Expression and Functional Analysis of the Fas-Associated Factor1 (*Faf1*) Gene

Dissertation

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ABBREVIATIONS

ABI	applied Biosystem Instrument
APS	ammonium peroxodisulfate
ATP	adenosintriphosphate
BCP	1-bromo-3-chloropropane
β -gal	β-galactosidase
bp	base pair
BSA	bovine serum albumin
°C	degree Celsius
cDNA	complementary DNA
Cy3	indocarbocyanine
dATP	desoxyriboadenosintriphosphate
dH_2O	distil Water
DAPI	diamidino-2-phenylindole dihydrochloride
dCTP	desoxyribocytosintriphosphate
DMSO	dimethyl sulfoxide
DEPC	diethylpyrocarbonate
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxynucleotidetriphosphate
dpc	day post coitus
DTT	dithiothreitol
EDTA	ethylene diamine tetraacetic acid
ES	embryonic stem
Faf1	Fas-associated factor-1
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
g	gravity
gm	gram
GST	glutathione S-transferase
HEPES	N-(-hydroxymethyl) piperazin, N'-3-propansulfoneacid

HPLC	high performance liquid chromatograpy
hr(s)	hour(s)
IPTG	isopropyl-ß-thiogalactopyranoside
IRES	internal ribosome entry site
IVF	in vitro fertilisation
JL	Jackson Laboratory
kb	kilobase pairs
LB	luria-Bertrani
LIF	recombinant leukaemia inhibitory factor
LPS	lipopolysaccharides
M	molarity
MEF	mouse embryonic fibroblast
MOPS	3-[N-Morpholino]-Propanesulfate
mRNA	messenger Ribonucleic acid
mg	milligram
ml	millileter
µl	microliter
µm	micrometer
min	minute
NaAc	sodium acetate
NBT	nitro-blue tetrazolium
NCBI	National Center for Biotechnology Information
Neo	*neomycin*
ng	nanogram
NLS	nuclear localization sequence
nm	nanometer
NTP	nucleotidetriphospate
UBA	ubiquitin binding domain
UBX	ubiquitin like domain
OD	optimal density
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
pH	preponderance of hydrogen ions

pmol	picomol
PBS	phosphatebuffersaline
PBT	phosphatebuffersaline + Tween 20
PMSF	phenylmethylsulfonyl fluoride
RNA	ribonucleic acid
Rnase	ribonuclease
RNasin	ribonuclease inhibitor
rpm	revolution per minute
RT	room temperature
RT-PCR	reverse transcriptase-PCR
SDS	sodium dodecylsulfate
SDS-PAGE	SDS-Polyacrylamide gel electrophoresis
sec	second
Taq	*thermus aquaticus*
TBE	tris-borate-EDTA-electrophoresis buffer
TE	tris-EDTA buffer
TEMED	tetramethylethylene diamine
Tris	trihydroxymethylaminomethane
U	unit
UV	ultra violet
V	voltage
w/v	weight/volume
X-Gal	5-bromo-4-chloro-3-indolyl

Symbol of amino acids

A	Ala	Alanine
B	Asx	Asparagine or Asparatic acid
C	Cys	Cystine
D	Asp	Asparatic 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 acid

A	Adenosine
C	Cytidine
G	Guanosine
T	Thymidin

1. INTRODUCTION

1.1 Gene trapping in the mouse genome

Manipulation of the genome for *in vivo* identification of gene functions requires reliable and predictable strategies to disrupt a given locus. Gene trap strategies have proven to be powerful tools not only to study the expression of genes *in vivo*, but also to identify their functions, because gene trap constructs commonly cause disruption of the targeted gene (Skarnes et al., 1992; Zambrowicz et al., 1998; Stanford et al., 2001). Gene trapping (GT) is a method of random insertional mutagenesis that uses a gene trap vector containing a DNA coding for a reporter or marker gene as a mutagen. Generally, a gene trap vector contains the intron and splice acceptor (SA) sequence from the mouse gene *engrailed-2* (En-2), joined upstream of promoterless reporter *ß- galactosidase* gene (*lacZ*) and *neomycin phosphotransferase* gene (*neo*) as selector, and is then followed by the SV40 polyadenylation signal at the 3' end. In some GT vectors, the internal ribosomal entry site (IRES) from the encephalomyocarditis virus is located in front of the reporter gene to allow the cap-independent translation of the *lacZ* from fusion transcript. Integration of the gene trap vector into the intron of a gene in the correct orientation was predicted to create *lacZ* fusion transcript, and if the reading frames of the endogenous genes and *lacZ* are the same, an active *ß*-galactosidase fusion protein should be produced. A gene trap vector was designed to generate a spliced fusion transcript between the reporter gene and the endogenous gene present at the site of integration (Fig. 1.1) (Brenner et al., 1989; Gossler et al., 1989; Kerr et al., 1989).

To generate a large scale of gene trapped cells, embryonic stem cells (ES) are electroporated with linearized GT vector. After culture of transfected cells in G418 selection medium, *lacZ* expressing clones are detected by staining the cells for ß-galactosidase activity. The *lacZ* reporter gene can only be expressed if it is inserted in an intron of a transcriptionally active gene. Using 5' rapid amplification of cDNA end (5'RACE), the 5' trapped exon can be easily determined from the spliced fusion transcript without having to clone the insertion site from the genomic DNA. Alignment of 5'RACE sequence in genomic, cDNA and EST databases will allow identifying a trapped gene.

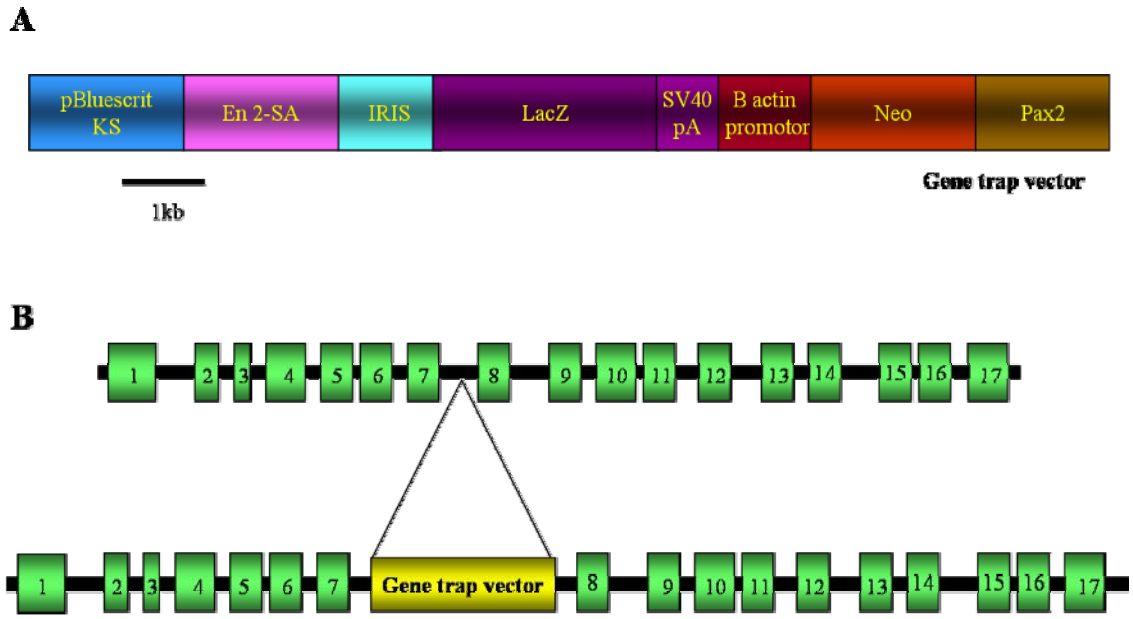

Figure 1.1 (A) Schematic representation of the gene trap vector used in this study. The gene trap vector contains the intron and splice acceptor (SA) sequence from the mouse gene *engrailed-2* (*En-2*) joined upstream of promoterless reporter *ß-galactosidase* gene (*lacZ*) and followed by the SV40 polyadenylation signal (pA) at the 3' end and the internal ribosomal entry site (IRES) from the encephalomyocarditis virus. ATG (translation initiation codon) is located in front of the reporter gene to allow the cap-independent translation of the *lacZ* from fusion transcript. The vector contains also the human ß-actin promoter and *neomycin phosphotransferase* (*neo*) gene as selector. In this vector transcription of *neo* is under the control of a constitutive promoter and the *LacZ* expression depends on the activity of the trapped gene. (B)The gene trap cassette is inserted in intron 7 of the *Faf1* gene.

To determine the function of a trapped gene, chimeric mice are produced by injection of gene trapped ES cells into blastocyts. The recombinant ES cells and the endogenous ES cells of blastocyts participate in the development of chimeric mice. If the gene trapped ES-cells are involved in development of germ cells of chimeras, then the trapped allele will be transmitted to their offspring. Breeding of heterozygous offspring will result in mice homozygous for the trapped gene in the F2 generation. Because *LacZ* expression in gene trapped mice is under control of the promoter of the trapped gene, monitoring the *lacZ* activity in heterozygous embryos should enable one to readily visualize the expression pattern of endogenous gene during development. Using the gene trap strategy (Fig.1.2), several groups were able to isolate and study new developmental regulatory genes and to produce the corresponding mutant mice.

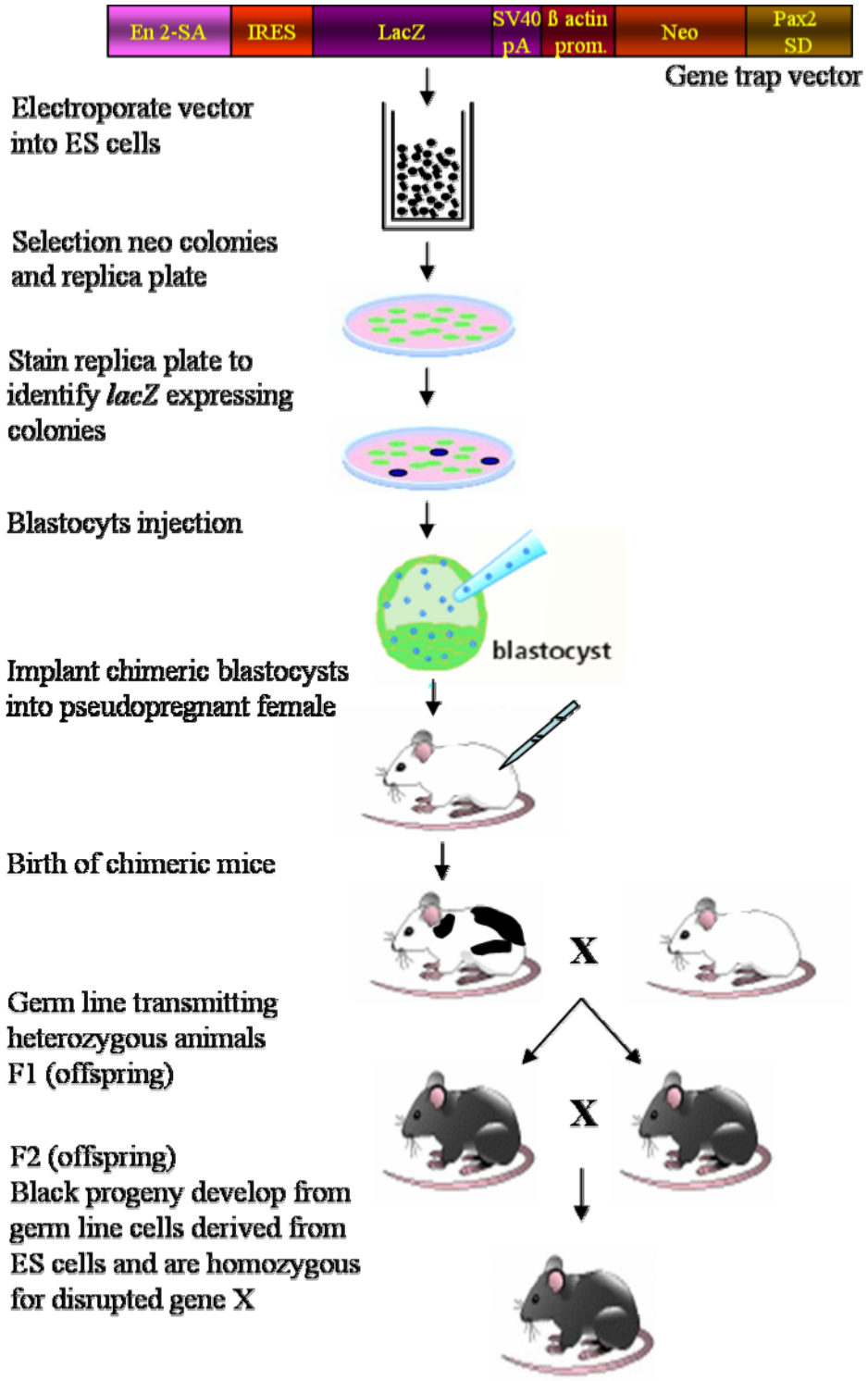

Figure 1.2 Generation of a gene trap mouse line.

1.2 Fas-associated factor 1 (Faf1)

Faf1 was first identified by yeast two hybrid assay using the cytoplasmic domain of FAS as bait (Chu et al., 1995). Fas, a member of the tumor necrosis factor receptor family (TNF-R), induces apoptosis when crosslinked with Fas ligand (Nagata, 1997). Fas mediated apoptosis has been implicated in the down regulation of the immune response, the depletion of peripheral autoreactive T lymphocytes and maintenance of sites of immune privilege (Abbas, 1996; Nagata, 1997). In testis, FasL is expressed in Sertoli cells (Suda et al., 1994; French et al., 1996) and has proven to be a major determinant in maintaining the immune privilege of testis (Bellgrau et al., 1995; Sanberg et al., 1996). Functional role for Sertoli cell-expressed FasL in inducing apoptosis of Fas-expressing germ cells has been recently demonstrated (Lee et al., 1997; 1999).

The specific binding of Faf1 to the cytoplasmic domain of Fas led to suggest that the Faf1 is a component of the death-inducing signaling complex in Fas-mediated apoptosis. Recently, several groups reported that overexpression of human *Faf1* can initiate apoptosis in transfected cell lines (Chu et al., 1995; Ryu et al., 2003).

Faf1 has been cloned in human (Walzak and Krammer 2000), in mouse (Chu et al., 1995) and in quail (Fröhlich et al., 1998). Human *Faf1* gene was localized to chromosome 1p32 (Ryu et al., 2000) and the mouse gene to chromosome 4C6 (Kikyo et al., 1996). A sequence similarity search revealed that the quail Faf1 shares an amino acid sequence similarity of 88% with mouse Faf1 and has 84% identical amino acids (Fröhlich et al., 1998). Mouse Faf1 amino acid sequence has 96% homology with that of human FAF1 (Ryu and Kim 2001).

Although Faf1 apoptotic potential has been demonstrated, sequence homology search in protein databases does not show typical death motifs such as the death domain and death effector domain (DED) in primary structure of Faf1. Instead, Faf1 has sequence motifs that are present in the proteins of the ubiquitination pathway. The Faf1 contains a UBX (ubiquitin-like) domain at the N-terminal end and the UBA (ubiquitin-associated) domain at the C-terminal end (Buchberger, 2002). The presence of both domains in Faf1 suggests that the Faf1 is involved in the ubiquitination pathway. Northern blot analysis revealed that the *Faf1* is expressed as a 2.8-kb mRNA in various human tissues. High expression of *Faf1* was seen in testis, brain and thymus. Western blot analysis of various human cell lines with a polyclonal antibody against Faf1 revealed protein products of two sizes, 74-kDa and 40-

kDa (Ryu et al., 1999). The 74-kDa protein corresponds well with the expected protein size of the predicted amino acid sequence of human FAF1. Expression analysis of Faf1 during embryonic development of quail revealed that Faf1 is bearly detected in pluripotent embryonic cells (Eo). After induction of differention by basic FGF, *Faf1* mRNA was detected as early as two hours after induction. The inducibility of quail Faf1 was restricted to the pluripotent embryonic cells. Whole mount in situ hybridization of E1 embryos (at the definitive primitive streak stage) revealed an intense and ubiquitous staining throughout the three embryonic germ layers (Fröhlich et al., 1998).

1.3 Faf1 gene is trapped in the mouse line 98-2C

In a large gene trap screen, the group of Prof. Dr. Peter Gruss in Max-Plank-Institute for biophysical Chemistry, Göttingen, has analysed the *LacZ* expression pattern in several mouse lines. Analysis of *LacZ* expression in the mouse 98-2C line revealed that the *LacZ* is highly expressed in brain and testis. 5' RACE method was not able to amplify the trapped sequence. Therefore, a genomic phage library of a heterozygous mouse was generated in the DASH-II vector .Two independent recombinant phage clones carrying inserts of 21- and 17.2-kb were isolated with *LacZ* and *neo*-specific hybridization probes. Regions flanking the integrated vector were sequenced and searched using Blast against mouse genome databases. Sequence alignment revealed that the gene trap vector is integrated in the intron 7 of the *Faf1* gene. To genotype animals of the 98-2C mouse line, a *LacZ* specific probe we used. Analyses of homozygous mutant mice, which have been genotyped with the quantative Southern blot, revealed that male and female mutant mice appear normal but mutant males are infertile.

Aims of this work

1. Analysis of *Faf1* expression during embryonic and germ cell development
2. Establishment of a direct method for genotyping the 98-2C gene trap line
3. Identification of the function of the *Faf1* gene

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals

Acrylamide	Serva, Heidelberg
Acetic acid	Merck, Darmstadt
Agar	Difco, Detroit, USA
Agarose	Invitrogen, Karlsruhe
Ammonium acetate	Fluka, Neu Ulm
Ammonium persulfate	Sigma, Deisenhofen
Ampicillin	Sigma, Deisenhofen
Ampuwa	Fresenius, Bad Homburg
Bacto-tryptone	Difco, Detroit, USA
Bacto-Yeast-Extract	Difco, Detroit, USA
BCIP	Boehringer, Mannheim
Bisacrylamide	Serva, Heidelberg
Blocking powder	Boehringer, Mannheim
BSA	Biomol, Hamburg
Coomasie G-250	Sigma, Deisenhofen
Choloroform	Baker, Deventer, NL
DAPI	Vector, Burfingame
Dextran sulfate	Amersham pharmalia, Freiburg
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
Ethanol	Baker, Deventer, NL
Ethidium bromide	Sigma, Deisenhofen
Ficoll 400	Amersham Pharmalia, Freiburg

FCS	Invitrogen, Karlsruhe
Formaldehyde	Invitrogen, Karlsruhe
Formamide	Fluka, Neu Ulm
Glutaraldehyde	Sigma, Deisenhofen
Glycerol	Invitrogen, Karlsruhe
Glycine	Biomol, Hamburg
Goat serum	Sigma, Deisenhofen
HCl	Merck, Darmstadt
H_2O_2	Merck, Darmstadt
HEPES	Merck, Darmstadt
IPTG	Biomol, Hamburg
Isopropanol	Merck, Darmstadt
KCl	Merck, Darmstadt
Lambda DNA	Roche, Penzberg
Methanol	Merck, Darmstadt
$MgCl_2$	Merck, Darmstadt
MOPS	Merck, Darmstadt
Methyl benzoat	Fulka, Neu Ulm
β-Mercaptoethanol	Serva, Heidelberg
Mineral oil	Sigma, Deisenhofen
NaCl	Merck, Darmstadt
Na_2HPO_4	Merck, Darmstadt
NaH_2PO_4	Merck, Darmstadt
$NaHCO_3$	Merck, Darmstadt
NaN_3	Merck, Darmstadt
NaOH	Merck, Darmstadt
NBT	Roche, Penzberg
Orange G	Sigma, Deisenhofen
PBS	Invitrogen, Karlsruhe
Phosphoric acid	Merck, Darmstadt
Picric acid	Fulka, Neu Ulm
Phenol	Invitrogen, Karlsruhe
Proteinase K	Roche, Penzberg

Protein marker	Biorad, Sigma
[α^{32}P]-dCTP	Amersham Pharmalia, Braunschweig
Rediprime™ II	Amersham Pharmalia, Freiburg
RNase Inhibitor	Roche, Penzberg
RNA length standard	Invitrogen, Karlsruhe
RNase away	Biomol, Hamburg
Salmon sperm DNA	Sigma, Deisenhofen
SDS	Serva, Heidelberg
Select Peptone	Gibco/BRL, Eggenstein
Sodium acetate	Merck, Darmstadt
Sodium citrate	Merck, Darmstadt
TEMED	Serva, Heidelberg
Triton X-100	Serva, Heidelberg
Tris	Sigma, Deisenhofen
Tween-20	Sigma, Deisenhofen
X-Gal	Biomol, Hamburg
Xylencyanol	Bio-Rad, München
Cell culture media	Invitrogen, Karlsruhe

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

2.1.2 Solutions, buffers and media

2.1.2.1 Agarose gel electrophoresis

5x TBE buffer	450 mM Trisbase 450 mM Boric acid 20 mM EDTA (pH 8)
Glycerol loading buffer –I	10 mM Tris/HCl (pH 7.5) 10 mM EDTA (pH 8) 0.025% Bromophenol blue 0.025% Xylenecyanol 30% Glycerol
Glycerol loading buffer –II	10 mM Tris/HCl (pH 7.5) 10 mM EDTA (pH 8) 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_2O
Sample buffer (2x)	0.5 M Tris/HCl (pH 6.8) 20% Glycerol 4% SDS 10% β-Mercaptoethanol

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

Denaturation solution	1.5 M NaCl 0.5 M NaOH
Denhardt´s solution (50x)	1% BSA 1% Polyvinylpyrrolidon 1% Ficoll 400
Denaturization solution	0.25 N HCl
E-buffer (10x)	300 mM NaH_2 PO_4 50 mM EDTA
Elution buffer	1.5 M NaCl 20 mM Tris/HCl (pH 7.5) 1 mM EDTA
Bouin's solution	15 volume of picric acid (in H_2O) 5 volumes Formaldehyde 1 volume Acetic acid

Hybridisation solution I	5x SSPE solution 5x Denhardt´s solution 0.1% SDS
Hybridisation solution II	5x SSC 5x Denhardt´s solution 10% Dextran sulfate 0.1% SDS
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
Lysis-buffer III(embryo)	50 mM Tris/HCl (pH 8.0) 0.5 mM EDTA (pH 8.0) 0.5% Tween 20 0.2 mg/ml proteinase K
Lysis-buffer IV	1% Tween20 1% Tritonx-100 5mM EDTA 10mM Tris pH7.5 2mM DTT

	20µl/ml Proteinase K
Lysis buffer A	10mM Tris/HCl pH 8.0 1mM EDTA 2.5% SDS 1mM PMFS
Lysis buffer B	50mM Tris-HCl, pH 7.5 150mM NaCl 1% Nonidet P40 0.5% sodium deoxycholate 1 Protease inhibitor cocktail tablet
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
Neutralisation solution	1.5 M NaCl 1 M Tris/HCl (pH 7.0)
PBS buffer	130 mM NaCl 7 mM Na_2 HPO_4 4 mM NaH_2 HPO_4
PBT buffer	0.1% Tween-20 in PBS (1x)
SSC (20x)	3 M NaCl 0.3 M Na_3 citrate (pH 7.0)
SSPE (20x)	0.02 M EDTA 0.2 M NaH_2 PO_4

	3.6 M NaCl (pH 7.0)
Stop-Mix I	95% Formamide 20 mM EDTA 0.05% Bromphenol blue 0.05% Xylene cyanol
Stop-Mix II	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).

Whatman blotting paper	Schleicher and Schüll, Dassel (GB 002, GB 003 and GB 004)
Cell culture flask	Greiner, Nürtingen
Culture slides	Falcon
Disposable filter Minisart NMI	Sartorius, Göttingen
Filter paper 0858	Schleicher and Schüll, Dassel
Hybond C	Amersham, Braunschweig

Hybond N	Amersham, Braunschweig
Petri dishes	Greiner, Nürtingen
Pipette tips	Eppendorf, Hamburg
Microcentrifuge tubes	Eppendorf, Hamburg
Transfection flasks	Lab-Tek/Nalge, Nunc, IL, USA
X-ray films	Amersham, Braunschweig
Superfrost Slides	Menzel, Gläser

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. Glasswares 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-trypton
	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

M2 and M16 media were purchased from Sigma, (Deisenhofen) and were used for washing and cultivation of mouse preimplantation embryos.

Embryonic stem (ES) cell medium:

DULBECCO´s MEM (DMEM)

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 (MEFs):

DULBECCO´s MEM (DMEM)

2 mM	L-Glutamine
10%	FCS

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

Freezing medium: DULBECCO´s MEM (DMEM)

50%	FCS
20%	DMSO

Embryo culture medium: M16 contains pyruvate and lactate as energy sources since preimplantation embryos do not utilize glucose efficiently. M2 Medium is a further modification of M16 that substitutes HEPES buffer in place of some of the bicarbonate. M2 is used for collecting and handling embryos for prolonged periods outside a CO2 incubator.

2.1.6 Antibiotics

Stock solutions were prepared for the antibiotics. The stock solutions were then 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

2.1.7 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.

2.1.8 Bacterial strains

E. coli DH5α	(Invitrogen, Karlsruhe)
E. coli BL21	(Novangen, Darmstadt)

2.1.9 Eucaryotic strains

F9 mouse teratocarcinoma cell line, Sherman et al., (1976), American Type Culture Collection (ATCC), Rockville, USA.
Tera1 human embryonic carcinoma cell line, Beatrice et al., (1997), American Type Culture Collection (ATCC), Rockville, USA.
Hela human cervical adenocarcinoma cell line, American Type Culture Collection (ATCC), Rockville, USA.

2.1.10 Plasmids

pBluesript SK (+/-)	(Stratagene, La Jolla, USA)
pGEM-T Easy	(Promega, Wisconsin, USA)
pET 41 (a-b)	(Novagen, Darmstadt)
pCS2	(Dr.H.Hammerschmidt, Freiburg) to generate epitop tagged fusion protein, expressed in mammalian cells

2.1.11 Synthetic oligonucleotides

The synthetic oligonucleotide primers used in this study were obtained from OPERON and dissolved in water to a final concentration of 100 pmol/µl.

L-40 WTF4:	5'CACGCTGGGACATACAAATG3'
L-40 WTR3:	5'TCACACAGAATTGTCAGAAAACAG 3'
L-40 MF3:	5'GCGTTGGGAAGACTACAGGA3'
L-40WTF5:	5'CCTTTCCTTCTACCCAGTTTGA3'
L-40MF5:	5'GGAACTCTGTGCTTCGGGT3'
L-40WTR5:	5'AAATTGCAATGACTTAAAGGTAGAA3'
F6	5'ACATCACTTTACCTGCTGAGC3'

R6	5’TCCCTGAAGCCTATTTAGAG3’
SB probe1F1:	5’TACTGCCCTGCAAGACACAG3’
SB probe1R1:	5’ATGTGGCATCGTGAGAAACA3’
SB probe2F2:	5’ATGTTTAAGGCCTGGCACTG3’
SB probe2R2:	5’TCAAATCCAATTCTGCCTGA3’
FafcF2:	5’ATGAGCTTCAGATACCTGTGC3’
Fafc R2:	5’GCATGGCATCATCTACCCTGA3’
FafcF4:	5’CTGTACAGACCCGTGAGCAA3’
FafcR4:	5’AGAAACCCTTTTCCTTCAAGCA3’
FafcF15:	5’GGGGATATCCCGCCATGGCGTCCAACATGG3’
FafcR15:	5’CCCCTCGAGGCTGGCCAGGAAACGCCGTTCC3’
UBAF1:	5’TTTGGGGATCCACTGTGGGAAGAAGGGCTTC3’
UBAR1:	5’TTCCCGAGCTCAAATGTCTTCCTGTTGTTG3’
UBXF1:	5’CCCAAGGATCCCCGAAGGAAGAAAATGCTGA3’
UBXR1:	5’GGGGGGGAGCTCGCTGCTTCCACCTCTCAGTC3’
UBAF2	5’TTTGGGTCGACTGTGGGAAGAAGAACTTC3’
UBAR2:	5’TTCCCGCGGCCGCTGTCTTCCTGTTGTTGAGC3’
UBXF2:	5’CCCAAGTCGACGAAGGAAGAAAATGCTGA3’
UBXR2:	5’GGGGGGGCGGCCGCTGCTTCCACCTCTCAGTC3’
Act F1:	5’CCCGAATTCGTGCAAAGCCGCCTTTGCTGG3’
Act R1:	5’CCCCTCGAGCTAGAAGCATTTGCGGTGGACG3’
SHIIR:	5’GAATAAGGCCGGTGTGCGTT3’
SHIIIR:	5’CCAACAGGTACCTGACAGAGCAGC3’
IRESF1:	5’TAACAAAGAGGACAAGCGGCCT3’
IRESF2:	5’TCTTCTTGACGAGCATTCCTAG3’
IRESF6:	5’CCCGAAAACCAAAGAAGAAG3’
IRESR6:	5’ATTCGATGATCTTCCGGGTA3’
T7:	5’TAATACGACTCACTATAGGG3’
T3:	5’ATTAACCCTTCACTAAAG3’
SP6:	5’AGGTGACACTATAGAATAC3’

2.1.12 Mouse strains

L-40 gene-trap line obtained from the group of Prof. Gruss, from MPI Göttingen.

2.1.13 Antibodies

Alkaline phosphatase-conjugated anti-goat	(Sigma, Deisenhofen)
FIFC-conjugated anti-mouse	(Sigma, Deisenhofen)
Cy3-conjugated anti-goat	(Sigma, Deisenhofen)
Mouse monoclonal antibody against *a*-tubulin	(Sigma, Deisenhofen)
Mouse monoclonal antibody against actin	(Sigma, Deisenhofen)
Goat polyclonal antibody against Faf1 C terminal	(Santa Cruz Biotechnology, Inc.)

2.1.14 Enzymes

Restriction enzymes (with supplied buffers)	(Invitrogen, Karlsruhe)
Collagenase (Type II)	(Sigma, Deisenhofen)
Klenow Fragment	(Invitrogen, Karlsruhe)
Proteinase K	(Sigma, Deisenhofen)
Platinum Taq polymerase	(Invitrogen, Karlsruhe)
RNase A	(Qiagen, Hilden)
RNase H	(Invitrogen, Karlsruhe)
RNase inhibitor	(Invitrogen, Karlsruhe)
Superscript-II	(Invitrogen, Karlsruhe)
Taq polymerase	(Invitrogen, Karlsruhe)
T4 DNA ligase	(Promega, Mannheim)
Trypsin	(Invitrogen, Karlsruhe)

2.1.15 Kits

BigDye Terminator Cycle	(Applied Biosystems)
Endo Free Plasmid Maxi Kit	(Qiagen, Hilden)
GST-Bind kit	(Novagen, Darmstadt)
Megaprime DNA Labeling Kit	(Amersham Pharmacia)
Maxi Plasmid Kit	(Qiagen, Hilden)
Mega Plasmid Kit	(Qiagen, Hilden)
Mini Plasmid Kit	(Qiagen, Hilden)
PCR Purification Kit	(Qiagen, Hilden)
QIAquick Gel Extraction Kit	(Qiagen, Hilden)
RNA Easy Kit	(Qiagen, Hilden)
Rediprime™ II Random Prime	(Amersham Pharmacia)
Labeling System	(Qiagen, Hilden)
Immunoprecipitation kit (protein G)	(Roche, Penzberg)
In Situ Cell Death Detection Kit, POD	(Roche, Penzberg)
One step RT PCR kit	(Qiagen, Hilden)

2.1.16 Instruments

Autoclave	(Webeco, Bad Schwartau)
Centrifuge 5415D	(Eppendorf, Hamburg)
Centrifuge 5417R	(Eppendorf, Hamburg)
Biophotometer	(Eppendorf, Hamburg)
DNA Sequencer Modell Megabace 1000	(Amersham, Freiburg)
Microscope BX60	(Olympus, München)
GeneAmp PCR System 9600	(Perkin Elmer, Berlin)
Histocentre 2 embedding machine	(Shandon, Frankfurt aM.)
Microtiterplate-Photometer	(BioRad laboratories, München)
Molecular Imager FX	(BioRad laboratories, München)

Phosphoimager Screen	(BioRad laboratories, München)
Semi-Dry-Blot Fast Blot	(Biometra, Göttingen)
Spectrophotometer Ultraspec 3000	(Amersham Pharmacia,Freiburg)
SpeedVac concentrator SVC 100H	(Schütt, Göttingen)
Thermomixer 5436	(Eppendorf, Hamburg)
Turboblotter™	(Schleicher & Schüll, Dassel)
UV Stratalinker™ 1800	(Leica, Nußloch)
X-Ray Automatic Processor Curix 60	(Agfa, München)

2.2 Methods

2.2.1 Isolation of nucleic acids

2.2.1.1 Isolation of plasmid DNA

(Sambrook et al., 1989)

2.2.1.1.1 Small-scale isolation of plasmid DNA

(adapted from Birnboim and Doly, 1979)

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 speed of 160 rpm. 1 ml of this saturated culture was used for making glycerol stocks and rest of the culture was centrifuged at 5000xg for 15 min. The pellet was resuspended in 150 µl of solution P1. The bacterial cells were lysed with 300 µl of P2 solution and then neutralised with 200 µl of solution P3. The precipitated solution was incubated on ice for 15 min, and centrifuged at 13000xg at 4°C. The supernatant was transferred 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 dissolved in 30 µl of TE buffer.

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

A single clone was inoculated in 2 ml LB medium with appropriate antibiotic as a pre-culture for 8 hrs in a 37°C shaker. In 100 ml LB medium with appropriate antibiotic, this pre-culture was added in a dilution of 1/100 fold and incubated overnight at 37°C with shaking. The saturated culture was centrifuged at 6000xg for 15 min. The pellet was resuspended in 5 ml of solution P1 and cells were lysed with P2 and P3 as described above. The precipitated solution was centrifuged at 20000xg for 30 min at 4°C. Meanwhile, the column (Qiagen-tip) that was provided with the midi preparation kit was equilibrated with 10 ml of QBT solution. After centrifugation, the lysate was poured into this equilibrated column to allow the DNA to bind with the resin present in the bed of the column. The column was then washed twice with 10 ml of solution QC. Finally, the DNA was eluted with 5 ml of QF solution. To precipitate the DNA, 3.5 ml of isopropanol was added and mixed thoroughly and centrifuged at 14000xg for 30 min at 4°C. The DNA pellet was washed with 70% ethanol and dissolved in 100 µl of TE.

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

2.2.1.2 Isolation of genomic DNA

2.2.1.2.1 Isolation of genomic DNA from tissue samples
(Laird et al., 1991)

The method employed was the same as that of Laird et al., (1991). 1 to 2 cm of the tail from a mouse was incubated in 700 μl of lysis buffer I containing 35 μl proteinase K (10μg/μl) at 55°C overnight in Thermomixer 5436. To the tissue lysate, equal volume of phenol was added, mixed by inverting several times, and centrifuged at 8000xg for 5 min at room temperature. 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. Finally, the DNA was precipitated with 0.7 volume of isopropanol, washed with 70% ethanol, and dissolved in 100-200 μl of TE buffer and incubated at 60°C for 15 min.

2.2.1.2.2 Isolation of genomic DNA from cultured cells

To isolate the DNA from cultured cells, cells in a 24 well plate were washed with PBS and incubated overnight in 500 µl of lysis buffer II at 37°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 60 µl of TE buffer and incubated at 60°C for 15 min.

2.2.1.3.1 Isolation of total RNA from tissue samples and cultured cells

Total RNA isolation reagent is an improved version of the single-step method for total RNA isolation. The composition of reagent includes phenol and guanidine thiocyanate in a mono-phase solution. 100-200 mg of tissue sample was homogenised in 1-2 ml of TRI Reagent by using a glass-teflon homogeniser. The sample volume should not exceed 10% of the volume of reagent used for the homogenisation. To isolate total RNA from cultured cells, 350 µl of reagent was added to the Petri dish (6 cm diameter). Cells were homogenised with a rubber stick and the lysate was transferred into a microcentrifuge tube. The homogenate was incubated at 4°C for 5 min to permit the complete dissociation of nucleoprotein complexes. Then, 0.2 ml of chloroform was added, mixed vigorously, and stored at 4°C for 10 min. After centrifugation at 12000xg for 15 min at 4°C, the colourless upper aqueous phase was transferred into a new tube. The RNA was precipitated by adding 0.5 ml of isopropanol. Finally, the pellet was washed twice with 75% ethanol and dissolved in 80-100 µl of DEPC-H_2O.

2.2.2 Determination of nucleic acid concentration

The concentration of nucleic acids was determined spectrophotometrically by measuring absorption of the samples at 260 nm. The quality of nucleic acids i.e. contamination with salt and protein was checked by the measurements at 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 mixtures of charged macromolecules, especially nucleic acids and proteins, are separated in an electrical field according to their mobility which is directly proportional to macromolecule's charge to mass ratio.

2.2.3.1 Agarose gel electrophoresis of DNA

Agarose gels are used to electrophorese nucleic acid molecules from as small as 50 bases to more than 20 kb, depending on the concentration of the agarose. Usually, 1 g of agarose was added to 100 ml of 0.5x TBE buffer and boiled in the microwave to dissolve the agarose, then cooled down to about 60°C before adding 3 µl of ethidium bromide (10 mg/ml). This 1% agarose gel was poured into a horizontal gel chamber.

2.2.3.2 Agarose gel electrophoresis of RNA
(Hodge, 1994)

Single-stranded RNA molecules often have complementary regions that can form secondary structures. Therefore, RNA was run on a denaturing agarose gel that contained formaldehyde, and before loading, the RNA was pre-treated with formaldehyde and

formamide to denature the secondary structure of RNA. 1.25g of agarose was added to 100 ml of 1x MOPS Buffer and dissolved by heating in a microwave. After cooling it to about 50°C, 25 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
10x MOPS Buffer
3 µl Formaldehyde
8 µl Formamide (40%)
1.5 µl Ethidium bromide

Samples were denatured at 65°C for 10 min and chilled on ice before loading into the gel. The gel was run at 40 V at 4°C for about 12 hrs.

2.2.3.3 SDS-PAGE for the separation of proteins
(Laemmli, 1970)

SDS gel electrophoresis is a method for separating proteins according to molecular weight. The proteins are denatured and rendered monomeric by boiling in the presence of reducing agents (β-mercaptoethanol or dithiotheitol) 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 boiled in sample buffer for 10 min at 95°C before loading into the gel. The gel was run at 15 mA for 1 hr and then at a constant current of 30 mA.

2.2.4 Isolation of DNA fragments after agarose gel electrophoresis

2.2.4.1 QIAquick gel extraction method

This method is designed to extract and purify DNA of 70 bp to 10 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 membranes. To the excised DNA fragment from agarose, and 3 volumes of QG buffer was added then incubated at 50°C for 10 min. After the gel slice was dissolved completely, it was applied over a QIAquick column and centrifuged for 1 min. The flow through was discarded and the column was washed with 0.75 ml of PE buffer. After drying the column, it was placed into a fresh microcentrifuge tube. To elute DNA, 50 µl of EB buffer was applied to the centre of the QIAquick membrane and centrifuged for 1 min.

2.2.5 Enzymatic modifications of DNA

2.2.5.1 Restriction of DNA

Restriction enzyme digestions were performed by incubating double-stranded DNA with an appropriate amount of restriction enzyme in its respective buffer as recommended by the supplier, and at the optimal temperature for the specific enzyme. Standard digestions included 2-10 U enzyme per microgram of DNA. These reactions were usually incubated for 1-3 hrs to ensure complete digestion at the optimal temperature for enzyme activity, which was typically 37°C. For genomic DNA digestion, the reaction solution was incubated overnight at 37°C.

2.2.5.2 Ligation of DNA fragments

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 room temperature for 2-4 hrs.

2.2.5.3 TA-Cloning

(Clark, 1988; Hu, 1993)

Taq polymerase and other DNA polymerases have a terminal transferase activity that results in the non-template addition of a single nucleotide to the 3' ends of PCR products. In the presence of all 4 dNTPs, dATP is preferentially added. This terminal transferase activity is the basis of the TA- cloning strategy. For cloning of PCR products, the pGEM-T or pGEM-T Easy vector systems that has 5‘ T overhangs were used.

The followings were mixed:

50 ng of pGEM-T Easy Vector

150 ng PCR product

1 µl of T4 DNA Ligase buffer (x10)

1 µl of T4 DNA Ligase

in a total volume of 10 µl

The content was mixed by pipetting and the reaction was incubated overnight at 16°C.

2.2.6 Preparation of competent *E.coli* bacteria

(Dagert and Ehrlich, 1979)

The competent bacterial cells are generated by a physical cell wall modification that facilitates DNA uptake. LB medium (100 ml) was inoculated with a single colony of *E.coli* (strain DH5α) and the culture was grown at 37°C to OD 600 = 0.6. Bacteria were centrifuged (10 min, 4°C, 3000xg) and the pellet was resuspended in 50 ml of sterile 50 mM $CaCl_2$ solution (4°C) and incubated on ice for 30 min. The suspension of bacteria

was centrifuged (10 min, 4°C, 3000xg) and the pellet was resuspended in 10 ml of sterile 50 mM $CaCl_2$ (4°C) with 15% glycerol. The mixture was dispensed into aliquots of 100 µl and stored at -80°C. Mostly, competent DH5α were purchased from Invitrogen.

2.2.7 Transformation of competent bacteria

(Ausubel et al., 1994)

Transformation of bacteria was done by gently mixing one aliquot of competent bacteria (50 µl) with 10 µl of ligation reaction. After incubation for 30 min on ice, bacteria were heat shocked for 45 sec at 42°C, cooled down for 2 min on ice. After adding 450 µl of LB medium, bacteria were incubated at 37°C, 200 rpm for 1hr to allow recovery of heat shocked bacteria and then plated out on LB-agar plates.

2.2.8 Polymerase Chain Reaction (PCR)

The polymerase chain reaction (PCR) represents the single 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.

2.2.8.1 PCR amplification of DNA fragments

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

10 ng	DNA
1 µl	forward primer (10pmol)
1 µl	reverse primer (10pmol)
1 µl	10mM dNTPs
5 µl	10x PCR buffer
1.5 µl	50mM $MgCl_2$
0.5 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. Thermal cycling was carried out for 35 cycles with denaturation at 97°C for 30 sec, annealing at 55-60°C for 30 sec, and extension at 72°C for 1 min.

2.2.8.2 Reverse transcription PCR (RT-PCR)

RT-PCR generates cDNA fragments from RNA templates and 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 (10pmol/µl) in a total volume of 12 µl. To avoid the possible secondary structure of the RNA, which might interfere with the synthesis, the mixture was heated to 70^0C 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 10mM dNTPs
1 µl RNasin (10U/µl)

The content of the tube was mixed gently and incubated at 42^0C for 2 min. Then, 1 µl of reverse transcriptase enzyme (Superscript II) was added and incubated at 42^0C for 50 min for the first strand cDNA synthesis. Next, the reaction was inactivated by heating at 70^0C for 15 min. One µl of the first strand reaction was used for the PCR reaction (2.2.8.1).

2.2.8.3 One-Step RT-PCR

To obtain specific RT-PCR products, the QIAGEN OneStep RT-PCR kit was employed which contains optimized components that allow both reverse transcription and PCR amplification to take place in what is commonly referred to as a ”one-step” reaction.

Master mix;	Per reaction
5 x Qiagen OneStep RT-PCR buffer	10 µl
dNTP mix (containing 10 mM of each dNTP)	2 µl
Forward primer (10 pmol)	1 µl
Reverse primer (10 pmol)	1 µl
Qiagen OneStep RT-PCR Enzyme Mix	2 µl
RNase inhibitor (20 units per l µl)	1 µl
RNase-free water	31 µl

2 µl (2 µg) of total RNA isolated from mouse tissues was added to 48 µl of prepared Master mix in a PCR tube, the sample was placed in the thermal cycler and the RT-PCR program run according to the user manual. Reverse transcription reaction was performed at 50°C for 30 min. To denaturate the DNA-RNA hybrid molecules, the reaction was heated to 94°C for 10 min. Thermal cycling was carried out for 35 cycles with denaturation at 94°C for 30 sec, annealing at 56°C-60°C for 40 sec, and extension at 72°C for 1 min. After the amplification step, the sample was checked on an agarose gel.

2.2.9 Protein methods

2.2.9.1 Isolation of total proteins

100 mg of tissue was homogenized in 500 µl Lysis buffer A with a Teflon-glass headed pestle. Then, homogenization samples were handled with ultrasound on ice 2x 2 min. The samples were centrifuged at 8000xg for 20 min at 4°C and supernatant was distributed in several microcentrifuge tubes. The tubes were frozen in liquid nitrogen and stored at –80°C. 5 x 10^6 cells/ml were washed with cold phosphate buffered saline and resuspended in 50 µl of lysis buffer B. The cells were allowed to swell on ice for 30 min, after which the cells were resuspended in 300 µl of lysis buffer B. After homogenization, samples were handled with ultrasound on ice 2 x 30 sec and centrifuged at 24000 x g for 20 min at 4°C. The protein extract was either used immediately or stored at –80°C for later use.

2.2.9.2 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 Coomassie Blue G-250 shifts from 494 to 595 nm when the binding to protein occurs. The 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 color reagent. The absorption of the colour reaction was measured at 595 nm in a microplate reader (Microplate Reader 450, Bio-Rad).

2.2.10 Blotting techniques

2.2.10.1 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. 20x SSC buffer, in which nucleic acids are highly soluble, is drawn up through the gel into the nitrocellulose membrane, taking with it the single-stranded DNA that becomes immobilised in the membrane matrix.

After electrophoresis of DNA, the gel was treated with 0.25 M HCl for depurination. It was followed by denaturation solution for 30 min and 45 min in neutralization solution. The transfer of the DNA to the nitrocellulose membrane was done in a Turbo-Blot-apparatus (Schleicher & Schuell, Dassel). About 20 Whatman filter papers (GB 003) were layered on a Stack Tray, followed by 4 Whatman filter papers (GB 002) and 1 Whatman filter paper GB 002 soaked with 2x SSC. The equilibrated nitrocellulose filter that was also soaked with 2x SSC was laid on the top. The agarose gel, which was treated as described above, was placed on the filter and was covered with 3 Whatman filter papers GB 002 soaked with 2x SSC. The buffer tray was placed and filled with 20x SSC. Finally a wick, which was soaked with 20x 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 2x SSC 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.

2.2.10.2 Northern blotting of RNA onto nitrocellulose filter

For the transfer of RNA onto a nitrocellulose filter, the same procedure as described above (2.2.10.1) was performed. In this case, however, the gel does not need to be denatured, but was transferred directly onto the filter.

2.2.10.3 Western blotting of protein onto PVDF membrane

(Gershoni and Palade, 1982)

Anode I buffer	0.3 M Tris/HCl, pH 10.4
	20 % Methanol
Anode II buffer	25 mM Tris/HCl, pH 10.4
	20 % Methanol
Cathode buffer	40 mM ε-Aminocaproic acid
	25 mM Tris/HCl, pH 9.4
	20 % 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 anode II buffer. Six pieces of GB004 Whatman filter paper were also cut at the size of the gel. Two pieces of filter papers were soaked in anode buffer I and one paper in anode II buffer. First, the papers soaked with anode I buffer were placed on semi dry transfer machine's lower plate and than papers soaked with anode II buffer were placed over it. The equilibrated membrane was placed over them and then the gel were placed avoiding any air bubbles. Another three Whatman papers soaked with cathode buffer was placed over to complete the sandwich model. The upper plate was placed over this sandwich and the transfer was carried out at 3.5 mA/cm^2 for 1 hr.

Membrane staining

Membrane was stained with Coomassie blue for 30 min in room temperature.

Coomassie blue:
- 0.1% coomassie
- 90% Methanol
- 10% CH_2COOH

Destaining of the membrane.

Destaining solution:
- 40% Methanol

-10% CH_2COOH

-up to H_20

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

(Denhardt, 1966; Feinberg and Vogelstein, 1989)

Rediprime™ II Random Prime Labeling System (Amersham Pharmacia) was used for labelling of DNA probes. The method depends on the random priming principle developed by Feinberg and Vogelstein (1989). The reaction mix contained dATP, dGTP, dTTP, Klenow fragment (4-8 U) and random oligodeoxyribonucleotides. Firstly, 25-50 ng of DNA were denatured in a total volume of 46 µl at boiling water for 10 min and quick chilled in ice for 5 min. After pipetting the denatured probe in Rediprime™ II Random Prime Labeling System cup, 4 µl of [α-^{32}P] dCTP (3000 Ci/mmol) were added to the reaction mixture. The labelling reaction was carried out at 37°C for 1 hr. The labelled probe was purified from uncorporated [α-^{32}P] dCTP by using microspin columns (Amersham Pharmacia).

2.2.12 Non-radioactive dye terminator cycle sequencing

Non-radioactive sequencing was performed with the Dye Terminator Cycle Sequencing-Kit (ABI PRISM). The reaction products were analysed with automatic sequencing equipment, MegaBace DNA Sequencer. 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 products, 10 pmol primer and 4 µl reaction mix (contains dNTPs, dideoxy dye terminators and *Taq* DNA polymerase). Elongation and chain termination take place during the following program in a thermocycler: 4 min denaturation followed by 25 cycles at 95°C, 30 sec; 55°C, 15 sec, annealing; 60°C, 4 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.

2.2.13 Hybridisation of nucleic acids

(Denhardt, 1966)

The membrane to be hybridised was equilibrated in 2x SSC and transferred to a hybridisation bottle. After adding 10 ml of hybridisation solution and sheared denatured 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 by chilled, and added to the hybridisation solution. The hybridisation was carried out overnight in the oven. Next day, the filter was washed for 10 min with 2x SSC at room temperature. Finally, was washed with 0.2x SSC containing 0.1 % SDS at the hybridisation temperature. After drying the filter, it was sealed in Saran wrap and exposed to autoradiography overnight at -80°C or to Phosphoimager screen for 1-4 hrs. The film was developed in X-Ray Automatic Processor Curix 60 or screen was scanned in Phosphoimager. For quantification of detected bands, the program Quantity One (Bio-Rad) was used.

2.2.14 Histological techniques

2.2.14.1.1 Tissue preparation for paraffin-embedding

The freshly prepared tissues were fixed in Bouin's solution or 4% (w/v) paraformaldehyde for 24 hrs to prevent alterations in the cellular structure. The tissue to be embedded in paraffin should be free of water. The dehydration process was accomplished by passing the tissue through a series of increasing alcohol concentrations. For this purpose, the tissue was let in 30%, 70%, 90%, and 100% (2x) ethanol for 1hr room temperature. Later, the alcohol

was removed from the tissue by incubating it in methylbenzoat overnight. It was then incubated in 5 ml of histoclear (Xylol) for 10-30 min at room temperature. The second histoclear was not discarded but 5 ml of paraplast were added and the incubation was continued at 60°C for another 30 min. The histoclear and paraffin mixture was discarded and the tissue was further incubated in 5 ml of paraplast at 60°C overnight. Before embedding, the paraffin was changed at least three times. Finally, the tissue was placed in embedding mold and melted paraffin was poured into the mold to form a block. The block was allowed to cool and was then ready for sectioning.

2.2.14.1.2 Sections of the paraffin block

The paraffin blocks were pre-cut to the optimal size and clamped into the microtome (Hn 40 Ing., Nut hole, Germany). The cut-thickness of the section was for 5 µm. The sections were floated on 40°C water to allow actual spread and subsequently put onto pre-treated slides. In order to achieve a better adhesion of the sections, the slides were treated with a drop of serum-formalin. A fine brush was used to transfer the sections to the pre-treated slides. After complete evaporation at 37°C for 2-5 days, slides were stored at 4°C for further analysis.

2.2.14.1.3 Preparation of paraffin sections

For X-gal staining, tissues were dehydrated for 1 hr each in an increasing series of ethanol (50%, 70%, 90%, and 96%). For the paraffin embedding, the alcohol must be removed from the tissue, which was achieved with an overnight incubation in methyl benzoate. Washing twice for 10 min with Roticlear, the methyl benzoate was also removed. The embedding was done with a paraffin mixture with the commercial name of ParaplastTM, which contains DMSO for a better infiltration in the tissue. The jars used in the procedure are temperated at 60° C. The ParaplastTM was melt at 60°C in an oven for 1hr. The Roticlear was replaced with a 1:1 mix of Roticlear and ParaplastTM, and the tissue was incubated at 60°C in this medium twice for 20 min. Then, the tissue was incubated 2x 20 min in ParaplastTM alone. The tissue was transferred to the embedding mould, which was

filled with liquid ParaplastTM and laid at room temperature to solidify. After this, the paraffin block was cut and mounted onto the microtome to make sections of 2-10 µm. The sections were mounted on superfrost slides. The paraffin sections were then stored at 4°C. Tissue sections were incubated twice for 10 min in Roticlear to remove the paraffin. Then, sections were re-hydrated in a decreasing ethanol series (100%, 96%, 70%, 50%, and 30%) for 2 min each, finally washed in PBS and air-dried. Now the sections were ready for light microscopical analysis.

2.2.14.1.4 X-gal staining

X-gal staining of adult mouse tissues

A histochemical staining procedure for *E. coli ß*-galactosidase activity in adult mouse tissue was used to detect Lac Z expression. Tissues were rinsed in cold PBS and fixed in FixA solution for 30 min on ice with shaking. Than the tissues were washed again in PBS and fixed in FixB solution for 1hr on ice shaking. After final washing with PBS, tissues were incubated overnight in X-gal staining solution at 30^0C under dark condition.

FixA	1% v/v formaldehyde
	0.2% v/v glutaraldehyde
	0.02% v/v NP-40
	in PBS
FixB	1% v/v formaldehyde
	0.2% v/v glutaraldehyde
	0.2% v/v NP-40
	0.1% w/v sodium deoxycholate
	in PBS

X-gal staining solution	1 mg/ml X-gal
	5 mM potassium ferricyanide
	5 mM potassium ferrocyanide
	2 mM $MgCl_2$
	in PBS

2.2.14.2.3 Immunofluorescence staining of mouse testes and tissues

Fixation and subsequent treatment of mouse testicular tissue was performed as described in section 2.2.15.1. Adult mouse testis cross sections (5-10µm) were dewaxed with roticlear solution and rehydrated by descending ethanol concentrations. For immunofluorescence staining, sections were washed in PBS and were then incubated with a blocking solution containing 5% horse serum and 0.02% Tween-20 in PBS for 1 hr at room temperature. The testis sections were incubated with affinity-purified primary polyclonal antibody for 16 hrs at 4°C. The tissue sections were rinsed four times in PBS and subsequently incubated with (Cy3)-conjugated mouse anti goat IgG (1:100) for 1 hr at room temperature. After the tissue sections were washed three times with PBS, the nuclei were counterstained with DAPI. Immunostaining of the sections was examined using a fluorescence equipped microscope (BX60; Olympus).

2.2.14.2.4 Immunofluorescence staining of cells

Cells were fixed in 4% paraformaldehyd in PBS for 20 min at room temperature, followed by 100% methanol at -20°C for 5 min. The cells were rinsed in PBS. An initial blocking step was performed with the blocking solution (2% horse serum and 0.5% Triton X-100 in PBS) for 1 hr. A mouse anti-goat Faf1 was applied for overnight at 4°C in a dilution of 1:100. Cells were subsequently incubated with Cy3- conjugated mouse anti-goat IgG for 1 hr at room temperature. One drop of mounting medium with DAPI was dispensed onto the slides after washing with PBS. Fluorescent cells were visualised with Olympus BX60 microscope using 20X or 60X Neofluor lens, photographed using digital camera and analysed by analysis 3.0 soft imaging system.

2.2.14.2.5 Immunocytochemical staining of germ cell suspensions.

Germ cell suspensions were prepared from mouse testes by using the collagenase/trypsin method according to published procedure (Romrell et al., 1976). Testes from 60 days old mice were collected in serum-free culture medium, rinsed in 0.1 M PBS, pH 7.2. After removal of the tunica albuginea, seminiferous tubules were enzymatically dissociated by the addition of 1 ml collagenase (1mg/ml). The slurry maintained at 37°C for 15 min, was triturated every 5 min. 5 ml of Hank's solution was added and then spun at 350 x g to sediment the dissociated cells. The pellet was resuspended in 3 ml trypsin and then trypsin was inactivated by adding 5 ml FKS. The slurry was passed through 80 µm nylon mesh. The filtrate was spun at 350 x g to sediment the cells. Cells were resuspended in 1 ml PBS and spread onto superfrost slides, air-dried and fixed in 100% methabol for 10 min. The immunocytochemical staining procedure was performed as described (2.2.15.4).

2.2.14.3 TUNEL-assay for detection of apoptotic cells.

Testes were fixed in 4% paraformaldehyde and embedded in paraffin. Sections were cut at 5µm. TdT- mediated nick labeling (TUNEL) staining was performed using the In Situ Cell Death Detection Kit (Roche Diagnostic GmbH, Mannheim) according to the manufacturer's instructions. After rehydration, the sections were incubated in 2 x SSC at 80°C for 20 min followed by washing twice with water and once with proteinase K buffer (29 mM Tris/HCl, pH7.5, 2mM CaCl) for 5 min each. The slides were then treated with proteinase K (10µg/ml) at 37°C for 30 min. An aliquot of 3'-end labeling reaction mixture containing 4 µl of 5x terminal deoxynucleotidyl transferase (TdT) buffer, 0.1 µl of digoxigenin-11-ddUTP (10 nmol/µl) 0,2 µl of ddATP (5 mM), 1 µl of TdT and 14.7 µl nuclease-free water was applied to one section. The slides were kept in a humidified box, incubated at 37°C for 1 h, and then washed three times with TBST buffer (10mM Tris/HCl, pH-8.0, 100mM NaCl, and 0.1%Tween-20) for 20 min each. An anti-digoxigenin-horseradish peroxidase monoclonal antibody (DAKO, 1:200 dilution in TBST containing 1% BSA) was applied, and the slides were incubated in the humidified box at room temperature for 1 h and then washed three times with TBST for 5 min each time. Finally,

the labeled cells were visualized by 3,3'-diaminobenzidine tetrahydrochloride for 0.5-2 min.

2.2.15. Culture and immunostaining of preimplantation embryos

2.2.15.1 Superovulation

Seven to eight week old female mice were superovulated by intraperitoneal injections of 5 IU of pregnant mare's serum gonadotropin (PMSG, Sigma St Louis, MO, USA) followed 44-48 hrs later by 5 IU of human chorionic gonadotrophin (HCG; Sigma). After the second injection, females were housed overnight with males and were checked by a vaginal plug the following morning. The E0.5 was considered to be 12^{00} noon at the day of vaginal plug.

2.12.15.2 Recovery of preimplantation embryos

Matings were set up between superovulated female and male Faf1 GT/+ mice. Pregnant females were killed by cervical dislocation at 0.5 days pc. 1-cell stage embryos (E-05) were isolated from oviducts and washed through several large volume changes of M2 medium to eliminate any contaminating maternal cells. Embryos were cultured in M16 medium. Embryos at 1-cell (18-20 hrs p.c.), 2-cell-(42-44 hrs p.c.), 4-cell-(50-52 hrs p.c.), 8-cell stage (66-72 hrs p.c.), morula and blastocyts (90-92 hrs p.c.) were washed and then individually treated into 5 µl drops of acide tyrode to remove the zona pellucida. After washing in two drops of PBS, embryos were treated with warm trypsin for 3 min at 37°C to separate the polar bodies from embryo. Embryos were washed into drop of PBS and then placed individually in a PCR tube (0.2 ml) with 5 µl PBS. Embryos were lysed by repeatedly (3 cycles) freezing on dry ice with methanol and thawing at 90°C. The entire lysate was used for PCR genotyping.

For isolation of total RNA from preimplantation embryos, cultured embryos were washed with M2 medium and PBS and then collected in an E-cup with RNAase free water. RNA preparation was performed according to QIAGEN® RNAeasy protocol supplied with the

kit.

2.2.15.3 In vitro culture of blastocyst stage embryos

Embryos were flushed out from the uteri of plugged females at day 3.5 and placed in gelatinized 96-well dishes. Embryos were cultured in ES cell medium without the addition of leukaemia inhibitory factor. The dishes were kept in a humidified incubator at 37^{o}C, 5% CO_2 and embryonic outgrowth was scored daily. Every second day, of fresh medium was added to the wells. DNA was prepared on the fourth day of culture.

2.2.15.4 Immunofluorescence staining of mouse preimplantation embryos

Preimplantation embryos were collected as described (2.12.16.2) and fixed in 4% paraformaldehyde in PBS for 30 min at 4°C. The fixed embryos were washed and transferred to block solution (PBS with 10% serum and 0.02% Triton-X) for 30 min. All subsequent incubations were carried out in block solution. The preimplantation embryos were incubated with anti-Faf1 antibody (diluted 1/100) overnight at 4°C, followed by three times (10 min) washing, and then incubate with 3 µg/ml of Cy3-conjugated mouse anti-goat IgG for 1h. Nucleus was counterstained with DAPI for 10 min, rinsed 3 times in PBS for 10 min. Immunostaining of the embryo was examined using a fluorescence-equipped microscope (BX60; Olympus).

2.2.16.1 Expression of recombinant proteins in the pET system (Novagen, Darmstadt)

Plasmid with pET41 constructs were transformed into the host bacterial strain *E.coli* BL-21(DE3). A single bacterial colony containing the vector with the fusion construct was picked from a freshly streacked plate and 50 ml BL culture with kanamycin was inoculated. Bacterial culture was incubated with shaking at 37°C until an OD600 of 0.4-1.0 was reached. A noninduced sample was removed as a control. To the remainder, IPTG from a 100 mM stock was added to a final concentration of 0.4 mM and the incubation step was

continued for 2-3 hrs. Then, the induced sample was removed and flasks were placed on ice for 5 min. Cells were harvested by centrifugation at 5000 x g for 5 min at 4°C, resuspended in 0.25 culture volume of cold 20 mM Tris-HCl, pH 8.0, and centrifuged as above. Finally, the supernatant was removed and cells were stored as a frozen pellet at -70°C or used directly for purification.

2.2.16.2 GST Pull-down assay

GST-fusion proteins were purified from bacterial cell extract using the GST-binding kit (Novagen, Darmstadt). Following reagent were combined in a 1.5 ml microcentrifuge tube on ice. Beads were mixed gently by inverting several times. A sufficient volume of beads was transferred to a clean 1.5 ml microcentrifuge tube. The beads were washed 3 times with 250 µl of GST Bind/Wash Buffer (4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.3) in a microcentrifuge tube and centrifuged at 800 x g for 30 sec. The supernatant was removed by aspiration with a micropipette. Steps were repeated and finally, the beads were resuspended to their original volume (i.e., the original beads volume was transferred to microcentrifuge tube) by adding GST Bind/Wash Buffer. 50 µl (100 µg) GST fusion proteins were added to the GST-beads. To ensure adequate mixing, the reaction tube was rotated at room temperature for 30 min. The tube was centrifuged at 800 x g for 1 min and the supernatant discarded. Beads were washed 2 times with lysis buffer B. Then, 100 µl of tissue extract (300-500 µg) were added to the reaction tube and the mixture was incubated for additional 2 hrs at 4°C on a rocking platform. Beads were washed 3 times with 250 µl of lysis buffer B for 10 min at 4°C. Finally the beads were resuspended in 50 µl SDS-PAGE-Loading Buffer, 6 µl DTT, and then denaturated and loaded onto a SDS-PAGE minigel. Integrity of the resulting proteins was checked by SDS-PAGE analysis.

2.2.17 Immunoprecipitation

Tissues (200 mg) were homogenized in 1 ml lysis buffer and incubated at 4°C for 30 min. Lysates were then centrifuged for 30 min at 4°C 12000 x g, the protein concentrations were

measured and equal amounts of protein were used for immunoprecipitation. 30 µl of protein-G-agarose slurry and 5 µg of antibody were suspended in 500 µl of PBS and an incubated for 1hr at 4°C. The beads were washed three times in lysis buffer, then 500 µg of lysate protein were added, and the mix was incubated for 3 hrs at 4°C. The immune complex on the beads was washed three times with washing buffer containing 500 mM NaCl and 0.1% Triton x-100 and once with PBS. Beads were boiled in 2 x SDS-PAGE sample buffer, and samples were electrophoresed on 10% SDS-PAGE gels (Invitrogen).

2.2.18 Transfection of HeLa cells

Approximately 4 x 10^5 Hela cells were plated in a cell chamber (with slide) (Lab-Tek, Wiesbaden) and cultured overnight in 5 ml DMEM medium containing 10% FCS and penicillin/streptomycin at 37°C and 5% CO2. 1 µg of DNA (*Myc* and *His fusion*) was diluted with the DNA-condensation buffer (buffer EC of the Effection Transfection kit, Qiagen, Hilden) to a total volume of 150 µl, 8 µl of enhancer was also added to DNA and mixed by vortexing for 1 sec. The mixture was incubated at room temperature for 5 min. 25 µl of Effectin Transfection Reagent was added to the DNA-Enhancer mixture, mixed by pipetting and incubated for 10 min at room temperature to allow complex formation. 1 ml of cell medium was added to the reaction tube, mixed and immediately added into the cell chamber. Cells were incubated for overnight at 37°C, 5% CO_2, to allow for gene expression. The glass slide of the culture chamber was rinsed with PBS and then fixed with methanol for 5 min. One drop of mounting medium with DAPI was dispensed onto the slide. Fluorescent cells were visualised with Olympus BX60 microscope using a 60X neofluor lens, photographed using digital camera and analysed using software 3.0, Soft Imaging System.

2.2.19 Computer analysis

For the analysis of the nucleotide sequences, programs like BLAST, BLAST2, MEGABLAST and other programs from National Center for Biotechnology Information (NCBI) were used (www.ncbi.nlm.nih.gov). Information about mouse alleles, phenotypes

and strains were used from Jackson Laboratory (www.informatics.jax.org). For protein studies ExPASy tools (www.expasy.ch) were used. Mouse genome sequence and other analysis on mouse genes, transcripts and putative proteins were downloaded from Celera discovery system (www.celera.com).

3. RESULTS

3.1 Expression of mouse *Faf1* gene

3.1.1 Expression of the *Faf1* gene in different tissues of the adult mouse.

To determine the expression of *Faf1* gene in different tissues of the adult mouse, 20 µg of total RNA from testis, ovary, brain, heart, kidney, liver, lung, thymus, skeletal muscle and spleen was size fractionated in 1% agarose gel containing formaldehyde and transferred to nitrocellulose membranes. The Northern blot was hybridized with ^{32}P-labeled mouse *Faf1* cDNA. Only a 2.8-kb transcript of the *Faf1* gene was detected in the RNA of all studied tissues. Equivalent loading and integrity of the RNA samples were confirmed after hybridization of the blot with human elongation factor 2 (hEF2) cDNA (Fig. 3.1).

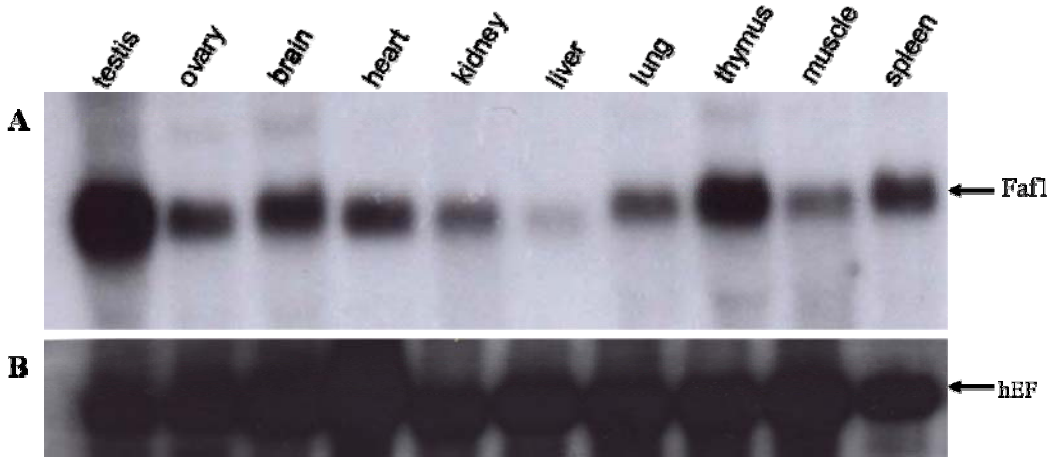

Figure 3.1 Northern blot analysis of *Faf1* gene expression in adult mouse tissues. Total RNA (20 µg) was extracted from different tissues (testis, ovary, brain, heart, kidney, liver, lung, thymus, muscle and spleen) of the adult mouse and subjected to Northern blot hybridization using the *Faf1* cDNA as a probe (A). Hybridization with the hEF2 cDNA demonstrated the integrity of the loaded RNA (B).

3.1.2 Expression of Faf1 protein in mouse tissues.

To determine the expression pattern of the Faf1 protein, Western blot analysis was performed. Protein was extracted from different tissues of adult mouse, separated on SDS PAGE and transferred onto a nitrocellulose membrane. The polyclonal anti-Faf1 antibody, which was raised against the C-terminal region of protein (sc-1887, Santa Cruz Biotechnology) recognizes a 74-kDa Faf1 protein in all studied tissues and a further 49-kDa isoform in testicular extract (Fig. 3.2). The expression level of the 49-kDa isoform was higher than the 74-kDa protein. The membrane was subsequently stripped and probed with anti-α tubulin for monitoring the protein loading.

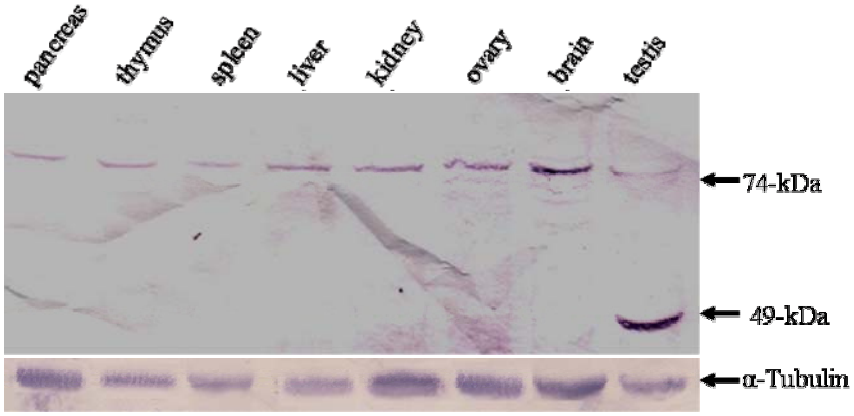

Figure 3.2 Expression of Faf1 protein in different mouse tissues. A polyclonal antibody raised against the C-terminus of Faf1 protein was used to probe the Western blot with protein extracted from different tissues. The 74-kDa band was found in all tissues, while the 49-kDa isoform was only detected in testis. The membrane was stripped and subsequently probed with an anti-α tubulin monoclonal antibody to monitor the loading.

3.1.3 Expression of *Faf1* gene during testicular development and in testes of different mutant mice

To evaluate the expression of the *Faf1* gene during testicular development, Northern blots with testicular RNAs from 5-, 10-, 15-, 20-, 25- and 60-day-old mice were hybridized with ^{32}P-labeled mouse *Faf1* cDNA. As shown in figure 3.3, a 2.8-kb transcript could be detected during the first three weeks of postnatal development. Thereafter, an increasing level of *Faf1* expression was observed. This result suggests that the expression of *Faf1* gene is increased in haploid spermatids.

To examine whether the expression of the *Faf1* gene is restricted to spermatogenic cells of testis, Northern blot analysis with testicular RNA isolated from *W/W^v, Tfm/Y, Insl3, qk/qk and olt/olt* mutant mice was performed. As shown in figure 3.4, the *Faf1* transcript was detected at low level in testis of *W/W^v , Tfm/Y* and at high level in testis of *Insl3, qk/qk* and *olt/olt* mice. This result suggests that the *Faf1* expression is increased in haploid spermatids.

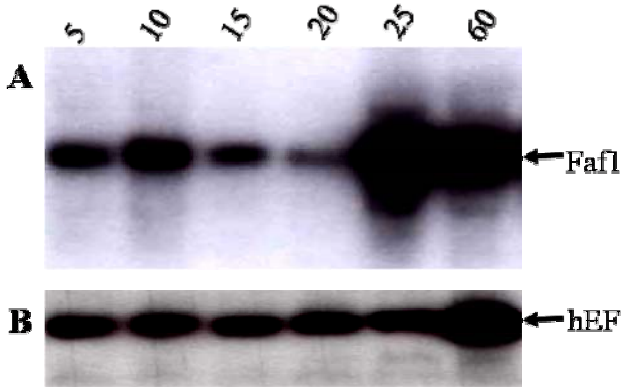

Figure 3.3 Northern blot analysis of *Faf1* expression during testicular development. (A) Testicular RNA was isolated from 5-, 10-, 15-, 20-, 25- and 60- day-old mice, separated in agarose gel, blotted and hybridized with mouse *Faf1* cDNA fragment. (B) Rehybridization with human elongation factor to confirm equal amount of RNA loading.

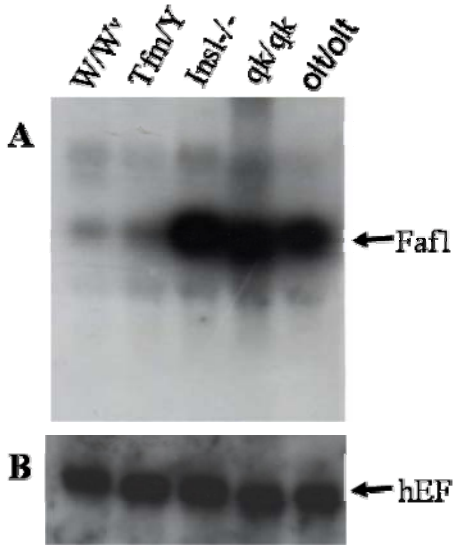

Figure 3.4 Northern blot analysis of *Faf1* expression in testis of different mutant mice. (A) Total testicular RNA isolated from *W/W^{v}, Tfm/Y, Insl3$^{-/-}$, qk/qk, olt/olt* mutant mice was hybridized with the mouse *Faf1* cDNA fragment. (B) Rehybridization of the membrane with human elongation factor2 (hEF2).

3.1.4 Expression of Faf1 protein during testicular development and in the testis of different mutant mice

To evaluate the expression of the Faf1 during testis development at protein level, total protein extracts from testes of mouse at different developmental stages were analyzed by Western blot. Western blot analysis showed that the 74-kDa isoform is present at equivalent amount throughout testicular development, while the 49-kDa protein is not detectable in mouse testis till postnatal day 21. The expression of the 49-kDa protein could be first detected at day 25 (Fig. 3.5). Thereafter, we observed an increasing level of the 49-kDa protein.

To verify the specificity of the anti-Faf1 polyclonal antibody, competition assay was performed. In this assay, Faf1 antibody was incubated with a Faf1 oligopeptide which was used to immunize a goat to generate Faf1 antibody. After 30 min of incubation, the solution of competition assay was probed in a Western blot containing protein extracts from testis of wild-type and *olt/olt* mice. As shown in figure 3.6, the 74-kDa and 49-kDa

isoforms are abolished in competition assay. This result indicates that the anti-Faf1 antibody specifically recognizes the 74- and 49-kDa proteins. The observed correlation between the increase of the *Faf1* transcript and the increase of the 49-kDa isoform in testis of 24- and 30-day-old mice suggests that the 49-kDa isoform is a result of posttranslational modification of the 74-kDa precursor.

The presence of the 49-kDa Faf1 was also examined in the testis of mouse mutants, in which spermatogenesis is arrested at different stages. As expected, the 74-kDa protein is found in testes of all mutants. The 49-kDa isoform is present in testes of *olt/olt* and *qk/qk* mutant mice (in which spermatogenesis is arrested at the spermatid stage), whereas the 49-kDa is not detectable in the testes of W/W^v mutant mice (which lack all germ cells), *Tfm/Y* mice (in which spermatogenesis is arrested at the primary spermatocyte stage) and in cryptorchid testes of $Insl3^{-/-}$ mutant mice (in which spermatogenesis is arrested at the stage of pachytene spermatocytes) (Fig. 3.7). This result indicates that the 49-kDa protein is a spermatid specific isoform.

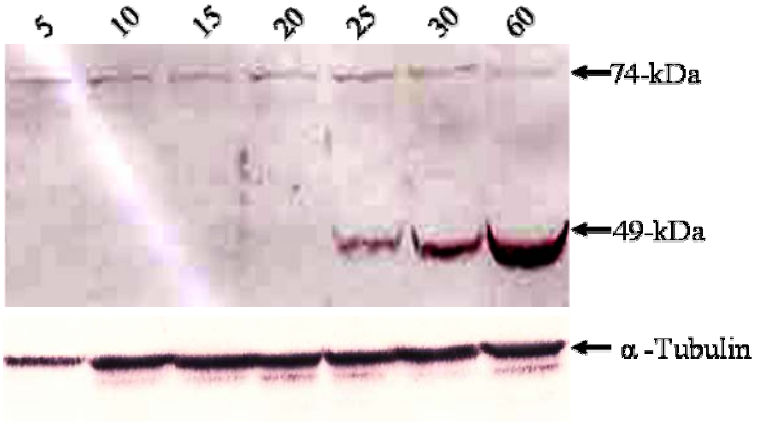

Figure 3.5 Western blot analysis of Faf1 expression during postnatal development of testis. Anti-Faf1 polyclonal antibodies detect a 74-kDa Faf1 protein in testicular lysates from all developmental stages, while 49-kDa Faf1 isoform is first detectable in testis of 25-day-old mice. α-tubulin in tissue lysates is shown as a control for protein loading.

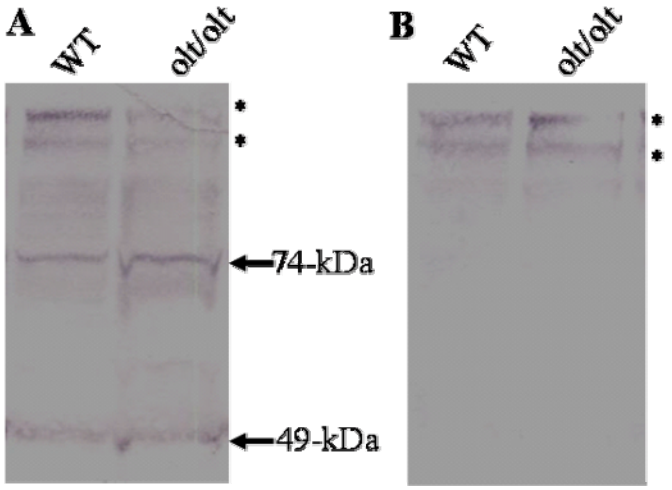

Figure 3.6 Competition assay to verify the specificity of Faf1 antibody. (A) Western blot with protein extracts from wild-type and *olt/olt* testis was probed with Faf1 antibody. The Faf1 antibody recognizes the 74-kDa and 49-kDa proteins and high molecular weight proteins (*). (B) In the competition assay, the 74-kDa and 49-kDa Faf1 isoforms were not detected. This result indicates that the anti-Faf1 antibody specifically recognizes the 74-kDa and 49-kDa isoforms.

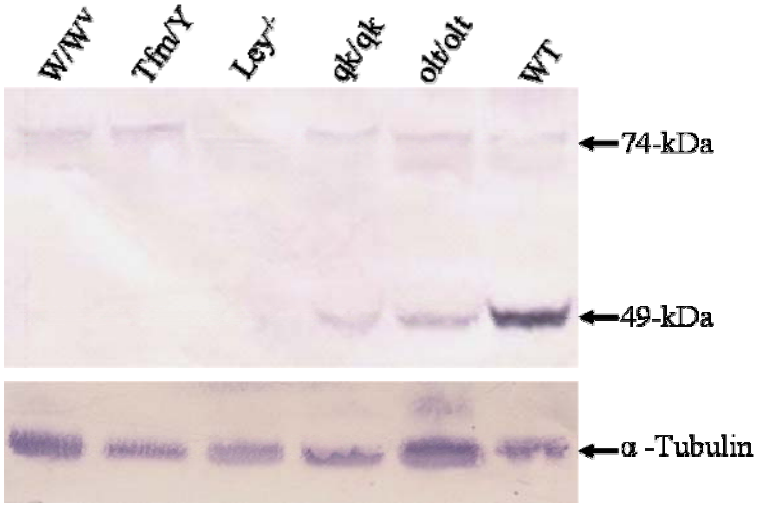

Figure 3.7 Western blot analysis of Faf1 expression in testes of different mutant mice. Anti-Faf1 antibodies detect 74-kDa protein in testicular lysates from all mutant mice, while the 49-kDa Faf1 isoform is present in testes of *qk/qk and olt/olt* mice.

3.1.5 Expression of *Faf1* in male germ cells

3.1.5.1 *Faf1* expression during male germ cell differentiation

The cellular localization of Faf1 protein in the testis was determined by immunohistochemistry. In testes of wild-type and *olt/olt* mice, the Faf1 immunoreactivity was mainly detected in the cytoplasm of diploid spermatocytes and haploid spermatids (Fig. 3.8 A, B). In wild-type testis, the highest level of Faf1 protein was observed in haploid spermatids (Fig. 3.8 A). In contrast to testes of *olt/olt* mutant mice, lower level of Faf1 protein was found in round spermatids (Fig. 3.8 B).

Northern blot analysis of *Faf1* expression revealed that the *Faf1* is highly expressed in cryptorchid testis of *Insl3*$^{-/-}$ mutant mice in which spermatogenesis is arrested in pachytene spermatocytes (Fig. 3.4). In Western blot, the Faf1 antibody recognized only the 74-kDa protein in testicular lysate of *Insl3*$^{-/-}$ mice (Fig. 3.7). Immunohistological staining of testicular sections of *Insl3*$^{-/-}$ mice revealed that the Faf1 protein is present at high level in cytoplasm of giant cells, which contain multiple nuclei (Fig.3.8 C, D).

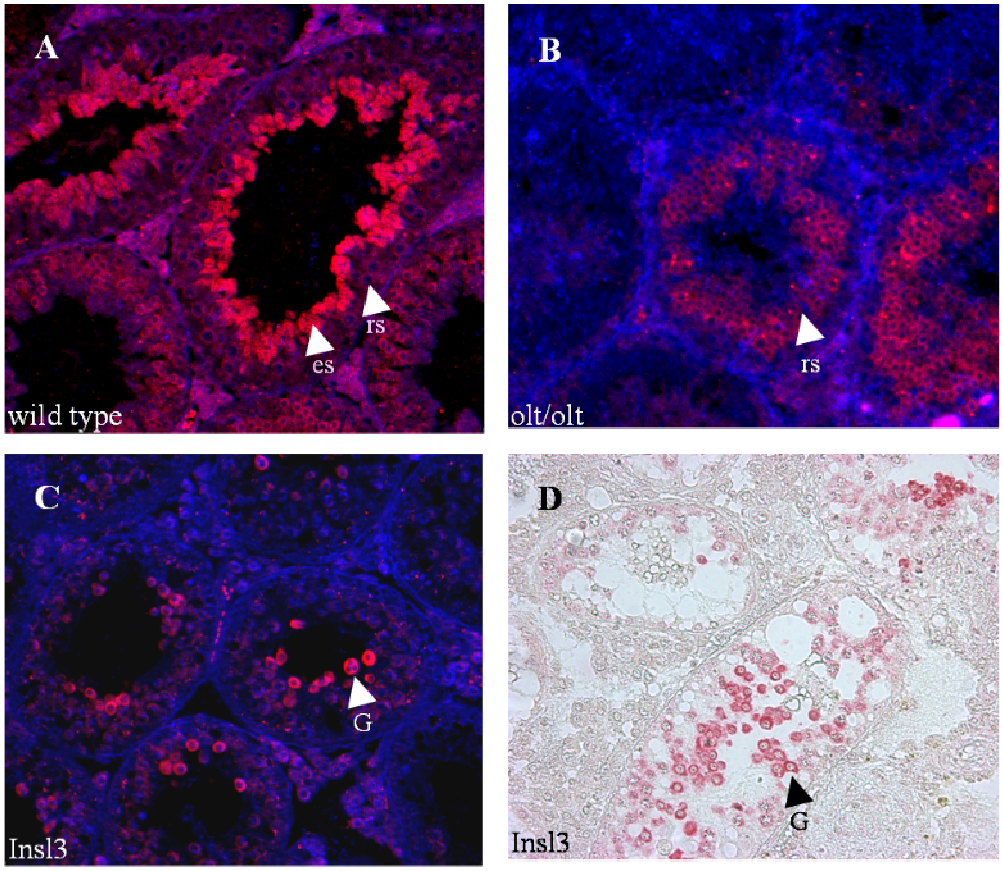

Figure 3.8 Immunohistochemical detection of Faf1 protein in testis of wild-type (A) and *olt/olt* mutant mice (B). Faf1 protein expression was at low level in diploid spermatocytes and higher in haploid spermatides. In cryptorchid testes of *Insl3*$^{-/-}$ mice (C, D) high expression of Faf1 protein is restricted to multinuclear giant cells. rs, round spermatid; es, elongated spermatid; G, giant cells.

3.1.5.2 Intracellular distribution pattern of Faf1

To determine the intracellular localization of Faf1 in male germ cells, germ cell suspension of adult mouse testis was prepared, spread on glass slides and stained with anti-Faf1 antibody. The nuclei were counterstained with DAPI. As can be seen in figure 3.9, extensive staining was found in cytoplasm of elongated spermatids and in germ cells containing multiple nuclei. No Faf1 expression was found in the mature spermatids. High accumulation of Faf1 in multinuclear germ cells was also observed in cryptorchid testes of Insl3 deficient mice (Fig. 3.8 C, D)

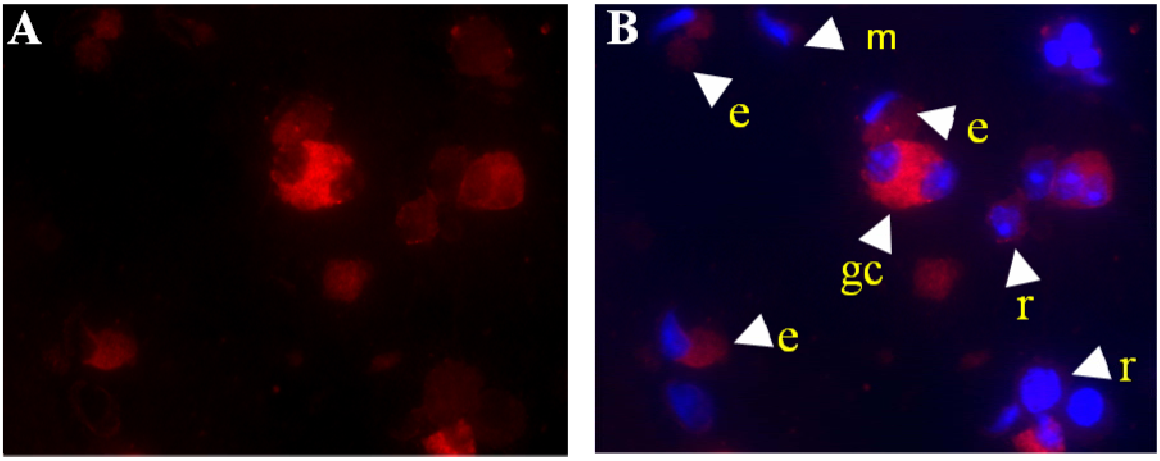

Figure 3.9 Expression pattern of Faf1 protein in male germ cells. In preparations of germ cells suspension, high level of Faf1 was found in cytoplasm of elongated spermatids and in multinuclear germ cells. No Faf1 protein was detected in mature spermatids. e, elongated spermatids; r, round spermatids; m, mature spermatids; gc, multinuclear germ cells.

3.1.6. Expression of Faf1 in ovary

To determine the expression of Faf1 protein in ovary, female mice were injected with 5 IU of human chorionic gonadotropin (HCG; Sigma). Two days later ovaries were isolated and fixed overnight in 4% paraformaldhyde, dehydrated and embedded in paraffin. Serial sections on glass slides were immunostained with Faf1 antibody.

As shown in figure 3.10 Faf1 was only detected in the cytoplasm of oocytes of all follicular stages including primary, secondary and antral follicles. Faf1 was not detectable in ovarian somatic cells.

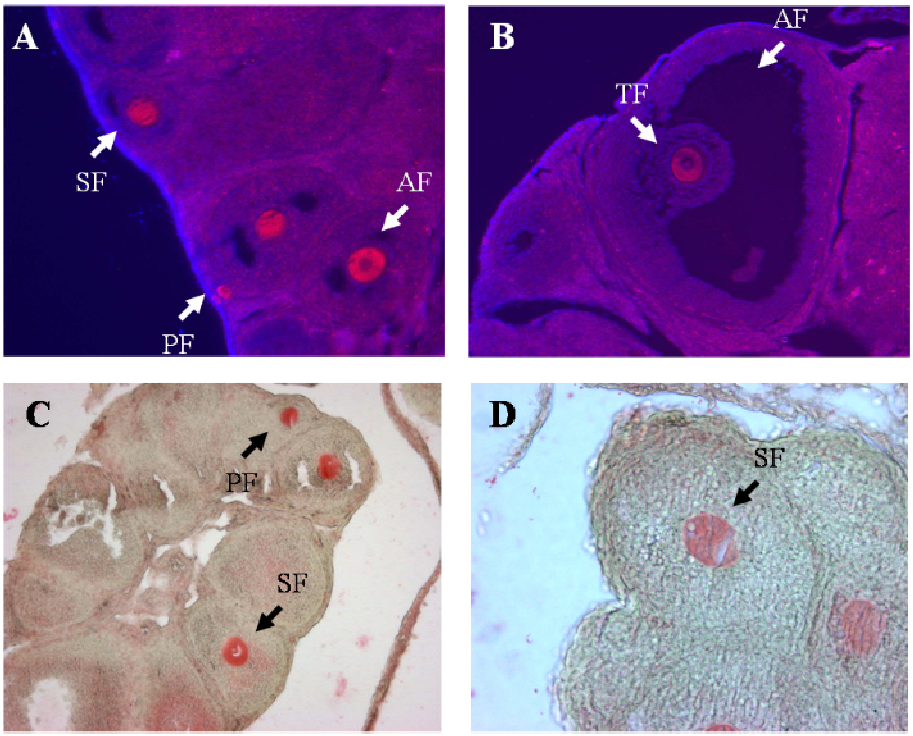

Figure 3.10 Immunohistochemical detection of Faf1 protein in mouse ovary. Faf1 immunoreactivity was detected in the cytoplasm of oocytes in the primary follicles (PF), secondary follicles (SF) and antral follicles (AF). In (A) and (B), second antibody was anti-goat Cy3 conjugated IgG, while in (C) and (D) it was anti-goat alkaline phosphatase.

3.1.7.1 Expression of the Faf1 protein in oocytes and early cleavage embryos

Indirect immunofluorescent labeling with the anti-Faf1 antisera was used to assess protein expression in oocytes as well as in preimplantation embryos (fertilized oocytes, 2-cell, 4-cell, 8-cell stage, morula and blastocyt).

Faf1 protein was localized throughout the cytoplasm in unfertilized oocytes (Fig. 3.11 A). This result is consistent with our immunohistochemistry results with ovarian follicles (Fig. 3.10). Faf1 protein assessment after fertilization demonstrates that the maternal protein persists in early zygotes (Fig. 3.11 B) as well as in early cleavage embryos (Fig. 3.11 C, D). Thus, Faf1 could theoretically function at any stage of oogenesis and in early embryos.

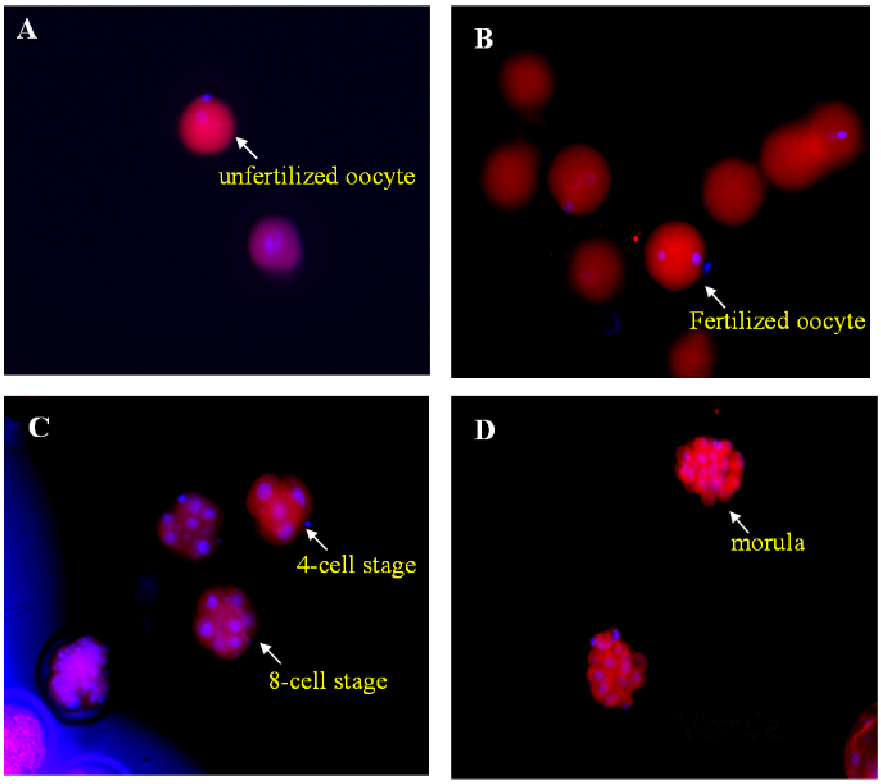

Figure 3.11 Faf1 protein was localized using an antibody against Faf1. We studied unfertilized oocytes, fertilized oocytes, 2-cell, 4-cell, 8-cell and morula stage wild-type embryos. Faf1 protein assessment after fertilization demonstrates that the protein persists in early zygotes.

3.1.7.2 Expression of the Faf1 in embryonic stem cells

The high expression of Faf1 protein in embryonic cells leads us to examine the expression of the *Faf1* in different cell lines. Northern blot with RNA from different cell lines was hybridized with *Faf1* cDNA. *Faf1* gene is highly expressed in embryonic stem cells (ES), murine embryonic teratocarcinoma (F9) and human Tera1 cell lines (Fig.3.12).

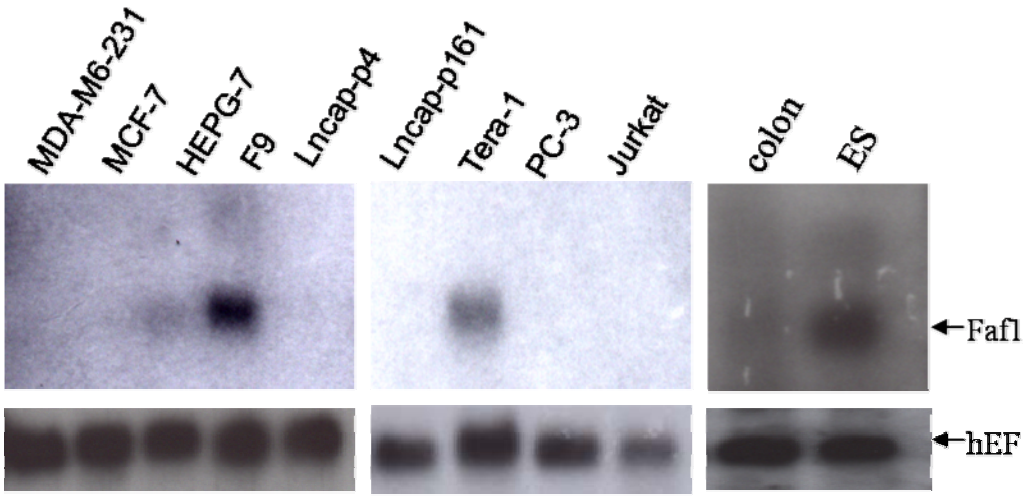

Figure 3.12 Northern blot with RNA from different cell lines reveals that the *Faf1* gene is highly expressed in embryonic stem cells (ES), murine embryonic teratocarcinoma, (F9), and human Tera1 cell lines. MDA-M6-231, human breast cancer; MCF-7, human breast cancer; HEPG-7, liver cancer; Lncap-p4, human prostate carcinoma; Lncap-p161, human prostate carcinoma; PC-3, human prostate carcinoma; Jurkat, human T cell leukemia.

3.2 Functional analysis of *Faf1* gene

3.2.1 Establishment of methods for genotyping the gene trap 98-2C line

Animals of the mouse line 98-2C have been genotyped in the group of Prof. Peter Gruss (Max-Planck-Institute for biophysical Chemistry, Göttingen) by quantitative Southern blot analysis using a LacZ-specific probe. Using this method, we were not able to distinguish between heterozygous and homozygous animals. Therefore, we have designed a breeding program to identify homozygous and heterozygous animals. Animals were first genotyped by PCR using LacZ-specific primers (Fig. 3.13). LacZ-positive mice were intercrossed and offspring were genotyped by PCR. In progeny of these intercrosses, we expected that LacZ-positive animals will be either heterozygous or homozygous for *LacZ* gene. To distinguish between heterozygous and homozygous LacZ-mice, LacZ-positive males and females were then backcrossed with-wild type animals. If the LacZ-positive parents would be homozygous, we expected that all offspring in each backcross should be LacZ-positive. Genotyping of progeny from 28 backcrosses revealed the presence of LacZ-positive and negative mice in offspring of all backcrosses. Ratio of the LacZ-positive and LacZ-negative offspring in backcross breeding was 1:1 (Table 3.1). This result suggests that all LacZ-positive parents of backcrosses were heterozygous for the trapped gene.

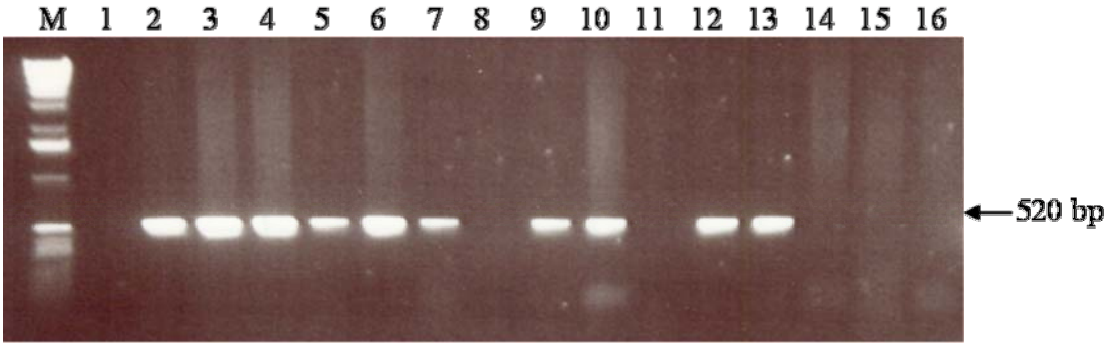

Figure 3.13 Genotyping of offspring in backcrosses between LacZ-positive and wild type mice. LacZ-gene was amplified using the primers LacZF and LacZR. PCR-cycle condition was 94°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec. The PCR products were

separated on a 1, 5% agarose gel and stained with ethidium bromide. The length of the amplified LacZ-fragment was 520-bp.

Genotype of parent	No of progeny with genotype	
	LacZ-pos.	LacZ-neg.
♂ LacZ- pos. x ♀ +/+	130	133
♂ +/+ x ♀ LacZ- pos.	83	82

Table 3.1 Genotypes of offspring in backcrosses between LacZ- positive and wild-type animals.

3.2.2 Identification of the integration site of gene trap vector in *Faf1* gene

The integration site of gene trap vector in the genome of the mouse 98-2C line was determined by the group of Prof. P.Gruss (Max-Planck-Institute for biophysical Chemistry, Göttingen). This group has cloned and sequenced a genomic fragment containing a gene trap sequence (Fig.3.14 A). Alignment of the genomic sequence in Database revealed the presence of 120-bp sequence of gene trap vector and a sequence of 350-bp located in intron 7 of the *Faf1* gene (Fig.3.14 B). The 120-bp sequence of the gene trap is located at 3' end of the vector and contains a sequence of intron and splice donor site of *Pax-2* gene.

Sequence analysis revealed that the gene trap vector was integrated in the 35-kb long intron 7 of *Faf1* gene, approximately 23-kb 3'of exon 7 (nucleotide position 108022268-108308418 bp of the chromosome 4 genomic counting sequence, GeneBank accession no. NT_039264). We referred the mutated allele in the gene trap 98-2C line as $Faf1^{GT}$.

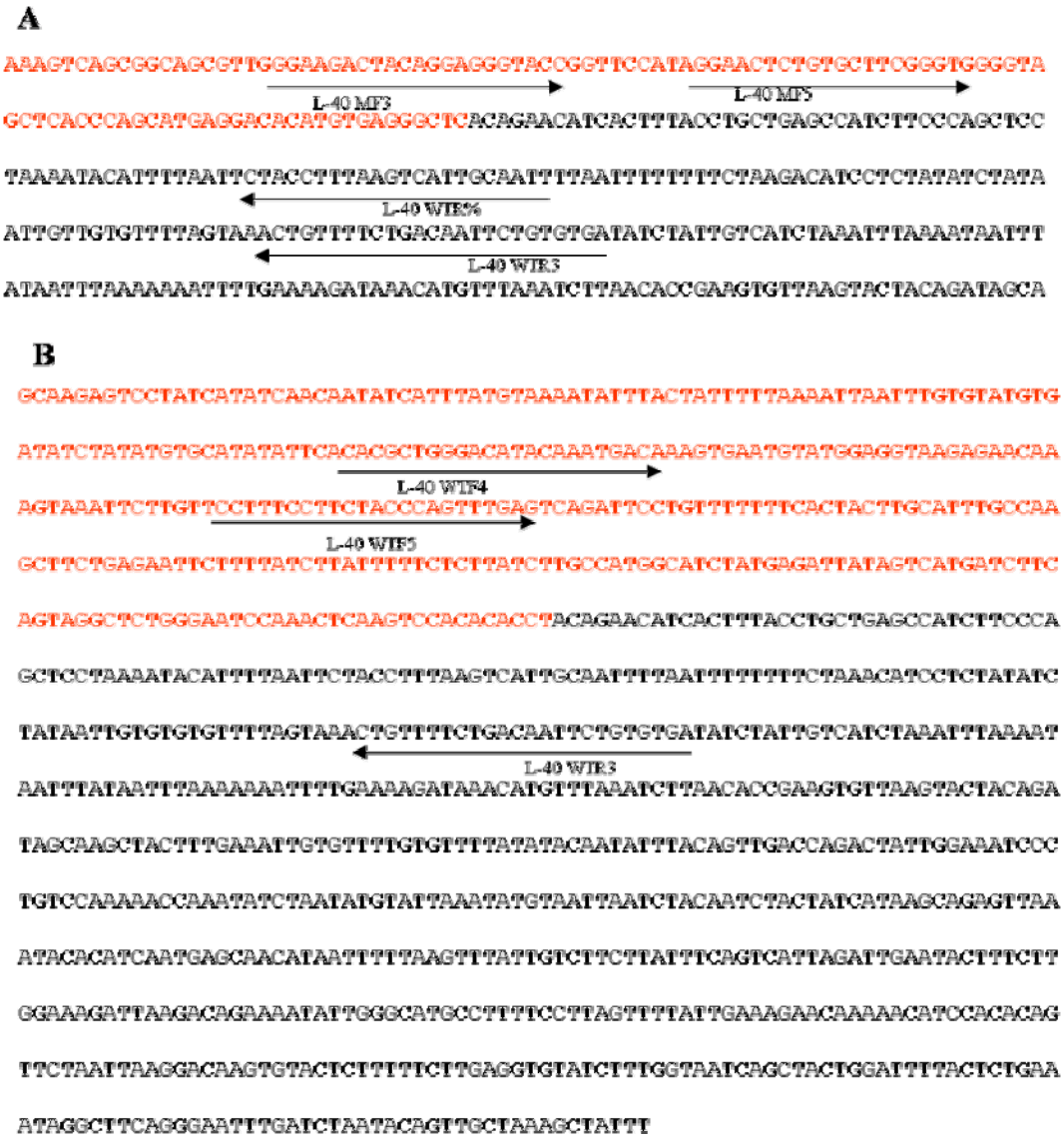

Figure 3.14 (A) Sequence of a genomic fragment containing the integration site of gene trap vector in the intron 7 of the *Faf1* gene. Red letters represent the 3' sequence of gene trap vector. Letter in black are the *Faf1* flanking sequence. Gene trap specific primers used for genotyping of the $Faf1^{GT}$ allele are indicated. **(B)** 5' and 3' sequences of *Faf1* gene, which are flanking the gene trap integration site, are given. Red letters are the 5' sequence, while black letters represent the sequence of 3' sequence of integration site. Locations of the primers, with are used for PCR genotyping of the wild-type and trapped allele, are indicated.

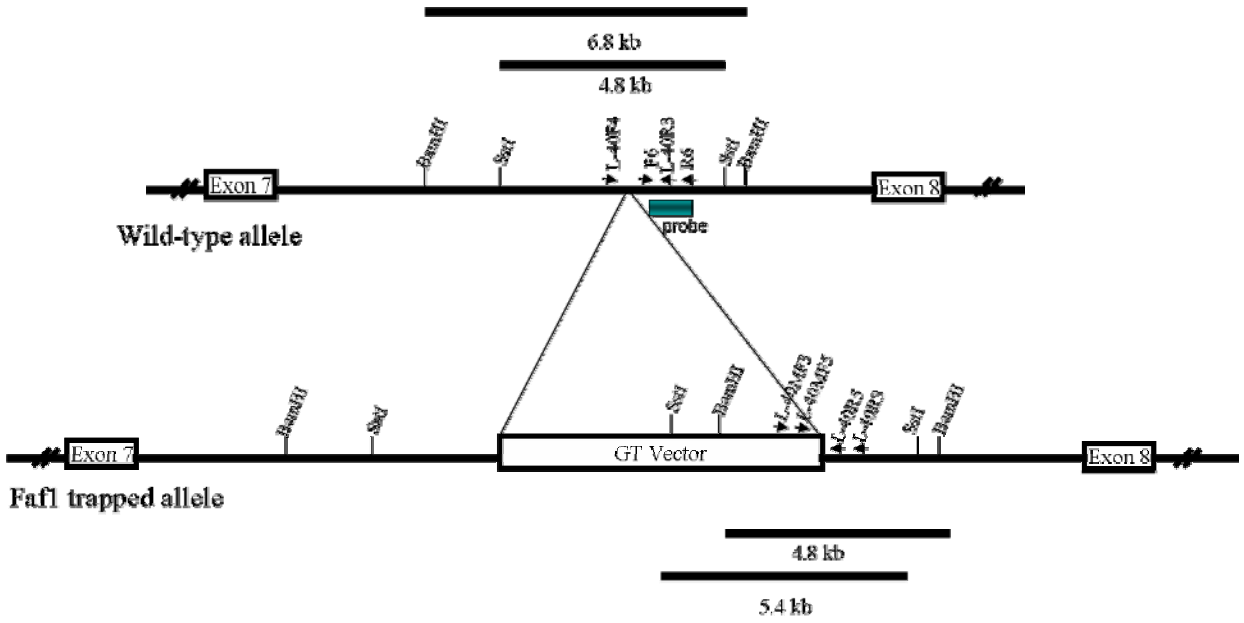

Figure 3.15 Schematic diagrams representing the *Faf1*^{GT} and the wild-type allele *Faf1*$^{+}$. The positions of the primers L-40 MF3 and L-40 WTR3 used to amplify the trapped allele and primers L-40 WTF4 and L-40 WTR3 used to amplify the wild-type allele are indicated. The primers F6 and R6 were used to amplify the genomic fragment (probe). The lengths of the *BamHI*- and *SstI*- wild-type and *Faf1*^{GT} genomic fragments, which were hybridized in Southern blot analysis with the probe, are indicated. GT, gene trap.

To establish a Southern blot analysis for genotyping, we have first established a restriction map for the *Faf1*$^{+}$ and *Faf1*^{GT} alleles (Fig. 3.15). Using primers F6 and R6, we have amplified a genomic fragment (probe in Fig. 3.15). This 0.6-kb genomic fragment (probe) was radioactively labeled and used to probe a Southern blot with *BamHI*- and *SstI*-digested DNA. As expected from restriction map of the *Faf1* gene, the probe recognizes a 6.8-kb *BamHI* and a 4.8-kb *SstI* fragment in DNA of wild-type. In genomic DNA of LacZ-positive mice, the probe detected additional 4.8-kb *BamHI* and 5.4-kb *SstI* mutant fragments (Fig.3.16 A). We have then used the Southern analysis to genotype 45 LacZ-positive mice including animals, which are genotyped as homozygous for trapped allele by using quantitative Southern blot analysis. All 45 mice were found to be heterozygous (*Faf1*$^{GT/+}$) (Fig. 3.16 B). These results clearly indicated that *Faf1* $^{GT/TG}$ homozygotes are lethal.

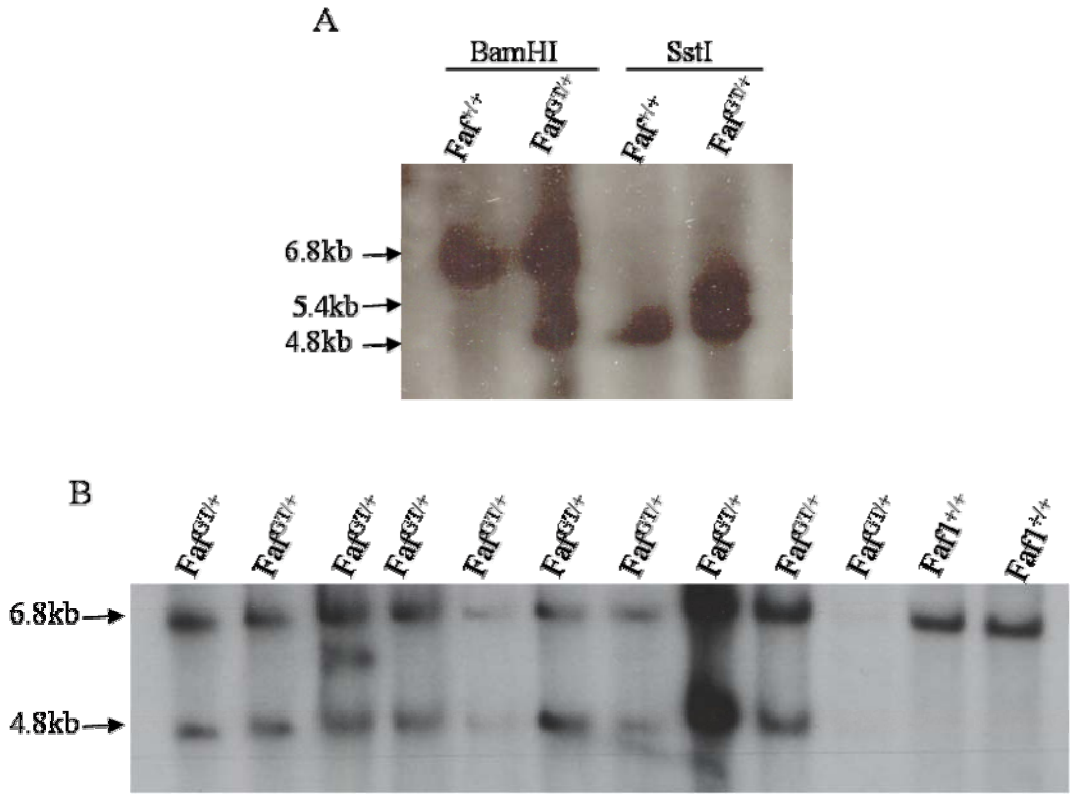

Figure 3.16 Southern blot analyses. (A) Genomic DNA of wild-type and heterozygous mice was digested with *BamHI* and *SstI* enzyme, separated on 1% agarose gel and transferred onto nitrocellulose membrane. The blot was hybridized with radioactively labeled 0.6-kb probe. The probe recognizes a 6.8-kb *BamHI* and a 4.8-kb *SstI* wild-type fragment in DNA of wild-type. In genomic DNA of LacZ-positive mice, the probe detected additional 4.8-kb *BamHI* and 5.4-kb *SstI* mutant fragments. (B) Genomic DNA from 45 LacZ-positive mice was digested with *BamHI* and the blots were hybridized with the probe. All analysed LacZ-positive animals were heterozygous for the trapped allele ($Fafl^{GT/+}$).

3.2.3 Developmental consequences of trapped *Faf1* gene

The *Faf1* trapped allele was maintained on the 129/Sv X NMRI hybrid and C57 Bl/6J inbreed background. Heterozygous mice were mated to obtain homozygous offspring. 291 offspring from 51 litters were analysed by PCR. No homozygous offspring were detected. Similar results were also obtained in the breeding of heterozygous mice on the C57 BL/6J inbreed background (Table 3.2). Because of these results, we have used animals with *Faf1* trapped allele on the 129/Sv X NMRI hybrid background for further experiments.

To investigate whether the homozygous $Faf1^{GT/GT}$ survived until birth, animals of 4 newborn litters obtained from heterozygous intercrosses were genotyped. We could not obtain any homozygous $Faf1^{GT/GT}$ mice. This result suggests that the homozygotes for the trapped *Faf1* allele are embryonic lethal. In crosses between $Faf1^{GT/+}$ and $Faf1^{+/+}$ mice in either gender combinations, the ratio of wild-type to heterozygous offspring was close to Mendelian ratio 1:1 (Table 2). All $Faf1^{GT/+}$ females used in the backcrosses were fertile. In contrast, 9% of $Faf1^{GT/+}$ males were infertile.

Genotyping analysis of offspring from different breedings				
	Genotyping			
Breeding	$Faf1^{+/+}$	$Faf1^{GT/+}$	$Faf1^{GT/GT}$	Litter size
♂GT/+ x ♀GT/+ (hybrid background)	93	198	0	6
♂GT/+ x ♀GT/+ (C57BL/6J inbreed background)	48	85	0	5
♂+/+ x ♀GT/+	29	27	0	7
♂GT/+ x ♀+/+	34	32	0	7
♂+/+ x ♀+/+ (NMRI)	100	-	-	10

Table 3.2 Genotypes of offspring from heterozygous and backcross breeding.

To assess the consequences of the *Faf1* mutation for embryonic development, embryos were collected from heterozygous intercrosses at different days of postimplantation development (E15.5, E12.5 and E9.5). Genomic DNA was isolated from whole embryos and genotyped by PCR assay using primers L-40WTF4, L-40WTR3 and L-40MF3 (Fig. 3.17 A, B). As shown in table 3.3, no $Faf1^{GT/GT}$ embryos at E15.5, E12.5 and E9.5 were

found. Heterozygous intercrosses segregated *Faf1*$^{GT/+}$ and *Faf1*$^{+/+}$ in a 2:1 ratio indicating that homozygous trapped *Faf1* allele results in early lethal phenotype. There was no indication of increased uterine resorption at E9.5 and 12.5, suggesting that *Faf1*$^{GT/GT}$ embryos do not implant. To investigate whether the *Faf1* deficient embryos die during preimplantation stages, blastocysts (E3.5) were isolated from heterozygous breedings and cultured for 4 days. Microscopic examination of embryos after four days of culture showed that cells of the inner cells mass (ICM) is able to proliferate in all cultured embryos. DNA was then extracted from cultured embryos and genotyped by PCR analysis. None of these embryos was homozygous for the trapped *Faf1* allele (data not shown). We have then isolated E0.5 (1-cell stage), E1.5 (2-cell stage), E2.5 (4 cell stage and morula) and E3.5 (blastocyts). To avoid a contamination of genomic DNA of embryos with that of polar bodies, single 1-, 2-, and 4-cell stages were treated with acid tyrode and then trypsin to remove the polar bodies. Single embryos were than genotyped by PCR (Fig. 3.17 A-D). As shown in table 3.3, all blastocyts, morula and 4-cell stages were either *Faf1*$^{+/+}$ or $^{Faf1GT/+}$, while *Faf1*$^{GT/GT}$ embryos were only detected in 1- and 2- cell stage embryos. These results demonstrate that the *Faf1*$^{GT/GT}$ embryos die between 2-cell and 4-cell stage.

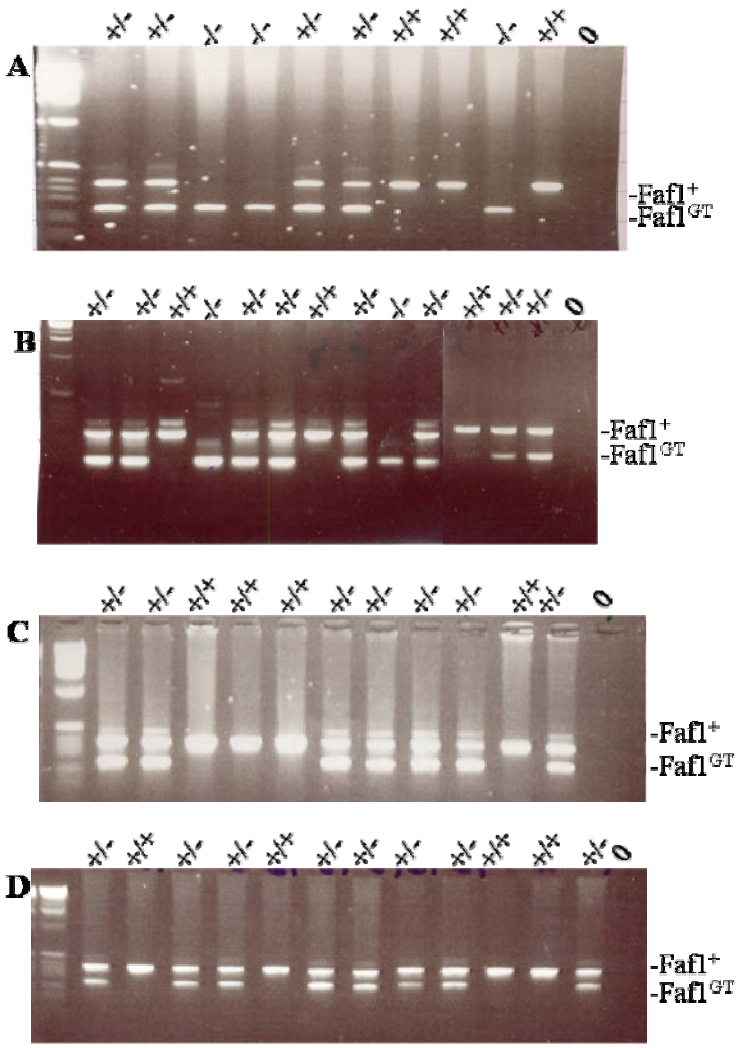

Figure 3.17 Results of the second-round PCR with DNA from E0.5 (A), E1.5 (B), E2.5 (C) and E3.5 (D) embryos which were derived from heterozygous intercrosses. The primers L-40WTF4, L-40MF3 and L-40WTR3 (Fig. 3.13 A, B) were used in the first round PCR. Cycle conditions were 94°C for 45 sec, 60°C for 45 sec, 72°C 45 sec. The nested primer L-40WTF5, L-40MF5 and L-40WTR5 were used in the second round PCR. Cycle conditions were 90°C for 30 sec, 60°C for 30 sec, 72°C 30 sec.

Stage	No. of progeny with genotyping		
	Faf1$^{+/+}$	Faf1$^{GT/+}$	Faf1$^{GT/GT}$
E15.5	7	14	0
E12.5	5	13	0
E9.5	8	15	0
in vitro culture of E3.5	6	14	0
E3.5	5	12	0
4-cell stage	9	22	0
2-cell stage	6	13	4
1-cell stage	10	18	8

Table 3.3 Genotyping of embryos of different age from heterozygous breedings.

To detect the cause for the embryonic lethality, 2-cell stage embryos obtained from heterozygous intercrosses and wild-type breedings, respectively, were isolated and cultured overnight in M16 medium. Of 75 isolated 2-cell embryos stage from heterozygous breedings, 21 embryos failed to divide after one day of culture and turned necrotic (28%), while only 12 of 96 2-cell embryos recovered from wild-type breedings failed to proceed beyond the 2-cell stage. After one day of culture, single embryos from heterozygous intercrosses were genotyped after removing the polar bodies. 4 embryos were wild-type and 7 were heterozygous. In all necrotic embryos, the polar bodies could not be distinguished from embryonic cells; therefore, the whole necrotic embryo was genotyped. Genotyping of the 13 necrotic embryos recovered after one day in culture revealed that 12 embryos were heterozygotes and 1 was of unknown genotype. The failure to detect *Faf1*$^{GT/GT}$ and *Faf1*$^{+/+}$ in necrotic embryos may be due to contamination of the embryonic genome with that of polar bodies. Increased number of embryos (28%) from heterozygous intercrosses, which failed to proceed beyond the 2-cell stage in vitro, as compared to 13% in wild-type breeding suggests that the *Faf1* is essential for cell viability or cell proliferation. To address the question, whether the lethality of the *Faf1*$^{GT/GT}$ is due to apoptotic cell death, E1.5 embryos were recovered from heterozygous and wild-type

crosses and stained by TUNEL assay. No embryo with intensely labeled nuclei, which is indicative of apoptotic cell death, was observed. One embryo which was isolated from wild-type breeding was arrested at one-cell stage and its nucleus was intensely stained (Fig. 3.19 C). This result suggests that the developmental arrest of *Faf1*$^{GT/GT}$ is not due to apoptotic cell death.

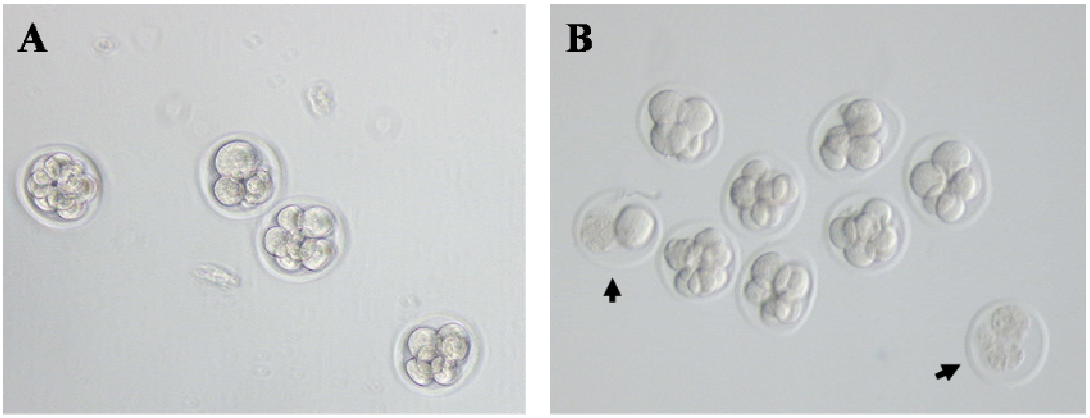

Figure 3.18 E1.5 embryos collected from plugged females of wild-type (A) and heterozygous (B) intercrosses were cultured in M16 medium with 5% CO_2 at 37°C. Pictures were taken 36h after culture. An increased number of necrotic embryos were observed from in vitro cultured of embryos recovered from heterozygous intercrosses. Arrows indicate necrotic embryos.

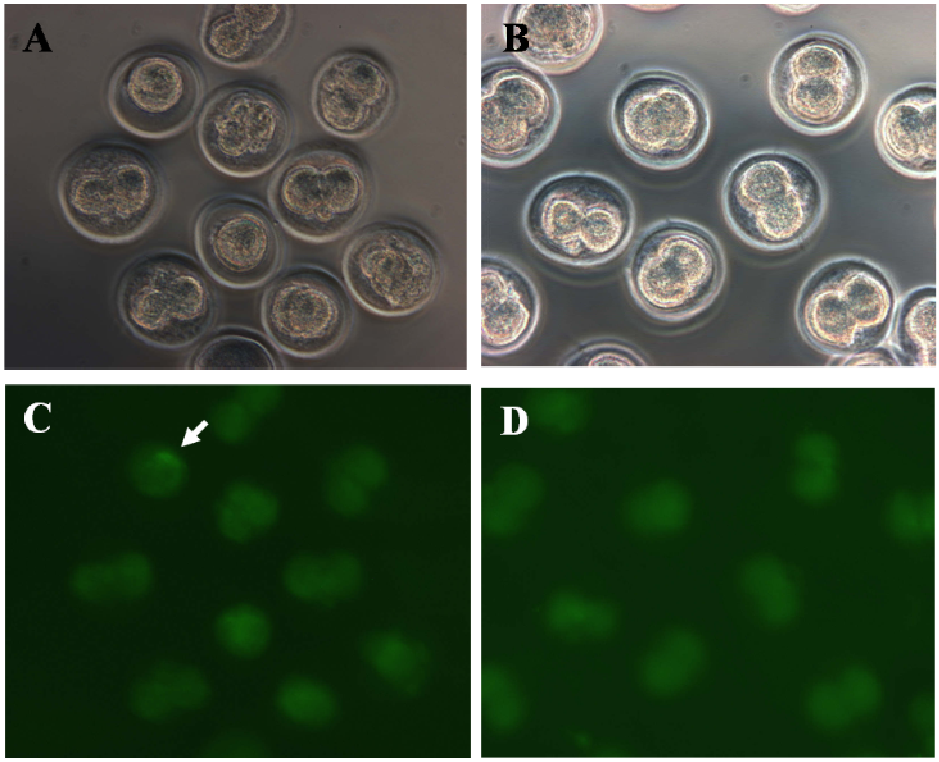

Figure 3.19 TUNEL staining of E1.5 embryos recovered from wild-type and heterozygous breedings. Phase contrast pictures of E1.5 derived from wild-type and heterozygous breedings are shown. (C) Nucleus of one wild-type embryo at 1-cell stage showed strong TUNEL staining (arrow). In contrast, all 2-cell stage embryos from wild-type (A) and heterozygous (B) intercrosses are unstained.

3.2.4 Expression of embryonic *Faf1* gene during preimplantation

Immunohistochemical staining of ovary and early embryonic stages revealed that Faf1 is highly expressed in oocytes of all follicle stages and in all preimplantation stages. This result demonstrates that the Faf1 protein in oocytes is present as maternal storage and may be transferred to and used by early embryonic cells during the first rounds of division. The persistence of maternally Faf1 protein in the embryos could explain the normal development of $Faf1^{GT/GT}$ to the 2-cell stage despite mutation of the *Faf1* gene in the embryos. To determine the preimplantation expression profile of *Faf1*, RT-PCR on total RNA prepared from 1-cell, 2-cell, 4-cell, morula and blastocyt stages was performed. To control for the relative abundance of *Faf1* transcript, we included RT-PCR analysis for the

ubiquitously expressed *Gapdh* gene. Whereas the *Gapdh* is expressed throughout preimplantation stages, *Faf1* transcripts are not detectable in 1- and 2-cell stages. The expression of embryonic *Faf1* starts at 4-cell stage and is increased thereafter (Fig. 3.20).

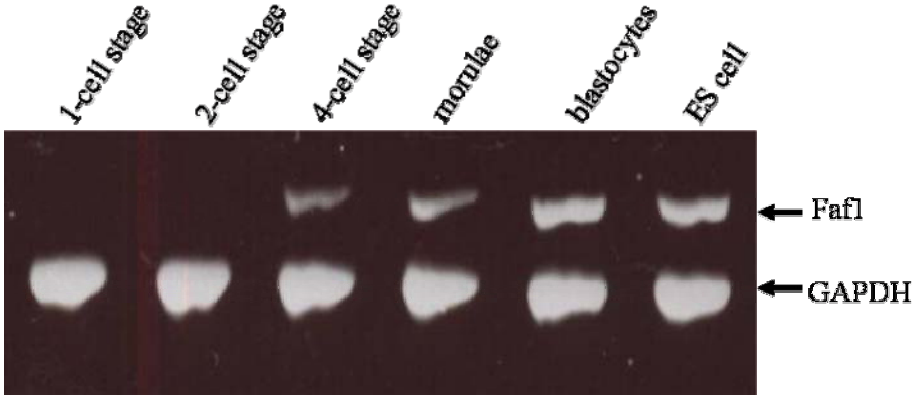

Figure 3.20 embryonic Faf1 expressions in preimplantation stages. RT-PCR was used to detect *Faf1* and *Gapdh* transcripts in total RNA preparations from wild-type 1-, 2-, 4-cell stage, morula, blastocysts and ES cells.

3.2.5 Reproductive functions of *Faf1* in male animals

3.2.5.1 Fertility test experiments

In breedings of $Faf1^{GT/+}$ males with wild-type females, two infertile males were found. We performed hematoxylin-eosin staining of testicular sections from infertile heterozygotes. Histological analysis revealed complete depletion of meiotic and postmeiotic germ cells in testes of both infertile males. By contrast, the full component of spermatogenic cells, including spermatogonia, spermatocytes and spermatids was detected in fertile heterozygous males (Fig. 3.21). The germ cell degradation in testis of some heterozygous animals lead us to determine whether apoptotic cell death in infertile testes of $Faf1^{GT/+}$ mice is responsible for impaired spermatogenesis. Testicular sections from fertile and

infertile *Faf1*$^{GT/+}$ mice were stained with TUNEL assay. For control, we stained sections from wild-type and from Apg$^{-/-}$ testes, which show high number of apoptotic cells. As shown in figure 3.22, the number of apoptotic cells in testes of fertile and infertile *Faf1*$^{GT/+}$ mice was not significantly different from that observed in sections of wild-type testis.

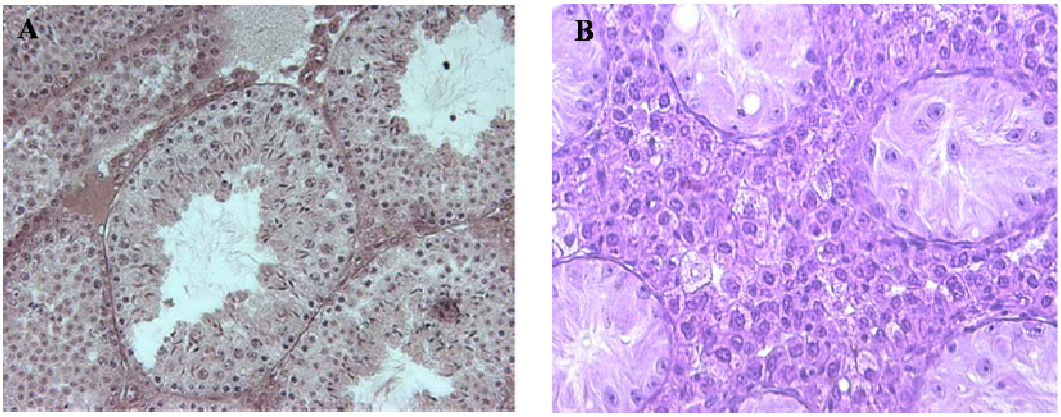

Figure 3.21 Hematoxylin-eosin staining of testicular sections from wild-type (A) and infertile *Faf1*$^{GT/+}$ mice (B). Histological analysis revealed complete depletion of meiotic and postmeiotic germ cells in testes of infertile *Faf1*$^{GT/+}$ males.

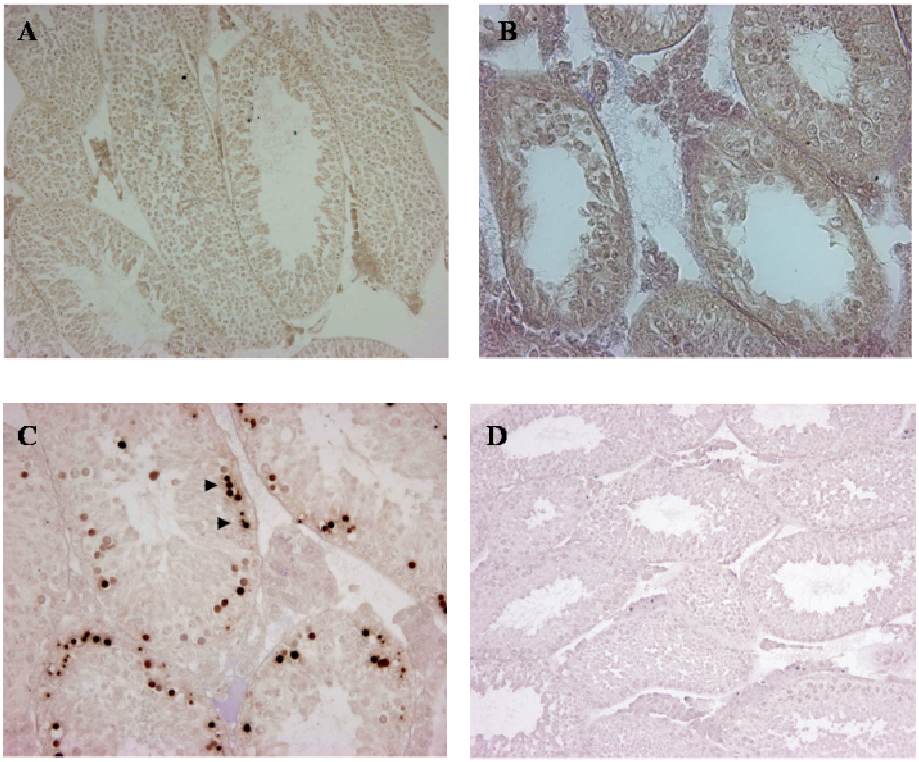

Figure 3.22 TUNEL staining of testicular sections from fertile *Faf1*$^{GT/+}$ mice (A) and infertile *Faf1*$^{GT/+}$ mice (B). For positive control, testicular sections of Apg$^{-/-}$ mice (C) and wild-type mice were used (D). No significant increase of TUNEL- positive cells was detected in fertile and infertile *Faf1*$^{GT/+}$ testes as compared to wild-type testes.

3.2.5.2. Expression analysis of the *Faf1* trapped allele

Gene trap vector, which is integrated in intron 7 of *Faf1* gene, contains the splicing acceptor site of mouse *engrailed-2* gene, followed with IRES sequence and *LacZ* gene. Therefore, we expected that the mRNA fusion transcript from the *Faf1* trapped gene contains the sequence of exon 1 to exon 7 of *Faf1* and the *LacZ* gene (Fig. 3.23). Furthermore, the vector contains the neomycin gene under the control of the *β*-actin promoter. The absence of the poly-A signal in the neomycin gene and presence of the splice donor site of *Pax2* gene at 3' end lead to expect that the *Faf1* trapped allele ubiquitously transcribe a second fusion mRNA. This fusion transcript should contain the sequence of the *β*-actin and the 3' located sequence of *Faf1* (Fig.3.23).

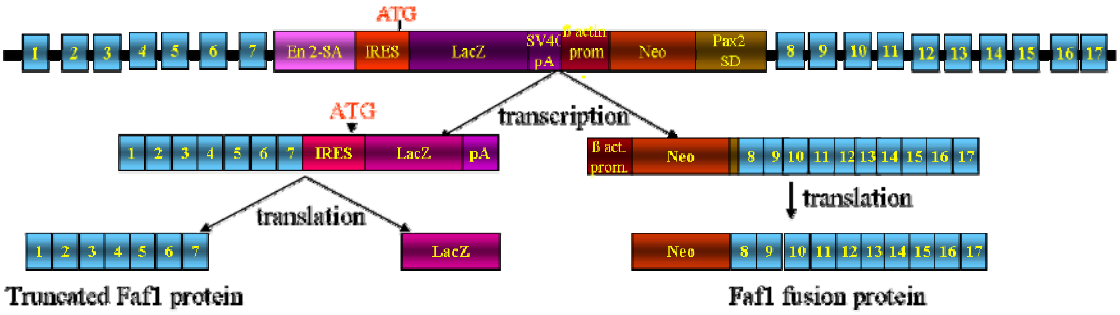

Figure 3.23 Schematic diagrams represent which the *Faf1* trapped allele, the expected fusion transcripts and proteins. En2-SA, mouse *engrailed-2* splice acceptor site; SD, mouse *Pax-2* splice donor site; IRES, internal ribosomal entry site from the encephalomyocarditis virus; ATG, translation initiation codon; *LacZ*, *β-galactosidase* (*βgal*) expressing gene and *neo, neomycin phosphotransferase* gene under control of the human *ß*-actin promoter; pA, SV40 polyadenylation signal.

To evaluate whether *Faf1* transcripts are affected by integration of the gene trap vector, Northern blot analysis was performed with testicular RNA from wild-type, fertile and infertile $Faf1^{GT/+}$ mice. By using cDNA probes localized 5' and 3' of the integration site, two transcripts of 2.8-kb (wild-type) and of 3.5-kb (trapped transcript) were detected in testis of $Faf1^{GT/+}$ mice (Fig. 3.25 A, B). Hybridization of the 3.5-kb trapped transcript with the 5' and 3' cDNA probes suggests a partial integration of the gene trap vector in the 3.5-kb transcript. The level of the 3.5-kb trapped transcript is lower than that of the 2.8-kb $Faf1^{+/+}$ transcript in testis of fertile $Faf1^{GT/+}$ mice (Fig. 3.25 A, B). In contrast, the level of $Faf1^{GT}$ transcript was prominent in testis of infertile $Faf1^{GT/+}$ animals. Rehybridization of the Northern blot with the LacZ probe revealed a weak hybridization of the 3.5-kb fusion transcript with the LacZ probe. This result suggests that a short sequence of the *LacZ* gene is integrated in the fusion transcript (Fig. 3.25 C). The variation in the expression of the gene trapped allele in testis of fertile and infertile $Faf1^{GT/+}$ mice lead us to study the expression of $Faf1^{GT}$ allele in different tissues of fertile and infertile $Faf1^{GT/+}$ mice. The expression level of the 2.8-kb $Faf1^{+}$ transcript is higher than that of the 3.5-kb $Faf1^{GT}$ transcript in all examined tissues of fertile $Faf1^{GT/+}$ mice (3.24 E). In contrast, the expression level of the 3.5-kb $Faf1^{GT}$ transcript is prominent in all studied tissues of infertile $Faf1^{GT/+}$ mice (Fig. 3.24 F).

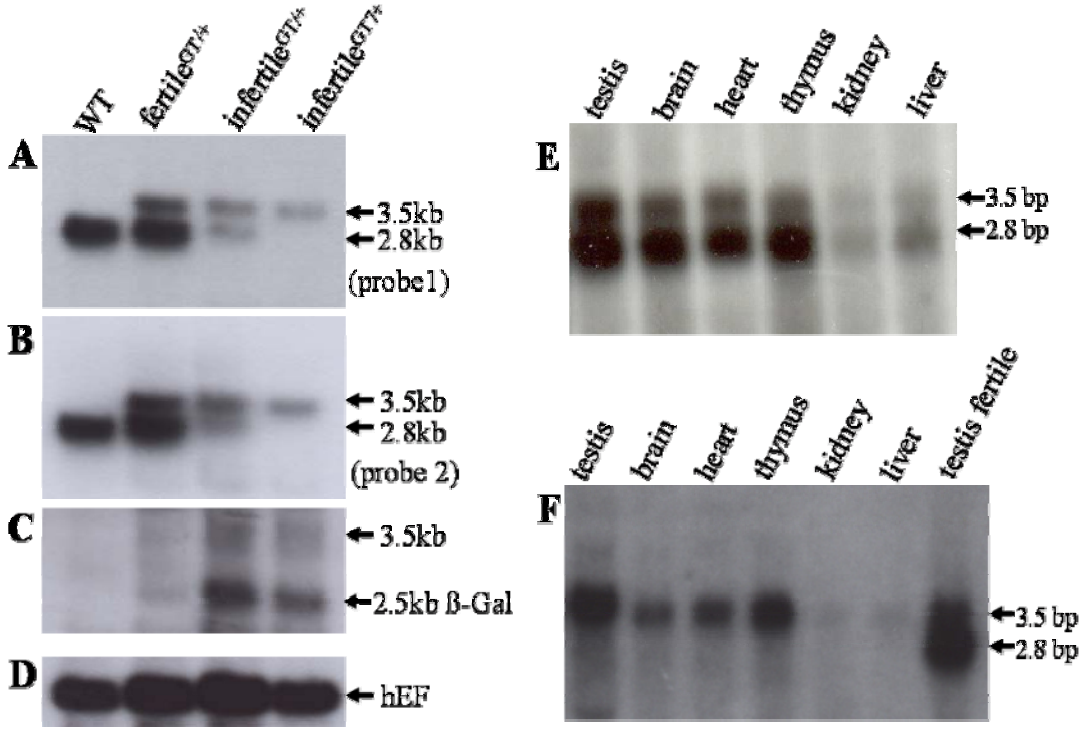

Figure 3.24 (A) Northern blot analysis with RNA from wild-type (WT), fertile and infertile *Faf1*$^{GT/+}$ animals. Testicular RNA was hybridized with *Faf1* cDNA probes locating at 5' (A) and 3' (B) of the integration site of gene trap vector. (C) Rehybridization of blot with LacZ probe. LacZ probe recognizes a very weak 3.5-kb *Faf1*^{GT} transcript and detects an additional transcript of 2.5-kb in RNA of fertile and infertile *Faf1*$^{GT/+}$ mice. (D) Rehybridization of Northern blot with the human elongation factor (EF) to confirm an equal amount of loading RNA. Expression of the 3.5-kb *Faf1*^{GT} and the 2.8-kb *Faf1*$^{+}$ transcript in different tissues of fertile (E) and infertile (F) *Faf1*$^{GT/+}$ mice.

To determine the molecular origin of the fusion transcript, RT-PCR analysis using primers located in the *Faf1* gene and in the gene trap vector was performed. Primers were located in IRESF1 of the gene trap vector and in exon 14 of the *Faf1* gene (Fig. 3.25 C). Using the primers UBAR and IRESF1 located in the IRES sequence of the gene trap vector and in exon 14 of *Faf1* gene, respectively (Fig. 3.25 A) a 920-bp PCR fragment could be amplified with testicular RNA of $Faf1^{GT/+}$ mice. The PCR fragment was then subcloned in to pGEMT easy vector and sequenced. Sequence analysis revealed that the amplified fragment contains an 82-bp of the IRES sequence of gene trap vector and 838-bp of the *Faf1* cDNA sequence (Fig. 3.25 A). Alignment of the 82-bp sequence with that of IRES sequence revealed the presence of cryptic exon/intron donor site (GT) in the IRES sequence. This cryptic donor site (GT) is located 3' downstream of the 82-bp in IRES sequence (Fig. 3.25 B). Alignment of the sequence of RT-PCR fragment with that of the *Faf1* gene showed that the sequence of *Faf1* in the amplified fragment starts with the sequence of exon 8. This result suggests that the amplified fusion transcript is the result of splicing the 82-bp of *IRES* sequence with exon 8 of the *Faf1* using a cryptic donor site in the IRES domain of the gene trap vector.

To identify the 5' sequence of gene trap vector in the 3.5-kb fusion transcript, several forward primers located in 5' exons of *Faf1*gene and reverse primers locating in the IRES domain of gene trap were used in RT-PCR assay with testicular RNA of $Faf1^{GT/+}$ mice. RT-PCR analysis could not detect any specific product.

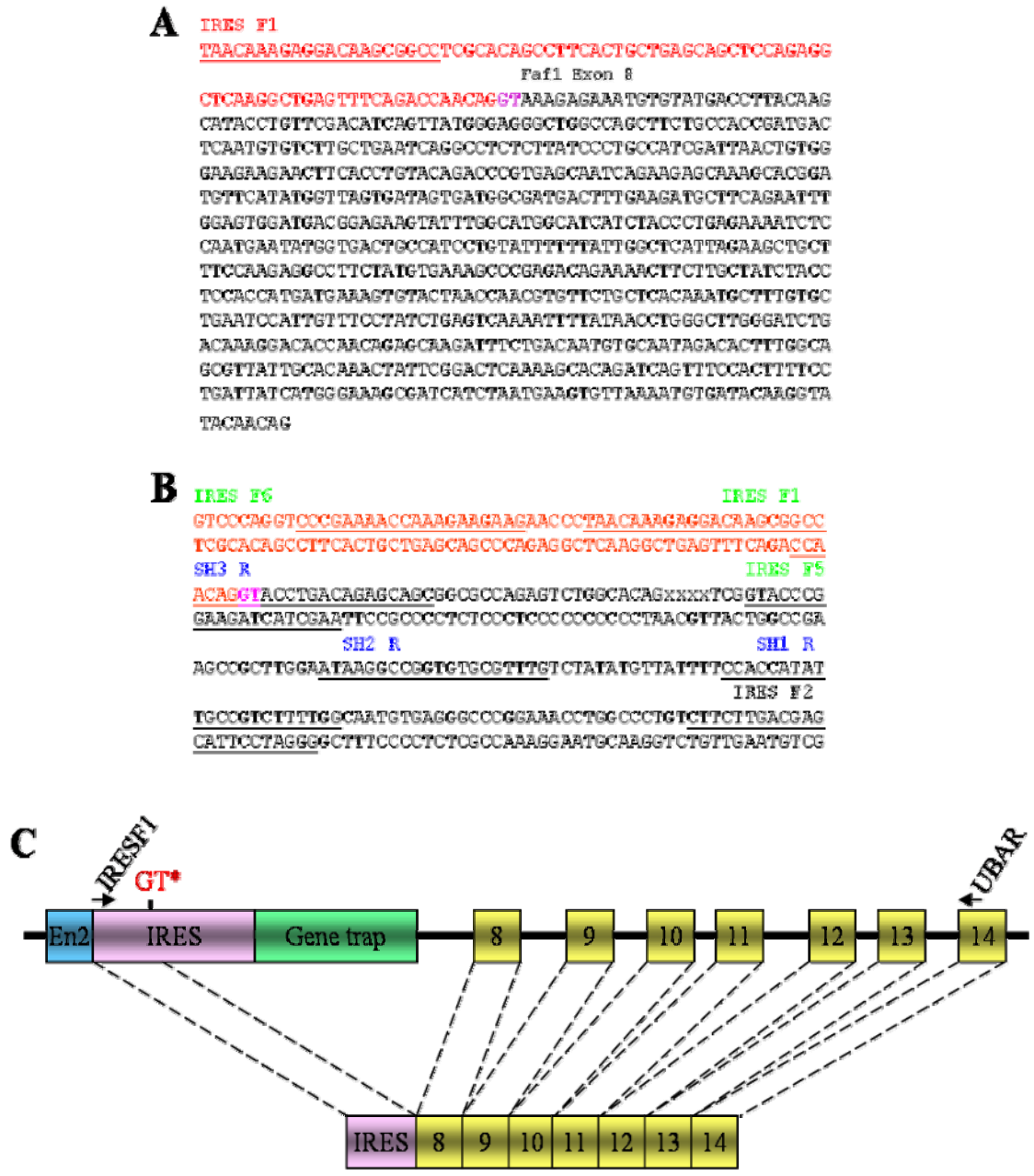

Figure 3.25 (A) Sequence of the amplified 920-bp fragment. Red letters represent the integrated *IRES* sequence. Letters in black are *Faf1* cDNA sequence. (B) A partial sequence of *IRES* region of gene trap vector. Red letters represent the integrated sequence in the amplified fragment. Unterlined GT represent the cryptic donor site. (C) Schematic diagrams represent the position of sequences in gene trap vector and *Faf1* gene, which are identified in the 920-bp amplified fragment.

3.2.6 Faf1 protein analysis

Identification of the interaction partner of Faf1

Alignment of the amino acid sequence in the Database reveals that Faf1 protein possesses UBA (ubiquitin-associated) and the UBX (ubiquitin like) domains. UBA and UBX domains are located at position 335 to 480 and 569 to 647 of the Faf1 sequence (Fig. 3.26), respectively.

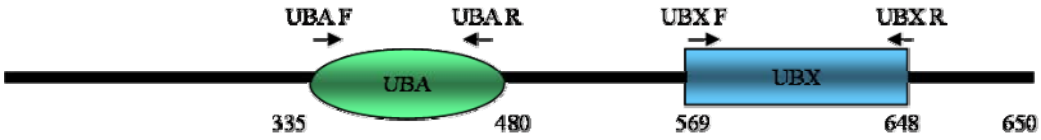

Figure 3.26 Schematic diagram represents the positions of the ubiquitin-associated (UBA) and the ubiquitin like domain (UBX) in the Faf1 protein. Position of primers used for the amplification of the UBA and UBX cDNA fragments are marked.

Three proteins, shp1, Ubx2 and Ubx5 in *Saccharomyces cerevisiae* and the p47 protein in mammalian species contain both domains. All these proteins have been shown to bind to ubiquitinated proteins through the UBA domain and UBX domain is utilized for interaction with ATPase p97/Cdc48 protein. The VCP/ATPase p97 is an abundant and widely expressed protein, and is a member of the AAA family of ATPases.

To identify the proteins that interact with the UBA and UBX domains of Faf1, GST-pulldown assay was performed. Using testis cDNA and primers shown in figure 3.26. cDNA fragments containing the sequences of UBA and UBX-domain were amplified and subcloned into *BamHI/SacI* digested pET41a vector for the expression of the GST-fusion protein in *E. coli*. The GST-UBA and GST-UBX fusion constructs were transformed into competent cells BL21 and expression was induced by IPTG for 6 h at 37°C. Recombinant GST-fusion proteins were purified on glutathione-sepharose beads. The expected 64-kDa GST-UBA and 45-kDa GST-UBX fusion proteins were separated by running the fusion proteins in SDS PAGE (Fig. 3.27 A). Then fusion proteins were transferred on nitrocellulose membrane. Membrane was incubated with the polyclonal anti-Faf1 antibody,

which recognizes the C-terminal domain of Faf1. As shown in figure 3.31 (B), the Faf1 antibody recognized the 45-kDa GST-UBX fusion protein, but not the GST-UBA protein. The results confirm that the commercial anti-Faf1 antibody specifically recognizes the C-terminal domain of the Faf1 protein. To identify UBX-binding protein, protein extracts from testis and brain were used. GST pulldown assay was performed by incubation of 500 µg total protein with 100 µg of GST-fusion protein, which was immobilized on glutathione-sepharose beads. After incubation for 4h at 4°C, the captured proteins were centrifuged and the beads were washed three times in lysis buffer. Samples were boiled in SDS electrophoresis sample buffer and centrifuged. Protein was analyzed on 10% SDS PAGE. Protein bands were visualized by Coomassie blue staining. As shown in figure 3.27 (C), the 45-kDa GST-UBX fusion protein interacts with a 96-kDa protein in both assays with the testis and brain protein extracts. In the control GST pulldown assay, the GST protein was not bound to the 96-kDa protein (Fig. 3.27 C). This result suggests that the UBX-domain interacts with the 96-kDa protein. To determine the sequence of the 96-kDa protein, fusion protein was transferred to a polyvinylidene difluoride membrane and stained with Coomassie blue. The 96-kDa protein band was eluted from membrane and subjected to digestion with trypsin. Tryptic peptides were analyzed by mass spectrometry (in collaboration with Dr. B. Schmidt, Institute of Biochemistry II, Göttingen). The 96-kDa protein was identified as Valosin-containing protein (VCP) (NCBI accession number 6005942) by peptide mass fingerprinting using MALDI-TOFMS and confirmed by sequencing using ESI-q-TOF tandem MS.

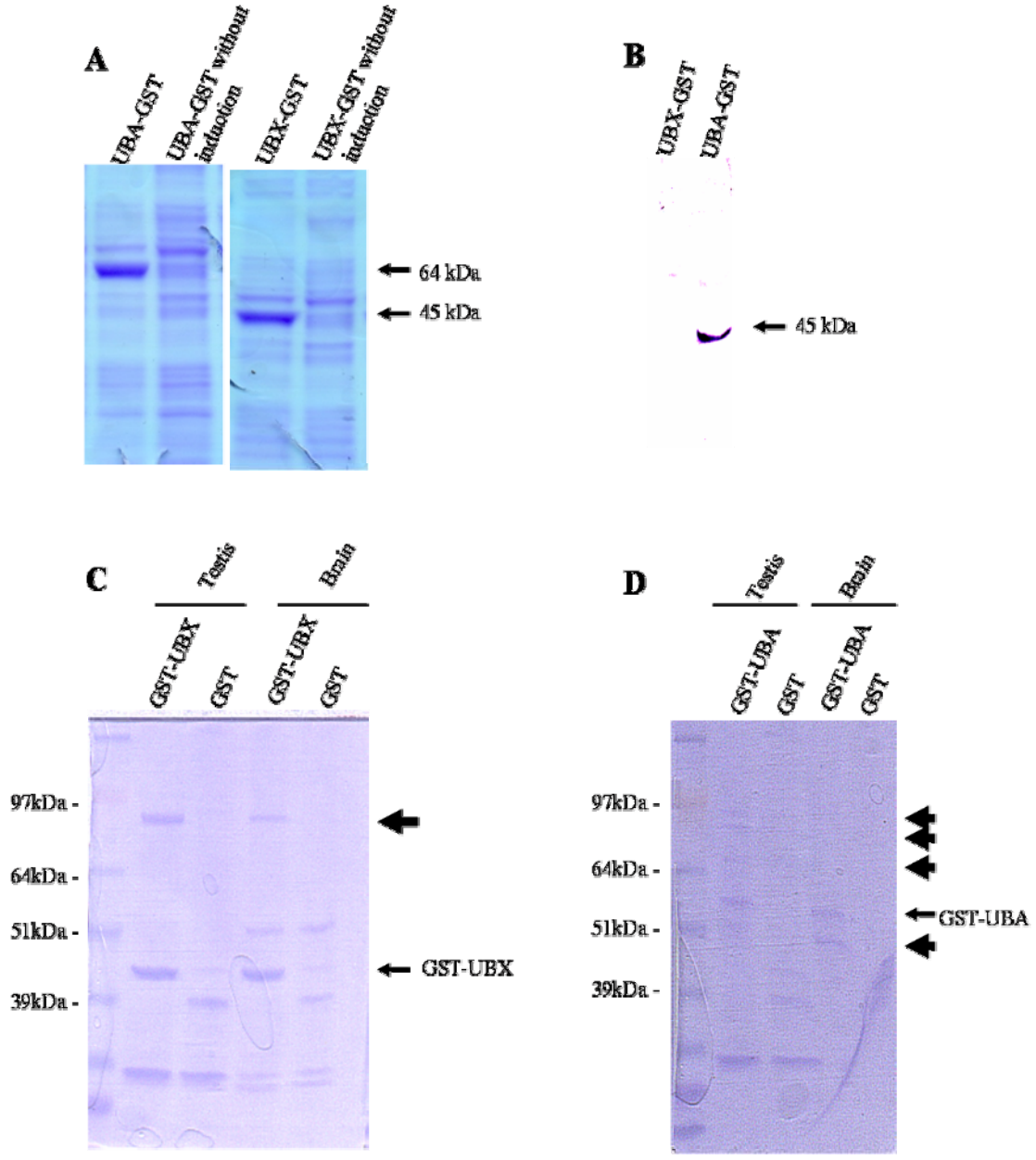

Figure 3.27 (A) SDS PAGE analysis of protein extract from recombinant UBA-GST and UBX-GST bacteria before and after induction of expression recombinant plasmid. (B) GST-UBA and GST-UBX fusion proteins were detected by the anti-Faf1 antibody. The anti-Faf1 antibody recognizes only the 45-kDa GST-UBX fusion protein. (C) SDS PAGE analysis of the GST pulldown assays shows that the GST-UBX fusion protein specifically interacts with a 96-kDa protein in protein extracts from testis and brain. (D) SDS PAGE analysis of the GST pulldown using the UBA-GST fusion protein shows the interaction of UBA-GST with multiple proteins in extracts of testis and brain.

4. DISCUSSION

Gene trap methodology involves the random integration of a gene trap vector into a host genome, thereby trapping a gene. Gene trap insertion is a powerful mutagenesis technique, which can provide data on gene function and expression. Most of gene trap vectors contain a strong splice acceptor site 5' of the reporter. Upon integration into a genomic locus, the gene trap vector acts as an artificial exon and is spliced to the endogenous gene to form a fusion transcript (Fig. 1.1). The fusion transcript between the reporter and the endogenous gene is a reporter of endogenous gene expression and aids gene identification. The *LacZ* gene trap vector, which contains a *ß-galactosidase* and *neomycin phosphotransferase* gene fusion transcript, is a widely used example of a gene trap vector.

The aim of this study was the characterization of gene trap line 98-2C, in which the trapped gene is highly expressed in testis. Screening of the genomic library, which has been constructed from DNA of gene trap line 98-2C, revealed that the integration site of gene trap vector is in the intron 7 of *Faf1* gene. This result lead us to determine the expression pattern of *Faf1* and consequence of *Faf1* gene trapped on the development of $Faf1^{GT/GT}$ animals.

4.1 Expression analysis of the *Faf1* gene

Northern blot analysis revealed that the *Faf1* gene transcribes a 2.8-kb mRNA in all studied tissues. The 2.8-kb *Faf1* transcript is present at high level in the testis and moderate levels in other tissues (Fig.3.1). *Faf1* transcripts shorter than 2.8-kb were not detected in any tissues by Northern blot hybridization using 5' and 3' cDNA probes and RT PCR assay. However, Western blot analysis revealed that the anti-Faf1 polyclonal antibody recognizes a protein with molecular weight of 74-kDa in extract of all tissues and a further smaller protein product of 49-kDa in testicular extract (Fig. 3.2). The 74-kDa protein corresponds well with the deduced protein size of 73.8-kDa based on the predicted amino acid sequences of mouse Faf1. Identification of only one transcript of *Faf1* suggests that the 49-kDa protein would be proteolytically cleaved product of 74-kDa Faf1 or it could be a Faf1 homologous protein. Using another polyclonal antibody against human FAF1, it was reported that anti-human FAF1 antibody recognized 74- and 40-kDa proteins in different cancer cell lines (Ryu et al., 1999) and in samples from human solid tumors. Jensen et al.

(2001) have overexpressed the human *Faf1* cDNA in bacteria. After protein purification, they found two Faf1 protein products of 74- and 40-kDa. Peptide sequence of the 40-kDa band revealed that 40-kDa contains sequence of the 74-kDa Faf1 protein. Furthermore, this group has found in phoshorylation assay that both Faf1 products are phosphorylated by protein kinase CK2. Identification of lower weight protein in testicular extract using the anti-mouse Faf1, which was raised against the C-terminal region, suggests that the 49-kDa protein would be proteolytically processed product of 74-kDa protein and contain the C-terminal region. Our approach to purify and sequence the 49-kDa protein by immunoprecipitation using Faf1 antibody was not successful.

Expression pattern of *Faf1* during testis development revealed that the expression level of the *Faf1* gene is highly increased at day 25 (Fig. 3.3). At the protein level, an equal expression of the 74-kDa isoform was detected throughout testicular development. In contrast, the 49-kDa protein could not be detected in mouse testes till postnatal day 21. The expression of the 49-kDa isoform could be first detected at day 25. Thereafter, an increasing level of 49-kDa protein was observed (Fig. 3.5). During mouse spermatogenesis, the first wave of spermatogonia enters meiosis and gives rise to spermatocytes at ~10 days after birth. The primary spermatocytes undergo two meiotic divisions at ~17 days of age. The correspondence of these events with the correlated increase of *Faf1* transcript and appearance of the 49-kDa protein suggests that the 49-kDa isform is a result of posttranslational modification of 74-kDa Faf1 precursor in haploid spermatid. Immunohistochemistry revealed that the most intense Faf1 immunoreaction was in haploid spermatids (Fig. 3.8). High level of Faf1 was found in cytoplasm of elongated spermatids, while Faf1 was diminished in mature spermatids (Fig. 3.9). These expression pattern and the results shown the high accumulation of Faf1 in multinuclear giant cells (Fig. 3.8; 3.9), which undergoe cell death, suggest that Faf1 protein is involved in the ubiquitin-dependent protein degradation pathway during spermatid differentation and in apoptotic germ cells.

4.2 Functional analysis of *Faf1* gene.

To investigate the physiological role of *Faf1*, the gene trap line 98-2C was analysed. Cloning of trapped allele of the mouse line 98-2C revealed that the gene trap vector is inserted in the intron 7 of *Faf1* gene. Genotyping of the progeny of heterozygous intercrosses indicated the absence of $Faf1^{GT/GT}$ pups and suggested an embryo-lethal

phenotype. Genotyping of preimplantated embryos from heterozygous intercrosses revealed that the early embryonic development failure of *Faf1*$^{GT/GT}$ can be detected past 2-cell stage. These results suggest the essential role of the *Faf1* for cell viability and/or cell division. The ability of the *Faf1*$^{GT/GT}$ to undergo cell division till 2-cell stage can be interpreted by presence of maternal Faf1 protein, which would replace the absence of zygotic Faf1 protein. Immunofluorescence staining showed that the Faf1 protein is present in oocytes in the ovary (Fig. 3.10), in unfertilized oocytes and all preimplantation stages of embryonic development (Fig. 3.11).This could indicate that the Faf1 protein is present in oocytes of mothers with an intact gene copy and may be transferred to and used by early embryonic cells during the first rounds of division. The persistence of maternally produced Faf1 protein in the embryo could explain the normal development of *Faf1*$^{GT/GT}$ to the 2-cell stage despite deletion of the *Faf1* gene in the embryo. Embryo expression of *Faf1* becomes appreciable at the 4-cell stage as assessed by RT-PCR (Fig. 3.20). Thus, the death of *Faf1*$^{GT/GT}$ embryos may coincide with depletion of maternal Faf1 in these embryos. Morphological analysis of embryos at early morula stage, which were isolated from heterozygous intercrosses, suggests that loss of *Faf1* leads to massive cell death (Fig. 3.18). However, TUNEL assay did not reveal TUNEL-positive cells in necrotic embryo. Therefore, it could not be excluded that Faf1 deficiency could led to cell death by a non-apoptotic pathway such as necrosis. Necrosis is known to follow rapid loss of cellular homeostasis and necrotic cells are characterized by swelling due to accumulation of water and electrolytes (Saraste et al., 2000). Apoptosis and necrosis share common mechanisms in the early phase of cell death, particularly the involvement of BCL-2 family members that control mitochondrial events, a critical step to commitment to both apoptotisis and non-apoptosis of cell death. Moreover, in some circumstance, apoptosis can progress into secondary necrosis (Saraste et al., 2000). Therefore, further investigations are needed to determine whether *Faf1* deficiency lead to apoptosis and/or to a non-apoptotic death of preimplantation embryos.

During breeding of heterozygous *Faf1* mutants, we found some infertile *Faf1*$^{GT/+}$ males. Histological analysis revealed that the diameter of the seminiferous tubules was significantly shorter in testis of the infertile males, and the luminas of the tubules were filled with a mass of Sertoli cells and germ cells were completely absent (Fig. 3.21). In addition, Leydig cell hyperproliferation was apparent. This is likely a secondary defect owing to shrinkage of seminiferous tubules. In some infertile *Faf1*$^{GT/+}$, which are younger than 6-month-old, all stage of spermatogenesis were observed in very few tubules (5-10%)

(data not shown). These observations suggest a gradual loss of germ cells in testis of infertile *Faf1*$^{GT/+}$ males feat and the germ cell depletion is not germ cell stage specific. Such pattern of germ cell loss has been reported to be due to a problem of supporting Sertoli cells rather than a specific and direct developmental arrest of germ cells (Russell et al., 1990). TUNEL assay did not shown an increase of apoptotic-positive cells in testis of infertile *Faf1*$^{GT/+}$ males (Fig. 3.22). This result suggests that the germ cell death like as the death observing in *Faf1*$^{GT/GT}$ embryos is due to non-apoptotic pathway. Non-apoptotic death of post-meiotic germ cells was observed in testes of mice lacking *CsnK2a2* gene, which encodes the CK2α subunit of protein kinase CK2 (Escalier et al., 2003). Interestingly, the protein kinase CK2 has been shown to interact with and phosphorylate the Faf1 protein (Kusk et al., 1999; Jensen et al., 2001). CK2 is a tetrameric protein and consists of beta-alpha dimer. The CK2 *ß* subunit is responsible for the interaction with the Faf1 (Kusk et al., 1999). Inactivation of the gene encoding the CD2 *ß* subunit leads to early embryonic lethality (Buchou et al., 2003). Therefore, it is interested to further investigate the interaction between CK2 and Faf1, and the significance of Faf1 phosphorylation for its function. To determine the cause of germ cell depletion in testes of infertile *Faf1*$^{GT/+}$ mice at molecular level, expression of wild-type and *Faf1*$^{GT/+}$ mice was analysed. These analyses revealed that the expression level of the fusion transcript deriving from trapped allele was higher than that of wild-type allele. In testes of some infertile heterozygotes, trapped transcript was prominent in testes (Fig. 3.24). In contrast, the expression level of the wild-type allele was higher than that of trapped allele in testis of fertile *Faf1*$^{GT/+}$ animals. These results suggest that the haploinsuficiency of Faf1 in some *Faf1*$^{GT/+}$ causes the germ cell loss.

4.3 Faf1 is involved in the ubiquitin-proteasome pathway

Faf1 was firstly identified by yeast two hybrid system using the cytoplasmic domain of Fas as bait (Chu et al., 1995). Its function has been described as potentiating Fas –induced apoptosis in a mouse fibroblast cell line and in a human T-cell tumor line (Jurkat) (Chu et al., 1995). However, Ryu and Kim (2001) reported that the Faf1 overexpression in BOSC23 cells can initiate apoptosis in the absence of an extrinsic death signal. Unlike other Fas-associating proteins, Faf1 does not contain a death domain, but has several homologous domains based on amino acid sequence analysis. The Faf1 protein contains an

UBA (ubiquitin- associated) domain and a UBX (ubiquitin-like) domain. Both domains are well characterized in mammalian protein p47 (Dreveny et al., 2004), and UBX proteins of *S.cerevisiae* (Buchberger et al., 2002; Schuberth et al., 2004). The UBA domain in p47 and in yeast UBX proteins binds ubiquitinated proteins *in vivo* (Meyer et al., 2002 and Schubert et al., 2004). The UBX domain in p47 and yeast proteins binds to the mammalian p97/VCP (Cdc 48 in yeast). VCP/p97 is an abundant and widely expressed protein, and is a member of the AAA family of ATPase. Disruption of the Cdc48 gene in yeast (Moir et al., 1982) or that encoding the *Drosophila* homologue TER94 (Leon and McKearin, 1999) is lethal, indicating that Cdc48 plays roles essential for cell growth and survival. VCP/p97 does not itself bind to polyubiquitinated proteins (Wilkinson et al., 2001). The interaction of the p47 with VCP/p97 and polyubiquitinated proteins led to suggest that p47 function as an adaptor to couple VCP/p97 to polyubiquitinated proteins and proteasome (Fig. 4.1). Polyubiquitinated proteins are themselves known to interact with the S5a subunit of the 19S component of the proteasome (Deveraux et al., 1994). The role of VCP/p97 itself may be to promote the ATP-dependent unfolding of polyubiquitinated proteins, thereby stimulating their distruction by the proteasome. The ATPase activity of VCP/p97 provides the energy required for this process (Golbik et al., 1999).

In this report, we found that the UBX domain of Faf1, like p 47, was able to bind to VCP/p97 in extracts of testis and brain (Fig. 3.2). While this work was in progress, it was reported that UBX and UBA domains interact with VCP/p97 and multiubiquitinated substrates, respectively (Song et al., 2005). These results suggest that Faf1 protein serves as an adaptor protein that regulates protein degradation in the ubiquitin proteasome pathway.

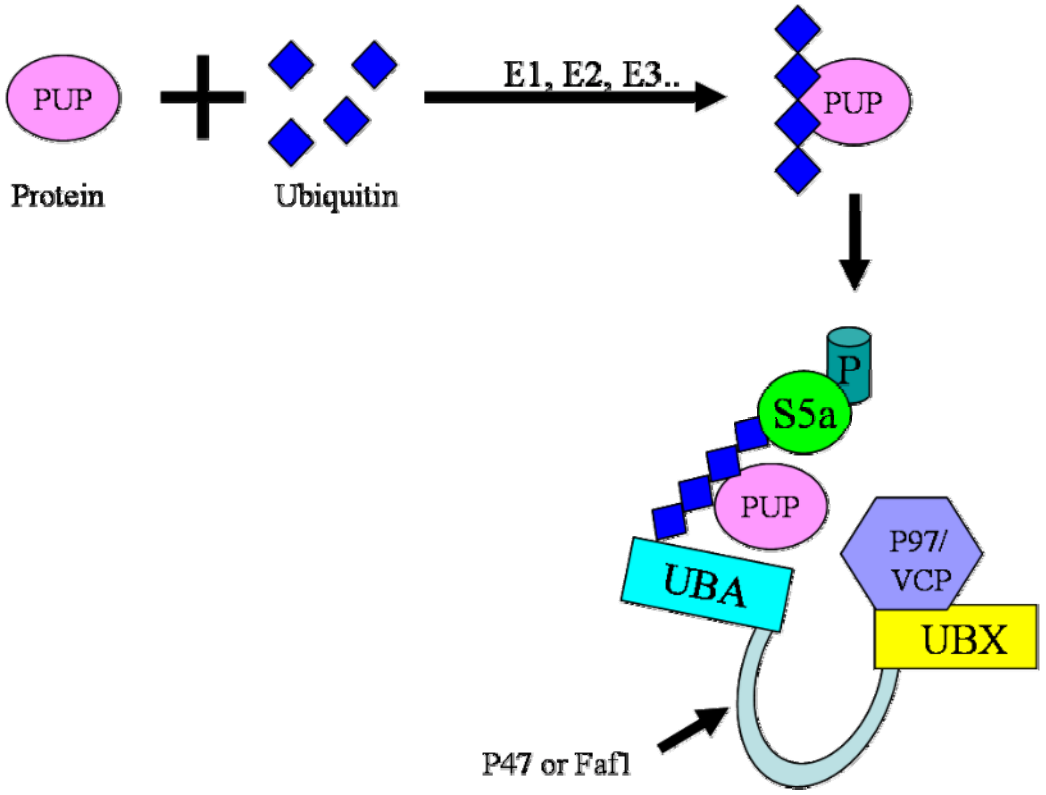

Figure 4.1 Schematic representation of the proposed role of p47 and Faf1 in localizing VCP/p97 to ubiquitinated proteins. Ubiquitin dependent protein degradation starts with labeling the protein with the ubiquitin. There are three types of enzymes E1, E2 and E3, which are involved in ubiquitination of proteins. The polyubiquitinated protein PUPs binds to the protein adaptor p47 or Faf1 via the polyubiquitin chain. On other side, the VCP/p97 interacts with the UBX domain of p47 or Faf1. The VCP/p97 is then directed to polyubiquitinated protein (PUPs) via the adaptor protein (p47 or Faf1), allowing VCP/p97 to unfold PUPs in an ATP-requiring process. PUPs can then be degraded by the proteasome, with which they interacts via S5a subunit of the proteasomal subunit (P).

4.4 Ubiquitin in preimplantation embryonic development

Ubiquitin is likely to be expressed in mammalian gametes and embryos at any given developmental step, but the information on ubiquitin dependence of gametogenesis and fertilization is sketchy. Mammalian preimplantation embryo development passes through four important stages following fertilization, degradation of oocyte transcripts, transcriptional activation of the zygotic genome, compaction, and differentiation into inner cell mass and trophectoderm. In mouse, the first two events occur at the 1-cell stage,

compaction occurs at the 8-cell stage, and differentiation at the blastocyst stage (Piko and Clegg, 1982; Schultz, 1993). Analysis of gene expression profiling (Meta-analysis) using the distribution of expressed sequence tags (EST) in cDNA libraries derived from mouse preimplantation embryos identified 11483 genes. These genes are expressed in preimplantation embryo development and included 1585 that are not expressed elsewhere in the mouse. Over 100 genes are expressed in a stage-specific manner (Stanton and Green, 2001). Ubiquitination is likely to be of major importance in early embryogenesis because of the need for each developmental stage to eliminate proteins that are destined for transient expression. Early developmental programs rely on maternal mRNAs and proteins that are synthesized during oogenesis. The regulated translation of maternal RNAs is essential for the proper deployment of regulatory factors during early embryogenesis. Recent studies suggest that the degradation of maternal proteins by the ubiquitin-proteasome pathway is also crucial during oocyte-to-embryo transition and degradation of germ line proteins is essential for remodeling the oocyte into a totipotent zygote that is capable of somatic development. The complex phenotypes observed in C. *elegans* zygotes that lack various components of the ubiquitination machinery suggest that protein degradation regulates many developmental events in early embryos (Feng et al., 1999; Kurz et al., 2002; Pintard et al., 2003; Sonneville and Gonezy, 2004). A mutation affecting the *PBF1* gene encoding the *β*6-subunit of the *Arabidopsis* 26S proteasome CP appears to be lethal, whereas the heterozygous plants are healthy, seeds homozygous for the *pbf1* mutation cannot be recovered. Loss of the *Arabidopsis* DUB that likely helps to disassemble free polyUb chains (*At*UBP14) induces early embryo arrest (Doelling et al., 2001; Tzafrir et al., 2002). The mutant embryos accumulate abnormally high levels of free polyUb chains, presumably inhibiting competitively substrate degradation by the 26S proteasome (Doelling et al., 2001).

4.5 Ubiquitin-proteasome degradation pathway and gametogenesis

Ubiquitin appears to have specialized functions in mammalian gametogenesis (Baarends et al., 1999). Ubiquitin mediated proteolysis is also critical for other aspects of reproduction, including the elimination of defective sperm in the epididymus, clearance of paternal mitochondria, and progression of embryonic development in mammals, as well as degradation of the vitelline coat during fertilization in ascidians (Sutovsky et al., 2000;

Sutovsky et al., 2001; Li et al., 2002; Sawada; 2002). There are tree well established types of enzymes involved in ubiquitination, termed E1, E2 and E3. E1 is the ubiquitin-activating enzyme, which forms a thiol-ester linkage with ubiquitin through its active site cysteine. Ubiquitin is subsequently transferred to an E2 ubiquitin-conjugating enzyme. The E3 enzyme is the ubiquitin protein ligase, which transfers ubiquitin from the E2 enzyme to lysines of a specific protein, targeting the protein for degradation by the proteasome. More recently, E4 enzymes have been described that appear to function in ubiquitn chain polymerization (2). Few E1 enzymes, several E2 enzymes, and hundreds of E3 enzymes have been identified. It is the E3 ubiquitin protein ligase that adds specificity to the process by interacting with specific target proteins. Included in the group of E3 enzymes are proteins such as the cancer-associated proteins, anaphase-promoting complex (APC), BRCA1, and MDM2, and the DNA repair proteins, RAD5 and RAD18 (Nobuhiro et al., 2003).

Mouse gene knockout studies have shown that inactivation of components of the ubiquitin system leads to impaired gametogenesis (Roest et al., 1996). Mutations in the human gene ubiquitin-specific protease 9 Y chromosome (USP9Y), which encodes a protein with a C-terminal ubiquitin hydroxlase domain, results in azoospermia and male infertility (Sun et al.,1999). Knockout mice lacking the E3 ubiquitin protein ligase SIAH1A or the E2 ubiquitin-conjugating enzyme HR6B demonstrate defects in meiosis and postmeiotic germ-cell development and have male infertility (Dickins et al., 2002; Roest et al., 1996). Yeast mutants heterozygous for a deletion of the polyubiquitin gene show reduced viability of spores, and homozygous mutants are sporulation defective (Finley et al., 1987). Ubiquitination of somatic histones has been implicated in their turnover during spermatogenesis (Baarends et al., 1999). Recently, Sutovsky et al., (1999) have shown that uniparental ubiquitin-tagging of mitochondria during spermatogenesis selectively targets sperm mitochondria for postfertilization degradation by proteasomes and lysosomes of the embryo.

Maturation-promoting factor (MPF), a heterodimer of p34Cdc2 kinase and cyclin B1, is a key regulator of oocyte meiosis that functions during discrete periods of the cell cycle. MPF activity increases during prophase and metaphase of meiosis I because of increased translation of cyclin B1 mRNAs and the dephosphorylation of p34Cdc2 in complexes with cyclin B1. As oocytes progress through meiosis I, MPF is transiently inactivated by the proteasomal degradation of cyclin B1 (Sawada, 2002; Holloway et al., 1993). Subsequently, translation of several key oocyte mRNA (e.g., cyclin B1 and *Mos*) and activation of Cdc2

kinase by Cdc25 phosphatase occur, so that MPF activity is high in metaphase II. Egg-spern fusion at fertilization releases the oocyte from metaphase II arrest by increasing Ca^{2+} levels, activating Ca^{2+}-calmodulin kinase II, and targeting cyclin B1 and MOS for degradation via the ubiquitin proteasome pathway (Glotser et al., 1991; Lorca et al., 1993; Tokumoto et al., 1997).These studies indicate that specific ubiquitination pathways regulate MPF at several key transitions in oocyte meiosis (Nobuhiro et al., 2003).

The high expression of *Faf1* gene in haploid spermatid suggests the role of Faf1 during spermatid maturation. The early embryonic death of $Faf1^{GT/GT}$ prevents us to study the role of Faf1 during spermatogenesis. The germ cell depletion shown in some $Faf1^{GT/+}$ can be explained by one of the following cases. 1-The haploinsufficiency of $Faf1^{/+}$ allele in some heterozygous animals. 2- The $Faf1^{GT}$ allele, in which the gene trap vector integrates in intron7, encodes a truncated protein lacking the UBA and UBX domain. This truncated protein might have negative dominant defect in some $Faf1^{GT/+}$ animals. Such negative dominant effect has been shown by overexpression of the truncated Faf1 protein lacking the UBA domain in the HEK293T cell line (Song et al., 2005). Overexpression of truncated Faf1 protein induces cell death. Such cell death has been attributed to inhibition of the degradation of ubiquitinated proteins in transfected cells (Song et al., 2005). However, truncated Faf1 protein was not detected by Western blot analysis with testicular protein extracted from the fertile and infertile $Faf1^{GT/+}$. Production of conditional knockout mice may be the most promising method for the direct assessment of the function of Faf1 during embryogenesis and spermatogenesis.

4.6 Mutation cause of preimplantation embryonic death in other genes

The very early preimplantation development depends entirely on maternal mRNAs and proteins deposited and stored in the oocyte prior to its ovulation. If oocyte is not sufficiently equipped with maternally stored products, or if zygotic gene expression does not commence at the correct time, the embryo will die. Many developmental targets and regulators are identified; it should become possible to separate the cell cycle and the developmental function of protein degradation. Mutations in some genes have been found, which lead to lethality in preimplantation development. Mice with a homozygous gene trap vector insertion in *mgcRacGap* die during preimplantation development. MgcRacGap protein is a GTPase-activating protein and was identified first in human. In the mouse,

maternally derived protein was found to be present in the oocyte and *mgcRacGap* transcription starts at the four-cell stage. E3.5 *mgcRacGap*$^{-/-}$ embryos display a dramatic reduction in cell number, but undergo compaction and form blastocyst. At 3.0-3.5, binucleated blastomers with nuclei partially interconnected are frequently observed, suggesting that mgcRacGap is required for normal mitosis and cytokinesis in the preimplantaion embryo. Severe phenotype of *null* embryos indicates that mgcRacGap is functionally non-redundant and cannot be substituted by other GTPase-activating proteins (Van et al., 2001). Targeted disruption of the *Rad51* gene leads to lethality in preimplantation embryos (Tsuzuki et al., 1996). The mouse Rad 51 gene is a mammalian homolog of the *E.coli recA* and yeast *RAD51* genes. These genes are known to be involved in homologous recombination and DNA repair. Domain II of RAD51, which is conserved in RecA-related proteins, was targeted by homologous recombination. The high level of mouse *Rad51* gene expression in testis, ovary, spleen, and thymus suggests that this protein might play an important role in meiosis (Shinohara et al., 1993). The neurogenic gene *brainiac* was first isolated in *Drosophila melanogaster*. It interacts with members of the Notch signalling cascade. The Notch signalling pathway has been implicated in cell fate decisions in a variety of developmental aspects in *Drosophila melanogaster, Caenorhabtidis elegans*, and in vertebrates. Targeted deletion of mouse *Brainaic 1* expression leads to preimplantation lethality.

Study of genes which cause embryonic lethality in the mouse could be of value to understand human embryonic failure. In human, most miscarriages occur due to chromosomal aberrations. The rate of chromosomal aberrations decreases during embryonic development, from 37% in the fertilized oocyte to 0.6% in the newborn (Plachot et al., 1987). Due to chromosomal aberrations, 16.4% of preimplantation embryos fail to develop to further stages. Spontaneous abortion in human pregnancy occurs during the preimplantation period with a frequency of ~30%. Embryonic lethality in human not only depends on the chromosomal aberrations but could also depend on monogenic defects. In table 4.1 represents a list genes, whose inactivation result in preimplantation death in case of mutation.

Name of the gene	Expression	Function	Day of the embryonic lethality	Reference
γ-adaptin	Widely expressed	Post-Golgi vesicular transport and sorting processes	E3.5	Ziziolo et al., 2000
ZarI	testis, ovary, oocytes		E0.5	Wu et al., 2001
mgcRacGAP	preimplantaion embryos, intra and extra embryonic tissues	GTP-ase activating	E3.5	Van et al., 2001
Rad51	testis, ovary, spleen, thymus	Cell proliferation	E2.5-E3.5	Tsuzuki et al., 1996
BrainicI	developing central nervous system, retina, adult hippocampus, kidney, ovary	Notch signalling	E3.5-4.5	V ollrath et al., 2001
FenI	epidermis, small intestinal epithelium	DNA repair as well as DNA replication	E.3.5	Larsen et al., 2003
muCdc7	ES cell, testis, spleen, lung, thymus, stomach, brain	DNA replication	E3.5-6.5	Kim et al., 2002
Ltb2	testis, lung, dermis, heart valves	structural role within elastic fibers	E3.5-6.5	Shipley et al., 2000
ICLn	ubiquitously expressed	regulation of the cell cycle and RNA processing	E3.5-7.5	Pu et al., 2000

Table 4.1 Mutations genes that result in preimplantation death.

5. Summary

The aim of this study was the characterization of mouse gene trap line 98-2C, in which the trapped gene is highly expressed in testis. Screening of the genomic library, which has been constructed from DNA of gene trap line 98-2C, revealed that the integration site of gene trap vector is in the intron 7 of *Faf1* gene. These results lead us to determine the expression pattern of the *Faf1* and consequence of *Faf1* gene trapped on the development of $Faf1^{GT/GT}$.

Northern blot analysis revealed that *Faf1* transcribes a 2.8-kb mRNA in all studied tissues. The 2.8-kb transcript is present at high level in testis and moderate level in other tissues. Western blot analysis revealed that the anti-Faf1 polyclonal antibody recognizes a protein with molecular weight of 74-kDa in extract of all tissues and a further smaller protein product of 49-kDa in testicular extract. Identification of only one transcript of *Faf1* suggests that the 49-kDa protein would be proteolytically cleaved product of 74-kDa Faf1.

Expression pattern of *Faf1* during testis development reveals that the expression level of the *Faf1* gene is highly increased at day 25. At the protein level, an equal expression of the 74-kDa isoform was detected throughout testicular development. In contrast, the 49-kDa protein could not be detected in mouse testes till postnatal day 21. The expression of the 49-kDa isoform could be first detected at day 25. Thereafter, an increasing level of 49-kDa protein was observed. The correlated increase of *Faf1* transcript and appearance of the 49-kDa protein suggests that the 49-kDa isform is a result of posttranslational modification of 74-kDa Faf1 precursor in haploid spermatid. Immunohistochemistry revealed that the most intense Faf1 immunoreaction was in haploid spermatids. High level of Faf1 was found in cytoplasm of elongated spermatids, while Faf1 was diminished in mature spermatids.

Analysis of the mouse gene trap line 98-2C revealed that the $Faf1^{GT/GT}$ are died during embryonic development. Northern blot analysis with RNA extracting from different tissues of $Faf1^{GT/+}$ animals revealed that the gene trap allele transcribes a fusion mRNA of 3.1-kb.

Genotyping of embryos from heterozygous intercrosses revealed that the early embryonic development failure of $Faf1^{GT/GT}$ can be detected past 2-cell stage. These results suggest the essential role of the *Faf1* for cell viability and/or cell division. The ability of the $Faf1^{GT/GT}$ to undergo cell division till 2-cell stage can be interpratated by presence of maternal Faf1 protein, which would replace the absence of zygotic Faf1 protein. Immunoflurescence staining shown that the Faf1 protein is presence in oocytes in the ovary,

in unfertilized oocytes and all preimplantation stages of embryonic development. Embryonic expression of *Faf1* becomes appreciable at the 4-cell stage as assessed by RT-PCR. Thus, the death of *Faf1*$^{GT/GT}$ may coincide with depletion of maternal Faf1 in these embryos. Morphological analysis of embryos at early morula stage, which are isolated from heterozygous intercrosses, suggests that loss of *Faf1* leads to massive cell death. However, TUNEL assay did not reveal TUNEL-positive cells in necrotic embryo. Therefore, it could not be excluded that Faf1 deficiency could led to cell death by a non-apoptotic pathway such as necrosis.

During breeding of heterozygous *Faf1* mutants, we found some infertile *Faf1*$^{GT/+}$ males. Histological analysis revealed that the diameter of the seminiferous tubules was significantly shorter in testis of the infertile males, and the lumens of the tubules were filled with a mass of Sertoli cells and germ cells were completely absent. TUNEL assay did not shown an increase of apoptotic-positive cells in testis of infertile *Faf1*$^{GT/+}$ males. This result suggests that the germ cell death like as the death observing in *Faf1*$^{GT/+}$ embryos is due to non-apoptotic pathway. In contrast, the expression level of the wild-type allele was higher than that of trapped allele in testis of fertile *Faf1*$^{GT/+}$ animals. These results suggest that the haploinsuficiency of Faf1 in some *Faf1*$^{GT/+}$ causes the germ cell loss.

Fas associated factor 1 (Faf1) was first identified by yeast two hybrid assay using the cytoplasmic domain of FAS as bait. Unlike the Fas associated factor 1 Faf1 does not a death domain but processes two ubiquitin homologous domains, the UBA (ubiquitin associated) and UBX (ubiquitin like).

To determine the interacting proteins that bind to the UBX domain, GST-Pulldown assay was done using the GST-UBX fusion protein and protein extracts from testis and brain. The GST-UBX protein interacts with a 96-kDa protein in both assays with testis and brain extracts. The 96-kDa protein was identified as Valosin containing protein (VCP) by peptide mass fingerprinting and confirmed by sequencing. The presence of UBA and UBX domains in primary structure of Faf1 suggest that Faf1 regulates protein degradation in the ubiquitin-proteasome pathway.

The early embryo death of the *Faf1*$^{GT/GT}$ prevent us to determine the role of Faf1 in the ubiquitin-proteasome pathway. Therefore, production of conditional knockout mice may be the most promising method for the direct assessment of the function of Faf1 during embryogenesis and spermatogenesis

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