

Expression and functional analysis of the testis expressed genes

ADAM 27 and testase 2

Dissertation



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Tag der mündlichen Prüfungen:

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ABBREVIATIONS

ABI	Applied Biosystem Instrument
ADAM	A Disintegrin And Metalloprotease
APS	Ammonium peroxydisulfate
ATP	Adenosintriphosphate
BCP	1-bromo-3-chloropropane
bp	base pair
BSA	Bovine serum albumin
°C	Degree Celsius
CASA	Computer Assisted Semen Analysis
cDNA	complementary DNA
dATP	Desoxyriboadenosintriphosphate
dH ₂ O	distilled water
D	Disintegrin
DCE	Disintegrin-Cys-rich-EGF-like
DAPI	Diamidino-2-phenylindole dihydrochloride
dCTP	Desoxyribocytosintriphosphate
DMSO	Dimethyl sulfoxide
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic acid
Dnase	Deoxyribonuclease
dNTP	deoxynucleotidetriphosphate
dpc	day post coitus
dT	deoxythymidinate
DTT	Dithiothreitol
CE	Cys-rich-EGF-like
EDTA	Ethylene diamine tetraacetic acid
ES	Embryonic stem
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
GST	Glutathione S-transferase
g	gravity

Gfp	Green fluorescence protein
gm	gram
HEPES	N-(β -hydroxymethyl)piperazin,N'-3-propansulfoneacid
hr(s)	hour(s)
IPTG	Isopropyl- β -thiogalactopyranoside
IVF	In vitro fertilization
kb	kilobase
LB	Luria-Bertrani
LIF	Recombinant leukaemia inhibitory factor
LPS	lipopolysaccharides
M	molarity
Mb	Mega base pair
MBP	Maltose Binding Protein
MOPS	3-[N-Morpholino]-Propanesulfate
mRNA	messenger Ribonucleic acid
mg	milligram
ml	milliliter
μ l	microliter
μ m	micrometer
min	minute
NaAc	Sodium acetate
NBT	Nitro-blue tetrazolium
NCBI	National Center for Biotechnology Information
<i>Neo</i>	<i>Neomycin</i>
ng	nanogram
nm	nanometer
NTP	Nucleotidetriphospate
OD	Optimal density
ORF	Open Reading Frame
Pa	Pascal
PAGE	Polyacrylamide Gel Electrophoresis
PCR	Polymerase chain reaction
PH	Prepondirance of hydrogen ions
Pmol	pmol

PBS	Phosphatebuffersaline
PBT	Phosphatebuffersaline + Tween
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
SV 40	Simian Virus 40
<i>Taq</i>	<i>Thermus aquaticus</i>
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- β -galactosidase
ZP	Zona Pellucida

Symbol of amino acids

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

Abbreviations

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	Thymidine
U	Uridine

1 INTRODUCTION

1.1 ADAM (A Disintegrin And A Metalloprotease) family of proteins

ADAM 27 and testase 2 investigated in this study belong to the ADAM family of proteins. This is a rapidly growing family. Currently, 34 distinct ADAM family members from different organisms (*Caenorhabditis elegans* to humans) have been reported (see reviews: Black and White, 1998; Schlondorff and Blobel, 1999; Primakoff and Myles, 2000; Evans, 2001; Kheradman and Werb, 2002). In mouse genome ADAM family members are distributed between almost all chromosomes. The highest number of ADAMs was mapped to chromosome 8 (9 genes, Figure 1.1 A). In a human and a rat, gene distribution is similar with accumulation of ADAMs on chromosome 16 in rat and 8 in human (Figure 1.1 B and C), which are in synteny with mouse chromosome 8. In a human a high number (8 genes) has been mapped also to chromosome 5, but these are members of the ADAM-TS subgroup of ADAM family with additional thrombospondin type 1 motif (Tang, 2001).

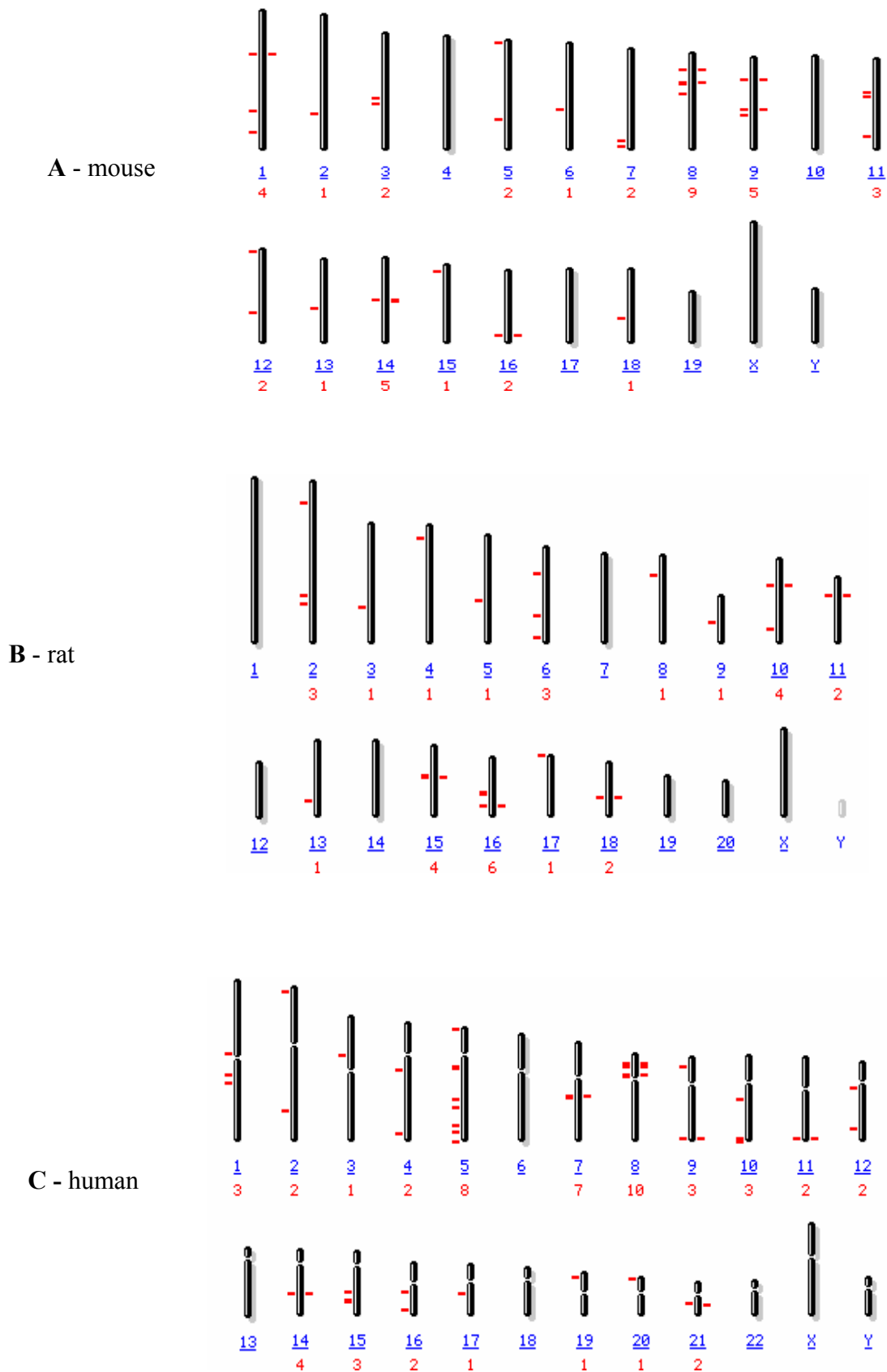


Figure 1.1 ADAM family genes distribution on mouse (A), rat (B) and human (C) chromosomes. ADAMs are indicated by red bars, chromosome numbers are in blue colour and amount of ADAM genes on single chromosomes is given in red colour numbers.

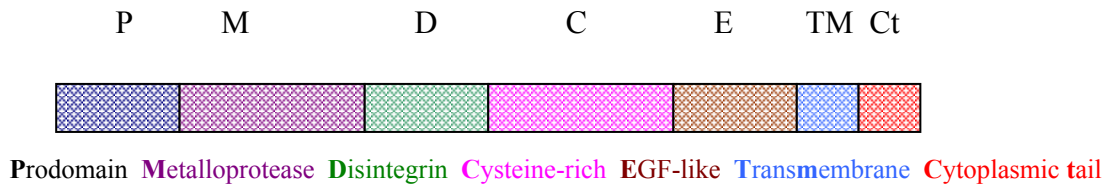


Figure 1.2 Domain organization of typical ADAM family member. The documented functions of the domains are: the pro-domain (P) blocks protease activity; metalloprotease domain (M) has protease activity; the disintegrin domain (D) has adhesion activity; cysteine-rich domain (C) has adhesion activity; the EGF-like (E) stimulates membrane fusion; the cytoplasmic tail (Ct) can be phosphorylated and regulates other ADAM activities.

Most of ADAM family members have unique domain structure composed of pro-domain (P), metalloprotease (M) – a catalytic domain with zinc-binding motif, disintegrin domain (D), cysteine rich domain (C), epidermal growth factor (EGF)-like domain (E), transmembrane (TM) and a cytoplasmic domain (Ct) (Figure 1.2). Thus, ADAMs are type 1 transmembrane proteins expressed on the cell surface. It is believed that ADAM proteins have potentially both, cell adhesion and a protease activity because they have high sequence homology with snake venom disintegrins and metalloproteases (Jia et al., 1996). Although all ADAMs have metalloprotease domain, about one third of the reported members have an amino acid sequence in the active site region that is incompatible with metalloprotease activity. Thus, these ADAMs are believed to lack protease activity (Figure 1.3). However, all ADAMs contain the disintegrin domain, which is approximately 80 amino acids in length with 15 highly conserved cysteine residues. The disintegrin domain of some or all ADAMs is likely to be ligand for integrins or other receptors. Soluble snake venom disintegrins are known as integrin ligands (Niewiarowski et al., 1994). These disintegrins interact with integrins through their disintegrin loop, a thirteen amino acid motif which contains an integrin binding sequence (e.g., RGD) at its tip (Niewiarowski et al., 1994). The disintegrin domains of ADAMs and snake venoms have a different tripeptide at the corresponding position (Blobel et al., 1990; Wolfsberg et al., 1995). Although all ADAMs share certain disintegrin loop residues, most notably cysteines, many residues are not conserved. In addition, not all ADAMs have the same number of residues in their disintegrin loop. This diversity of the sequence suggests three hypotheses. First, different ADAMs may interact with different integrins and/or other receptors. Second, different ADAMs might interact with the same or highly related receptors. Finally, only a subset of ADAMs may be functional adhesion molecules.

ADAMs were found in mammals as well as in *C. elegans*, *Drosophila* and *Xenopus* but they are not present in *E. coli* and *S. cerevisiae* or plants. Some ADAMs are ubiquitously expressed such as *ADAM 9*, *ADAM 10*, *ADAM 15* and *ADAM 17* and may have pleiotropic effects, as has been found for *ADAM 15* and *17* (Bohm et al., 1999; McCulloch et al., 2000; Bosse et al., 2000; Al-Fakhri et al., 2003; Shi et al., 2003; Moro et al., 2003;). Many of other ADAMs show tissue specific expression: *ADAM 12* and *19* in muscle (Yagami-Hiromasa et al., 1995), *ADAM 22* in brain and *ADAM 23* in brain and heart (Sagane et al., 1998). They are involved in such processes as muscle cell differentiation (Yagami-Hiromasa et al., 1995; Gilpin et al., 1998), asthma pathology (Van Eerdewegh et al., 2002; FitzGerald et al., 2002) and cancer pathology (O'Shea et al., 2003; Karan et al., 2003). The largest group of ADAMs (1, 2, 3, 5, 6, 16, 18, 20, 21, 24, 25, 26, 27, 29, 30, and 34) is specifically or predominantly expressed in testis and is thought to be involved in spermatogenesis and/or fertilization. Up-to-date registries of all ADAM family members in different species can be found at: <http://www.uta.fi/~loiika/ADAMs/HADAMs.htm>, <http://www.uta.fi/%7Eloiika/ADAMs/MMADAMs.htm>, and http://www.people.virginia.edu/%7Ejw7g/Table_of_the_ADAMs.html.

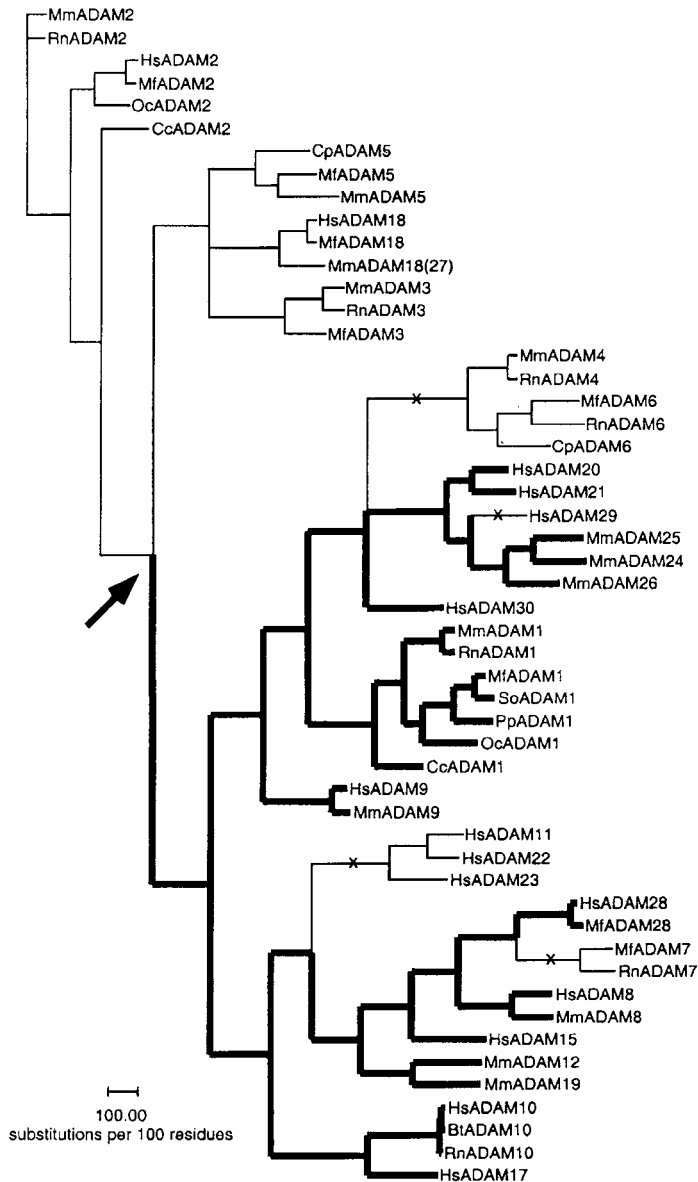


Figure 1.3 The Mammalian ADAM family maximum parsimony tree. The phylogeny was inferred using the PILEUP and PAUP programs and is unrooted, with branch lengths drawn proportional to the number of inferred substitutions along each lineage. Branches marked with heavy lines indicate ADAM family members with a consensus zinc-binding motif (HEXGHXXGXXHD). The arrow indicates the probable zinc-binding motif containing a common ancestor. Lineages with the zinc-binding site were subsequently lost and are denoted with an X. Species abbreviations: Mm, *Mus musculus*; Rn, *Rattus norvegicus*; Hs, *Homo sapiens*; Mf, *Macaca fascicularis*; Oc, *Oryctolagus cuniculus*; Cc, *Cavia cobaya*, Cp, *Cavia porcellus*; So, *Saguinus oedipus*; Pp, *Pongo pygmaeus*; Bt, *Bos taurus*.

1.2 Adhesion and protease activities of ADAM family proteins

An ADAM contains a disintegrin and a metalloprotease domain, and therefore it potentially has both cell adhesion and protease activities. Nineteen of the known ADAMs are predicted to be active proteases (ADAMs 1, 8, 9, 10, 12, 13, 15, 16, 17, 19, 20, 21, 24, 25, 26, 28, 30 and 34) but only for a few their biological roles have been elucidated. One of the proposed roles of disintegrin metalloproteases is "ectodomain shedding" (Moss and Lambert. 2002), the process by which biologically active, soluble forms of cytokines, growth factors, and their receptors are released from membrane-bound precursors. For example *TACE/ADAM 17* - tumor necrosis factor- α converting enzyme was isolated as the proteinase required for the shedding of *TNF- α* from the plasma membrane (Moss et al., 1997; Black et al., 1997). Later on, *ADAM 17* has been reported also to be required for the ectodomain shedding of other cell surface proteins including Alzheimer precursor protein (Lammich et al., 1999), L-selectin and TGF- α (Peschon et al., 1998).

Beside proteolysis, ADAMs are proposed to be cell adhesion molecules. Among all ADAMs there is no basis for predicting how many of them will be active in cell adhesion because the required active site residues in the disintegrin domain have not been defined as yet. For some of them, their integrin ligands have been identified by in vitro studies. All tested ADAMs except *ADAM 10* and *17* can bind to $\alpha 9\beta 1$ integrins (Eto et al., 2002), but they can be also associated with other integrin receptors. For example, *ADAM 28* binds $\alpha 4\beta 1$ integrin (Bridges et al., 2002) *ADAM 15* associates with $\alpha v\beta 3$ and $\alpha 5\beta 1$ (Nath et al., 1999; Eto et al., 2002) and several ADAMs can associate with $\alpha 6\beta 1$ integrin receptors (Chen et al., 1999a,b,c; Nath et al., 2000).

ADAM disintegrin and metalloproteases could have also dual adhesion/proteolytic activity. *Meltrin α* (*ADAM 12*) could serve as an example. First described for the role of its disintegrin domain in the promotion of myoblast fusion into myotubes (Yagami-Hiromasa et al., 1995), later on, biochemical work has shown that meltrin α does have also a protease activity (Loechel et al., 1998). It is possible that the ADAM adhesion and protease domains cooperate. Initially, ADAM might adhere to its substrate or a substrate associated protein using its disintegrin domain and subsequently cleave the substrate proteolytically. However, this model has no experimental support and it must be tested whether both, the metalloprotease and the disintegrin domains are active in the same ADAM at the same time.

1.3 ADAM family genes and their involvement in spermatogenesis and fertilization

Expression studies of the known ADAMs have shown that a bulk of ADAMs is expressed in the testis. This suggests a relationship between ADAM function and the processes of spermatogenesis and fertilization. Among testicular ADAMs, *fertilin* and *cyritestin* are the best studied members of the family. *Fertilin* is a heterodimer of two subunits *fertilin* α and *fertilin* β (*ADAM* 1 and 2).

It was found that sperm bind to the integrin $\alpha 6 \beta 1$ on the plasma membrane of the mouse oocyte (Almeida et al., 1995). Some experiments support the idea that sperm ADAM-*fertilin* and egg integrins $\alpha 6 \beta 1$ are adhesion partners (Bigler et al., 2000). Recently, members of the $\alpha 4 / \alpha 9$ subfamily of integrin receptors and *fertilin* have been implicated in fertilization (Zhu and Evans, 2002). These uncertainties deserve attention because a sperm has many ADAMs on its surface and an egg has at least several integrins, different approaches are required to define interactions between sperm ADAMs and integrins or other egg receptors.

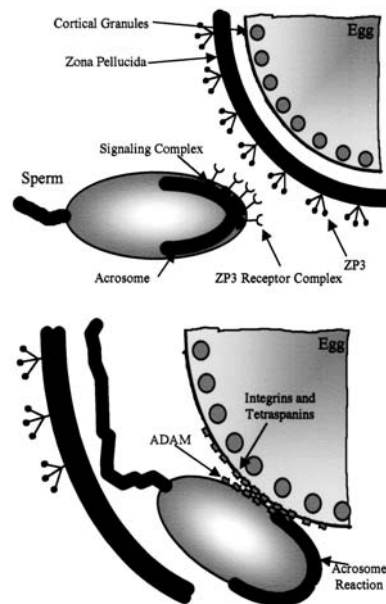


Figure 1.4 The schematic working model of fertilization. Acrosome-intact sperm complete capacitation and bind to the zona pellucida. ZP3 binds mouse sperm through a receptor or receptor complex and triggers the exocytosis of the sperm acrosome. Once the fertilizing sperm completes the acrosome reaction, it migrates through the zona pellucida and binds and fuses with the oocyte plasma membrane, using ADAM family members on sperm that interact with integrins in a complex with tetraspanins on the

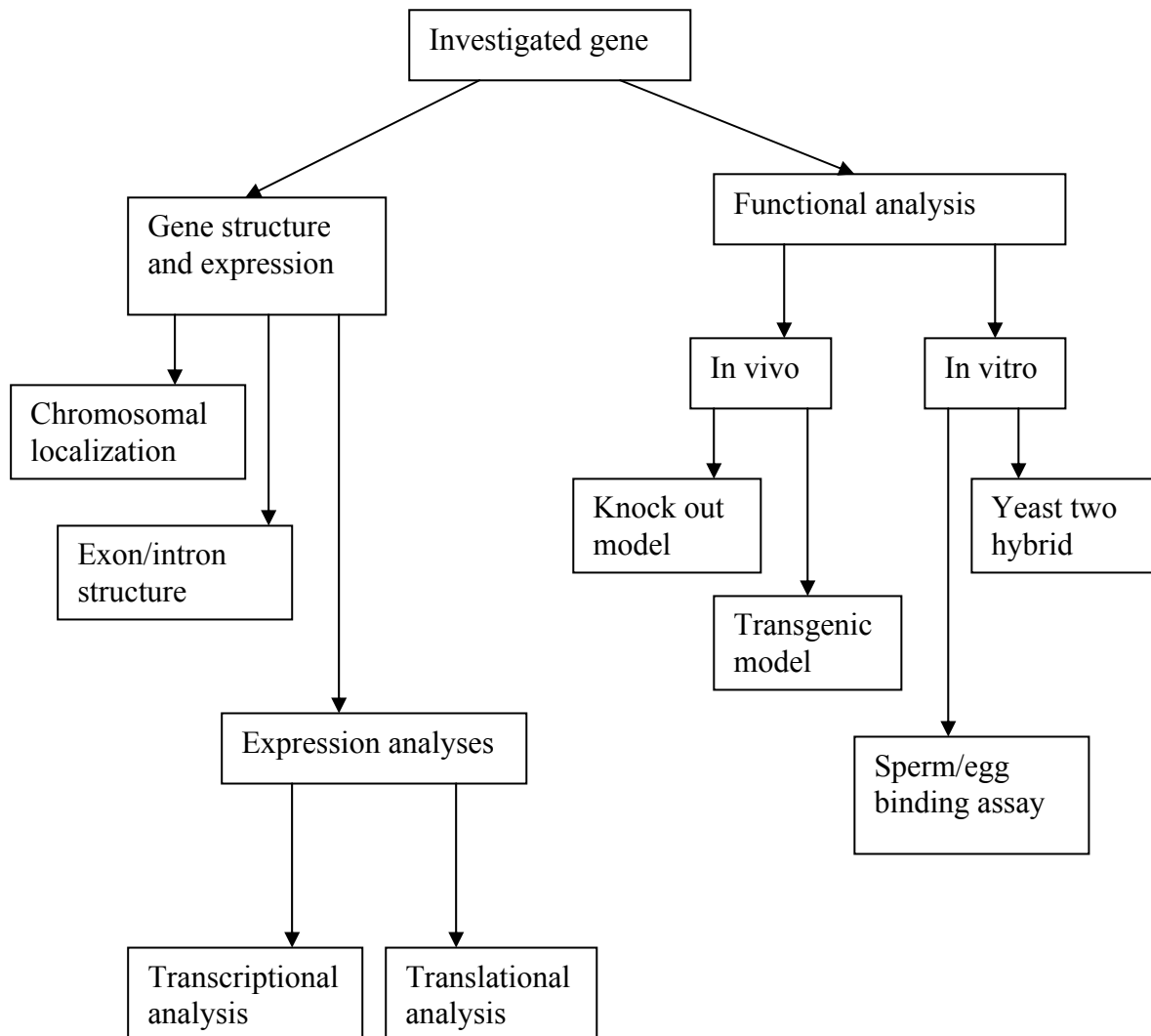
oocyte plasma membrane (lower panel). Fusion activates the oocyte, releasing cortical granules whose secretion modifies the zona pellucida so that additional sperm do not bind to the zona.

To elucidate the functions of fertilin and cyritestin proteins, different approaches have been undertaken. Peptide mimetics of the disintegrin domain active sites of *fertilin* β and *cyritestin* inhibit sperm-egg plasma membrane adhesion and fusion (Myles et al., 1994; Almeida et al., 1995; Evans et al., 1995; Yuan et al., 1997; Linder and Heinlein, 1997; Bronson et al., 1999). These results suggested that fertilin β and cyritestin function in sperm-egg plasma membrane adhesion and fusion (Figure 1.4). This model is contradicted by gene-knockout data on these proteins. *Fertilin* β -null sperm fuse at ~50%, and *cyritestin*-null sperm at 100% of the wild-type rate (Shamsadin et al., 1999). Sperm from the double knockout (lacking *fertilin* β and *cyritestin*) also fuse at ~50% of the wild-type rate (Nishimura et al., 2001). These findings show that *fertilin* β and *cyritestin* are not individually or together required for gamete membrane fusion but they are important for binding to zona pellucida (Cho et al., 1998; Nishimura et al., 2001). In addition, eggs carrying a deletion of the gene for the $\alpha 6$ integrin subunit can bind to and fuse normally with sperm (Miller et al., 2000). Thus, none of the specific proteins acting in the current *ADAM*-integrin model for adhesion/fusion are required for sperm-egg fusion, and other molecules must exist on the surface of gametes that can act in sperm-egg fusion. These could be other members of the *ADAM* and integrin families or entirely different proteins. It demands further studies to understand how, 15 testis specific or predominant *ADAM*s (including *ADAM 27* and *testase 2*) might be required in spermatogenesis and/or fertilization.

Testase 2 and *ADAM 27* are two not yet characterized *ADAM* family members. *Testase 2* is predicted metalloprotease, however could be also an active adhesion protein, *ADAM 27* lacks proteolytic activity and is a potential adhesion molecule. Both genes are expressed specifically in testis and therefore might have a function during processes of spermatogenesis and fertilization.

1.4 Aims of the study

The aim of this study was to elucidate the functions of ADAM 27 and testase 2 proteins in the fertilization and/or spermatogenesis. Experimental approaches undertaken in the present study can be placed in several categories, which are schematically shown below:



2 MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals

Acrylamide/Bisacrylamide	Roth, Karlsruhe
Acetic acid	Merck, Darmstadt
Agar	Roth, Karlsruhe
Agarose	Invitrogen, Karlsruhe
Ammonium acetate	Fluka, Neu Ulm
Ammonium persulfate	Sigma, Deisenhofen
Ampicillin	Sigma, Deisenhofen
Ampuwa	Fresenius, Bad Homburg
Bacto-tryptone	Roth, Karlsruhe
Bacto-Yeast-Extract	Roth, Karlsruhe
BCIP	Applichem, Darmstadt
Blocking powder	Roth, Karlsruhe
Bromophenol blue	Sigma, Deisenhofen
BSA	Biomol, Hamburg
Cell culture media	Invitrogen, Karlsruhe
Coomasie G-250	Sigma, Deisenhofen
Choloroform	Merck, Darmstadt
Vectashield (DAPI)	Vector, Burlingame
Dextran sulfate	Amersham, Freiburg
Dextrose (glucose)	Fisher
Diethyl pyrocarbonate (DEPC)	Sigma, Deisenhofen
Dimethyl sulfoxid (DMSO)	Merck, Darmstadt
Dithiothreitol	Sigma, Deisenhofen
DNA Markers	Invitrogen, Karlsruhe
dNTPs (100 mM)	Invitrogen, Karlsruhe

Dropout mix (-his -ura -trp -leu)	BIO101
Dye Terminator Mix	Applied Biosystems
Ethanol	Baker, Deventer, NL
Ethidium bromide	Roth, Karlsruhe
Ficoll 400	Applichem, Darmstadt
FCS	Invitrogen, Karlsruhe
Formaldehyde	Merck, Darmstadt
Formamide	Sigma, Deisenhofen
Galactose	Sigma, Deisenhofen
Glutaraldehyde	Sigma, Deisenhofen
Glycerol	Invitrogen, Karlsruhe
Glycine	Biomol, Hamburg
Goat serum	Sigma, Deisenhofen
HCl	Roth, Karlsruhe
Histidine	Fisher
H ₂ O ₂	Merck, Darmstadt
HEPES	Merck, Darmstadt
Ionophore A23187	Calbiochem, Bad Soden
IPTG	Biomol, Hamburg
Isopropanol	Merck, Darmstadt
IVF Media	Medicult, Berlin
KCl	Merck, Darmstadt
Leucine	Sigma, Deisenhofen
Methanol	Merck, Darmstadt
MgCl ₂	Merck, Darmstadt
MOPS	Applichem, Darmstadt
Methyl benzoat	Fulka, Neu Ulm
β-Mercaptoethanol	Serva, Heidelberg
Mineral oil	Sigma, Deisenhofen
Na acetate	Merck, Darmstadt
Na citrate	Merck, Darmstadt
NaCl	Merck, Darmstadt
Na ₂ HPO ₄	Merck, Darmstadt
NaH ₂ PO ₄	Merck, Darmstadt

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NaHCO ₃	Merck, Darmstadt
NaN ₃	Merck, Darmstadt
NaOH	Merck, Darmstadt
NBT	Applichem, Darmstadt
NuPAGE LDS sample buffer (4x)	Invitrogen, Karlsruhe
Orange G	Sigma, Deisenhofen
Peptone	Roth, Karlsruhe
PBS	Invitrogen, Karlsruhe
Phosphoric acid	Merck, Darmstadt
Picric acid	Fulka, Neu Ulm
Phenol	Biomol, Hamburg
Proteinase K	Applichem, Darmstadt
Protein marker	Invitrogen, Karlsruhe
Radioactive substances:	
[γ ³² P]-ATP [α ³² P]-dCTP	Amersham, Braunschweig
Raffinose	Sigma
Rediprime™ II	Amersham, Freiburg
RNase Inhibitor	Boehringer, Mannheim
RNA length standard	Invitrogen, Eggenstein
RNA reagent	Biomol, Hamburg
RNase away	Biomol, Hamburg
Salmon sperms DNA	Sigma, Deisenhofen
SDS	Serva, Heidelberg
Select peptone	Invitrogen, Eggenstein
TEMED	Serva, Heidelberg
Triton X-100	Serva, Heidelberg
Tris	Sigma, Deisenhofen
Tryptophan	Sigma, Deisenhofen
Tween-20	Promega, Mannheim
Uracil	Sigma, Deisenhofen
X-Gal	Biomol, Hamburg
Yeast nitrogen base	BIO 101

All those chemicals which are not mentioned above were ordered from either 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)
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Glycerol loading buffer -I	10 mM Tris/HCl (pH 7.5) 10 mM EDTA (pH 8) 0.025% Orange G 30% Glycerol
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2.1.2.2 SDS-PAGE

40% Acrylamide stock solution	Acrylamide 29.2% (w/w) Bis-acrylamide 0.8% (w/w)
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10% Ammonium persulfate solution in H₂O

NuPAGE LDS sample buffer (4x)

Running buffer (5x)	25 mM Tris/HCl (pH 8.3) 192 mM Glycine 0.1% SDS
---------------------	---

Stacking gel buffer (4x)	0.5 M Tris/HCl (pH 6.8) 0.4% SDS
--------------------------	-------------------------------------

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

2.1.2.3 Frequently used buffers and solutions

Denaturation solution	1.5 M NaCl 0.5 M NaOH
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Denhardt's solution (50x)	1% BSA 1% Polyvinylpyrrolidon 1% Ficoll 400
---------------------------	---

Depurization solution	0.25 M HCl
-----------------------	------------

E-buffer (10x)	300 mM NaH ₂ PO ₄ 50 mM EDTA
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Elution buffer	1.5 M NaCl 20 mM Tris/HCl (pH 7.5) 1 mM EDTA
----------------	--

AP buffer	100 mM Tris-HCl (pH 9.5) 100 mM NaCl 50 mM MgCl ₂
-----------	--

NBT- Solution	75 mg/ml NBT 70% Dimethyl formamide
---------------	--

BCIP-Solution	50 mg/ml BCIP 70% Dimethyl formamide
---------------	---

Bouin's solution	15 volume of picric acid (in H ₂ O) 5 volume Formaldehyde
------------------	---

Materials and Methods

	1 volume Acetic acid
Kinase buffer (10x)	100 mM Tris/HCl (pH 7.4) 100 mM MgCl ₂ 100 mM DTT 1 mM ATP
Ligation buffer (10x)	600 mM Tris/HCl (pH 7.5) 80 mM MgCl ₂ 100 mM DTT
Lysis buffer I	100 mM Tris/HCl (pH 8.0) 100 mM NaCl 100 mM EDTA 0.5% SDS
Lysis-buffer II	100 mM Tris/HCl (pH 8.0) 5 mM EDTA 200 mM NaCl 0.2% SDS 100 µg/ml proteinase K
10 X MOPS buffer	41.8 gms MOPS 16.6 ml 3 M Sodiumacetate 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

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PBT buffer	0.1% Tween-20 in PBS (1x)
1x PBS II	4 mM KH_2PO_4 16 mM Na_2HPO_4 115 mM NaCl
Blocking solution II	5% Skimmed milk powder in washing stock 1x 0.5% Tween 20 in 1x PBS II
PBT II	0.1% Tween 20 in 1x PBS II
10x washing stock:	1.4 M NaCl 5 mM MgCl_2 100 mM Tris/HCl, pH 7.5 0.5% Tween 20
Washing buffer:	2% Skimmed milk powder in 1x washing buffer
SSC (20x)	3 M NaCl 0.3 M Na_3 citrate (pH 7.0)
Stop-Mix	15% Ficoll 400 200 mM EDTA 0.1% Orange G
TE-buffer	10 mM Tris/HCl (pH 8.0) 1 mM EDTA
Washing solution I	2x SSC 0.1% SDS
Washing solution II	0.2x SSC

10 X LiOAc

50 % PEG-3350

1 X TE/LiOAc

Right before use, 1 part 10 X TE, 1 part 10 X LiOAc, and 8 parts sterile distilled water were mixed.

1 X TE/LiOAc/PEG

Right before use, 1 part 10 X TE, 1 part 10 X LiOAc, and 8 parts 50 % PEG-3350 were mixed.

Carrier DNA

sonicated salmon sperm DNA, 5 mg/ml

2.1.3 Laboratory Materials

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

Whatman blotting paper
(GB 002, GB 003 and GB 004)

Schleicher and Schüll, Dassel

Cell culture flask

Greiner, Nürtingen

Dialysis hoses

Serva, Heidelberg

Disposable filter Minisart NMI

Sartorius, Göttingen

Filter paper 0858

Schleicher and Schüll, Dassel

Hybond C

Amersham, Braunschweig

Hybond N

Amersham, Braunschweig

Petri dishes

Greiner, Nürtingen

Pipette tips

Eppendorf, Hamburg

Microcentrifuge tubes

Eppendorf, Hamburg

Transfection flask

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, 10⁵ Pa for 60 min in an autoclave (Webeco, Bad Schwartau). Heat sensitive solutions were filtered through a disposable sterile filter (0.2 to 0.45 µm pore size). Plastic wares were autoclaved as above. Glassware were sterilised overnight in an oven at 220°C.

2.1.5 Media, antibiotics and agar-plates

2.1.5.1 Media for bacteria

LB Medium (pH 7.5):

- 1% Bacto-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 Yeast growth media

YPD (rich medium):

- 2% peptone
- 1% yeast extract
- 2% glucose
- one pellet (0.1 g) NaOH [if for plates]
- 2% agar [if for plates]

YNB-ura-his-leu-trp (selective medium):

- 0.17% yeast nitrogen base w/o amino acids
- 0.5% ammonium sulfate
- 0.6 g -his-ura-trp-leu dropout mix per 1 liter
- 2% glucose (or 2% galactose + 1% raffinose for gal/raff media)
- 2% agar (if for plates)

other YNB (selective) media:

following reagents were added to the YNB-ura-his-leu-trp medium to make the appropriate medium.

- trp 0.04 mg/ml.
- ura 0.02 mg/ml.
- leu 0.06 mg/ml.
- his 0.02 mg/ml.

2.1.5.3 Media for cell culture

ES-cell medium:

DULBECCO's MEM (DMEM)

0.1 mM Non essential amino acids

1 mM Sodium pyruvate

10 μ M β -Mercaptoethanol

2 mM L-Glutamine

20% Fetal calf serum (FCS)

1000 U/ml Recombinant leukaemia inhibitory factor (LIF)

Fibroblast cell medium (EmFi):

DULBECCO's MEM (DMEM)

2 mM L-Glutamine

10% FCS

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

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

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

2.1.5.4 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 ₂ O	50 $\mu\text{g/ml}$
Kanamycin	25 mg/ml	H ₂ O	50 $\mu\text{g/ml}$
G 418	40mg/ml	PBS	400 $\mu\text{g/ml}$
Gancyclovir	100 mM	PBS	2 μM
Mitomycin C	1 mg/ml	PBS	10 $\mu\text{g/ml}$

2.1.5.5 IPTG / X-Gal plate

LB-agar with 50 $\mu\text{g/ml}$ ampicillin, 100 μM IPTG and 0.4% X-Gal was poured into petri dishes. The dishes were stored at 4°C .

2.1.6 Bacterial strains

E. coli DH5 α

Invitrogen

E. coli BL21

Novagen

2.1.7 Yeast strains

EGY48 **MATa** *trp1 his3 ura3 leu2::6 LexAop-LEU2* (high sensitivity)

EGY194 **MATa** *trp1 his3 ura3 leu2::4 LexAop-LEU2* (medium sensitivity)

EGY188 **MATa** *trp1 his3 ura3 leu2::2 LexAop-LEU2* (low sensitivity)

2.1.8 Plasmids

pBluescript SK (+/-)	Stratagene
pBluescript KS (+/-)	Stratagene
pGEM-T Easy	Promega
pTKneo	Dr. N. Brose, MPI für Experimentelle Medizin, Göttingen
pZERO-2	Invitrogen
pEGFP-N1	Clontech
pMAL	New England BioLabs
pET	Novagen
Lawrist 7	RZPD, Berlin
pSH18-34	Origene
pJK103	Origene
pRB1840	Origene
pJK101	Origene
pEG202	Origene
pRHFM1	Origene
pSH17-4	Origene

2.1.9 Synthetic oligonucleotide primers

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

DelTM RP	5' TTC TCC CTG AGG CAC GAT TT 3'
DelTM FP	5' TTG CTA CAA AGC GCC TGA GT 3'
ADAM27FP	5' GCT GCG ACC ATG AAA CGT G 3'
ADAM27RP	5' CCC AAC CTG CAC AAA TGG 3'
E1AD27FP	5' TTC TTG CAC TTG GAC CCA ACC A 3'
E1AD27RP	5' ATA CCA ACA CCC GCA ACA TAG C 3'
BigAD27FP	5' GCC ATG TTT GGC AGA CAG ACC AGC 3'
BigAD27RP	5' CCA GGA TCA CCC AGC TGT CCT CAT 3'
KOTMFP	5' TAG ACT GCA CCA AGA GCT GAC 3'
KOTMRP	5' GAA TGA GAG CTG GAT CCA CAC 3'
TM3	5' GCC CTG TGC ACT TGT CTA C 3'
TM4	5' TTA GGT GGG AAC CCA GCA TC 3'
EX4FP	5' ATG TGA CTC TGA CTG ACC AG 3'
EX4RP	5' ATG GTC TGG ATG CTG CCA TG 3'
JKAD27RP	5' CCC TTT GCA CTT CTT AGT GGC GTT GC 3'
JAD27FP	5' GGG AAA TTA GCT TGT TTT CGG CCG CCC 3'
JAD27RP	5' CAT TTG TGG TCC ACA CAC AGT GCC GTC 3'
A27G1FP	5' GCT ACA AAG CGC CTG AGT AAG 3'
A27G2FP	5' CCA TTC ATT GCA CCA GAA ATC 3'
A27G1RP	5' GAT TTA AAG TGT ATC CAC CGG 3'
Y1FP	5' ACC CAT GGC TGA GCT TCT TGC ACT TG 3'
Y1RP	5' GTG TCA GGA ACA CAG TGG CTA 3'
Y2FP	5' AGC TCG AGT GTA TCC ACC GGT AAC AG 3'
Y2RP	5' CTC GAG TGT ATC CAC CGG TAA CAG 3'
Y3FP	5' GGA TCC AAT GCT TTC CTG ACT ACA GG 3'
Y4FP	5' GGA TCC TGA GTA AGA ATG AGG ACA GC 3'
Y5RP	5' AGC TCG AGA ACA TCC TGC TGT CCG C 3'

Y6RP	5' AGC TCG AGT GGC TGT GCA GTC ATT TCC TG 3'
DCEfp(Xba I)	5' AGT CTA GAC AAC CAA AGC AAG CCG TGT GTG 3'
DCErp(Pst I)	5' GTC TGC AGT CAA GCC GAC TCA GTT CTG AGA GTG 3'
Drp(Pst I)	5' GTC TGC AGT CAC AAA TGG CCG TTC AGT GCA 3'
Tesfp	5' CAC TTC ACC TGC TAT CCT GCT ACT C 3'
Tesrp	5' TCT CCA TCG TCA ACC ACT CCA TCC 3'
expT2fp	5' GGT GAG ACC TAG ATT CTC GA 3'
expT2rp	5' GAA GCA ACA GTC ATT GCT 3'
KOATGfp	5' AAG TGC AAG AAG CTC AGC CGA 3'
KOATGrp	5' CCT GAG CTG GTA GTT CAG AAC 3'
MalE	5' GGT CGT CAG ACT GTC GAT GAA GCC 3'
pTKNf:	5' ATT GTC TGA GTA GGT G 3'
pTKNr:	5' GCG CGA ATT CGA TGA TCC TGA ACG GC 3'
pTKR:	5' AAC AGC TAT GAC CAT GAT TAC G 3'
NeoRI:	5' AGG AGC AAG GTG AGA TGA CAG 3'
T7:	5' TAA TAC GAC TCA CTA TAG GG 3'
T3:	5' ATT AAC CCTT CAC TAA AG 3'
SP6:	5' AGG TGA CAC TAT AGA ATA C 3'
Poly T:	TTTTTTTTTTTTTTTTTTTT

2.1.10 cDNA probes

Human <i>EF-2</i> cDNA	Hanes et al.,1992
<i>β-actin</i> cDNA	Clontech
BigAD27	Generated in present study
5'AD	Generated in present study
TM3/external probe	Generated in present study
ATG/external probe	Generated in present study
Tes2	Generated in present study
expT2	Generated in present study

2.1.11 Eukaryotic cell lines

RI mouse embryonic stem cell line (Passage 11), Dr. A. Nagi, Toronto, Canada

MA10- Leydig cell line

15P1- Sertoli cell line (Rassoulzadegan et al., 1993)

2.1.12 Mouse strains

Mouse strains C57BL/6J, 129X1/SvJ, C3H/J and DBA/2J were initially ordered from Charles River Laboratories, Wilmington, USA and further bred in Animal facility of Institute of Human Genetics, Göttingen.

2.1.13 Antibodies

Mouse monoclonal against γ -Tubulin (Sigma)

Goat anti-rabbit alkaline phosphatase conjugated (Sigma)

Goat anti-rabbit FITC and Cy3-conjugated (Sigma)

Rabbit polyclonal anti-GST (Novagen)

Mouse monoclonal anti-MBP (New England BioLabs)

Rabbit anti-mouse ADAM27 polyclonal antibodies were generated in present study, anti ppADAM27 designates anti peptide antibody and anti fpADAM27 anti fusion protein antibody.

2.1.14 Enzymes

Restriction enzymes (with supplied buffers) (Invitrogen, NEB)

Klenow Fragment (Invitrogen)

Mung bean exonuclease (Invitrogen)

Proteinase K (Sigma)

Platinum Taq polymerase (Invitrogen)

<i>Pfx Platinum</i> polymerase	(Invitrogen)
RNase A	(Qiagen)
Rnase H	(Invitrogen)
Rnase inhibitor	(Invitrogen)
Superscript-II	(Invitrogen)
<i>Platinum Taq</i> polymerase	(Invitrogen)
T4 DNA ligase	(Promega)
T4 RNA ligase	(Invitrogen)
Trypsin	(Invitrogen)

2.1.15 Kits

BigDye Terminator Cycle Sequencing Ready Reaction Kit	(Applied Biosystems)
DYEnamic ET-Terminator mix	(Amersham Pharmacia)
Endo Free Plasmid Maxi Kit	(Qiagen)
Megaprime DNA Labeling Kit	(Amersham Pharmacia)
Maxi Plasmid Kit	(Qiagen)
Mega Plasmid Kit	(Qiagen)
Mini Plasmid Kit	(Qiagen)
QIAEX II	(Qiagen)
QIAquick Gel Extraction Kit	(Qiagen)
Rediprime TM II Random Prime Labeling System	(Amersham Pharmacia)
Bug Buster GST-bind purification kit	(Novagen)
pMAL Protein Fusion and Purification System	(New England BioLabs)
pET GST Fusion Systems 41	(Novagen)
SulfoLink Kit	(Pierce)

2.1.16 Instruments

ABI PRISM 377 DNA Sequencer	(Applied Biosystem)
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ABI 3100 Genetic Analyzer	(Applied Biosystem)
Microscope BX60	(Olympus)
GeneAmp PCR System 9600	(Perkin Elmer)
Microtiterplate-Photometer	(BioRad)
Molecular Imager FX	(BioRad)
Phosphoimager Screen	(Kodak)
Semi-Dry-Blot Fast Blot	(Biometra)
Spectrophotometer Ultraspec 3000	(Amersham Pharmacia)
SpeedVac concentrator SVC 100H	(Schütt)
Thermomixer 5436	(Eppendorf)
Turboblotter™	(Schleicher & Schüll)
UV Stratalinker™ 1800	(Leica)
Video-Documentationsystem	(Herolab, Heidelberg)
X-Ray Automatic Processor Curix 60	(Agfa)
Autoclave	(Webeco, Bad Schwartau)
Neubauer cell chamber	(Schütt Labortechnik, Goettingen).

2.2 Methods

2.2.1 Isolation of nucleic acids

2.2.1.1 Isolation of plasmid DNA

(Sambrook et al., 1989)

2.2.1.1.1 Small-scale isolation of plasmid DNA

A single *E.coli* colony was inoculated in 5 ml of LB medium with the appropriate antibiotic and incubated in a shaker for 16 hrs at 37°C with a speed of 160 rpm. 1 ml of this saturated culture was used for making glycerol stock 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 P3 solution. The

precipitated solution was 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 was dissolved in 30 µl of TE buffer (adapted from Birnborn and Doly, 1979).

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 37°C shaker. This pre-culture was added in a dilution of 1/100 fold to 100 ml LB medium with appropriate antibiotic and incubated overnight at 37°C with shaking. The culture was centrifuged at 6000xg for 15 min. Plasmid DNA preparation was performed according to QIAGEN® Plasmid Purification Protocol supplied with the kit.

2.2.1.1.3 Endotoxin free preparation of plasmid DNA

Endotoxins, also known as lipopolysaccharides (LPS), are cell membrane components of Gram-negative bacteria (e.g. *E.coli*). During lysis of bacterial cells for plasmid preparations, endotoxin molecules are released from the outer membrane into the lysate. Endotoxins strongly influence transfection of DNA into primary cells and sensitive cultured cells like embryonic stem (ES) cells, increased endotoxin levels lead to sharply reduced transfection efficiencies. Endofree plasmid preparation kit integrates endotoxin removal into standard plasmid preparation procedure. The neutralised bacterial lysate was filtered through a QIAfilter cartridge (provided in kit) and incubated on ice with a specific Endotoxin Removal buffer (patented by Qiagen). The endotoxin removal buffer prevents LPS molecules from

binding to the resin in the columns (QIAGEN-tips), thus allowing purification of DNA containing less than 0.1 endotoxin units per μg plasmid DNA.

2.2.1.2 Isolation of genomic DNA from tissue samples

(Laird et al., 1991)

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

The method was performed according to Laird et al. (1991). 1 to 2 cm of the tail from a mouse was incubated in 700 μl of lysis buffer containing 35 μl proteinase K (10 $\mu\text{g}/\mu\text{l}$) 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 at room temperature for 5 min. After transferring the upper aqueous layer into a new tube, the same procedure was repeated, first with 1:1 ratio of phenol and chloroform and then with chloroform. 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.

2.2.1.3 Isolation of genomic DNA from ES cells

Lysis-buffer II:	100 mM Tris-HCl (pH 8.5)
	5 mM EDTA
	200 mM NaCl
	100 $\mu\text{g}/\text{ml}$ proteinase K
	0.2% SDS

To isolate the DNA from the ES cells, cells in a 24 well plate were washed with PBS and incubated overnight in 500 μl lysis buffer II at 55°C. Equal volume of isopropanol was added and mixed for 15 min to precipitate the DNA. After washing with 70% ethanol, the DNA was

transferred into a microcentrifuge cup containing 60 µl TE buffer and incubated at 60°C for 15 min.

2.2.1.4 Isolation of total RNA from tissue samples and cultured cells

(modified Chomczynski and Sacchi, 1987).

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 tissue sample was homogenised in 1-2 ml of RNA reagent by using a glass-teflon homogeniser. The sample volume should not exceed 10% of the volume of reagent used for the homogenisation. To isolate total RNA from cultured cells, 350 µl of reagent was added to the 6 cm diameter petri dish. 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 centrifuging 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 DEPC-H₂O.

2.2.2 Determination of the nucleic acid concentration

The concentration of nucleic acids was determined spectrophotometrically by measuring absorption of the samples at 260 nm. The quality of nucleic acids i.e. contamination with salt and protein was checked by the measurements at 230, 280, and 320 nm. The concentration was calculated according to the formula:

$$C = (E_{260} - E_{320})fc$$

C = concentration of sample (µg/µl)

E₂₆₀ = ratio of extinction at 260 nm

E₃₂₀ = ratio of extinction at 320 nm

f = dilution factor

c = concentration (standard) / absorption (standard)

for double stranded DNA : $c = 0.05 \mu\text{g}/\mu\text{l}$

for RNA : $c = 0.04 \mu\text{g}/\mu\text{l}$

for single stranded DNA : $c = 0.03 \mu\text{g}/\mu\text{l}$

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 can be used to electrophorese nucleic acid molecules from as small as 50 bases to more than 50 kilobases, depending on the concentration of the agarose and the precise nature of the applied electrical field (constant or pulse). Usually, 1 g of agarose was added in 100 ml 0.5x TBE buffer, and boiled in the microwave to dissolve the agarose, then cooled down to about 60°C before adding 3 μl ethidium bromide (10 mg/ml). This 1% agarose gel was poured into a horizontal gel chamber.

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. 1.25g of agarose was added in 100 ml of 1x MOPS buffer and dissolved by heating in 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 10 x MOPS Buffer

3 μ l Formaldehyde
8 μ l Formamide (40%)
1.5 μ l 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 can be used for separating proteins for analysis and molecular weight determination. The proteins are denatured and rendered monomeric by boiling in the presence of reducing agents (β -mercaptoethanol or dithiothreitol) and negatively charged detergent (SDS). The proteins, which normally differ according to their charges, are all coated with the SDS molecules, which are negatively charged. Hence, all the proteins in the sample become negatively charged and achieve constant charge to mass ratio. In this way, the separation is according to the size of the proteins. A SDS-PAGE consists of two gels; firstly, a 10-12 % separating gel was poured. In order to achieve a smooth boundary between separating and stacking gel, the separating gel was covered with a layer of water. After polymerisation of the separating gel, a 4 % stacking gel was poured over it. The samples were heated in 70°C in LDS sample buffer for 10 min before loading into the gel. The gel was run at 15 mA for 1 hr, then at a constant current of 30 mA.

2.2.4 Isolation of DNA fragments from agarose gel

2.2.4.1 Glass silica method

(Vogelstein and Gillespie, 1979)

For the isolation of DNA fragments of 300-4000 base pairs (bp) in length from agarose gels, the QIAEX II Gel Extraction System kit from Qiagen was used. The principle of this method depends on the binding capacity of DNA to silica in high salt concentrations and elution in

low salt solutions. After separation of DNA on an agarose gel, the DNA fragment to be isolated was excised with a razor blade and weighed. DNA isolation was performed according to protocol in QIAEXII handbook supplied with the kit.

2.2.4.2 QIAquick gel extraction method

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

2.2.5 Enzymatic modifications of DNA

2.2.5.1 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 that specific enzyme. Standard digestions include 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. However, for genomic DNA digestion the reaction solution was incubated overnight at 37°C.

2.2.5.2 Ligation of DNA fragments

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

30 ng vector DNA (digested)
50-100 ng insert DNA (1:3, vector: insert ratio)

1 μ l ligation buffer (10x)
1 μ l T4 DNA ligase (5U / μ l)
in a total volume of 10 μ l

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 and other polymerases have a terminal transferase activity that results in the non-template addition of a single nucleotide to the 3' ends of PCR products. In the presence of all 4 dNTPs, dATP is preferentially added. This terminal transferase activity is the basis of the TA-cloning strategy. For cloning of PCR products, 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
PCR product (1:3, vector to insert ratio)
1 μ l T4 DNA Ligase 10x buffer
1 μ l T4 DNA Ligase
in a total volume of 10 μ l

The content was mixed by pipetting and the reaction was incubated overnight at 4°C. For transformation of the ligation reaction, DH5 α competent cells were used (Invitrogen).

2.2.6 Transformation of competent bacteria

(Ausubel et al., 1994)

Transformation of the bacteria was done by gently mixing one aliquot of competent bacteria (50 μ l) with 10 μ l 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 μ l of LB medium, bacteria were incubated at 37°C, 200 rpm for 1hr to allow recovery of heat

shocked bacteria and were plated out on LB-agar plates containing appropriate antibiotic (50µg/ml), and whenever required 1 mM IPTG and X-Gal 40mg/ml, X-Gal for “Blue-White” selection.

2.2.7 Polymerase Chain Reaction (PCR)

Without a doubt, 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.

2.2.7.1 PCR amplification of DNA fragments

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

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
1 µl	<i>Taq</i> DNA Polymerase (5U/µl)

Up to 50 μ l H₂O

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

Initial denaturation	95 ⁰ C	5 min
Elongation	95 ⁰ C	30 sec (denaturation)
30-35 cycles	58 ⁰ C	45 sec (annealing)
	72 ⁰ C	1-2 min (extension)
Final extension	72 ⁰ C	10 min

2.2.7.2 Genotyping of knock out mice by using PCR

The genotypes of all offspring of ADAM 27 transmembrane knock out mutant mice were analysed by polymerase chain reaction (PCR). For amplification of the wild-type and the mutant allele, the DNA was extracted from mouse tails as described in 2.2.1.2 and pipetted to the following reaction mixture:

1 μ l DNA (300-500 ng)
1 μ l KOTMFP (10 pmol/ μ l)
1 μ l KOTMRP(10 pmol/ μ l)
0.4 μ l NeoRI (10 pmol/ μ l)
1 μ l dNTPs (10 mM)
5 μ l *Taq* Polymerase buffer (10x)
0.5 μ l Platinum *Taq* Polymerase (5 U/ μ l, Invitrogen)
1.5 μ l magnesium chloride 50 mM
Up to 50 μ l H₂O

The mixture was subjected to the following program in the thermocycler:

Denaturation	94°C for 4 min	
Elongation (for 35 cycle)	94°C for 30 sec 60°C for 45 sec 72°C for 30 sec	Denaturation Annealing Elongation
Extension	72°C for 5 min	

2.2.7.3 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°C for 10 min, and then quickly chilled on ice. After a brief centrifugation, the followings were added to the mixture:

- 4 µl 5x First strand buffer
- 2 µl 0.1 M DTT
- 1 µl 10mM dNTPs
- 1 µl RNasin (10U/µl)

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

2.2.8 Generation of constructs for recombinant fusion proteins

A number of systems exist for overexpressing specific polypeptides in bacterial cells. Fusion vectors facilitate the purification of the required protein. The sequence of interest is cloned behind the gene for a protein such as maltose-binding protein (MBP) or glutathione-S-

transferase (GST), and introduced into bacteria. Commercial vectors are available for the production of fusion constructs. Expression is usually under the control of an inducible promoter. Production of the fusion protein is induced by the addition of IPTG, and purification from bacterial protein is aided by a property of the fusion partner. MBP fusions will bind to amylose. Similarly, GST will bind to glutathione-sepharose beads. The fusion pair is eluted from the immobilized matrix by the addition of maltose in the case of MBP fusions or reduced glutathione in the case of GST.

In this chapter, the generation of a range of partial ADAM27 cDNA constructs for expression of protein is summarized. The preparation of defined cDNA fragments by polymerase chain reaction (PCR) amplification and subsequent cloning of the fragments into expression vectors is described.

2.2.8.1 Production of GST-ADAM27 fusion protein construct.

GST (glutathione-S-transferase) fusion construct containing fragment of ADAM 27 sequence coding for end part of Disintegrin domain, Cys-rich domain, EGF-like domain, Transmembrane domain and Cytoplasmic Tail was designed (Figure 2.1).

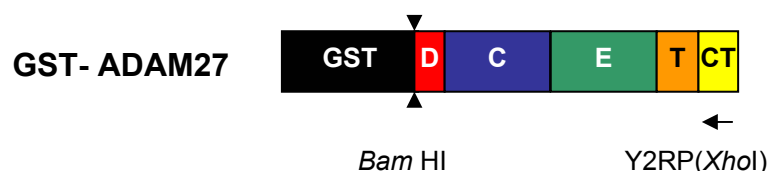


Figure 2.1 Schematic representation of GST-ADAM27 fusion protein. Abbreviations of domains: Disintegrin, Cys-rich, EGF-like, Transmembrane domains and Cytoplasmic Tail.

2.2.8.1.1 Amplification of ADAM27 cDNA.

ADAM27 cDNA fragments were amplified using primers with introduced convenient restriction sites for subsequent cloning. Fragments were designed to be cloned into ORF of GST gene to make fusion proteins. PCR was performed using Platinum Pfx DNA polymerase with proof-reading activity to avoid mismatches in amplification.

PCR conditions:

94⁰C - 4 min
 94⁰C - 30 sec
 50⁰C - 30 sec } 35 cycles
 72⁰C - 1 min
 72⁰C - 5 min

2.2.8.1.2 Sub-cloning and sequencing of PCR product.

PCR using DisFP/Y2RP(*Xho* I) primers was done on the template of testicular cDNA. PCR product was digested with *Bam* HI (1443 cDNA) and *Xho* I (introduced in primer) and then purified from a 1% agarose gel by QiaQuick method 2.2.4.2. The purified fragment was ligated between the *Bam* HI and *Xho* I sites of pET41a+ expression vector. The ligations were transformed into competent *E.coli* DH5a cells. Plasmid DNA from few colonies was sequenced with vector specific primers to confirm correct ORF and to check for mismatches. One GST-AD27 clone was sequenced completely. When compared with the mouse *ADAM 27* cDNA sequence, no mismatches were detected.

2.2.8.2 Generation of MBP-AD27 fusion protein constructs.

MBP (maltose-binding protein) fusion constructs containing different combinations of ADAM 27 domains were designed (Figure 2.2).

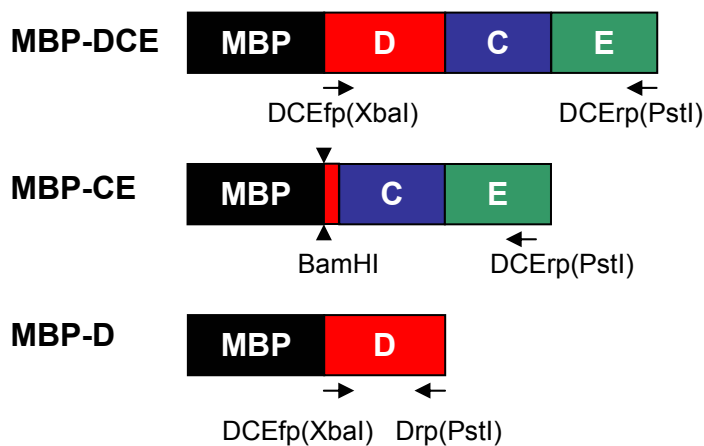


Figure 2.2 Schematic representation of MBP-ADAM27 fusion proteins. Abbreviations of domains: Disintegrin, Cys-rich, EGF-like

2.2.8.2.1 Amplification of ADAM27 cDNA fragments.

Testicular cDNA was used as a template for PCR, as described above 2.2.8.1.1. Modified primers with introduced restriction sites were used for PCR as it is shown in Figure 2.2. The reverse primer was designed such that it included introduced stop codon. Primers were designed that PCR fragments fit into OFR of maltose binding protein and give MBP fusion proteins. Exceptionally fragment CE is derived from DCE by digestion with *Bam* HI enzyme.

2.2.8.2.2 Sub-cloning and sequencing of PCR products.

PCR products were digested with *Xba* I and *Pst* I for DCE and D fusion proteins, and *Bam* HI and *Pst* I for CE fusion protein, fragments were then purified from a 1% agarose gel by QiaQuick method. The purified fragments were ligated between the corresponding sites of pMAL-p2E expression vector. The ligations were transformed into competent *E.coli* DH5 α cells. Plasmids DNA from few colonies of each fusion protein construct were sequenced with vector specific primers to confirm correct ORF and to check for mismatches. One clone of each MBP-AD27 constructs was sequenced completely. When compared with the mouse ADAM27 cDNA sequence, no mismatches were detected.

2.2.9 Expression of recombinant proteins.

2.2.9.1 Preparation for induction

Plasmids with fusion protein constructs (GST-ADAM27, MBP-DCE, MBP-CE, and MBP-D) isolated from DH5 α cells were transformed to expression host bacterial strain *E.coli* BL21 (DE3). Single colony of bacteria containing vector with fusion construct was picked from a freshly streaked plate and 50 ml LB culture with kanamycin / ampicillin was inoculated.

2.2.9.2 Sample induction protocol

1. Bacterial culture was incubated with shaking at 37°C until OD600 reached 0.4–1.

2. Uninduced sample was removed for the control (see TCP sample protocol). To the remainder, IPTG from a 100 mM stock was added to a final concentration of 0.4 mM and incubation was continued for 2–3 hrs.
3. Induced sample was removed (see TCP sample protocol). Flasks were placed on ice for 5 min and then cells were harvested by centrifugation at 5000 x g for 5 min at 4°C.
4. Cells were resuspended in 0.25 culture volume of cold 20 mM Tris-HCl pH 8.0, and centrifuged as above.
5. Finally supernatant was removed and cells were stored as a frozen pellet at –70°C or used directly for purification according to BugBuster method or using amylose resin.

2.2.9.3 Analysis of protein from bacterial cultures.

2.2.9.3.1 Total cell protein (TCP) sample

The expression of target genes was assessed by analysis of total cell protein on a SDS-polyacrylamide gel followed by Coomassie blue staining.

1. Prior to harvesting the cells, 1 ml sample of well-mixed culture was taken and centrifuged at 10,000 x g for 1 min.
2. Pellet was resuspended by mixing in 150 µl of 1X phosphate-buffered saline (PBS).
3. 50 µl of 4X sample buffer and fresh DTT was added and sample was sonicated for 20 sec.
4. Proteins were heated for 10 min at 70°C to denature and then stored at –20°C until SDS-PAGE analysis.
5. When required, detection of the expressed protein was achieved by Western blotting.

2.2.9.3.2 Preparation of cell extracts with BugBuster™ protein extraction reagent

a. Soluble fraction

1. Bacteria were harvested from liquid culture by centrifugation at 6,500 x g for 5 min. Pellet was drained to remove as much liquid as possible.
2. Cell pellet was resuspended in the BugBuster at room temperature, using 2 ml reagent for cells from a 50 ml culture.

3. Cell suspension was incubated on a shaking platform at a slow setting for 10 min at room temperature.
4. Insoluble cell debris was removed by centrifugation at 16,000 x g for 20 min at 4°C. Pellet was saved for inclusion body purification as described below.
5. Supernatant - soluble extract was transferred to a fresh tube.
6. Sample was subjected to SDS-PAGE electrophoresis.

b. Inclusion body purification

1. Induced culture was processed according to steps 1–4 above for the soluble protein fraction.
2. Pellet from step 4 above was resuspended in the same volume of BugBuster reagent that was used to suspend the cell pellet. Pellet material was vortexed to obtain an even suspension.
3. Lysozyme was added to a final concentration of 200 µg/ml. Sample was mixed by vortexing and incubated at room temperature for 5 min.
4. 6 volumes of 1:10 diluted BugBuster reagent (in deionized water) was added to the suspension and vortexed for 1 min.
5. Suspension was centrifuged at 16,000 x g for 15 min at 4°C to collect the inclusion bodies. Supernatant was removed.
6. Inclusion bodies were resuspended in ½ the original culture volume of 1:10 diluted BugBuster, mixed by vortexing, and centrifuged as in step 5. Wash step was repeated 2 more times.
7. The final pellet of purified inclusion bodies was resuspended in 1X PBS.
8. Sample was subjected to SDS-PAGE electrophoresis.

2.2.9.4 Purification of MBP fusion proteins using amylose resin.

1. 1 liter LB + glucose and ampicillin was inoculated with 10 ml of an overnight culture of cells containing the fusion plasmid.
2. Bacterial culture was induced as described above.
3. Cells were harvested by centrifugation at 4000 x g for 20 min. Cell pellet was resuspended in 400 ml 30 mM Tris-HCl, 20% sucrose, pH 8.0. EDTA was added to 1 mM and sample was incubated for 5–10 min at room temperature with shaking.

4. After centrifugation at 8000 x g for 20 min at 4°C, supernatant was removed; pellet was resuspended in 400 ml of ice-cold 5 mM MgSO₄ and shaken for 10 min in ice bath.
5. Sample was centrifuged at 8000 x g for 20 min at 4°C. Collected supernatant was the cold osmotic shock fluid.
6. 8 ml of 1 M Tris-HCl, pH 7.4, was added to the osmotic shock fluid.
7. Amylose resin was poured in a 2.5 x 10 cm column. Column was washed with 8 volumes of column buffer.
8. Osmotic shock fluid from step 6 was loaded at a flow rate of about 1 ml/min.
9. Column was washed with 12 column volumes of column buffer.
10. Fusion protein was eluted with column buffer + 10 mM maltose. 10 to 20 fractions of 3 ml each were collected (fraction size = 1/5th column volume). The fraction with fusion protein was detected by Bradford protein assay.
11. The protein-containing fractions were pooled and concentrated using Amicon Centricon concentrator.
12. Sample was subjected to SDS-PAGE electrophoresis.

2.2.9.5 Reduction of recombinant proteins.

MBP-CE and -DCE recombinant proteins are rich in cystein residues and during expression in bacteria intermolecular disulfide bonds could be formed resulting in large multimeric aggregates. It was described (Evans et al., 1997) that nonreduced multimers did not bind to eggs; in order to have egg-binding activity of recombinant proteins, reduction protocol was necessary.

To prepare protein for egg-binding studies, recombinant ADAM27-CE, and -DCE was reduced by treating with 20 mM DTT for 30–60 min at room temperature. The sample was then dialyzed extensively against a Hepes-buffered Whitten's medium compatible dialysis buffer (WHITCO; 109.5 mM NaCl, 4.7 mM KCl, 1.2 mM KH₂PO₄, 0.23 mM sodium pyruvate, 4.8 mM sodium lactate, 7mMNaHCO₃, 15mMHepes, 0.1 mg/ml gentamicin). The protein was then concentrated to 1 mg/ml; protein concentration was determined using Bradford method.

2.2.10 Protein and biochemical methods

2.2.10.1 Isolation of total protein

Proteins were extracted from mouse testis, kidney and other tissues by homogenization in protein lysis buffer (150mM NaCl, 10mM EDTA, 50mM Tris/HCl, pH7.6, 1% Triton X-100 and 1% sodium deoxycholate) containing protease inhibitors (1 μ g/ μ l leupeptin, 3 μ g/ μ l aprotinin). Lysates were sonicated and centrifuged at 7000 x g for 10 min at room temperature, and supernatants were collected.

2.2.10.2 Isolation of detergent (membrane proteins) and aqueous (soluble proteins) protein fractions

(modified Bordier, 1981)

A solution of the nonionic detergent Triton X-114 is homogeneous at 0 °C, but separates in an aqueous phase and a detergent phase above 20°C. Hydrophilic proteins are found exclusively in the aqueous phase and integral membrane proteins with an amphiphilic nature are recovered in the detergent phase. Triton X-114 is used to solubilize membranes and whole cells, and the soluble material is submitted to phase separation. Integral membrane proteins can thus be separated from hydrophilic proteins and identified as such in crude membrane or cellular detergent extracts.

1. Tissue was homogenized in small amount of 2% Triton X-114 in PBS, resuspended in 50 ml of 2% Triton X-114 in PBS and proteins were extracted over night in 4°C with agitation.
2. Sample was centrifuged 10 min at 13,000 g in 4°C to remove cell debris.
3. Supernatant was transferred to a new tube and incubated 30 min in 37 °C followed by centrifugation for 10 min at 13,000 x g in RT.
4. The aqueous phase was transferred to a new tube.
5. The detergent phase was mixed with 30 ml of PBS at 0°C, next warmed up to 37°C for 30 min and centrifuged as before, aqueous phase was discarded.

6. Detergent phase (about 5 ml) and 5 ml of aqueous phase were precipitated with 10 volumes of acetone in -20°C for 1 hr, pelleted by centrifugation for 10 min at 13,000 x g.
7. Protein pellet was resuspended in PBS.

2.2.10.3 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 H₂O 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).

2.2.10.4 Coupling of the synthetic peptide to BSA

20 mg of BSA (~ 0.3 µmol) were dissolved in 0.5 ml of 0.4 M PBS, pH 7.5. 10 µmol of synthetic peptide was dissolved separately in 1.5 ml of water and the pH was adjusted to 7.5. The solutions of BSA and peptide were mixed and added drop by drop during a course of 5 min time into a solution of 10 µM glutaraldehyde under continuous stirring. This composite mixture was stirred further for 30 min. The unincorporated glutaraldehyde was inactivated by adding 0.1 volume of 1 M glycine solution and the sample was stirred for 30 min and finally dialysed against PBS for overnight.

2.2.11 Blotting techniques

2.2.11.1 Southern blotting of DNA to nitrocellulose filters

(Southern, 1975)

In Southern blotting, the transfer of denatured DNA from agarose gels to nitrocellulose membrane is achieved by capillary flow. 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.

2.2.11.2 Northern blotting of RNA onto nitrocellulose filters

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

2.2.11.3 Western blotting of protein onto PVDF membrane

(Gershoni and Palade, 1982)

Semidry buffer (1x): 25 mM Tris pH 8.3

150 mM Glycin

10 % Methanol

After the electrophoresis of proteins on a SDS-PAGE, the gel and the PVDF membrane, which was cut at the size of the gel, was first moistened with methanol and then equilibrated in semi dry transfer buffer. Six pieces of GB004 Whatman filter paper were also cut at the size of the gel. First, three papers soaked with transfer buffer were placed on semi dry transfer machine's lower plate and then equilibrated membrane was placed over them. Next the gel was placed avoiding any air bubbles. Another three Whatman paper soaked with transfer buffer were placed over to complete the sandwich model. The upper plate was placed over this sandwich and the transfer was carried out at 3.5 mA/cm^2 for 1 hr.

2.2.12 “Random Prime” method for generation of ^{32}P labelled DNA

(Denhardt, 1966; Feinberg and Vogelstein, 1989)

Rediprime™ II Random Prime Labeling System (Amersham Pharmacia) was used for labelling of DNA probes. The method depends on the random priming principle developed by Feinberg and Vogelstein (1989). The reaction mix contained dATP, dGTP, dTTP, 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 to Rediprime™ II Random Prime Labeling System cup, 4 μl of [α - ^{32}P] dCTP (3000 Ci/mmol) was added to the reaction mixture. The labelling reaction was carried out at 37°C for 1 hr. The labelled probe was purified from unincorporated [α - ^{32}P] dCTP by using microspin columns (Amersham Pharmacia).

2.2.13 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, namely 377 DNA Sequencer (ABI PRISM). 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 μ l containing 1 μ g plasmid DNA or 100-200 ng purified PCR products, 10 pmol primer and 4 μ l 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 μ l of loading buffer, denatured at 95°C for 3 min, and finally loaded into the sequence gel.

2.2.14 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 salmon DNA, the membrane was incubated for 2 hrs in the hybridization oven at an appropriate temperature, which was usually 65°C. Then, the labelled probe was denatured at 95°C for 10 min, quick chilled, 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 it 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.

2.2.15 Generation of polyclonal antibody against peptide

2.2.15.1 Peptide analysis

Different computational tools were applied to select potential antigenic peptides. Before synthesis of the peptide, a hydrophilicity/hydrophobicity profile analysis was carried out and for further confirmation antigenicity prediction was performed. In next step, predictions of secondary structure such as β - turns and α -helices in combination with the surface probability

of the protein region were the parameters which enabled us to select the best peptides. In the last step, we compared primary sequence of our protein with international data bank to select unique sequence for antibody generation. Two peptides were selected and synthesised. The sequences of peptides are as follows:

Peptide EP010132 : N₂H-CRP PKN YKS PSQ SVV Y-CONH₂ (16 aa)

Peptide EP010133: N₂H-CKR NER KIV PQG EHK I-CONH₂ (16 aa)

2.2.15.2 Immunisation of rabbit

Eurogentec Company did immunisation under DOUBLE X program. Two peptides were selected and synthesised instead of one. Using modern algorithms for peptide selection, the success rate for peptide immunization can be as high as 75 %. This still means a 25 % chance of failure. Under DOUBLE X program the success rate is increased to $1-25\%^2 = 93.75\%$. 5 mg of each peptide were conjugated with carrier protein molecules (KLH) as described in 2.2.10.4 and mixed together before immunisation. Two rabbits were immunised with 100 µg of antigen mixed with Freund's complete adjuvant in 1:1 ratio. Before injection, pre-immune sera were collected from the animals. After 14 days a second booster immunisation was performed with 1:1 ratio of antigen with Freund's incomplete adjuvant. A third booster was given after 28 days and final bleeding was done after 36 days. The antiserum was aliquoted and stored at -80°C.

2.2.15.3 Determination of titre of polyclonal antibody

After SDS-PAGE and electrotransfer of the total mouse proteins to a PVDF membrane, the membrane was blocked with 20 ml of blocking solution II for 1 hr at RT. Then the filter was cut and each lane was incubated with different dilutions of antiserum (1:25, 1:100, 1:500) in washing buffer for overnight at 4°C. Thereafter, the unbound antiserum was removed by washing the membrane 3 times for 20 min with washing buffer. The secondary antibody coupled with alkaline phosphatase was diluted 1:10000 in washing buffer, added to the blot and incubated for 1 hr. Again the unbound antibodies were removed by washing 4 times for 15 min with washing buffer. The chromogenic reaction was performed with 33 µl of NBT and

66 µl of BCIP solution in buffer AP until chromogenic precipitate develops. The reaction was stopped by washing the membrane several times with water. The membrane was air-dried and stored in the dark.

2.2.15.4 Affinity purification of polyclonal antibody against peptide

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

2.2.15.4.1 Immobilization

Sample preparation buffer:	0.1 M sodium phosphate 5 mM EDTA-Na, pH6.0
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Coupling buffer:	50 mM Tris 5 mM EDTA-Na, pH 8.5
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The peptide (10mg) was dissolved in 1 ml of sample preparation buffer. The solution was added to vial containing 6 mg of 2-mercaptoethylamine (2-MEA) and incubated at 37°C for 1.5 hrs. The mixture was cooled to room temperature and applied to the 5 ml desalting column which was equilibrated with 30 ml of coupling buffer to remove excess 2-MEA from the reduced sample. 1 ml fractions were collected and fractions 4, 5 and 6 were pooled for coupling to gel.

2.2.15.4.2 Coupling to gel and blocking nonspecific binding sites on gel

Reduced protein mixture (3 ml) was added to 2 ml SulfoLink[®] Coupling Gel column after equilibrating with 12 ml of coupling buffer. The column was mixed at room temperature for 15 min, and then incubated for 30 min without mixing. After that, the column was washed with 6 ml of coupling buffer. 2 ml of 0.05 M cysteine in coupling buffer was

applied to the column. The column was mixed for 15 min at room temperature, and then incubated for 30 min without mixing

2.2.15.4.3 Washing and deactivation

Washing buffer A:	1.0 M NaCl
Washing buffer B:	1.0 M NaCl 0.05% sodium azide in PBS

A series of alternate washings with buffer A (injection 4x4 ml) and buffer B (injection 3x4 ml) were done. Finally, 4 ml of 0.05% degassed sodium azide in PBS was injected, and then the top porous disc was inserted to the column.

2.2.15.4.4 Purification

The column was equilibrated with 6 ml of PBS. The antiserum (8 ml) was applied onto the column. The column was incubated at room temperature for 1 hr. During pumping a constant flow rate of 0.5 ml/min was maintained. The column was washed with 16 ml of PBS. Elution was done with 8 ml of glycine buffer (100 mM, pH 2.5-3.0). 1 ml fractions were collected and monitored by A280. Fractions 3 and 4 were pooled and 0.05% sodium azide was added. Purified antiserum was stored -20°C and the column was re-equilibrated with 10 volumes of PBS.

2.2.16 Generation of polyclonal antibody against fusion protein

2.2.16.1 Immunisation of rabbit

Fusion protein GST-AD27 produced in bacteria 2.2.9.3.2 due to the content of hydrophobic transmembrane domain was insoluble and was located in structures called inclusion bodies. This insoluble kind of protein serves as well as good immunogenic and was used for immunization. One rabbit was immunised with 200 µg of antigen mixed with Freund's

complete adjuvant in 1:1 ratio. Before injection pre-immune sera were collected from the animals. After 14 days a second booster immunisation was performed with 1:1 ratio of antigen with Freund's incomplete adjuvant. A third booster was given after 28 days and final bleeding was done after 36 days. The antiserum was aliquoted and stored at -80°C .

2.2.16.2 Purification of anti fusion protein antibody.

1. Antiserum was purified by adsorption to the immunizing protein.
 - a. Sera was prepared from whole blood
 - b. Protein was prepared from bacterial cultures as in 2.2.9.3.2. 0.5 - 1.0 mg protein was separated through a one well 1.5 mm thick minigel. Protein was transferred to PVDF membrane as described in 2.2.11.3. The membrane was cut on the margins and incubated with antiserum followed by alkaline phosphatase conjugated secondary antibody. Signal was developed by staining NBT/BCIP solution as described in 2.2.15.3 and the appropriate protein band was cut out from the rest of the PVDF membrane.
 - c. Serum was adsorbed onto the appropriate nitrocellulose strip by incubating the strip in 4 ml serum with agitation at 4°C overnight. The serum was removed and the strip washed twice with PBS.
 - d. Antiserum was eluted from the PVDF membrane in 5 ml 3M KSCN (0.1 % BSA) in PBS for 15 min at room temperature with strong agitation. The antisera solution was then dialysed twice for 24 hrs against 2 litres of 0.5 x PBS at 4°C .
 - e. The antiserum solution was recovered from the dialysis tubing and concentrated using Centriscart columns to about 0.5 ml.

2.2.17 Histological techniques

2.2.17.1 Tissue preparation for paraffin-embedding

Bouin's solution	15 ml Picric acid
	5 ml 37% Formaldehyde
	1 ml Acetic acid

The freshly prepared tissues were fixed in Bouin's solution for 24 hrs at RT to prevent the alterations in the cellular structure. The tissue to be embedded in paraffin should be free of water. The dehydration process was accomplished by passing the tissue through a series of increasing alcohol concentrations. For this purpose, the tissue was let in 50%, 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 was 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 mould and melted paraffin was poured into the mould to form a block. The block was allowed to cool and was then ready for sectioning.

2.2.17.2 Sections of the paraffin block

The paraffin blocks were pre-cut to the optimal size and clamped into the microtom (Hn 40 Ing., Nut hole, Germany). The cut-thickness of the paraffin embedded was for 7 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.

Serum-Formalin:	15 ml	Fresh serum
	15 ml	Glycerin
	6 ml	Formalin 6%

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

The stored slides with the paraffin sections were stained by the following method:

1. Slides were incubated three times in histoclear (Xylol) for 3 min.
2. Incubation in 100%, 96%, 80%, 70% and 50% ethanol for 2 min in each.
3. 1 min in H₂O and then 15 min in Hematoxylin.
4. Running tap water (control) for 10 min, then 1 min in dH₂O.
5. Eosin 0.1% + 2 drops acetic acid for 5 min, then in dH₂O for 1 min.
6. Incubation in 50%, 70%, 80%, 90%, 96% and 100% ethanol for 2 min in each.
7. Slides were incubated two times in histoclear (Xylol) for 3 min.

2.2.18 Indirect immunohistochemistry staining of mouse testis

Fixation and subsequent treatment of mouse testicular tissue was performed as described in 2.2.17. Adult mouse testis cross sections (7-10 μ m) were deparaffinized with roticlear solution (Roth) and rehydrated by descending ethanol concentrations. For immunostaining, sections were washed in PBS and were then incubated with a blocking solution containing 5 % sheep serum PBS for 1 hr at RT. The testis sections were incubated with affinity-purified primary polyclonal antibody for 16 hrs at 4⁰C. The tissue sections were rinsed four times in PBS and subsequently incubated with Alkaline Phosphatase-conjugated goat anti rabbit Ig (1:100; Sigma) for 1 hr at RT. After incubation with secondary antibody sections were washed again in PBS and then were covered with approximately 200 μ l color solution (45 μ l NBT /35 μ l BCIP solution in AP buffer) and incubated in a humid chamber for 2 – 24 h in the dark. Color reaction was stopped by incubating the slides in TE buffer and dipping slides briefly in distilled water. Sections were counterstained for 1 – 2 min in 0.1% nuclear Fast Red in distilled H₂O and mounted prior to microscope analysis. Immunostaining of the sections was examined using a microscope (BX60; Olympus). In case of anti-peptide antibody, antigen retrieval was needed as follows: slides were immersed into citrate buffer (0.01 M, pH 6.0), microwaved for 5 min high power, 5 min medium power, 5 min low power and than immersed in cold PBS. Prior to localisation of ADAM 27 on mature sperm, epididymal sperm suspension was isolated as described in paragraph 2.2.22. Sperm suspension were spread onto

pre-treated glass slides, air-dried and fixed in acetone:methanol (1:1) fixative for 10 min at 4⁰C, next washed in PBS and immunostained as described above.

2.2.19 Fluorescence in situ hybridization (FISH)

DNA of the ADAM 27 genomic cosmid clone was labeled with digoxigenin-11-dUTP by nick translation and hybridized in situ to metaphases of the WMP-1 cell line (Zörnig et al., 1995) from newborn WMP mice carrying Robertsonian translocation chromosomes (Said et al., 1986). Signal detection via fluorescinated avidin (FITC-avidin) was performed as described (Lichter et al., 1988). Chromosomes were counterstained with 4',6'-diamidino-2-phenylindole dihydrochloride (DAPI). Images of emitted light were captured separately by use of the DAPI and FITC filter set and subsequently merged and aligned.

2.2.20 Techniques for production of targeted mutant mice

(Joyner, 2000)

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

2.2.20.1 Production of targeted embryonic stem cell clones

2.2.20.1.1 Preparation of EMFI feeder layers

A frozen vial of EMFI cell was quickly thawed at 37°C and transferred to 10 ml EMFI medium. After centrifugation at 270xg for 5 min, the cell pellet was gently resuspended in 10 ml EMFI medium and plated on a 50mm culture flask. Cells were incubated at 37°C, 5% CO₂. When the cells formed a confluent monolayer (three days), they were either trypsinized, transferred to five 150 mm dishes and grown until they formed confluent monolayer, or directly treated with mitomycin C. To treat the EMFI with mitomycin C, the medium was removed and 10 ml fresh medium containing 100 µl mitomycin C (1mg/ml) was added. After 2-3 hrs of incubation, the monolayer of cells was washed twice with 10 ml PBS. The cells were then resuspended with 10 ml medium, and gentle pipetting dissolved any cell aggregates. The cells were centrifuged, resuspended in EMFI medium and plated onto dishes, which were treated with 0.1% gelatine for 30 min. The feeder cells were allowed to attach by incubation overnight at 37°C, 5% CO₂ or used after 2 hrs of incubation. Before adding ES cells on the feeder layer, the medium was changed to ES cell medium.

2.2.20.1.2 Growth of ES cells on feeder layer

One vial of frozen ES cells was quickly thawed and cells were transferred to a 12 ml tube containing 6 ml ES cell medium. After centrifugation, the cell pellet was resuspended in 5 ml ES cell medium and plated on 60 mm dishes containing EMFIs at 37°C, 5% CO₂. Next day the medium was changed. The second day, cells were washed with PBS, treated with 2 ml trypsin/EDTA at 37°C, 5% CO₂ for 5 min. The cells were gently pipetted up and down to dissolve cell clumps, resuspended with 5 ml ES medium and centrifuged. The cell pellet was resuspended in 10 ml ES cell medium and distributed either to 5 or 6 dishes (60 mm) or to 2 dishes (100 mm) containing feeder layers. The cells were passaged every second day as described above.

2.2.20.1.3 Electroporation of ES cells

ES cells, which have grown for two days on 100 mm dishes, were trypsinized. The cell pellet was resuspended in 20 ml PBS and centrifuged. The cell pellet was then resuspended in 1 ml PBS. 0.8 ml of cell suspension was mixed with 40 µg of linearized DNA-construct and

transferred into an electroporation cuvette. The electroporation was performed at 240 V, 500 μ F with the BIO RAD gene pulser™. After electroporation, the cuvette was placed on ice for 20 min. The cell suspension was transferred from cuvette into 20 ml of ES cell medium and plated onto two 100 mm dishes containing feeder layers. The medium was changed every next day. Two days after the electroporation, the drugs for the selection were added (active G418 at 150-250 μ g/ml and gancyclovir at 2 μ M). The medium was changed every day. After about eight days of selection, drug resistant colonies have appeared and were ready for screening by Southern blot analysis.

2.2.20.1.4 Growing ES cells for Southern blot analysis

The drug resistant colonies that were formed after about eight days of selection were picked with a drawn-out Pasteur pipette under a dissecting microscope. Each colony was transferred into a 24 well plate containing feeders and ES cell medium. After 2 days, the ES cells were trypsinized with 100 μ l trypsin for 5 min and resuspended in 500 μ l ES cell medium. Half of the cell suspension in each well was transferred to a well on two different 24 well plates, one gelatinised plate, and the other containing feeder cells (master plate). The gelatinised plate was used for preparing DNA and the master plate was kept frozen.

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

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

2.2.20.3 Detection of chimerism and mice breeding.

The most convenient and readily apparent genetic marker of chimerism is coat colour. Chimeric males (and sometimes females) are test bred to ascertain contribution of the ES cells to germ line. Once a germ line chimera has been identified, the first priority will be to obtain and maintain the targeted allele in living animals (inbred background). The chimeras were bred with C57BL/6J and with 129X1/SvJ background mice to compare the phenotype in two different genetic backgrounds.

2.2.21 Generation of transgenic mouse.

Generation of transgenic mice was performed by “Transgenic Service” of Max Planck Institute for Experimental Medicine in Goettingen. Method for transgenic animal production was based on Hogan et. al. 1986.

2.2.21.1 Preparation of DNA for pronuclear microinjection.

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

2.2.22 Determination of sperm parameters

2.2.22.1 Sperm count in epididymes, uterus and oviduct.

Epididymes of mice were dissected under aseptic condition and put in 0.5 ml of IVF medium. Spermatozoa were allowed to swim out of the epididymes for 1 hr at 37⁰C, 5 % CO₂. Sperm suspension was diluted 40 times with PBS before counting. 5 µl of this suspension was put into Neubauer counting chamber and sperms were counted in 8 independent fields (each having an area of 0.0025 mm²) under the microscope (Olympus BX60) with 20X magnification. Total sperms were calculated by following formula:

Total Sperm = average of sperms x 10 x 40 x 500

For determination of sperm number in the uterus and the oviduct female wild type mice were mated with ADAM 27 -/- males. The uteri and oviduct of those mice, which were positive for vaginal plug, were dissected in IVF medium and the sperm were flushed out.

2.2.22.2 Sperm motility

10 µl of sperm suspension was put on a dual sided sperm analysis chamber. Sperm motility was quantified using the computer assisted semen analysis (CASA) system (CEROS version 10, Hamilton Thorne Research). Then, 6,000-10,000 spermatozoa from 4 mice of mutant line and wild-type were analyzed using the following parameters: straightness (STR), path velocity (VAP), straight line velocity (VSL) For statistical analysis, frequencies of the six sperm motility parameters VAP, VSL, VCL, ALH, BCF and STR were examined by probability plots categorised by mouse type (wild-type/mutant) and by time of observation (1.5, 3.5 and 5.5 h after preparation).

2.2.22.3 Acrosome reaction

Sperms were isolated and capacitated by incubating for 1 hr at 37⁰C, 5 % CO₂. The sperms were transferred into two microcentrifuge tubes and centrifuged for 2 min at 3000xg. The

supernatant was aspirated, leaving only 50 µl for resuspension of sperms. 2.5 µl of Ionophore A23187 (end concentration 10 µM in DMSO) was added to sperm suspension, for negative control 2.5 µl of phosphoric acid (5 mM) was added and incubated at 37°C for 1 hr. The sperms were then fixed in 500 µl of 2 % formaldehyde (in PBS) for 30 min at 4°C. After completion of fixation sperms were centrifuged at 4000 x g for 2 min. Sperms were further washed twice with 0.15 mM ammonium acetate. Finally they were resuspended in 100 µl of PBS and 30 µl of suspension was spread on superfrost slide and air-dried. The slides were stained with coomasie G-250 in 3.5 % H₂O₂ for 2.5 min, unbound dye was removed by washing several times with water. The slides were mounted with 30 % glycerol and observed under microscope. Sperms with and without blue head were counted at least for 100 sperms. Here, blue head sperms means those sperms which failed to undergo acrosome reaction. The acrosome reaction was calculated as follows:

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

2.2.23 Sperm egg binding assay.

2.2.23.1 Oocyte isolation and Zona pellucida removal

Mature oocytes were collected from 8-12-week-old superovulated NMRI or CD-1 female mice by intraperitoneal injections of 5 IU of pregnant mare serum gonadotrophin (PMSG) followed by 5 IU of human chorionic gonadotrophin. Eggs were incubated with hyaluronidase (3 mg/ml) in embryo culture medium (M16) for 5-10 min at 37°C to dissociate cumulus cells, and washed through three 200-µl drops of fresh M16. To prepare zona-free eggs, oocytes were treated for 15 to 30 sec in acidic Tyrode's solution, pH 2.7. Eggs were transferred into IVF medium, overlaid with light mineral oil, and incubated for 1 hr at 37°C in a 5% CO₂ incubator before use.

2.2.23.2 Sperm-egg binding assays

Eggs with or without Zona pellucida were placed in 50- μ l drops of IVF medium supplemented with test proteins to final concentration of 0.5 μ g/ μ l . Eggs were incubated with test proteins for 1 hr prior to the addition of sperm. Capacitated sperm were added to the drops at final concentration of 500 000 sperm and incubated for 30 min at 37 °C in a 5% CO₂ incubator for Zona-free eggs and 1 hr for eggs with Zona. Eggs were washed three times in fresh medium to remove loosely attached sperm. All oocytes from one experiment were collected in 10 μ l of IVF medium; sperms were released by gentle pipetting and were counted as described in 2.2.22. Sperm number per oocyte was calculated.

2.2.24 Yeast two hybrid using DupLEX-A system.

The DupLEX-A™ system is a LexA-based version of the yeast two-hybrid system originally developed by Fields and Song. The yeast two-hybrid system has proven to be a powerful tool for identifying proteins from an expression library which can interact with the protein of interest. The DupLEX-A system was developed as a more versatile and more accurate version of the yeast two-hybrid system. The two-hybrid system of Fields and Song exploits the fact that a yeast transcriptional activator protein, Gal4p, has a separable DNA binding domain and activation domain; neither domain can activate transcription on its own. Transcriptional activation is detected only when the binding domain is bound to its DNA recognition sequence and is also tethered to the activation domain. The two-hybrid system involves fusing the Gal4p binding domain with a protein “X” and the Gal4p activation domain with a protein “Y” (Figure 2.3). If “X” and “Y” interact, then a functional Gal4p is restored and transcriptional activation can be detected (Figure 2.4). If binding sites for Gal4p are placed upstream of a reporter gene (such as lacZ), transcriptional activation can be monitored easily. The DupLEX-A system utilizes the same basic idea except that the DNA binding protein is the *E. coli* LexA protein while the activation protein is the acid blob domain B42. Neither LexA protein bound upstream of a reporter gene nor B42 alone can activate transcription of the reporter, but if brought together via fusions with two interacting proteins, reporter gene expression can be detected.

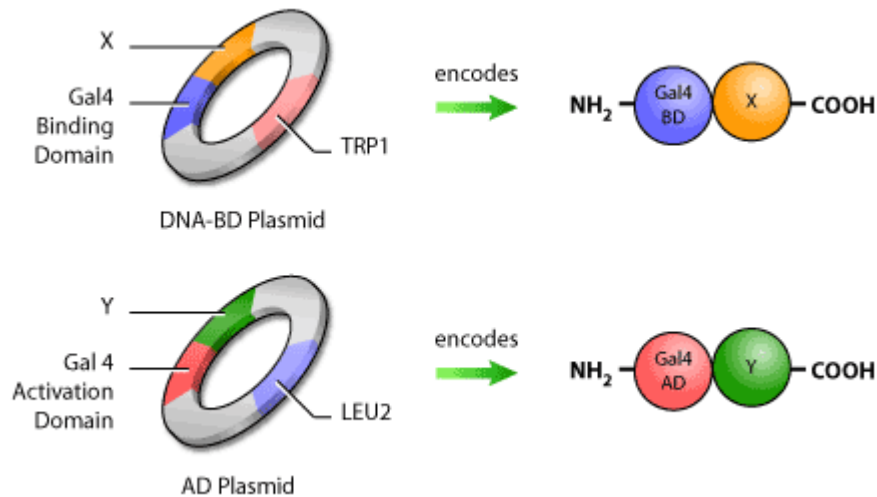


Figure 2.3 The yeast two-hybrid plasmid constructs: the bait plasmid, which is the protein of interest (X) fused to a GAL4 binding domain, and plasmid which is the potential binding partner (Y) fused to a GAL4 activation domain.

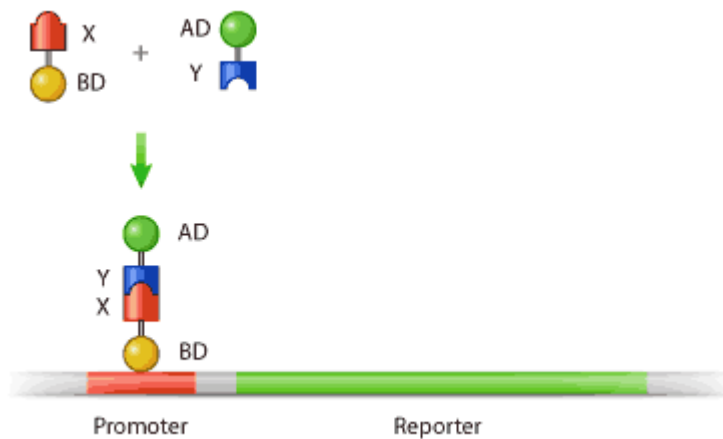


Figure 2.4 Yeast two-hybrid transcription. The yeast two-hybrid technique find protein-protein interactions by triggering transcription of a reporter gene. If protein X and protein Y interact, then their DNA-binding domain and activation domain will combine to form a functional transcriptional activator (TA). The TA will then proceed to transcribe the reporter gene that is paired with its promoter.

2.2.24.1 Small-scale yeast transformation protocol

1. 5 ml culture of EGY48 was grown in YPD at 30°C with shaking (overnight) by inoculating with a colony from freshly streaked plate of EGY48.

2. OD600 of a 1:10 dilution of the overnight culture was measured. OD600 of the 5 ml overnight culture was calculated and appropriate amount was used to inoculate a 60 ml YPD culture to an OD600=0.1. Next, culture was grown at 30°C with shaking.
3. When the OD600 =0.5-0.7 (approximately 4-6 hours after inoculation), cells were pelleted by spinning the culture at 1500 x g for 5 minutes. Pellet was resuspended in 20 ml of sterile distilled water, spun again, and resuspended in 0.3 ml of 1 x TE/LiOAc. 100 µl was put into each of three sterile 1.5 ml eppendorf tubes.
4. Carrier DNA was boiled 5 minutes and quickly chilled on ice.
5. 100 ng of each plasmid DNA and 50 µl of denatured carrier DNA was added to each tube and mixed.
6. 0.3 ml of 1 x TE/LiOAc/PEG was added, mixed by inversion, and the tubes were placed at 30°C for 30 minutes.
7. 70 µl of DMSO (dimethyl sulfoxide) was added to each tube, mixed by inversion, and tubes were incubated at 42°C for 15 minutes.
8. Tubes were spun at 1500 x g in a microcentrifuge for 10 seconds, the supernatant was poured off, and each pellet was resuspended in 0.5 ml of sterile distilled water.
9. 50-100 µl of each was spread onto separate YNB(glu)-his-ura plates and incubated at 30°C for 2-3 days. 8 colonies from each plate were used for β-galactosidase chloroform-agarose overlay assay described below.

2.2.24.2 Chloroform-agarose β galactosidase overlay assay (*Duttweiler 1996*).

1. Eight colonies from each tested transformation were resuspended in distilled water, plated on Glu-Ura-His and Gal-Ura-His plates and grown 1-2 days in 30°C (Figure 2.5).

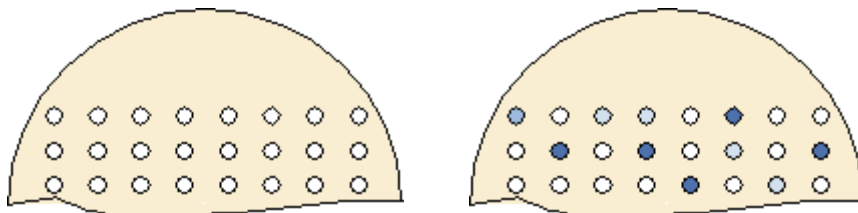


Figure 2.5 Graphical representation of overlay assay technique.

2. Plates were overlaid with chloroform and incubated for 5 min.
3. Remaining chloroform was decanted and plates were dried for 10 min in the hood.

4. Colonies were overlaid with 7-10 ml of X-Gal agarose (1% low-melting agarose, 0.1 M NaHPO₄ buffer pH7.0, 0.25 mg/ml X-Gal).
5. After agarose hardened plates were incubated at 30°C until development of color (Figure 2.5).

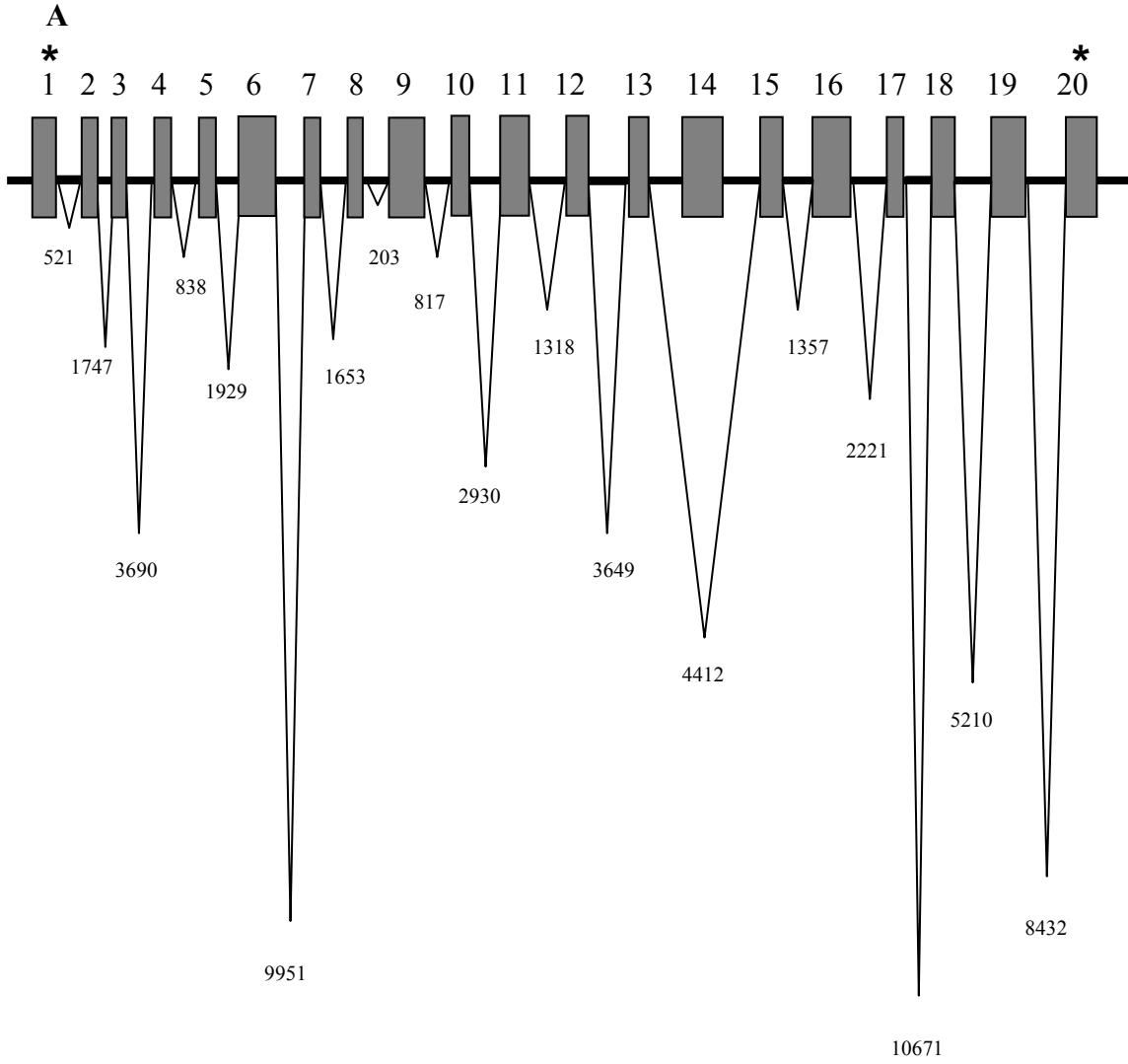
2.2.25 Computer analysis

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 proteins studies ExPASy tools (www.expasy.ch) were used. Mouse genome sequence and other analysis on mouse genes, transcript and putative proteins were downloaded from Celera discovery system (www.celera.com).

3 RESULTS

3.1 Introduction to result section of *ADAM 27*

ADAM 27 gene was published by Zhu et al. in 1999 (GI:6753683). By comparison of cDNA and mouse genomic sequence available in internet databases (NCBI, Celera) we were able to determine exon-intron structure of the gene. *ADAM 27* is composed of 20 exons, in size from 70 to 200 bp, separated by introns of different length (Figure 3.1A). Genomic structure of human orthologue gene *ADAM 18* is identical to mouse; both genes have 20 exons of similar size (Figure 3.1B). The gene was localized to mouse chromosome 8A2 using FISH hybridization technique (2.2.19) with cosmid clone containing *ADAM 27* genomic sequence (Figure 3.2). Human *ADAM 18* locus is found in syntenic region to mouse on chromosome 8 p11.22. Most of the members of ADAM family are located on chromosome 8. *ADAM 27* is localized at the end of ADAM gene cluster, containing genes for *ADAM 9*, *ADAM 32*, *ADAM 5* and *cyritestin*. *ADAM 27* is around 3 kb far away from end of *cyritestin* gene. In cytogenetic band 4 at least three more genes are localized, namely *ADAM 24-26* also known as *testases 1-3*. It was shown that at least *testase 2* exists as tandem duplication (Bolcun et al., 2003).



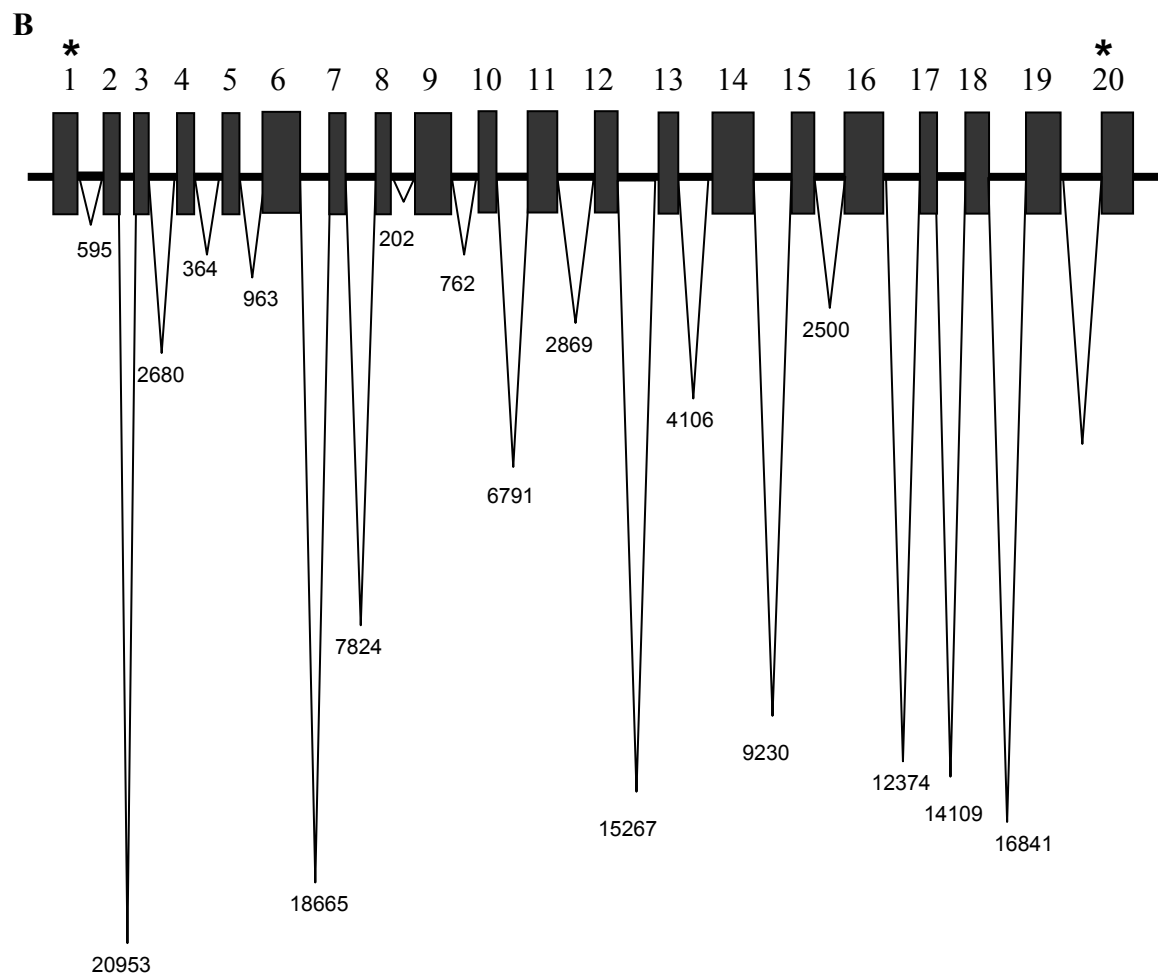


Figure 3.1 Schematic representation of the exon-intron structure of mouse *ADAM 27* (A) and human orthologue *ADAM 18* (B). Translational start and stop sites are marked by asterisks. Numbers on the top of the pictures indicate exons, numbers on the bottom indicates length of the introns.

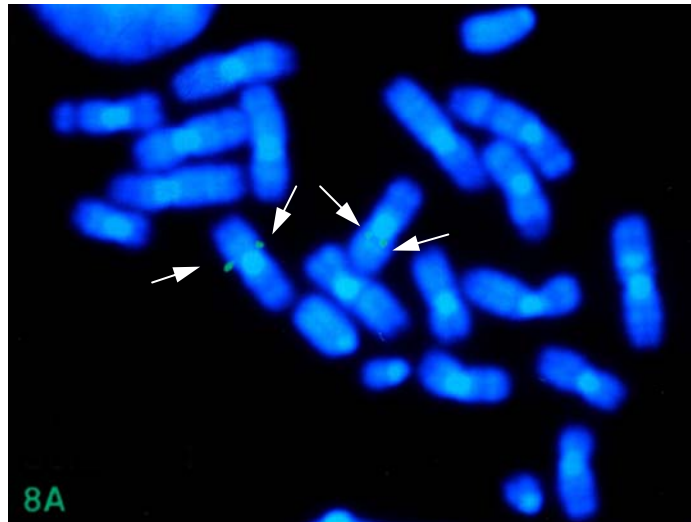


Figure 3.2 Chromosomal localization of the *ADAM 27* gene by fluorescence in situ hybridization (FISH) to mouse metaphase chromosomes. Arrows point to specific *ADAM 27* signals on chromosome 8A.

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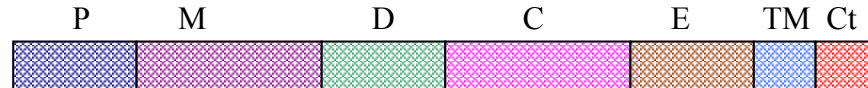
*
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M P L L F I L A E L A M L F A R L D S E G I C L H I T V P Q
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Results

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1317aggctaaaaggctcagcacaatgtggatctggagcttgcctgcatgccacgtgtgagctgtcagcatcaggcacgacctgttagaaagct
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                                EPO10132 synthetic peptide
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    I F L P F I V T F L V G I M K R N E R K I V P Q G E H K I *
                                EPO10133 synthetic peptide
2217gggccattcattgcaccagaaatcgccaaggaagctagcagttgatggacccaaggaggaaagtgtgtgttctaaactgctctgttaccgg
    tggatacactttaaatctcagacttttgaaaaataaaactgatatatgcacgaaaaaaaaaaaaaaaaaaaaa
  
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Prodomain Metalloprotease Disintegrin Cystein-rich EGF-like Transmembrane Cytoplasmic tail

Figure 3.3 Nucleotide sequence, deduced amino acid sequence and schematic view of *ADAM 27* cDNA.

Numbering of nucleotide is given on the right, translation start and end codons are bold and indicated by asterisks. In deduced amino acid sequence, domains are marked with different colors. The amino acid sequences of synthetic peptides used for generation of polyclonal antibody are underlined.

3.1.1 *ADAM 27* expression analysis

3.1.1.1 Transcriptional analysis

ADAM 27 was first described by Zhu et al. (1999) as a testis specific gene. In Northern blot analysis using RNA from testes of males of increasing age (8-40 days) they found that transcription starts at day 20 postnatal. In order to confirm these results, more sensitive

method (RT-PCR) was used. RT-PCR was performed on testicular RNA from 5 to 25 days old and adult males. Additionally, RT-PCR was done using RNA from testes of different mutants with spermatogenesis defects at different stages of germ cells development. w/w^v mutant is characterized by lack of germ cells in testes, in *Tfm* and *Insl3* mutant mice spermatogenesis is arrested at spermatocyte stage whereas in *olt* and *qk* at spermatid stages. *ADAM 27* transcript is detectable by RT-PCR already at day 15 (Figure 3.4), but expression level is increased in developing testes. Presence of transcript in *Tfm* and *Insl3* mutants indicates that *ADAM 27* transcription starts as early as in spermatocytes. Lack of the detectable transcript in w/w^v mutant shows that gene expression is restricted to germ cells. To check this, RT-PCR was performed on the template of RNA isolated from MA10 and 15P1 cell lines corresponding to Leydig and Sertoli cells, respectively. Negative result for both cell lines confirms germ cell specific expression of the *ADAM 27* gene (Figure 3.5).

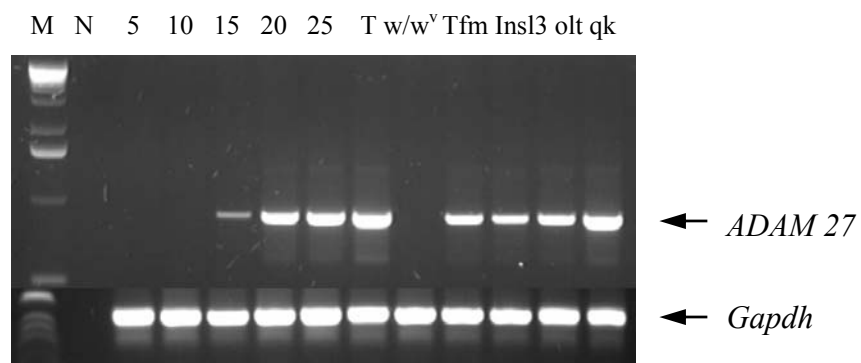


Figure 3.4 RT- PCR analysis on total RNA isolated from various developmental stages of mouse testis development and testes of different mutants using *ADAM 27*-specific primers. Expression is present from day P15 and continues during testicular development. Expression was detected in all mutants except w/w^v mice, indicating germ cell specific expression of the gene. M: standard molecular weight marker, N: negative control (H_2O). RT-PCR for *GAPDH* transcript was performed as positive control.

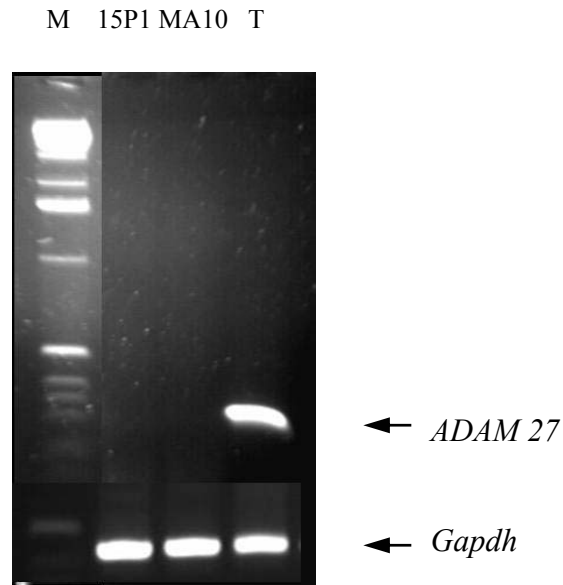


Figure 3.5 RT-PCR analysis of *ADAM 27* transcription in Sertoli and Leydig cell lines performed on total RNA isolated from cultured cells. Expression was detected only in testicular RNA. M: marker, 15P1: Sertoli cell line, MA10: Leydig cell line and T: testis. *Gapdh*: control for RNA quality.

3.1.1.2 Translational analysis

3.1.1.2.1 Expression pattern of ADAM 27 in adult mice

In order to analyze the translation of *ADAM 27*, antibodies against this protein were generated. Polyclonal antibodies against two *ADAM 27* synthetic peptides were generated as described in Materials and Methods section 2.2.15. The positions and sequences of the peptides are shown in Figure 3.3. Both peptides were checked for immunogenicity by “dot blot” hybridization with antiserum, different amounts of synthetic peptides were spotted on the Hybond-C membrane and were let to dry. After that, membrane was incubated with sera as in classical Western blot procedure. EPO10132 peptide localized in Cys-rich membrane is not recognized by antiserum whereas using peptide EPO10133 localized in cytoplasmic domain, immunoreactivity was detected (Figure 3.6), indicating that only the second peptide was immunogenic. In order to check affinity of generated antibodies, Western blot analysis was performed. Total protein extracts from testis and kidney, as control, were separated on SDS-PAGE gel and transferred to PVDF membrane. Western blots were incubated with different dilutions of antiserum (1:50, 1:100 and 1:500). Specific and unspecific bands in both

protein extracts were detected (Figure 3.7). Selected peptides had low immunogenicity and gave rise to unspecific antibodies as well.

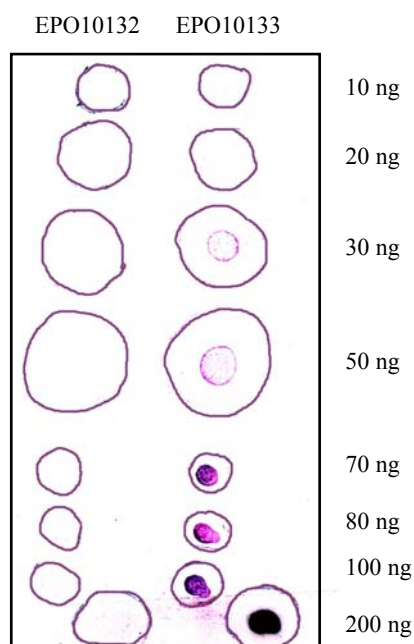


Figure 3.6 Peptide dot blot hybridization with polyclonal anti *ADAM 27* antiserum. EPO10132 and EPO10133 - synthetic peptides are localized in Cys-rich and cytoplasmic domains, respectively. Amount of spotted peptide is given on the right side.

To improve the affinity of antibodies against ADAM 27 protein, purification of the antibody was performed (2.2.15.4). An affinity column (Sulfolink, Pierce), conjugated with ADAM 27 peptide EPO10133, was used for affinity purification. After binding of antiserum to the column, fractions of purified antibody were eluted. Fraction with the highest antibody concentration was used in experiments. Western blot was performed with testicular and kidney protein extracts with 1:100 dilution of purified antibody. Signal was obtained only in testicular protein extracts but not in the kidney extracts. Additionally, testicular membranes and cytoplasmic protein fractions were included in the Western blot analysis. A protein band of 80 kDa was detected in Western blot analysis, which is in agreement with predicted molecular weight of ADAM 27. ADAM 27 band is found as expected in membrane fraction but not in cytoplasmic(Figure 3.7 C). An additional band was detected in total testicular cell extract as well as in cytoplasmic fraction (Figure 3.7). This could be result of unspecific

binding of antibody to other members of this family. Anti peptide antibodies are named anti ppADAM27 antibody in the rest of the study.

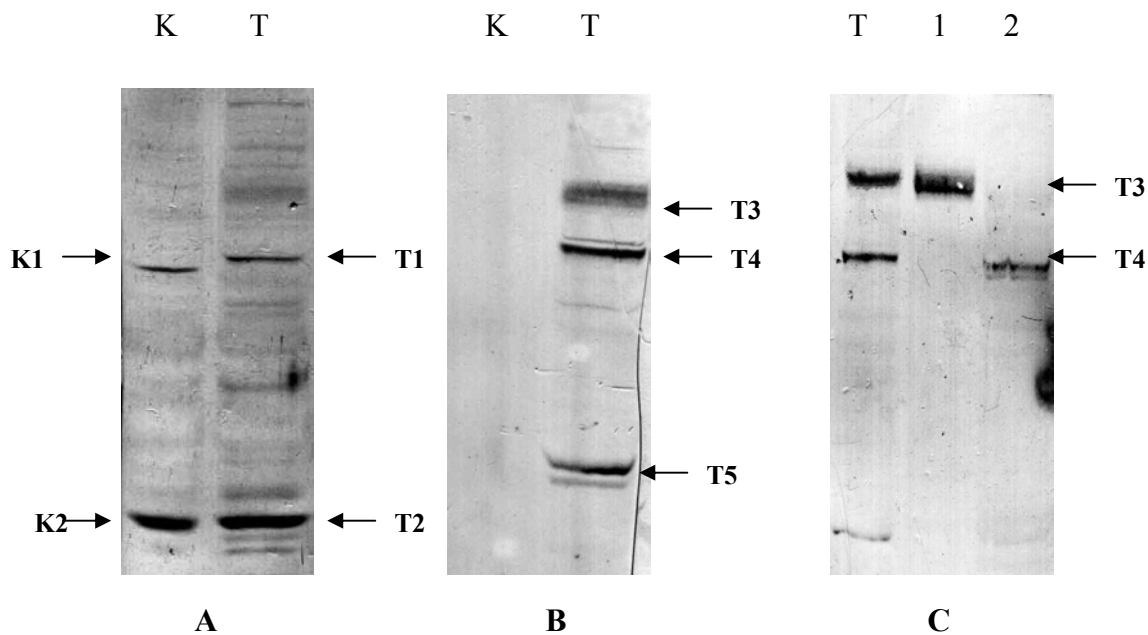


Figure 3.7 Western blot analyses with polyclonal antibody against *ADAM 27* protein. In this analysis an anti-peptide antibody in dilution of 1:100 was used. (A) Unpurified antiserum showed cross-reactivity to lots of testicular and kidney proteins (T1, T2, K1, K2). (B) Affinity purified antibody detects a strong band corresponding to 80 kDa size in testicular extract but not in kidney (T3). Purified antibody detects also additional smaller bands (T4, T5). (C) Western blot with membrane (1) and cytoplasmic (2) fractions shows that the 80 kDa band representing *ADAM 27* is a membrane protein (T3) whereas the smaller band reflects some soluble cytoplasmic protein (T4). K: kidney, T: testis.

Antibodies raised against peptide localized in cytoplasmic domain would not recognize mutated protein, if it is produced in transmembrane domain knock out mice. Therefore another antibody was generated as described in section 2.2.16 by immunization with GST-ADAM27 fusion protein. GST-ADAM27 fusion construct used to produce recombinant protein (2.2.9) was generated as described in section 2.2.8.1. Purified protein was subjected to SDS-PAGE electrophoresis, blotted to PVDF membrane and immunostained with antibodies. To detect fusion protein, antibodies against fusion tag (GST) were used, obtained signal corresponded to predicted fusion protein size. In order to check if expressed protein belongs to ADAM 27, immunodetection using anti ppADAM27 antibody was performed. As it is shown on Figure 3.8 specific anti ADAM 27 antibody recognizes the same protein band as anti GST antibody confirming that the produced protein represents a part of ADAM 27

protein. Generated GST-ADAM27 fusion protein was used for immunization of rabbit to obtain polyclonal antibody (anti fpADAM27) as described in section 2.2.16.1.

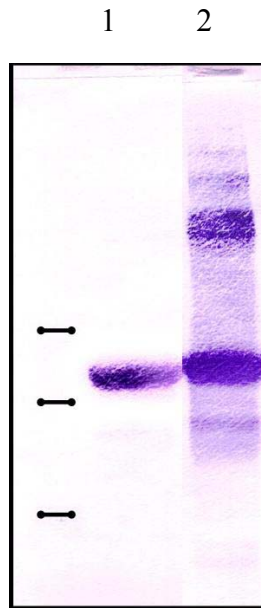


Figure 3.8 Western blot analysis using (1) anti GST and (2) anti ppADAM 27 antibodies of produced and purified recombinant GST-ADAM27 protein. Each line contains equal amount of recombinant protein.

Affinity of newly generated antiserum was checked by Western blot analysis with protein extracts from testis and kidney as negative control (Figure 3.9), as described earlier. Protein extracts were incubated with 1:100 dilution of antiserum. Antibody generated against fusion protein gave some unspecific bands in testicular extract and few in kidney (Figure 3.9 A). To improve specificity of antibody, affinity purification of serum was performed (2.2.16.2). Purified antibody (1:100) was used in Western blot analysis and signal was obtained only in testis (Figure 3.9B). In contrast to ppADAM27 antibody, this antibody did not detect the cytoplasmic protein. The size of protein band, when compared with molecular markers was 80 kDa, which was in agreement with the predicted molecular weight of ADAM 27 protein. Anti fusion protein antibodies are named anti fpADAM27 in the rest of the study.

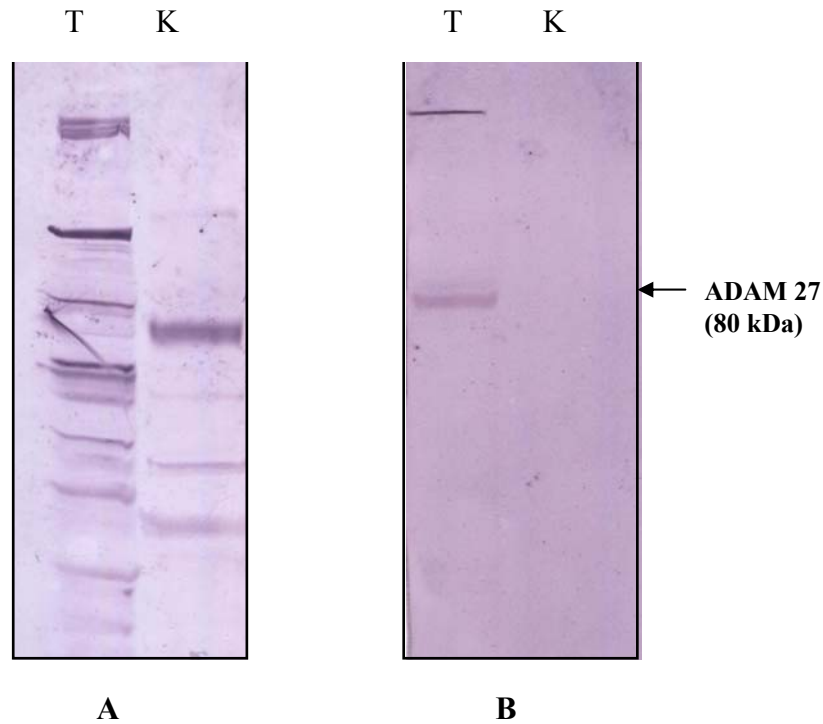


Figure 3.9 Western blot analyses with anti fpADAM27 polyclonal antibody against ADAM 27 protein. In this analysis an anti fusion protein antibody dilution of 1:100 was used. (A) Unpurified antiserum showed cross-reactivity to lots of testicular proteins. (B) Affinity purified antibody detects a band corresponding to 80 kDa size in testicular extract but not in kidney. K: kidney, T: testis.

3.1.1.2.2 ADAM 27 protein expression during different stages of mouse testis development

Total protein extracts from testes of mouse from different developmental stages were prepared, namely from postnatal stages P10, P15, P20, P25, P30, and from adult mouse. Western blot was performed with purified antibody (1:100 dilutions). ADAM 27 expression could be detected as a weak band already at P15 of mouse testicular development. The expression becomes stronger with ascending developmental stages and the expression is comparable with adult mouse at stage P30. After P30 the expression remains constant during further testicular development (Figure 3.10).

The observation that ADAM 27 protein expression starts around P15 of mouse development also supports RT-PCR analysis result, where expression was first detected at the same day. At day 15 of postnatal development, testis contains only diploid germ cells like spermatogonia,

early to late pachyten spermatocytes, so the protein is present already in diploid cells. This indicates that the expression of ADAM 27 is not under translational control.

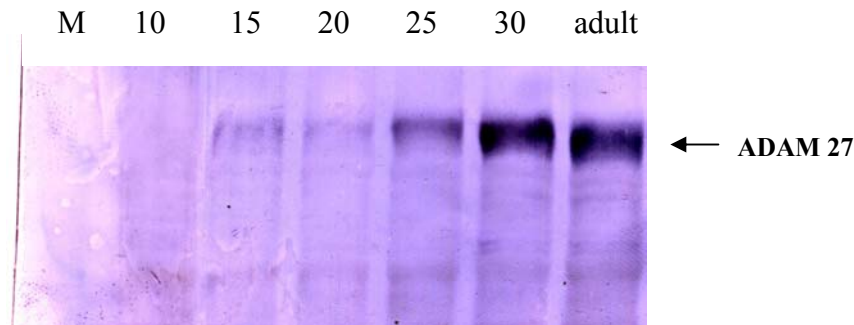


Figure 3.10 Western blot analysis using affinity purified anti ppADAM27 antibody (1:100) on total testicular protein from various developmental stages of mice: P10, P15, P20, P25, P30 and adult.

3.1.1.2.3 ADAM 27 expression during male germ cell differentiation

To determine the expression pattern of ADAM 27 during spermatogenesis, paraffin embedded adult mouse testes sections were immunostained with antibody against ADAM 27. After an initial blocking step with goat serum in PBS, the slides were incubated with affinity-purified antibody against ADAM 27 in a dilution of 1:100 followed by secondary alkaline phosphatase conjugated antibody. Specific immunostaining as dark blue color (arising from precipitation of alkaline phosphatase substrates NBT/BCIP) was observed in the center of tubule (Figure 3.11A, B). However, no specific staining was observed in Sertoli cells or in Leydig cells or when testis sections were incubated only with secondary antibody (Figure 3.11D). Strong signal corresponds to early round to elongated spermatid stages. Dot like signal in the tubule belongs to formatting Golgi apparatus in spermatocytes (Figure 3.11C, E-G).

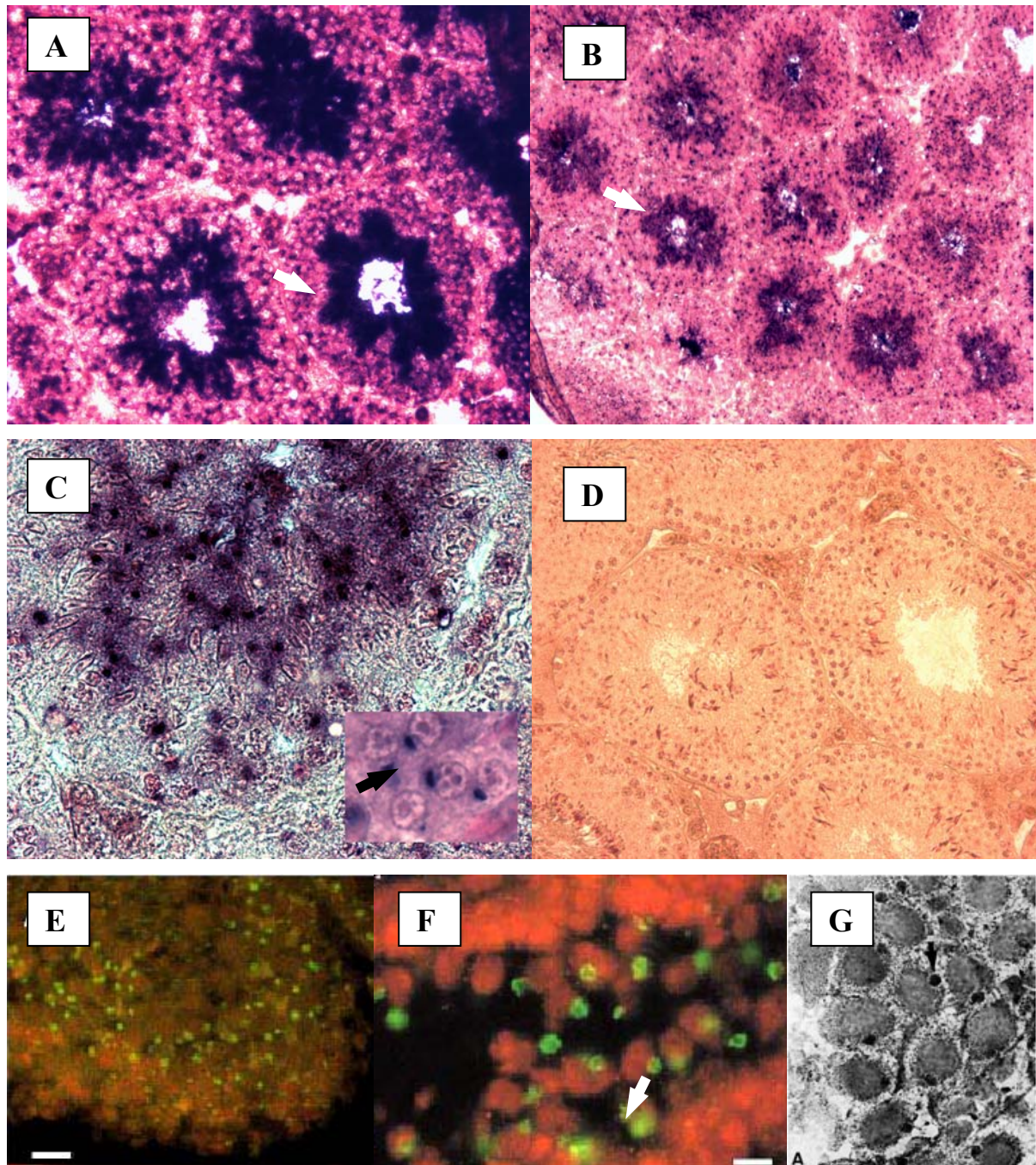


Figure 3.11 Tissue distribution of ADAM 27 protein in adult testis. (A,B) Immunostaining using anti ppADAM27 antibody followed by alkaline phosphatase conjugated secondary antibody detects strong expression of ADAM 27 in the center of tubule. (C) Higher magnifications of the tubule, vesicle like signal observed indicating presence of ADAM 27 protein in forming Golgi apparatus, right bottom magnification of spermatocytes with Golgi apparatus. (D) Incubation with only secondary antibody was performed as control for background staining. Sections were counterstained with fast red. Mea2 (E, F) and p21 marker proteins (G) localize to the Golgi complex of primary spermatocytes at the pachytene stage.

3.1.1.2.4 Localization of the ADAM 27 on mouse sperm

Three parts of epididymes were isolated from wildtype mice: caput, corpus and cauda epididymis and protein extracts were prepared. Proteins were isolated also from sperm squeezed from epididymes of wildtype mice. Protein extracts were separated on SDS-PAGE gel and transferred to PVDF membrane. Western blot was then incubated with anti ppADAM27 antibodies as described above. Protein was detected in epididymal and sperm protein extracts (Figure 3.12A), on Western blot shown in Figure 3.12B it was possible to detect protein modification during maturation of sperm in epididymis.

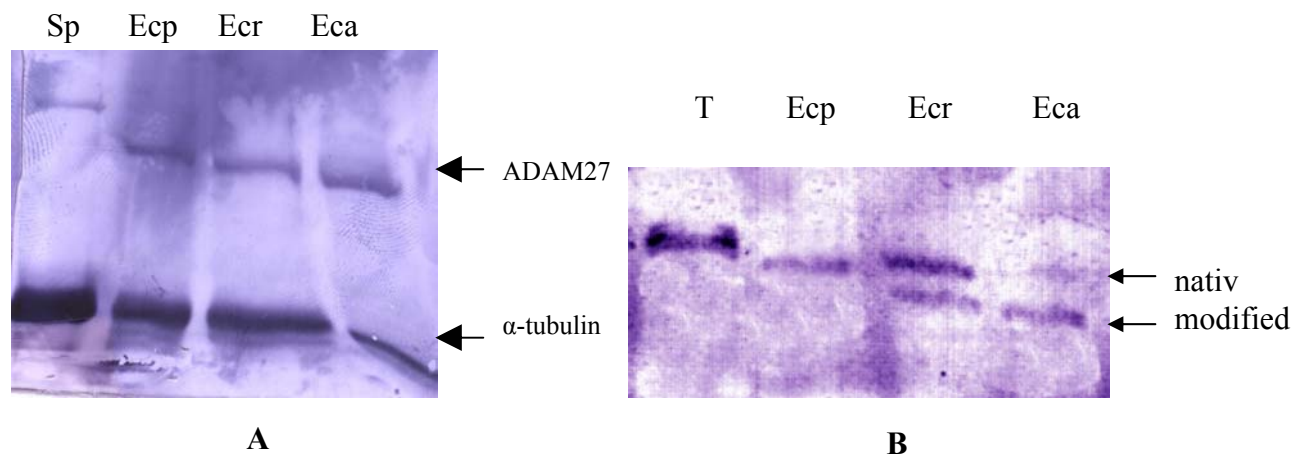


Figure 3.12 Western blot analyses of epididymal and sperm protein extracts using anti ppADAM 27 antibodies. In experiments antibody dilution of 1:100 was used. (A) ADAM 27 protein is detected in all three parts of epididymis and on sperm. (B) Posttranslational modification of ADAM 27 protein during sperm maturation in epididymis. Sp: sperm extract, Ecp: epididymis caput, Ecr: epididymis corpus, Eca: epididymis cauda and T: testiculat extract

Epididymal mouse sperm were used for immunofluorescence experiments using anti ADAM 27 antibody (anti-peptide and anti-fusion protein). Isolated sperms were incubated with anti ADAM 27 antibody, followed by incubation with FITC or alkaline phosphatase conjugated secondary antibody. No specific signal was obtained in comparison with only secondary antibody control. This result could be due to masking of antigens, or antibody is not able to detect native protein on the sperm surface.

3.1.2 Targeted inactivation of mouse *ADAM 27* gene

One of the best ways to elucidate gene function is analysis of an animal model deficient in the gene of interest. In this study such a model mouse has been generated by targeted inactivation of the *ADAM 27* gene. Phenotypic analysis would help us to understand the importance of *ADAM 27* gene in spermatogenesis or/and fertilization.

3.1.2.1 Isolation of a cosmid clones from mouse genomic DNA library

The mouse RZPD (The Resource Center and Primary Database) genomic library 129 ola was screened using a fragment of *ADAM27* cDNA (BigAD27) and 5 positive clones were obtained: MPMGc121J18198Q2; MPMGc121L12665Q2; MPMGc121A07446Q2; MPMGc121E11618Q2; MPMGc121B17781Q2. Hybridization with 5'probe showed that none of these clones contained 5' region of the gene. Clone B17781Q2 contained 3' region and was used for generation of targeting construct according to hypothesis described below. As mentioned earlier *ADAM27* protein is a membrane protein with functional domains presented on the cell surface. Targeting construct was designed to replace exon coding for transmembrane domain by *Neomycin* resistance gene (*Neo*). The mutant mouse obtained from this construct would produce mutated protein lacking hydrophobic transmembrane domain and therefore, the protein would not be integrated into membrane, but either secreted or degraded in the cell. Mouse generated by this approach would not possess functional *ADAM27* domains on the surface of the germ cells.

3.1.2.2 Genomic structure and restriction digestion analysis of the 3' end of *ADAM27* gene

The cosmid clone containing the murine *ADAM 27* gene was restricted with the enzymes *Bam* HI, *Pst* I, *Xba*I, *Not*I, *Kpn*I, *Sst*I, *Sst*II, *Eco*RI, *Xho*I and *Apa*I, blotted and radioactively hybridized with *ADAM 27* 3' fragment (Figure 3.13A). The size of fragments was calculated and compared to the known genomic structure.

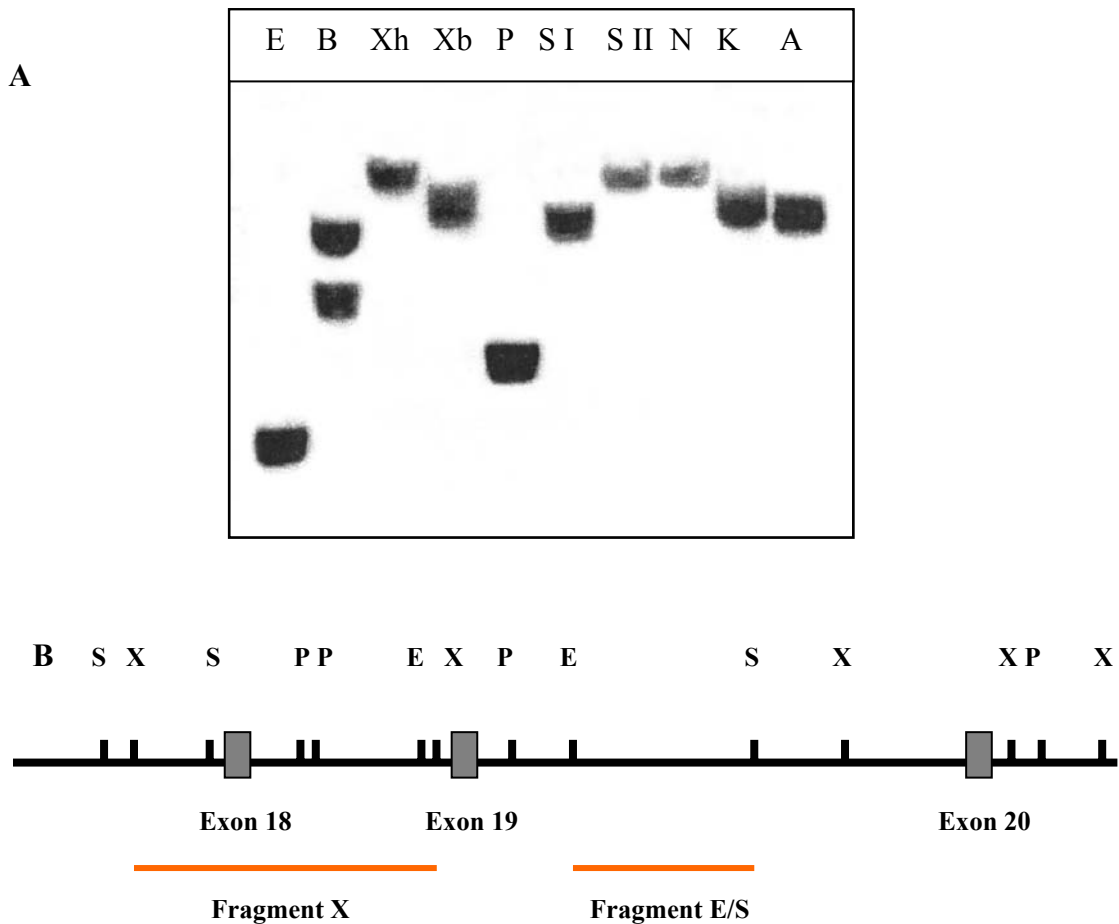


Figure 3.13 (A) Radioactive hybridization of cosmid clone B17781Q2 digested with different restriction enzymes. (B) Restriction digestion map of *ADAM27* genomic DNA and fragments which were cloned, abbreviations are: B: *Bam* HI; ERI: *Eco* RI; ERV: *Eco* RV; P: *Pst* I; SI: *Sst* I; Sp: *Spe* I; X: *Xba* I.

3.1.2.3 Generation of transmembrane knock out construct

A knock out construct was generated in which 5' wing 5 kb *Xba* I fragment was cloned into *Not* I/ *Bam* HI site of pTKNeo vector and 3' wing 3.1 kb *Eco* RI/ *Sst* I fragment was cloned into *Cla* I site of the targeting vector (Figure 3.13 B, Figure 3.14). After homologous recombination genomic fragment of about 2.4 kb containing exon 19 coding for transmembrane domain was replaced by *Neomycin* resistance gene. Neomycin was used as positive selection marker and two copies of herpes virus *Thymidine kinase* (Tk) as a negative selection marker. The correct orientation of both 5' and 3' wings was confirmed by sequencing of the construct with vector specific primers pTKNf, pTKNr, pTKr.

3.1.2.4 Generation of the 5' external probe

A fragment about 0.6 kb downstream to transmembrane domain was amplified by PCR using TM3FP and TM4RP primers. External probe sequence was selected so that the probe detects in Southern blot hybridization *Pst* I wildtype fragment specifically (Figure 3.14). This PCR product was subcloned into pGEM-TEasy and cut out with *Eco*RI enzyme. The 0.6 kb fragment was extracted from the agarose gel and used as 3' external probe for hybridization in the Southern blot with DNA extracted from the transfected ES-cells clones.

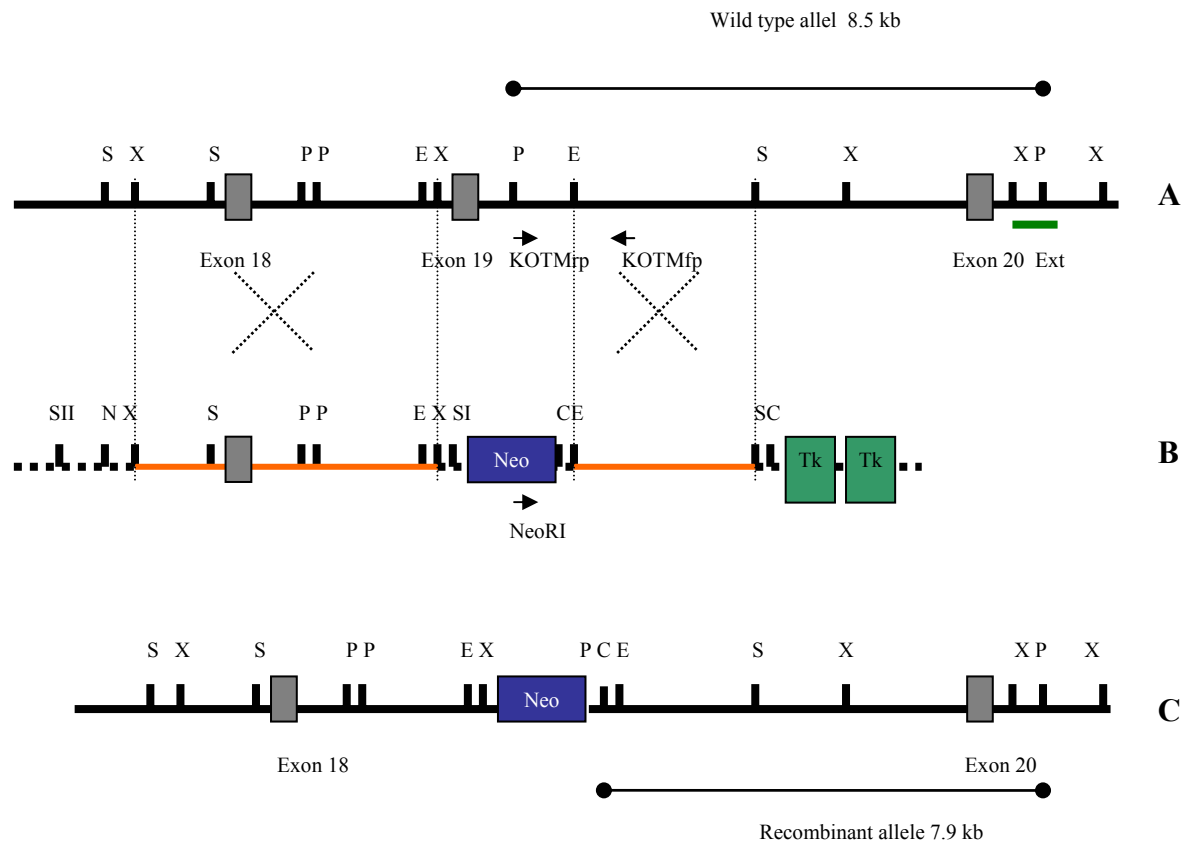


Figure 3.14 Schematic representation of the targeting strategy. Wildtype *ADAM 27* locus (A), targeting vector (B) and mutated allele (C). Exon 19 was replaced by the *Neomycin* resistance gene cassette. External probe is marked as green bar, which enables to recognize an 8.5 kb *Bam* HI fragment in wildtype DNA and a 7.9 kb fragment in recombinant DNA. The gray boxes represent exons of *ADAM27* gene, green boxes stand for *Thymidine kinase* and blue box stands for *Neomycin*. KOTMfp, KOTMrp and NeoRI are

the primers used for genotyping. The restriction site abbreviations are: B: *Bam* HI; C: *Cla* I; EI: *Eco* RI; EV: *Eco* RV; P: *Pst* I; SI: *Spe* I; SII: *Sst* II; SA: *Sal* I; Ext: external probe.

3.1.2.5 Electroporation of the RI ES- cells and screening of ES- clones for homologous recombination events

The *ADAM27* targeting vector was linearised by digesting with *Sst* II restriction enzyme and 50 µg of linearised purified DNA was electroporated into RI embryonic stem cells as described in section 2.2.20.1. The cells were plated on fibroblast feeder layer and after 10 days of selection 56 individual *Neomycin* resistant clones were picked in 24 well plates and replicated. Genomic DNA was prepared from these ES clones for Southern blot analysis. DNA of individual ES clones was digested with *Pst* I, electrophoresed into 0.6 % agarose gels and blotted onto Hybond C membranes. The blots were hybridized with ³²P-labelled 0.6 kb 3' external probe. In the case of homologous recombination event two bands were expected, a wildtype allele of 8.5 kb and recombinant allele of 7.9 kb. However, in the event of a random integration, only wildtype allele will be detected (Figure 3.14). After screening of all the ES clones, five recombinant clones were identified: TM31, TM36, TM14, TM83 and TM56. Southern blot for recombinant clone TM31 is shown in Figure 3.15.

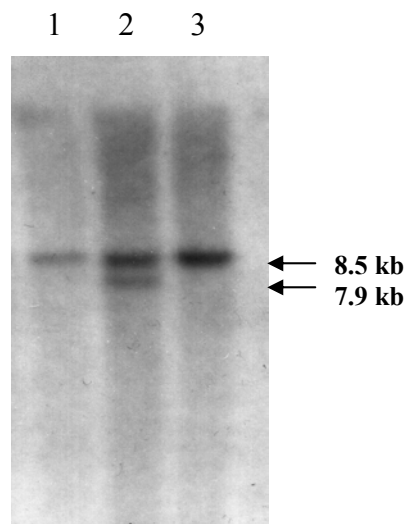


Figure 3.15 Genomic Southern blot analysis. Genomic DNA of ES clones was digested with *Pst* I, separated on 0.6 % agarose gel and transferred onto Hybond C membrane. The blot was hybridized with radioactive labeled 0.6 kb external probe. In case of wildtype allele (ES clones 1 and 3) one band of 8.5 kb was observed and in case of recombinant allele (ES clone 2) two bands were observed, in addition to wildtype allele, a recombinant allele of 7.9 kb.

3.1.2.6 Generation of chimeric mice

ES cells from the recombinant clones were injected into 3.5 dpc blastocyst derived from C57BL/6J mice. The blastocysts were implanted into pseudopregnant CD1 mice to generate chimeric mice. Eight chimeras were obtained after 2 independent injections of recombinant ES cell clones TM31 and TM36. The chimeras were scored according to coat colour (in percentage): two males with 99%, two with 95%, 85%, 45%, 20% and 10 % males. Two chimeras with 99 % of chimerism were bred with C57BL/6J and 129X1/SvJ mice to obtain F1 animals in respective background namely C57BL/6J x 129X1/SvJ and in 129X1/SvJ. The germline transmission of *ADAM27* recombinated allele was checked by genomic PCR with KOTMfp, KOTMrp and NeoRI primers (Figure 3.14) with genomic DNA isolated from tail biopsies of the mice (Figure 3.16).

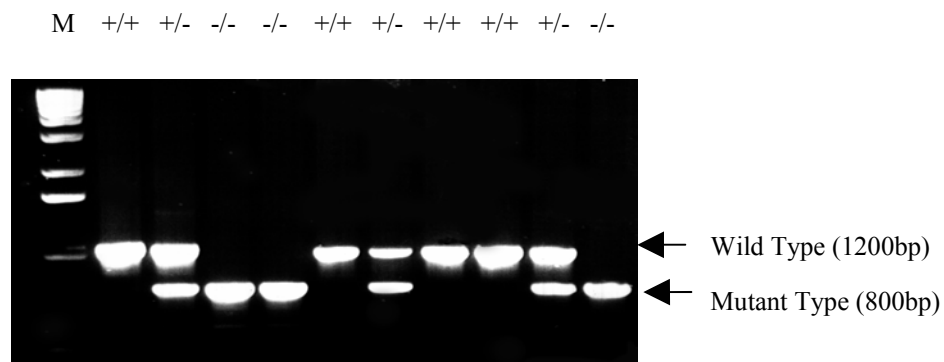


Figure 3.16 PCR genotyping of mice. The wildtype allele for *ADAM 27* is amplified by KOTMfp and KOTMrp primers resulting in a PCR product of 1.2 kb. The mutated *ADAM 27* allele is expected to generate a fragment of 800 bp using the primers KOTMfp and NeoRI. M, Molecular weight marker; +/+, wildtype; +/-, heterozygous; -/-, homozygous.

3.1.3 Generation and analysis of the murine *ADAM27* deficient mice

F1 animals, which were heterozygous at the *ADAM27* locus, were intercrossed to obtain F2 animals. The breeding strategy was undertaken in such a way that the knock out line was established in both C57BL/6J x 129X1/SvJ and in 129X1/SvJ genetic backgrounds.

3.1.3.1 Analysis of *ADAM 27* expression in transmembrane knock out mice

3.1.3.1.1 Transcriptional analysis

Total RNA from testes of wildtype, heterozygous (*ADAM27^{+/-}*) and homozygous mice (*ADAM27^{-/-}*) was prepared. Northern blot analysis was performed using E1AD27 probe localized before deletion and DelTM probe localized in deleted exon 19. Blots were re-hybridized with β -actin to show the integrity of RNA. Northern blot analysis using E1ADA27 probe showed that a signal was observed in wildtype and heterozygous as well as in homozygous mutant (Figure 3.17) with different intensity. When DelTM probe was used, no specific hybridization was obtained probably due to short length of the probe (135 bp). To check the transcript in knock out mice, RT-PCR was performed using two pairs of primers. One pair (DelTMfp and DelTMrp) localized in deleted exon 19 and second outside of deletion in neighboring exons (Y3fp,Y2rp Figure 3.18A).

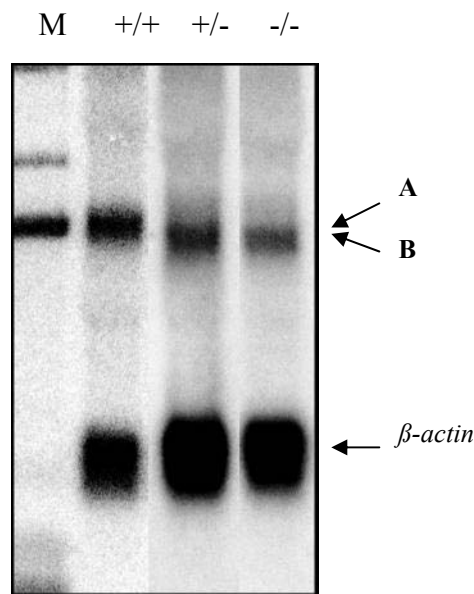


Figure 3.17 Northern blot analysis, testicular total RNA of wildtype (+/+), heterozygous (+/-), and homozygous (-/-) mice were hybridized with E1AD27 probe, signals with different intensity and size (A,B) can be seen in +/+, +/- and in -/-mice. β -actin-hybridization as a control for integrity of RNA.

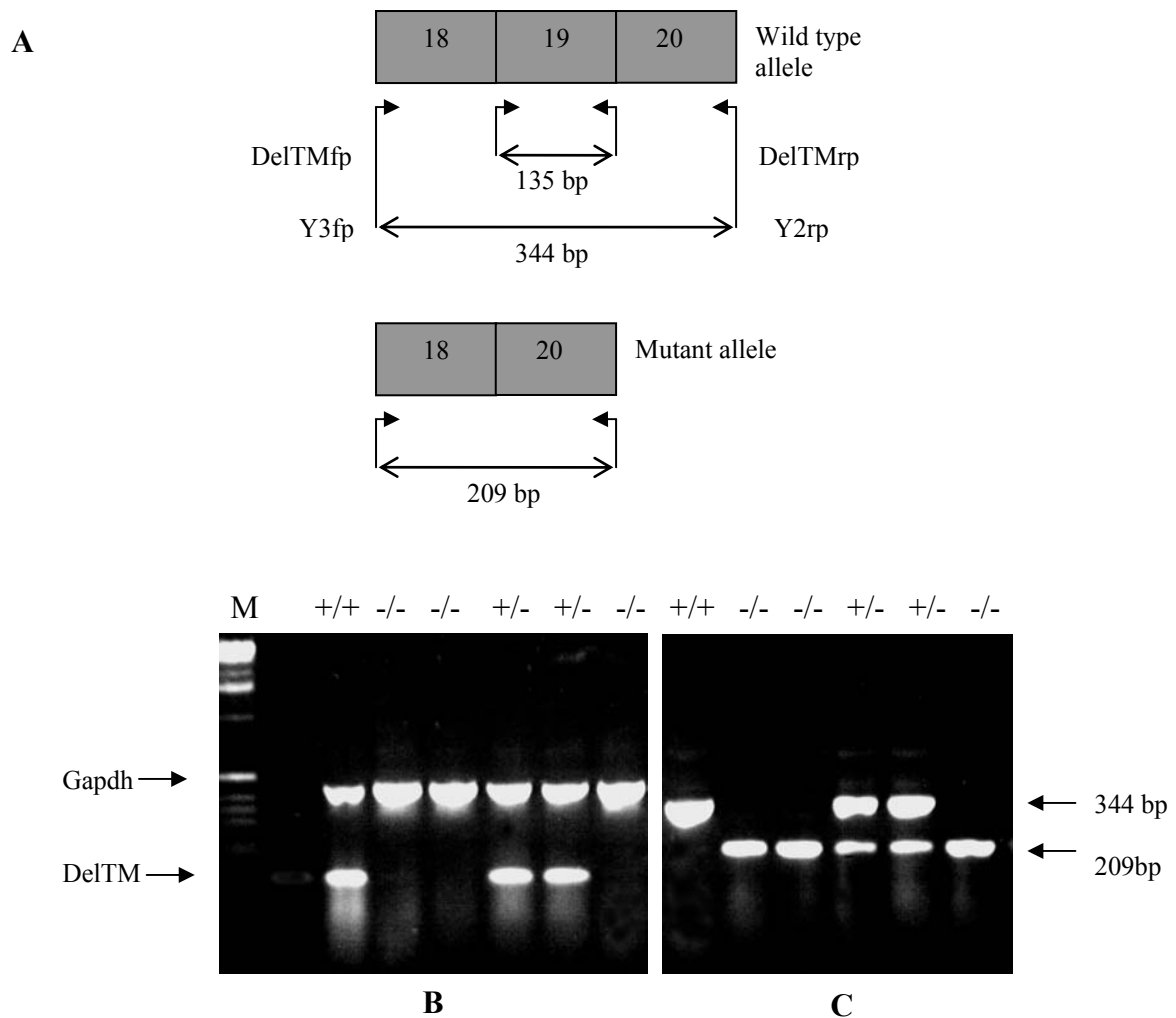


Figure 3.18 RT-PCR analysis of mutated *ADAM 27* transcript. (A) Scheme of primer localization and the sizes of RT-PCR products in wildtype. (B) RT-PCR performed using DelTMfp and DelTMrp primers which are able to amplify specifically transmembrane domain (135 bp) in wildtype and heterozygous mice but not in homozygous, Gapdh RT-PCR performed to check quality of RNA. (C) RT-PCR analysis using Y3fp and Y2rp primers resulting in 344 bp wildtype fragment detected in +/+ and +/- animals and 209 bp mutant fragment detected in -/- and +/- mice.

In wildtype and heterozygous mice using DelTMfp/DelTMrp primers we were able to amplify exon 19 with transmembrane domain but not in homozygous mutant, as expected (Figure 3.18B). Second pair of primers in wildtype amplifies fragment of 344 bp and in homozygous mice mutants a shorter fragment of 209 bp lacking exon 19 (Figure 3.18B). 209 bp fragment was sequenced and in fact exon 19 was deleted, in mutant RNA exon 18 is followed by exon 20. Deletion caused open reading frame change after exon 18 with new stop codon and new protein sequence on 3' end (Figure 3.19).

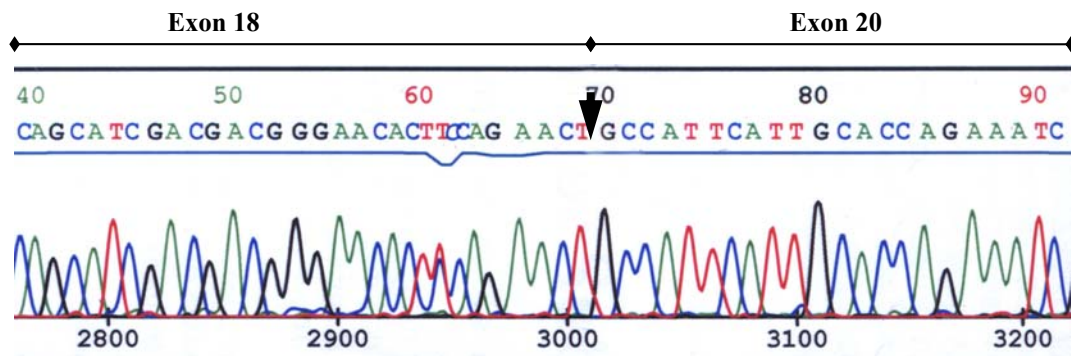


Figure 3.19 Sequencing result of mutant RT-PCR fragment of 209 bp amplified with Y3fp and Y2rp primers. Black arrow indicates new junction between exons 18 and 19.

3.1.3.1.2 Translational analysis

In the generated knock - out mice it was shown that the transcript with deleted transmembrane domain exist (Figure 3.18). Using ORF finder (NCBI) open reading frame of the mutated transcript was predicted and translated to protein. Mutated protein has different amino acid sequence in the 3' end and therefore it is not recognized by anti ppADAM27 antibody. We analyzed mutated form using available online software (ExPASy tools) and found that the mutated protein would have molecular weight of 76kDa and would not be membrane protein (Figure 3.20)

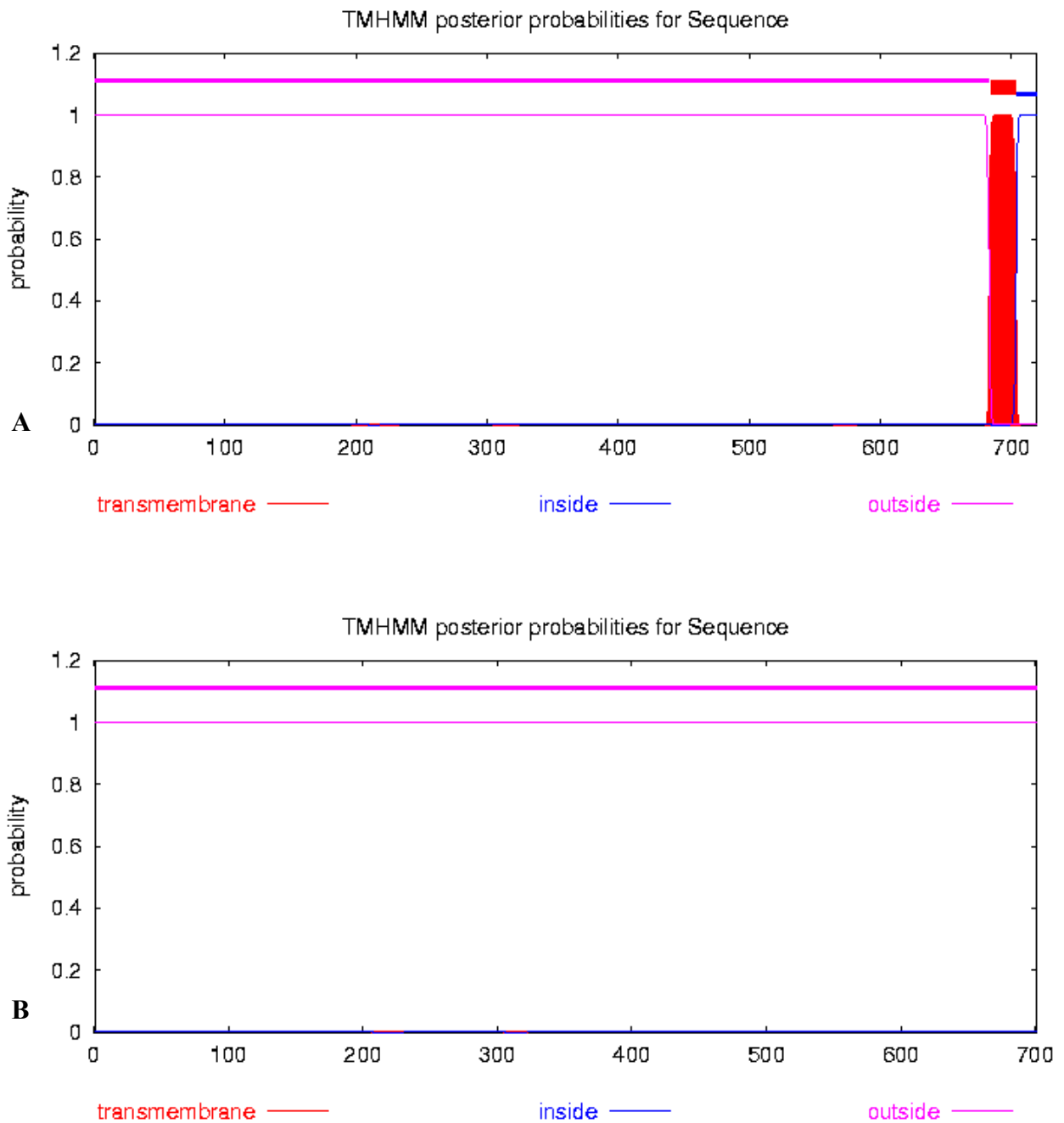


Figure 3.20 Results of TMHMM software analysis predicting transmembrane helices in proteins. (A) Wildtype ADAM 27 protein possesses one transmembrane helical at the 3' end, N-terminus is located outside whereas C-terminus inside the cell. (B) Mutated ADAM 27 has no potential transmembrane helices and could be secreted outside of the cell.

Total testicular protein extracts, membrane and cytoplasmic protein fractions from wildtype and mutant mice were prepared and subjected to SDS-PAGE electrophoresis. Western blot analyses were performed using anti ADAM27 antibodies. Both antibodies detect ADAM 27 in wildtype mouse testis. Anti ppADAM27 antibody did not detect mutated protein in total testicular protein extracts from ADAM 27 deficient mice (Figure 3.21A). However, anti fpADAM27 antibody was able to detect ADAM 27 protein still in membrane fraction (Figure 3.21B). These results suggest that mutated transcript without exon 19 is translated to protein, and still have some hydrophobic properties.

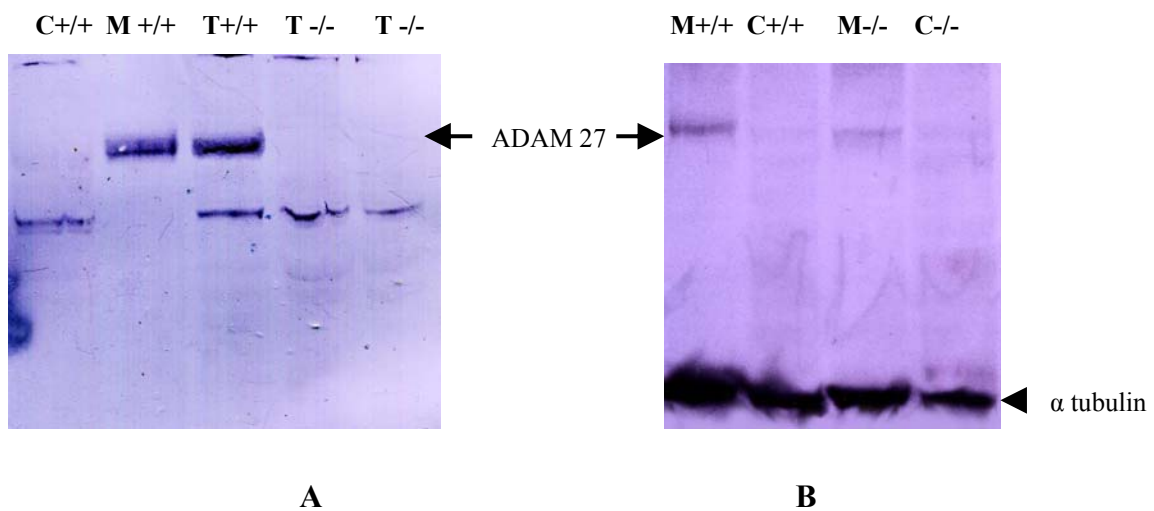


Figure 3.21 Western blot analysis of ADAM 27 protein expression in TM knock out mice. (A) Blot incubated with 1:100 dilution of anti ppADAM27 antibody, protein is detected in membrane fraction (M) and total testicular extracts of wildtype testis but not in total testicular extracts of mutant testes. (B) Blot incubated with 1:100 dilution of anti fpADAM27 antibody, protein is detected in membrane fractions of wildtype as well as mutant testes. α tubulin hybridization was performed to check quantity of proteins. C: cytoplasmic fraction, M: membrane fraction, T: total testicular extracts.

3.1.3.2 Phenotypic analysis of murine *ADAM 27* transmembrane knock out mice

ADAM 27 mutant mice with deleted transmembrane domain are fertile, with no visible morphological abnormalities in the testis as shown in Figure 3.22. All developmental stages of spermatogenesis are present and cell organization in tubule is not changed.

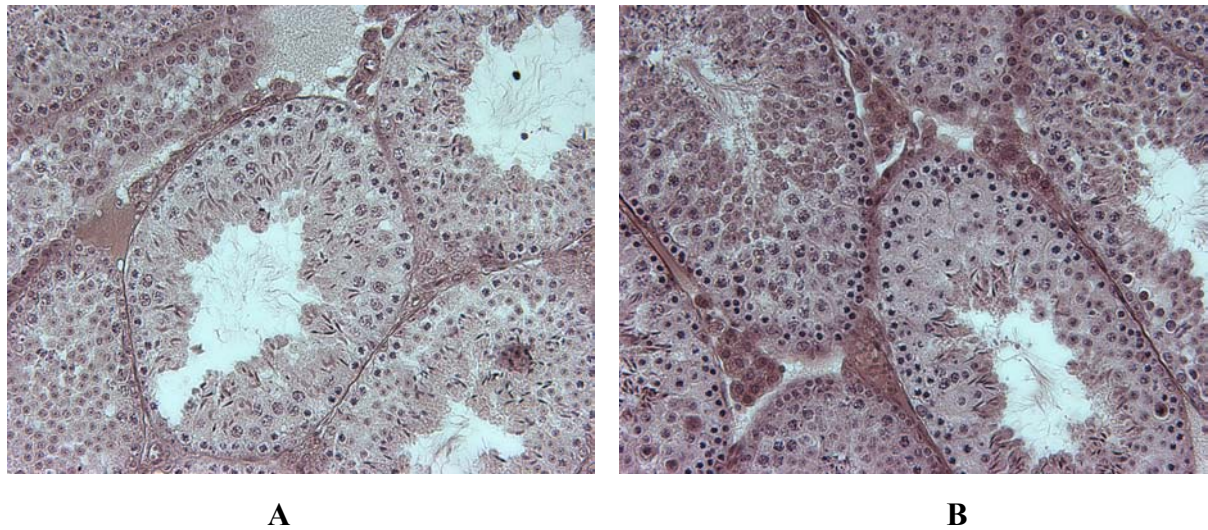


Figure 3.22 Hematoxylin-Eosin staining of testicular sections. A: Wildtype, B: Mutant homozygous mice.

3.1.3.2.1 Mode of inheritance

In order to determine the mode of inheritance of *ADAM 27* deleted allele in mice, F1 heterozygous mice were intercrossed to obtain F2 generation. The mice were genotyped by genomic PCR on DNA isolated from tail biopsies. The results of this statistical analysis are summarized in Table 3-1. On both backgrounds Mendelian inheritance pattern was not affected. Small under representation of wildtype males (C57Bl) and heterozygous males (129Sv) was observed. Average litter size was 7.6 (+/- x +/-) and 7.4 (-/- x -/-) on the C57BL/6J x 129X1/SvJ background, whereas 5 (+/- x +/-) and 4.8 (-/- x -/-) on the 129Sv/6J x 129X1/SvJ background.

A

AD27/36 C57BL/6J x 129X1/SvJ					
+/+		+/-		-/-	
M	F	M	F	M	F
12	24	32	26	14	13
36		58		27	
Total 121					

B

AD27/36 129Sv/6J x 129X1/SvJ					
+/+		+/-		-/-	
M	F	M	F	M	F
15	12	15	25	13	13
27		40		26	
Total 93					

Table 3-1 Statistical analysis of genotype distribution of *ADAM 27* locus within mice from F2 generation. (A) Line AD27/36 C57BL/6J x 129X1/SvJ was in accordance with Mendelian inheritance, in total 121 animals were genotyped. (B) Line AD27/36 129X1/SvJ showed small deviation from Mendelian segregation, here 60 animals were genotyped and number of wildtype was slightly increased. M: male; F: female.

3.1.3.2.2 Sperm count and sperm motility analysis of *ADAM 27* mutant males

We have determined the total sperm count in the cauda epididymes as well as in uteri and oviducts of vaginal plug positive females mated with *ADAM 27* -/- males (Table 3-2). Total sperm number in cauda epididymes for *ADAM 27* -/- males was in average $1.87 \pm 0.21 \times 10^7$ on the AD27/36 C57BL/6J x 129X1/SvJ background and $1.3 \pm 0.26 \times 10^7$ for AD27/36 129X1/SvJ background and therefore not significantly different than wildtype. Number of sperm in uterus and oviduct was not altered as well. To address the question whether the

fertility of *ADAM 27* mice might be influenced by an acrosomal defect, we examined the response of spermatozoa from *ADAM 27^{-/-}* and wildtype mice to the calcium ionophore A23187. There was no significant difference in the assay of acrosome reaction between *ADAM 27^{-/-}* (80%) and wildtype spermatozoa (85%).

	AD27/36 C57BL/6J x 129X1/SvJ					AD27/36 129X1/SvJ		
Uterus (10 ⁶)	9.7	8.4	8.5	9.4	7.4	1.6	2	1.9
Oviduct	187	150	475	187	3600	1200	1600	300

Table 3-2 Sperm count in uterus and oviduct of vaginal plug positive females mated with *ADAM27^{-/-}* males on both backgrounds.

Analysis of sperm motility was done only on the C57Bl/6J x 129X1/SvJ background, because there was not enough 129Sv *-/-* males, most of them were in breeding tests. We measured sperm motility of six wildtype and five mutant males after 1.5, 3.5, and 5.5 hrs incubations in vitro. After 1.5 h, the proportion of motile spermatozoa of *ADAM 27^{-/-}* mice was reduced compared with wildtype, 41.7% versus 65% and the proportion that exhibited progressive movement in *ADAM 27^{-/-}* mice was also reduced compared with those of wildtype mice, 24.5% versus 44.2% (Table 3-3). After 3.5 and 5.5 h, the proportion of motile and progressively motile spermatozoa of *ADAM 27^{-/-}* versus wildtype mice was highly decreased (Table 3-3). The following parameters were evaluated in more detail and statistically analyzed: curvilinear velocity (VCL), average path velocity (VAP), straight line velocity (VSL), beat cross frequency (BCF), straightness (STR), and amplitude of the lateral head displacement (ALH) (Figure 3.23). For the beat cross frequency and straightness reductions were small however significant, with 2 exceptions (see Figure 3.23). For all other parameters, a significant reduction for *ADAM 27^{-/-}* spermatozoa in comparison to the wildtype sperm was observed ($P < 0.001$). A decrease of 30 to 50% was found for the ALH and velocity parameters VAP, VSL, VCL. After 5.5 h of incubation time, sperm motility of *ADAM 27^{-/-}* in comparison with wildtype mice was found to be highly reduced. However these high reductions of sperm motility didn't affect the fertility of mutant mice.

Results

Genotype of mice	Incubation time (h)	Total no. of measured spermatozoa	No. of motile spermatozoa (%)	No. of spermatozoa with progressive movement (%)
+/+	1.5	7017	(65.6)%	(44.2)%
	3.5	7164	(69)%	(45.2)%
	5.5	8913	(54.4)%	(33.4)%
-/-	1.5	9083	(41.7)%	(24.5)%
	3.5	9015	(34.5)%	(18.5)%
	5.5	11211	(27.7)%	(13.7)%

Table 3-3 Analysis of sperm motility of *ADAM 27*^{+/+} and *ADAM 27*^{-/-} mice

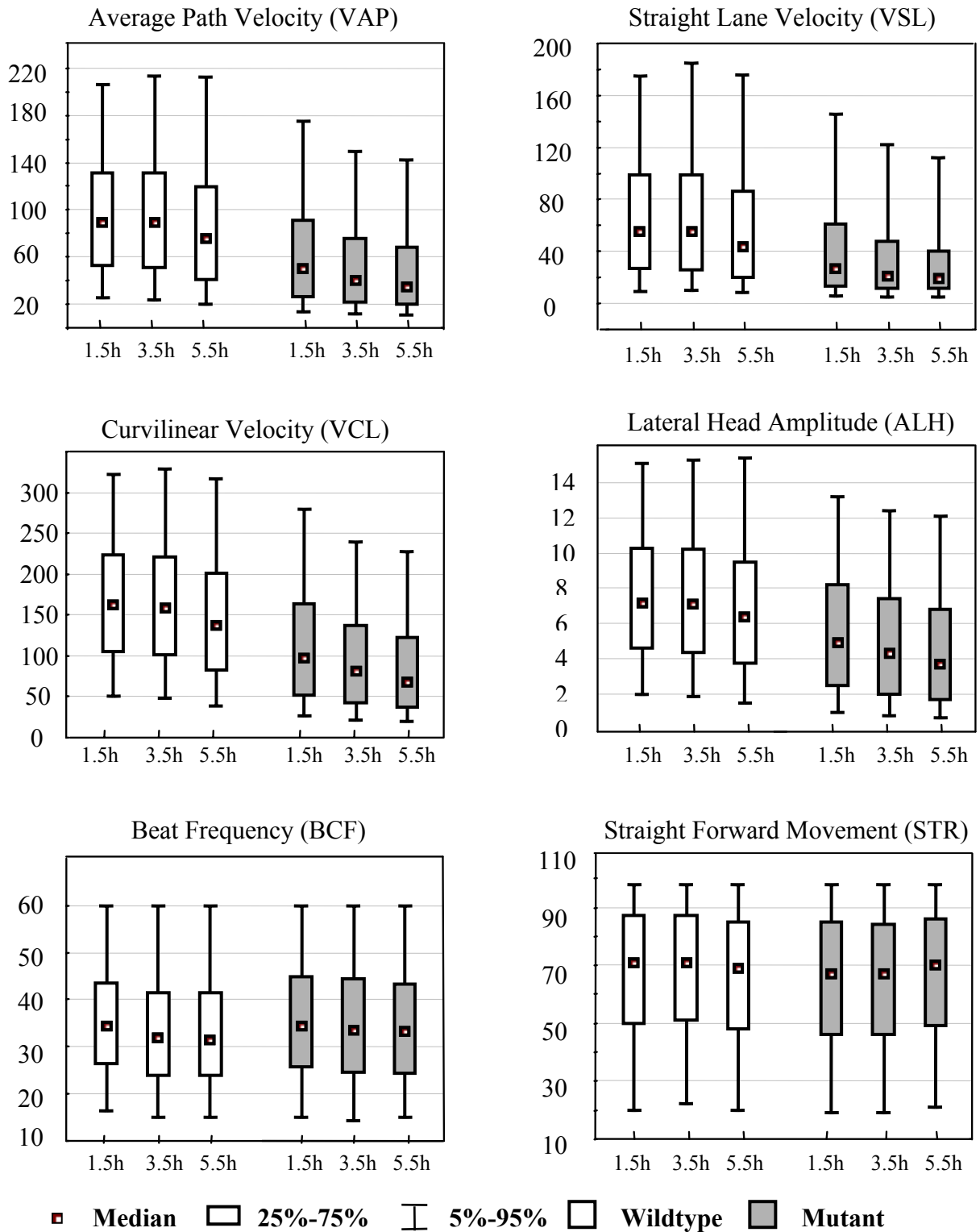


Figure 3.23 Computer-assisted analysis of sperm motility. The results of analyses of wildtype and *ADAM 27*^{-/-} spermatozoa are shown. Sperm velocities (micrometers/second), forward movement (percent), lateral amplitude of the sperm head (micrometers), and beat frequency (hertz) were measured after 1.5, 3.5 and 5.5 h. For all parameters the medians and percentiles are shown. The *ADAM 27*-deficient spermatozoa exhibit highly significantly reduced velocities, amplitude, beat frequency and forward movement in

comparison to wildtype sperm ($P < 0.001$ by Mann-Whitney U-Test). With exceptions for BCF at 1.5 h and STR at 5.5h of incubation, where differences are not significant.

3.1.4 Deletion of 5' region of *ADAM27* gene using homologous recombination

3.1.4.1 Isolation of cosmid clones containing 5' region of the gene.

Second targeting vector for disruption of *ADAM27* gene was designed to delete ATG start codon and to eliminate by this way ADAM27 protein completely. New screening of the cosmid genomic library was done to identify cosmid clones that contain 5' end of the gene. For this purpose radioactively labeled cDNA probe containing 5' region (ex 1-5) was used for hybridization of 11 filters with cosmid clones. Three clones were identified and confirmed to be positive and contain *ADAM 27* genomic fragments representing 5' region of the gene: RZPD clone ID: MPMGc121N16522Q4, MPMGc121H2441Q4, MPMGc121O14783Q4. Last two clones were also identified by cyritestin probe. This indicates that the 5' end must be present in these clones.

3.1.4.2 Genomic structure and restriction digestion analysis of the 5' end of *ADAM 27* gene

Cosmid clones containing the murine *ADAM 27* gene were restricted with the enzymes *EcoRI* and *Pst I*, blotted and radioactively hybridized with *ADAM 27* 5' fragment (Figure 3.24A). The size of fragments was calculated and compared to the known genomic structure (Figure 3.24B).

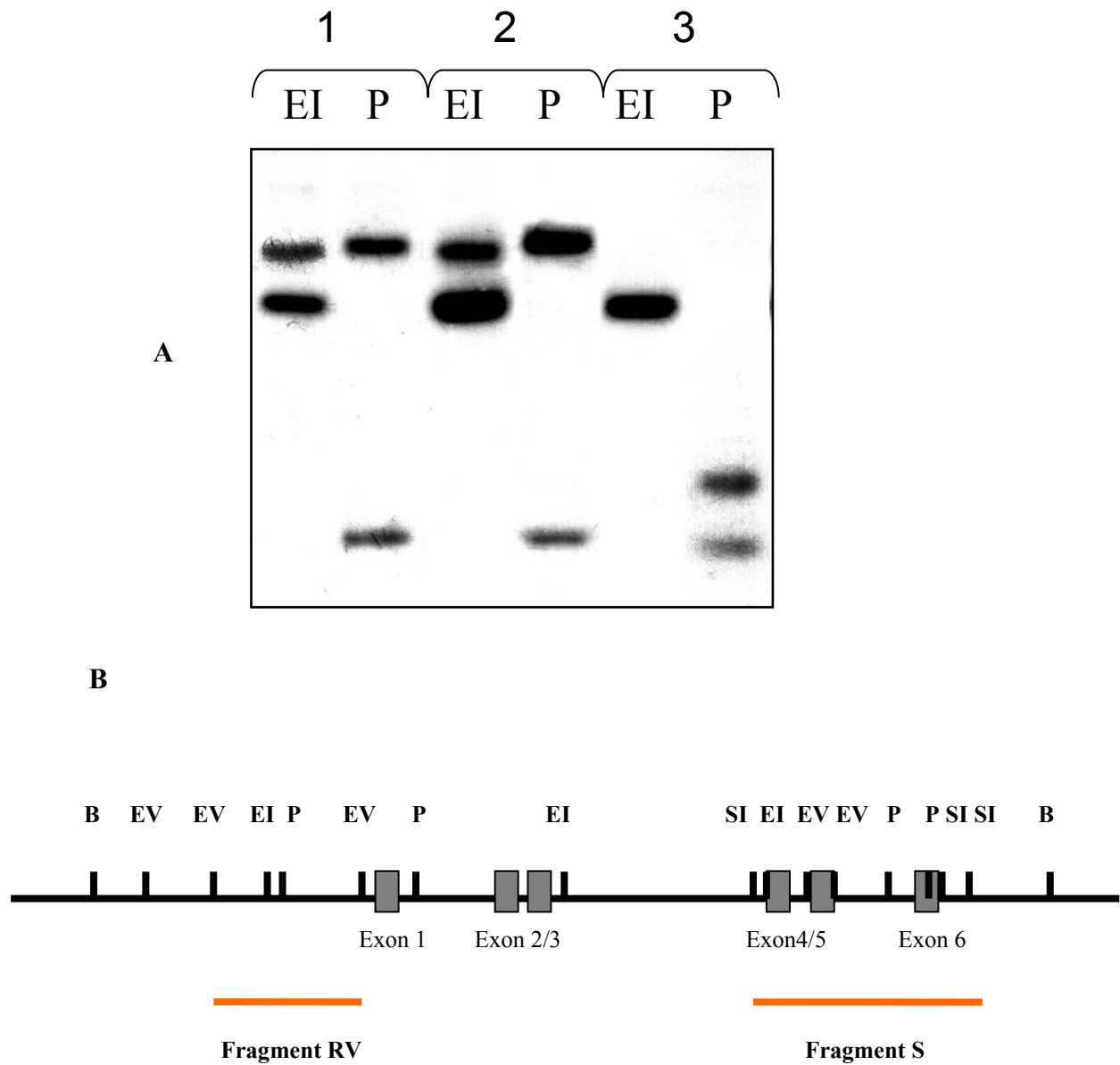


Figure 3.24 (A) Radioactive hybridization of cosmid clone B17781Q2 digested with different restriction enzymes. (B) Restriction digestion map of *ADAM27* genomic DNA and fragments which were cloned, abbreviations are: B: *Bam* HI; ERI: *Eco* RI; ERV: *Eco* RV; P: *Pst* I; SI, *Sst* I; Sp: *Spe* I; X: *Xba* I. Numbers represent cosmid clones 1: MPMGc121H2441Q4, 2: MPMGc121N16522Q4, 3: MPMGc121O14783Q4

3.1.4.3 Construction of *ADAM 27* gene targeting vector

A new knock out construct was generated in which a 2.8 kb *Eco* RV fragment containing 5' flanking region of the *ADAM 27* gene was cloned into *Sal* I/*Cla* I site of the pTKneo vector and 3.3 kb *Spe* I fragment containing exons 4-6 was cloned into *Sal* I site of pTKneo targeting vector (Figure 3.25). This new construct was designed to replace 6.8 kb of the *ADAM 27* gene containing exons 1-3 by *Neomycin* (Neo) gene. The orientation of both 5' and 3' wings was confirmed by sequencing of the construct with vector specific primers pTKNf, pTKNr, pTKr.

3.1.4.4 Generation of the 5' external probe

A fragment about 0.6 kb was amplified by PCR using 27FP and 27RP primers. Fragment is localized upstream of *ADAM 27* gene and was designed to detect *Bam* HI wildtype fragment in Southern blot hybridization (Figure 3.25). This PCR product was subcloned into pGEM-TEasy and cut out with *Eco* RI. The 0.6 kb fragment was extracted from the agarose gel and used as 5' external probe for hybridization in the Southern blot with DNA extracted from the recombinant ES-clones.

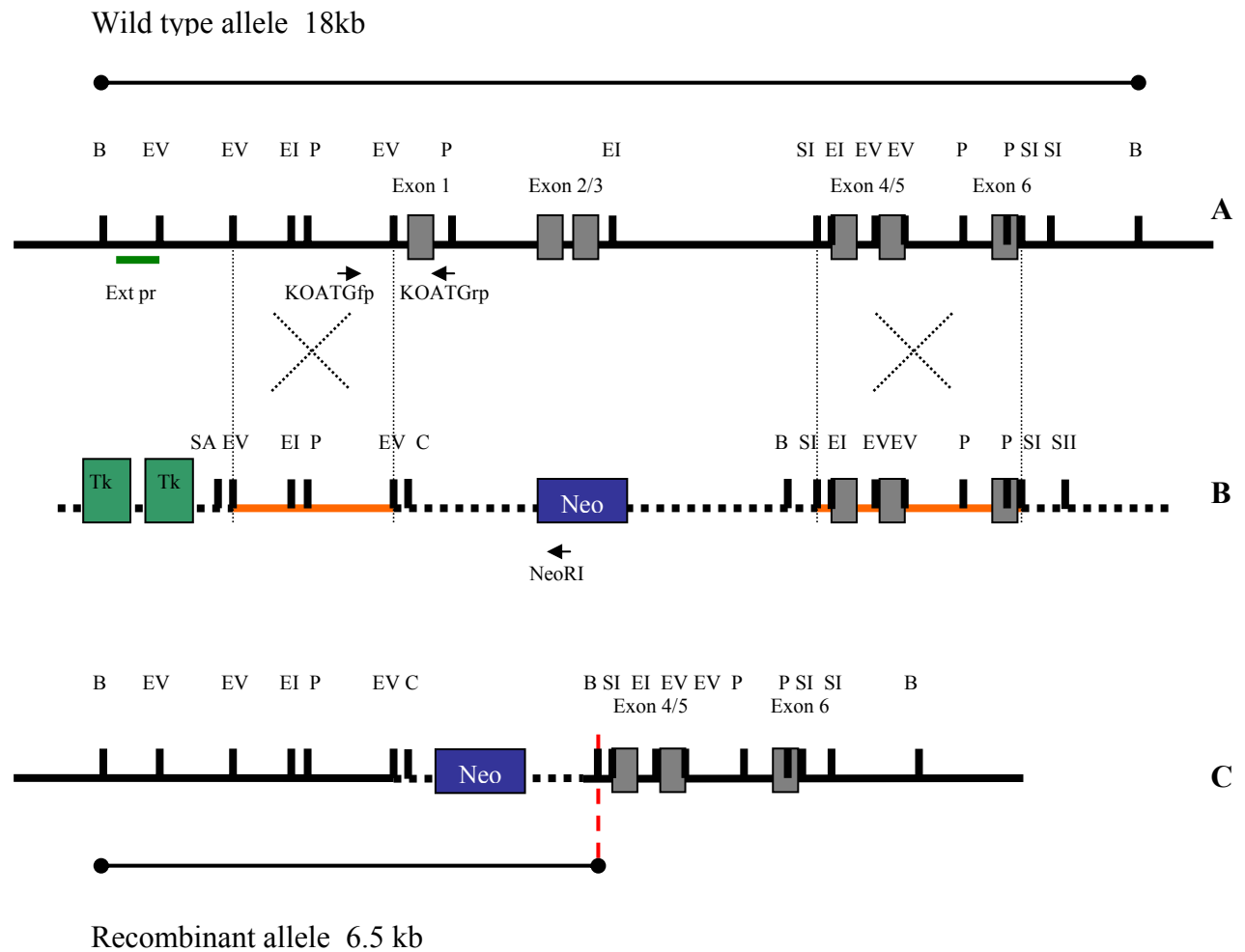


Figure 3.25 Schematic representation of the targeting strategy. Wildtype *ADAM 27* locus (A), targeting vector (B) and mutated allele (C). Exons 1, 2 and 3 were replaced by the Neomycin resistance gene cassette. External probe is marked as green bar, which enables to recognize 18 kb *Bam* HI fragment in wildtype DNA and 6.5 kb fragment in recombinant DNA. The gray boxes represent exons of *ADAM 27* gene, green boxes stand for Thymidine kinase and blue box stands for Neomycin. KOATGfp, KOATGrp and NeoRI were the primers used for genotyping. The restriction site abbreviations are: B: *Bam* HI; C: *Cla* I; EI: *Eco* RI; EV: *Eco* RV; P: *Pst* I; SI: *Spe* I; SII: *Sst* II; SA: *Sal* I;

3.1.4.5 Electroporation of the RI ES-cells and screening of ES-clones for homologous recombination events

The *ADAM 27* targeting vector was linearised by digesting with *Sst* II restriction enzyme and 50 µg of linearised DNA was electroporated into RI embryonic stem cell line as described in

section 2.2.20.1. The cells were plated on fibroblast feeder layer and after 10 days of selection around 200 individual *Neomycin* resistant clones were picked in 24 well plates and replicated. Genomic DNA was prepared from these ES clones for Southern blot analysis. DNA of individual ES clones was digested with *Bam* HI and electrophoresed into 1% agarose gels and blotted onto Hybond C membranes. The blots were hybridised with 32 P-labelled 0.6 kb 5' external probe. In the case of homologous recombination event two bands were expected, a wildtype allele of 18 kb and recombinant allele of 6.5kb. However, in the event of a random integration, only wildtype allele will be detected (Figure 3.25). After screening of all ES cell clones one recombinant clone was identified, namely EW50. Southern blot for recombinant clone EW50 is shown in Figure 3.26.

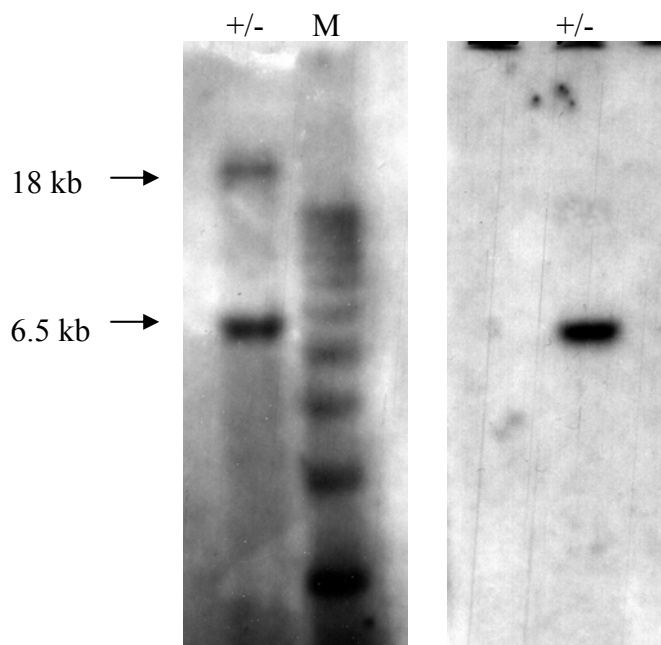


Figure 3.26 Genomic Southern blot analysis. Genomic DNA of ES clones was digested with *Bam* HI, separated on 1% agarose gel and transferred onto Hybond C membrane. (A) The blot was hybridized with radioactive labeled 0.6 kb external probe. In case of wildtype allele band of 18 kb was observed and in case of recombinant allele two bands were observed, in addition to wildtype allele, a mutant allele of 6.5 kb. (B) Blot was hybridized with neomycin probe to proof if the construct was integrated into mouse genome by single homologous recombination. In homologous recombination event only one band of 6.5 kb is obtained.

3.1.4.6 Generation of chimeric mice

ES cells from the recombinant clone were injected into 3.5 dpc blastocysts derived from C57BL/6J mice. The blastocysts were implanted into pseudopregnant CD1 mice to generate chimeric mice. Four chimeras were obtained after 2 independent injections of recombinant ES clone EW50. The chimeras were scored according to coat colour (in percentage) two 80%, 75% and 15 % males were obtained. Chimeras were bred with C57BL/6J and 129X1/SvJ mice to obtain F1 animals in respective background namely C57BL/6J x 129X1/SvJ and in 129X1/SvJ. Until now 15% chimera had two litter with no transmission of deleted allele, other three chimeras were not fertile. Six females, vaginal plug positive, were tested for presence of sperm in uterus and oviducts. No sperm was found. First, 75% chimera was sacrificed and a few organs were isolated, including testis, for DNA preparation. One testis was fixed in Bouin's solution and paraffin sections were prepared. Hematoxylin-Eosin staining was performed to check the morphology of the testis. From second testis DNA and RNA were isolated. Testes of the 75% chimera were significantly smaller than wildtype (Figure 3.27 A). Testis of the analyzed chimera was fixed, paraffin embedded and sections were stained with Hematoxylin-Eosin (Figure 3.27B). Tubules were mostly abnormal with vacuoles and symplastic cell agglomerations in the lumen and hypertrophies of the interstitial tissue.

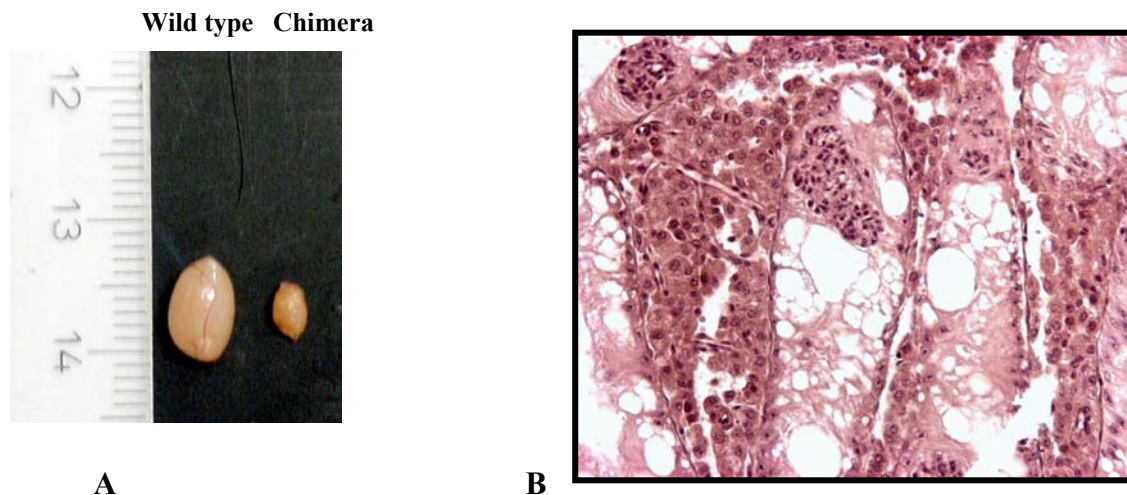


Figure 3.27 Size comparison of the wildtype and 75% chimera testes (A). Hematoxylin-Eosin stained section of the chimera testis demonstrating high testicular abnormalities (B).

DNA of different organs was used in genomic PCR, using primers KOATGfp, KOATGrp and Neo RI (Figure 3.25), to proof whether recombinant ES cells contribute to these tissues. Other PCR was performed using strain specific microsatellite markers to compare contribution of ES cells of both strains. Among tested organs only liver had very low contribution of recombinant ES cells (Figure 3.28), other organs demonstrated proportionally different contribution of wildtype ES cells (C57Bl) and injected recombinant ES cells (129 Sv) (Figure 3.28). In heart and specially lungs wildtype originated cells dominate, in muscle higher percentage of cells come from ES cells carrying ADAM 27 mutation. The most important tissue in this case testis, shows equal contribution of both types of ES cells (Figure 3.28).

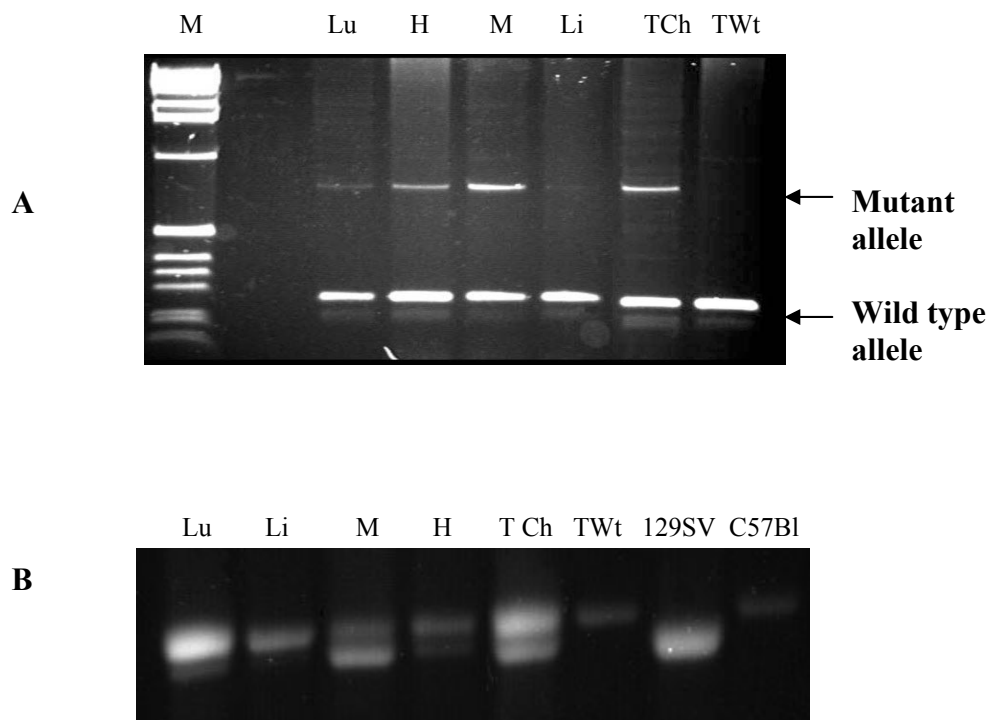


Figure 3.28 Analysis of 75% chimera obtained from microinjection of *ADAM 27* mutated ES cells into blastocyst. (A) Genomic PCR using KOATGfp, KOATGrp and Neo RI primers amplifying Mutated and Wildtype alleles, (B) Genomic PCR for strain specific microsatellite markers. Lu: lung; Li: liver; M: muscle; H: heart; T Ch: testis of chimera; TWt: testis of wildtype: 129SV and C57Bl mouse strains.

RT-PCR analysis of the expression of few testicular genes including *ADAM 27* was performed. We were not able to detect *ADAM 27* transcript, whereas two early spermatogenic markers (*Stra 8* and *Piwil*) had significantly lower expression and meiotic marker *Scp3*

transcription was close to the wildtype level (Figure 3.29). Therefore, indicating presence of early spermatogenesis stages.

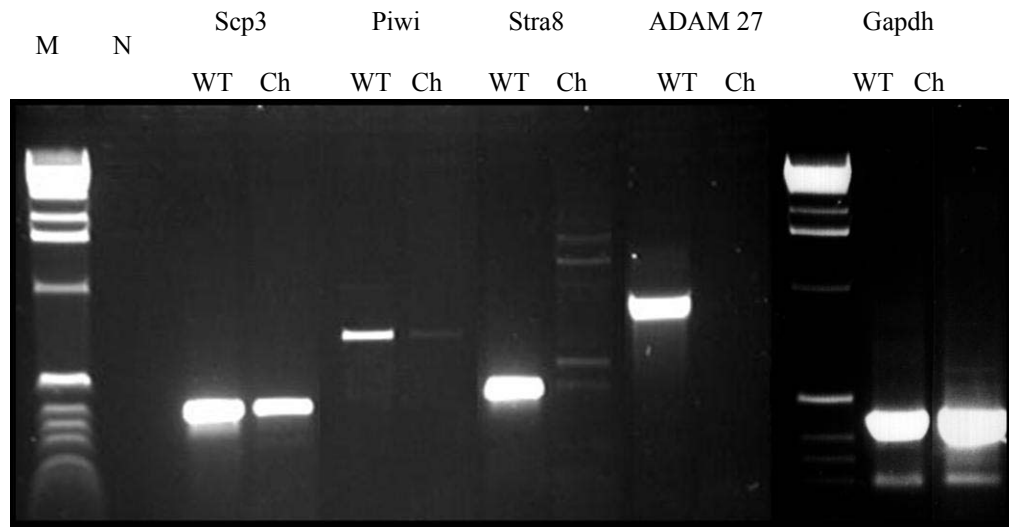


Figure 3.29 RT-PCR analysis of the expression of some spermatogenic markers, performed on RNA isolated from testis of chimeric mouse in comparison to wildtype. WT-wildtype; Ch-chimera; M-size marker; N-negative control.

Two other chimeras (80%) were not fertile. In case of chimera A we checked 5 vaginal plug positive females and did not find sperm in uterus, chimera B was mated in the same period of time as A but did not give any vaginal plug positive females. Both chimeras were sacrificed and reproductive organs were analyzed. Interestingly, in both animals we found that right testis and epididymis are abnormal whereas left looks normal but is smaller than wildtype (Figure 3.30). Additionally, we have found sperm in the left epididymis of chimera B but not in the right as well as in chimera A epididymes. Gross anatomy of the testes showed certain level of abnormality, in chimera A both testes had arrested spermatogenesis, none of the spermatids or spermatozoa were found (Figure 3.31). In chimera B, in testes we have found tubules with normal spermatogenesis and presence of spermatozoa, as well as tubules with different degree of spermatogenesis degeneration (Figure 3.32). However in the right testis abnormality degree was higher what coincidences with higher chimerism ratio.



Figure 3.30 Testis abnormalities found in 80% chimeras generated with ADAM 27 homologues recombinant ES cells.

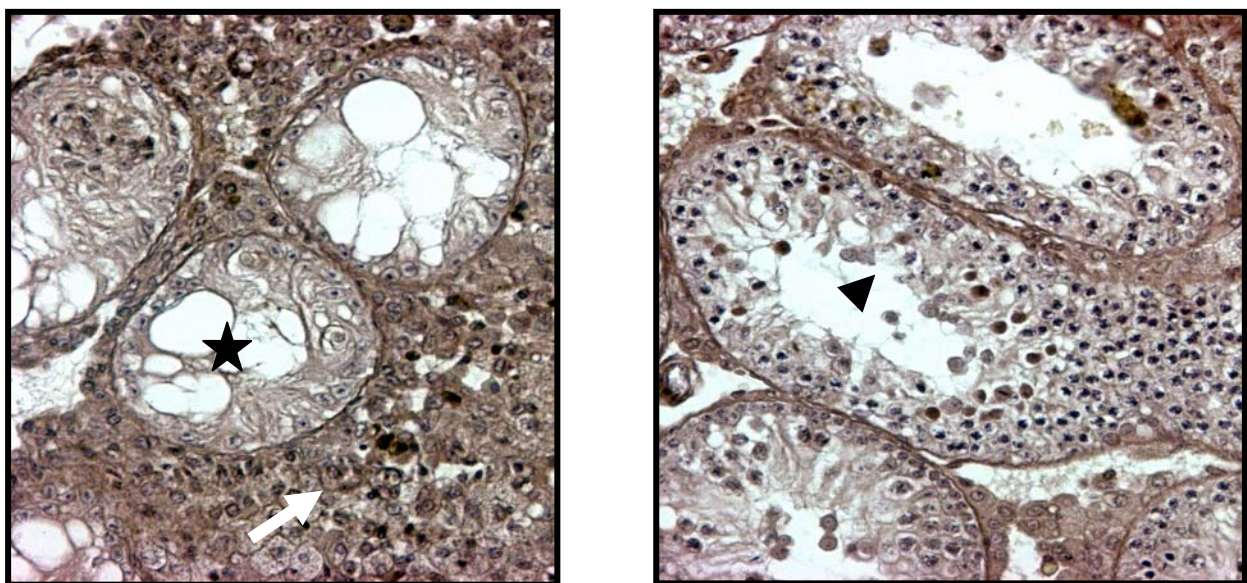


Figure 3.31 Testicular sections of chimera A presenting tubules with typical abnormalities of spermatogenesis: empty tubules (asterisk) and hypertrophy of interstitial tissue (white arrow) on the left panel, abnormal tubules with spermatogenesis defects (arrow head) on the right panel.

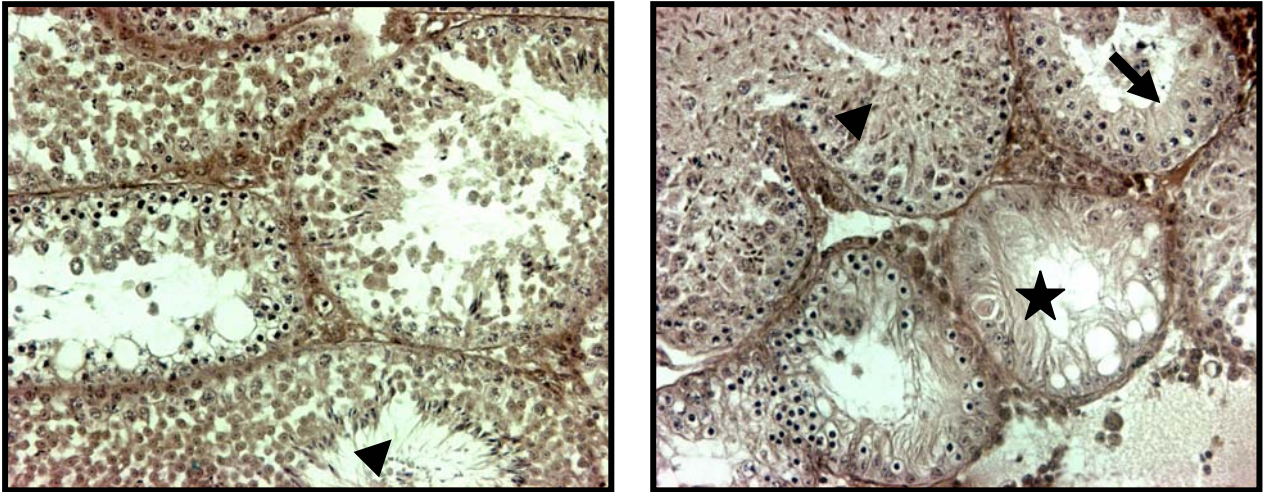


Figure 3.32 Gross anatomy of the testes of chimera B. Typical tubules appearance found in left testis (left panel) and right testis (right panel), showing empty tubules (asterisk), some tubules with spermatogenesis arrested at different stages (arrow) and some with spermatids and spermatozoa (arrow heads).

We have performed similar experiments with DNA and RNA samples from 80% chimera B as earlier for 75%. Genotyping PCR designed to recognize wildtype and mutated allele showed that in all tested organs cells derived from injected ES cells has been found (Figure 3.33A). However PCR using strain specific primers indicated that kidney and right testis (abnormal) have higher chimerism ratio than other tissues (Figure 3.33B). RT-PCR analysis of the expression pattern of genes specifically expressed in the testis (*Piwil*, *Tp2* and *Pgk2*) and *ADAM27* revealed decrease in *ADAM 27* transcription level in comparison to wildtype, more drastically in right testis, although expression level of other genes was not affected (Figure 3.34). Here again, decrease of *ADAM 27* expression correlates with the chimerism ratio.

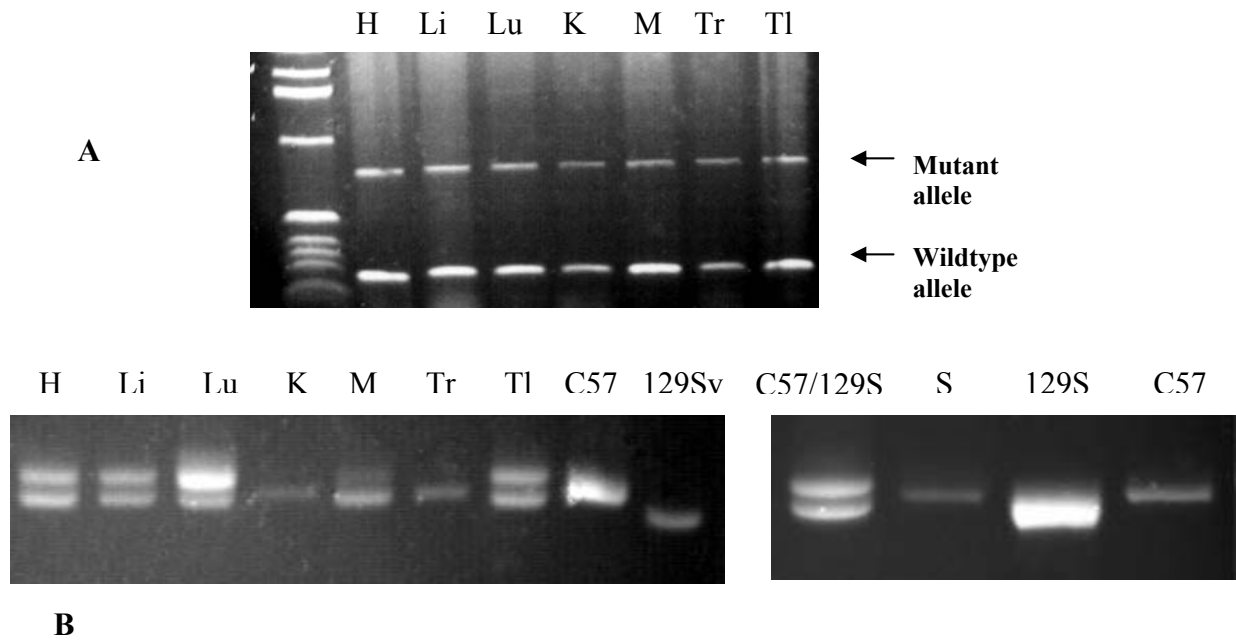


Figure 3.33 Analysis of 80% chimera B obtained from microinjection of *ADAM 27* mutated ES cells into blastocyst. (A) Genomic PCR using KOATGfp, KOATGrp and Neo RI primers amplifying Mutated and Wildtype alleles, (B) Genomic PCR for strain specific microsatellite markers. H: heart; Li: liver; Lu: lung; K: kidney; M: muscle; Tr: right testis of chimera; Tl: left testis of chimera; S: sperm of chimera B; 129SV and C57Bl mouse strains.

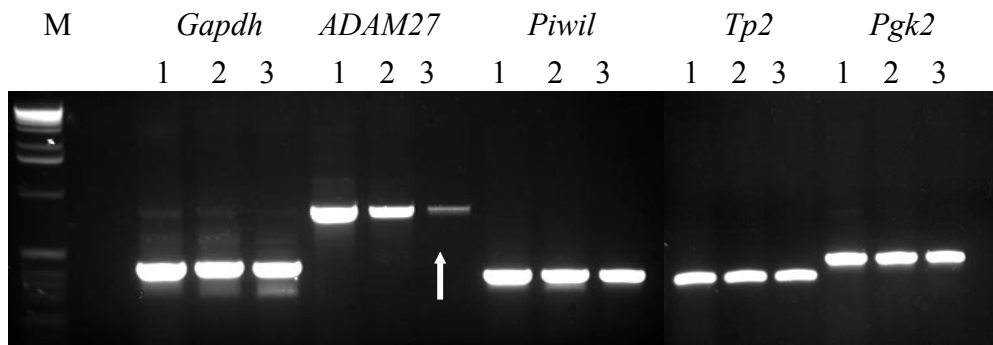


Figure 3.34 RT-PCR analysis of the expression of some testis specific genes, performed on RNA isolated from both testes of chimera B in comparison to wildtype. M-size marker; 1-wildtype testis; 2-left testis and 3 right testis of chimera B.

3.1.5 Analysis of sperm-egg interaction mediated by ADAM 27

One of the proposed functions of the ADAM 27 molecule is participation in the process of fertilization as an adhesion molecule. The interactions between mammalian sperm and egg plasma membranes are believed to occur via a multi-step process mediated by multiple cell adhesion molecules on the surfaces of both gametes. ADAM 27 has a region with homology to the family of integrin ligands known as disintegrins, which has been hypothesized to be involved in the binding of sperm to the egg surface. To investigate this hypothesis and determine what role ADAM 27 plays in fertilization, we have expressed the putative extracellular domain of mouse ADAM 27 in bacteria as a fusion protein with maltose-binding protein and used in *in vitro* assay to characterize its binding to mouse eggs. In the sperm-egg binding assay ligands for ADAM 27 molecule localized on the oocyte surface were blocked by recombinant proteins and thereafter the effect of these blocking of sperm-egg binding was analyzed.

3.1.5.1 Generation of MBP-ADAM27 fusion proteins

MBP fusion constructs generated as described in 2.2.8.2 were used to transform expression bacterial host BL21 cells and to produce recombinant proteins (2.2.9). Isolated and purified proteins were subjected to SDS-PAGE electrophoresis, blotted to PVDF membrane and immunostained with antibodies. For MBP fusion proteins anti MBP antibodies were used to detect recombinant proteins. To confirm that produced proteins in fact belong to ADAM 27, blots were incubated with anti fpADAM27 antibodies. Primary antibodies were followed by secondary anti rabbit alkaline phosphatase conjugated antibody. Size of the fusion proteins correspond to predicted ones and was determined by comparison with molecular marker. Produced MBP fusion proteins: MBP-D, MBP-DCE and MBP-CE (Figure 3.35A) were detected by MBP as well as by ADAM 27 specific antibodies confirming that produced proteins represent ADAM 27 (Figure 3.35B). Size of proteins is similar to the predicted by software analysis. Produced MBP-fusion proteins were used then in sperm-egg binding assay.

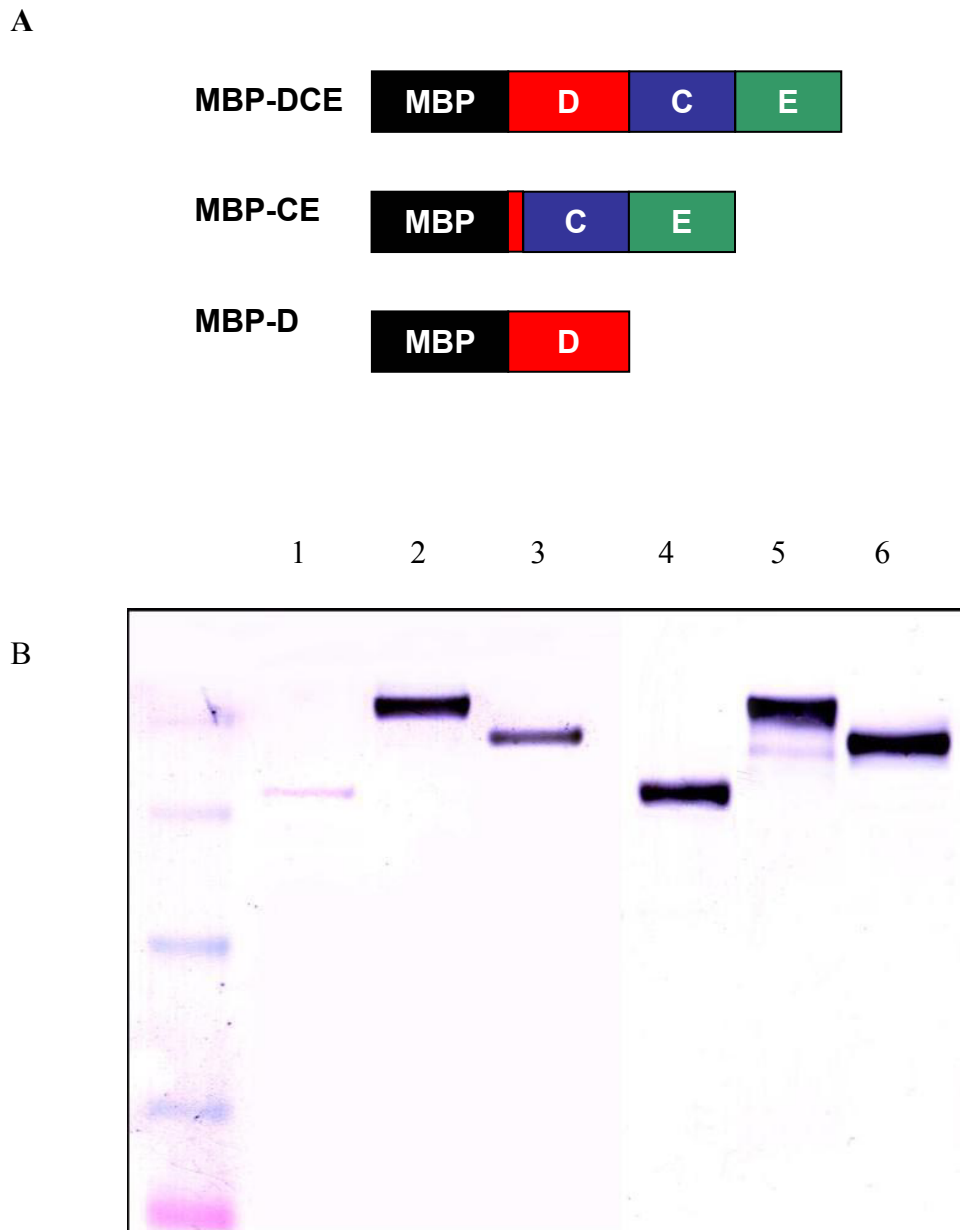
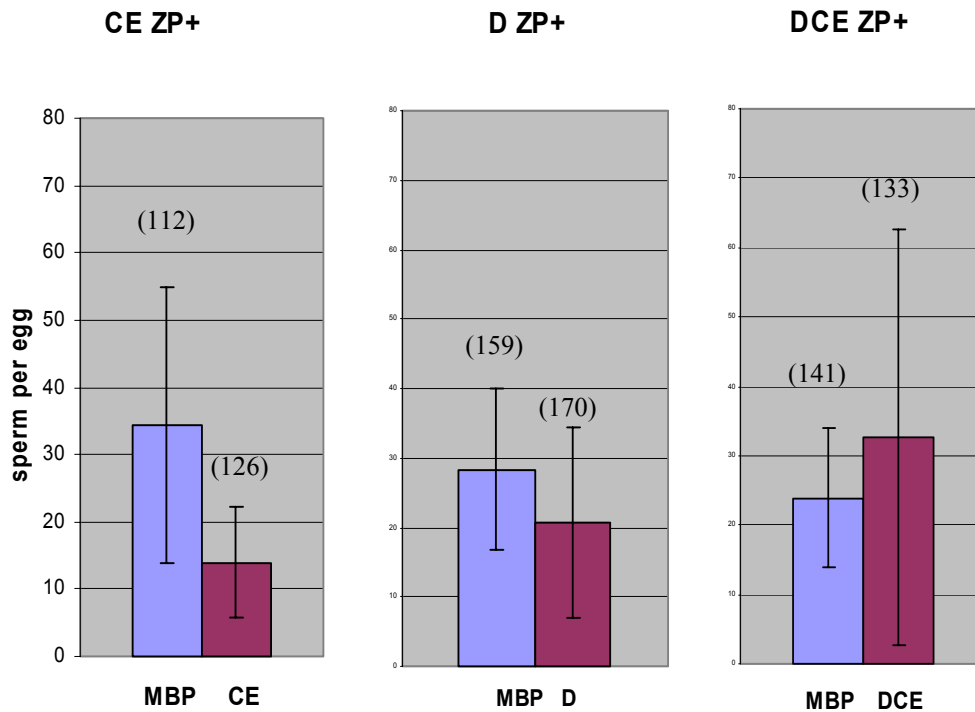


Figure 3.35 (A) Graphical representation of ADAM 27 fusion proteins. (B) Western blot analysis of produced MBP recombinant proteins using anti MBP (1-3) and anti fpADAM27 antibodies (4-6). Lines 1 and 4 represent MBP-D, lines 2 and 5 MBP-DCE and lines 3 and 6 MBP-CE proteins.

3.1.5.2 Effect of different recombinant forms of ADAM 27 on sperm-egg binding.

Recombinant proteins MBP-DCE, MBP-CE and MBP-D produced as described in 2.2.8.2 were used in sperm-egg binding assays. For each protein 4 to 5 independent experiments were done using 20 - 40 oocytes. MBP protein was used in the study as control, known not to affect

gamete binding. Figure 3.36 is a graphic illustration of the results. In experiments using recombinant proteins we observed that sperm-egg binding was reduced in the presence of recombinant ADAM 27 forms: CE and D as compared with sperm-egg binding in the presence of the control protein, MBP. The decrease was higher in experiments with oocytes with zona pellucida, sperm binding with zona free eggs was not changed in presence of MBP-CE and slightly decreased in case of MBP-D. Opposite results were obtained when eggs were incubated with recombinant DCE form of ADAM 27, in both groups of oocytes sperm binding was increased.



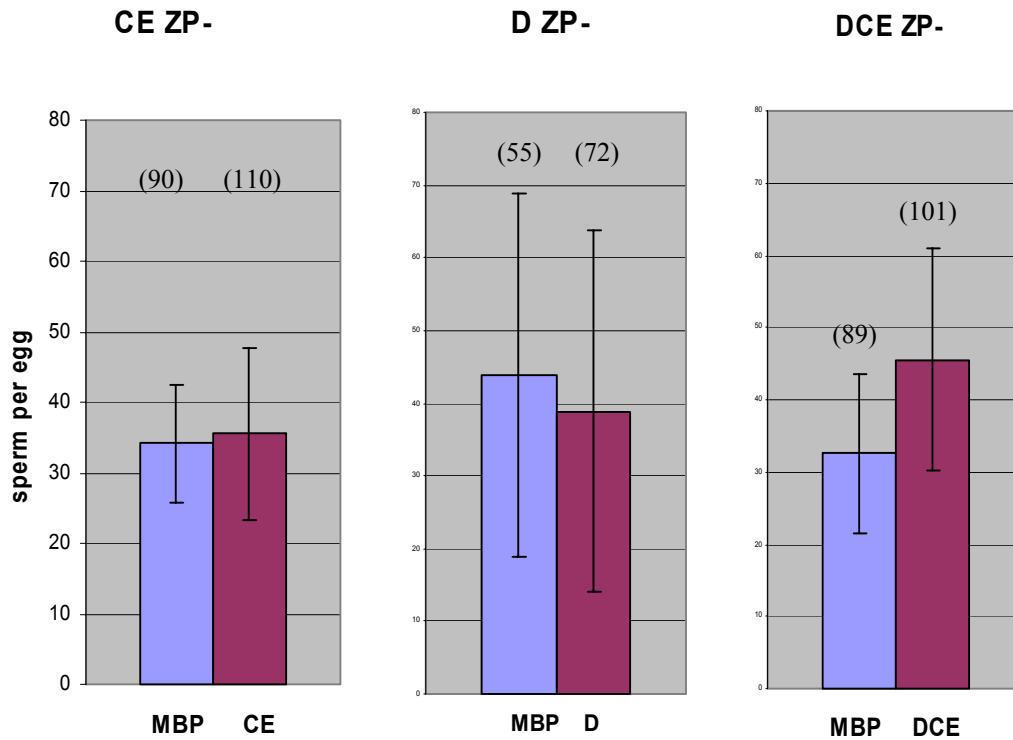


Figure 3.36 Sperm - egg binding assay. Oocytes with Zona Pellucida (ZP+) and without (ZP-) were incubated with recombinant proteins (MBP-CE, MBP-D and MBP-DCE) followed by incubation with sperm. Graphs show number of sperm bound per oocyte. Number of oocytes tested are given in brackets.

3.1.6 Plasmid construction for the transgenic mice

Since membrane proteins form specialized network of interacting molecules on the sperm surface, it is an interesting question whether disruption of this network by overexpressing one of the proteins will affect the sperm. To answer the question we started to generate mouse overexpressing *ADAM 27* gene. To generate transgenic mouse overexpressing *ADAM 27* gene two constructs were generated and simultaneously injected into pronuclei (Figure 3.37). One construct was designed to express EGFP protein under the control of PGK-2 promoter in order to monitor the promoter activity. Second construct for overexpressing *ADAM 27* contained full coding sequence of *ADAM 27* gene with SV40 polyadenylation signal, under the control of PGK-2 promoter as well. PGK-2 promoter was cut out from the donor vector (generously provided by R.P. Erickson) using *Xho I/Hind III* enzymes and cloned into multi cloning site of pEGFP vector and by this way the first construct PGK-2/pEGFP was

generated. cDNA of *ADAM 27* was amplified using Y1fp/Y2rp primers and cloned into pGEMTEasy vector. *ADAM 27* coding sequence was cut out from cloning vector using *Sac I/Not I* enzymes and cloned replacing EGFP gene in the pEGFP vector, giving second construct PGK-2/*ADAM 27*. Constructs were cut from vectors using *ApaLI/BglII* enzymes and DNA was purified prior to microinjection as described in Methods 2.2.21.1. For the microinjection, 4.9 kb PGK-*ADAM27* and 2.7 kb PGK-2 EGFP *ApaLI/BglII* fragments were used.

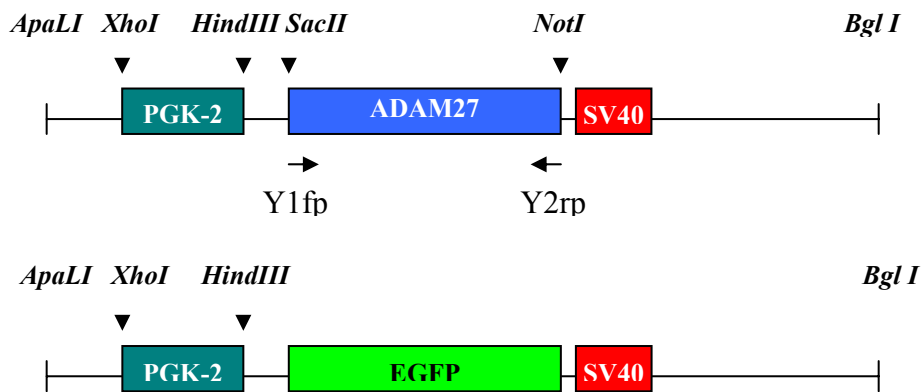


Figure 3.37 Transgenic constructs for *ADAM 27* were generated; *ADAM 27* and EGFP (enhanced green fluorescent protein) genes will be expressed under control of PGK-2 promoter (phosphoglycerate kinase 2), SV40- SV40 polyadenylation signal.

3.1.7 Screening for ADAM 27 interaction partner

In order to find out interaction partner of ADAM 27 protein on the surface of oocyte, yeast two hybrid screening using ovarian cDNA library could be used. Potential interacting domains: disintegrin, Cys-rich and EGF-like were amplified using DisFP(*Nco I*)/DisRP(*Xho I*) modified primers (Figure 3.38). RT-PCR products were cloned into pEG202 vector between *Nco I* and *Xho I* restriction sites, primers were designed that cloned fragment fits to open reading frame of LexA protein. Plasmid containing correct insert was sequenced to verify open reading frame of LEX A-*ADAM 27* junction.

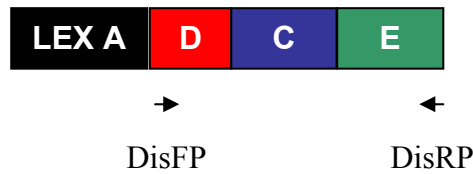


Figure 3.38 Schematic representation of LEX A-*ADAM 27* bait construct with primer location.

ADAM 27 bait before library screening was tested for reporter genes autoactivation properties. For this purpose three, yeast strain EGY48, transformations were done (2.2.24.1):

1. pEG202-*ADAM27* + pSH18-34 (test)
2. pSH17-4 + pSH18-34 (control for strong activation)
3. pRFHM1 + pSH18-34 (control for no activation)

In β -galactosidase assay transformation nr 2 should turn blue while transformation nr 3 should stay white. β -galactosidase assay showed that yeast colonies containing tested bait protein became blue what indicates that *ADAM 27* bait protein containing disintegrin, Cys-rich and EGF-like domains can autoactivate *LacZ* reporter gene (Figure 3.39A). Growth of yeast transformations on selective medium (Gal)-U-H-L showed that test *ADAM 27* bait is able to grow on -Leucin selective medium which indicates autoactivation of *Leu2* reporter gene as well (Figure 3.39B).

To overcome autoactivation problem DupLEX A system provides yeast strain with less sensitive reporter genes (EGY194). Test for autoactivation was performed as described before using EGY194 yeast strain and pJK103 reporter plasmid instead EGY48 and pSH18-34, respectively. Test for autoactivation in medium sensitivity strain EGY194 was again positive indicating that *ADAM 27* bait protein still autoactivates *LacZ* and *LEU2* reporter genes (Figure 3.40)

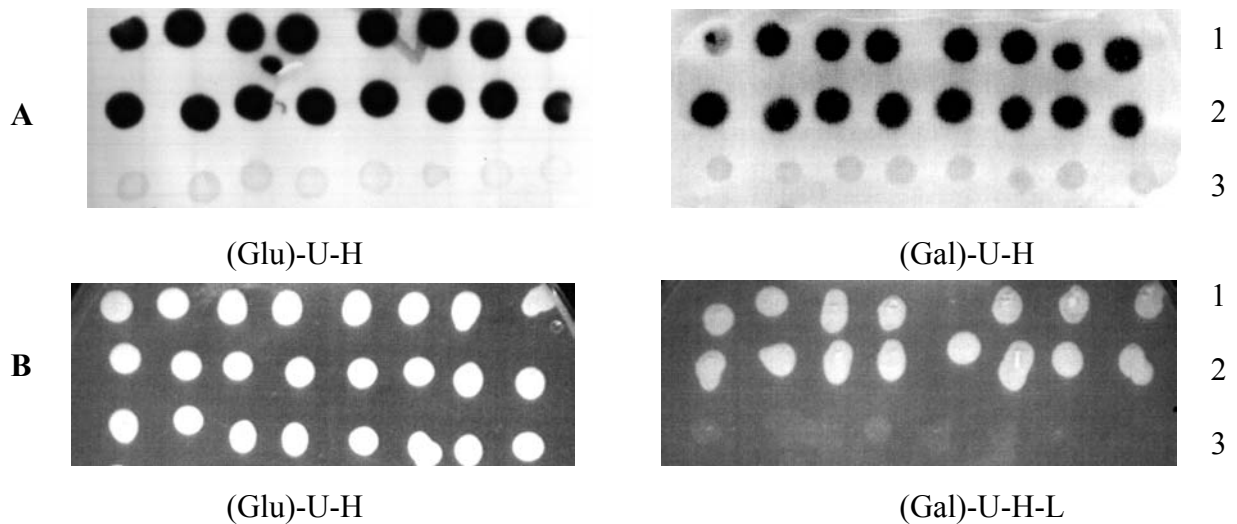


Figure 3.39 Test for autoactivation of reporter genes by ADAM 27 bait protein in the high sensitivity yeast strain EGY48. (A) β -galactosidase overlay assay testing bait autoactivation of LacZ gene. (B) Growth test on selective medium -Leucine, testing bait autoactivation of LEU2 gene. 1- ADAM 27 bait test, 2- control for activation, 3- control for no activation. Dark color on panel A represents blue color in the test; white color in panel B indicates grown colonies.

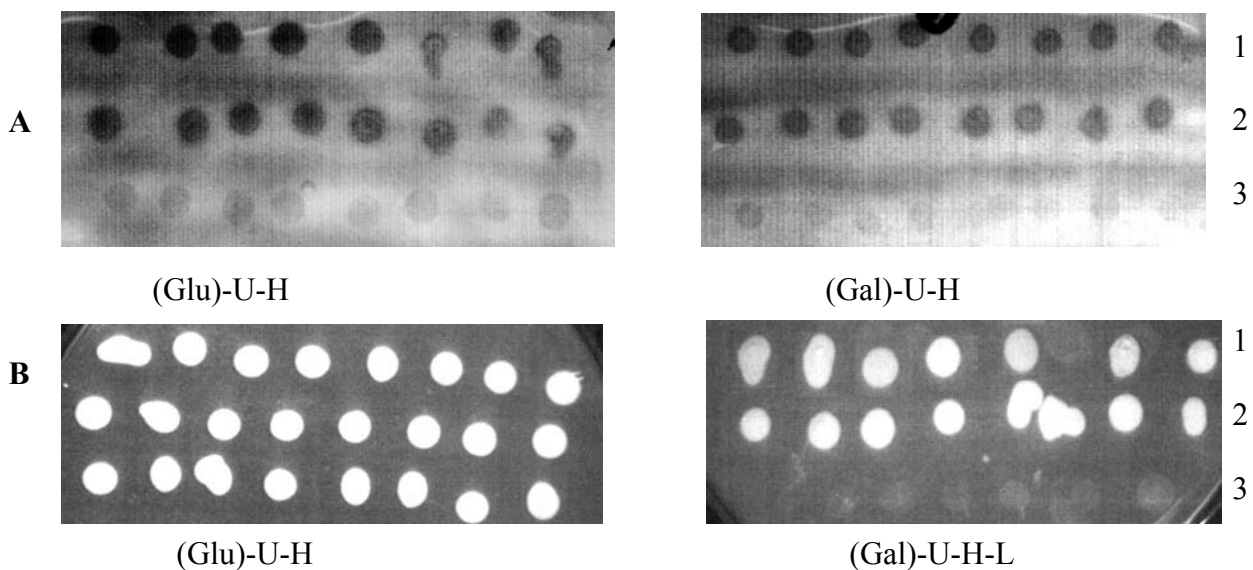


Figure 3.40 Test for autoactivation of reporter genes by ADAM 27 bait protein in medium sensitivity yeast strain EGY194. A. β -galactosidase overlay assay. B. Growth test on selective medium -Leucine. 1- ADAM 27 bait test, 2- control for activation, 3- control for no activation. Dark color on panel A represents blue color in the test; white color in panel B indicates grown colonies.

Autoactivation of ADAM 27 bait protein, containing three functional domains, makes it impossible to perform the yeast two hybrid screening. Bait protein was truncated to disintegrin domain, which is considered as main interacting part of the protein and tested again for autoactivation of reporter genes as described above using medium sensitivity strain EGY194 (Figure 3.41). Truncated protein showed decreased autoactivation properties on LacZ gene showing only bluish staining of tested yeast colonies (Figure 3.41A), but it still autoactivates LEU2 promoter (Figure 3.41B).

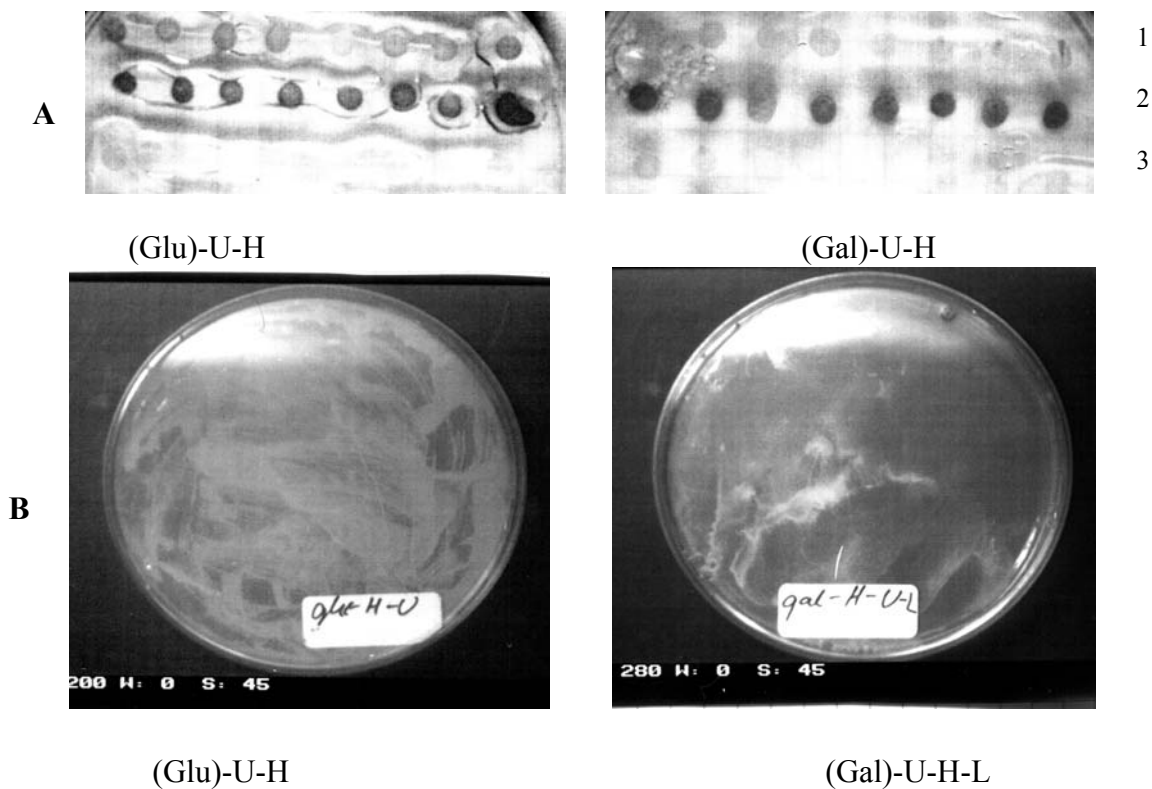


Figure 3.41 Test for autoactivation of reporter genes by truncated ADAM 27 bait protein in the medium sensitivity yeast strain EGY194. (A) β -galactosidase overlay assay testing bait autoactivation of LacZ gene. (B) Growth test of transformation 1 with bait plasmid on selective medium -Leucine, testing its autoactivation of LEU2 gene. 1- truncated ADAM 27 bait test, 2- control for activation, 3- control for no activation. Dark color on panel A represents blue color in the test.

In all three test experiments described above ADAM 27 proteins autoactivated LacZ and LEU2 reporter genes, making it impossible to proceed with interaction screening. ADAM 27 is a surface protein and its interaction take place outside of the cell, in classical yeast to hybrid system interaction takes place in nucleus. In this abnormal localization, ADAM 27 protein could demonstrate these unexpected gene activating properties. It is possible now to

Results

specifically look for interactions between membrane proteins in the membrane based yeast two hybrid screening, which was not available before. To find out ADAM 27 ligand this membrane based screening must be performed.

3.2 Introduction to result section of *testase 2*

Mouse *testase 2* gene was published by Zhu et al. in 1999, (GI:6752969). At the beginning of the study we considered *testase 2* as a single copy gene and we decided to make its knock out to find out the gene function. Based on the published sequence we generated probes used for cosmid library screening, as a result we obtained two positive clones which we used for generation of knock out construct. During restriction analysis of these two clones we found some discrepancies, which we initially explained as different genomic fragment content in both cosmids. We focused then only on one clone and used that cosmid for knock out construct. By Southern blot analysis we obtained results which were indicating the possibility that *testase 2* is not a single copy gene. Cloning and restriction digestion of cDNA fragment showed 2 different restriction patterns, confirming that in fact at least 2 copies of *testase 2* gene exists. Later on, with the access to Celera mouse genomic database (Celera Genomics) our results were confirmed. *Testase 2* gene was duplicated in mouse genome and two copies exist on chromosome 8 in distance of around 24 kb, both genes have two exons with ATG codon in second exon (Figure 3.42). This intron-exon structure is exceptional among known ADAM family members, they are typically composed of about 20 exons, like *ADAM 27* gene. We designated both copies as *testase 2 α* and *testase 2 β* , respectively (Bolcun et al., 2003). In the results part we will describe expression studies, knock out generation and finding the duplication of *testase 2* gene.

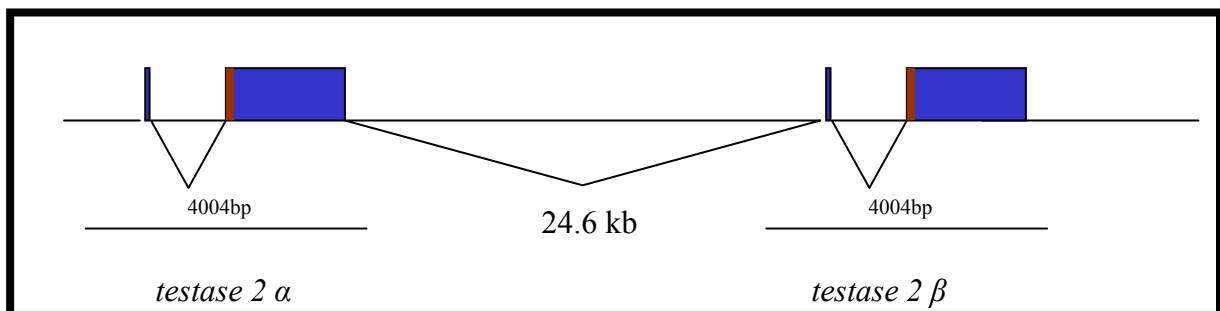


Figure 3.42 Exon - intron structure of *testase 2* genes.

3.2.1 Transcriptional analysis of *testase 2*

It was shown earlier by Northern blot analysis (Zhu et al., 1999) that *testase 2* expression reaches adult level at day 25 of postnatal testis development. We used more sensitive method (RT-PCR) to study gene expression. RT-PCR was performed on testicular RNA from 5 to 25 days old and adult males. Additionally, RT-PCR was done using RNA from testes of different mutants as described before (3.1.1). *Testase 2* transcript is detectable by RT-PCR at day 20 (Figure 3.43A) and increases later on, presence of transcript only in *olt* and *qk* mutants (Figure 3.43 B) indicates that the expression is restricted to post meiotic stages of spermatogenesis - spermatids. To check if transcription is germ cell specific, RT-PCR was performed on RNA from 15P1 and MA10 cell lines (Figure 3.44). Detectable expression only in total testis RNA but not in Sertoli or Leydig cell lines indicates germ cell specific expression of *testase 2* gene.

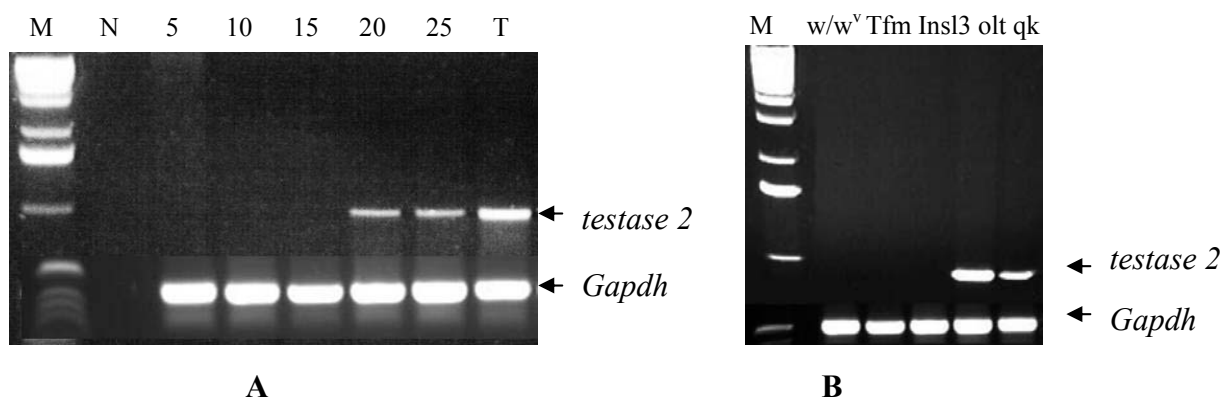


Figure 3.43 RT- PCR analysis on total RNA isolated from various developmental stages of mouse testis development (A) and testes of different mutants (B) using *testase 2*-specific primers. Expression is present from day P20 and continues during testicular development. Expression was detected in *olt* and *qk* mutants indicating germ cell specific expression of the gene. M: standard molecular weight marker, N: negative control (H₂O), T: adult testis. RT-PCR for *GAPDH* transcript performed as positive control.

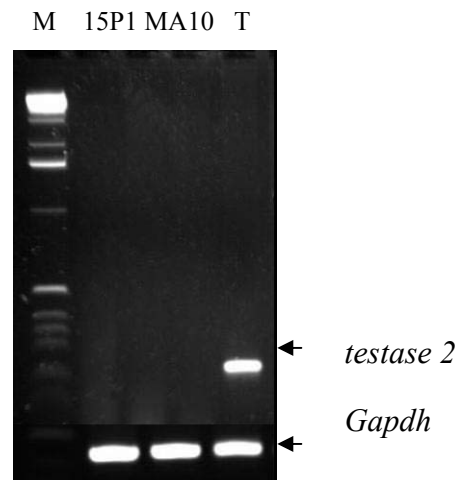


Figure 3.44 RT-PCR analysis of *testase 2* transcription in Sertoli and Leydig cell lines performed on total RNA isolated from cultured cells. Expression was detected only in testicular RNA. M: marker, 15P1: Sertoli cell line, MA10: Leydig cell line and T: testis. *Gapdh*: control for RNA quality.

3.2.2 *Testase 2* is a duplicated gene

Using primers Tesfp/Tesrp a 1.3kb fragment was amplified (Figure 3.45 A) and cloned into pGEMtEasy vector. After plasmid preparation, DNA of few clones was digested with *Eco* RI to check for presence of *testase 2* fragment (Figure 3.45 B). Two different restriction patterns were observed. The sequence analysis of these two types of clones revealed two different transcripts with high similarity to the known transcript for *testase 2*. Comparison of the two full-length cDNAs with that of *testase 2* showed high homology of 87.8 and 95.5%, respectively, and were therefore designated as *testase 2 α* and *2 β* .

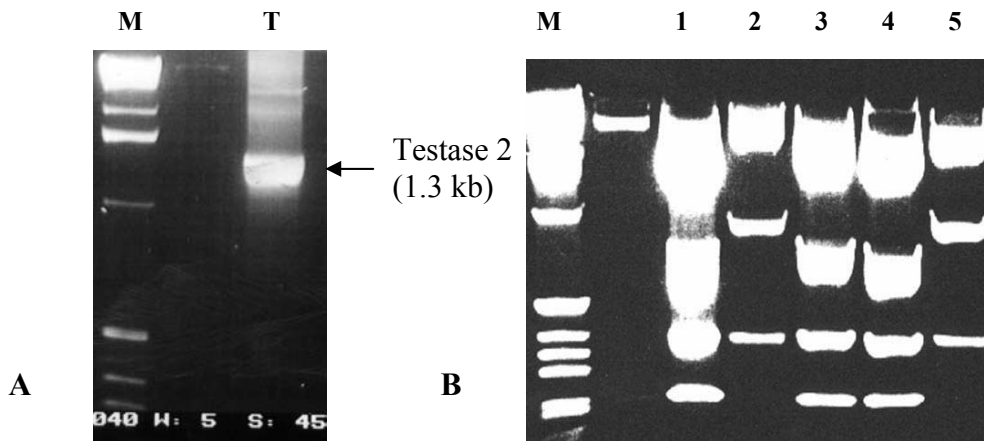


Figure 3.45 (A) Product of RT-PCR using Tesfp and Tesrp primers (1.3 kb). (B) DNA of minipreps with cloned fragment of *testase 2* gene digested with *Eco* RI. Two different restriction patterns are observed: clones 1, 3 and 4 represent pattern expected from published sequence, clones 2 and 5 new pattern.

Testase 2 1.3 kb fragment was radioactively labeled and used in Southern blot hybridization with genomic DNA digested with *Bgl* I, *Spe* I, *Bam* HI and *Eco* RV. As it is shown in Figure 3.46 A, double or triple bands were detected. Restriction analysis of genomic fragment containing both genes α and β was performed using online software (NEB cutter). Restriction analysis enabled to partially explain the results of hybridization. Obtained signals 1, 3, 5 and 7 belong to *testase 2* α and 2, 4, 6 and 8 to *testase 2* β (Figure 3.46 B). Additional bands could arise from cross hybridization with other ADAM family genes.

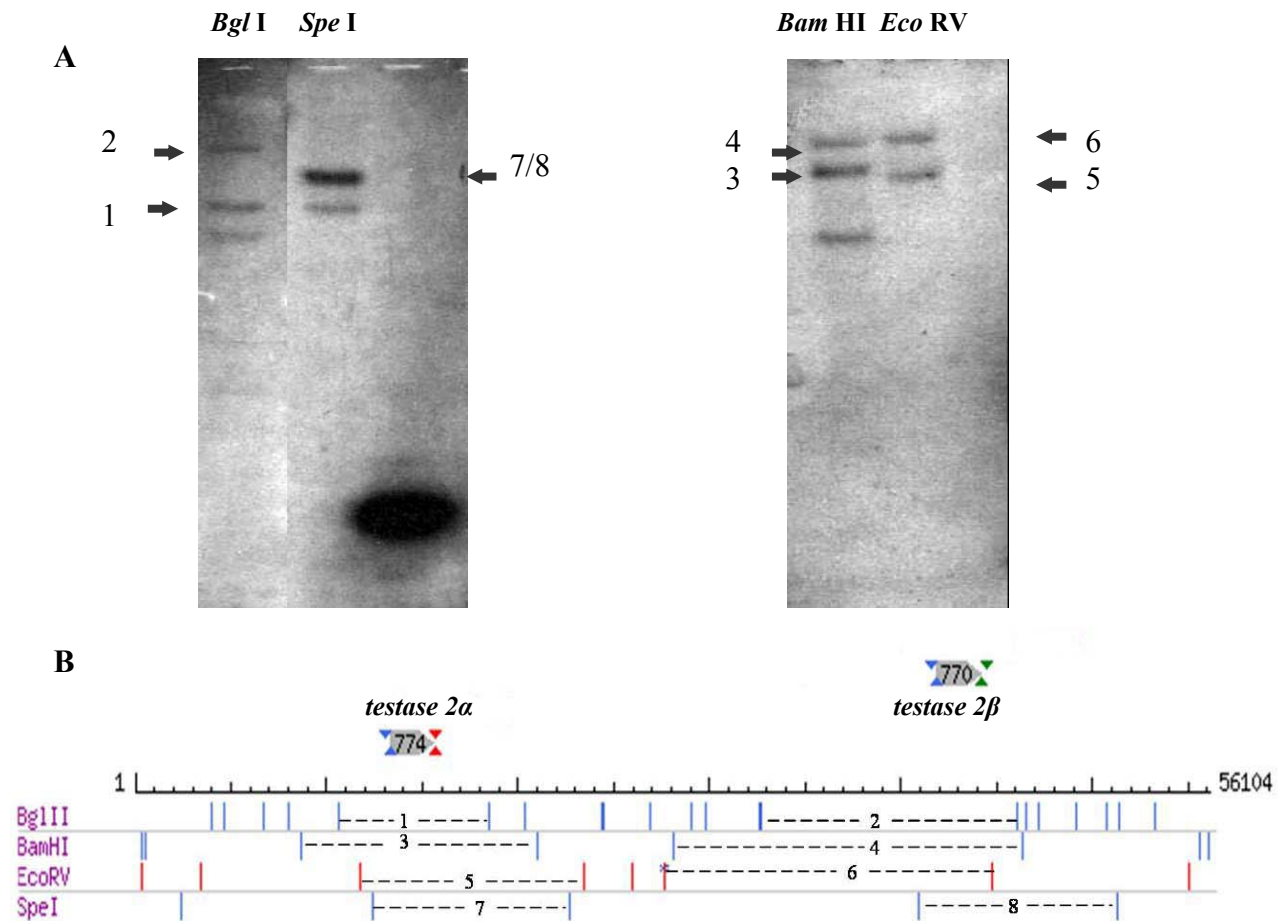


Figure 3.46(A) Southern blot hybridization of genomic DNA digested with *Bgl*I, *Spe*I, *Bam*HI and *Eco*RV, with radioactively labeled 1.3 kb *testase 2* fragment. (B) Restriction digestion analysis of genomic fragment containing both *testase 2* genes. Numbers represent corresponding restriction fragments.

Since we know that there are two transcripts for *testase 2*, we were interested to check whether both genes have the same expression pattern. To achieve this, RT-PCR on testicular RNA from 5 to 25 days old, adult males and mutant mice was repeated, RT-PCR products were then digested with *Xba*I enzyme, to distinguish both copies (Figure 3.47 A), and separated by agarose gel electrophoresis (Figure 3.47 B). *Testase 2α* and *2β* are simultaneously expressed during mouse testis development and in testes of mutant mice (Figure 3.47 B).

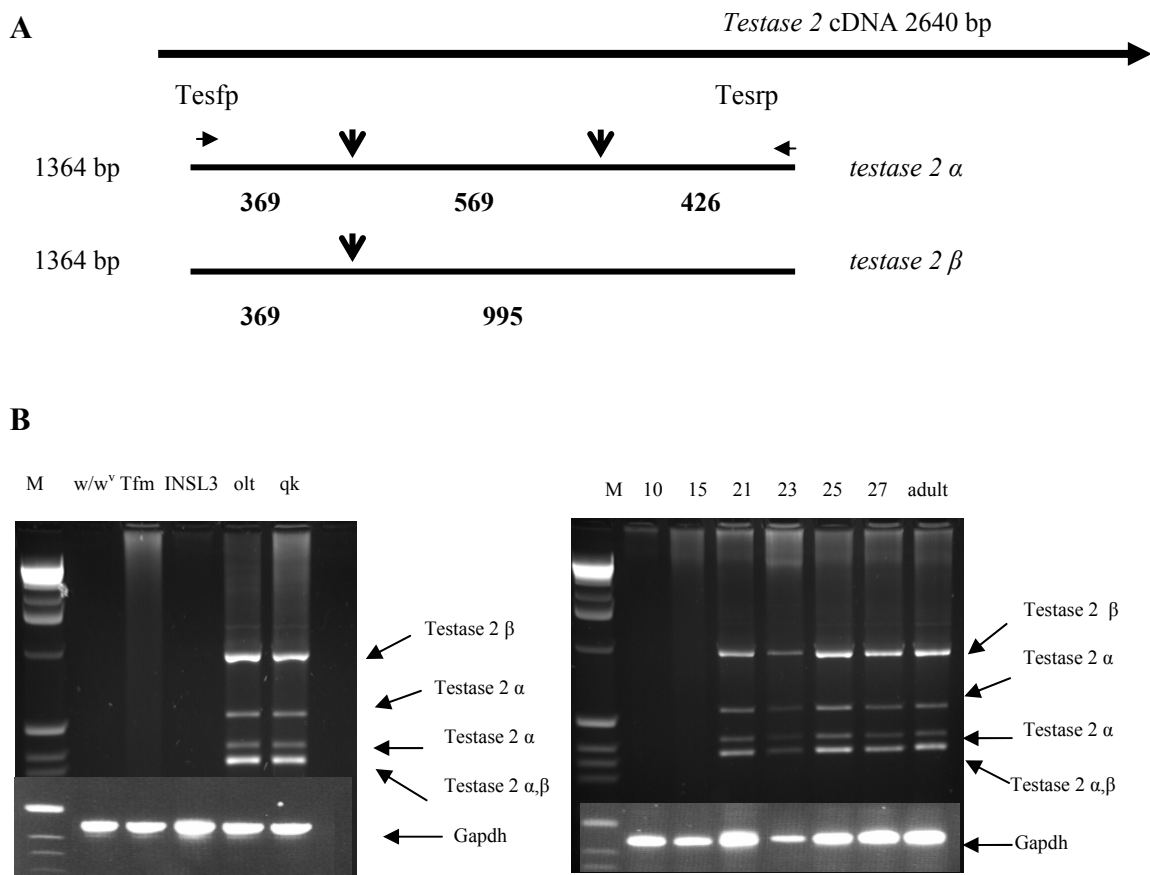


Figure 3.47 (A) Schematic representation of digestion strategy. Vertical arrows represent primers (TesFP nt 71-96 and TesRP nt 1412-1435) and horizontal arrows *Xba* I recognition sites. For *testase 2 α* and *2 β* , RT-PCR products of 1364 bp were obtained. Numbers represent the length of fragments after *Xba* I digestion in bp. (B) Expression pattern of *testase 2 α* and *2 β* during male germ cell differentiation. Reverse transcriptase-polymerase chain reaction (RT-PCR) products were digested with *Xba* I, and four fragments were obtained which were originated from the *testase 2 α* and *2 β* transcripts. Reverse transcriptase-polymerase chain reaction (RT-PCR) products were observed only using testicular RNA from *qk* and *olt* mice indicating the haploid expression of *testase 2 α* and *2 β* genes. Haploid expression was confirmed by RT-PCR analysis using testicular RNA of mice in different developmental stages. RT-PCR products were observed from day 21, which correlates with appearance of first haploid germ cells in testis.

3.2.3 Targeted inactivation of *testase 2* gene

3.2.3.1 Isolation of cosmid clones with *testase 2* genomic DNA.

The mouse RZPD ([The Resource Center and Primary Database](#)) genomic library 129 ola was screened using a fragment of *testase 2* cDNA (Tes2) and 2 positive clones were obtained: MPMGc121H24624Q2 and MPMGc121H20723Q2. These two cosmid clones were digested with restriction enzymes, blotted to Hybond C membrane and hybridized with 5'probe (Figure 3.48 A). Cosmids showed slightly different restriction patterns indicating different genomic content of both clones. Clone MPMGc121H20723Q2 was used for generation of knock out construct. During the time knock out construct was generated it was not known that there are two copies of the *testase 2* gene. Sequencing results and restriction analysis have suggested that cosmid MPMGc121H20723Q2 contains *testase 2 α* genomic fragment whereas clone MPMGc121H24624Q2 contains genomic fragment with *testase 2 β* .

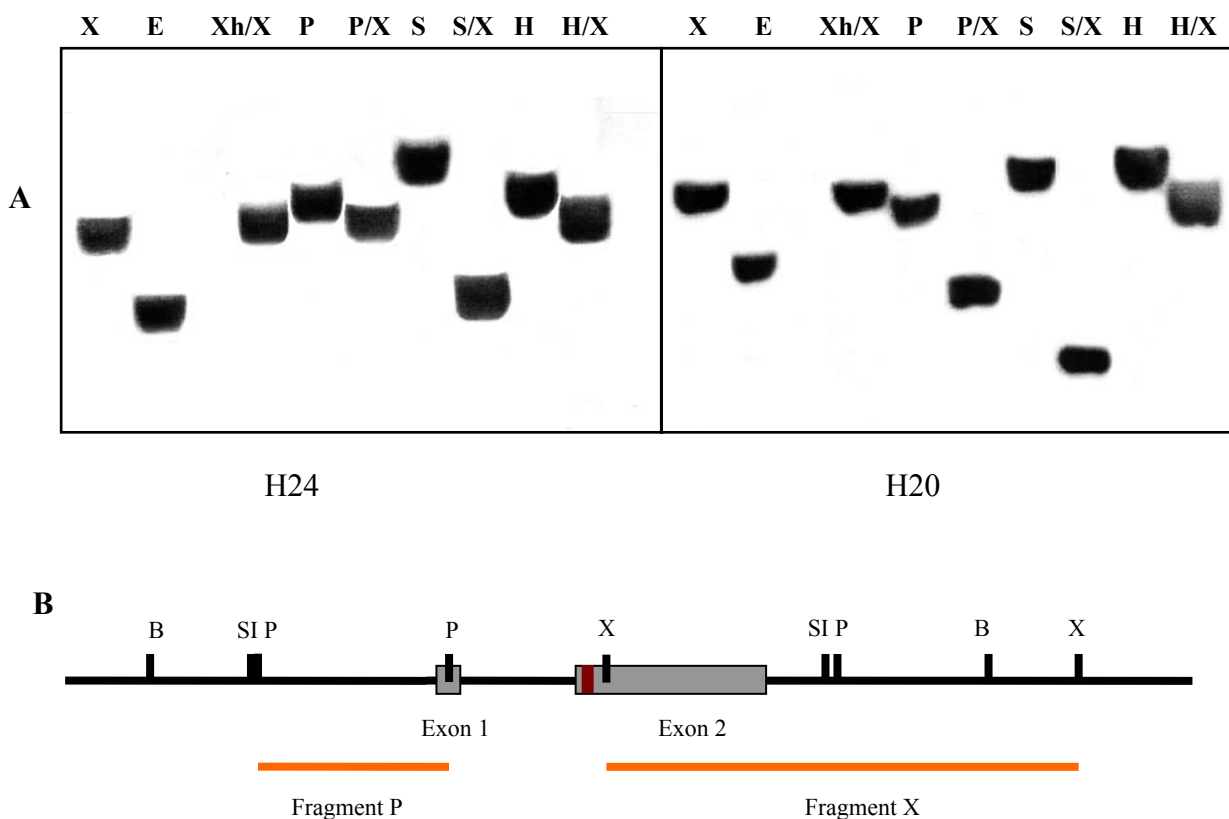


Figure 3.48 (A) Radioactive hybridization of cosmid clone H24624Q2 and H20723Q2 digested with different restriction enzymes. **(B)** Restriction digestion map of *testase 2* genomic DNA and fragments which were cloned, abbreviations are: B: *Bam* HI; E: *Eco* RI; H: *Hind* III; P: *Pst* I; SI: *Sst* I; X: *Xba* I; Xh: *Xho* I.

A knock out construct was generated in which 5' wing 2 kb *Pst* I fragment was cloned into *Sal* I/*Cla* I site of pTKNeo vector and 3' wing 5 kb *Xba* I fragment was cloned into *Spe* I/ *Bam* HI site of the targeting vector (Figure 3.49). After homologous recombination genomic fragment of about 1.8 kb was replaced by *Neomycin* resistance gene. Neomycin was used as positive selection marker and two copies of herpes virus *Thymidine kinase* (Tk) as a negative selection marker. The correct orientation of both 5' and 3' wings was confirmed by sequencing of the construct with vector specific primers pTKNf, pTKNr, pTKr.

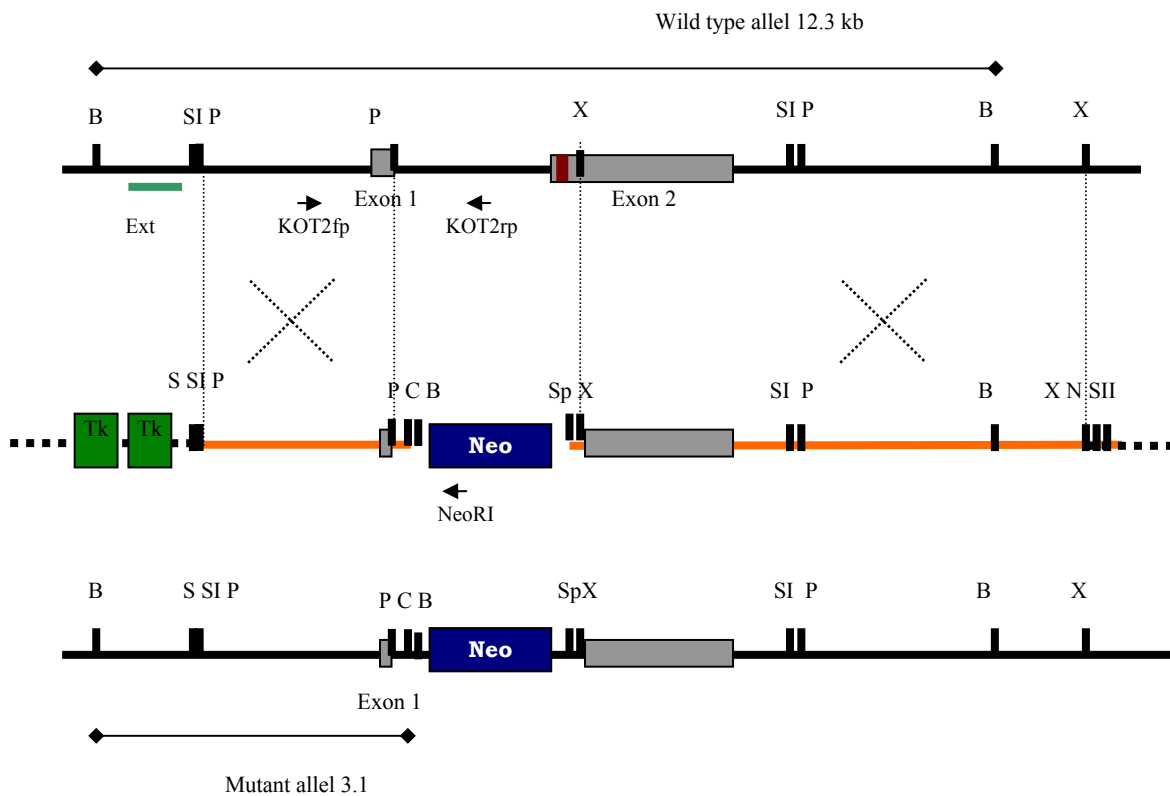


Figure 3.49 Schematic representation of the targeting strategy. Wildtype *testase 2* locus (A), targeting vector (B) and mutated allele (C). Parts of exon 1 and 2 were replaced by the *Neomycin* resistance gene cassette. External probe is marked as green bar, which enables to recognize a 12.3 kb *Bam* HI fragment in wildtype DNA and a 3.1 kb fragment in recombinant DNA. The gray boxes represent exons of *testase 2* gene, green boxes stand for *Thymidine kinase* and blue box stands for *Neomycin*. KOT2fp, KOT2rp and NeoRI are the primers used for genotyping. The restriction site abbreviations are: B: *Bam* HI; C: *Cla* I; P: *Pst* I; SI: *Sst* I; SII: *Sst* II; Sp: *Spe* I; S: *Sal* I; X: *Xba* I; N: *Not* I; Ext: external probe.

3.2.3.2 Generation of the 5'external probe

A fragment about 0.5 kb was amplified by PCR using expT2fp and expT2rp primers. Fragment is localized upstream of *testase 2* gene and was designed to detect *Bam* HI wildtype fragment in Southern blot hybridization (Figure 3.49). This PCR product was subcloned into pGEM-TEasy and cut out with *Eco* RI. The 0.5 kb fragment was extracted from the agarose gel and used as 5' external probe for hybridization in the Southern blot with DNA extracted from the recombinant ES-clones. Generated external probe was detecting exclusively genomic fragment belonging to *testase 2 α*.

3.2.3.3 Electroporation of the RI ES-cells and screening of ES-clones for homologous recombination events

The *testase 2* targeting vector was linearised by digestion with *Sst* II restriction enzyme and 50 µg of linearised DNA was electroporated into RI embryonic stem cell line as described in section 2.2.20.1. The cells were plated on fibroblast feeder layer and after 10 days of selection individual *Neomycin* resistant clones were picked in 24 well plates and replicated. Genomic DNA was prepared from these ES clones for Southern blot analysis. DNA of individual ES clones was digested with *Bam* HI and electrophoresed into 1% agarose gels and blotted onto Hybond C membranes. The blots were hybridised with ³²P-labelled 0.5 kb 5' external probe. In the case of homologous recombination event two bands were expected, a wildtype allele of 12.3 kb and a recombinant allele of 3.1 kb. However in the event of a random integration, only wildtype allele will be detected (Figure 3.50). By screening of more than 200 clones no homologues recombination was found. Further transfections were not performed due to the fact, that there is gene duplication and knocking out both copies, would be impossible because of the close distance between genes. Furthermore, knocking out only one copy would not give clear information about gene function. Other question also arises: if these two genes are functional or these are pseudogenes.

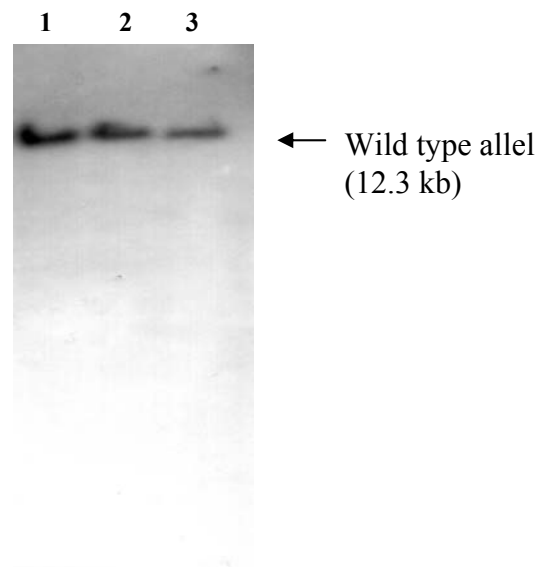


Figure 3.50 Genomic Southern blot analysis. Genomic DNA of ES clones was digested with *Bam* *HI*, separated on 1 % agarose gel and transferred onto Hybond C membrane. The blot was hybridized with radioactive labeled 0.5 kb external probe. In case of wildtype allele (ES clones 1,2 and 3) one band of 12.3 kb was observed and in case of recombinant allele two bands would be observed, in addition to wildtype allele, a recombinant allele of 3.1 kb.

4 DISCUSSION

ADAM is an interesting family of proteins, characterized by having two potential activities, proteolytic and adhesive. Many of the ADAM family genes are expressed in the testis indicating the important role of these molecules in germ cell differentiation and in participation in gamete interaction during fertilization. The functions of two murine proteins expressed in testis, namely cyritestin and fertilin β were already elucidated, indicating their role in the fertilization process (Shamsadin et al., 1999; Cho et al., 1998). It requires further investigations to understand the functions of all testis specifically expressed ADAMs in spermatogenesis and fertilization. We focused our work on two germ cell specific genes, *ADAM 27* and *testase 2*, and their possible functions. In the first part of this thesis, the results concerning *ADAM 27* gene are discussed. Its genomic structure, chromosomal localization and expression pattern are characterized. Two different types of approaches in vitro (sperm-egg binding assays and yeast two hybrid screen) and in vivo (knock out and transgenic animal models) are described with their implications for *ADAM 27* function. In the second part of the thesis I discuss duplication, genomic structure and expression of *testase 2 α* and *testase 2 β* genes. And finally, the generation of the knock out construct, difficulties in producing *testase 2 α* and *β* null mice, and possible functions of *testase 2 α* and *β* proteins are discussed.

4.1 ADAM 27 gene structure and chromosomal localization

We have analyzed the genomic structure of *ADAM 27* in the mouse. *ADAM 27* gene is composed of 20 short exons, which is similar to other known ADAM family genes. Two well known testis specific ADAMs: *cyritestin* and *fertilin β* are composed of 22 and 21 short exons (Cho et al., 1997), respectively. Other genes beside mentioned above that share this genomic structure are *ADAM 32* (22 exons), *ADAM 9* (22), *ADAM 19* (24) and *ADAM 28* (24). Human orthologue of mouse *ADAM 27*, *ADAM 18* has preserved this genomic structure during evolution. Human *ADAM 18* is also composed of 20 exons, human and mouse genes share 79% identity on the cDNA level and 62% on the protein level.

FISH hybridization mapped *ADAM 27* to mouse chromosome 8, where it is located in close proximity to four other members of ADAM family, namely *ADAM 3* (*cyritestin*), *ADAM 5*, *ADAM 32* and *ADAM 9*. In the rat, except *ADAM 9* all above mentioned genes are located on chromosome 16, the synteny region to mouse chromosome 8. *ADAM 18*, *ADAM 3*, *ADAM 5*, *ADAM 9* and *32* are clustered also on the human syntenic region on chromosome 8. This indicates that these ADAM genes have been generated by gene duplication during evolution and that the region of the ancestor chromosome containing clustered ADAM genes has been preserved.

4.2 Expression of *ADAM 27* gene

4.2.1 Transcriptional analysis

Expression analysis of *ADAM 27* gene showed that this gene is expressed specifically in testis as described earlier (Zhu et al., 1999). Thus, *ADAM 27* joins a subgroup of 15 ADAMs that are specifically or predominantly expressed in testis. Furthermore, we have shown that *ADAM 27* gene is exclusively expressed in germ cells, since no transcript was detected in somatic testicular cells. By RT-PCR method first appearance of transcription was detected at day 15 of postnatal development. At this stage about 82% of tubules contain pachytene spermatocytes (Nebel et al., 1961), indicating that expression of *ADAM 27* gene takes place at meiotic stages of spermatogenesis. Few other ADAM genes have been reported to be expressed at the same stage of spermatogenesis. *Fertilin β* was detected by in situ hybridization in early pachyten spermatocytes, whereas *cyritestin* and *ADAM 5* are expressed in mid to late pachytene spermatocytes (Wolfsberg et al., 1995). Some other testicular genes are transcribed in later postmeiotic stages of germ cells differentiation, for example *ADAM 4* (Wolfsberg et al., 1995) or testases 1-3 (Zhu et al., 1999) are expressed in spermatids.

The orthologues to mouse *ADAM 27* gene have been described, rat *tMDCIII*, macaque *tMDCIII* and human *ADAM 18 (tMDCIII)* (Frayne et al., 1997; Frayne et al., 1998; Frayne et al., 2002). Rat *tMDCIII* as well as human and mouse genes were found to be also expressed exclusively in testis, while macaque *tMDCIII* transcript is expressed in a range of different tissues (including muscle, lung and kidney), albeit at a lower level when compared to the testis (Frayne et al., 1998). There is no information about time of expression during spermatogenesis for human and macaque *tMDCIII* genes. Expression of the rat *tMDCIII* was correlated with the presence of diplotene spermatocytes, either being expressed by these cells, or being expressed in pachytene spermatocytes but not being detected until the numbers of such cells have increased. Similar to mouse, rat *tMDCIII* expression corresponds with the presence of spermatocytes although very low levels of transcript were also detected from day 1 postpartum, possibly indicating a degree of species variation in the onset of expression (Frayne et al., 1997). Expression analysis of *ADAM 27* has indicated that this gene is specifically expressed in germ cells with the onset of expression in pachytene spermatocytes.

4.2.2 Characterization of the ADAM 27 protein and its potential role in the testis and/or sperm surface

Polyclonal antibody against mouse ADAM 27 was able to detect the protein in testicular extracts that was in agreement with predicted size for ADAM 27. Testicular proteins were extracted with Triton X-114 and aqueous and detergent phases were analyzed, ADAM 27 signal appeared in detergent phase indicating that ADAM 27 is in fact a transmembrane protein. In similar experiments it was shown that cyritestin, an other ADAM is also a membrane protein (Linder et al., 1995).

Translational analysis of ADAM 27 protein revealed that ADAM 27 is produced directly after transcription. The protein was detected at day P15 of postnatal testis development, when it coincidences with onset of transcription. Therefore, post-transcriptional regulation can not be postulated. A different situation was reported for cyritestin. By RT-PCR the mRNA was detected at day P14 whereas protein was detected first around P18, suggesting that cyritestin mRNA is stored for about 4 days prior to translation (Linder et al., 1995).

Guinea pig fertilins α and β (Blobel et al., 1990), mouse cyritestin (Linder et al., 1995), rat fertilin β (McLaughlin et al., 1997) and macaque MDC proteins (Frayne et al., 1998) have all been shown to undergo endoproteolytic processing during epididymal sperm transport. ADAM 27 was detected by Western blot method in epididymal sperm extracts. When proteins from three parts of epididymis: caput, corpus and cauda were used for analysis, processing of ADAM 27 was also detected. However, only a minor modification was detected in comparison to macaque tMDCIII or human ADAM 18 proteins (Frayne et al., 1998; Frayne et al., 2002). When spermatozoa leaves the testis it is not yet capable of fertilizing an oocyte. This fertilizing ability is acquired during epididymal transit where extensive remodeling of the plasma membrane and modification of membrane proteins take place. Was is interesting is that, many of the sperm surface proteins, that are post-testicular processed, are proposed to be important in gamete interaction during fertilization (Jones et al., 1996). Therefore a number of testicular ADAMs processed in the epididymis and maintained on the sperm may be involved in some aspects of gamete interaction, possibly via an integrin receptor on the oocyte plasma membrane as has been shown for mouse fertilin (Evans et al., 1997a,b). Thus, ADAM 27 is an attractive candidate protein for sperm-egg interactions.

The intratesticular distribution of ADAM 27 protein was determined by immunohistochemistry staining of mouse testis sections. ADAM 27 protein was detected in

spermatocytes as a round signal above the nucleus. This localization corresponds to Golgi complex that participates in biogenesis of the acrosome (Figure 4.1). However, in later stages of spermatogenesis the signal is still round or dispersed in differentiating spermatids and rather localized to polar lobe than to the acrosome. Golgi-ER complex in spermatocytes is very active, synthesis and maturation of future acrosomal proteins take place there. Once these proteins are produced and translocated to the acrosome, the Golgi complex is not any more functional. Infact, after the stage of early spermatids it becomes segregated and restricted to the posterior portion of the spermatid in a polar lobe. Later, the polar lobe together with the excess-cytoplasmic matrix are dissociated from the spermatid as a residual body (Figure 4.2).

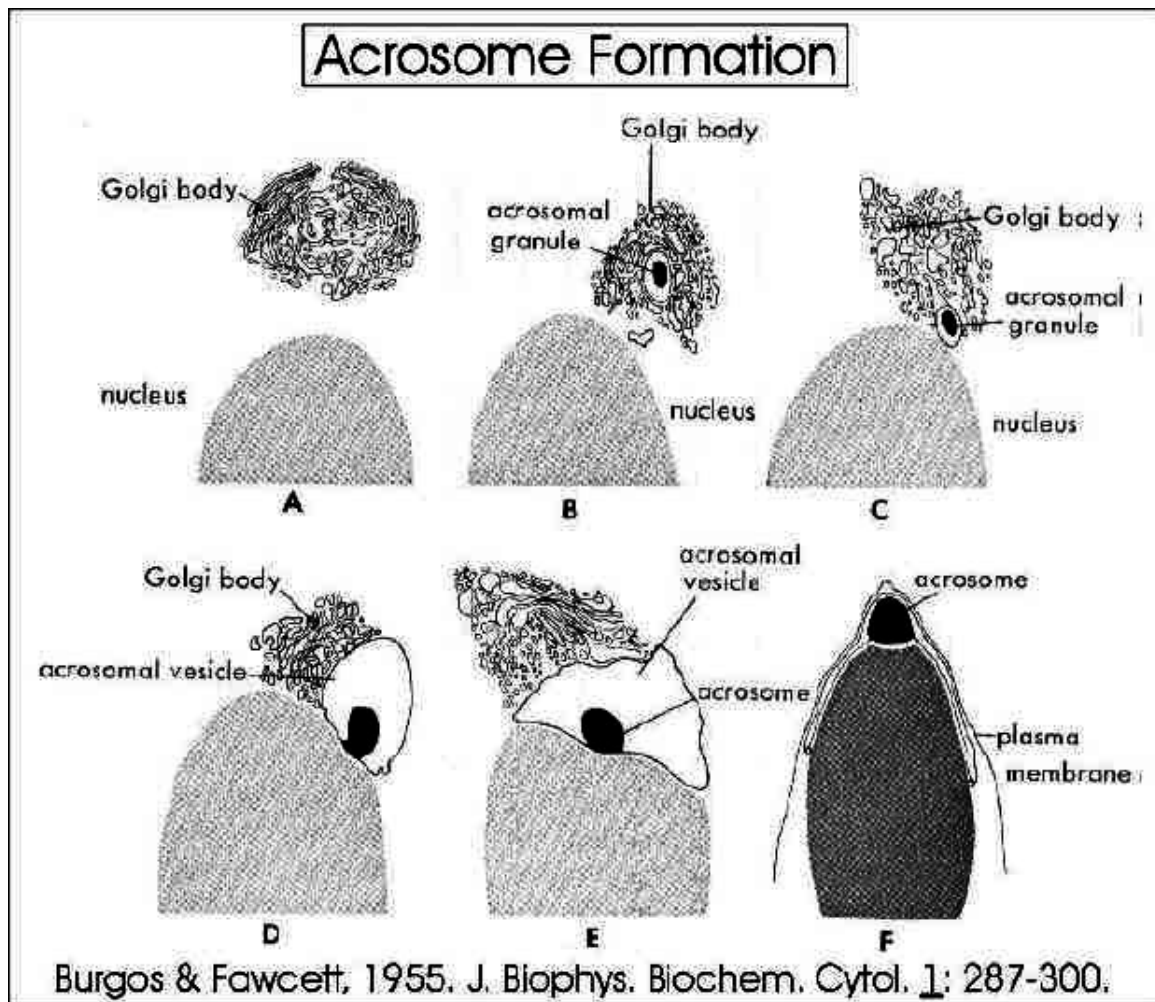


Figure 4.1 Schematic view of acrosome formation and participation of Golgi complex in this process A-F represents different stages of acrosome biogenesis.

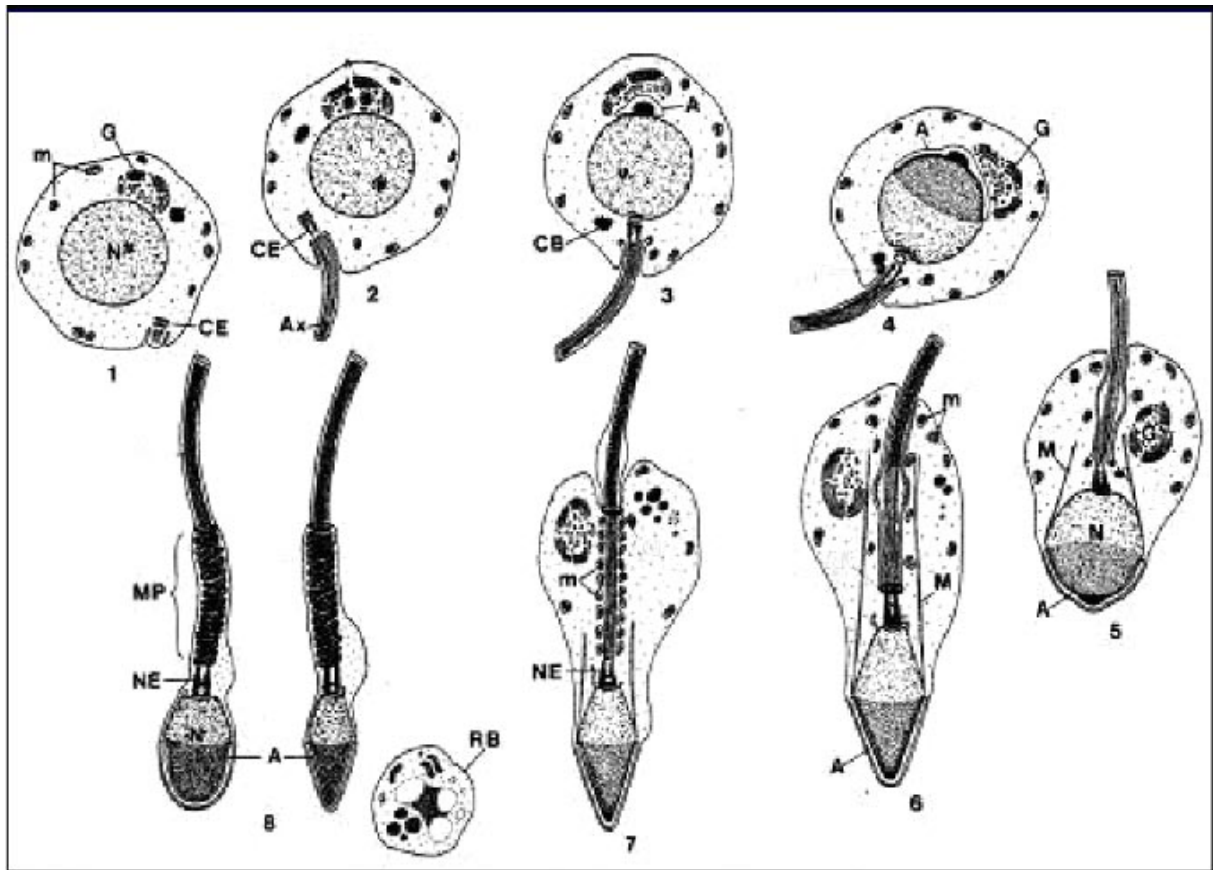


Figure 4.2 Differentiation of spermatid into mature sperm, acrosome and tail formation can be seen. Golgi complexes, ribosomes, specialized membranous organelles, and long strands of smooth endoplasmic reticulum, these organelles are abundant at the early spermatid stage of sperm development, Golgi complexes, ribosomes, and excess ER, which are not functional after this stage, become segregated and confined to the posterior portion of the spermatid in a polar lobe. Later, the polar lobe together with excess cytoplasmic matrix is bound by a membrane and dissociated from the spermatid as a residual body. The spermatid is then devoid of Golgi complexes and ribosomes. m: mitochondria, G: Golgi, CE: centriole, A: acrosome, N: nucleus, M: manchette, RB: residual body, NE: neck, MP: midpiece; 1-8 stages of spermiogenesis.

It is possible that ADAM 27 initially linked to Golgi apparatus, later becomes dispersed in the cytoplasm and is finally relocated and removed with the residual body, as it is schematically proposed in Figure 4.3. We cannot conclude now, whether all of ADAM 27 protein is placed later in the residual body, or only that part which is not placed in the developing sperm.

Appearance of the late signal does not resemble typical formation and flattening of the acrosome during spermiogenesis. Thus from these results we cannot say whether ADAM 27 is located in the acrosome or not. It is also possible, that once ADAM 27 is packed into the

acrosome, immunodetection is not possible any more. From the other hand strong staining near the lumen of tubule, colocalizing with residual bodies of differentiated sperm, might indicate that ADAM 27 is not at all localized to acrosome and sperm head. It is known, that some epididymal sperm possess also residual bodies and that could explain signal obtained in Western blot analysis of epididymal sperm extracts.

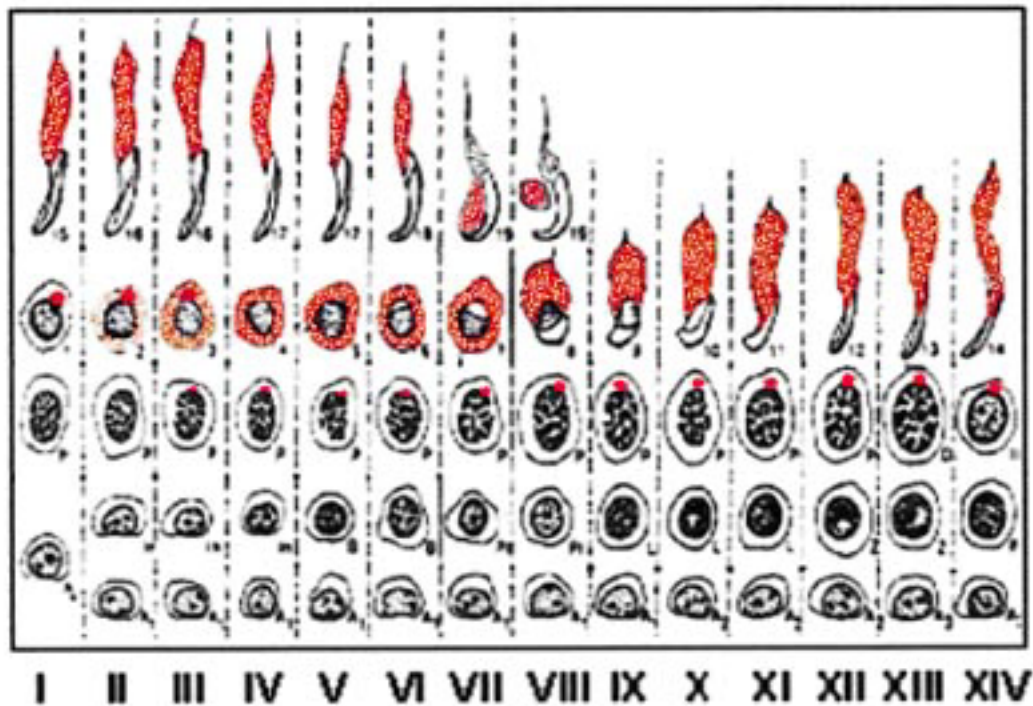


Figure 4.3 Schematic representation of proposed model for ADAM 27 relocation during spermatogenesis, red colour represents ADAM 27 protein.

Furthermore, the fact that we were not able to detect ADAM 27 protein on the mature sperm by immunolocalization might also support this suggestion.

However, a similar situation was reported for human ADAM 18. Specific antibodies detected protein in the sperm extracts by Western blot method, while they failed to localize it by indirect immunofluorescence (Frayne et al., 2002). The same instance was reported for macaque tMDCI and tMDCIII antibodies (Frayne et al., 1998). This is not an unusual problem. There are many documented examples of antisera which are usable for Western blotting but fail to work in immunolocalization studies. It can be attributed to extremely cysteine-rich nature of ADAM proteins. As the specific disulfide bond configuration is

undoubtedly important in the proteins native conformation. Bacterially expressed ADAM proteins will have a large proportion of either free cysteine residues or inappropriate disulfide bonds. Immunization of rabbits with these proteins may generate antibodies with low activity and specificity towards native ADAM proteins. We encountered this problem with polyclonal antibodies generated against recombinant ADAM 27 protein. These antibodies were capable to detect ADAM 27 protein in testicular extracts by Western blot. We have also produced recombinant ADAM 27 proteins (see MBP-DCE, -CE and D) in bacterial systems and the antibody was able to detect them. However, the antibody failed in all attempts of indirect immunofluorescence and immunohistochemistry. The other anti ADAM 27 antibody generated against synthetic peptide was capable to detect the protein in Western Blot approach as well as in immunohistochemistry on paraffin testis sections. Although, the antibody also failed to immunolocalize ADAM 27 on the sperm by indirect immunofluorescence. Thus, it remains questionable whether ADAM 27 is present on the mature sperm, and further experiments are necessary to answer this question.

Initially we expected that ADAM 27 as a membrane protein can be found on the membrane of developing germ cells or at least on the membranes of sperm as it was shown for cyritestin or fertilin. Cyritestin was immunolocalized to the acrosomal region of the sperm head (Linder et al., 1995; Forsbach and Heinlein, 1998), other mouse sperm specific ADAM - Testase 1 was found in the equatorial region of the sperm head (Zhu et al., 2001).

The localization process of the Guinea pig fertilin is well described in the literature. After synthesis, fertilin is not directly inserted into the membrane but is intracellularly present in spermatocytes and round spermatids (Carroll et al., 1995). The incorporation into the plasma membrane takes place at late stages of spermatogenesis. Temporal regulation of the surface expression of the germ cell surface antigens, has been reported for other species (mouse: O'Brien and Millette, 1984, rabbit: O'Rand and Romrell, 1980). It is possible that other components required for the transport and processing of plasma membrane proteins are inactive during spermatid elongation, therefore, it requires that proteins destined for the plasma membrane are synthesized at an earlier stage and stored until the appropriate time for surface expression comes. In the differentiated testicular sperm of Guinea pig, fertilin is present in the whole head plasma membrane. During the epididymal passage, fertilin becomes concentrated in the posterior head plasma membrane, and after the acrosome reaction it remains in the same position (Hunnicuttt et al., 1997; Cowan et al., 2001). Localization of Guinea pig fertilin to sperm surface is a complicated process, requiring intracellular storage of protein prior to membrane insertion and relocalization during epididymal maturation of

sperm. It is therefore possible, that ADAM 27 is indeed present on the sperm surface, but our results are insufficient to understand the path of ADAM 27 between synthesis and integration to sperm plasma membrane.

All studies on ADAM family proteins have focused on their role during fertilization process as adhesion proteins on the sperm surface. However, it cannot be excluded that ADAM 27 is important during spermatogenesis. As an adhesion protein it might participate in Sertoli-germ cells interactions. Developing germ cells are attached to Sertoli cells which provide them with critical factors necessary for the successful progression into spermatozoa (Griswold, 1998). The adhesion with Sertoli cells is important also for sperm release and movement of spermatocytes from basal to adluminal compartments of the tubule. Integrins- potential interaction partners for ADAMs- were found on the Sertoli cells at the specific sites of cell-to-cell contact between germ cells and supportive Sertoli cells (Lustig et al., 1998; Giebel et al., 1997). Therefore, it might be possible that the disintegrin domain of ADAM27 interacts with integrin receptors on the Sertoli cell and participate in movement of developing germ cells and release of spermatozoa into the lumen of tubules.

Concluding from the expression studies of ADAM 27 we suggest, that this is a protein present in the plasma membrane of sperm head with potential function in sperm-egg interaction. Or that ADAM 27 is participating in the biogenesis of the acrosome and itself is not present on the acrosome and sperm surface. On the other hand, ADAM 27 might be involved in the cell-to-cell contact with Sertoli cell in the process of spermatogenesis. To answer these questions we have undertaken few approaches. To investigate the potential role in gamete interaction we studied sperm-egg interactions in vitro in the presence of ADAM 27 recombinant proteins. Simultaneously, we started with yeast two hybrid screening in order to identify ADAM 27 interaction partners on the egg surface. However, the best way to find out the function of the gene is its knock out. Therefore, we decided to generate a knock out mouse for *ADAM 27* to analyze the phenotype of mouse lacking functional ADAM 27 protein and to answer the question; What is the biological role of ADAM 27 protein?

4.3 Functional studies: ADAM 27, a candidate molecule for mediating the binding and fusion of the sperm and egg plasma membranes during fertilization.

4.3.1 Introduction to fertilization process

Mammalian sperm interact with oocytes on three different levels during fertilization: (a) the cumulus layer, (b) the zona pellucida (ZP) which induces exocytosis of the acrosome contents and (c) the oocyte plasma membrane. Sperm-ZP interactions are important, because the binding of the sperm to specific ZP-glycoproteins (ZP3 in the mouse) induces the sperm to undergo the “acrosome reaction”, the exocytosis of the acrosome vesicle on the head of the sperm. The acrosome reaction has two important results. First, enzymes released from the acrosome allow the sperm to penetrate ZP to gain access to perivitelline space. Secondly, new portions of the sperm membrane are exposed or modified upon the acrosome reaction, including the inner acrosomal membrane and the equatorial segment, regions of the sperm head that can participate in initial gamete membrane binding or subsequent sperm-egg membranes fusion (Yanagimachi, 1994; Huang and Yanagimachi, 1985). The interactions of the gamete plasma membranes appear to involve multiple ligands and receptors. In addition the sperm and egg not only adhere to each other but also undergo membrane fusion, ultimately making one cell. Finally, sperm-egg interactions lead to the series of signal transduction events in the egg, known as egg activation. The events associated with egg activation include the oscillations in intracellular calcium concentration, the exit from meiosis, the entry into the first embryonic mitosis, and the formation of a block to polyspermy.

Development of IVF (In Vitro Fertilization) assay systems provided a tool for studying gamete interactions. One commonly adopted approach is based on the principle that sperm oocyte interactions can be significantly decreased in vitro when eggs are preincubated with a peptide mimic or recombinant protein based on the sperm protein implicated in oocyte binding. During the past years, a model for sperm-egg binding has developed that suggests that sperm-egg binding results from the adhesion between an integrin on the egg and an integrin ligand (i.e., the disintegrin domain of a member of the ADAM family) on the sperm. Relatively little is known about the biochemical and biological properties of ADAM

disintegrin domain. What is known can be summarized as follows: peptide analogues of the disintegrin loops of several sperm ADAMs inhibit sperm-egg binding and fusion (Almeida et al., 1995; Bronson et al., 1999; Evans et al., 1995a; Myles et al., 1994; Yuan et al., 1997). Recombinant proteins encoding the extracellular domains of mature mouse ADAM 1 (fertilin α) and ADAM 2 (fertilin β) fused to maltose-binding protein, have been expressed in *E.coli*. When added to mouse eggs, the fertilin α and β recombinant proteins inhibited sperm-egg binding and fusion in vitro (Evans et al., 1997 a,b). IVF experiments can be performed with either ZP-intact eggs or egg from which ZP have been removed (ZP-free eggs) to examine the interactions on the level of zona pellucida or plasma membrane, respectively.

4.3.2 Evaluation of ADAM 27 role in sperm-egg interaction and implications for binding with egg integrin ligands

Out of those ADAMs identified in the reproductive tract, fertilin β , cyritestin, tMDCII and tMDCIII are exclusively expressed by spermatogenic cells and found on the spermatozoa, and are therefore potential oolemma binding candidates. However, most functional studies to date concentrated on fertilin β and cyritestin.

In the present study we examined the possible role(s) of ADAM 27 in mouse sperm-egg interaction, by using expressed recombinant forms of the extracellular domain of the mouse ADAM 27 in bacteria as fusion proteins with maltose-binding protein (MBP). We generated three constructs: MBP-DCE containing disintegrin, cys-rich and EGF-like domains; MBP-B with only disintegrin domain and MBP-CE with cys-rich and EGF-like domains (Figure 3.35). However, it should be noted that a cysteine rich character of EGF-like and cys-rich domains might cause inappropriate folding of bacterially expressed recombinant proteins in comparison to native proteins expressed in eukaryotic cells. Recombinant proteins produced in bacteria and renatured as described in methods section 2.2.9.5, might be folded differently, and thus these proteins may have additional or different properties.

MBP recombinant proteins were used in in vitro sperm-egg binding assays to examine if they could perturb the interaction between gametes. We studied ZP-intact and ZP-free oocytes where ZP was removed by incubation in acidic Tyrode's solution. We demonstrated that the recombinant MBP-CE and MBP-D when incubated with ZP-intact eggs showed inhibition of sperm-egg binding, to 40% and 73% of the control level, respectively (Figure 3.36). The same proteins, MBP-CE and -D, when incubated with ZP-free eggs showed no or moderate

inhibition (89%) of sperm binding, respectively (Figure 3.36), suggesting that ADAM 27 protein might react on the zona pellucida level. Unexpected and contradictory results were obtained when MBP-DCE recombinant protein was used in incubation experiments. In both cases, eggs with and without zona pellucida, an increased level of binding was observed (136% and 139% of the control level, respectively). This situation never happened in experiments with recombinant fertilin α or β and therefore is difficult to explain.

There is a possibility that the bacterially produced fragment of ADAM 27 protein containing these three domains had been incorrectly folded and the protein acquired new properties. However it is also possible that ADAM 27 protein on the sperm is composed of all domains including metalloprotease, which is removed in mature fertilins and cyritestin (Frayne et al., 1998; Linder and Heinlein, 1997), as only minor protein modification in epididymis was observed. Correct conformation of incomplete protein, necessary for its activity, could not be achieved in bacterial cells. It might be also suggested that obtained inhibition or enhancement results can be explained by lack of sequence specificity of binding of ADAM 27 derived proteins to the oocyte. It is known that ADAMs share high similarity among disintegrin, cysteine-rich and EGF-like domains and this homology is one of the weak points of all inhibition studies using synthetic peptides as reported by McLaughlin et al., 2001. Unless recombinant proteins are correctly folded (which could be difficult to establish), they may simply behave as “long peptides” with lack of binding specificity.

Experiments with in vitro inhibition of sperm-egg binding and fusion by recombinant proteins or synthetic peptides showed that cyritestin and fertilin α and β are heavily implicated in sperm-egg binding and fusion with oocyte. Results from mouse specific deletions of fertilin β or cyritestin yielded surprising results. Fertilin β knock out mice are infertile, but due to defective binding to zona pellucida and sperm migration from the uterus into the oviduct. However, in vitro experiments showed also that sperm fail to adhere to the egg plasma membrane (13% of wild type level) and have reduced ability to fuse with oocyte (45-50% of wild type level), although some fusion occurs (Cho et al., 1998). Cyritestin knock out mice are also infertile, they have severely reduced ZP binding, but these defects did not affect fusion with the oocyte membrane and fertilization of ZP-free eggs (Shamsadin et al., 1999; Nishimura et al., 2001).

The protein partner on oocytes to which fertilin β binds was believed to be $\alpha 6\beta 1$ integrin (Almeida et al., 1995). Antibodies to $\alpha 6$ blocked binding of sperms, although this appeared to depend on conditions used (Almeida et al., 1995). Later, it was discovered that oocytes lacking $\alpha 6$ are still able to bind and fuse with sperm (Miller et al., 2002). After this report it

was believed that other integrins that are reported to bind ADAM, found on the sperm surface, could fulfill that function (Almeida et al., 1995; Evans et al., 1995b; Tarone et al., 1993). It has been proposed that a web of ADAMs and multiple $\beta 1$ integrin associated proteins are formed to mediate sperm-oocyte membrane interaction (Takahashi et al., 2001; Zhu and Evans, 2002).

With the very recent report of He et al., (2003), the widely accepted theory, that sperm binding to an egg integrin is a prerequisite adhesion step for sperm-egg membrane fusion has collapsed. An elegant study with integrin subunit knock outs demonstrate that none of the integrins known to be present on the mouse egg or to be ADAM receptors are essential for sperm-egg binding and fusion, thus egg integrins may not play the role in gamete fusion as previously attributed to them. Since sperm bound and fused normally to the eggs with the integrin knock-out, there certainly could be other egg molecule(s) that function as an ADAM receptor. Therefore search for other possible ADAM partners in gamete binding/fusion must be performed.

Comparing the results of in vitro experiments and knock out analysis it can be concluded that in vitro studies can not always reflect the real function of the molecule in living organism. Therefore the ADAM 27-null mouse must be generated and analyzed to clarify ADAM 27 function.

4.3.3 Looking for ADAM 27 interaction partner(s) – application of yeast two hybrid method

In order to identify interaction partners for ADAM 27 protein, we have chosen the yeast two-hybrid system for the detection of protein-protein interactions. As it was mentioned before the main interaction domains of ADAM proteins are: metalloprotease (which seems to be inactive in ADAM 27), disintegrin, cys-rich and EGF-like domains. Cytoplasmic tail of ADAMs can be implicated in signal transduction pathways. To find the interaction partners of ADAM 27 protein on the egg surface we have decided to use mouse ovarian library (mouse oocyte library was not available at that time). Domains that were predicted to act in adhesion process are disintegrin, cys-rich and EGF-like, thus we used a fragment of protein containing these three proteins in screening. Unfortunately we had to face with the autoactivation problem in the performed pre-screening tests. This autoactivation of reporter genes made it impossible to

proceed with the screening. The autoactivation indicated that the protein produced in yeast, somehow had the property to activate the reporter genes LEU2 and LacZ. This autoactivation problem is mostly encountered in screens with transcription factors. However, ADAM 27 does not belong to this group of proteins. To overcome autoactivation of the reporter genes by our bait protein containing three functional domains we have undertaken two steps (3.1.7). First, we used less sensitive yeast strains as recommended in the manual. Second, we truncated the bait protein to disintegrin domain only and tested again. Both trials to overcome reporter genes autoactivation failed and therefore we were unable to perform screening for adhesion interaction partner of ADAM 27. Until now, there is no report that describes interaction partners for extracellular domains of ADAM family proteins. However, there are yeast two hybrid experiments that focused on the cytoplasmic tails of ADAMs: 5, 9, 19 and 22 (Howard et al., 1999; Huang et al., 2002; Zhu et al., 2003). For example, interaction partner screening for ADAM 22 revealed that it interacts with intracellular signaling molecule 14-3-3 zeta protein (Zhu et al., 2003), for ADAM 5 and 9, SH3PX1 protein was found to interact with their cytoplasmic tails (Howard et al., 1999). Recently, a new system for yeast two hybrid screen has been developed, designed to detect membrane proteins interaction in vitro. This system would be convenient for study the interaction partners of ADAM 27 and therefore a new screening for ADAM 27 partners must be performed in the future with the use of membrane-based yeast two hybrid system.

4.4 Functional studies of *ADAM 27* in vivo: its role in spermatogenesis and/or fertilization

4.4.1 Generation of *ADAM 27* mutant mice with transmembrane domain deletion

The fact that two other members of ADAM family cyritestin and fertilin α participating in fertilization process, as it was shown in mouse models, are not functional in human makes *ADAM 27* an interesting target to investigate. It was shown that human ortholog ADAM 18 is a functional gene (Frayne et al., 2002). To elucidate function of *ADAM 27* gene in a mouse, a targeted mutation of the gene was generated by homologous recombination in ES cells. Deletion embraced exon 19, coding for the part of the protein that has hydrophobic properties and is responsible for transmembrane localization of ADAM 27. The heterozygous animals appeared phenotypically normal with no fertility disturbances. Mice homozygous for *ADAM 27* mutation were generated on two different genetic backgrounds, namely, inbred strain 129X1/SvJ and in hybrid strain C57Bl/6Jx129X1/SvJ.

In the heterozygous and homozygous mutant mice we were looking for the transcription of mutated allele. In the Northern blot hybridization we used a probe that is localized upstream to deletion, in heterozygous as well as in homozygous mice we detected *ADAM 27* transcript (Figure 3.17). Presence of the transcript in -/- mice indicates that mutated allele transcription is not altered. Using RT-PCR approach and primers designed to detect both transcripts, (wildtype and mutated) we were able to amplify two fragments 344 bp (WT) and 209 bp (mutant) (Figure 3.18B). RT-PCR product of 209 bp was sequenced and indicated that the mutated transcript is lacking exclusively exon 19, and the deletion cause ORF change at the carboxyl end of the protein (Figure 3.19). Using primers located within the deleted exon there was no amplification in -/- mice as expected (Figure 3.18B). Western blot analysis were performed to check whether the transcript is translated and for the protein properties. In the experiments we have used two different antibodies. Anti ppADAM27 antibodies generated against synthetic peptide, localized at the carboxyl end of the protein that is being changed in the mutant mice, were not able to detect ADAM 27 because the epitope is not present in the mutated form (Figure 3.21A). Second antibody anti fpADAM27 raised against fusion protein composed of extracellular domains of ADAM27, suppose to detect wildtype protein as well as

mutated. Figure 3.31B shows that indeed mutated protein can be detected also in *ADAM 27*^{-/-} animals, but unexpectedly is still present in the membrane fraction, although the transmembrane domain has been removed.

Homozygous mice were fertile with normal reproduction parameters, the litter size on both backgrounds was not significantly affected. We have determined the total sperm count in the cauda epididymis as well as in uteri and oviducts of female inseminated by *ADAM 27*^{-/-} mice, and found that they are not significantly different from wildtype (3.1.3.2.2). As *ADAM 27* was detected by antibody in the Golgi complex, participating in the acrosome biogenesis, mutation in *ADAM 27* might influence acrosomal reaction in mutants, therefore we examined the acrosome reaction in response to calcium ionophore A23187. There was no significant difference in the assay of acrosome reaction between wildtype and *ADAM 27* mutant spermatozoa as well. Motility of the *ADAM27*^{-/-} sperm on the C57Bl/6Jx129X1/SvJ was analyzed with the CASA (Computer Assisted Sperm Analysis) system, several motility parameters were determined (Table 3-3, Figure 3.23). Homozygous mice showed a highly reduced motility compared to wildtype (in total path velocity, progressive velocity and truck speed), however reductions did not affect sperm ability to fertilize oocytes. *ADAM 27* mutation did not show manifesting phenotype, although, we are not sure whether the function of *ADAM 27* was destroyed by deletion of transmembrane domain. In our understanding of *ADAM* proteins function, their activity determined by presence of disintegrin and/or metalloprotease domains, take place extracellularly, thus mutated *ADAM 27* should not be functional. The first unexpected result, that *ADAM 27* might be associated with Golgi complex but not with plasma membrane or acrosome, suggest that *ADAM 27* protein might have a different role that has been proposed, or other *ADAMs* (cyritestin and fertilin) localized on the sperm have. Considering the fact that at least fertilin is a heterodimeric protein, it might be possible that *ADAM 27* built also a heterodimer, and its association with some other membrane protein preserves the localization and therefore function of *ADAM 27* protein. However this is only a hypothesis, not supported by any experimental results.

In comparison, knock out male mice for cyritestin are infertile due to the drasticall defects in adhesion to zona pellucida (Shamsadin et al., 1999), although earlier reports suggested that cyritestin is involved in sperm-egg plasma membrane binding and fusion. The binding suppose to involve disintegrin domain on the sperm and integrin receptor on the egg. It was shown that cyritestin predicted active site peptide and an antibody to a peptide from active site region could inhibit sperm-egg binding and fusion strongly (80-90%) (Yuan et al., 1997).

However, the cyritestin-null sperm fuse with the egg plasma membrane at 100% of the wildtype rate.

A similar situation has been encountered during elucidation of fertilin β function. Many publications supported the model which demonstrated that fertilin α/β dimer is a key molecule in gamete binding and fusion (Evans et al., 1997 a, b; Evans et al., 1998; Bigler et al., 2000; Wong et al., 2001; Zhu et al., 2002; Zhu and Evans, 2002). Again, synthetic peptides corresponding to the predicted active site as well as fusion proteins and antibodies, were strongly inhibiting gamete binding and fusion, but the fertilin β -null males exhibited a more complicated phenotype. Infertility was caused by defective binding to zona pellucida and migration into oviduct (Cho et al., 1998). However, reduced binding to egg plasma membrane (13% of the wildtype level) and less extreme reduction in the rate of mutant sperm fusion (45-50% of the wildtype rate) was also observed, using in vitro sperm-egg adhesion and fusion assays. Results of the analysis of the knock out animals were contradictory to the theory based on the previous studies. Integrins have not been found on the zona pellucida and therefore an interaction of ADAMs with zona pellucida was not earlier postulated.

Analysis of the knock out mice for cyritestin and fertilin β showed a new phenomenon; loss of the multiple gene products, resulting from deletion of one gene for a membrane protein. In the cyritestin-null mature sperm, fertilin α was not detectable and fertilin β was reduced to 60% but the fertilin α and β precursors were present on normal levels, indicating loss of the already produced proteins. Similarly, in fertilin β -null sperm, fertilin α was absent and cyritestin was decreased to 11% of the wildtype level (Nishimura et al., 2001). The findings that deletion of the single gene results in loss of more than one gene product was reported before, this type of result exist when the deleted gene encodes; for example, a transcriptional factor whose activity is needed to express various other gene products. However this is a new situation for the membrane protein and driven by novel and unknown mechanism.

Therefore, fertilization phenotype of the cyritestin $-/-$, fertilin β $-/-$ and future any ADAM $-/-$ animals might be due to the simultaneous loss of few other members of ADAM family. Concluding results form *ADAM 27* knock out mice with deletion of exon 19 with transmembrane domain, we can not postulate critical role for ADAM 27 neither in spermatogenesis nor fertilization processes.

4.4.2 Generation of *ADAM 27* new knock out mice

ADAM 27 knock out mice with deletion of transmembrane domain were not helpful to elucidate function of ADAM27, therefore we have decided to generate new knock out mice. To completely remove ADAM 27 protein we have designed a construct replacing the first three exons including translational start, by neomycin cassette. Homologous recombination with this construct gave us a guarantee for *ADAM 27*-null mice. ES cells were transfected with the knock out construct, and only one homologous recombination clone among over 200 tested was identified (Figure 3.26). This might be caused by a large size of deletion (6.8 kb) and not long enough 5' and 3' wings of the construct (2.8 kb + 3.3 kb). The positive clone was tested for any random integration by hybridization with neomycin probe (Figure 3.26).

As only homologous integration was found, the ES cells were used to generate chimeric mice. In the result of blastocyste injection we obtained four chimeras (15%, 75% and two 80%), however they failed to transmit further the mutated allele. 15% had only C57Bl offspring and other chimeras appeared to be infertile. All three infertile chimeras were high percentage (75-80%). It was reported for some other genes (*Zfp148* by Takeuchi et al., 2003; and *Hmgal* by Liu et al., 2003) that males with high percentage of chimerism are infertile due to the haploinsufficiency of the knocked out gene. However, infertility of chimeras without any reason (not dependent on gene knock out) has been reported. Because we have not expected such a drastical phenotype for the *ADAM 27* knock out mouse, we decided to analyze the infertile chimeras. It was shown that all three chimeras had abnormal testes (Figure 3.27; 3.30; 3.31 and 3.32) and that in all cases injected recombinant cells have participated in testis development of chimeras (Figure 3.28 and 3.33). The 75% chimeric male had small testes with highly abnormal morphology, with some symplastic multinucleated cell agglomerations or Sertoli cell debris detached from the basal membrane. Among these cells there were also some germ cells. Expression of germ cell specific genes was detectable (Figure 3.29), however we were not able to detect *ADAM 27* transcript. Other two 80% chimeras were analyzed. Although sperms were found in the left epididymis of chimera B, the male was not fertile. In chimera A no sperm at all have been found. Histological analyses of the testes of chimera B demonstrated obvious pathological defects when compared with wildtype (Figure 3.32). The abnormalities were manifested as a partial to total deficiency of germ cells within some seminiferous tubules. However, sections with normal spermatogenesis were also observed. Within the abnormal tubules we observed those with total lack of germ cells to

tubules with undifferentiated germ cells. We speculate that these germ cells might be derived from ES cells with disrupted *ADAM 27* gene. The sperm found in epididymis of chimera B, were tested for the strain origin, and it was found that all of the differentiated spermatozoa were derived from the host C57Bl cells (Figure 3.33B). Indicating that the cells derived from injected recombinant ES cells present in the testis of chimera B, were not able to differentiate into spermatozoa. Interestingly, in the highly abnormal testis of chimera B, expression level of *ADAM 27* was decreased whereas other genes transcripts remained unaffected (Figure 3.34). This indicates that disruption of the *ADAM 27* locus by knock out technology causes high decrease of *ADAM 27* expression even in the heterozygous state. Low amount of ADAM 27 protein might cause the phenotypic changes in chimeric mice. The observed phenotype might correlate with postulated functions of ADAM 27 in germ cell-germ cell or germ cell-Sertoli cell interactions. It might be also possible, that the decreased amount of *ADAM 27* causes decrease of any other important ADAM, as it was shown in knock outs of cyritestin and fertilin β , and results in this infertile phenotype. Generation and analysis of other chimeras generated with *ADAM 27* recombinant ES cells would hopefully answer the question whether the phenotype of chimera could be caused by *ADAM 27* gene disruption or if this infertility has been arisen independently.

4.4.3 Analysis of *ADAM 27* by transgenic approach

Our next approach to elucidate *ADAM 27* function was generation of transgenic mice in which we overexpressed *ADAM 27* in testis. In the cell membrane different types of proteins are localized (Figure 4.4). They are important for transport inside and outside of the cell, some are linkers for other proteins or receptors for extracellular ligands and others are enzymes associated with the membrane. Our idea was to overexpress ADAM 27 transmembrane protein, which may cause abnormalities of the membrane structure and protein network by excess of ADAM 27 protein inserted into membrane, and to investigate how it influences the function of testis.

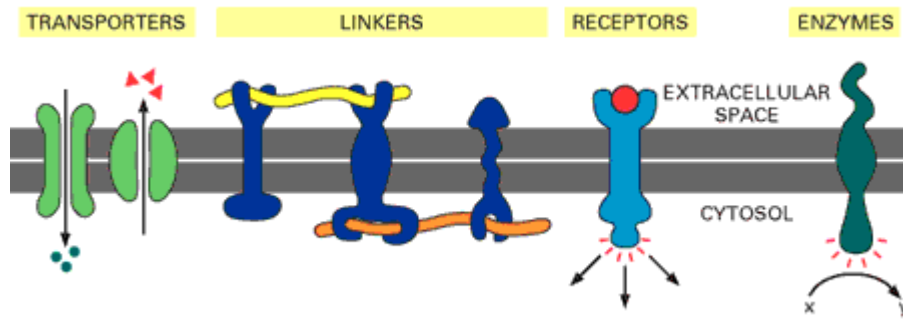


Figure 4.4 Common types of proteins found in cell membrane.

We have generated two transgenic constructs one overexpressing *ADAM 27* and second overexpressing GFP protein in intention to coinject them into the same pronucleus (Figure 3.37). Simultaneous integration of both constructs into the genome gives us the opportunity to correlate GFP fluorescence with expression of the *ADAM 27* transgene, due to the fact that expression of both genes is driven by the same promoter of P_{gk-2}. Constructs were injected and we are waiting for the founder animals. Therefore the analysis of the *ADAM 27* transgenic animals will be performed in the future.

4.5 *Testase 2* gene structure and chromosomal localization

Testase 2 also known as *ADAM 25* is another member of ADAM family. We found that *testase 2* is in fact a gene that exists in the mouse genome in two highly similar copies which we named *testase 2 α* and *testase 2 β* (Bolcun et al., 2003). *Testase 2 α* and *2 β* genes belong to the ADAM gene family but they significantly differ in genomic structure as well as in protein properties. In contrast to *ADAM 27*, *testase 2 α* and *2 β* are composed of 2 exons, first one 85 bp short and second one over 2.5 kb with translational start. *Testases 2* show a high similarity to two other ADAM family members namely, *testase 1* and *testase 3*. We performed sequence analysis of these two genes and found that both have two exons as well. There are other ADAMs beside testases composed of only 2 exons (mouse *ADAM 21* and mouse and human *ADAM 29*), there are also members having only one exon (mouse *ADAM 4, 6, 16, 30* and human *ADAM 20* and *21*). However, most of the ADAM family genes are characterized by multiexonic structure (>20 exons) as described earlier for *ADAM 27* or *cyrtestin*. Duplication of *testase 2* gene seems not to be unique among ADAM family. *Testase 4 (ADAM 34)* published by Brachvogel et al, (2002) is highly similar to *testase 3* (90% on DNA level) indicating possible, similar to *testase 2*, duplication of *testase 3* gene during evolution. We cannot exclude that *testase 1* shares this characteristic with two other *testases*. It was also reported that in a mouse there are two genes (*ADAM1a* and *ADAM 1b*) coding for *ADAM 1 (fertilin α)* located on the chromosome 5 (Nishimura et al., 2002). In a human it was shown that there are two *cyrtestin* genes. However they are localized on different chromosomes and are pseudogenes (Adham et al., 1998; Grzmil et al., 2001). Both *testase 2 α* and *2 β* are localized on the chromosome 8 A4 in tandem repeat, in the head to tail orientation (Figure 3.42). *ADAM 24 (testase 1)*, *ADAM 26 (testase 3)*, *ADAM 34* and *29* are localized in the close distance to these two genes (Figure 4.5). As mentioned earlier chromosome 8 is an ADAMs-rich chromosome. In total 13 genes are located on it, in two cytogenetic bands A2 and A4.

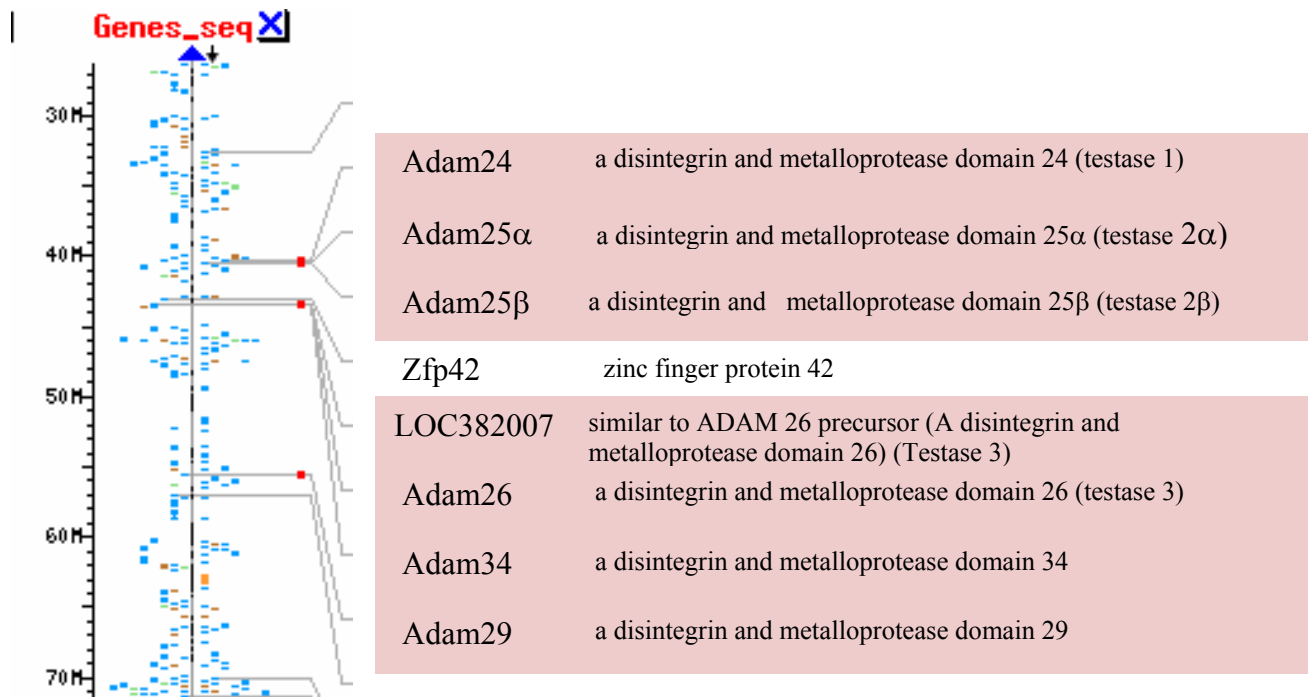


Figure 4.5 Localization of ADAM family members on chromosome 8 cytogenetic band A4

4.6 Expression of *testase 2 α* and *β* genes

We demonstrated that both *testase 2 α* and *β* share the same spatial and temporal expression pattern, transcripts were not detected in the testicular somatic cell lines (Figure 3.44). Transcripts of both genes were found in the olt and qk mutants (Figure 3.47B), which suggests that their expression appears with the first wave of spermatids. First presence of the transcripts at day 20 of postnatal development confirms spermatid specific expression of both genes (Figure 3.47B). It was recently shown that fertilin α (ADAM1), which makes heterodimeric complex with fertilin β (ADAM2) has two isoforms, originated from two intron-less genes localized on chromosome 5 (Kim et al., 2003). Both precursors ADAM1a and ADAM1b form heterodimers with ADAM 2 in the endoplasmic reticulum of germ cells in testis, however, isoform 1b is found on the sperm surface only. The authors propose that these two isoforms ADAM1a and 1b play potential roles in spermatogenesis and fertilization. Until now we don't know what the functions of *testase 2 α* and *β* proteins are and whether both copies play the same role during processes of spermatogenesis and fertilization. One of the proteins from *testase* sub-group, *testase 1*, is a functional protein found on the surface of mouse sperm (Zhu et al., 2001). Identity on the protein level between *testase 2 α*, *testase 2β*,

testase 1 and testase 3 is about 51% however, we don't know whether all of them are functional giving high abundance of testis specific proteases.

4.7 Functional studies of *testase 2* genes

Testase 2 α and β proteins belong to a metalloprotease and disintegrin domain proteins. Therefore they are postulated to have dual activity proteolysis/adhesion. Protein sequence of both testase 2 copies contains the putative zinc binding domain. This domain is characteristic for members of the zinc dependent metalloprotease astacin family suggesting that testases 2 α and β are active metalloproteases. For some members of the ADAM family such a consensus zinc binding motif was demonstrated (ADAM 1, 9, 12, 15 and others), some of the others lost it during evolution (ADAM 4, 6, 7, 11, 22, 23 and 29) and the rest did not have it (ADAM 2, 3, 5 and 18) (Cerretti et al., 1999) (Figure 1.3). However, testase 2 protein might also be a functional adhesion protein although responsible for adhesion residues have not been defined yet.

In order to elucidate the function of the *testase 2* gene we decided to generate a mouse lacking testase 2 protein. Unfortunately, at that time we believed that *testase 2* is a single copy gene. We generated ES knock out cells, which we found later to be specific for *testase 2* α copy. The few attempts we made to obtain recombinant clones with gene deletion were unsuccessful. When we found that there are in fact two genes that possibly must be deleted to find out the function of testase 2 proteins we stopped further trials to generate testase 2 mutant mice. Even if we were able to delete one copy, there would be a still functional second one. Both genes are separated only by 24 kb of genomic fragment, thus giving us a very small probability to obtain the double knock out by breeding mice mutants with specific deletions of both copies.

The closest to testase 2 protein, testase 1, was partially described (Zhu et al., 2001). It was shown that testase 1 is a monomeric glycoprotein, present on the surface of mature sperm, in the equatorial region of the acrosome intact sperm. Testase 1 is being processed in the caput epididymis where only pro-domain is removed, no further modifications were observed during capacitation and acrosome reaction. Thus, testase 1 is a plasma membrane anchored protease on fertilizing sperm. Authors suggest that testase 1 might participate in sperm penetration of the zona pellucida. Until now, soluble acrosome proteases are the main candidates for ZP penetration, however one of them – acrosin has been found not critical for

this process. Acrosin-null mice can penetrate the zona pellucida (Baba et al., 1994; Adham et al., 1997), therefore plasma membrane-anchored proteases like testase 1 should be considered in zona pellucida penetration. Another function proposed for testase 1 is participation in gamete fusion. There are few studies suggesting importance of metalloprotease activity in the gamete fusion (Diaz-Perez et al., 1988; Diaz-Perez and Meizel, 1992; Roe et al., 1988; Correa et al., 2000). High similarity of the *testase 1* and *2* genes might indicate that they possess similar function(s). There is no report up to date about *testase 1* duplication, therefore mouse model lacking testase 1 is awaited to elucidate function of at least one of the protein from the testase sub-group of ADAM family members.

5 Synopsis

There is more interest today in the molecular biology of gamete interaction than in the past. An understanding of the mechanism that underlies fertilization events has provided insights into important aspects of early development. In addition, many emerging strategies for contraception and treatment of infertility are based on the mechanism of gamete interaction. Therefore, an expanded knowledge about proteins involved in the differentiation of gametes and fertilization event and their functions is needed. In the present study we report the results of the experiments we have undertaken to elucidate functions of two ADAM family genes in spermatogenesis and fertilization events. In the first part, the germ cell specific gene *ADAM 27* is characterized. The gene is located on chromosome 8 A2 of the mouse, composed of 20 short exons and codes for a multidomain transmembrane protein of about 80 kDa. *ADAM 27* is expressed in the testis with an onset of expression at day P15, correlating with the appearance of a high number of pachytene spermatocytes. Using immunodetection techniques it was shown that *ADAM 27* is localized in the membrane of Golgi apparatus, which is participating in biogenesis of the acrosome. However, in the later stages of spermatogenesis, in elongating spermatids, the protein seems to have been relocated into the cytoplasm, and removed with the excess of cytoplasm from spermatozoa in residual bodies. Using Western blot experiment the *ADAM 27* protein can be detected in the epididymal sperm extracts. To elucidate the function of *ADAM 27* protein, in vitro and in vivo experiments were performed. In vitro sperm-egg binding assays were performed in the presence of recombinant proteins representing functional domains of *ADAM 27* (Disintegrin, Cys-rich, EGF- like). The results indicate that *ADAM 27* is more likely to interact with Zona pellucida via cys-rich domain and less via disintegrin domain or at very low level with oolemma. We presume that the real interaction level will definitely be observed in the mouse model lacking *ADAM 27*. Therefore, we have decided to generate mutant mice with targeted disruption of *ADAM 27* gene. The first knock out was designed to delete specifically the transmembrane domain and to deprive the protein of transmembrane properties. As a result of this knock out approach, we have obtained fertile animals with mutated form of *ADAM 27* and with no obvious abnormalities in the reproductive phenotype. We observed significant decrease of sperm

motility. However, it did not affect the fertility of mutant mice. Lack of the phenotype might be explained by two hypotheses. First one postulates the redundancy of *ADAM 27* for spermatogenesis and fertilization. The second hypothesis considers that the generated mutation did not affect the properties and function of the protein. We generated a second knock out, with deletion of the first three exons including translation start codon, and therefore we expect total absence of the protein in the mutant mice. We have generated four chimeras using targeted ES cells, but we did not obtain any germ line transmission of the targeted allele. Three out of four male chimeras appeared to be infertile, all of them were characterized by chimerism ratio higher than 70%. We found that they have testes with different degree of abnormalities: from seminiferous tubules sections with normal spermatogenesis to sections with partial to total deficiency of germ cells. In the testis with sperm, both types of cells were detected by PCR, those derived from C57Bl strain and those from injected targeted ES cells of 129Sv strain. By PCR genotyping, we found only wildtype C57 Bl sperms, indicating that germ cells derived from ES cells, where one allele for *ADAM 27* has been disrupted, might not be able to differentiate into spermatozoa. We have also observed that there is a decreased level of *ADAM 27* transcription when compared with wildtype and expression of other germ cell specific markers. The decrease of *ADAM 27* expression can be correlated with percentage of abnormal seminiferous tubules. The phenotype of chimeras might indicate the haploinsufficiency of *ADAM 27* gene and its role in germ cell differentiation rather than in fertilization. This striking phenotype must be proven in other chimeras. Targeted ES cells microinjections have been repeated and further chimeras are awaited to elucidate the function of *ADAM 27*.

The second gene that has been investigated in the present study was *testase 2*, also known as *ADAM 25*. With few other genes it belongs to a subgroup of testases, ADAMs with potential proteolytic activity expressed in testis. Initially, *testase 2* was considered as a single copy gene expressed in haploid germ cells. Later on, we found that the gene underwent duplication event during evolution, and now two very similar copies, *testase 2 α* and *β* , are present on mouse chromosome 8. Both copies have two exons, are orientated tail-to-head and 24 kb distant from each other. Small differences in the coding sequence cause different restriction patterns and therefore we were able to study expression of both copies of *testase 2*.

We have observed that *testase 2 α* and *β* are transcribed simultaneously in mouse testis. Expression of both genes is detectable from day 20 postnatal pointing appearance in first round spermatids.

We have started to generate knock out mice lacking testase 2 at the time we thought there is only one gene. The knock out construct was generated and transfected into ES cells but no homologous recombination was found. When we discovered the existence of duplication we checked the construct and found it belongs to *testase 2 α* . This duplication makes it impossible to generate knock out mice lacking both *testase 2 genes* and therefore we have stopped our attempts. Another method other than disruption by homologues recombination must be applied to turn off the expression of *testase 2 α* and *β* . A lot of enthusiasm is now given to siRNA, and in the future this method might be used to elucidate the function of testase 2 α and testase 2 β proteins.

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