2 and EGFPR2, localised in EGFP cassette (Fig 3.41).

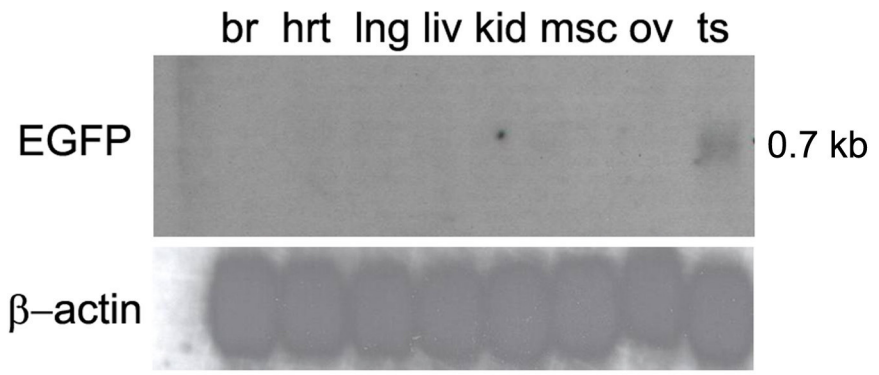


Figure 3.40 Northern blot analysis of different tissues of Stra8/17 transgenic line using EGFP probe. Abbreviations are: br: brain, hrt: heart, lng: lungs, liv: liver, kid: kidney, msc: muscle, ov: ovary, ts: testis. β-actin served as a positive control.

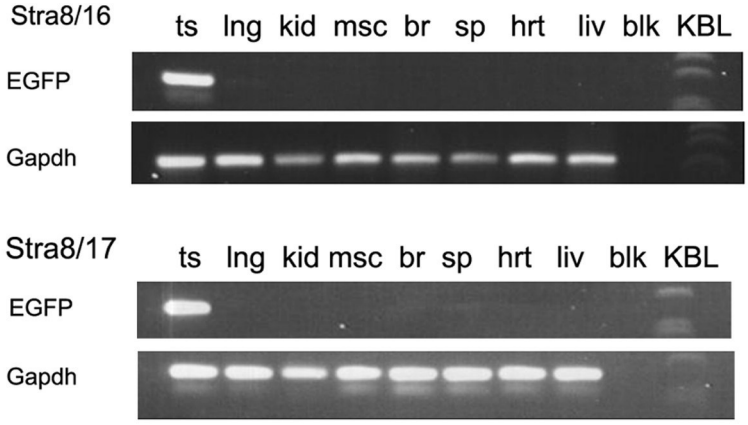


Figure 3.41 RT-PCR analysis of two independent transgenic lines Stra8/16 and Stra8/17 using EGFP primers. Total RNA from different organs was used. Abbreviations are: ts: testis, lng: lung, kid: kidney, msc: muscle, br: brain, ov: ovary, hrt: heart, liv: liver, blk: blank = no-template probe. Gapdh served as a control.

Histological sections of testis from Stra8/17 transgenic line were performed. Green signal was observed under UV light in premeiotic cells localised close to the basal lamina of seminiferous tubules (Fig. 3.42). This signal reminiscent result of immunohistochemistry performed with testis sections from wild type males with *Stra8* antibody, shown in Figure 3.37.

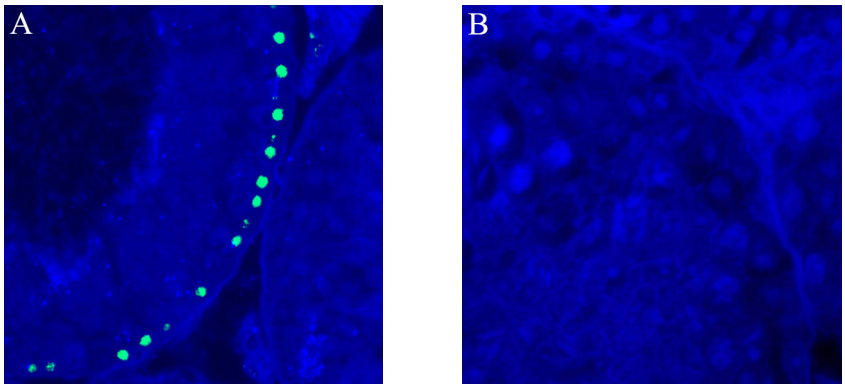


Figure 3.42 Histological analysis of testis from transgenic line Stra8/17. Green signal emitted from EGFP is localised in early premeiotic germ cells (A). No green signal was observed in wild type control (B). Blue colour represents DAPI staining

3.2.3 Targeted inactivation of mouse *Stra8* gene

3.2.3.1 Isolation of cosmid clones from mouse genomic DNA library

Mouse RZPD (The Resource Center and Primary Database, Berlin) genomic library 129 ola was screened with cDNA probe developed with Stra8R and Stra8R primers, including *Stra8* exons 2-5. Three positive clones were obtained: MPM Gc 121A05192Q2, MPM Gc 121B15401Q2 and MPM Gc 121J13186Q2. Dot blot hybridization of these clones revealed that only one clone was positive. This clone was used for restriction analysis. During analysis of restriction fragments digested with different enzymes and comparing them with known genomic structure it was realized, that cosmid clone MPM Gc 121A05192Q2 do not contains sequences upstream from exon 2. Second screening of cosmid library was then performed, using 5' probe containing promoter region of *Stra8* gene. Again three positive clones were recognized: MPM Gc 121M14699Q2, MPM Gc 121E24561Q2 and MPM Gc 121K13513Q2 and all of them were used for restriction analysis.

3.2.3.2 Restriction digestion analysis of cosmid clones

Cosmid clone MPM Gc 121A05192Q2 was digested with enzymes: *Xba* I, *Bam* HI, *Eco* RI, *Not* I and *Xho* I, blotted and radioactively hybridized with *Stra8* cDNA probe. The size of fragments was calculated and compared with the known genomic sequence.

The *Bam* HI fragment, containing exon 9, was chosen as 3' wing of the targeting construct (Fig. 3.43). Subsequently, cosmid clone MPM Gc 121M14699Q2 was restricted with the following enzymes: *Eco* RI, *Pst* I, *Apa* I and *Hind* III, blotted and hybridized with 5' probe. After calculating of the fragments' sizes and comparing them with the known genomic structure of *Stra8*, *Pst* I fragment, which contains exon 1 was chosen as 5' wing of the construct (Fig. 3.44).

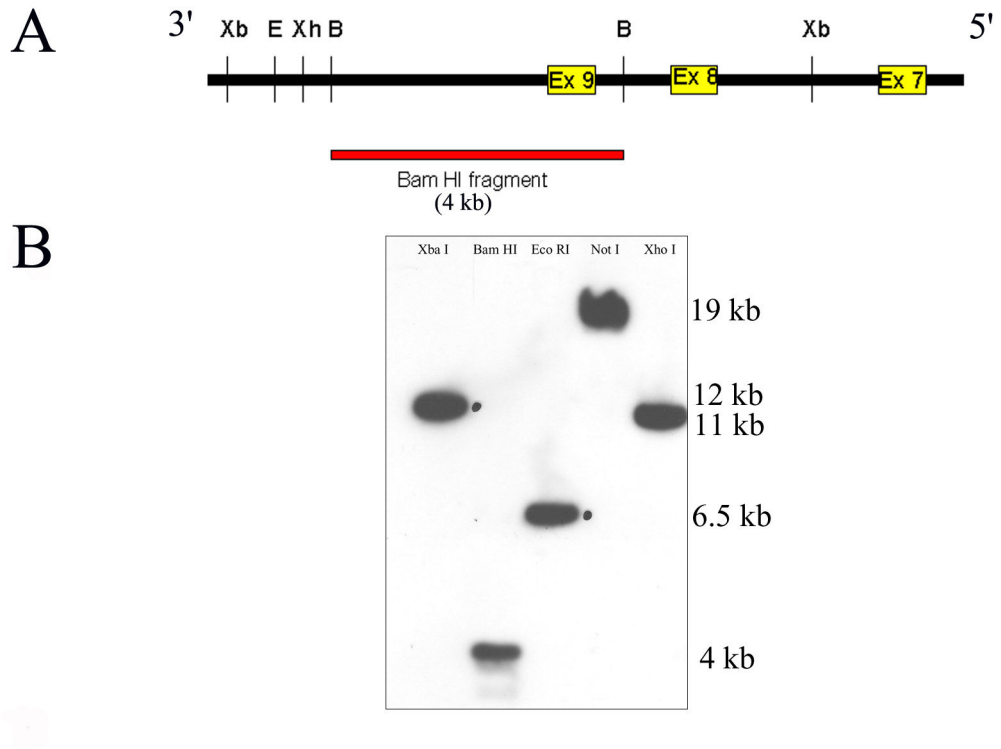


Figure 3.43 (A) Restriction digestion map of 3' fragment of *Stra8* gene and fragment used as 3' wing of targeting vector. Abbreviations are: Xb: *Xba* I, E: *Eco* RI, Xh: *Xho* I, B: *Bam* HI. (B) Southern blot hybridization of cosmid clone MPM Gc 121A05192Q2 digested with different enzymes.

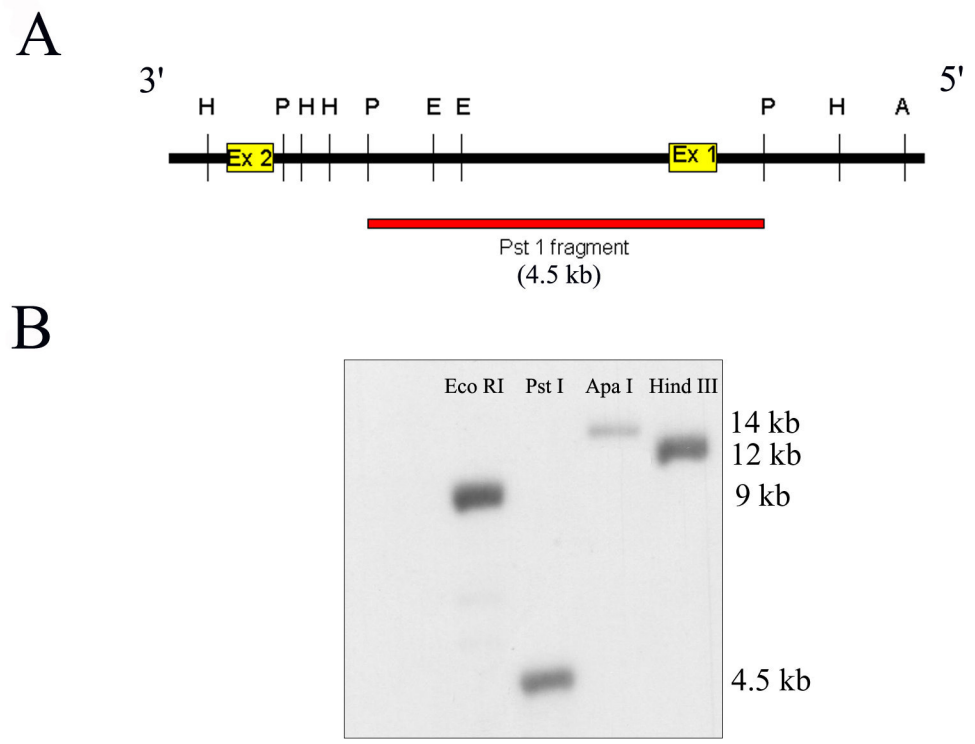


Figure 3.44 (A) Restriction digestion map of 5' fragment of *Stra8* gene and fragment used as 5' wing of targeting vector. Abbreviations are: H: *Hind* III, P: *Pst I*, E: *Eco* RI, A: *Apa* I. (B) Southern blot hybridization of cosmid clone MPM Gc 121M14699Q2 digested with different enzymes.

3.2.3.3 Generation of the *Stra8* knock – out construct

The 5' wing of the knock – out construct was generated by subcloning of the *Pst* I fragment into pZERO-2 vector and subsequent cutting of 4.5 kb *Not* I fragment and cloning it in pPNT-Neo targeting vector (Fig. 3.44 A and 3.45). For the generation of 3' wing, *Bam* HI fragment was cloned in pBS II SK vector and then 4 kb *Eco* RI/*Xba* I fragment was cloned in pPNT-Neo vector (Tybulewicz et al., 1991)(Fig. 3.43 A and 3.45). In this way, exons from 2 to 8 of *Stra8* gene will be disrupted and replaced with *Neo* cassette in homologous recombinant clones. *Neomycin* can be used as marker for positive selection, while two copies of *thymidine kinase* from Herpes virus serve as a negative selection marker. The correct orientations of both wings were checked by sequencing them with pPNTF1, pPNTF2, pPNTR1 and pPNTR2 primers.

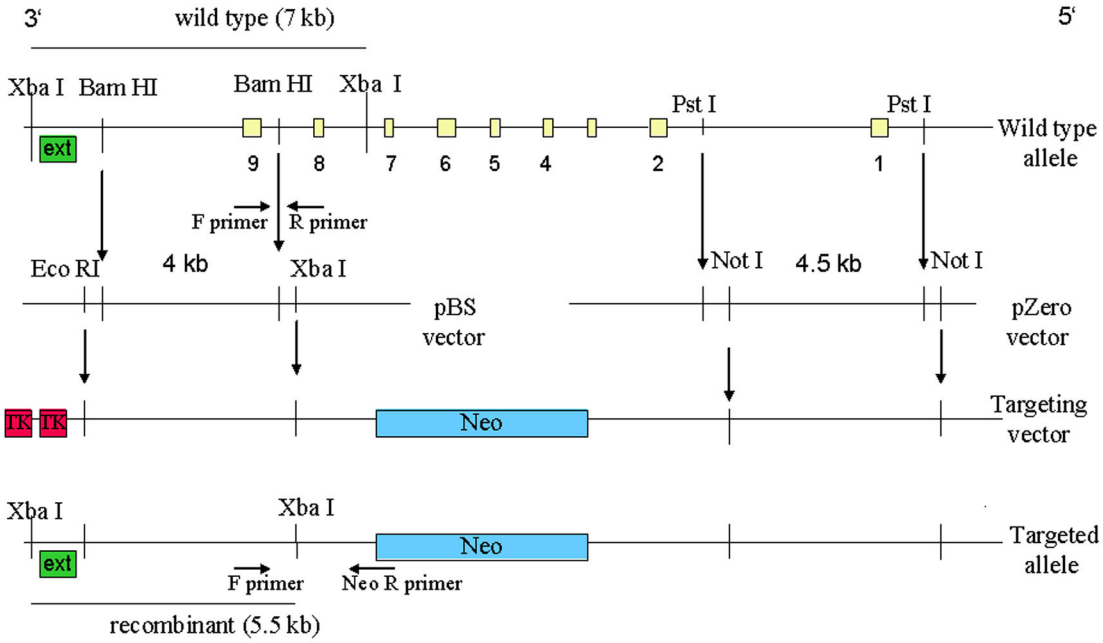


Figure 3.45 Schematic representation of the targeting strategy of *Stra8* gene. The structure of the wild type allele, targeting vector and mutant allele are shown together with the relevant restriction sites. Exons from 2 to 8 were replaced by *Neo* selection cassette. Position of primers StraPCRF (F primer), StraPCRR (R primer) and NeoRStra2 (Neo R primer) used for genotyping of mice and position of external probe used for screening of ES clones and lengths of fragments recognized by this probe by Southern blot in wild type and targeted allele are indicated. TK stays for *thymidine kinase* cassette

3.2.3.4 Generation of the 3' external probe

A fragment of 0.6 kb downstream to *Stra8* gene was amplified by PCR using primers StrextF and StrextR. External probe was generated in such a way, that it recognizes in Southern blot hybridization *Xba* I fragments specifically (Fig. 3.45). This PCR product was cloned in pGEM-TEasy vector and cut out with *Eco* RI enzyme. It was used then for Southern blot screening of ES cells for recombinants.

3.2.3.5 Electroporation of the ES –cells and screening of ES – cells for homologous recombination.

The *Stra8* targeting construct was linearised with *Pvu* I enzyme and 50 µg of purified DNA was electroporated into RI embryonic stem cells as it was described in section 2.2.19.1. Cells were plated on fibroblast layer and after 10 days of selection 97 individual Neomycin resistant clones were picked in 24 well plates and replicated. Subsequently DNA from ES cell clones was isolated, digested with Xba I enzyme, electrophoresed and blotted onto Hybond C membrane. Blots were hybridized with radioactively labelled 3' external probe. In case of wild type allele only one band of 7 kb was expected, in the event of homologous recombination an additional band of 5.5 kb should be obtained. In case of non – homologous recombination only wild type band will be visible (Fig. 3.46). DNA of only 51 ES cells was of good quality and two positive clones were found, namely clones 19 and 21. They were checked again by Southern blot using external probe as well as with Neo probe (in this case band of 2 kb was expected). Clone number 21 was used for blastocyst injection.

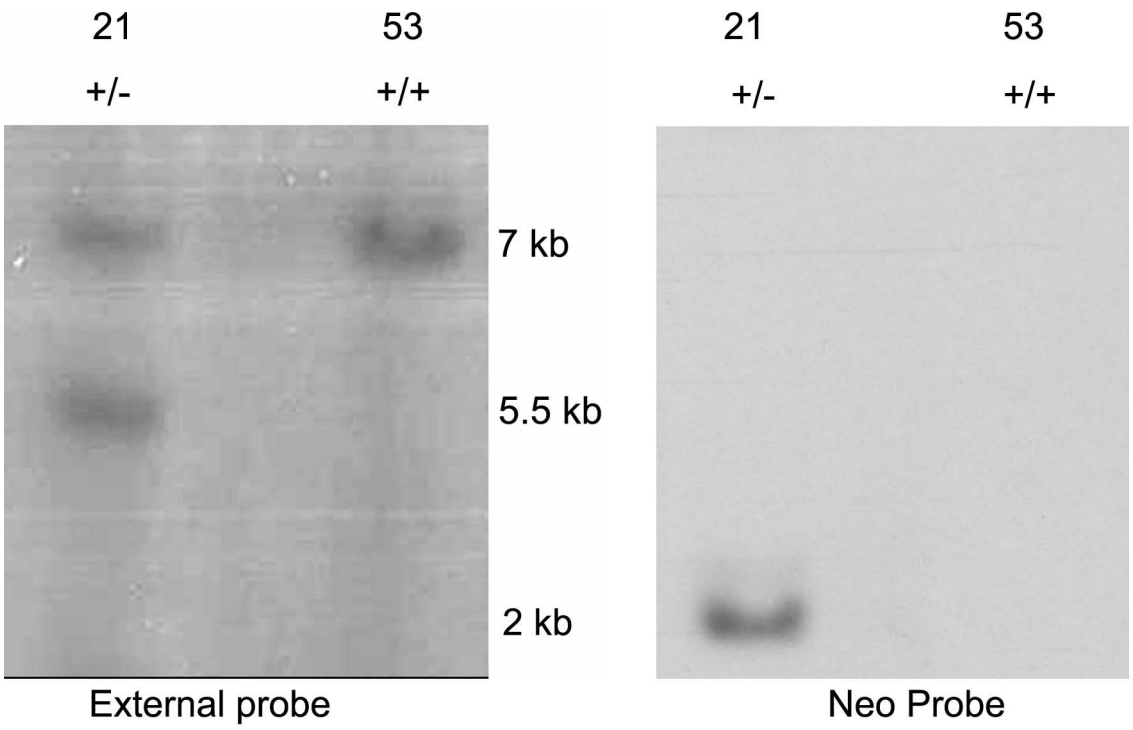


Figure 3.46 Genomic Southern blot analysis. Genomic DNA of ES clones was digested with *Pvu* I enzyme, separated on 0.6% agarose gel and transferred onto Hybond C membrane. The blot was hybridized with radioactively labelled 0.6 kb 3' external probe. In case of wild type allele (+/+) one

band of 7 kb was observed, while in case of recombinant allele (+/-) an additional band of 5.5 kb was found. Clones' numbers are given.

3.2.3.6 Generation and analysis of chimeric mice

ES cells from clone number 21 were injected into 3.5 dpc blastocyst derived from C57 BL/6J mice. They were implanted into pseudopregnant CD 1 mice to generate chimeric mice. Altogether 5 chimeras were obtained; they were scored according to coat colour (in percentage): one male with 10%, one with 20%, two males with 5% and one female with 35%. All of them were bred with C57 BL/6J mice to obtain F1 generation. Genotyping strategy by PCR, using primers StraPCRF, StraPCRR and NeoRStra2 (F primer, R primer and Neo R primer in Figure 3.45) to distinguish between wild type and recombinant alleles was designed. In case of wild type allele a fragment of about 250 bp will be obtained, while in the case of recombinant allele a fragment of around 700 bp will be visible (Fig. 3.47).

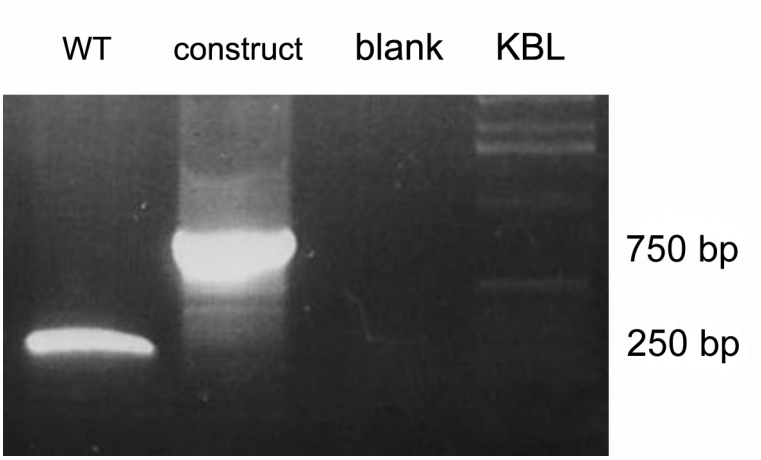


Figure 3.47 Strategy for genotyping of *Stra8* deficient mice. Wild type allele of *Stra8* gene amplified by primers StraPCRF and StraPCRR results in 250 bp PCR product (WT). For recombinant allele a PCR fragment of around 700bp amplified with primers StraPCRF and NeoRStra2 is expected (construct). Blank is no template probe.

Two males: with 10% and 20% of chimerism and one female with 35% of chimerism gave few offspring, but all of the mice were black, therefore no transmission of

recombinant allele occurred. Two males with 5% of chimerism did not give any offspring, although they were bred for almost three months. No sperm was observed in uterus and oviducts in vaginal plug positive females bred with 5% males. DNA from different tissues of two 5% males and DNA from sperm from all chimeric males was isolated. PCR using primers ST F1 and ST R1 was applied – it enables to distinguish between DNA of strains C57 BL/6J and 129/Sv, since the PCR products from both strains differ in lengths (recombinant allele is derived from 129/Sv strain, while recipient embryo is from C57 BL/6J background). Low chimerism was observed in different tissues of both 5% chimeras, but only in one of them, when DNA from sperm was used for PCR. No PCR marker specific for 129/Sv strain was observed on PCR on sperm DNA from chimeras with 10% and 20 %, (Fig. 3.48).

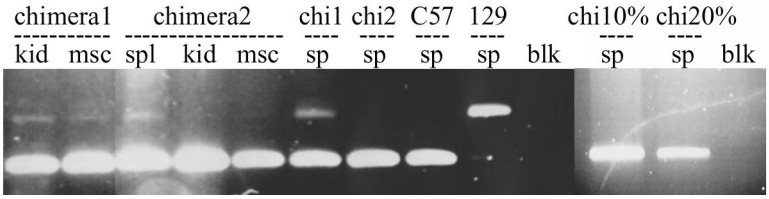


Figure 3.48 PCR analysis of *Stra8* chimeras. Low chimerism was found in some tissues from both 5% chimeras, as a weak band of 129/Sv marker is observed. Low chimerism is observed also in sperm of first 5% chimera. No chimerism was detected in case of second chimera with 5% and in chimeras of 10% and 20% of chimerism. Abbreviations are: chi1: first 5% chimera, chi2: second 5% chimera, chi10%: 10 % chimera, chi20%: 20 % chimera, kid: kidney, msc: muscle: spl: spleen, sp: sperm, blk: blank = no template probe.

Histological sections were performed on testes from both 5% chimeras. Microscopical observations of slides stained with hematoxylin/eosin revealed arrest of spermatogenesis in testis of first chimera, as in many seminiferous tubules no spermatozoa and spermatids were observed (Fig. 3.49). No abnormalities were found in second 5% chimera. Although no detailed conclusions can be made from analysis of chimeras, it is obvious that *Stra8* gene has an important role in spermatogenesis, as its function was disrupted even in male of 5% chimerism.

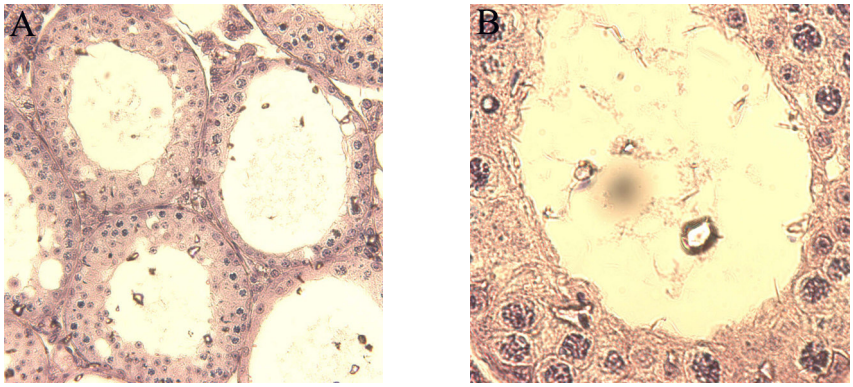


Figure 3.49 Histological analysis of testis of *Stra8* 5% chimera. Paraffin sections of testis revealed arrest of spermatogenesis. No spermatozoa and spermatids were observed in the lumen of most of the seminiferous tubules.

Since all of the chimeras could not be used for breeding in order to obtain F1 generation, new blastocyst injection was planned. During this time, scientific report on conference in Cold Spring Harbor revealed that other scientific group generated *Stra8* deficient mice and both sexes with knock – outed *Stra8* gene are infertile. Because these data were expected to be published soon, our *Stra8* project was stopped.

4 DISCUSSION

Spermatogenesis is a complex process, which is essential for male reproductive success. It requires coordination and proper function of a number of testis-expressed genes. Although much effort is given in recent years into elucidation of molecular mechanisms governing spermatogenesis, the functions of many genes remain unclear. One of the crucial steps in the developing of male germ cells is when spermatogonial stem cells (A_s spermatogonia) have to either self-renew themselves and to retain their stem cell character or to differentiate into a further stage (A_{pr} spermatogonia) of spermatogenesis.

The initial aim of the present study was the elucidation of molecular mechanisms of proliferation and differentiation of spermatogonial stem cells. For this purpose genes which are expressed in these cells were taken into consideration. *Tex18* and *Stra8* are two recently discovered genes. Their expression was described to be restricted to the premeiotic stages of spermatogenesis. It was hypothesized, that they may play roles in the determination of fate decision of spermatogonial stem cells; therefore they were chosen as a subject of this study.

In the first part of the discussion, results concerning expression analysis of *Tex18*, its subcellular localisation as well as results obtained by the analysing of *Tex18* transgenic and knock-out mice are considered. Generation of the knock-out model and obtained interesting phenotype is also widely discussed. In the second part, expression of *Stra8* gene, generation and analysis of transgenic animals and comparison of our data with published results are discussed. Examples of practical implications of the knowledge obtained from *Stra8* studies are also given. Expression pattern of human ortholog of *Stra8* gene is presented as well. Finally, generation of knock-out model for *Stra8* and problems which were faced during this work are discussed.

4.1 Expression analysis of *Tex18* gene

4.1.1 RT-PCR analysis of *Tex18* gene

Tex18 gene (**T**estis **ex**pressed gene **18**) was described for the first time, as it was mentioned before, by Wang et al. (2001). As it was previously shown by RT-PCR, expression of *Tex18* is restricted to the testis. We have confirmed these data, performing RT-PCR on RNA isolated from different tissues (testis, ovary, lung, kidney, spleen, heart, brain, muscle, liver). RT-PCR signal was observed exclusively in testis. In addition, RT-PCR was performed on RNA isolated from the testes of different developmental mutants. W/W^V mice, which have the mutation in the dominant white-spotting allele are characterized by lack of any germ cells, because of disruption of the c-kit/SCF pathway (review de Rooij and Boer, 2003). In Tfm/y (Testicular Feminization) and $Leyl^{-/-}$ ($Insl3^{-/-}$) mutants spermatogenesis is arrested at the stage of primary spermatocytes (Lyon and Hawkes, 1970; Zimmermann et al., 1999). In olt/olt mutants spermatogenesis is arrested at the stage of round spermatid, while in qk/qk at elongated spermatids (Moutier, 1976; Bennett et al., 1971). Expression of *Tex18* was observed in the testes of all mutants, except W/W^V mice. This result indicates, that expression is restricted to germ cells. RT-PCR analysis performed on RNA isolated from the testes of postnatal males of different ages revealed that *Tex18* is expressed constantly after birth, from the time point when spermatogenesis starts (Fig. 3.2). Prenatal stages of 15.5 and 18.5 dpc (days post coitum) males were evaluated, and in both cases expression of the gene was observed. Interestingly, *Tex18* RT-PCR signal was obtained also with RNA isolated from embryonic stem cells (ES cells). ES cells are derived from inner cell mass (ICM), a cluster of pluripotent stem cells, which exist only temporarily in blastocysts and have the capacity to differentiate into all types of embryonic tissues (Temple, 2003). Because ES cells are obtained from blastocysts cells, it suggests that *Tex18* could be expressed in such an early stage of mouse development. However, primordial germ cells are not recognizable until 7.5 dpc (Matsui, 1998). Possibility of early expression is supported by reports, that male and female germ cells can be derived from ES cells (Geijsen et al., 2003; Toyooka et al., 2003; Huebner et al., 2003). A similar situation is, for example, in the case of transcription factor *Oct4*, which is initially expressed in ICM, and then the expression is restricted to the germ cells

(Schoeler et al., 1990). However, expression of *Oct4* was observed also in preimplantation stages, which was not the case for *Tex18* (Fig.3.2). This early expression suggests, that *Tex18* might have function during the origination of primordial germ cells.

4.1.2 Generation and analysis of *Tex18* transgenic animals

To investigate *Tex18* expression more deeply, we designed a transgenic construct, in which a 1.6 kb promoter region upstream from the *Tex18* gene was cloned into pEGFP-1 vector, containing a coding region of enhanced green fluorescent protein (EGFP). We expected expression of the EGFP under the control of *Tex18* promoter in the same manner as endogenous gene (Fig. 3.7). After injection of the transgene DNA into the pronuclei of fertilized 1-cell embryos, which were transferred into pseudopregnant females, we obtained two lines of transgenic animals. Northern blot and RT-PCR revealed, that expression of the EGFP under the *Tex18* promoter is restricted to the testis, similarly like in the case of the endogenous gene (Fig. 3.9 and 3.10). It was a little bit surprising that the EGFP signal was visible in Northern blot analysis. It was not achieved in case of endogenous gene, despite many trials. One possible explanation for this discrepancy is that Tex18-EGFP transgene is integrated in the genome in high copy number and the expression level of Tex18-EGFP transgene is higher than endogenous *Tex18*. Therefore this expression can be detected by Northern blot analysis. Other possibility is the stability of EGFP RNA, not observed for *Tex18* RNA.

Histological sections of testes from transgenic males showed green signals emitted from germ cells, predominantly from postmeiotic cells, which was not observed in wild type control (Fig. 3.11). This observation indicates that *Tex18* expression is not restricted to the spermatogonia (as it was postulated by Wang et al., 2001), but occurs also in other stages of male germ cells differentiation. Expression of EGFP under *Tex18* promoter was evaluated in blastocysts. A green signal was observed in some cells of ICM of 3.5 dpc transgenic embryos, and was not observed in wild type control (Fig. 3.12). Result of this experiment supports data obtained by RT-PCR analysis of *Tex18* expression in ES cells.

4.1.3 FACS analysis of testicular cells of transgenic animals

To isolate EGFP- positive cells from testicular cell suspension, fluorescent activated cell sorting (FACS) was applied. We observed a distinct population of EGFP-positive cells after FACS analysis (Fig. 3.13). Interestingly, two different groups of males could be found, according to their age. The percentage of FACS-positive cells was significantly different in testes of younger (less than 20 days old) as compared to older (more than 20 days old) animals (Fig. 3.14). About ten times more EGFP positive cells were observed in testes of older animals. It is known, that postmeiotic germ cells are present in mouse testis at about postnatal day 20 (Shoji et al., 2005). Such a great change in the number of germ cells expressing EGFP suggests, that *Tex18* is expressed predominantly in postmeiotic germ cells and supports results obtained from histological analysis of testes of transgenic mice. Additional proof was given by the measurement of the DNA content of the EGFP-positive cells and testicular cell suspension from the wild type control. This analysis showed enrichment in the number of haploid cells in the EGFP-positive cells as compared to the wild type control (Fig 3.15). FACS assay using testicular cell suspension from *Tex18* transgenic mice cannot be used for the purification of the postmeiotic male germ cells, since the gene is expressed also in earlier stages. However, predominant expression of the gene in haploid cells is evident. Genes expressed in the spermatids are often involved in fertilization process. One example is a serine proteinase acrosin (ACR), a hydrolytic enzyme, localised in acrosome. It is involved in the recognition and binding of the sperm to the zona pellucida of the oocyte and the sperm penetration through the zona pellucida. Although sperm of the homozygous Acr -/- mice are able to penetrate the zona pellucida, fertilize the oocyte, and produce viable offspring, spermatozoa lacking acrosin protein show a delayed fertilization. Therefore Acr -/- sperm have a selective disadvantage when they are in competition with Acr +/+ sperm (Adham et al., 1997). Genes expressed in postmeiotic germ cells are involved also in the maturiation of spermatids. For example, histones are replaced by transition proteins and then by protamines during spermiogenesis. Disturbances in time of expression of either transition proteins or protamines lead to spermatid arrest (Escalier, 2001). Predominant expression of *Tex18* in haploid germ cells suggest its role in fertilization or spermiogenesis.

4.2 Subcellular localisation of the *Tex18* protein

For the translational expression analysis of *Tex18*, subcellular localisation and to prove, that the Tex18 protein is absent in the -/- mice, generation of antibody was planned. Unfortunately, obtained Tex18-GST fusion protein was not stable, as mainly GST protein and not fusion protein was detected on the Western blots (Fig 3.4). It could be possible, that immunized rabbits would generate antibody against GST protein, and not against fusion (and therefore Tex18) protein. This antibody would be useless, therefore it's generation was not continued. Another method had to be chosen for the subcellular localisation of the protein. Construct, which enables expression of *Tex18* together with enhanced green fluorescent protein (EGFP) as a fusion protein was designed. Microscopical observation of transiently transfected NIH 3T3 cells emitting a green signal and immunostained with 1D4B antibody, directed against lysosomal protein α-Lamp 1 (Chen et al., 1985), was performed. It revealed colocalisation of Tex18-EGFP fusion protein and α-Lamp 1 (Fig. 3.6). Lysosomes are organelles rich in hydrolytic enzymes and are responsible for the degradation of macromolecules from the extracellular environment through phagocytosis and endocytosis for reutilization. They can degradate macromolecules also from the cytosol through autophagy. They contain various acid hydrolases that are capable of degrading proteins, lipids, polysaccharides and other macromolecules (Sun-Wada et al., 2003). There are some organelles, termed as lysosome-related, and acrosome is one of them. This is a unique organelle, which is formed during spermatogenesis. During acrosome biogenesis, many small proacrosomal vesicles fuse together, forming an acrosomal vesicle. Finally, the acrosomal vesicle spreads radially over the lengthening and compacting nucleus. The acrosome lumen is acidic and contains hydrolytic enzymes involved in acrosome reaction – because of this characteristic acrosome is a lysosome-related organelle (Sun-Wada et al. 2003). It is possible that lysosomal localisation of the Tex18-EGPF fusion protein in NIH 3T3 cells correspond to acrosome localisation of the Tex18 protein in the spermatozoa. *Tex18* could be then involved in the fertilization process.

4.3 Functional studies of *Tex18* gene - generation and analysis of *Tex18*-deficient mice

4.3.1 Generation of *Tex18* knock-out mice and fertility analysis

To elucidate the role of the *Tex18* gene we decided to generate knock – out mice. Because *Tex18* is very short, it was possible to delete the whole gene. 5 kb and 2.5 kb fragments, flanking *Tex18* were cloned into pTK-Neo targeting vector and the gene was replaced with a *Neo* cassette (Fig 3.17). The targeting vector was transfected into the ES cells and several positive recombinant clones were obtained. One of them was chosen and injected into blastocysts. Four male chimeras were obtained, from which two were fertile and both of them transmitted recombinant allele on both C57BL/6J x 129Sv and 129Sv backgrounds. Obtained heterozygous mice were further intercrossed, in order to obtain *Tex18*-deficient homozygous mice. RT-PCR performed on RNA isolated from testes of homozygous, heterozygous and wild type males showed that *Tex18* was fully disrupted in the -/- males, as no *Tex18* specific band was obtained (Fig. 3.20). Fertility of -/- males from both backgrounds were tested for three months, when they were bred with -/- and +/+ females. A small underrepresentation of heterozygous offspring was observed, when heterozygous males were crossed with wild type females, as it was shown by a chi^2 test. Disruption of Mendelian ratio was observed also when heterozygous males were bred with heterozygous females. Both situations respect 129/Sv strain (Table 3.1). Average litter sizes from every type of breeding are summarized in the Table 3.2. The average litter size from breeding of homozygous mice was significantly smaller comparing to other results, especially in case of 129/Sv background, because typical litter size is 7 on C57 Bl/6J and 5.9 on 129/Sv backgrounds (Silver, 1985). 8 males from mixed background and twelve from 129/Sv background were used for fertility tests. All the males from the C57 BL/6J x 129/Sv background were fertile, while four males from the 129-Sv background were fully infertile, since they did not give any offspring in the three month long breeding period. The remaining 8 males gave not more than 2 offspring per litter. It indicates fertility problems of *Tex18* knock-out males. To check if subfertility of *Tex18*-deficient mice is due to a reduced number of sperm in the cauda epididymes, we counted the sperm number from the testes of -/-, +/- and +/+ males. No significant differences in sperm number were

observed, indicating that the production of spermatozoa was not affected in knock-out mice and therefore oligozoospermia was not the cause of subfertility (Table 3.3).

4.3.2 Significant increase in the number of abnormal spermatozoa in the *Tex18*-deficient males

During the sperm count an increased number of abnormal spermatozoa was observed, therefore this characteristic was evaluated in detail. Typical sperm head abnormality, shown in Figure 3.21, was observed in about 46% of spermatozoa from a mixed background and in about 30% from a 129/Sv background. Only a few percent of spermatozoa with an abnormal head shape was found in the case of +/- and +/+ males. Results of immunostaining revealed that also the shape of the acrosome is disrupted (Fig. 3.22). This observation was supported by results of electron microscopy (Fig. 3.23). Response of spermatozoa from mutant and wild-type mice to the calcium ionophore showed no significant differences in the acrosome reaction between *Tex18* -/- and wild-type spermatozoa. No differences were observed as well for sperm – egg binding assay (which depends on acrosome reaction) between normal and *Tex18*-deficient sperm. Therefore it seems that abnormal acrosome shape is probably due to the abnormal structure of sperm head and has no serious effect for its function.

Such abnormal structure of spermatozoa, called teratozoospermia, is a typical cause of male reduced fertility or infertility. Good examples are mice carrying autosomal recessive mutation “abnormal spermatozoon head shape” (azh), caused by the mutation in the *Hook1* gene. All spermatozoa display a highly abnormal head morphology that differs drastically from the compact and hook-shaped head of the normal murine sperm. Coiled sperm tails and decapitation of the tail from the flagellum are also often visible (Mendoza-Lujambio et al., 2002). This phenotype could be contributed to the reduced fertility, which is only between 5% and 10% of wild-type or heterozygous mice (Meistrich et al., 1994). Total infertility on 129/Sv background (but not on C57BL/6Jx129/Sv) is observed in mice lacking *Tnp2* gene. It encodes for the transition protein 2, which participates in the removal of the nucleohistones and in the initial condensation of the spermatid nucleus. 24% of the sperm from *Tnp2*-deficient mice show abnormal head shape, which make them similar to *Tex18* null mice. Attachment of the acrosome membrane to the nucleus is impaired in the *Tnp2* -/- sperm, and a reduced

number of spermatozoa undergoing acrosome reaction is observed (Adham et al., 2001). Above examples of male mouse infertility caused by abnormalities in spermatozoa shape suggest, that abnormal head shape of sperm from *Tex18* deficient mice might have the influency for the efficiency of sperm migration in the female genital tract and fertility.

4.3.3 Reduced motility of sperm from *Tex18* -/- males

To check, if abnormal head shape has a real effect on sperm migration in the female genital tract, vaginal plug tests were applied. A strong reduction in the sperm number in the uteri of the females was observed. Sperm number was reduced or even no sperm was seen in the oviducts (Table 3.4). These effects were seen when males from both backgrounds were used. Problems with the migration in female genital tracts suggests reduced sperm motility, therefore motility tests using a computer assisted semen analysis (CASA) system were applied. Strong reduction in sperm motility and progressive movement on both backgrounds was observed, when compared with wild type controls from both strains (Table 3.5). Detailed analysis, with the following parameters: curvilinear velocity (VCL), average path velocity (VAP), straight-line velocity (VSL), beat frequency (BCF), straight forward movement (STR) and lateral head amplitude (ALH) was applied (Fig. 3.24). Statistically significant differences were observed for each parameter, especially high for all velocities and lateral head amplitude, as it was tested by the Mann-Whitney U- Test. All of these results suggest that reduced sperm motility contributes to reduced fertility. However, reduced motility affects males' fertility much stronger on the 129/Sv background, than on the mixed background.

Such a reduction in sperm motility, named asthenozoospermia, is reported, for example in mice lacking the *MDHC7* gene, coding for mouse dynein heavy chain 7, a component of the inner dynein arm. No sperm is observed in oviducts of the females bred with males deficient for *MDHC7*, which is caused by the severe reduced sperm motility. Drastic reduction in the average path velocity and straight-line velocity is observed, being the main cause of males' infertility (Neesen et al., 2001). Another example is the disruption of *Smcp* gene, coding for sperm mitochondrion-associated cysteine rich protein. SMCP is a cysteine and proline-reach structural protein that is

closely associated with the keratinous capsules of the sperm mitochondria in the mitochondrial sheath surrounding the outer dense fibers and axoneme. No abnormalities are found in the structure of sperm head, mitochondria or tail. *Smcp*-deficient males from 129/Sv background are totally infertile, probably because of reduced motilility, together with decreased ability of the spermatozoa to penetrate oocytes (Nayernia et al., 2002). Similarity of the phenotypes in given examples and *Tex18* knock-out mice makes asthenozoospermia one of the possible causes of *Tex18* -/- males subfertility.

4.3.4 Histological evaluation of the testes of *Tex18*-deficient mice

Histological sections of testes from *Tex18*-deficient mice were performed, slides were stained with hematoxylin/eosin and microscopical analysis was made in order to investigate the testes morphology. Histological sections of homozygous males' testes from both backgrounds revealed abnormalities in structure of seminiferous tubules. Spermatogenesis was disturbed, as it was often arrested at the stage of round spermatid. Sometimes vacuoles were observed in spermatids (Fig. 3.25) No spermatozoa and/or elongated spermatids were observed in many tubules as well. Interestingly, this phenotype has variable penetrance. Animals were affected to a different extent, in some evaluated testis sections hardly any tubule showed arrest of spermatogenesis, in some animals most of them were disrupted. Also the degree of the spermatogenesis disruption was different in the seminiferous tubules, as it is shown in Figure 3.25. Some tubules showed round spermatids and a small number of elongated spermatids, some only round spermatids. Immunostaining of the *Tex18*-deficient testis with antibody directed against Stra8 protein revealed no disruption of premeiotic germ cells, because the pattern showed no differences as compared to wild type control (Fig. 3.26).

Spermatogenesis is a very strictly regulated process, which in the case of mammals is additionally cyclic. Time between the formation of subsequent cohorts of new A1 spermatogonia is always similar in particular species and is called the epithelial cycle. Based on the differentiation of spermatids into spermatozoa - which is divided into 16 distinct steps - it is possible to divide the epithelial cycle into stages. In most species 12 stages are distinguished, with particular types of spermatogonia, spermatocytes and spermatids present at each stage of epithelial cycle. Therefore a stage of a given seminiferous tubule is defined by a specific association of germ cells types (de Rooij,

1998) (Fig 4.1). Since every testis section can be subordinate to particular stage of epithelial cycle, eventual deviation from the known pattern can be observed. Differences from the described model were seen in many epithelial stages in testes of *Tex18*-deficient mice. Abnormalities in spermatogenesis start already at the spermatocytes stage, as apoptotic cells were seen regularly in many seminiferous tubules. Apoptotic round spermatids and rarely elongated spermatids with abnormal morphology were present as well. Diploid spermatids, indicating problems with second meiotic division were also observed. Relatively often, round spermatids did not start the elongation process or were retarded. In some stages, spermatids from earlier developmental steps delayed in elongation process were seen (Fig. 3.27).

Such an impairment in testis structure was seen in some knock-out models for testis expressed genes. For example, apoptosis of germ cells is shown in mice lacking zinc finger transcription factor *Egr4*, expressed mainly in the brain but also at a low level in germ cells. Apoptotic changes are found in early mid-pachytene spermatocytes in stage VI-VII in some tubules. However, arrest of spermatogenesis is not complete, some spermatocytes escape apoptosis and a small number of mature spermatozoa showing a variety of structural abnormalities are observed (Tourtellotte et al., 1999). Another example are mice deficient of *RXRβ* gene coding for one of the retinoid receptor. Some of the late spermatids from testes of *RXRβ*-deficient males fail to align at the luminal side of the tubules at late stage VII. Almost all stage IX tubules as well as some stage X tubules contain retained spermatids from step 16, together with spermatids from steps 9 or 10. In stage IX, heads of these spermatids delayed in spermatogenesis are scattered throughout the seminiferous tubule epithelium, often lying close to the basal lamina and are then phagocytosed by Sertoli cells (Kastner et al., 1996). A phenotype similar to that of *Tex18* -/- mice is observed in the testes of mice lacking *Cnot7*. This is a testicular somatic cell expressed gene coding for a transcriptional cofactor. Histological analysis shows reduction in the number of late spermatids, in some tubules germ cells are absent. Multiple generations of elongated spermatids in the same section of testis are often observed, indicating that maturation of spermatids is unsynchronised. An increased number of apoptotic cells is also observed (Nakamura et al., 2004). Above results suggests that unsynchronised spermatogenesis in some tubules of *Tex18*-deficient males could be the reason for not proper differentiation of spermatozoa: sperm head abnormalities, reduced motility and subfertilty.

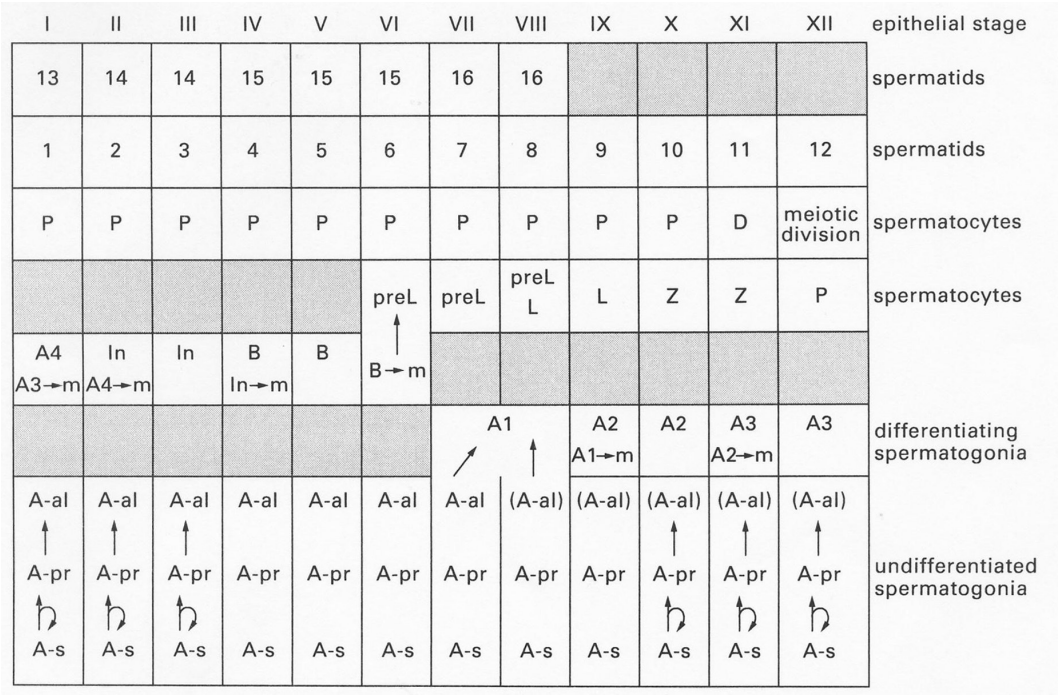


Figure 4.1 Diagram showing the cellular associations (epithelial stages) during cycle of the seminiferous epithelium of the mouse. Undifferentiated spermatogonia consist of A_s, A_{pr} and A_{al} spermatogonia that divide during stages X-III, as indicated by the arrows between the cells. During stages VIII to XII A_{al} are rare as indicates by the brackets. In the category of differentiating spermatogonia the moments of divisions are indicated by an m. The spermatocytes go through a G1 and S phase, during which these cells are indicated as preL (preleptotene), subsequently they go into meiotic prophase through leptotene (L), zygotene (Z), pachytene (P) and diplotene (D) phases. Spermatids develop in 16 steps into spermatozoa, which are shed into the lumen of the seminiferous tubules. The first 12 well recognizable steps of spermatid development are used to divide the epithelial cycle into 12 stages. The cell types in each column are always found together (from de Rooij, 1998).

4.3.5 *Tex18* null mice as a model of asthenoteratozoospermia

A wide range of abnormalities observed in the testes of *Tex18*-deficient mice, together with asthenoteratozoospermia suggests that *Tex18* might interact with other testis expressed genes. Different fertility on both backgrounds suggests that *Tex18* locus interacts with as-yet-unknown modifying genes. Interestingly, highly variable penetrance of male infertility on different genetic backgrounds has also been reported

for mice carrying targeted null mutations of other genes affecting spermatogenesis, including *Smcp* (sperm mitochondrion-associated cystein-rich protein) (Nayernia et al., 2002) and *Tnp2* (transition protein 2) (Adham et al., 2001). Except the above-described examples of teratozoospermia and asthenozoospermia, mouse models showing both of these effects, together with oligozoospermia, are known. Previously mentioned mice lacking receptor *RXRβ* display oligoasthenoteratozoospermia. These mice show a reduced number of spermatozoa in the seminiferous tubules, immobility of sperm, coiling of the tail and partial detachment of the acrosome from the nuclear envelope. Such abnormalities lead to the death of about 50% of the mutants around birth and sterility of males, which survived (Kastner et al., 1996). A similar phenotype is observed in *Cnot7* –deficient mice. *Cnot7* -/- males produced only 7% of the sperm as compared to *Cnot7* +/- or +/+ males. Sperm motility is highly reduced and almost all of the spermatozoa have irregularly shaped heads, abnormally arranged mitochondria and wrongly attached flagella. All of the *Cnot7*-deficient males are infertile. Interestingly, it was shown that *Cnot7* contributes to *RXRβ* function (Nakamura et al., 2004). As it was mentioned before, dysfunction of both genes leads also to misregulation of spermatogenesis. As some of the symptoms observed in *Tex18* null males are similar to these previously described in literature (especially concerning *Cnot7* and *RXRβ* genes), *Tex18*-deficient mice provide interesting experimental model to study the mechanisms of asthenoteratozoospermia and spermatid differentiation. It would be very interesting, if human ortholog of murine *Tex18* could be found. Screening of the patients suffering from infertility caused by asthenoteratozoospermia might reveal mutations in human *Tex18* gene. However, as for now no such a gene was found in known databases.

4.4 *Stra8* as a retinoic acid inducible gene

Stra8 gene (**St**imulated by **r**etinoic **a**cid gene **8**) belongs to the big group of genes whose expression is controlled by retinoids. Retinoids have been shown to regulate different physiological functions. Effects of retinoid treatment are mediated through two families of receptors, which act as ligand-inducible transcriptional regulatory proteins. There are three retinoic acid (RA) receptors: RARα, β and γ which bind all-trans (T-RA) and 9-cis retinoic acid (9C-RA); and three retinoid X receptors: RXRα, β and γ which bind only 9C-RA (review Chambon, 1996). It was shown, that RXR-RAR heterodimers are the functional units transducing the retinoid signal. Experiments using RXR-RAR mutant teratocarcinoma F9 cells (in which certain receptors are lacking) indicate that *Stra8* induction is preferentially mediated by RXR(β+γ)-RARγ pairs (Chiba et al., 1997). Retinoids are required for vertebrate reproduction and for the maintenance of normal testicular structure and function. Retinol deficiency leads to cessation of spermatogenesis and to seminiferous tubules degeneration (Thompson et al., 1964). Degeneration of testis was observed also in males deficient for RARα (Lufkin et al., 1993). Retinoic acid has also a role in survival and proliferation of primordial germ cells (Koshimizu et al., 1995).

Stra8 gene was identified by a differential subtractive hybridization cloning strategy, together with fifty other RA induced genes, from which forty were novel (Bouillet et al., 1995a). P19 embryonal carcinoma cells were used for this purpose. Several putative phosphorylation sites for protein kinase A and C, casein 2 and proline-dependent kinases are present in the Stra8 protein. It contains also a 51-amino acid domain rich in glutamic acid – such domains are found in proteins like centromere autoantigen protein B, troponin T or neurofilaments L, M and H (Oulad-Abdelghani et al., 1996).

4.5 Expression analysis of *Stra8* gene

4.5.1 Transcriptional analysis

It was shown by RT-PCR, that *Stra8* is upregulated in P19, F9 and in ES cells treated with RA. RT-PCR revealed also, that expression is restricted to the testis, because the signal was not observed in any other tissue (Oulad-Abdelghani et al., 1996). Our results confirm these data. Using RNA isolated from testis, ovary, lung, kidney, spleen, brain, heart, muscle and liver we showed by RT-PCR *Stra8* expression exclusively in testis (Fig. 3.32). Additionaly, RT-PCR was performed on RNA isolated from testes of different developmental mutants. Because the RT-PCR product was observed in all of the mutants except W/W^V , it proves that expression of *Stra8* is restricted to germ cells and no expression in Sertoli or Leydig cells occurs (Fig. 3.32). We performed RT-PCR also on RNA isolated from the testes of males of different postnatal stages. *Stra8* signal was detected in testes of 5, 10, 15, 20 and 25 days old males. Because male germ cells resume proliferation and re-entry into spermatogenesis shortly after birth to give rise to spermatogonial stem cells, it suggests that *Stra8* is expressed from the early, premeiotic stages of spermatogenesis. It was shown previously by in situ hybridization that in prenatal development, expression of *Stra8* starts at 12.5 dpc in male embryos in the genital ridges. At 14.5 dpc *Stra8* transcripts were detected in some cells of developing gonads. No signal was detected in developing ovaries at 16.5 dpc (Oulad-Abdelghani et al., 1996). Contrary data were obtained by Menke et al. (2003). Using RT-PCR they detected *Stra8* transcript in female, but not in male embryos from 12.5 to 14.5 dpc. Whole mount mRNA in situ hybridization of male and female gonads at 13.5 dpc revealed *Stra8* positive cells only in developing ovaries. Furthermore, whole mount in situ experiments have shown, that *Stra8* is expressed in embryonic ovarian germ cells in anterior to posterior wave, which lasts from 12.5 to 16.5 dpc. No positive signals were observed before embryonic day 12,5 and after 16,5 or postnatal ovaries (Menke et al., 2003). Our RT-PR analysis, which was performed on 15.5 and 18.5 male embryos, seems to support data obtained by Oulad-Abdelghani (expression of *Stra8* in male developing gonads, Fig. 3.32). However, a definitive answer cannot be given and further experiments should prove if the gene is expressed in prenatal developmental stages in both sexes, or only in one. Expression of *Stra8* was detected by in situ

hybridization in trophoblastic giant cells of mouse placenta between embryonic days 10.5 – 15.5. Giant cells play a role during placentation by producing hormones, which are required for the maintenance of pregnancy, and by modulating immunity between maternal and fetal tissues (Sapin et al., 1999). It proves, that *Stra8* expression is not restricted to testis only. However, the role of the gene in mouse placentation remains unknown.

Other *Stra* genes are expressed in greater variety of tissues, for example *Stra1* (known also as *LERK-2* or *Eplg2*) was found to be expressed in tissues like brain, heart, lung, liver, kidney, spleen, female genital tract and testis (Bouillet et al., 1995a). *Stra7* (known also as *Gbx-2*) transcripts were found in the adult brain, spleen, and female genital tract, whereas no expression could be observed in heart, liver, lung, kidney, or testis (Bouillet et al., 1995b). *Stra10* (*Meis-2*) expression was found in brain and female genital tract, it was less abundant in lung and only a basal level of expression was detected in heart, liver, kidney, spleen and testis (Oulad-Abdelghani et al., 1997).

4.5.2 Translational analysis

Stra8 protein was previously detected in cytosolic extract from RA treated P19 cells – a band of molecular mass of about 46 kDa was observed in Western blot using anti *Stra8* polyclonal antibodies. This molecular weight was coincident with the expected, deduced protein mass of 45 kD. As glutamic acid rich domains are found in proteins of cytoskeleton, such as neurofilaments or troponin T, it was examined if Stra8 protein could be a component of cytoskeleton. Detergent-soluble and insoluble (cytoskeletal) fractions isolated from P19 cells with Triton X-100 were subjected to Western blot and the *Stra8* signal was detected in a soluble fraction only. This suggests that the protein is not included in the cytoskeleton (Oulad- Abdelghani et al., 1996). Since Stra8 protein contains several putative serine and threonine phosphorylation sites, it is possible that different phosphorylated forms of the protein exist. Oulad-Abdelghani et al. (1996) showed by two-dimensional gel electrophoresis and Western blot that nine phosphorylated forms of Stra8 protein can be detected when T-RA was applied. However, only two of them were present, when cells were treated with 9C-RA. It is known, that T-RA in higher concentration can be converted into 9C-RA isomer, therefore it is possible that these two forms are in fact induced specifically by 9C-RA

and not by T-RA. All of the phosphorylated forms disappeared when P19 cells were treated with alkaline phosphatase.

For the analysis of *Stra8* expression on the protein level in detail, we generated an antibody against Stra8 protein. No successful amplification of *Stra8* cDNA was achieved. Therefore antibodies against two Stra8 peptides were ordered from Eurogentec Company. We obtained two purified antibodies designated as directed against EP034149 and EP034150- peptides which are included in the amino acid sequence of Stra8 protein (Fig. 3.29). Western blots using protein extracts from different mouse tissues and both antibodies were performed. When the antibody directed against EP034150 was applied, many unspecific bands of different sizes were observed in Western blots, using protein extracts from testis and other tissues. When antibody directed against EP0341490 was applied, not any band or bands of unexpected sizes were obtained. Both situations suggested no proper antibodies purification. However, purification of *Stra8* antibody against EP034150 did not improve Western blot quality.

Stra8 antibodies were applied for immunohistochemistry of paraffin embedded mouse testis sections. The green signal emitted by secondary FITC antibodies was observed in some seminiferous tubules in cells localised close to the basal lamina of the tubules (Fig 3.37). This characteristic pattern indicates that *Stra8* expression in the testis is localised in spermatogonia. Because *Stra8* signal was observed in not all of the seminiferous tubules, it is justified to assume that gene expression depends on the stage of the spermatogenic cycle (Russel et al., 1990). The results of our expriment are in accordance with published data (Oulad-Abdelghani et al., 1996) – they showed the same localisation of Stra8 protein in the testis by immunohistochemistry and in situ hybridization. The discrepancy between Western blot analysis and immunohistochemistry is a common phenomenon. It can be explained by the fact, that proteins prepared for Western blot are sonicated and their native structure can be disrupted. Intact epitops recognizable by *Stra8* antibodies during immunohistochemistry on testis sections could be damaged during sonication. Therefore, their detection could be not possible in Western blot.

4.5.3 Generation and analysis of *Stra8* transgenic mice

For a detailed analysis of *Stra8* expression we have generated transgenic mice. The construct harbouring 1.4 kb promoter region upstream from *Stra8* gene and a coding region of enhanced green fluorescent protein was generated and injected into the pronuclei of fertilized 1-cell eggs. Subsequently, they were transferred into FvB pseudopregnant females and two lines of transgenic animals were generated. As it was shown by Northern blot and RT-PCR, expression of the EGFP under the *Stra8* promoter was restricted to the testis – the same pattern as for the endogenous gene (Fig. 3.40 and 3.41). Testis sections of the transgenic males (Fig. 3.42) revealed, that the green signal is emitted from cells localised in periphery of the seminiferous tubules. Since this pattern is reminiscent of results of testis immunostained with *Stra8* antibodies, it can be concluded that expression of EGFP in transgenic animals resembles truly *Stra8* expression in normal males. Another group previously reported generation of *Stra8* transgenic mice (Giuili et al., 2002). They cloned a 400 bp *Stra8* promoter fragment into the luciferase reporter vector pXp. Three different lines of transgenic animals showed the same expression pattern – high luciferase activity was observed in testis, but also in brain. A limited number of positive cells (average 0.1 cell per section) were detected in the periphery of the tubules. In our transgenic lines about 11% of positive EGFP cells per tubule were detected (Nayernia et al., 2004a). It is possible that the expression of luciferase not only in testis, but also in the brain, and the more restricted expression profile of the luciferase in testcular cells, comparing to endogenous *Stra8* expression, is due to the truncated promoter. No such restrictions were observed in our transgenic model.

4.6 *Stra8* as a premeiotic marker

Stra8 is commonly used as a premeiotic specific marker. One example is its use in characterization of “side population” cells. Testicular cell suspension display “side population” - cells, which exhibit a low Hoechst 33342 fluorescence after DNA staining and which are highly enriched in stem cells, as it was shown by in vivo repopulation assay in busulfan-treated recipient mice. Since *Stra8* is expressed in “side population”

cells, it indicates that the gene is expressed in spermatogonial stem cells, because only SSCs are able to repopulate busulfan-treated testes (Lassalle et al., 2003). Another example is TAF4B knock-out mice, which lacks a component of TFIID, a transcriptional regulator enriched in mouse testis. *Taf4b*-deficient mice are initially fertile, but display multiple reproductive defects by 11 weeks of age. However, expression of *Stra8* in Taf4b knock-out mice is decreased as early as at the day of birth, when the number of germ cells in their testes is comparable with wild type and heterozygous males. This expression remains lower at day 8 after birth and in the adult testis. It suggests also, that infertility of Taf4b –deficient males could be caused in part by a decrease of *Stra8* expression (Falender et al., 2005). Mauduit et al. (2001) investigated the effects of radiation on radioresistant (somatic) and radiosensitive (premeiotic germ) cells in mice in term of genes expression. They analysed mRNA level of *Stra8* and three different genes expressed in premeiotic cells (*c-kit*, *Fas* and *LIF-R*) together with genes expressed in somatic cells (*SCF*, *TNF-R55* and *FAS-L*) and a gene expressed in both somatic and germ cells (*TGFβR1*). *Stra8* expression decreased in a time- and dose-dependent manner after testicular irradiation. Expression of *Stra8* was the most sensitive for irradiation, as a decrease occurred earlier (4 hours after 2-Gy) and significantly with the lowest dose (0.5-Gy), comparing to the decrease in expression of other genes. Although it is not clear how the changes in the expression of *Stra8* may explain the testis response to irradiation, it could be used as a marker to evaluate the level of the response in premeiotic germ cells (Mauduit et al., 2001). Other group showed that *Stra8* expression was also significantly decreased in busulfan-treated mice in the first week after treatment. It was closely associated with busulfan-induced death of differentiating spermatogonial cells and preleptotene spermatocytes. Although differentiating spermatogonia from busulfan-treated males were repopulated from stem cells, which survived, *Stra8* expression was downregulated until six weeks after busulfan treatment. It indicates that *Stra8* function is not important for spermatogonial differentiation, but for premeiotic cells (Choi et al., 2004).

4.7 Practical application of *Stra8* gene

Generation of *Stra8* promoter constructs is followed by practical applications, because of specific expression of *Stra8* in SSCs. Giuili et al. (2002) generated double transgenic Stra8-CD4Haglo;ROSA26 animals, in which *Stra8* promoter directs the expression of

the recombinant gene. Paramagnetic beads coated with anti-CD4 monoclonal antibodies were used to sort the positive cells from transgenic testis cells suspension and a high percentage of purity was obtained. Purified cells showed known spermatogonia (RBM) and stem cell (α6 and β1 integrins) markers. Subsequently, they were injected into the seminiferous tubules of irradiated males. ROSA26 β-galactosidase staining of the tubules revealed a 700 – fold increase in colonization efficiency, comparing with testis where total testis cell suspension was injected into irradiated mouse testes (Giuili et al., 2002). In vitro generation of a germ cell line (SSC1) from the pluripotent teratocarcinoma F9 cell line by *Stra8*- promoter based sequential selection strategy was shown in our group (Nayernia et al., 2004a). *Stra8* - EGFP construct was transfected into F9 cells, which were then induced with RA, cultured and selected by FACS. After repeating of this procedure, 86% of the cells showed EGFP expression. They showed also expression of known molecular markers for germ cells. The cells, designated as SSC1, were transplanted into the testis of germ cell depleted recipient mice. Restoration of spermatogenesis was observed. Although an increased number of sperm with abnormal morphology was observed, SSC1 derived sperm were able to fertilize oocytes by intracytoplasmic sperm injection (ICSI). These results suggests that *Stra8* is expressed at least in spermatogonial stem cells, since teratocarcinoma cells expressing EGFP under the *Stra8* promoter are able to develop into functional spermatozoa.

The same mouse *Stra8*-EGFP construct was used in our group for establishing spermatogonial stem cell lines from ES cells. They were able to undergo meiosis in vitro and to activate early embryonic development. For this purpose cultured ES cells were transfected with the Stra8-EGFP construct. After neomycin selection, positive clones were induced with RA and FACS analysis was applied. FACS positive clones were cultivated and a second transfection with the Prm1-RED-construct was applied. In this construct red fluorescent protein is expressed under the control of the promoter of Prm1 gene. Prm1 – protamin 1, is a gene expressed in haploid cells and codes for a protein that replaces histones and transition proteins in spermatids. Neomycin selection was performed, positive clones were cultivated, RA induction and FACS analysis for RED were applied. RT-PCR, analysis of haploidisation and immunocytochemistry revealed that the cells showed the phenotype of mature sperm. As it was shown by ICSI, injection of these cells into mouse oocytes induced embryogenesis to the stage of 8 cells embryos. Results of this experiment prove expression of *Stra8* in stem cells and reveal its practical role in establishing of spermatogonial stem cell line from ES cells.

4.8 Expression analysis of human *STRA8* gene

There is a human ortholog of the *Stra8* gene (Fig. 3.30 and 3.31). It is localised in human chromosome 7 and includes also 9 exons, however the encoded protein is only 330 amino acids long and does not contain a glutamic acid rich domain. We showed by RT-PCR, that expression of *STRA8* in human is also restricted to testis (Fig.3.33), which is in accordance with published data (Miyamoto et al., 2002). However, we have used greater variety of tissues for RT-PCR analysis. To check if *STRA8* expression is induced by RA treatment, like in the case of mouse *Stra8*, we used human teratocarcinoma Tera1 cells. Higher expression of *STRA8* in RA treated Tera1 cells was observed by RT-PCR as compared to non-treated cells (Fig. 3.34). It suggests, that generation of a germ cell line from the human pluripotent teratocarcinoma cells by promoter based sequential selection strategy, like it was done from murine F9 teratocarcinoma cell line, is theoretically possible (Nayernia et al., 2004a). It could be an interesting approach in treatment of males with testis carcinoma and maybe help in restoring their fertility. The function of the gene is still unclear, however screening of infertile patients for mutation in *STRA8* gene and comparing phenotypes with it's mouse model would be helpful for the elucidation of some causes of human male infertility.

4.9 Generation and analysis of *Stra8* deficient mice

To elucidate role of the mouse *Stra8* gene, we decided to generate knock – out mice. The best results would be obtained, if the whole gene could be disrupted, because truncated protein can interfere with interpretation of the phenotype, or even no phenotype can be observed. Because the first intron of *Stra8* gene is relatively long (more than 6 kb) and ATG is localized in the second exon we decided to disrupt exons from 2 to 8. In such a way no truncated protein should be obtained. Fragments of 4.5 kb (containing exon 1) and 4 kb (containing exon 9) were cloned into pPNT-M1 vector – in this way a genomic fragment of about 14 kb will be deleted in the targeted allele (Fig. 3.45). During the screening of transfected ES cells two positive recombinant clones were found and one of them was used for blastocyst injection and the generation of chimeras. In total, 5 *Stra8* chimeras were obtained: two males with 10% and 20% of

chimerism, two males with 5% of chimerism and one female with 35% of chimerism. All of them were bred with C57Bl/6J mice to obtain F1 generation. Few offsprings were obtained from male chimeras with 10 and 20 % of chimerism and from the female chimera, but unfortunately all of them were black. Both 5% male chimeras were bred with females for three months, but no offspring was obtained. They were sacrificed and DNA from different tissues, including testis, was isolated. DNA from sperm of all the males was isolated too. Using primers specific for defined microsatellite regions it is possible to distinguish between C57Bl/6J and 129/Sv mouse strains, because lengths of the PCR products are different in both strains. PCR analysis revealed that chimerism, corresponding to 5% estimated on the coat basis was observed in some tissues of both males. However, in the case of sperm, band specific for 129/Sv strain occurred only in DNA from sperm of the first 5% chimera. No such band was observed in PCR product obtained from DNA of the second 5% chimera and from the 10 % and 20 % chimeras (Fig.3.48). These results explain why no transmission of recombinant allele could be obtained from 10% and 20% male chimeras. It is still uncertain, why the other two males were infertile. Testes of both 5% males were sectioned and slides were stained with hematoxylin/eosin. Not suprisingly, no abnormalities were found in the testicular structure of the second chimera (which did not reveal 129/Sv strain specific band). On the contrary, structures of all of the seminiferous tubules of first chimera were abnormal (Fig. 3.49). Spermatogenesis was arrested at early stages, degeneration of the tissue, enlarged lumen of the seminiferous tubules and lack of the spermatids and sperm were observed in most of the tubules. It was reported for other genes, that chimeric males were infertile due to haploinsufficiency of the knock-outed genes (Takeuchi et al., 2003; Liu et al., 2003). Such a phenotype was observed also in our group in the case of ADAM 27 knock-out male chimeras. However, in all of these cases high chimerism was observed, therefore haploinsufficiency could be a likely explanation for the phenotypes. *Stra8* chimeras have only 5% chimerism, therefore such a strong phenotype (seminiferous tubules degeneration and infertility) suggests important role of the gene in fertility and spermatogenesis. Because no definitive explanation of the role of *Stra8* gene could be given, a second blastocyst injection and generation of new chimeras was planned. At this time we obtained information that David Page's group generated also *Stra8* knock - out mice, as it was reported on the Germ cells conference in Cold Spring Harbor in October 2004. Both male and female *Stra8* deficient mice were infertile. The females' germ cells do not initiate meiotic chromosome condensation, form DNA

double strand breaks or express *DmcI* (which is required for DNA double strand break repair). *Stra8* deficient germ cells maintain a nuclear morphology consistent with that of premeiotic germ cells for several days and then die around birth. In *Stra8* deficient males, spermatogonial divisions proceed normally with premeiotic spermatocytes appearing as expected. However, *Stra8* deficient spermatocytes do not undergo phosphorylation of the histone variant H2AX, a marker of premeiotic S-phase. Additionally, neither meiotic chromosome condensation nor DNA double strand breaks are observed in *Stra8* deficient spermatocytes, which subsequently undergo apoptosis. Therefore the authors concluded that *Stra8* deficient germ cells are unable to initiate prophase of meiosis I in both male and female mice (Baltus et al., 2004). These results are in accordance with our observation in the first 5% chimera (lack of the postmeiotic germ cells), supporting hypothesis that *Stra8* is required for the initiation of meiosis of germ cells. Results of Page's group are still not yet published after one year. Therefore, we decided to generate further *Stra8* chimeras. The elucidation of the gene's role hopefully will be given in a near future.

5 SUMMARY

The subject of this study was an expression and functional analysis of two testis specific genes, namely *Tex18* and *Stra8*.

Tex18 was described for the first time by the group of D. J. Page in 2001 in a systematic search for genes expressed in mouse spermatogonia, but not in somatic tissues. Our RT-PCR analysis confirmed that the *Tex18* gene is expressed exclusively in testis, namely in germ cells. Expression of *Tex18* was observed also in testes developmental mutants, with exception of W/W^V mice, which lack any germ cells. Expression was found also in postnatal and prenatal stages. Interestingly, expression of *Tex18* was found also in ES cells. For additional expression analysis transgenic mice, in which EGFP was expressed under the control of 1.6 kb *Tex18* promoter, were generated. Histological sections of these mice showed that *Tex18* is expressed predominantly in the postmeiotic germ cells. FACS analysis performed with testicular cell suspension of transgenic mice confirmed these data. An increased percentage of EGFP positive cells was observed in the case of older animals, in which postmeiotic cells are already present. It was also shown, that FACS positive cells contain an increased ratio of haploid cells, comparing to wild type control. These data prove that *Tex18* is expressed predominantly in haploid germ cells.

For the functional analysis, knock-out mice were generated on C57 BL/6J x 129/Sv and on 129/Sv backgrounds. Males from the first background were fertile, but 1/3 of the males on 129/Sv background were fully infertile in three months breeding, and others have reduced number of offsprings and a reduced number of litter size. The number of sperm in cauda epididymes is not disturbed as compared to the wild type controls, but both backgrounds showed a reduced number of sperm in uteri and oviducts in VP tests. Statistically reduced motility and progressive movement of sperm on both backgrounds was observed in comparison to wild type controls. An increased number of sperm with abnormal head shape was found in cauda epididymes. Although acrosome shape was shown to be changed in spermatozoa with head abnormality, its function was not disturbed since spermatozoa showed normal acrosome reaction. Histological sections of homozygous male testes revealed abnormalities in seminiferous tubules structure. Efficiency of spermatogenesis was disturbed – it was very often arrested at the stage of round spermatid (spermatids did not start the elongation process or were delayed in this

process – therefore asynchronous spermiogenesis is concluded). Reduced number (or sometimes none) of elongated spermatids and mature sperm were observed in some seminiferous tubules. Apoptotic spermatocytes and spermatids and diploid spermatids, which skipped the second meiotic division, were seen regularly in many seminiferous tubules. Rarely morphologically abnormal elongated spermatids were seen. From these data it can be concluded that *Tex18* gene is essential for spermatid differentiation rather than for spermatogonial differentiation. Asthenoteratozoospermia is likely the cause of the infertility of *Tex18*-deficient males.

In the second part of this study, expression and function of *Stra8* was analysed. This gene was described by Oulad-Abdelghani (1996) as a premeiotic specific gene. RT-PCR confirmed that expression of *Stra8* gene is also restricted to germ cells. Expression was observed also in developmental mutants, with the exception of W/W^V mice. Expression was found in postnatal and prenatal stages. Immunostaining, using *Stra8* specific antibody showed that the expression of the gene is restricted to the premeiotic stages of spermatogenesis, as the signal was visible only in cells localised close to the basal lamina of seminiferous tubules. These data were confirmed in transgenic animals, in which EGFP was expressed under the control of 1.4 *Stra8* promoter. EGFP signal was observed also in the same manner. Expression of the human *STRA8* gene was found to be restricted to testis, too. It was shown also that the expression of the *STRA8* is increased in the RA treated Tera1 human teratocarcinoma cells.

For the functional analysis a knock-out strategy was designed, in which exons 2-8 (from 9 existing) were disrupted. Five chimeras were obtained. One female and two male chimeras did not transmit the knock-outed gene to the offspring. Another two males, both showing 5% chimerism, were infertile. Strain specific PCR revealed that in the sperm of one of them the disrupted gene is present. The testis histological section of this male showed serious degeneration of the testis structure and the arrest of spermatogenesis in all seminiferous tubules. These results suggest that *Stra8* might be essential for meiotic differentiation.

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