Expression and functional analysis of murine *Brunol1* and *Brunol4*, members of Elav/Bruno family

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Tag der mündlichen Prüfungen

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ABBREVIATIONS

ABI	Applied Biosystem Instrument
AP	Alkaline Phosphatase
ATP	Adenosinetriphosphate
BCIP	1-bromo-3-chloropropane
bp	base pair
BSA	Bovine Serum Albumin
°C	Degree Celsius
CASA	Computer Assisted Semen Analysis
cDNA	complementary DNA
dATP	desoxyriboadenosintriphosphate
dH_20	distilled water
DAPI	Diamidino-2-phenylindole dihydrochloride
dCTP	Desoxyribocytosinetriphosphate
DMEM	Dulbecco's Modified Eagle Medium
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic acid
DNAse	Deoxyribonuclease
dNTP	deoxynucleotidetriphosphate
dpc	day post coitum
dT	deoxythymidinate
DTT	Dithiothreitol
EDTA	Ethylene Diamine Tetraacetic Acid
EGFP	Enhanced Green Fluorescence Protein
ES	Embryonic Stem
EtBr	Ethidium Bromide
FCS	Fetal Calf Serum
g	gravity
HBSS	Hanks' Balanced Salt Solution
HE	Heterozygote

HEPES	N-(-hydroxymethyl)piperazin,N'-3-propanesulfoneacid
hr(s)	hour(s)
HO	Homozygote
ICM	Inner Cell Mass
ICSI	Intracytoplasmic Sperm Injection
IPTG	Isopropyl-ß-thiogalactopyranoside
kb	kilobase
Kda	Kilodalton
mRNA	messenger Ribonucleic acid
mg	milligram
ml	millilitre
µl	microlitre
µm	micrometre
min	minute
NaAc	Sodium acetate
NBT	Nitro-blue tetrazolium
NCBI	National Center for Biotechnology Information
Neo	Neomycin
ng	nanogram
nm	nanometer
OD	Optimal Density
ORF	Open Reading Frame

Symbols of nucleic acids

A	Adenosine
C	Cytidine
G	Guanosine
T	Thymidine
U	Uridine

Symbols of amino acids

A	Ala	Alanine
B	Asx	Asparagine or Aspartic acid
C	Cys	Cysteine
D	Asp	Aspartic acid
E	Glu	Glutamic acid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophan
Y	Tyr	Tyrosine
Z	Glx	Glutamine or Glutamic acid

1. INTRODUCTION

The post-transcriptional regulation of gene expression by RNA-binding proteins is an important element in controlling both normal cell functions and animal development. Proteins that contain RNA recognition motifs (RRMs) represent a large family with diverse functions in RNA metabolism (Burd and Dreyfuss, 1994). Many of these proteins have normal 'housekeeping' functions such as rRNA processing, translation initiation, constitutive RNA processing and RNA transport to the cytoplasm. Others are expressed in a tissue- or developmental specific manner, suggesting a role in regulating gene expression in specialized cells (Antic and Keene, 1997).

1.1 Elav/Bruno/CELF RNA binding protein family

Elav is the superfamily of RNA binding proteins; Bruno is a subfamily of the elav family. The members of the Bruno family have been predicted from database searches; They are named bruno-like genes due to their relation to the *bruno* sequence (Good et al., 2000). The founder member of Bruno-like family (BRUNOL) is the *Drosophila* protein, Bruno (Timchenko et al., 1996). Since then, other members have been identified mainly by sequence similarity. In fact, the founder member was cloned or identified in a number of laboratories which lead to profusion of names and the separate name systems. The other member of Bruno family, ETR (Embryonic lethal abnormal vision Type RNA-binding protein3) has 76% sequence identity with CUGBP1 (CUG repeats Binding Protein1) and was independently cloned during a screen for the genes expressed in apoptic neuroblastoma cells and from mouse liver using a probe to CUGBP1 and was named NAPRO and CUGBP2, respectively. The rapid increase in the amount of available sequence data allowed four other proteins with related sequences to be identified and the grouping of these seven gene products in families was proposed by Good et al. (2002) and Ladd et al. (2001). These authors suggested two different systems. Good's system is based on the similarity to the *Drosophila* protein, Bruno and the proteins are called Bruno-like (BRUNOL 1, 2 etc) whereas that of Ladd uses the similarity of the CUG-BP1 and ETR-3 proteins and the members are called CUG-BP1 and ETR-3 Like Factors (CELF). Neither system is complete as BRUNOL1 is not represented in the CELF family and similarly CELF3 is absent from BRUNOL system. The correspondence between these two systems and certain information on the expression pattern is given in Table1.0.

Species	BRUNOL/CELF family members						
Homo sapiens	*BRUNOL1* Brain	CUG-BP1 CELF1	*CUG-BP2* *CELF2* *ETR-3* *BRUNOL3* Human: heart, skeletal muscle and brain	*CELF3* Brain	*CELF4* *BRUNOL4* Many tissues	*CELF5* *BRUNOL5* Brain	*CELF6* *BRUNOL6* Many tissues, (Mainly, kidney, brain and testis)
Mus musculus	*Brunol1* *CAGH4* *Etr-1* Brain and testis	*Brunol2* Ubiquitous	*ETR-3* ubiquitous (Mainly brain heart, lung, skeletal muscle)		*Brunol4* Brain	*Brunol5* Brain	*Brunol6* Brain and testis
Xenopus laevis	*Etr-1* *BrunoL-1* Nervous system	*EDEN-BP* *BrunoL-2* Embryos	*Etr-3* *BrunoL-3*				
Rerio danio	*Etr-1* Nervous system in embryos	*Brul* Vegetal pole in oocytes and early embryos	*Etr-3* Nervous system in embryos				

Table 1.0 Correspondence between the CELF/Bruno-like genes identified in vertebrates. The different names of the BRUNOL/ CELF family members cloned in vertebrates are given. The tissues in which the expression of each member has been observed is indicated.

1.2 The modular domains of Elav/Bruno RNA binding protein family

A model for the functional domains of ELAV/Bruno like RNA binding proteins is presented in Fig 1.0. All proteins within this family have the same domain structure: an amino terminal domain, two consecutive RRMs, a 50-70 amino acid tether or linker domain and a carboxyterminal third RRM (Burd and Dreyfuss, 1994). The most highly conserved sequences within the RRMs are the ribonucleoprotein 1 (RNP1 and RNP2) motifs that are signature sequences for the RRM and have been shown to specifically

interact with RNA (Merrill et al., 1987). The bipartite structure of Bruno- like RNA binding proteins allows for two target RNAs to bind to the proteins, either in *cis*, allowing for interactions within the same RNA, or in *trans*, bringing two RNAs together. The binding of Bruno/ ELAV like RNA binding proteins to RNA could be accompanied by binding to other proteins to form a functional RNP complex. Protein interaction domains could be anywhere, including within the RRMs. Finally, this model proposes that ELAV-like proteins bind to specific target RNAs and regulate their expression by formation of a functional RNP complex.

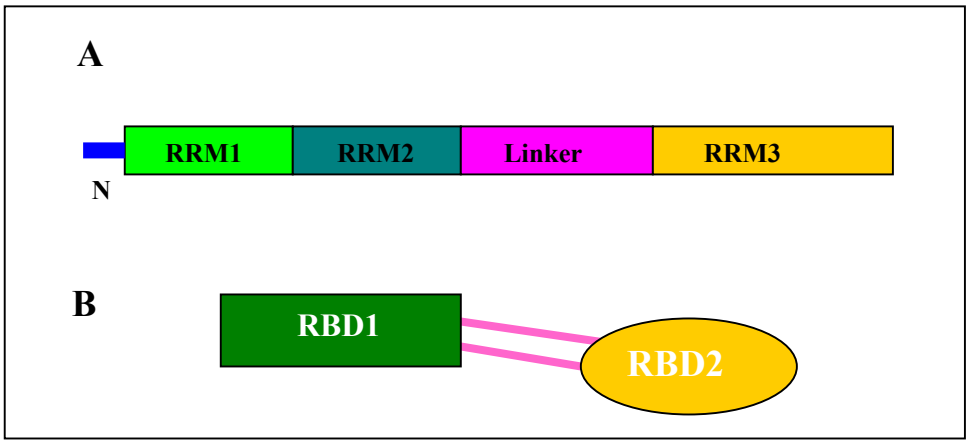


Figure 1.0. A model for the domain organization of a Bruno-like protein. (A) Linear representation of an ELAV-like protein is on top with the labeled boxes representing different domains. The box labeled N is the amino terminus while other boxes are labeled as described in the text. (B) The cartoon on the bottom is a proposed structure with two RNA-binding domains RBD separated by a linker region.

1.3 Functional role of ELAV/Bruno like RNA binding proteins

The importance of RNA binding proteins (RBPs) in development is underscored by the isolation of mutants with interesting developmental phenotypes where the defective gene encodes a RBP. In *Drosophila melanogaster* (Yao et al., 1993), *Caenorhabditis elegans* (Lundquist et al., 1996), mouse (Ebersole et al., 1996), and *Arabidopsis thaliana* (Macknight et al., 1997), mutants with defects in RBPs are defective in cell growth and differentiation.

An example of a RBP that regulates development is provided by the Bruno protein and its role as a translational repressor of *oskar* mRNA. In *Drosophila*, *oskar* is required for formation of germ cells and positioning of the posterior of the embryo (Lehmann and

Nüsslein-Volhard, 1996). Both *oskar* mRNA and the encoded protein must be properly localized to the posterior pole of the oocyte for correct development (Ephrussi and Lehmann, 1992; Kim-Ha et al., 1995). Localized expression of Oskar protein is determined in part by translational silencing of the *oskar* mRNA outside of the posterior of the oocyte. This repression is mediated by *cis*-acting sequences in the 39-untranslated region (UTR) of *oskar* mRNA, called Bruno response elements (BREs) and a corresponding *trans*-acting factor, the Bruno protein. Deletion of these BREs results in inappropriate translation of oskar mRNA in the anterior end of the oocyte leading to embryos with two posterior poles. The Bruno protein is a RRM-containing protein present in oocytes. Extracts prepared from *Drosophila* ovaries recapitulate this Bruno-dependent translational repression of *oskar* mRNA *in vitro* (Lie and Macdonald,1999). By regulating the localized expression of oskar, Bruno has a key role in germ cell formation and early embryogenesis.

Members of the Elav family play roles in regulating differentiation because overexpression of different family members enhances the differentiation of 3T3-L1 cells into adipocytes (Jain et al., 1997), the teratocarcinoma cell line N-Tera2 into neurons (Antic and Keene, 1998; Antic et al., 1999), chicken neural crest stem cells into neurons (Wakamatsu and Weston, 1997) and the PC12 pheochromocytoma cells into neurons (Kasashima et al., 1999). In embryos, overexpression of Elav-like proteins results in altered neural differentiation in both frogs (Perron et al., 1999) and mice (Akamatsu et al., 1999). Thus, the Elav family has diverse roles in regulating development through several different mechanisms.

The *Xenopus laevis Etr-1* gene was previously identified as a marker of the developing nervous system and is distantly related to the *elav* gene (Knecht et al., 1995). Subsequently, the Etr-1 protein was shown to be related to the *Drosophila* Bruno protein (Webster et al., 1997). Good et al. (2000) describe a family of human genes related to both Etr-1 and Bruno. The corresponding proteins have three RRMs and share a domain structure with the Elav family of proteins. The authors characterized in detail two members of this family, the *BRUNOL2* gene, which is ubiquitously expressed, and the *BRUNOL3* gene, which is expressed preferentially in muscle, heart, and the nervous system.

The BRUNOL2 and BRUNOL3 proteins bind to the same RNA sequence as the *Drosophila* Bruno protein, demonstrating a conservation of both protein sequence and RNA binding specificity. This binding occurs through the first two consecutive RRMs. The BRUNOL2 protein is identical to the CUGBP1, a RBP that binds to CUG repeats and is

implicated in the etiology of the triplet repeat expansion disease myotonic dystrophy (Timchenko et al., 1996). Thus, members of this gene family may be involved in human disease as well as in differentiation of specific cell types.

1.4 Isolation and characterisation of murine *Brunol1* and *Brunol4* gene, members of Elav/Bruno family

The isolation of the genes, which play a role in human diseases, is of great importance for understanding and elucidating the molecular pathway that mediates progression in disease. Identifying the gene responsible for a human disease and elucidating its function not only help to determine the pathogenesis of the disease but also offer a possible therapy. Until now, there is a row of well-known genes like SCA7 (David et al., 1997), SCA2 (Sanpei et al.,1996) or Huntington (The Huntington `s disease Collaborative Research Group 1992), which play a role in the emergence of neurodegenerative diseases. A common characteristic of these diseases is the expansion of short tandem repeats of three (CAG or CTG) or five (ATTCT for SCA10) nucleotides (Orr et al., 1993; Nakamura et al., 2001). However, the genetic loci for at least 20% of familial autosomal dominant cerebellar ataxia (ADCSA) have yet to be identified, implying the presence of other unidentified responsible genes (Takano et al., 1998; Devos et al., 2001; Soong et al., 2001)

Thus, to identify a new candidate gene for neurodegenerative diseases, brain cDNA libraries of human and mouse were screened with 63 CAG repeats containing cDNA probes. The complete ORF of human and mouse *Brunol1* consisting of 16 CAG repeats was isolated (Wilhelm et al 2001). When the cDNA and deduced amino acid sequences of Brunol1 were compared with the Genebank NCBI /EMBL and EST database, *Brunol1* was shown to encode a novel protein belonging to the Elav/Bruno family. Further, Wilhelm et al. (2001) studied the patients, whose clinical and genetic findings indicated the absence of extended CAG repeats in the genetically characterized known genes responsible for neurodegenerative diseases. Then they examined, whether the *Brunol1* cDNA of these patients have the variation in CAG repeats but none of them was found to have extended CAG repeats in *Brunol1* cDNA.

Meins et al. (2002) had screened adult mouse brain library with a probe generated from conserved parts of mouse *Brunol1* cDNA sequence to identify other novel members of the elav/bruno family expressed in mouse neural tissues. Analysis of the positive clone DKFZp598E0330Q2 revealed major parts of the cDNA sequence of a novel elav-type

related gene *Brunol4*, which proves to be the murine homologue to human BRUNOL4. A sequence was predicted from database searches (Good et al., 2000). To further identify a new member of Bruno family we have searched the mouse genome database for proteins, which have homology with Bruno family. A previously uncharacterized gene *Brunol6* was identified.

1.5 Aim of the study

The aim of this study was the expression and functional analysis of *Brunol1*and *Brunol4* genes. Scientific approaches undertaken in this study are as follows:

1. An expression study of *Brunol1, Brunol4 and Brunol6* gene by RT-PCR and Northern blot.
2. Subcellular localisation of Brunol1 protein in the cell.
3. Functional analysis of *Brunol1* and *Brunol4* genes in knockout mouse models.
4. Study of the embryonic genome activation of *Brunol4* gene.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals

Acrylamide/Bisacrylamide	Roth, Karlsruhe
Acetic acid	Merck, Darmstadt
Agar	Roth, Karlsruhe
Agarose	Invitrogen, Karlsruhe
Ammonium acetate	Fluka, Neu Ulm
Ampicillin	Sigma, Deisenhofen
Ampuwa	Fresenius, Bad Homburg
Bacto-tryptone	Roth, Karlsruhe
Bacto-Yeast-Extract	Roth, Karlsruhe
BCIP	Applichem, Darmstadt
Blocking powder	Roth, Karlsruhe
Boric acid	Scharlau Chemie, Barcelona
BSA	Biomol, Hamburg
Cell culture media	Invitrogen, Karlsruhe
Clonfectin	BD Clontech, Palo Alto
Coomasie G-250	Sigma, Deisenhofen
Chloroform	Merck, Darmstadt
Crystal violet	Sigma, Deisenhofen
Vectashield (DAPI)	Vector, Burlingame
Diethyl pyrocarbonate (DEPC)	Sigma, Deisenhofen
Dulbecco's Modified Eagle Medium (DMEM)	Merck, Darmstadt
Dimethyl sulfoxid (DMSO)	Sigma, Deisenhofen
Dithiothreitol	Invitrogen, Karlsruhe
DNA Markers	Invitrogen, Karlsruhe
dNTPs (100 mM)	Invitrogen, Karlsruhe
Dye Terminator Mix	Applied Biosystems
EDTA	Sigma, Deisenhofen
Ethanol	Baker, Deventer, NL
Ethidium bromide	Roth, Karlsruhe

Ficoll 400	Applichem, Darmstadt
FCS	Invitrogen, Karlsruhe
Formaldehyde	Merck, Darmstadt
Formamide	Sigma, Deisenhofen
Glutaraldehyde	Serva, Heidelberg
Glycerol	Invitrogen, Karlsruhe
Glycine	Biomol, Hamburg
HBSS medium	Sigma, Deisenhofen
Horse serum	Sigma, Deisenhofen
HCl	Roth, Karlsruhe
H_2O_2	Merck, Darmstadt
HEPES	Merck, Darmstadt
Ionophore A23187	Calbiochem, Bad Soden
IPTG	Biomol, Hamburg
Isopropanol	Merck, Darmstadt
IVF Media	Medicult, Berlin
1 kb DNA Ladder	Gibco BRL, Karlsruhe
0.24-9.5 RNA Ladder	Gibco BRL, Karlsruhe
KCl	Merck, Darmstadt
M16 medium	Sigma, Deisenhofen
Methanol	Merck, Darmstadt
$MgCl_2$	Merck, Darmstadt
MOPS	Applichem, Darmstadt
β-Mercaptoethanol	Serva, Heidelberg
Mineral oil	Sigma, Deisenhofen
Na azide	Sigma, Deisenhofen
Na acetate	Merck, Darmstadt
Na citrate	Merck, Darmstadt
Na deoxycholate	Merck, Darmstadt
NaCl	Merck, Darmstadt
Na_2HPO_4	Merck, Darmstadt
NaH_2PO_4	Merck, Darmstadt
NaN_3	Merck, Darmstadt

NaOH	Merck, Darmstadt
NBT	Applichem, Darmstadt
Neomycin (G-418)	PAN, Aidenbach
NuPAGE LDS sample buffer (4x)	Invitrogen, Karlsruhe
NuPAGE MOPS SDS running buffer	Invitrogen, Karlsruhe
Orange G	Sigma, Deisenhofen
OPTI-MEM I	Invitrogen, Karlsruhe
Penicillin/Streptomycin	PAN, Aidenbach
PBS	Invitrogen, Karlsruhe
Phosphoric acid	Merck, Darmstadt
Picric acid	Fluka, Neu Ulm
Phenol	Biomol, Hamburg
Proteinase K	Applichem, Darmstadt
Protein marker	Invitrogen, Karlsruhe
Radioactive substances: $[\gamma^{32}P]$-ATP $[\alpha^{32}P]$-dCTP	Amersham, Braunschweig
Rediprime™ II	Amersham, Freiburg
RNase Inhibitor	Boehringer, Mannheim
RNA length standard	Invitrogen, Eggenstein
RNA reagent	Biomol, Hamburg
RNAse away	Biomol, Hamburg
Saccharose	Roth, Karlsruhe
Salmon sperm DNA	Sigma, Deisenhofen
SDS	Serva, Heidelberg
S.O.C Medium	Invitrogen, Karlsruhe
Triton X-100	Serva, Heidelberg
Tris	Sigma, Deisenhofen
Tween-20	Promega, Mannheim
X-Gal	Biomol, Hamburg
Xylol	Merck, Darmstadt

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

2.1.2 Solutions, buffers and media

2.1.2.1 Agarose gel electrophoresis

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

2.1.2.2 SDS-PAGE

40% Acrylamide stock solution	Acrylamide 29.2% (w/w) Bis-acrylamide 0.8% (w/w) 10% Ammonium persulfate solution in H_2O
NuPAGE gel SDS sample buffer (4x)	10% Glycerol 62.5 mM Tris/HCl (pH 6.8) 2% SDS 0.01 mg/ml BPB
Running buffer (5x)	25 mM Tris/HCl (pH 8.3) 192 mM Glycine 0.1% SDS
Stacking gel buffer (4x)	0.5 M Tris/HCl (pH 6.8) 0.4% SDS
Separating gel buffer (4x)	1.5 M Tris/HCl (pH 8.3) 0.4% SDS

2.1.2.3 Frequently used buffers and solutions

AP buffer	100 mM Tris-HCl (pH 9.5) 100 mM NaCl 50 mM $MgCl_2$
BCIP-Solution	50 mg/ml BCIP 70% Dimethyl formamide
Blocking solution (immunostaining)	60 µl of horse serum, 150 µl of 10%Triton X-100 2790 µl PBS
Bouin's solution	15 volume of picric acid (in H_2O) 5 volume 37% formaldehyde 1 volume acetic acid
Carrier DNA	sonicated salmon sperm DNA, 5 mg/ml
Denaturation solution	1.5 M NaCl 0.5 M NaOH
Depurination solution	0.25 M HCl
E-buffer (10x)	300 mM NaH_2PO_4 50 mM EDTA
Elution buffer	1.5 M NaCl 20 mM Tris/HCl (pH 7.5) 1 mM EDTA
Ligation buffer (10x)	600 mM Tris/HCl (pH 7.5) 80 mM $MgCl_2$

	100 mM DTT
Lysis-buffer I	100 mM Tris/HCl (pH 8.0) 100 mM NaCl 100 mM EDTA 0.5% SDS
Lysis-buffer II	100 mM Tris/HCl (pH 8.0) 5 mM EDTA 200 mM NaCl 0.2% SDS 100 µg/ml proteinase K
10 X MOPS buffer	41.8 g MOPS 16.6 ml 3 M Sodium acetate 20 ml 0.5 M EDTA in 1 liter of DEPC Water adjust pH to 6.75
NBT- Solution	75 mg/ml NBT 70% Dimethyl formamide
Neutralisation solution	1.5 M NaCl 1 M Tris/HCl (pH 7.0)
PBS buffer	130 mM NaCl 7 mM Na_2HPO_4 4 mM NaH_2HPO_4
Protein lysis buffer	150 mM NaCl 10 mM EDTA 50 mM Tris/HCl pH7.6 1% Triton X-100

	1% sodium deoxycholate
Semidry transfer buffer (1x)	25 mM Tris pH 8.3 150 mM Glycin 10 % Methanol
SSC (20x)	3 M NaCl 0.3 M Na_3 citrate (pH 7.0)
Stop-Mix	15% Ficoll 400 200 mM EDTA 0.1% Orange G
TE-buffer	10 mM Tris/HCl (pH 8.0) 1 mM EDTA
Washing solution I	2x SSC 0.1% SDS
Washing solution II	0.2x SSC

2.1.3 Laboratory materials

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

Cell culture flasks	Greiner, Nürtingen
Dialysis hoses	Serva, Heidelberg
Disposable filter Minisart	NMI Sartorius, Göttingen
Filter paper 0858	Schleicher and Schüll, Dassel
Hybond - C	Amersham, Braunschweig
Hybond - N	Amersham, Braunschweig
Microcentrifuge tubes	Eppendorf, Hamburg
Petri dishes	Greiner, Nürtingen

Pipette tips	Eppendorf, Hamburg
Roti-plast paraffin	Roth, Karlsruhe
Superfrost slides	Menzel, Gläser
Culture slides	Lab-Tek/Nalge, Nunc, IL, USA
Whatman blotting paper (GB 002, GB 003 and GB 004)	Schleicher and Schüll, Dassel
X-ray films	Amersham, Braunschweig

2.1.4 Sterilisation of solutions and equipments

All solutions that are not heat sensitive were sterilised at 121°C, 10^5 Pa for 60 min in an autoclave (Webeco, Bad Schwartau). Heat sensitive solutions were filtered through a disposable sterile filter (0.2 to 0.45 µm pore size). Plastic wares were autoclaved as above. Glassware were sterilised overnight in an oven at 220°C.

2.1.5 Media, antibiotics and agar-plates

2.1.5.1 Media for bacteria

LB Medium (pH 7.5):
- 1% Bacto-tryptone
- 0.5% Yeast extract
- 1% NaCl

LB-Agar:
- 1% Bacto-tryptone
- 0.5% Yeast extract
- 1% NaCl
- 1.5% Agar

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

2.1.5.2 Mammalian cell culture media

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

Dulbecco's MEM (DMEM) medium containing:
- 10% fetal calf serum (FCS)
- 2 mM L-Glutamine
- 1% of penicillin (6 mg/ml)/streptomycin (5 mg/ml) solution

This medium was used to culture the NIH 3T3 cells. For long time storage of the cells in liquid nitrogen, the following freezing medium was used:

90% culture media
10% DMSO

2.1.5.3 Antibiotics

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

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

2.1.5.4 IPTG / X-Gal plate

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

2.1.6 Bacterial strains

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

2.1.7 Plasmids

pTK-Neo	Prof. N. Brose, MPI für Experimentelle Medizin, Göttingen
pPNT-M1	Prof. R. Mulligan, Children's Hospital, Boston, USA; modified by Prof H. Hahn, Institut für

	Humangenetik Göttingen.
pZERO-2	Invitrogen
pBluescript SK (+/-)	Stratagene, Amsterdam
pBluescript KS (+/-)	Stratagene, Amsterdam
pGEM-T Easy	Promega, Mannheim
pEGFP 1	Clontech, Palo Alto
pEGFP-N1	Clontech, Palo Alto
pET 41a+	Novagen

2.1.8 Synthetic oligonucleotide primers

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

Bru-4 Geno-F	5'GAGAGCCCAGAGCAGAGCAAGGTGTAGGAA3'
Bru-4 Del-R	5'TGAGAGGGAAAGGTGCTCTCACCTTTGTG3'
GFP-1405-R	5'TGTGGTATGGCTGATTATGATCTAGAGTCG3'
Bru-4 Geno-F3	5'AAGATGGCCACGTTAGCAAACGGA3'
Bru-4 Geno-F4	5'CTGACAACGCGAGCCTCAGTACCAA3'
Bru-4 Del-R-3	5'TGAGAGGGAAAGGTGCTCTCACC3'
Bru-4 Del-R-4	5'GTGAACCTGTCCTTCAGAACCGTA3'
GFP-916-R	5'AGTCGTGCTGCTTCATGTGGTC3'
GFP-936-R	5'AGCCTTCGGGCATGGCGGACTTGAA3'
Bru4-Ext-Probe-F	5'AATACCCTGGCATCCTGCTTCGCAA3'
Bru4-Ext-Probe-R	5'GGCCACAATTATTCATCCAC 3'
Bru4-Ntern-Probe-F	5'TTTATAAATGAGAACTGTTGGACGAC3'
Bru4-Ntern Probe-R	5'CCATTCCACCTCTAATTGACAGAG3'
Bru-1Geno-F	5'TGGCTGTTGAGCTCACTCCTCTCCAGCAA3'
Bru-1 Del-R	5'TGTTCAGGTCCATCCCCCTCATTAACAGTC3'
PGK-352-R	5'GCCAGAAAGCGAAGGAGCAAAGCT3'
Bru1-Ext-Probe-F	5'GTGTTTCATGGGCCTGTCTC3'
Bru1-Ext-Probe-R	5'CCTGACCCCCAGAATATGTG3
Bru4-cDNA-F	5'ATGAAGGACCACGATGCCATCAAGCT 3'

Bru4-cDNA-R	5'TCAGTACGGGCGATTGGCGTCT3'
Bru1-Notrn-Probe-F	5'CTTCAGGTCTGGAGATACCAGAGG3'
Bru1- Notrn Probe-R	5'TGAGAGCCCGGGGTTTGGAGTTC3'
Bru-1GFP-F	5'CAAGCTTCTATGAAGGAGCCAGATGCCAT3'
Bru-1GFP-R	5'TGGATCCCCACCGTAGGGCCTGTTTGCATCCTT3'
Bru1-cDNA-F	5'AATGAAGGAGCCAGATGCCATCAAGCTG3'
Bru1-cDNA-R	5'GGACCATGAAGCAGCTCTTGCCCCAGAGTC3'
Bru1-CAG-F	5' TC5'CACAAAGCCAAAACATTTGC3'
Bru1-CAG-R	ATG5'ATACCAGAGGAAGGGGCACTTCA3'
Bru1-3´UTR-F	GCTTGTGATGCTCTC3'
Bru1-3´UTR-R	5'GGAGATCTCCTACCTCTAGCT3'
pET41-R	5'ATGCTAGTTATTGCTCAGCGG3'
T7:	5'TAA TAC GAC TCA CTA TAG GG 3'
T3:	5'ATT AAC CCT TCA CTA AAG 3'
SP6	5'AGG TGA CAC TAT AGA ATA C 3'

2.1.9 cDNA probes

β-actin cDNA	Clontech
Neo probe	Generated in present study
GFP probe	Generated in present study
Brunol4 3' cDNA probe	Generated in present study
Brunol4 external probe	Generated in present study
Brunol1 3' cDNA probe	Generated in present study
Brunol1 external probe	Generated in present study

2.1.10 Eukaryotic cell lines

RI mouse embryonic stem cell line	Dr. A. Nagi, Toronto, Canada
NIH 3T3	S.A. Aaronson, Bethesda, U.S.A.

2.1.1.1 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.

2.1.12 Enzymes

Alkaline phosphatase	New England Biolabs, Frankfurt am Main
Platinum Taq polymerase	Invitrogen, Karlsruhe
Proteinase K	Sigma, Deisenhofen
Restriction enzymes (with supplied buffers)	Invitrogen, Karlsruhe,
RNase A	Invitrogen, Karlsruhe
RNase H	Invitrogen, Karlsruhe
RNase inhibitors	Invitrogen, Karlsruhe
Superscript-II	Invitrogen, Karlsruhe
Taq Polymerase	Invitrogen, Karlsruhe
T4 DNA ligase	Promega, Mannheim
T4 RNA ligase	Invitrogen, Karlsruhe
Trypsin	Invitrogen, Karlsruhe

2.1.13 Kits

Dye Terminator Cycle Sequencing-Kit	Applied Biosystem
DYEnamic ET-Terminator mix	Amersham Pharmacia
Endo Free Plasmid Maxi Kit	Qiagen, Hilden
Lipofectamine 2000	Invitrogen, Karlsruhe
Megaprime DNA Labelling Kit	Amersham Pharmacia,
Maxi Plasmid Kit	Qiagen, Hilden
Mega Plasmid Kit	Qiagen, Hilden
Mini Plasmid Kit	Qiagen, Hilden

QIAquick Gel Extraction Kit	Stratagene, Amsterdam
Rediprime™ II Random Prime Labeling System	Qiagen, Hilden
RNeasy Minikit	Qiagen, Hilden

2.1.14 Instruments

ABI PRISM 377 DNA Sequencer	Applied Biosystem
ABI 3100 Genetic Analyzer	Applied Biosystem
Autoclave	Webeco, Bad Schwartau
Centrifuge 5415 D	Eppendorf, Hamburg
Centrifuge 5417 R	Eppendorf, Hamburg
Biophotometer	Eppendorf, Hamburg
GeneAmp PCR System 9700	Perkin Elmer
Histocentre 2 embedding machine	Shandon, Karlsruhe
Inverted Microscope IX81	Olympus, Planegg
Megafuge 1.0 R	Heraeus, Hanau
Microscope BX60	Olympus, Planegg
Microtom Hn 40 Ing.,	Nut hole
Microplate-Reader, Model 450	BioRad, München
Neubauer cell chamber	Schütt Labortechnik, Göttingen
Pipette	Eppendorf, Hamburg
Power supply	Gibco BRL, Karlsruhe
Refrigerated Superspeed Centrifuge RC-5B	Sorvall, Langenselbold
Semi-Dry-Blot Fast Blot	Biometra, Göttingen
Spectrophotometer Ultraspec 3000	Amersham Pharmacia, Braunschweig
SpeedVac concentrator SVC 100H	Schütt Labortechnik, Göttingen
Thermomixer 5436	Eppendorf, Hamburg
Turboblotter™	Schleicher & Schüll, Dassel
UV Stratalinker™ 1800	Leica, Bensheim
X-Ray Automatic Processor Curix 60	Agfa, Köln

2.2 Methods

2.2.1 Isolation of nucleic acids

2.2.1.1 Isolation of plasmid DNA (Sambrook et al., 1989)

2.2.1.1.1 Small-scale isolation of plasmid DNA

A single *E.coli* colony was inoculated in 5 ml of LB medium with the appropriate antibiotic and incubated in a shaker for 16 hrs at 37°C with a vigorous shaking. 0.5 ml of this culture was used for making glycerol stock (0.5 ml of culture and 0.5 ml of glycerol) and rest was centrifuged at 2000 x g for 10 min. The pellet was resuspended in 100 µl of solution P1. The bacterial cells were lysed with 200 µl of P2 solution and then neutralised with 150 µl of P3 solution. The precipitated solution was centrifuged at 10,000 x g at 4°C. The supernatant was transferred into a new tube and centrifugation was done again. The supernatant was transferred into a new tube and 1 ml of 100% ethanol was added to precipitate the DNA. It was then stored on ice for 15 min, centrifuged at full speed for 20 min, and finally the pellet was washed with 70% ethanol and after air-drying was dissolved in 30 µl of sterile water.

P1	50 mM Tris-HCl, pH 8.0
	10 mM EDTA
	100 µg/ ml RNase A
P3	200 mM NaOH
	1% SDS
P2	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 into 100 ml LB medium with appropriate antibiotic and incubated overnight at 37°C with shaking. The culture was centrifuged then at 6,000 x g for 15 min. The pellet was resuspended in 4 ml of solution P1 and cells were then lysed with 4 ml of P2 and incubated on ice for 5 min. 4 ml of P3 buffer was added, mixed and incubated on ice for 15 min. The precipitated solution was centrifuged at 20,000 x g for 30 min at 4°C. Meanwhile, the column (Qiagen-tip) that was provided with the midi preparation kit was equilibrated with

10 ml of QBT solution. After centrifugation the lysate was poured into this equilibrated column, thus, allowing the DNA to bind with the resin present in the bed of the column. The column was then washed twice with 10 ml of solution QC. Finally, the DNA was eluted with 5 ml of QF solution. To precipitate the DNA, 3.5 ml of isopropanol was added, mixed thoroughly and centrifuged at 14000 x g for 30 min at 4°C. The DNA pellet was washed with 70% ethanol and dissolved in 100 µl of TE buffer.

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

2.2.1.1.3 Endotoxin free preparation of plasmid DNA

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

2.2.1.2 Isolation of genomic DNA from mouse tail 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 mouse tail was incubated in 700 µl of lysis buffer I containing 30 µl Proteinase K (10 µg/µl) at 55°C for overnight in Thermomixer 5436. To the tissue lysate, equal volume of phenol was added, mixed by inverting several times, and centrifuged at 10000 x g at RT for 5 min. After transferring the upper aqueous layer into a new tube, the same procedure was repeated, first with 1:1 ratio of phenol and chloroform and then with chloroform alone. Finally, the DNA was precipitated with 700 µl of isopropanol, washed with 500 µl of 70% ethanol, dissolved in 100-200 µl of sterile water and incubated at 60°C for 10 - 20 min. DNA was then stored at 4°C.

2.2.1.3 Isolation of genomic DNA from ES cells

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

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

2.2.1.4 Isolation of genomic DNA from cultured blastocysts

Culturing of blastocysts is described in 2.2.14.3 After culturing, DNA was prepared by incubating the individual embryos with 20 µl of lysis buffer II for 4 hr at 60^{o}C followed by incubation at 90^{o}C for 30 min. Three microliters of embryonic DNA was used for PCR.

Lysis-buffer II

50 mM Tris/HCl (pH 8.0)

0.5 mM EDTA (pH 8.0)

0.5% Tween 20

0.2 mg/ml Proteinase K

2.2.1.5 Preparation of genomic DNA from preimplantation embryos

Embryos were obtained from superovulated and plugged female mice (2.2.14.1) at the following stages: 1-cell (18-20 hrs p.c.), 2-cell (42-44 hrs p.c.), 4-cell (50-52 hrs p.c.), 8-cell (66-72 hrs p.c.), morulae and blastocysts (90-92 hrs p.c.). Individual embryos were collected in a PCR tube (0.2 ml) containing 5 µl Ampuwa H_2O. Samples were repeatedly (2-3 cycles) frozen and thawed (– 80^0C and 95^0 C). Then samples were directly used for genotyping by using PCR approach.

2.2.1.6 Isolation of total RNA from tissue samples and cultured cells

(according to Chomczynski and Sacchi, 1987)

Total RNA isolation reagent (Biomol) is an improved version of the single-step method for total RNA isolation described first by Chomczynski and Sacchi (1987). The composition of reagent includes phenol and guanidine thiocyanate in a monophase solution. In order to avoid any RNase activity, homogeniser used for RNA isolation was previously treated with RNase away and DEPC water and special RNase free Eppendorf cups were used during the procedure. 100 mg tissue sample was homogenised in 1 ml of RNA reagent by using a glass-teflon homogeniser. The sample volume should not exceed 10% of the volume of reagent used for the homogenisation. The homogenate was vortexed and incubated on ice for 5 min to permit the complete dissociation of nucleoprotein complexes. Then, 0.2 ml of chloroform was added, vortexed and incubated on ice for 5 min. After centrifuging at 8000 x g for 15 min at 4°C, the colourless upper aqueous phase was transferred into a new tube. 500 µl of isopropanol was added, solution was mixed by vortexing and RNA was precipitated by centrifugation at 10000 x g for 1 min. Finally, the pellet was washed with 75% ethanol, and dissolved in 50-100 µl DEPC-H_2O. The RNA was stored at -80°C. To isolate total RNA from cultured cells, 350 µl of reagent was added to the 6 cm diameter Petri dish. Cells were collected with a rubber stick and the lysate was transferred into a QIA shredder in 2 ml cup. Probe was then centrifuged for 2 min at 10000 x g in order to homogenise. 350 µl of 70% ethanol was added and mixed. Mixture was put in RNeasy mini spin column, centrifuged for 15 sec at 6000 x g and washed with 700 µl RWI buffer. Filter was put into new cup, 500 µl of RPE was added and centrifuged for 2 min at 6000 x g. After removing of supernatant, washing with buffer RPI was repeated. Finally filter was put into new 1.5 ml cup, 30- 50 µl of DEPC treated H_2O was added and centrifuged for 1 min at 6000 x g.

2.2.2 Determination of nucleic acid concentrations

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

C = (E 260 – E 320) fc

C = concentration of sample (µg/µl)

E 260 = ratio of extinction at 260 nm

E 320 = ratio of extinction at 320 nm

f = dilution factor

c = concentration (standard) / absorption (standard)

for double stranded DNA : c = 0.05 µg/µl

for RNA : c = 0.04 µg/µl

for single stranded DNA : c = 0.03 µg/µl

2.2.3 Gel electrophoresis

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

2.2.3.1 Agarose gel electrophoresis of DNA

Agarose gels are used to electrophorese nucleic acid molecules from as small as 50 base pairs to more than 50 kilobases, depending on the concentration of the agarose and the precise nature of the applied electrical field (constant or pulse). Usually, 1 g of agarose was added in 100 ml 0.5x TBE buffer, 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. 0.5x TBE buffer was used as electrophoresis buffer. Before loading the samples, about 0.1 volume of loading buffer was added and mixed. The samples were then loaded into the wells of the gel and electrophoresis was carried out at a steady voltage (50 - 100 V). Size of the DNA fragments on agarose gels was determined using 1 kb DNA ladder, which was loaded with samples in parallel slots. DNA fragments were observed and photographed under UV light.

2.2.3.2 Agarose gel electrophoresis of RNA (Hodge, 1994)

Single-stranded RNA molecules often have complementary regions that can form secondary structures. Therefore, RNA was run on a denaturing agarose gel that contained formaldehyde, and before loading, the RNA was pre-treated with formaldehyde and formamide to denature. 2 g of agarose was added to 20 ml of 10x MOPS buffer and 148 ml of DEPC water and dissolved by heating in microwave oven. After cooling it to about 50°C, 33.2 ml of formaldehyde (37%) was added, stirred and poured into a horizontal gel chamber. RNA samples were prepared as follows:

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

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

2.2.3.3 SDS-PAGE of proteins (Laemmli, 1970)

SDS-Page (Sodium Dodecylsulfate-Polyacrylamide 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 NuPage SDS sample buffer for 10 min before loading into the gel. The gel was run in NuPage MOPS SDS running buffer at 15 mA for 1 hr, then at a constant current of 30 mA.

2.2.4 Isolation of DNA fragments from agarose gel

2.2.4.1 Glass silica method (Vogelstein and Gillespie, 1979)

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

2.2.4.2 QIAquick gel extraction method

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

2.2.5 Enzymatic modifications of DNA

2.2.5.1 Digestion of DNA using restriction enzymes

Restriction enzymes are class of bacterial enzymes that cut DNA at specific sites. In bacteria their function is to destroy foreign DNA, such as that of bacteriophages. This attribute of restriction endonucleases is widely utilized in molecular biology. Restriction enzyme digestions were performed by incubating double-stranded DNA with an appropriate amount of restriction enzyme in its respective buffer as recommended by the supplier, and at the optimal temperature for that specific enzyme. Standard digestions include 2-10 U enzyme per microgram of DNA. 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 µl ligation buffer (10x)
1 µl T4 DNA ligase (5U / µl)
in a total volume of 10 µl

Blunt-end ligations were carried out at 16°C for overnight, whereas overhang-end ligations were carried out at 4°C overnight.

2.2.5.3 Phenol-chloroform extraction and ethanol precipitation

Protein impurities were removed by vigorous shaking of nucleic acid solution with an equal volume of phenol/chloroform/isoamyl alcohol mixture (25:24:1). The emulsion was then centrifuged for 5 min, 10000 x g, at RT, and the upper aqueous phase was collected, mixed with an equal volume of chloroform and centrifuged (5 min, RT, 10000 x g). Finally, the upper aqueous phase was collected for precipitation. Nucleic acids were precipitated by addition of NaAc (final conc. 0.3 M) and 2.6 volume of absolute ethanol. The mixture was then vortexed and centrifuged (5 min, 4°C, 10000 x g). The pellet was washed with 70% ethanol and centrifuged (5 min, RT, 10000 x g). After washing, the supernatant was aspirated and the pellet was air dried. The dried pellet was redissolved in sterile RNase free water or TE buffer.

2.2.5.4 TA-Cloning (Clark, 1988; Hu, 1993)

Taq and other polymerases have a terminal transferase activity that results in the non-template addition of a single nucleotide to the 3' ends of PCR products. In the presence of all 4 dNTPs, dATP is preferentially added. This terminal transferase activity is the basis of the TA- cloning strategy. For cloning of PCR products, pGEM-T Easy vector systems that have 5’ T overhangs were used. The followings were mixed:
50 ng of pGEM-T Easy Vector
PCR product (1:3, vector to insert ratio)
1 µl T4 DNA Ligase 10x buffer
1 µl T4 DNA Ligase
in a total volume of 10 µl.

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

2.2.5.5 Filling-up reaction (Costa and Weiner, 1994)

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

2.2.6 Transformation of competent bacteria (Ausubel et al., 1994)

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

2.2.7 Polymerase Chain Reaction (PCR)

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

2.2.7.1 PCR amplification of DNA fragments

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

1 µl DNA

1 µl Forward primer (10 pmol)

1 µl Reverse primer (10 pmol)

1 µl 10 mM dNTPs

5 µl 10x PCR buffer

1.5 µl 50 mM $MgCl_2$

1 µl *Taq* DNA polymerase (5U/µl)

Up to 50 µl H_2O

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

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

2.2.7.2 Reverse transcription PCR (RT-PCR)

RT-PCR is a technique, which generates cDNA fragments from RNA templates, and thereafter amplifies it by PCR. It is very useful to determine the expression of genes in specific tissues or in different development stages. 1-5 µg of total RNA was mixed with 1 µl of oligo $(dT)_{18}$ primer (10 pmol/µl) and sterile water was added to total volume of 12 µl. To avoid the possible secondary structure of the RNA, which might interfere with the synthesis, the mixture was heated to 70°C for 10 min, and then quickly chilled on ice. After a brief centrifugation, the following were added to the mixture:

4 µl 5x First strand buffer

2 µl 0.1 M DTT

1 µl 10 mM dNTPs

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

2.2.8 Protein methods

2.2.8.1 Isolation of total protein

Proteins were extracted from fresh or frozen mouse tissues by homogenization in protein lysis buffer (150 mM NaCl, 10 mM EDTA, 50 mM Tris/HCl, pH7.6, 1% Triton X-100 and 1% sodium deoxycholate) containing protease inhibitors (1µg/µl leupeptin, 3µg/µl aprotinin, 1µg/µl pepstatin). Lysates were sonicated on ice (about 20 impulses) and centrifuged at 12000 x g for 10 min at 4°C. Supernatant, containing membrane, organelles and cytosol proteins was collected and stored at -80°C, or used immediately for Western blot.

2.2.8.2 Determination of protein concentration (Bradford, 1976)

To determine the protein concentration, Bio-Rad protein assay was employed which is a dye-binding assay based on the differential colour change of a dye in response to various concentrations of protein. The assay is based on the observation that the absorbance maximum for an acidic solution of Coomasie Blue G-250 shifts from 494 to 595 nm when the binding to protein occurs. The bovine serum albumin (BSA) stock solution of 1 mg/ml was diluted in order to obtain standard dilutions in range of 10 µg/ml to 100 µg/ml. The Bio-Rad's color reagent was diluted 1:5 with H_2O and filtered through 0.45 µm filters. In a 96-well microtiter plate, 20 µl of each standard dilution and the samples to be measured were pipetted with 280 µl of the colour reagent. The absorption of the colour reaction was measured at 595 nm in a microplate reader (Microplate Reader 450, Bio-Rad).

2.2.9 Blotting techniques

2.2.9.1 Southern blotting of DNA to nitrocellulose filters (Southern, 1975)

In Southern blotting, the transfer of denatured DNA from agarose gels to nitrocellulose membrane is achieved by capillary flow. 20 x SSC buffer, in which nucleic acids are highly soluble, is drawn up through the gel into the nitrocellulose membrane, taking with it the single-stranded DNA that becomes immobilised in the membrane matrix. After electrophoresis of DNA, the gel was treated for 10 min with 0.25 M HCl for depurination. It was followed by denaturation solution for 30 min and 30 hrs in neutralization solution. The transfer of the DNA to the nitrocellulose membrane was done in a Turbo-Blot-apparatus (Schleicher & Schuell, Dassel). About 24 Whatman filter papers (GB 003) were layered on a Stack Tray followed by 3 Whatman filter papers (GB 002) and 1 Whatman

filter paper GB 002 soaked with 2 x SSC. The equilibrated nitrocellulose filter that was also soaked with 20 x SSC was laid on the top. The agarose gel, which was treated as described above was placed on the filter, and was covered with 3 Whatman filter papers GB 002 soaked with 20 x SSC. The buffer tray was placed and filled with 20 x SSC. Finally a wick, which was soaked with 20 x SSC, and the wick cover were put on the top of the blot. The transfer was carried out for overnight. Finally, after disassembling of the blot, the filter was dried on air 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.

2.2.9.2 Northern blotting of RNA onto nitrocellulose filters

For the transfer of RNA onto a nitrocellulose filter, the same procedure as described above (2.2.9.1) 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 2.2.9.1.

2.2.9.3 Western blotting of protein onto PVDF membrane (Gershoni and Palade, 1982)

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

After the electrophoresis of proteins on a SDS-PAGE, the gel and the PVDF membrane, which were cut of the size of the gel, were first moistened with methanol and then equilibrated in semidry transfer buffer. Six pieces of GB004 Whatman filter paper were also cut of the size of the gel. First, three papers soaked with transfer buffer were placed on semi dry transfer machine's lower plate and then 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 10 W (150 - 250 mA, 39 V) for 1 hr. For protein dot blot, specified amounts of protein solution in different concentrations were poured onto PVDF membrane. Membrane was air-dried and used for incubation with antibodies. To confirm transfer efficiency of proteins onto nitrocellulose membranes, the gel was incubated for 30 min in Coomassie blue solution at RT

2.2.9.4 Incubation of protein-bound membranes with antibodies

The membrane was first incubated in P1 buffer with 5 % non-fat dry milk for 1 hr at RT in order to block unspecific binding sites, followed by incubation with 2% milk in P1 buffer for 5 min. Membrane was then incubated with a primary antibody at the recommended antibody dilution in P1 buffer with 2 % non-fat dry milk for overnight at 4°C. Then, the membrane was washed 4 times in P1 buffer with 2% dry milk for 5 to 10 min and then incubated with the alkaline phosphatase conjugated secondary antibody in P1 buffer with 2% non-fat dry milk for 1 hr at RT. After this step, the membrane was washed 4 times in P1 with 2% dry milk, one time in P1 without dry milk and one time in AP buffer for 5 min at RT. Finally, the proteins on the membrane were visualized by an incubation step in the dark with 10 ml of staining solution (alkaline phosphatase substrate solution) for 15 min and rinsed with water to stop the reaction.

P1 buffer:	1 .5M NaCl
	1 M Tris/HCl pH 7.5
AP buffer:	100 mM Tris-HCl (pH 9.5)
	100 mM NaCl
	50 mM $MgCl_2$
Staining Solution:	66 µl NBT
	33 µl BCIP
	in 5 ml of AP buffer

2.2.10 "Random Prime" method for generation of ^{32}P labelled DNA (Denhardt, 1966; Feinberg and Vogelstein, 1989)

Rediprime™II Random Prime Labeling System (Amersham Pharmacia) was used for labelling of DNA probes. The method depends on the random priming principle developed by Feinberg and Vogelstein (1989). The reaction mix contained dATP, dGTP, dTTP, dCTP, Klenow fragment (4 - 8 U) and random oligodeoxyribonucleotides. Firstly, 10 - 25 ng of DNA were denatured in a total volume of 46 µl in boiling water for 10 min and quick chilled on 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 0.5 - 1 hr. The labelled probe was purified from unincorporated [α-^{32}P] dCTP by using microspin columns (Amersham Pharmacia).

2.2.11 Hybridization of nucleic acids (Denhardt, 1966)

The membrane to be hybridised was equilibrated in 2 x SSC and transferred to a hybridisation tube. After adding 8 ml of hybridisation solution and 150 µl of sheared salmon DNA, the membrane was incubated for 6 to 8 hrs in the hybridization oven at an appropriate temperature, which was usually 65°C. Then, the labelled probe was denatured at 95°C for 10 min, quick chilled on ice, and added to the hybridisation solution, together with 150 µl of sheared salmon DNA. The hybridisation was carried out overnight in the oven. Next day, the filter was washed for 10 min with 2 x SSC at RT. Finally it was washed with 0.2 x SSC containing 0.1 % SDS and then with 0.02 x SSC at the hybridisation temperature. After drying the filter, it was sealed in plastic foil and exposed to autoradiography overnight to few days (depending on the value of radioactive signal) at -80°C. The film was developed in X-ray automatic processor Curix 60. If membrane has to be used again, it was stripped in 0.2 x SSC at 80°C, until radioactive signal was no longer detected.

2.2.12 Non-radioactive dye terminator cycle sequencing

Non-radioactive sequencing was performed with the Dye Terminator Cycle Sequencing-Kit (Applied Biosystem). The reaction products were analysed with automatic sequencer (Mega Base 1000). For the sequencing reaction, four different dye labelled dideoxy nucleotides were used (Sanger et al., 1977), which, when exposed to an argon laser, emit fluorescent light which can be detected and interpreted. The reaction was carried out in a total volume of 10 µl containing 1 µg plasmid DNA or 100 - 200 ng purified PCR products, 10 pmol primer and 4 µl reaction mix (contains dNTPs, dideoxy dye terminators and *Taq* DNA polymerase). Elongation and chain termination take place during the following program in a thermocycler: 4 min denaturation followed by 25 cycles at 95°C, 30 sec, denaturation; 55°C, 15 sec, annealing; 60°C, 4 min, elongation. After the sequencing reaction, the DNA was precipitated with 1/10 volume 3 M sodium acetate and 2.5 volume 100% ethanol and washed in 70% ethanol. The pellet was dissolved in 4 µl of loading buffer, denatured at 95°C for 3 min, and finally loaded into the sequence gel.

2.2.13 Transfection of NIH 3T3 cells with the Brunol1-EGFPC-1and Brunol1-EGFPN-1 constructs

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

Solution A:

2–4 mg Brunol1-EGFPC-1

100 ml Serum-free medium

Solution B:

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

100 ml Serum-free medium

Solutions A and B were combined into one tube, mixed gently and incubated at room temperature for 10–30 min. Then 1.8 ml of serum-free medium was added to tube containing the CLONfectin/DNA solution and mixed gently. Old medium was removed from the culture and CLONfectin/DNA/media solution was applied. Cell culture slides were gently moved back and forth to distribute transfection solution evenly. Slides were incubated at 37°C for 4 hrs in a CO_2 incubator, after this time CLONfectin/DNA containing medium was removed and cells were washed with PBS prewarmed to 37°C. About 2 ml of fresh complete growth medium was applied and cells were incubated at 37°C. After 24 hrs, cell culture slides were fixed and immunostaining was applied.

2.2.14 Techniques for recovery and culture of preimplantation embryos

2.2.14.1 Superovulation

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

2.2.14.2 Recovery of preimplantation embryos

1-cell to 8-cell stage embryos were flushed from the oviduct of superovulated females. Plugged female mice (1.5-2.5 days pc) were killed by cervical dislocation. The skin and peritoneum were opened with the large transverse incision to expose the abdominal cavity. The oviducts with the upper part of the uterus were dissected and placed into a drop of M2 medium. Under dissection microscope, the needle attached to a 1 ml syringe was inserted in the fimbrial end of the oviduct. The needle was then held with forceps and oviducts were flashed with 0.05 ml of M2 medium (2.1.5.2.). Embryos were collected with a pipette and washed through several M2 drops. To remove the zona pellucida, embryos were treated in one drop of Tyrode's acid solution and transferred into the drop of M2-medium as soon as their zona pellucida was dissolved. 2 cell and 4 cell stage embryos were incubated in a drop of acutase in order to remove the polar bodies. The collected embryos were washed five times in a drop of PBS and each single embryo was transferred into a PCR cup (0.2 ml) containing 5 µl dH_2O. Those samples were used for genotyping. In order to decrease the possible contamination with maternal cells, 2 cell stage embryos were cultivated in M16 medium at 37^0C and 5% CO_2 until they developed to expected developmental stages as a 4-cell to blastocyst.

2.2.14.3 In vitro culture of blastocyst stage embryos

Embryos were flushed out from the uteri of plugged females at day 3.5 and placed in gelatinized 96-well dish. Embryos were cultured in ES cell medium without the addition of leukemia inhibitory factor. The dish was kept in a humid incubator at 37 ^{0}C, 5% CO_2 and embryonic outgrowth was scored daily. Every second day, 5 µl of fresh medium was added to the wells. DNA was prepared on the fourth day of the culture (2.2.1.4).

2.2.15 Histological techniques

2.2.15.1 Tissue preparation for paraffin embedding

The freshly prepared tissues were fixed in Bouin's solution for 24 - 72 hrs to prevent the alterations in the cellular structure. The tissue to be embedded in paraffin should be free of water. The dehydration process was accomplished by passing the tissue through a series of increasing alcohol concentrations. For this purpose, the tissue was let in 70%, 80%, 90%, 96% and 100% ethanol for at least 1 hr at RT. Later, the ethanol was removed from the tissue by incubating it in isopropanol overnight. Tissue was then incubated in different

mixtures of isopropanol/xylol (histoclear) in ratios 3:1, 1:1 and 1:3 for 30 min - 1 hr at RT. Then tissue was incubated in 100% xylol overnight. Further, tissue was incubated in paraplast at 60°C overnight. Before embedding, paraplast was changed at least three times. Finally, the tissue was placed in embedding mould and melted paraffin was poured into the mould to form a block. The block was allowed to cool and was then ready for sectioning or stored at 4°C.

2.2.15.2 Sections of the paraffin block

The paraffin blocks were pre-cut to the optimal size and clamped into the microtom (Hn 40 Ing., Nut hole, Germany). The cut-thickness of the paraffin embedded was for 3 - 7 µm. The sections were floated on 40°C water to allow actual spread and subsequently put onto Superfrost slides. A fine brush was used to transfer the sections to slides. Slides were then dried at 40°C and incubated in 80°C for about 15 min to get rid off excess of paraffin. Slides were then stored at RT for further analysis.

2.2.15.3 Hematoxylin-eosin staining of the histological sections

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 each for 2 min.
3. 1 min in H_2O and then stained for 15 min in hematoxylin.
4. Running tap water (control) for 10 min, then 1 min in dH_2O.
5. Eosin 0.1% + 2 drops acetic acid for 5 min, then in dH_2O for 1 min.
6. Incubation in 50%, 70%, 80%, 90%, 96% and 100% ethanol each for 2 min.
7. Slides were incubated two times in histoclear (Xylol) for 3 min.

2.2.16 Techniques for production of targeted mutant mice (Joyner, 2000)

The discovery that cloned DNA introduced into cultured mouse embryonic stem cells can undergo homologous recombination at specific loci has revolutionized our ability to study gene function in vitro and in vivo. This technique allows us to generate any type of mutation in any cloned gene. Over twenty years ago, pluripotent mouse embryonic stem (ES) cells derived from inner cell mass cells of mouse blastocysts were isolated and cultured (Martin, 1981; Evans and Kaufman, 1981). Using stringent culture conditions, these cells can maintain their pluripotent developmental potential even after many passages

and following genetic manipulations. Genetic alterations introduced into ES cells in this way can be transmitted into the germ line by producing mouse chimeras. Therefore, applying gene targeting technology to ES cells in culture gives the opportunity to alter and modify endogenous genes and study their functions in vivo.

2.2.16.1 Production of targeted embryonic stem cell clones

2.2.16.1.1 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 2700 X g for 5 min, the cell pellet was gently resuspended in 10 ml EmFi medium and plated on a 50 mm culture flask. Cells were incubated at 37°C, 5% CO_2. When the cells formed a confluent monolayer (three days), they were trypsinized, transferred to five 150 mm dishes and grown until they formed confluent monolayer, or directly treated with mitomycin C. To treat the EmFi with mitomycin C, the medium was removed and 10 ml fresh medium containing 100 µl mitomycin C (1mg/ml) was added. After 2-3 hrs of incubation, the monolayer of cells was washed twice with 10 ml PBS. The cells were then resuspended with 10 ml medium, and gentle pipetting dissolved any cell aggregates. The cells were centrifuged, resuspended in EmFi medium and plated onto dishes, which were treated with 0.1% gelatine for 30 min. The feeder cells were allowed to attach by incubation overnight at 37°C, 5% CO_2 or used after 2 hrs of incubation. Before adding ES cells on the feeder layer, the medium was changed to ES cell medium.

2.2.16.1.2 Growth of ES cells on feeder layer

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

2.2.16.1.3 Electroporation of ES cells

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

2.2.16.1.4 Analysis of recombinant ES cells

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

2.2.16.2 Production of chimeras

The ability of mammalian embryos to incorporate foreign cells and develop as chimeras has been exploited for a variety of purposes including the perpetuation of mutations produced in embryonic stem (ES) cells by gene targeting and the subsequent analysis of these mutations. The standard procedure is to inject 10-20 ES cells, which are recombinant for a targeted locus into the blastocoel cavity of blastocysts that have been recovered by flushing the uteri of day 3.5 pregnant mice (C57BL/6J). After injection, embryos are cultured for a short period (2-3 hrs) to allow re-expansion of the blastocoel cavity, which collapses upon injection. Then the embryos were transferred to the uterine horns of day 3

CD1 pseudopregnant mice. Pseudopregnant females were obtained by mating 6-8 weeks old oestrous females with vasectomized males.

2.2.16.3 Detection of chimerism and mice breeding

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

2.2.17 Determination of sperm parameters

2.2.17.1 Sperm count in epididymes, uterus and oviducts

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

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

For determination of sperm number in the uterus and the oviducts, *Brunol1* deficient males were mated with wild type females. The uteri and oviducts of those mice, which were positive for vaginal plug, were dissected in IVF medium and the spermatozoa were flushed out.

2.2.17.2 Determination of sperm abnormalities

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

normal or abnormal (normal or unusual sperm head shape). Percentage of abnormal sperm was determined.

2.2.17.3 Sperm motility

10 µl of sperm suspension was put on a dual sided sperm analysis chamber. Sperm motility was quantified using the computer assisted semen analysis (CASA) system (CEROS version 10, Hamilton Thorne Research). Then, 5000-10000 spermatozoa from 3 mice of mutant line and 2 of wild type were analyzed using the following parameters: average path velocity (VAP), straight line velocity (VSL), curvilinear velocity (VCL), lateral head amplitude (ALH), beat frequency (BCF) and straight forward movement (STR). Frequencies of these six sperm motility parameters were examined by probability plots categorised by mouse type (wild-type/mutant) and by time of observation (1.5, 3.5 and 5.5 hrs after preparation) for statistical analysis.

2.2.17.4 Acrosome reaction

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

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

2.2.18 Computer analysis

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

3. RESULTS

3.1 Isolation of the *Brunol1* cDNA

To identify new candidate genes for neurodegenerative diseases, the fetal cDNA library was screened with a probe containing 63 CAG repeats. The complete ORF frame of mouse *Brunol1* was isolated.The cDNA of *Brunol1* has 1485 nucleotides, consisting of 13 exons and it encodes a protein consisting of 495 amino acids. On position 1161 to 1209, it contains a 16 amino acid long stretch of glutamine. *Brunol1* has been mapped on mouse chromosome 1q23. The size of *Brunol1* is around 18 kb due to large size of several introns. Like the other member of elav family, *Brunol1* also has three RNA recognition motifs (RRM) and one variable motif (Fig. 3.1 A).

A

B

```
  1  atgaaggagccagatgccatcaagctgtttgtggggcagatcccg
     M  K  E  P  D  A  I  K  L  F  V  G  Q  I  P
 46  aggcatctggaggaaaaggacctgaagcccatcttcgagcagttt
     R  H  L  E  E  K  D  L  K  P  I  F  E  Q  F
 91  ggtcggatcttcgagctgactgtcatcaaggacaagtacaccggg
     G  R  I  F  E  L  T  V  I  K  D  K  Y  T  G
136  ctgcacaagggatgtgctttcctgacgtactgtgctcgcgattca
     L  H  K  G  C  A  F  L  T  Y  C  A  R  D  S
181  gccctgaaggcccagagtgccctgcacgaacagaagactctccca
     A  L  K  A  Q  S  A  L  H  E  Q  K  T  L  P
226  gggatgaacaggccgatccaggtcaagccagccgacagcgagagt
     G  M  N  R  P  I  Q  V  K  P  A  D  S  E  S
271  cgaggagaagaccggaagctctttgtgggcatgctaggaaagcag
     R  G  E  D  R  K  L  F  V  G  M  L  G  K  Q
316  cagacagatgaggatgtccggaagatgtttgaaccatttgggact
     Q  T  D  E  D  V  R  K  M  F  E  P  F  G  T
361  atagacgagtgcactgtgctccgggggccagacggtaccagcaaa
     I  D  E  C  T  V  L  R  G  P  D  G  T  S  K
406  ggctgtgcctttgtgaagttccagactcacgctgaggcccaggca
     G  C  A  F  V  K  F  Q  T  H  A  E  A  Q  A
451  gccatcaacacccttcacagcagccggaccctaccgggtgcctca
     A  I  N  T  L  H  S  S  R  T  L  P  G  A  S
496  tccagcctggtggtaaagtttgctgacacggagaaggagcgaggt
     S  S  L  V  V  K  F  A  D  T  E  K  E  R  G
541  ctccgtcgaatgcagcaggtggctacccagctgggcatgttcagc
```

```
    L  R  R  M  Q  Q  V  A  T  Q  L  G  M  F  S
586 ccgatcgccctccagtttggagcctacagcgcctacacccaggcc
    P  I  A  L  Q  F  G  A  Y  S  A  Y  T  Q  A
631 ctgatgcagcagcaggcggccctggtagcagctcacagtgcctac
    L  M  Q  Q  Q  A  A  L  V  A  A  H  S  A  Y
676 ctcagccctatggccaccatggctgccgtgcagatgcagcacatg
    L  S  P  M  A  T  M  A  A  V  Q  M  Q  H  M
721 gctgccatcagtgccaatggcctcatcgccacccccatcactcca
    A  A  I  S  A  N  G  L  I  A  T  P  I  T  P
766 tcctcaggaaccagcacccctcctgccattgctgccacgcccgtc
    S  S  G  T  S  T  P  P  A  I  A  A  T  P  V
811 tctgccatccctgctgccttgggcgtcaacggctacagcccggtg
    S  A  I  P  A  A  L  G  V  N  G  Y  S  P  V
856 cccacccagcctacagggcagcctgccccggatgctctgtatccc
    P  T  Q  P  T  G  Q  P  A  P  D  A  L  Y  P
901 aacggggttcacccttacccagatgaggctctgtctgctgagaga
    N  G  V  H  P  Y  P  D  E  A  L  S  A  E  R
946 agtgctggcggggttcccataatgtcccaggcccactcatggctt
    S  A  G  G  V  P  I  M  S  Q  A  H  S  W  L
991 gtgatgctctctgcagcccagagcccggcagcccccgtggaccct
    V  M  L  S  A  A  Q  S  P  A  A  P  V  D  P
1036 ctccagcaggcctatgcaggaatgcagcactacacagcagcgtac
    L  Q  Q  A  Y  A  G  M  Q  H  Y  T  A  A  Y
1081 cccgcagcctacagcctggttgcacctgcgttcccgcagcctcca
    P  A  A  Y  S  L  V  A  P  A  F  P  Q  P  P
1126 gccctggttgcccagcagcccccaccaccacctcagcaacagcag
    A  L  V  A  Q  Q  P  P  P  P  P  Q  Q  Q  Q
1171 cagcagcagcagcagcaacagcaacagcagcagcaacgggaaggc
    Q  Q  Q  Q  Q  Q  Q  Q  Q  Q  Q  Q  R  E  G
1216 cctgatggctgcaacatcttcatctaccacctgccccaggagttc
    P  D  G  C  N  I  F  I  Y  H  L  P  Q  E  F
1261 acagactcagagatcctccagatgtttgtccctttggtcatgtc
    T  D  S  E  I  L  Q  M  F  V  P  F  G  H  V
1306 atctcagccaaagtctttgttgaccgggccaccaatcagagcaaa
    I  S  A  K  V  F  V  D  R  A  T  N  Q  S  K
1351 tgttttggctttgtgagtttcgacaatccggccagtgcccaggct
    C  F  G  F  V  S  F  D  N  P  A  S  A  Q  A
1396 gccatccaggctatgaacggtttccagattggcatgaagcgcctc
    A  I  Q  A  M  N  G  F  Q  I  G  M  K  R  L
1441 aaagtccagctaaagcggcctaaggatgcaaacaggccctactaa 1485
    K  V  Q  L  K  R  P  K  D  A  N  R  P  Y  *
```

Figure 3.1 (A) Schematic representation of Brunol1 protein. (B) Nucleotide sequence of *Brunol1* cDNA (from NCBI). ATG codon, indicating start of translation and TAA terminating codon are bold and coloured in blue.

3.1.1. Expression analysis of mouse *Brunol1* gene

3.1.1.1 Analysis of *Brunol1* during embryonic development

To examine the expression of *Brunol1* during embryonic development, RT-PCR analysis was performed with RNA range of different embryonic stages. *Brunol1* expression was detected as early as 9.5 days post coitum (dpc) stage (Fig. 3.2 B). Interestingly, expression of *Brunol1* was detected in ES cells, but not in early preimplantation stages (2-, 4- and 8-cell stages). Taken together, these data indicate that *Brunol1* gene expression starts at 9.5 dpc and is also observed in embryonic stem cells (Fig. 3.2 A).

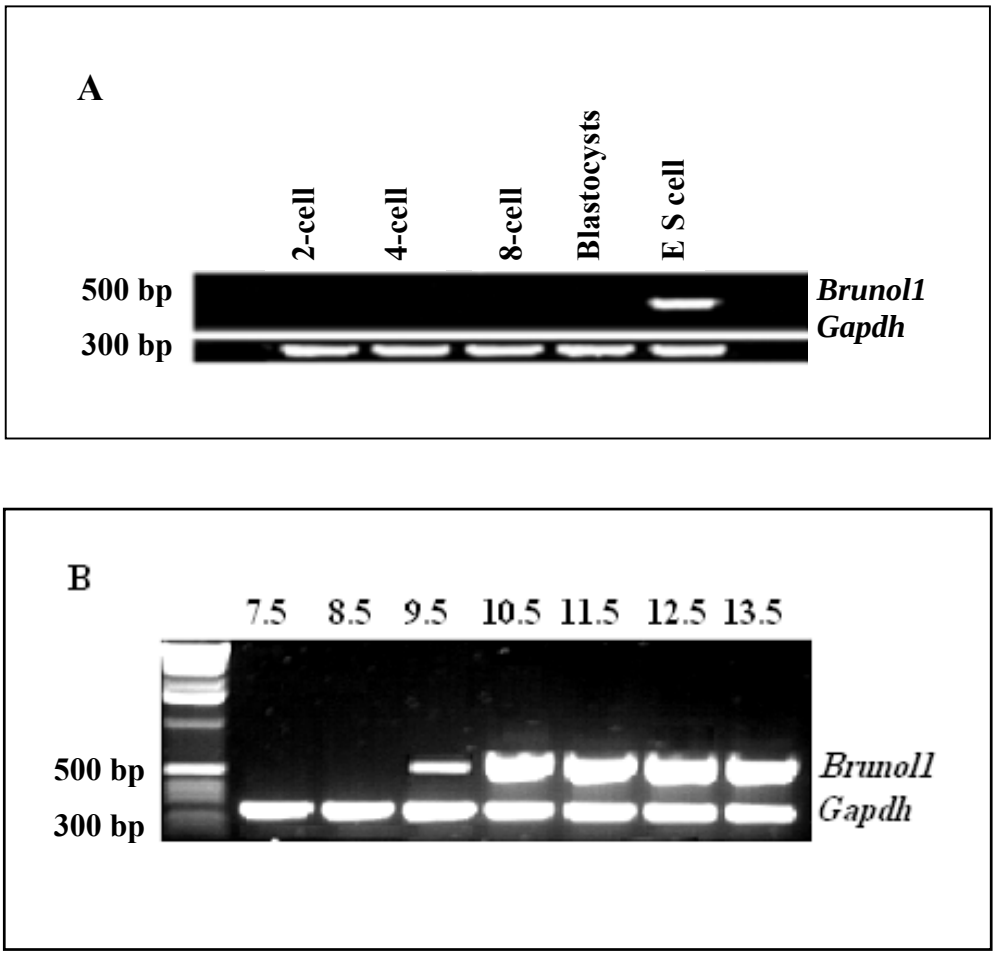
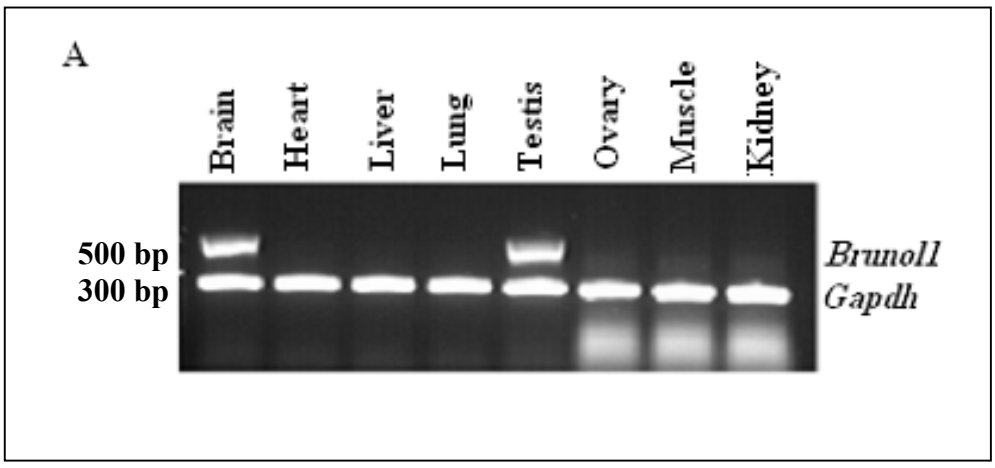
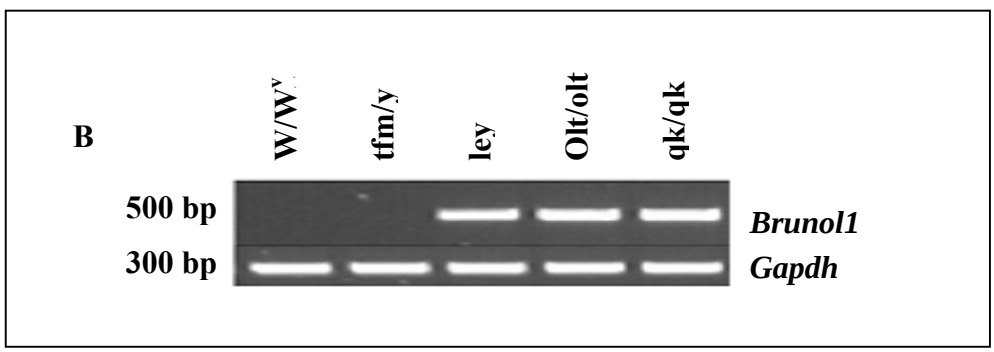


Figure 3.2 RT-PCR expression analysis of *Brunol1* during embryonic development. (A) Preimplantation stages of development. Expression of *Brunol1* was detected in ES cells but not in 2-, 4- and 8-cell stages. (B) Postimplantation stages. Expression of *Brunol1* starts at early stage 9.5 dpc. *Gapdh* served as a control.

3.1.1.2 RT-PCR analysis of *Brunol1* in adult mouse

To analyse the expression of *Brunol1* in adult mouse at the RNA level, total RNA was isolated from different adult mouse tissues including brain, heart, liver, lung, testis, ovary, muscle and kidney. By RT-PCR analysis, *Brunol1* transcript could be detected from samples of brain and testis. Integrity of the RNA used for RT-PCR was proven by amplification of the *Gapdh* transcript (Figure 3.3 A). RT-PCR was done also with RNA from testes of different mutants with spermatogenesis defects: W/W^V, Tfm/y, Leyl-/-, olt/olt and qk/qk. W/W^V mice were characterised by lack of any germ cell (Rooij and Boer, 2003), in Tfm/y and Leyl-/- mutants spermatogenesis is arrested at spermatocyte stage (Lyon and Hawkes, 1970; Zimmermann et al., 1999). Spermatogenesis arrest is known in olt/olt at round spermatid stage and in qk/qk at elongated spermatid stage, respectively (Bennett et al., 1971; Moutier, 1976). *Brunol1* transcript was detectable in Leyl-/-, olt/olt and qk/qk mutants but not in W/W^V and Tfm/y mutants (Figure 3.3 B). This indicates that expression starts as early as in spermatocyte stage. Because there is no expression in W/W^V and Tfm/y mutants, it can be concluded that expression of the gene is restricted to germ cells. Expression studies were also done in postnatal stages. *Brunol1* transcript was detectable from day P20 to P40 in all tested postnatal stages (Figure 3.3 C).





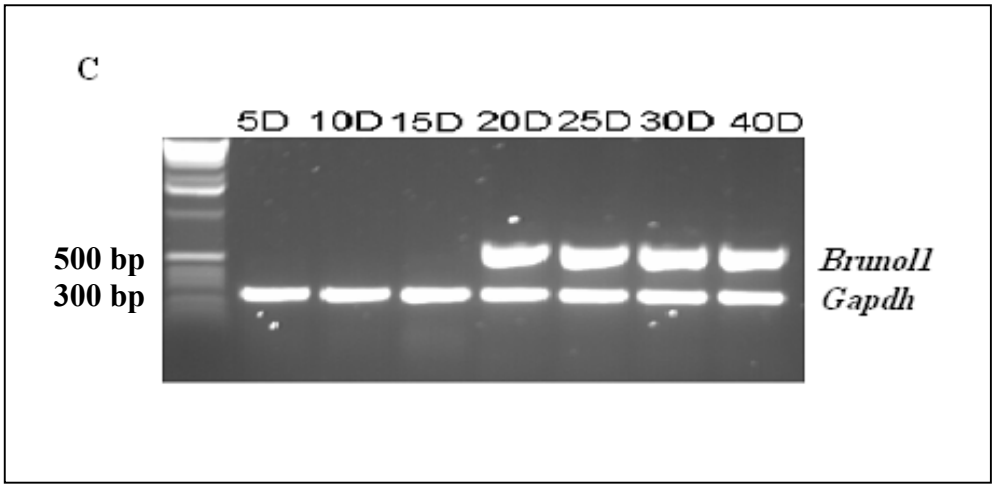


Figure 3.3 RT-PCR expression analysis of *Brunol1*, (A) in different tissues, (B) in testes of mutants and (C) in postnatal testis development.

3.1.1.3 Northern Blot analysis of *Brunol1* in adult mouse

To determine the expression of *Brunol1* at the Northern blot level, total RNA from heart, liver, lung, muscle, kidney, ovary, testis and brain was size fractionated in a 1 % agarose/MOPS gel containing 5.5 % formaldehyde and blotted onto Hybond-XL membrane. The Northern blot was hybridised with a ^{32}P-labelled 500-bp fragment from the 3′ UTR of the murine *Brunol1* cDNA probe, which detected approximately 3.0 kb and 4.0 kb strong hybridisation signals in whole brain sample and testis. No signal was visible in other adult tissues tested. Integrity of RNA was checked by hybridization with HEF probe (Figure 3.4)

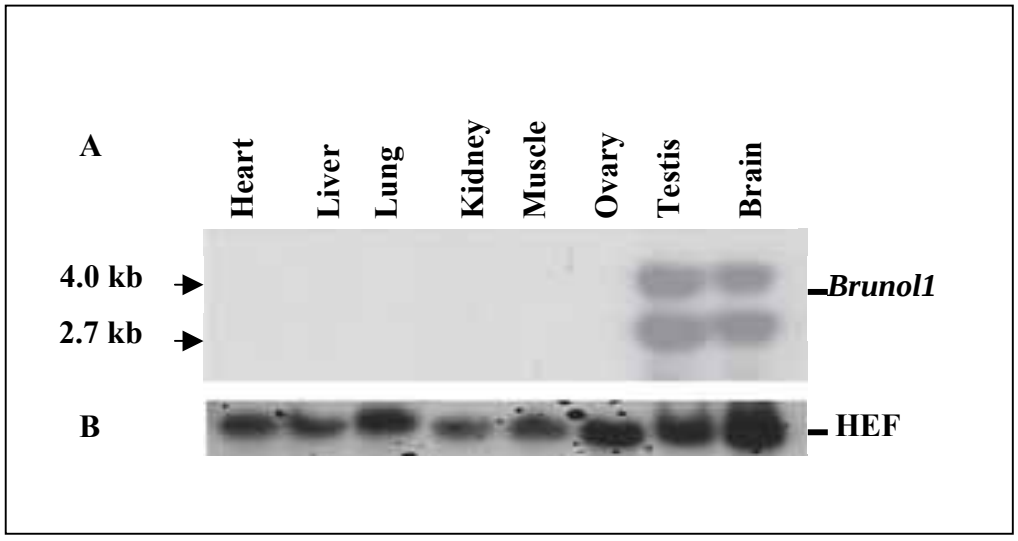


Figure 3.4. Northern blot analysis of *Brunol1* gene in different adult mouse tissues. (A) Total RNA (20 µg) was extracted from various tissues, heart, liver, lung, muscle, kidney, ovary, testis and brain of the adult mouse and subjected to Northern blot hybridization using the 500-bp fragment from the 3´ UTR of *Brunol1* as a probe. (B) The integrity of the RNA was checked by hybridization with HEF probe.

3.1.1.4 Localisation of Brunol1 protein in the cell

In order to study the subcellular localization of Brunol1 protein, Brunol1-EGFPC-1and Brunol1-EGFPN-1 fusion protein was generated. Coding region of *Brunol1* gene was amplified with Brunol1GFPF and Brunol1GFPR primers and PCR product was checked for mismatches by sequencing. PCR product which showed no mutation was digested with *EcoR*I and *BamH*I, as restriction sites for these enzymes were introduced in Brunol1GFPF and Brunol1GFPR primers sequences, respectively. Vector pEGFPN-1 and pEGFPC-1 were digested with the same enzymes and cDNA was cloned. Brunol1EGFP fusion protein is expressed in this case under the control of CMVIE promoter (human cytomegalovirus immediate early promoter) (Fig 3.5). Construct was transiently transfected into cultured NIH3T3 fibroblast cells using Lipofectamin2000 kit, as it was described in section 2.2.13. After 24 hrs, cells were fixed and observed under the microscope using UV light. Taking advantage of green signal emitted from EGFP, we were able to localise Brunol1 protein, since Brunol1 and EGFP build fusion protein. Brunol1 is localised in the cytoplasm and nucleus (Figure 3.6)

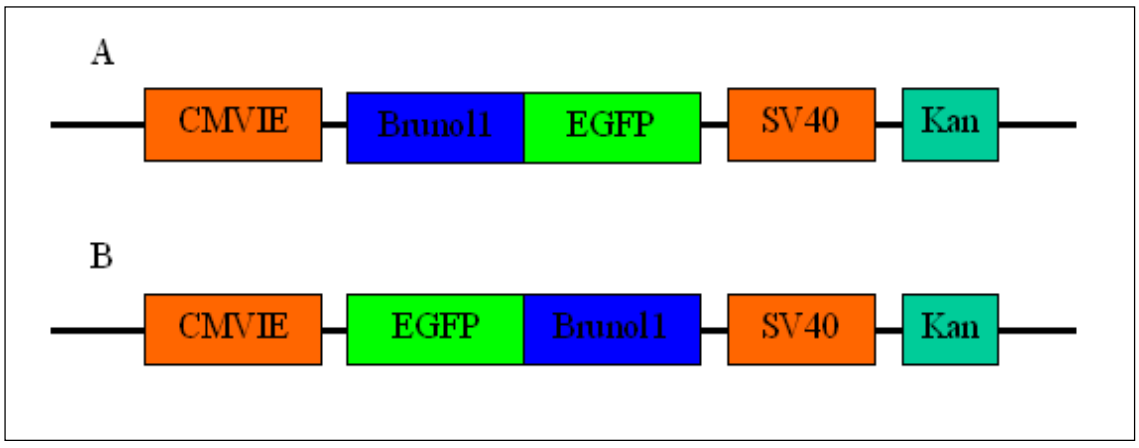


Figure. 3.5 Schematic representation of (A) Brunol1-EGFPC-1 and (B) Brunol1-EGFPN-1 construct. Fusion protein was expressed under the control of CMVIE promoter.

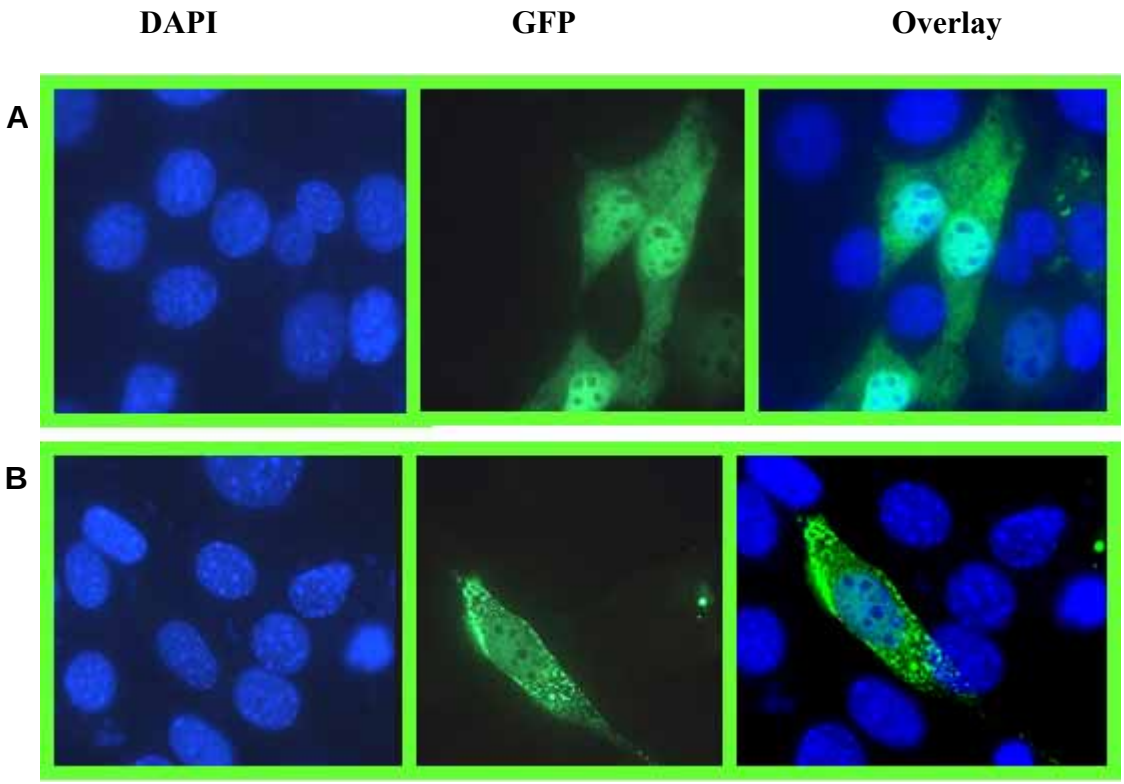


Fig.3.6 Subcellular localisation of Brunol1 protein. Microscopic fluorescence overlay image of transfected NIH3T3 cell expressing Gfp-tagged mouse Brunol1 protein. Green represents Gfp fluorescence from expressed mouse Brunol1 protein, blue represents DAPI-stained cell nuclei. (A) Brunol1-EGFPC-1 and (B)Brunol1-EGFPN-1

3.1.2 Targeted inactivation of mouse *Brunol1* gene

One of the best ways to elucidate gene function is the generation of a knockout animal model. For this purpose, *Brunol1* knockout mice were generated in this study. Analysis of the phenotype of mice with targeted disruption of *Brunol1* gene could help in understanding the role of the *Brunol1* gene during development and spermatogenesis.

3.1.2.1 Isolation and characterization of the cosmid clone with mouse genomic DNA

Cosmid clone *Mcos115* containing the complete *Brunol1* gene was isolated by Schlickum; 1999. We characterized the cosmid clone by restriction digestion and Southern blot analysis. The cosmid clone *Mcos115* was restricted with the enzymes *Xba*I and *Xho*I, blotted and radioactively hybridized with *Brunol1* cDNA probe (Fig 3.7A and 3.7 B).

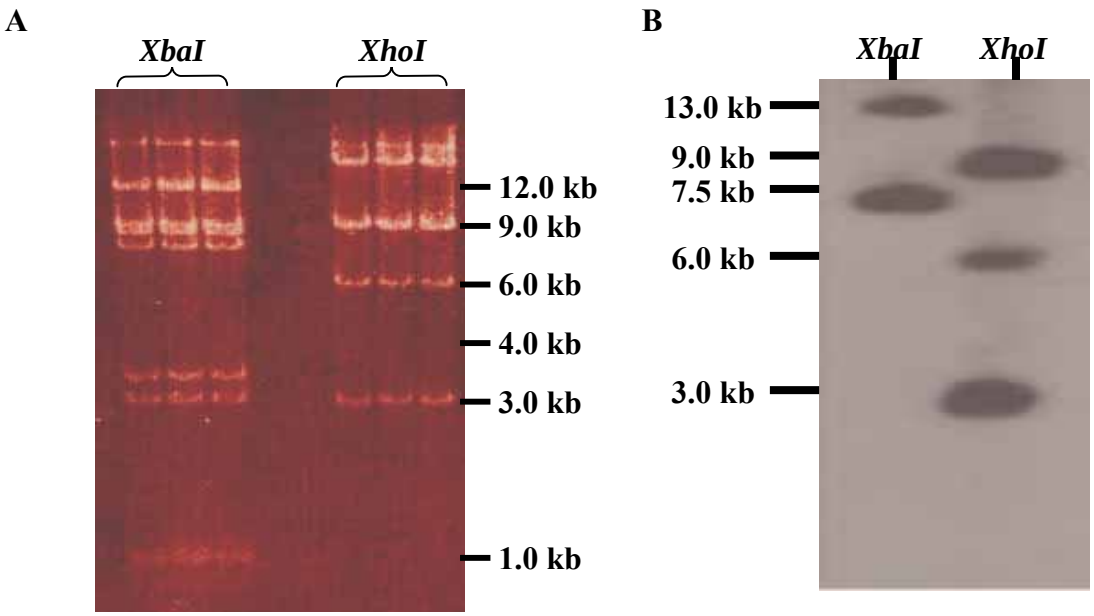


Figure 3.7 (A) Enzymatic digestion of a cosmid clone *Mcos115* with *Xba*I and *Xho*I. (B) The clone of *Mcos115* was digested with *Xba*I and *Xho*I enzymes and hybridized with *Brunol1* cDNA probe.

3.1.2.2 Construction of the *Brunol1* knockout construct

In order to generate the *Brunol1* targeting vector, a region of 7.5 kb of the *Brunol1* gene consisting of exon 3 to exon 12 was replaced by Neomycin phosphotransferase gene cassette under the control of phosphoglycerate kinase promoter. In this construct, neomycin was used as marker for positive selection while two copies of thymidine kinase from Herpes simplex virus was used as a negative selection marker.

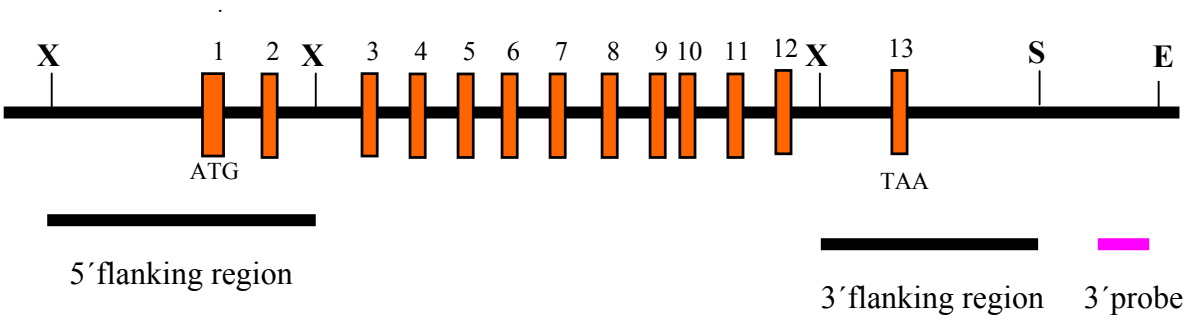


Figure 3.2 Restriction digestion map of *Brunol1* genomic DNA and fragments, which were cloned. The cloning strategy for subcloning the 5' and 3' regions of the *Brunol1* gene and subcloning of the 3' external probe are designated. Abbreviations are: E, *EcoRI,* X, *Xho*I and S, *Sac*I.

3.1.2.3 Subcloning of 5' wing of the *Brunol1* knockout construct into the pGATA vector

The clones which were designated as positive during cosmid library screening were digested with *XhoI* enzyme. A 6.0 kb *XhoI* fragment containing the 5'-flanking region with exon-1 and exon-2 of the *Brunol1* gene (Figure 3.8) was isolated from the cosmid clone (Mcos115 from R.Z.P.D) and purified from the agarose gel. This fragment was subcloned into pGATA vector using the *XhoI* site. The fragment was again extracted from pGATA vector by *XhoI* enzyme and cloned into the pPNTM-1 vector using the same restriction site.

3.1.2.4 Subcloning of 3' wing of the *Brunol1* knockout construct into pBlueScriptII SK

For the generation of 3' wing of the *Brunol1* knockout construct, a 9.0 kb *Xho*I fragment containing the 3'-flanking region of the *Brunol1*gene with exon13 (Figure 3.8) was isolated from the cosmid clone (Mcos115 from R.Z.P.D) and purified from the agarose gel. This fragment was subcloned into pBlueScript SK vector using the *Xho*I site and then 5.0 kb *Sac*I fragment was extracted from pBlueScript and ligated into pZero vector. Then 5.0 kb *EcoR*I fragment from pZero vector was cloned into pPNT-M-1 Neo targeting vector. The replacement vector BrunolI-Neo-Tk; pPNTM-1 Neo vector containing 5'- 6.0 kb arm and 3'- 5.0 kb arm) was sequenced in order to check right orientation of both wings in the vector. Bru-1-6kb-F; Bru-1-6kb-R and Neo-R primers, specific for construct, were used for sequencing and the construct was linearized at the unique *Nhe*I site present at the 5´multiple cloning site.

3.1.2.5 Generation of the 3' external probe

The 3' external probe for screening of ES cells was generated to distinguish between wild type and recombinant clones. For this purpose, a fragment of 600 bp was amplified by PCR, using primers Bru-1-Ext-Probe-F and Bru-1-Ext-Probe-R. The PCR fragment was cloned in pGEM T-Easy vector and then extracted with *Eco*RI restriction enzyme. This external probe recognizes a 16 kb fragment in case of wild type and 11kb in case of

recombinant clone in Southern blot hybridization after digestion of genomic DNA with *Eco*RI enzyme (Fig 3.7).

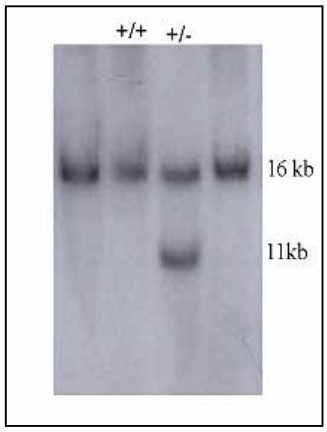


Figure 3.7. Southern blot analysis of ES clones by using 3´external probe. Genomic DNA extracted from ES clones was digested with EcoRI. The external probe recognises a 16 kb band in case of wild type and 11 kb in recombinant.

3.1.2.6 Electroporation of the ES cells and screening of ES cells for homologous recombination

Brunol1 targeting vector was linearized with *Nhe1* enzyme and 50 µg of purified DNA was electroporated into RI embryonic stem cells, as it was described in section 2.2.15.1.3. Cells were plated on feeder layer and after 10 days of selection 98 clones resistant for neomycin were selected and cultured on 24 well plates. Genomic DNA was isolated from ES cells, as it was described in section 2.2.1.3, and used for Southern blot hybridization. DNA from each clone was digested with *EcoRI* enzyme, electrophoresed and blotted onto Hybond-XL membrane. Blots were then hybridized with radioactively labelled 3´external probe. Two bands were recognized in case of homologous recombination (16 kb wild type allele and 11 kb recombinant allele). When no recombination has occurred or non-homologous recombination had taken place, only wild type band could be detected (Fig 3.8 B). Total 290 clones were screened, out of which three were recombinant. These results were confirmed using Neo probe, which detected 13.5 kb expected recombinant band. Clone No.128, rehybridized with external probe again and was chosen for blastocyst injection.

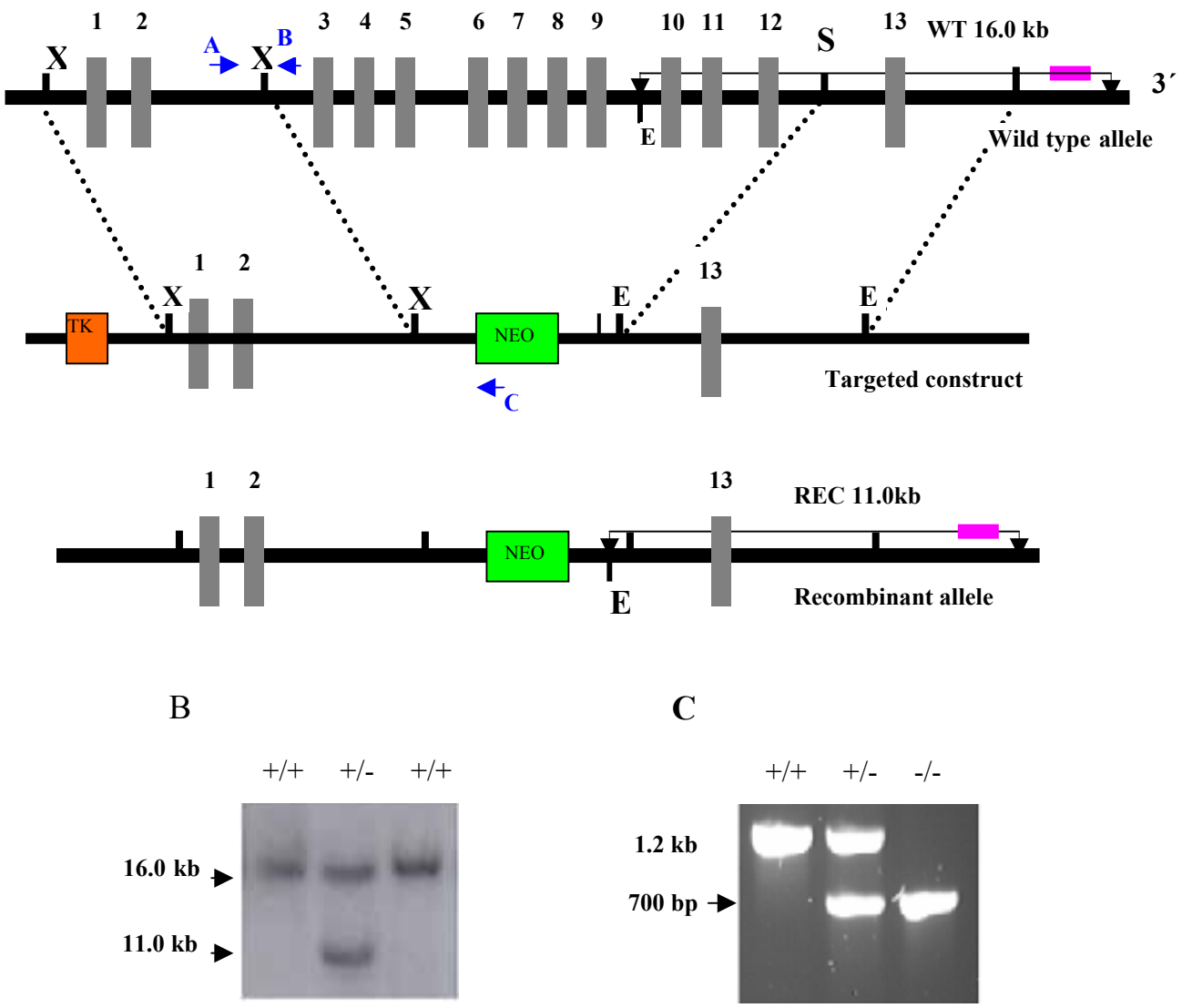


Figure 3.8 Generation of *Brunol1 knock*out line. A: Schematic representation of the targeting strategy for *Brunol1*construct. Wild-type *Brunol1* locus (top), targeting construct (middle), and mutated allele (bottom) are shown. The Pgk-Neo cassette replaced exon 3 to exon 12 of the *Brunol1*. The external probe (Ext-probe) shown as a pink block at the 3′ end of *Brunol1* locus. The location of various primers are represented by blue arrows, A; Brunol1-F; B, Brunol1-R; C. pGK-NeoR. The exons are shown as numerical numbers. Neo stand for Pgk-Neo cassette The restriction site abbreviations are. X, XhoI; E. EcoRI; S. SacI

3.1.2.7 Generation of chimeric mice

The recombinant ES cells were injected into 3.5 dpc blastocysts derived from C57BL/6J mice. The blastocysts were implanted into pseudopregnant CD-1 mice to generate the chimeric mice. Total 29 chimeras were obtained by four independent injections of recombinant ES clones. The chimeras were scored according to the coat color (in percentage), 22 male chimeras with 1x 95%, 3x 85%, 1x 75%, 6x 50%, 3x 25%, 2x 20%, 2x 15%, 4x 10% and 7 female chimeras with 1x 45%, 1x 25%, 2x 20%, 1x 20% 2x 15%, and 2 x 10% were obtained. Four high percentage male chimeras 1x 95%, 3x 85%, were bred with C57BL/6J and 129X1/SvJ mice, respectively to obtain F1 animals in respective background (C57BL/6J x 129/Sv) and in (129X1/SvJ). Germ line transmission was detectable by all the four male chimeras on C57BL/6J x Sv/129 and on inbred 129/Sv background after one and half month breeding. The germ line transmission of the mutant allele was verified by genomic PCR. Mutated allele generates an 700 bp PCR product by using Bruno1-Geno-F, Bru-1 Del-R and pGKK-325R primers and wild type yield a PCR product of 1.2 kb by using the Bruno1-Geno-F, Bru-1 and Del-R primers on DNA isolated from tail biopsies of the offspring (Fig 3.8C).

3.1.2.8 Generation and analysis of *Brunol1* knockout mice

F1 animals, heterozygous for *Brunol1* were used for further crossing in order to obtain F2 animals and to establish knockout lines on both C57 BL/6J x 129/Sv and 129/Sv background.

3.1.2.9 Transcriptional analysis

In order to assess the *Brunol1* transcript in the *Brunol1* knockout mice, a Northern blot analysis was performed using testicular RNA from wildtype, heterozygous and homozygous mice of about 50 days of age. The filter was hybridized with the *Brunol1* 3′ UTR cDNA probe. To check the integrity of RNA, the filter was rehybridized with a HEF probe. Northern blot analysis showed the absence of *Brunol1* transcript in the *Brunol1*$^{-/-}$ mice, indicating that, owing due to the integration of the *neomycin* cassette, the expression of *Brunol1* is hampered (Figure 3.9A). By RT-PCR analysis using the testicular RNA from *Brunol1* $^{-/-}$, heterozygous and wild type mice, PCR product was obtained when RNA from wild type and heterozygous males was used, but not any product was visible in case of RT-

PCR performed with RNA from homozygous mice. It proves that in homozygous *Brunol1* knockout mice the whole *Brunol1*gene was disrupted (Fig 3.9 B).

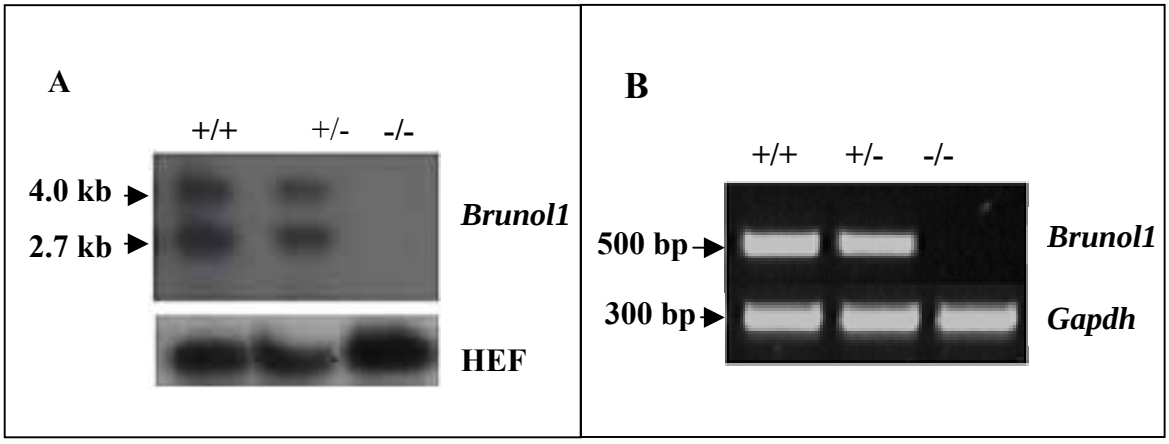


Figure 3.9 Transcriptional analyses of *Brunol1* deficient mice. (A) Northern blot analysis of testicular RNA from *Brunol1* wild type, heterozygous and homozygous, 50 days old mice. The *Brunol1* 3´UTR cDNA probe (500 bp) detected a specific transcript of about 3.0 and 4.0 kb. Variation of the loaded RNA was checked by hybridising the same blot with a HEF cDNA probe. (B) RT-PCR analysis for *Brunol1* expression in testis. *Brunol1* specific product (500 bp) was detected both in the wild type and heterozygous mice, while in *Brunol1*$^{-/-}$ mice no PCR product was detected. Integrity of RNA was verified by *Gapdh* primers amplification.

3.1.3 Phenotypic analysis of *Brunol1* knockout mice

3.1.3.1. Mode of inheritance

F1 heterozygous mice were intercrossed to obtain F2 generation. Mice were genotyped using DNA obtained from tail biopsies. Breeding of heterozygous mice with wild type mice were performed for a control. No change in the sex ratio of the offspring of the heterozygous matings was noted in both backgrounds (C57BL/6J x 129/Sv and129/Sv), (Table 3.1A and 3.1C). In addition, there seemed to be no reduction in litter size 9.1 on C57BL/6J x 129/Sv and 5.9 on 129/Sv in comparison to matings of wildtype animals. The ratio of wildtype, heterozygous and homozygous was approximately 1:2:1 (Table 3.1B). Among the 107 offspring, 35 (31%) were wild type, 52 (47%) were heterozygous and 20 (18%) were homozygous mice (Table 3.1B) in the C57BL/6J x 129/Sv genetic background,

while in case of 129/Sv inbred background, 27 were wild type, 42 were heterozygous and 30 were homozygous mice out of 97 animals genotyped (Table 3.1D).

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

	female	male
WT	14 = 53 %	12=46%
	26=44%	
HE	13=39%	20=63%
	33=55%	
	27=45%	32=54%

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

	female	male
WT	16 = 16%	18 = 16%
	35 = 31%	
HE	29=15%	23= 24%
	52 = 47%	
HO	13 = 12 %	10= 17%
	20 = 18 %	
	59 = 53 %	51 = 46 %

C HE x WT on 129 SV background

	female	male
WT	12 = 22%	10 = 34 %
	22 = 43%	
HE	17= 19%	11 = 25%
	28 = 54 %	
	29 = 56 %	21 = 41 %

D HE x HE on 129 SV background

	female	male
WT	12 = 44 %	15 = 56 %
	27=27%	
HE	17 = 40 %	25 = 60 %
	42=42%	
HO	18 = 60 %	12 = 40 %
	30=30%	
	47 = 48 %	52 = 52 %

Table 3.1 Statistical analysis of genotype distribution of *Brunol1* in HE x WT and HE x HE breeding on C57 BL/6J x 129/Sv and 129/Sv backgrounds. (A) Breeding of heterozygous animals with wild type ones on mixed background did not show significant statistical differences, as it was shown by chi^2 test. 59 animals in 8 throws were genotyped. (B) Crossing of heterozygous animals on mixed background also did not show deviation from Mendelian ratio. 107 animals in 12 throws were analyzed. (C) On 129/Sv background no deviations from expected numbers were observed when heterozygous animals were crossed with wild type ones. 50 animals in 7 throws were genotyped. (D) No statistically significant difference was observed by chi^2 test, when breeding of heterozygous animals on 129/Sv background was analyzed. 99 animals in 17 throws were genotyped. Abbreviations are: WT: wild type, HE: heterozygous, HO: homozygous.

3.1.3.2 Analysing the fertility of *Brunol1* deficient males

To evaluate consequences of *Brunol1* disruption, fertility of homozygous males on both backgrounds were tested by mating them with homozygous and wild type females. All of the matings were performed for four months and usually after one month first litter was obtained. Number of born mice and mean litter size of these breedings together with results of breedings of heterozygous mice are summarized in Table 3.2. Average litter size of offspring of HO x HO mating on both backgrounds was not significantly different from litter size of HE x WT and HO x WT matings on the mixed background and from HE x WT mating on 129/Sv background. The average litter size on C57 Bl/6J was 7.9 and 7.6 on

129/Sv background. Eight males from C57 BL/6J x 129/Sv background and 5 males from 129/Sv background were used for the fertility test. All of the males from both backgrounds were fertile

Type of breeding	No. of mice born	No. of litters	Average litter size
C57BL/6J x 129Sv			
+/-♀ x +/+♂	59	8	7.3
+/- x +/-	110	12	9.1
-/- ♂ x +/+♀	71	8	8.8
-/- x -/-	79	10	7.9
129/ Sv			
+/- x +/+	51	7	7.2
+/- x +/-	117	17	6.8
-/-♂ x +/+♀	99	16	6.1
-/- x -/-	61	8	7.6

Table 3.2 Fertility of *Brunol1* +/- and *Brunol1* -/- mice on C57BL/6J x 129/Sv and 129/Sv backgrounds. Numbers of born mice, number of litters and average litter sizes are given. Average litter size from both backgrounds was not affected.

3.1.3.3 Sperm count, acrosome reaction and analysis of sperm morphology of *Brunol1* deficient mice

Total sperm count in the cauda epididymes of *Brunol1* homozygous, heterozygous and wild type males was determined. Four homozygous, three heterozygous and three wild type males from both genetic backgrounds were used for sperm count. A statistically significant difference in total sperm count was observed between knockout and wild type animals (Table3.3), as determined by Mann-Whitney U-test with a confidence value of < 0.01. To analyse sperm morphology, slides were prepared as it was described in section 2.2.17 No abnormalities were observed in sperm shape in *Brunol1* mutants as compared to wild type

(Figure 3.10A & 3.10B). To analyse the acrosome reaction, we examined the response of spermatozoa of *Brunol1*$^{+/+}$ and Brunol1$^{-/-}$ to the calcium ionophore A 23187. There was no significant difference in the assay for acrosome reaction between *Brunol1*$^{+/+}$ (84%) and Brunol1$^{-/-}$ (79%) mice.

No of sperm	Genotype of male	
	+/+	-/-
C 57BL/6J x 129Sv Epididymis	$7.0 \pm 0.8 \times 10^7$	$2.0 \pm 0.4 \times 10^7$
129/Sv Epididymis	$7.2 \pm 1.3 \times 10^6$	$3.0 \pm 1.8 \times 10^6$

Table 3.3 Sperm count in the cauda epididymes of -/- and +/+ mice from both C 57BL/6J x 129Sv and 129/Sv backgrounds. Statistically significant differences were observed as compared to wild type.

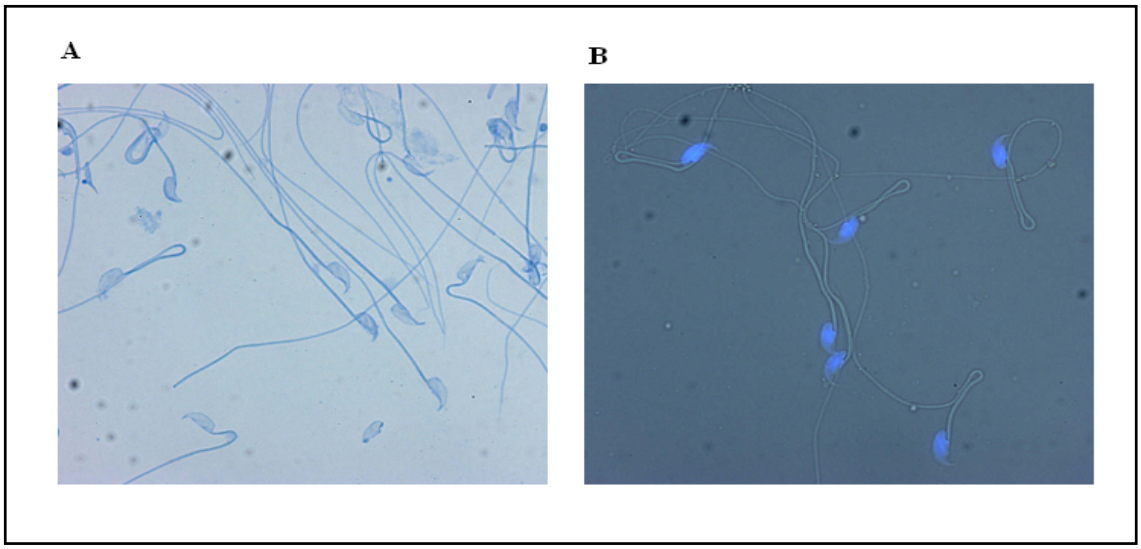


Figure 3.10. No sperm morphology abnormalities were observed with sperm isolated from cauda epididymes of males from (A) C 57BL/6J x 129/Sv and (B) 129/Sv backgrounds

3.1.3.4 Sperm motility assay

Analysis of sperm motility of *Brunol1*$^{-/-}$ mice was done on the C57BL/6J x 129/SvJ and 129/Sv backgrounds (Table 3.5). The sperm motility of 2 wildtype males and four mutant males was measured at 1.5, 3.5 and 5.5 hrs incubation *in vitro*, using the Hamilton Thorne computer assisted sperm analyser, as it was described in section 2.2.22.3. Significant differences in motility and progressive movement of sperm of *Brunol1* deficient mice were observed, as compared to wild type. Percentage of motile sperms and sperms showing progressive movement of mutant mice was always 15 – 20 % lower than wild type like 24% of motile sperm of mutant vs. 58% of wild type at 1.5 hrs and 36% of progressive moving sperm of mutant vs. 65. % of wild type at 3.5 hrs on mixed background. On 129/Sv background 44% of motile sperm of mutant vs.70% of wild type at 3.5 hrs and 27 % of progressive moving sperm of mutant vs. 38 % of wild type at 3.5 hrs was observed. This data is summarized in Table 3.5.

For further investigation of sperm motility, the following parameters were evaluated more in details: straight forward movement (STR), lateral head amplitude (ALH) curvilinear velocity (VCL), average path velocity (VAP), straight line velocity (VSL) and beat frequency (BCF) (Fig. 3.11). Mann-Whitney U-Test was done and statistically significant differences were observed for almost each parameter with $p<0.001$, with few exceptions. On C57 BL/6J x 129/Sv background significant differences were observed for BCF with $p=0.004$ at 1.5, 3.5 and at 5.5 hrs, significant differences were found for STR at 3.5 hrs with $p=0.004$. On 129/Sv background statistically significant differences were found for STR at 1.5 hrs, BCF at 3.5 hrs and STR at 5.5 hrs. Differences were especially high for all velocities and lateral head amplitude. This finding indicates that motility of *Brunol1* deficient mice sperm is significantly reduced.

A

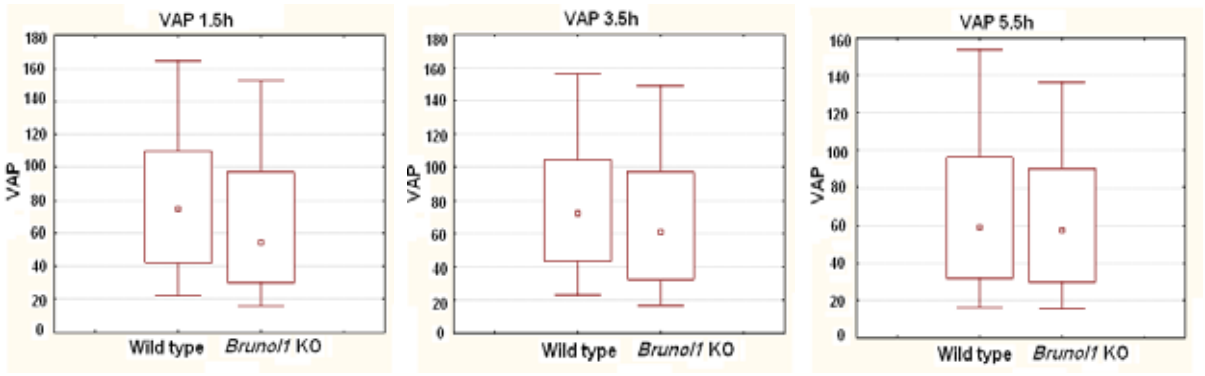
Genotype	Incubation time (hr)	Percentage of motile spermatozoa	Percentage of spermatozoa with progressive movement
-/-	1.5	24	25
	3.5	53	36
	5.5	58	30
+/+	1.5	58	46
	3.5	64	65
	5.5	71	49

B

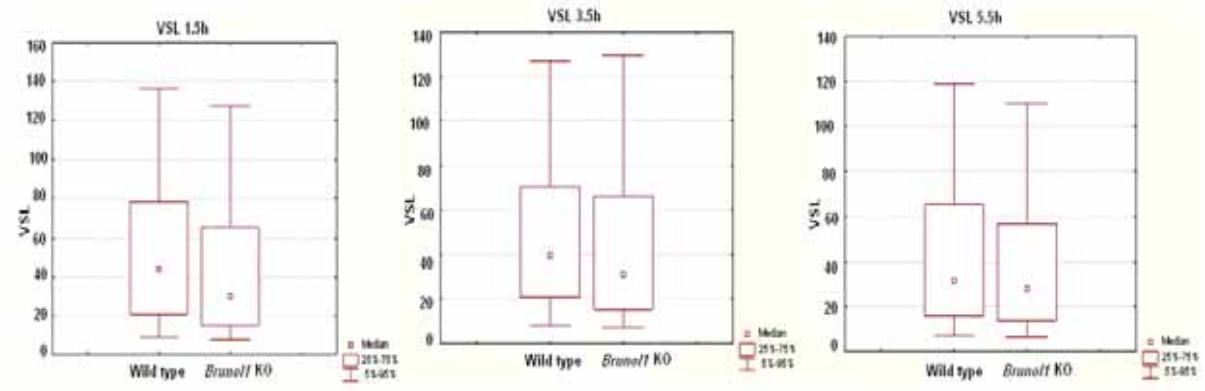
Genotype	Incubation time (hr)	Percentage of motile spermatozoa	Percentage of spermatozoa with progressive movement
-/-	1.5	48	30
	3.5	44	27
	5.5	35	20
+/+	1.5	70	42
	3.5	67	38
	5.5	71	27

Table 3.5 Motility analysis of sperm from *Brunol1* deficient males on (A) C57 BL/6J x 129/Sv background and (B) 129/Sv background.

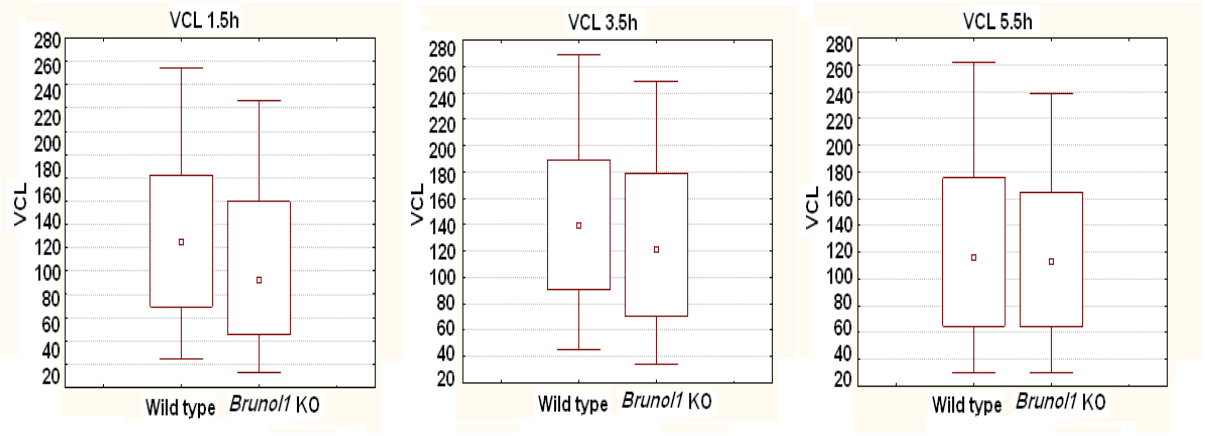
Average path velocity (VAP)



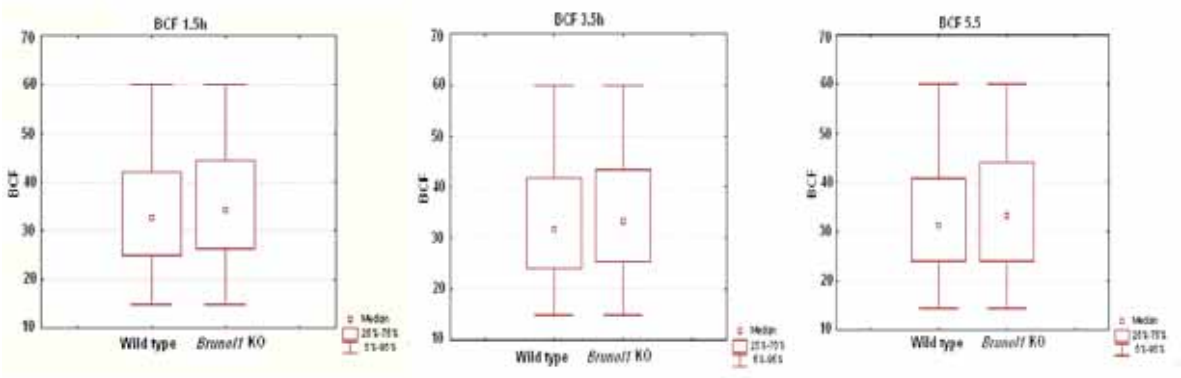
Straight Line Velocity (VSL)



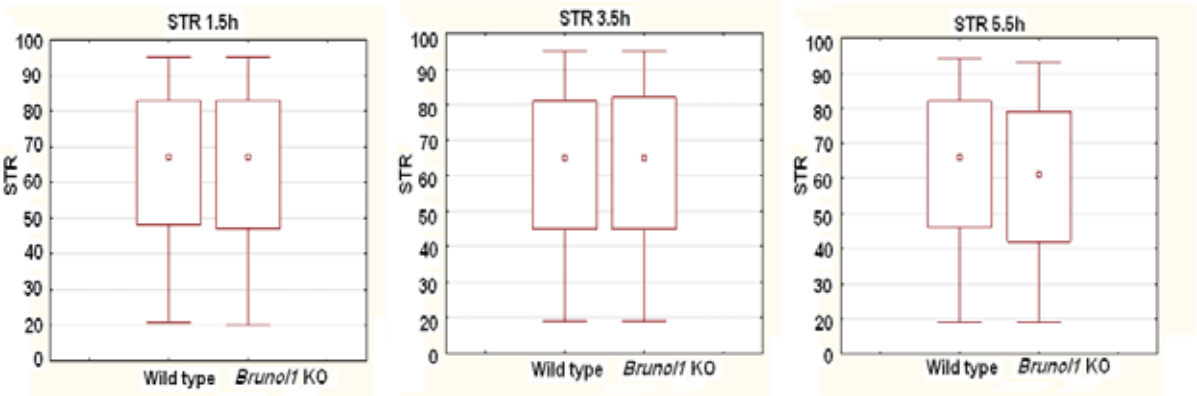
Curvilinear Velocity



Beat Frequency (BCF)



Straight Forward Movement



Lateral Head Amplitude

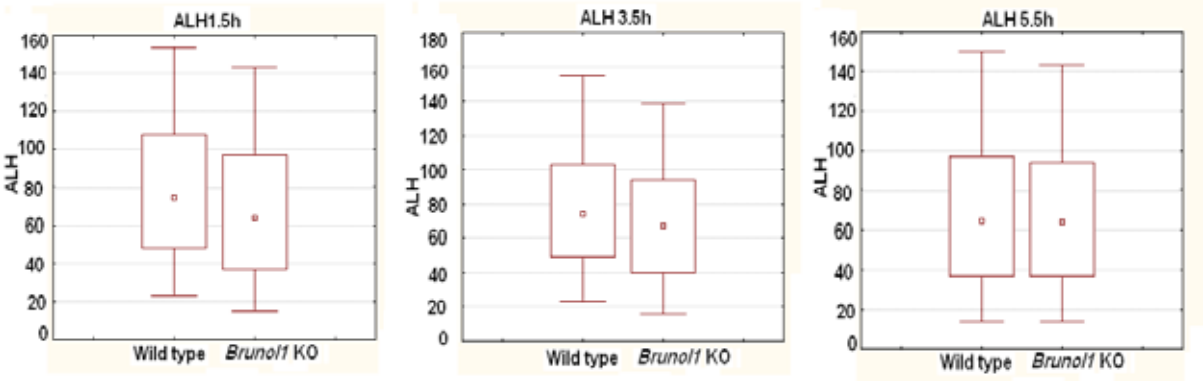


Figure 3.11 Computer assisted analysis of sperm motility. The results of analysis of wild type and knockout spermatozoa on C57 BL/6J x 129/Sv and 129/Sv backgrounds are shown. Sperm velocities (micrometers/second), forward movement (percent), lateral amplitude of the sperm head (micrometers) and beat frequency (hertz) were measured after 1.5, 3.5, and 5.5 hrs. The means and appropriate standard deviations for each parameter are shown. The *Brunol1*-deficient spermatozoa exhibit statistically significant reduction in all parameters, as compared to wild-type sperm, as it was shown by Mann – Whitney U Test ($p < 0.001$). Average Path Velocity (VAP), Straight Line Velocity (VSL), Curvilinear Velocity (VCL), Beat Frequency (BCF) and Lateral Head Amplitude (ALH) , Straight Forward Movement (STR).

3.1.3.5 Histological analysis of *Brunol1* deficient males testes

Testes of *Brunol1*$^{-/-}$ and *Brunol1*$^{+/-}$ males were of normal size and weight. No abnormalities were noticed in their shape. Testes of three *Brunol1*$^{-/-}$ and two wild type males were fixed in Bouin's solution; paraffin sections were prepared and stained with hematoxylin/eosin, as it was described in section 2.2.14. Histological sections of homozygous male testes on C57 BL/6J x 129/Sv and 129/Sv (Fig 3.12A & 3.12B) backgrounds revealed normal spermatogenesis; no difference in cellular type or cell number was observed in *Brunol1* mutant mice as compared to wild type.

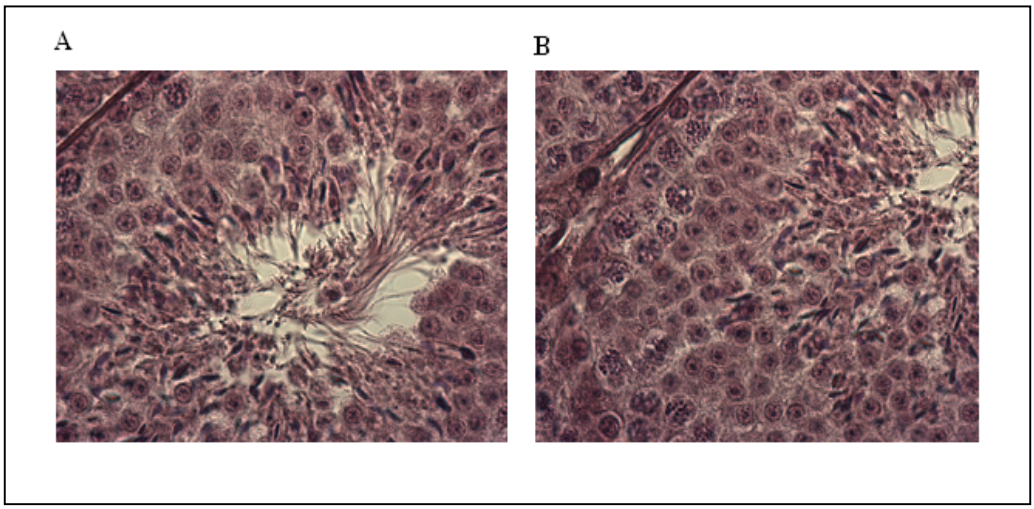


Figure 3.12 Histological sections of testes of *Brunol1* mutant males. Paraffin sections of testes of *Brunol1* deficient mice reveal normal spermatogenesis. No difference in cellular type or cell number was observed in *Brunol1* knockout mice as compared to wild type. Section of testes of *Brunol1* deficient male of (A) C 57 Bl/6J x 129/Sv background and (B) 129/Sv background.

3.1.3.6 Expression analysis of *Brunol6* by Northern blot in *Brunol1* deficient mice testis

In order to determine whether the expression of the *Brunol6* (a member of Elav/Bruno family expressed in testis) is increased in testis of the *Brunol1* $^{-/-}$ mice, we performed Northern blot analysis on total testicular RNA isolated from the *Brunol1* $^{+/+}$, *Brunol1* $^{+/-}$ and *Brunol1* $^{-/-}$ mice by using 500 bp long 3´UTR of *Brunol6* cDNA as probe. Same expression of *Brunol6* was detected in all RNA samples and level of expression in the *Brunol1* $^{-/-}$ was not significantly different from that of the *Brunol1* $^{+/+}$ mice (Figure.3.13). Integrity of RNA of Northern blot was shown by 28S and 18S RNA signals on Northern gel picture.

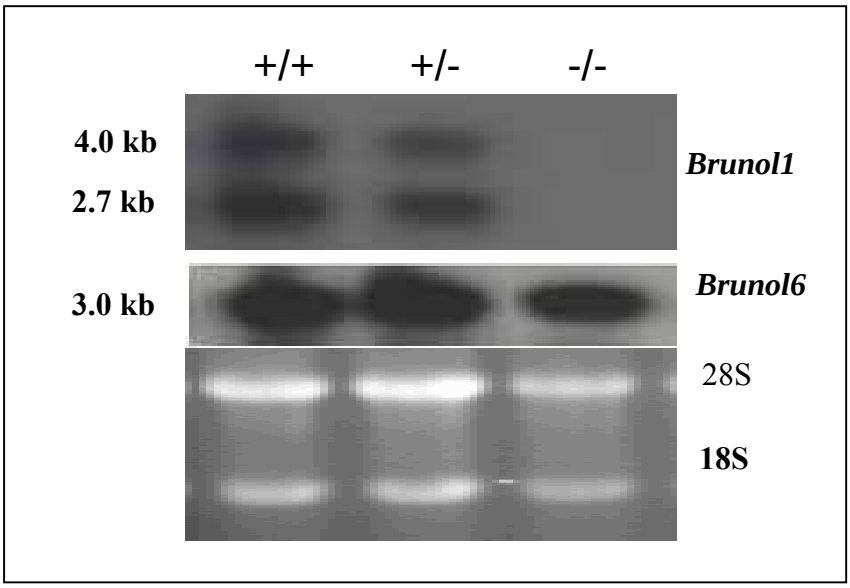


Figure 3.13 Testicular total RNA of the *Brunol1* $^{+/+}$, *Brunol1* $^{+/-}$ and *Brunol1* $^{-/-}$ mice was hybridized with ^{32}P-labeled 500 bp long 3´UTR of *Brunol1* cDNA. Strong expression of *Brunol1* can be seen in *Brunol1* $^{+/+}$ and *Brunol1* $^{+/-}$ but not in *Brunol1* $^{-/-}$ mice. Northern blot analysis with total RNA from testes of *Brunol1* $^{+/+}$, *Brunol1* $^{+/-}$ and *Brunol1* $^{-/-}$ mice using 600 bp long 3´UTR of *Brunol6* cDNA as a probe showed no

significant difference in *Brunol6* expression between *Brunol1* $^{+/+}$, *Brunol1* $^{+/-}$ and *Brunol1* $^{-/-}$ mice.

3.1.3 Histological analysis of brain of *Brunol1* deficient mice

Macroscopic inspection of *Brunol1* deficient mice brain showed a normal brain size. Histological section of neuronal tissue derived from *Brunol1* deficient mice were made by Prof Schwegler at the Institute of Anatomy University of Madeburg. When paraffin embedded brain sagittal sections were stained with crystal violet and observed under microscope, all morphological subdivisions of brain were observed. No difference in cellular type or cell number was observed in *Brunol1* mutant mice as compared to wild type. (Fig 3.14)

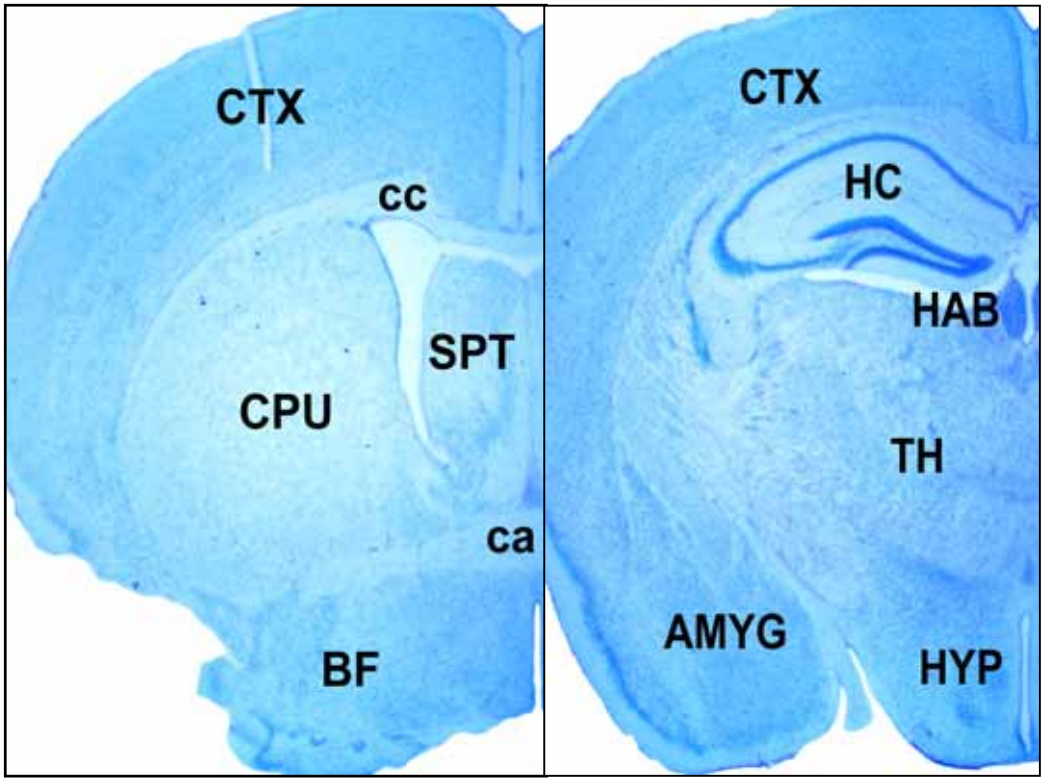


Figure 3.14 Nuclear staining of neuronal tissue derived from *Brunol1* deficient mice. Abbrevations are CTX, Cortex; HC, Hippocampus; HAB, Habenula; TH, Thalamus; HYP, Hypothalamus; AMYG, Amygdala; cc, Corpus callosum; CPU, tamen/Nucleus caudatus = Striatum; SPT, Septum; ca, Commissura anterior; BF, Forebrain.

3.2 Isolation and characterization of the *Brunol4* cDNA

Brunol4 gene (AC010381) was cloned and characterized by Meins et al. (2002). Murine *Brunol4* is a novel gene closely related to elav-type family of genes, which encodes for RNA binding protein. *Brunol4* is localised in mouse near the centromere of chromosome 18. The *Brunol4* cDNA has 13 exons. It encodes a protein consisting of 495 amino acids. The size of *Brunol4* is around 250 kb due to large size of several introns. Like the other member of elav family, Brunol4 protein also has three RNA recognition motifs (RRM) and one variable motif (Fig.3.15A).

A

B

```
   1 atgtatataaagatggccacgttagcaaacggacaggctgacaacgcgagcctcagtacc
  61 aacgggctaggcagcagcccgggcagcgccgggcatatgaacggattaagccacagcccg
 121 gggaacccgtcgaccattcccatgaaggaccacgatgccatcaagctgttcattgggcag
 181 atccccccgaaacctggatgagaaggacctcaagcccctcttcgaggagttcggcaagatc
 241 tacgagcttacggttctgaaggacaggttcacaggcatgcacaaaggctgcgctttcctc
 301 acctactgcgagcgtgagtcagcgctgaaggcccagagcgcgctgcacgagcagaagacc
 361 ctgcccgggatgaaccggccgatccaggtgaagcctgcggacagcgagagccgaggaggt
 421 agtagctgcctgcgccagccccttcacaagatagaaaactcttcgtgggtatgctcaac
 481 aagcaacaatctgaggacgacgtgcgccgcctcttcgaggccttcgggaacatcgaggag
 541 tgcactatcctgcgcgggccggacggcaacagcaaggggtgcgcctttgtgaagtactcc
 601 tcccatgccgaggcacaagccgccattaacgctctacatgcagccagaccatgcctggaa
 661 gcctcctccagcctggtggtcaagtttgcagacactgacaaggagcgcacaatgcgacgg
 721 atgcagcagatggctggccagatgggcatgttcaaccccatggccatccccttcggagcc
 781 tatggcgcctatgctcaggcactgatgcagcagcaagcagccctcatggcatcggtcgcg
 841 caaggaggctacctgaatcccatggctgccttcgctgccgcccaaatgcagcagatggcg
 901 gccctcaacatgaatggcctggcagccgcacctatgaccccaacctcaggtggcagcacc
 961 cctccaggcatcactgcaccagctgtgcctagcatcccatcccccattggggtgaacggc
1021 ttcacgggcctccccccctcaggccaatgggcagcctgctgcggaagctgtgtttgccaat
1081 ggcattcacccctacccagcacagagccccaccgcagccgacccccctgcagcaggcctac
1141 gctggagtgcagcagtatgcaggacctgcctaccctgctgcctatggtcagattagccag
1201 gcctttcctcagccaccgccaatgattccccagcaacagagagaagggcccgagggctgt
1261 aacctgctcatctaccatctgccccaggagtttggggacgctgagctgatgcagatgttc
```

```
1321 ctccctttcggcttcgtgagcttcgacaacccggccagcgcacagaccgccatccaggcc
1381 atgaacggcttccagataggcatgaagaggctcaaggtgcagctgaagcggcccaaagac
1441 gccaatcgcccgtactga
```

Figure 3.15 (A) Schematic representation of Brunol4 protein domain. (B) Nucleotide sequence (from NCBI) of *Brunol4* cDNA. ATG codon, indicating start of translation and TGA terminating codon are bold and coloured blue.

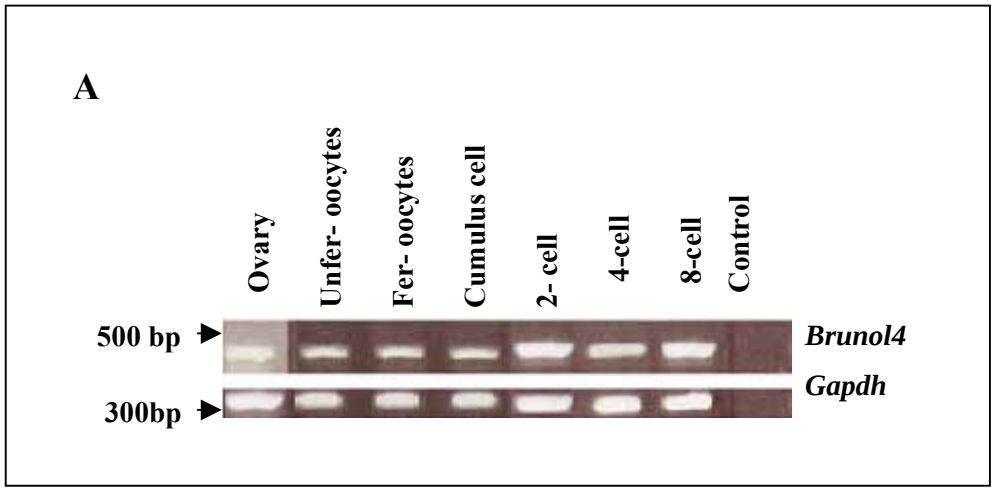
3.2.1 Expression analysis of mouse *Brunol4* gene

3.2.1.1 RT PCR analysis of *Brunol4* during development

To examine the expression of *Brunol4* during development, RT-PCR analysis was performed with RNA range from embryonic stages including preimplantation stages (2, 4 and 8 cell stages) (Fig 3.16 A). *Brunol4* expression was detected as early as two cell stage and continued up to blastocysts stage. In postimplantation stages, *Brunol4* expression is detected as early as 7.5 days post coitum (dpc) and continues to express at all the stages of embryonic development (Fig. 3.16 B).

3.2.1.2 RT PCR analysis of *Brunol4* in adult mouse

To analyse the expression of *Brunol4* in adult mouse at the RNA level, total RNA was isolated from different adult mouse tissues including brain, eye, muscle, kidney, liver, heart, lung, spleen, testis and ovary. By RT-PCR analysis, a *Brunol4* transcript could be detected from samples of brain and ovary, Integrity of the RNA used for RT-PCR. was proved by amplification of the *Gapdh* transcript. (Fig. 3.16 C).



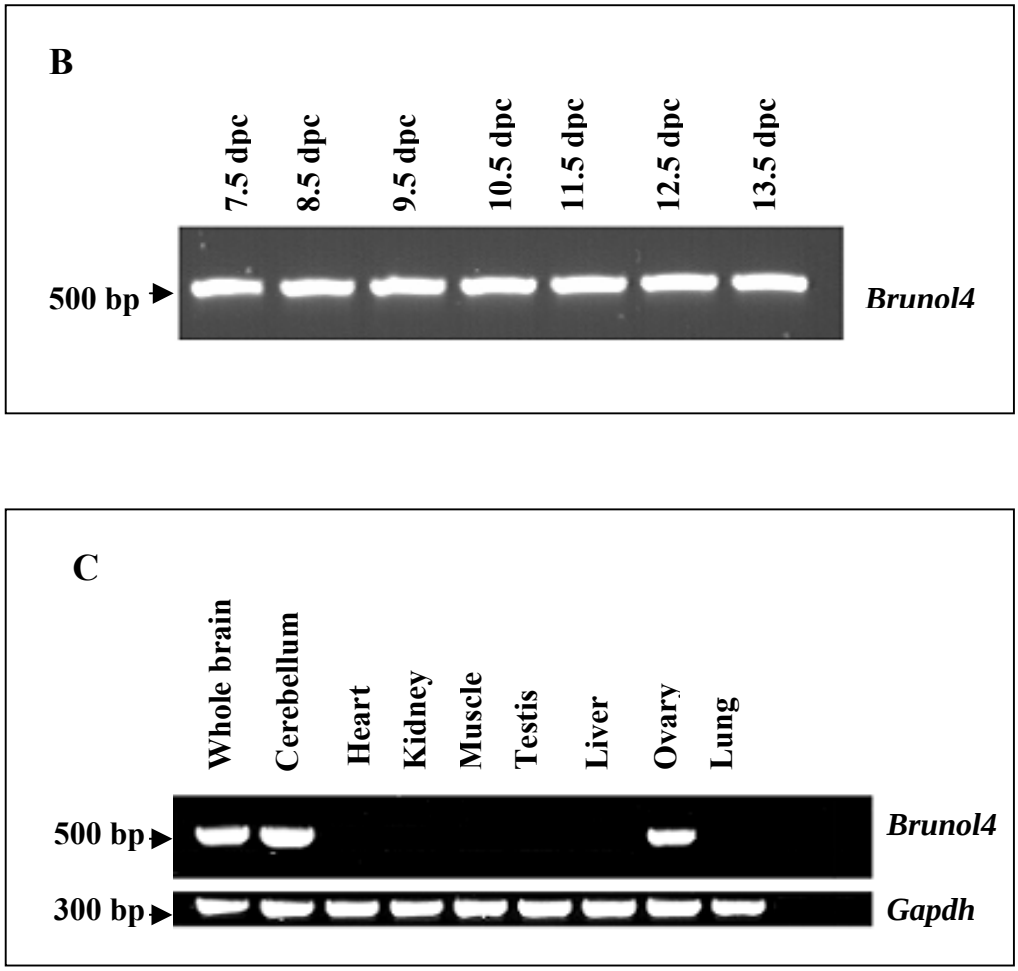


Figure 3.16 RT-PCR expression analysis of *Brunol4* during development by using *Brunol4F* and *Brunol4R* specific primers (A) Preimplantation stages of development. (B) Postimplantation stages of development. (C) Different adult tissues. Expression of *Brunol4* is restricted to brain and ovary. Gapdh served as a control.

3.2.1.3 Northern Blot analysis of *Brunol4* in adult mouse

To determine the expression of *Brunol4* at the Northern blot level, total RNA from heart, lung, muscle, testis, liver, kidney, ovary, cerebellum and whole brain was size fractioned in a 1% agarose/MOPS gel containing 5.5% formaldehyde and blotted onto Hybond-XL membrane. The Northern blot was hybridised with a ^{32}P-labelled 500-bp fragment from the 3′-end of the murine *Brunol4* cDNA probe, which detected approximately 3.0 kb strong hybridisation signal in whole brain sample and cerebellum.

No signal was visible in other adult tissues tested. Integrity of RNA was checked by hybridization with HEF probe (Fig. 3.17).

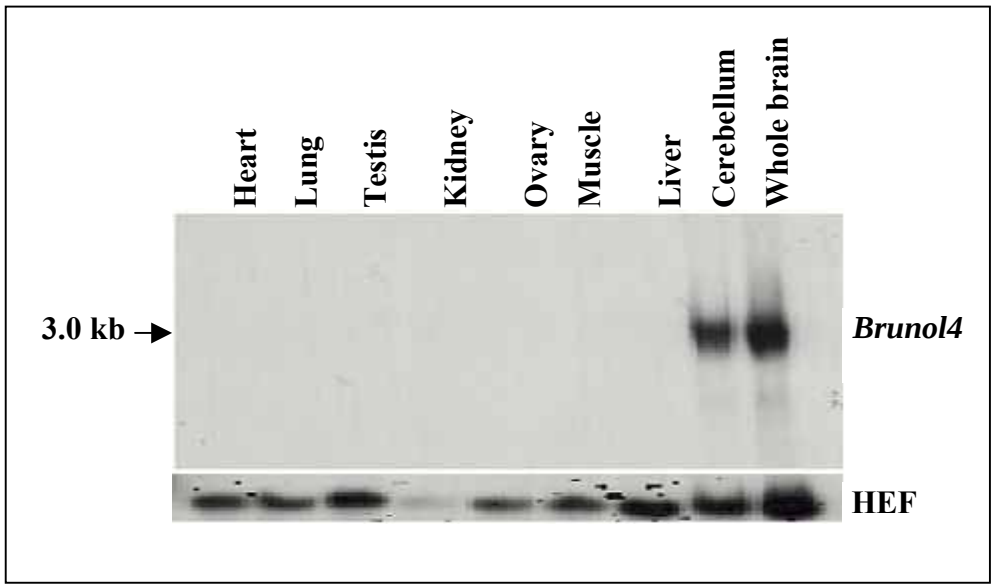


Figure 3.17 Northern Blot analysis of *Brunol4* in different tissues, using 3´ UTR specific probe. Expression of *Brunol4* is restricted to the brain.

3.2.2 Targeted inactivation of mouse *Brunol4* gene

One of the best ways to elucidate gene function is the generation of a knockout animal model. For this purpose, *Brunol4* knockout mouse was generated in this study. Analysis of the phenotype of mice with targeted disruption of *Brunol4* gene could help in understanding the role of the *Brunol4* gene during development.

3.2.2.1 Generation of the *Brunol4* knockout construct

A cosmid clone (MPMGc121D15262Q2) containing the 5´ region of *Brunol4* gene was isolated and characterised by restriction analyses and sequencing reactions (Meins et al., 2002). In order to generate the *Brunol4* targeting vector, a region of 800 bp of the *Brunol4* gene containing part of exon1 and intron1 was replaced by GFP and Neomycin phosphotransferase gene cassette under the control of phosphoglycerate kinase promoter. In this construct, neomycin was used as marker for positive selection while two copies of thymidine kinase from Herpes simplex virus were used as a negative selection marker (Fig 3.18).

3.2.2.2 Subcloning of 5' wing of the *Brunol4* knockout construct into the pBlueScriptII SK vector

The clones that were designated as positive during cosmid library screening were digested with *KpnI* and *SalI* enzyme. A 3.8 kb *KpnI* and *SalI* fragment containing the 5'-flanking region with part of exon1 and intron1 of the *Brunol4* gene (Figure 3.18) was isolated from the cosmid clone (MPMGc121D15262Q2) and purified from agarose gel. This fragment was subcloned into pBlueScript SK vector using the *KpnI* and *SalI* sites. To tag the EGFP open reading frame with 5´wing of *Brunol4* knockout construct, primers were designed having *XhoI* restriction site at the 3´end. The complete ORF of EGFP was amplified and the fully sequenced EGFP PCR product was cloned in to pBlueScriptII SK vector by using the *XhoI*. Finally the 5´ fragment fused with EGFP was digested with *XhoI* and *SalI* from pBlueScriptII *SK+* vector and was cloned into pTKNeo vector.

3.2.2.3 Subcloning of 3' wing of the *Brunol4* knockout construct into the pBlueScriptII SK vector

For the generation of 3' wing of the construct, a 5.3 kb *XbaI* fragment containing a part of intron1 of the *Brunol4* gene (Figure 3.18) was isolated from the cosmid clone (MPMGc121D15262Q2) and purified from the agarose gel. This fragment was subcloned into pBlueScript SK vector using the *XbaI* site. Then the 5.3 kb fragment was again extracted from pBlueScript vector by *SpeI* and *NotI* enzyme and cloned into the pTKNeo vector using the same sites. In order to check positive orientation of both wings in the vector, GFP-1374-F, Bru4-3´GenomicR, Neo-R and Neo-F primers, specific for the construct, were used for sequencing. The construct was linearized at the *PvuI* site present at the backbone of pTKNeo vector and in ampicillin cassette.

3.2.2.4 Generation of the 5' external probe

The 5' probe for screening of ES cells was generated to distinguish between wild type and recombinant clones. For this purpose, a fragment of 900 bp was amplified by PCR, using primers Bru-4-Ext-Probe-F and Bru-4-Ext-Probe-R.The PCR fragment was cloned in pGEM T-Easy vector and sequenced, then digested with *Eco*RI restriction enzyme. This external probe recognizes a 7.6 kb fragment in case of wild type and a 6.0 kb fragment in case of recombinant clone in Southern blot hybridization after digestion of genomic DNA with *AflII* enzyme (Fig 3.18B).

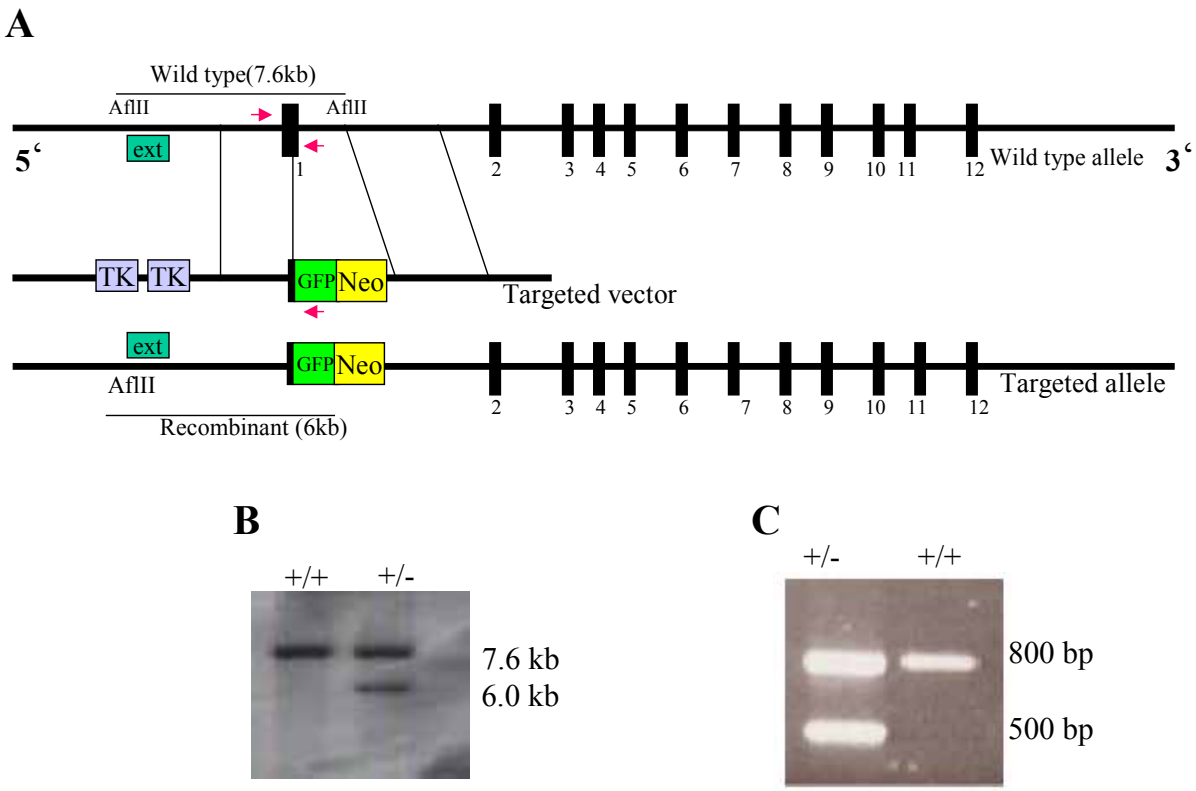


Figure 3.18 Mouse *Brunol4* gene and targeting vector. (A) Upon homologous recombination of the 5´and 3´arm of vector with the *Brunol4* locus (top), a part of exon 1 is replaced by the coding sequence of GFP and Neo cassette. (B) Southern blot analysis of genomic DNA isolated from recombinanat ES cell upon digestion with *AflII*. Wild type (+/+) allele gives rise to a 7.6-kb fragment, while the digested mutant allele (+/-) generates 7.6-kb and 6-kb fragments detected with a 5´external probe. (C) PCR genotyping of wild type (800 bp) and heterozygous (800bp + 500 bp) mutant mice. Red arrows show the location of the primers for genotyping.

3.2.2.5 Electroporation of the ES cells and screening of ES cells for homologous recombination

Brunol4 targeting vector was linearized with *PvuI* enzyme and 50 µg of purified DNA was electroporated into RI embryonic stem cells, as it was described in section 2.2.19.1. Cells were plated on feeder layer and after 10 days of selection 98 clones resistant for neomycin

were selected and cultured on 24 well plates. Genomic DNA was isolated from ES cells, as it was described in section 2.2.1.4, and used for Southern blot hybridization. DNA from each clone was digested with *Afll* II enzyme, electrophoresed and blotted onto Hybond-XL membrane. Blots were then hybridized with radioactively labelled 5´external probe. Two bands were recognized in case of homologous recombination, 7.6 kb wild type allele and 6.0 kb recombinant allele. When no recombination has occurred or non-homologous recombination had taken place, only wild type band could be detected (Fig.3.18 B). Total 90 clones were screened, out of 400 clones only one of them was recombinant, these results were confirmed, using GFP probe, which detected 6.0 kb expected recombinant band. Clone No. 55, rehybridized with external probe again and was chosen for blastocyst injection.

3.2.2.6 Generation of chimeric mice

The recombinant ES cells were injected into 3.5 dpc blastocysts derived from C57BL/6J mice. The blastocysts were implanted into the pseudopregnant CD1 mice to generate the chimeric mice. Total 4 chimeras were obtained by three independent injections of recombinant ES clones. The chimeras were scored according to the coat color (in percentage), 5%, 2x15% and 25%. All chimeras were bred with C57BL/6J mice, to obtain F1 animals in C57BL/6J background. Germ line transmission was detectable only by one 25% chimera after one and half month of breeding. The germ line transmission of the mutant allele was verified by genomic PCR by using Bruno4-Geno-F, Bru-4 Del-R and GFP-1405-R primers on DNA isolated from tail biopsies of the offspring (Fig 3.18 C).

3.2.2.7 Generation and analysis of *Brunol4* knock out mice

F1 heterozygotes were intercrossed to produce the homozygous offspring. No change in the sex ratio of *Brunol4* mice for the offspring of the heterozygous matings was noted in C57BL/6J background. Surprisingly, no homozygous (*Brunol4*$^{-/-}$) mice were notified out of 255 offspring, derived from the heterozygous intercrosses. In addition, there seemed to be no reduction in litter size (7.5) in comparison to matings of wildtype animals. The ratio of wildtype to heterozygotes was approximately 1: 3 and among the 255 live-born offspring, 90 were wildtype and 165 were heterozygous mice in the C57BL/6J x129/Sv genetic background (Table 3.6)

C57BL/6J x 129/Sv	Female	Male
WT	49 = 54.5%	41 = 45.5%
	90 = 35%	
HE	94=56%	71= 43%
	165 = 65%	
HO	00 = 00 %	00= 00%
	00 = 00 %	
	143 = 56 %	112 = 45 %

Table 3.6 Result of the genotype analysis of the F2 generation in C57BL/6J x 129/Sv background. Abbreviations are WT (wild type), HE (heterozygous) and HO (homozygous).

3.2.3 Determination of the stage of embryonic death of *Brunol4* homozygous mice

While *Brunol4* heterozygous mice were apparently healthy and fertile, no *Brunol4* homozygous mice were identified. This result revealed that homozygous embryos might be lethal. To determine the stage when development of homozygous embryos is arrested, a detailed analysis of embryos at different stages of development was done including pre- and post-implantation stages. To analyse the post-implantation stages of embryonic death, *Brunol4+/-* mice were mated and the pregnant mice were sacrificed at 13.5 dpc. Among 31 embryos obtained, 10 were wild type and 21 were *Brunol4*$^{+/-}$, but no *Brunol4*$^{-/-}$ mice were obtained. We also examined 10.5 and 8.5 dpc but none of embryo was homozygous (Table 3.7). To examine the embryos at 2-cell (E1.5) stage, 4-cell (E2.5) stage, 8-cell (E3.0) stage and blastocyst stage (E3.5), *Brunol4*$^{+/-}$ mice were mated and the embryos were collected at two-cell stage (E1.5). To avoid any maternal contamination, embryos were cultured with the M16 medium (Sigma) to 4-cell stage (E2.5) and 8-cell stage (E3.0). From individual 2, 4 and 8-cell stage embryos, zona pellucida was dissolved and polar bodies were removed,

before genotyping (Figure 3.19). Embryos were individually photographed and transferred directly to PCR cups and subjected to PCR. Genotyping results of embryos showed wild type and heterozygous embryos but none of them was homozygous (Table 3.7). Further we analysed one-cell stage embryos. To genotype the embryos at one cell stage, individual 1-cell embryos were microscopically analysed to differentiate between fertilized and unfertilised embryos. From individual one-cell embryos, zona pellucida was dissolved and polar bodies were removed, before genotyping (Figure 3.19). We genotyped 61 one-cell stage embryos, all of them were heterozygous (Table 3.7) (Figure 3.20). To genotype the blastocysts, embryos at E3.5 were flushed out from the uteri of *Brunol4*$^{+/-}$ female mice and mated with *Brunol4*$^{+/-}$ males. To avoid any maternal contamination, blastocysts were cultured for six days in ES cell medium containing leukaemia inhibitory factor (LIF). Under these conditions, embryos hatched from their zona pellucida and attached to the gelatine-coated dishes. All the blastocysts were morphologically normal and indistinguishable from wild type (Figure 3.21). Among the 52 blastocysts genotyped, 15 were wild type and 37 were heterozygous (Table.3.7)

Embryonic development stage	**Genotyping**		
	+/+	+/-	-/-
13.5 dpc	**10**	**21**	**00**
10.5 dpc	**07**	**20**	**00**
8.5 dpc	**15**	**24**	**00**
E 3.5 (Blastocysts)	**15**	**37**	**00**
8-cell stage	**11**	**37**	**00**
4-cell stage	**15**	**77**	**00**
2 cell stage	**37**	**151**	**00**
1 cell stage	**00**	**61**	**00**

Table 3.7 Genotyping of embryos obtained from intercroses between *Brunol4*$^{+/-}$ mice. Embryos were examined by PCR analysis at different stages of embryonic development, 13.5, 10.5, 8.5, 3.5 dpc, 8-cell stage, 4-cell stage, 2-cell stage and 1-cell stage. Wild type (+/+) and heterozygous (+/-) embryos were obtained from *Brunol4*+/- intercroses but none of them was homozygous (-/-)

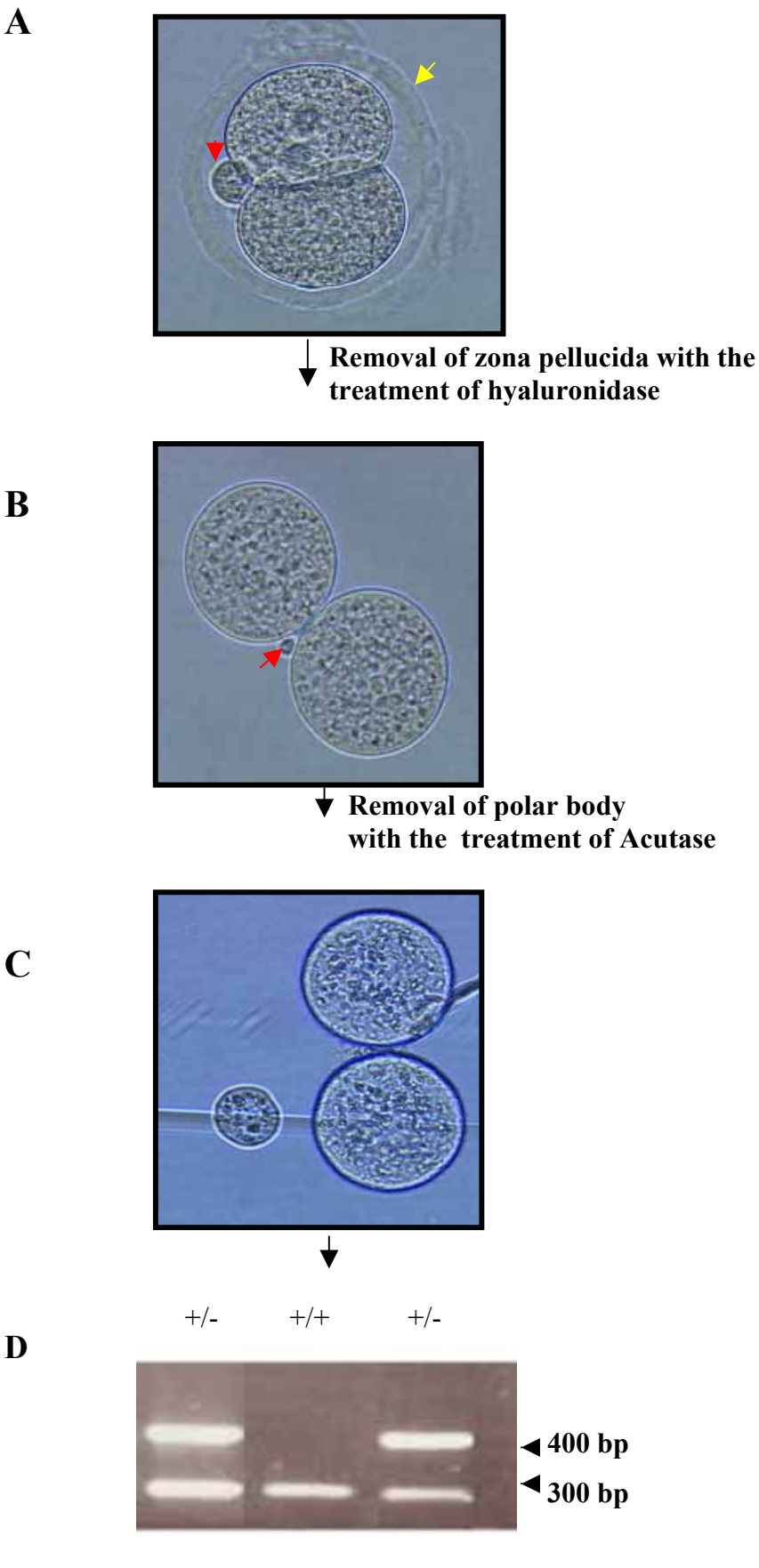

A
Removal of zona pellucida with the
treatment of hyaluronidase
B
Removal of polar body
with the treatment of Acutase
C
+/-
+/+
+/-
D
400 bp
300 bp

Figure 3.19 Genotyping of two-cell stage embryos. (A) 2-cell stage embryo with polar body and zona pellucida. (B) 2-cell stage embryo after removal of zona pellucida. (C) 2-cell stage embryo after removal of polar body. (D) PCR results of 2-cell stage embryos genotyping, wild type (400bp) and heterozygous (400bp + 300 bp). Red arrows represents the polar body, yellow arrow represents the zona pellucida.

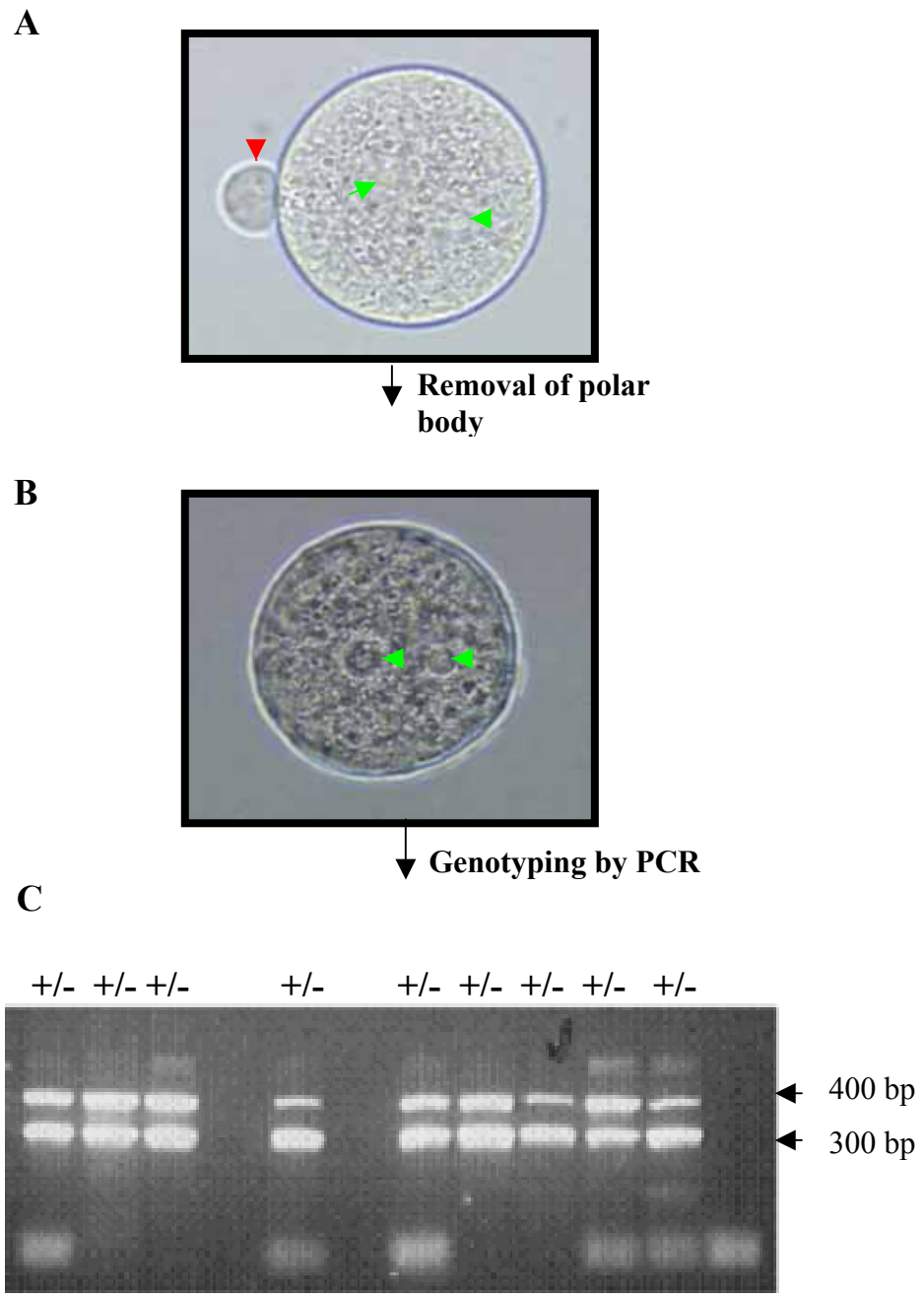


Figure 3.20 Genotyping of one-cell stage embryos. (A) One-cell stage embryo with maternal and paternal nucleus. (B) One-cell stage embryo after removal of polar body. (C) PCR results of one-cell stage embryos genotyping. Green arrows represents the maternal and paternal nuclei, red arrow represents the polar body.

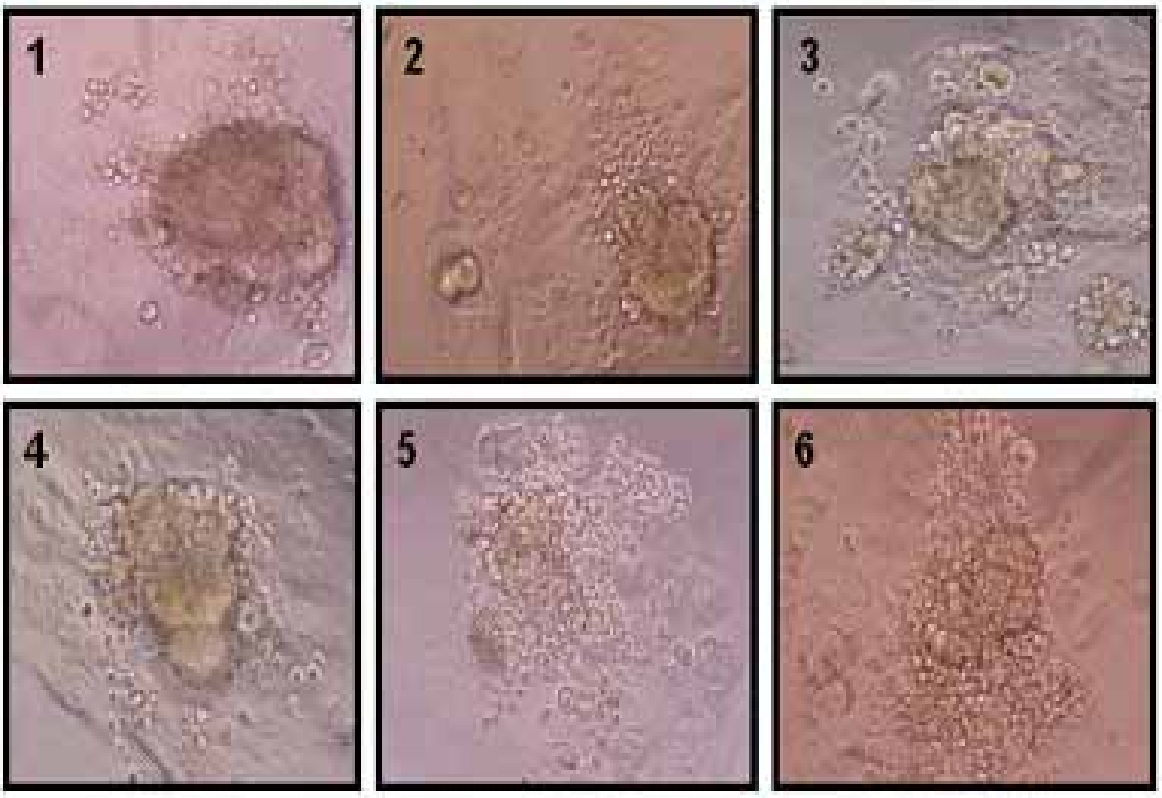


Figure 3.21 *In vitro* outgrowth of cultured blastocyst (3.5 + 6). Isolated blastocysts were grown *in vitro*. Representative heterozygous blastocysts (1,2,3,4,5) and wild type blastocyst (6) is shown.

3.2.4 Murine *Brunol4* is present in more than one copy

Embryological analysis of *Brunol4*$^{+/-}$ mating showed that there were wild type and heterozygous embryos but homozygous embryos were selectively missing even at one cell stage (Table 3.7). This result suggested that there might be two copies/duplication of *Brunol4* gene. *Brunol4* homozygous mutant was not identified by genotyping (PCR and Southern Blot). Then, 10 *Brunol4* heterozygous mice were bred with wild type and four pairs gave all heterozygous offspring (Table 3.8). These results clearly indicated that there are in fact *Brunol4* homozygous mice among heterozygous but they could not be identified

by genotyping. Further, these homozygous mice were analysed by Northern Blot analysis but there was no reduction in *Brunol4* transcript compared to wild type (Figure 3.22) Taken together these observations suggest that there are two copies of *Brunol4* gene and the one, which is not transcribed, was deleted.

Parents		**Offspring**
36♂	WT ♀	HE & WT
37♂	WT ♀	HE & WT
39♂	WT ♀	HE
92♂	WT ♀	HE & WT
93♂	WT ♀	HE & WT
45♀	WT ♂	HE & WT
49♀	WT ♂	HE
53♀	WT ♂	HE & WT
77♀	WT ♂	HE
83♀	WT ♂	HE

Table 3.8. Result of breeding of *Brunol4*$^{+/-}$ with wild type. Ten *Brunol4*$^{+/-}$ mice (36♂, 37♂, 39♂, 92♂, 93♀, 45♀, 49♀, 53♀, 77♀ and 83♀) were bred with wild type mice. Out of 10 breeding pairs, 4 pairs (93♂, 49♂, 53 ♀ and 77♀) gave only heterozygous offspring.

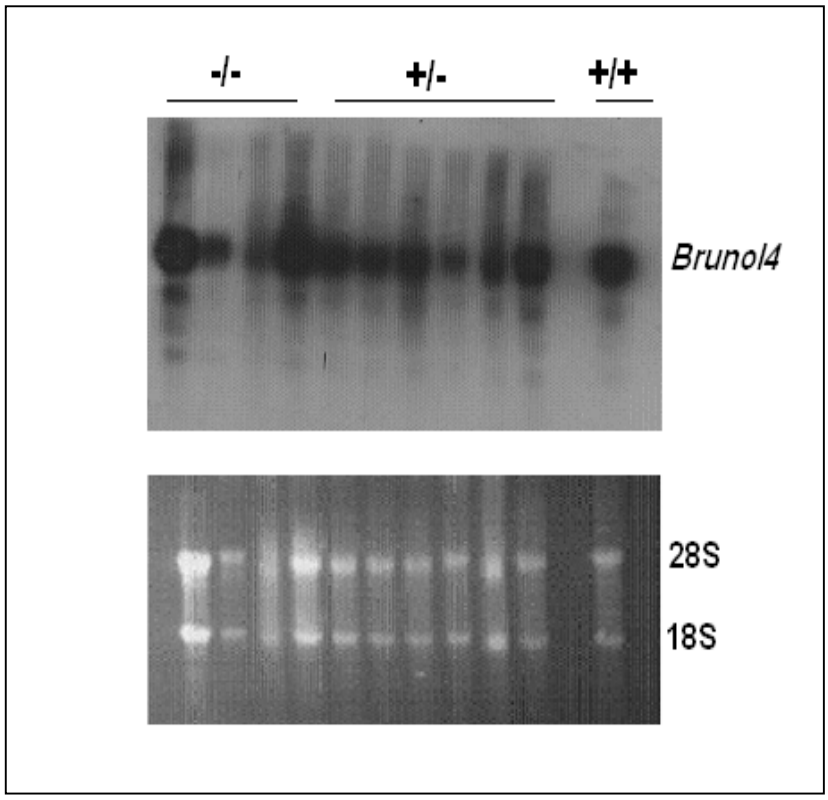


Figure 3.22 Northern blot analysis with total RNA of brain of different *Brunol4* heterozygous, so-called homozygous and wild type mice by using 3´ UTR specific probe. There was no reduction of *Brunol4* transcript in *Brunol4* homozygous and heterozygous as compared to wild type mice.

3.3 Isolation of the *Brunol6* cDNA

Brunol6 gene (accession number BC052406) is a novel member of Elav/ Bruno family, encoding RNA binding protein. To obtain the actual coding region of *Brunol6*, full-length *Brunol6* ORF was amplified from mouse brain cDNA by PCR using the primers that contained the predicted start and stop codon. *Brunol6* has 13 exons. It encodes a protein consisting of 460 amino acids. *Brunol6* is localised on mouse chromosome 9. As the other Brunol family members, the domain structure of *Brunol6* is also the same, having three RNA recognition motifs (RRMs) and a divergent domain (linker) separating RRM2 and RRM3 (Fig 3.23 A)

A

B

```
  1 atggccgcggcgcctggagggtctgcgccgcccgccggccccagc
    M  A  A  A  P  G  G  S  A  P  P  A  G  P  S
 46 ccgcgcttggctttcagcaccgcggacagcggcggcggcatgagc
    P  R  L  A  F  S  T  A  D  S  G  G  G  M  S
 91 gggctgaacccaggtcccgcggtgcccatgaaggaccacgacgcc
    G  L  N  P  G  P  A  V  P  M  K  D  H  D  A
136 atcaagctcttcgtggggcagatcccgcggggcttggacgagcag
    I  K  L  F  V  G  Q  I  P  R  G  L  D  E  Q
181 gacctcaagccgctgttcgaggagttcggccgcatctacgagctg
    D  L  K  P  L  F  E  E  F  G  R  I  Y  E  L
226 acggtgctgaaggaccggctcaccggcctccacaaaggctgtgcc
    T  V  L  K  D  R  L  T  G  L  H  K  G  C  A
271 ttcctcacctactgcgcccgggactctgccctcaaggcccagagt
    F  L  T  Y  C  A  R  D  S  A  L  K  A  Q  S
316 gcactgcatgagcagaagaccctgccagggatgaatcgtccgatc
    A  L  H  E  Q  K  T  L  P  G  M  N  R  P  I
361 caagtgaagccggctgccagtgagggccgaggagaggaccggaag
    Q  V  K  P  A  A  S  E  G  R  G  E  D  R  K
406 ctgtttgtggggatgttgggcaagcagcagggtgaggaggatgtc
    L  F  V  G  M  L  G  K  Q  Q  G  E  E  D  V
451 agacgtctgttccagcccttcggccatatcgaggagtgcactgtc
    R  R  L  F  Q  P  F  G  H  I  E  E  C  T  V
496 ctgcggagtccggacggtaccagtaaaggctgtgcctttgtgaaa
    L  R  S  P  D  G  T  S  K  G  C  A  F  V  K
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    G  S  R  T  M  T  G  A  S  S  S  L  V  V  K
```

```
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     L  A  D  T  D  R  E  R  A  L  R  R  M  Q  Q
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     M  A  G  Q  L  G  A  F  H  P  A  P  L  P  L
 721 ggggcctgtggcgcctataccactgcgatcctacagcaccaggca
     G  A  C  G  A  Y  T  T  A  I  L  Q  H  Q  A
 766 gcgttgctggccgcagcgcagggtccggggttaggccaggtggcc
     A  L  L  A  A  A  Q  G  P  G  L  G  Q  V  A
 811 gcggtggccgcccagatgcagcacgtggcggccttcagcttggtg
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     N  G  P  G  A  L  P  G  L  P  A  P  M  G  V
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     N  G  F  G  S  L  T  P  Q  S  N  G  Q  P  G
 991 tccgacacgctctataacaacggggtttcccccttacccagcagcc
     S  D  T  L  Y  N  N  G  V  S  P  Y  P  A  A
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     Y  P  S  A  Y  A  P  A  S  T  A  F  S  Q  Q
1081 ccttcagctctgcctcaacaacaaagagaaggccccgaaggctgt
     P  S  A  L  P  Q  Q  Q  R  E  G  P  E  G  C
1126 aacctcttcatctatcacctgcctcaggagtttggggatgcagaa
     N  L  F  I  Y  H  L  P  Q  E  F  G  D  A  E
1171 ctcatacagacattcctgcccttcggagctgttgtctctgccaaa
     L  I  Q  T  F  L  P  F  G  A  V  V  S  A  K
1216 gtttttgtggaccgtgccaccaatcagagcaagtgttttgggttt
     V  F  V  D  R  A  T  N  Q  S  K  C  F  G  F
1261 gttagttttgacaatccaaccagtgcccagaccgccatccaggcc
     V  S  F  D  N  P  T  S  A  Q  T  A  I  Q  A
1306 atgaatggctttcaaatcggcatgaagaggctcaaggtccagcta
     M N  G  F  Q  I  G  M  K  R  L  K  V  Q  L
1351 aagagacctaaggatgccaacaggccttactga 1383
     K  R  P  K  D  A  N  R  P  Y  *
```

Figure 3.23 (A) Schematic representation of Brunol6 protein domain. (B) Nucleotide sequence (from NCBI) of *Brunol6* cDNA. ATG codon, indicating start of translation and TGA terminating codon are bold and coloured in blue.

3.3.1. Expression analysis of mouse *Brunol6* gene

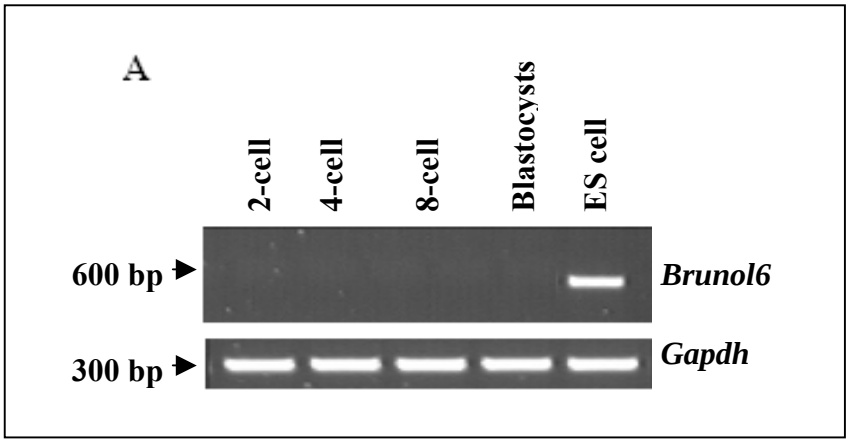
3.3.1.1. Expression analysis of *Brunol6* gene during embryonic development

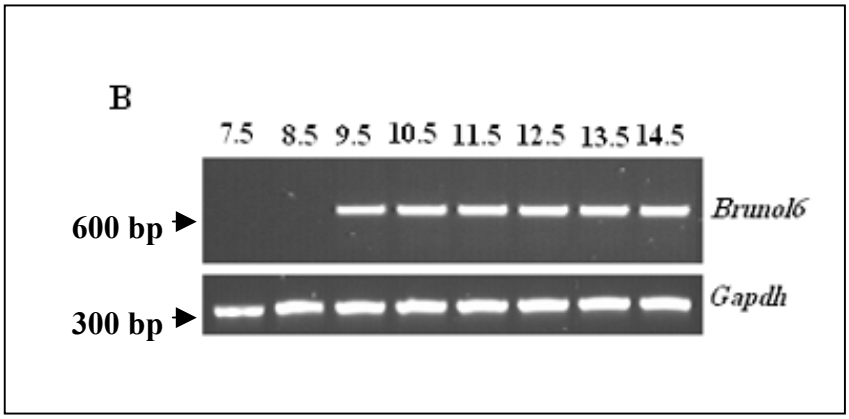
To examine the expression of *Brunol6* gene during embryonic development, RT-PCR analysis was performed on RNA range of embryonic stages. *Brunol6* expression was detected as early as 9.5 days post coitum (dpc) stages (Fig. 3.24 B). Interestingly, expression of *Brunol6* was detected in ES cells, but not in early preimplantation stages; 2

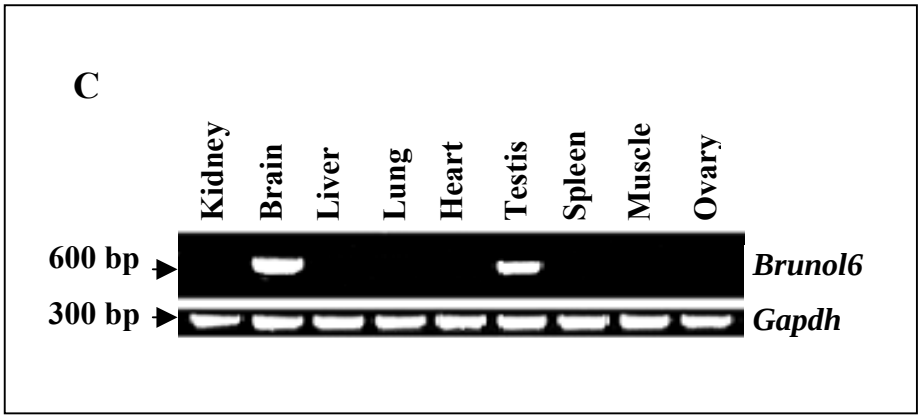
cell, 4-cell and 8-cell stages (Fig 3.24 A). Taken together, these data indicate that *Brunol6* gene expression starts at 9.5 dpc and is also observed in embryonic stem cells.

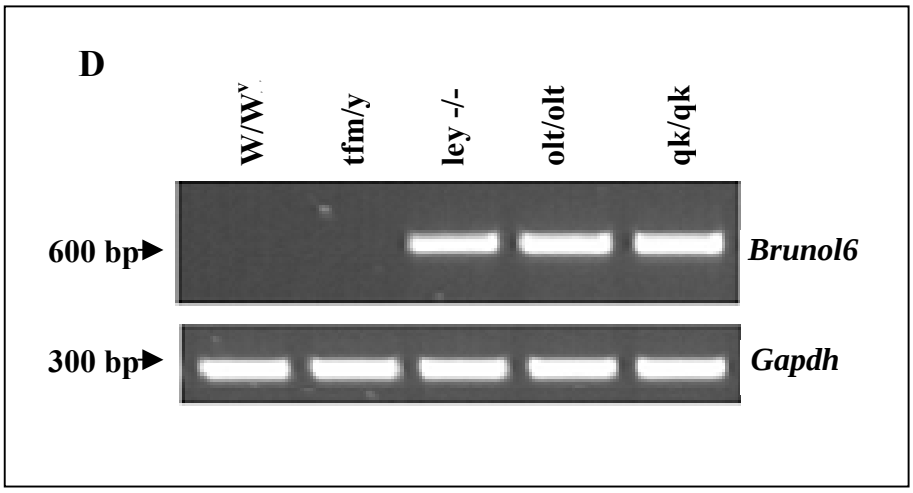
3.3.1.2. RT- PCR analysis of *Brunol6* in adult mouse

To analyse expression of *Brunol6* in adult mouse at the RNA level, total RNA was isolated from multiple adult mouse tissues including brain, heart, liver, lung, testis, ovary, muscle and kidney. By RT-PCR analysis, *Brunol6* transcript could be detected from RNA samples of brain and testis. Integrity of the RNA used for RT-PCR was proven by amplification of the *Gapdh* transcript (Figure 3.24 C). RT-PCR was done also with RNA from testes of different mutants with spermatogenesis defects. *Brunol6* transcript was detectable in Leyl-/-, olt/olt and qk/qk mutant but not in W/W^V and Tfm/y mutants (Figure 3.24 D). This indicates that expression starts as early as in spermatocyte stage. Because there is no expression in W/W^V and Tfm/y mutants, it can be concluded that expression of the gene is restricted to germ cells. Expression studies were also done in postnatal stages. *Brunol6* transcript was detectable from day P20 to P40 in all tested postnatal stages (Figure 3.24 E).






C
Kidney
Brain
Liver
Lung
Heart
Testis
Spleen
Muscle
Ovary
600 bp
300 bp
Brunol6
Gapdh


D
W/W^v
tfm/y
ley -/-
olt/olt
qk/qk
600 bp
300 bp
Brunol6
Gapdh

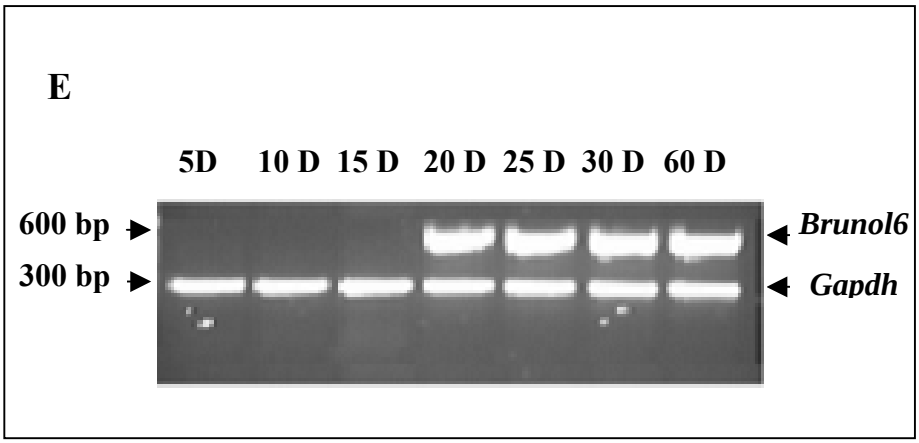

E
5D
10 D
15 D
20 D
25 D
30 D
60 D
600 bp
300 bp
Brunol6
Gapdh

Figure 3.24 RT-PCR expression analysis of *Brunol6,* (A) In preimplantation stages of development, expression of *Brunol6* was detected in ES cells but not in 2, 4 and 8-cell stages. (B) During embryonic development *Brunol6* gene expression starts at least at 9.5 dpc (C) In different adult tissues, *Brunol6* expression is restricted to the brain and testis, (D) In testes of mutants, expression of *Brunol6* is observed in testes of all mutants except W/W^{V} and Tfm/y indicating that expression is restricted to germ cells. (E) In postnatal testicular developmental stages, *Brunol6* expression is present from P20 onward and was observed in testes of all later stages (P20 to P40).

3.3.1.3 Northern Blot analysis of *Brunol6* in adult mouse

To determine the expression of *Brunol6* at the Northern blot level, total RNA from brain, liver, lung, muscle, heart, kidney, ovary and testis was size fractionated in an 1 % agarose/MOPS gel containing 5.5 % formaldehyde and blotted onto Hybond-XL membrane. The Northern blot was hybridised with a ^{32}P-labelled 500 bp fragment from the 3´ UTR of the murine *Brunol6* cDNA probe, which detected approximately 3.0 kb strong hybridisation signal in whole brain sample and testis. No signal was visible in other adult tissues (Fig 3.25).

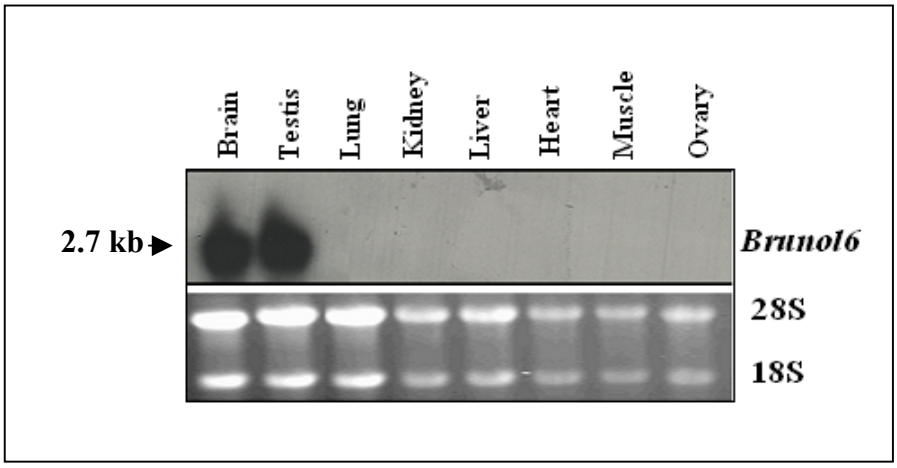


Figure 3.25. Northern Blot analysis of *Brunol6* gene in different adult mouse tissues. Total RNA (20 µg) was extracted from various tissues including muscle, kidney, ovary, lung, liver, heart, brain and testis of the adult mouse and subjected to Northern Blot hybridization using the 500 bp fragment from the 3´ UTR of *Brunol6* as a probe.

3.4 Study of the activation of the *Brunol4* gene in preimplantation development

3.4.1 Expression study of *Brunol4* during embryonic development

Expression analysis of *Brunol4* during embryonic development showed that expression of *Brunol4* starts at one cell stage and continue to all the stages of embryonic development including 2-, 4-, 8-cell stages and blastocysts (Fig 3.16 A). It is well known that transcripts are synthesised and stored during oogenesis for later use at various stages of oocytes maturation and early embryonic development. To differentiate between maternal transcripts and newly synthesised transcripts of *Brunol4* at two-cell stage embryo, complete cDNA of *Brunol4* of different mice strains (CD-1, C57 BL, NMRI, 129/Sv and FVB) was sequenced to search for any polymorphism that could differentiate between maternal and paternal transcript. One single nucleotide polymorphism was identified at the 3´UTR of *Brunol4* transcript that could differentiate between C57BL and CD-1 mice strains. This polymorphism results in the restriction site for *PmI1*. By using the *PmI1* restriction enzyme it is possible to differentiate between transcript arising from CD-1 or C57 BL mouse strains (Fig 3.26).

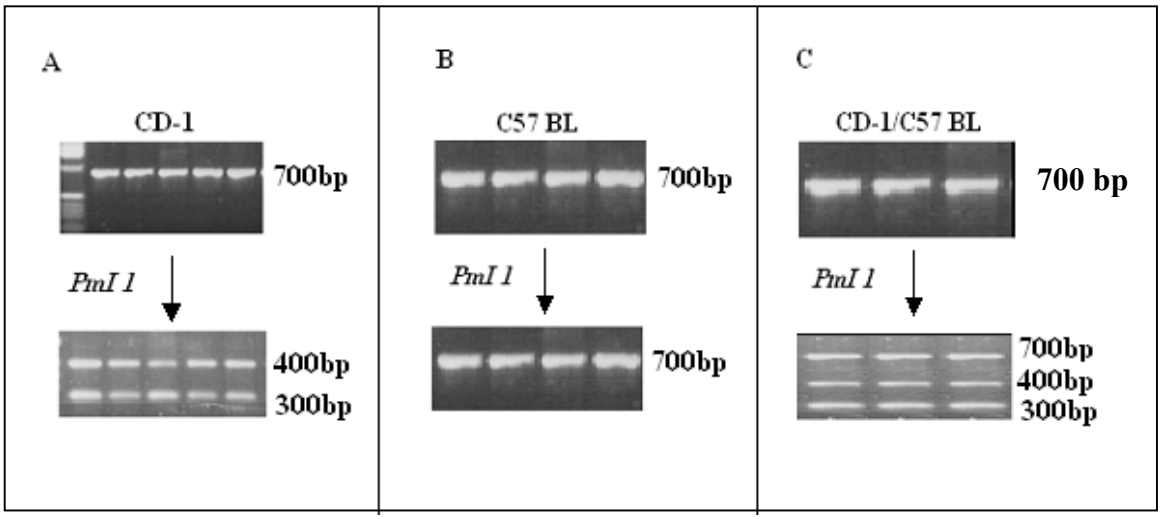


Figure 3.26 Strategy to differentiate between CD-1 and C57BL mouse strains. To differentiate between CD-1 and C57 BL mouse strains, cDNA of CD-1 and C57 BL was subjected to RT- PCR by using Bru4-Exo-13F and Bru4-3´UTR-R primers, which results in a 700 bp long PCR product. (A) When RT-PCR product of CD-1 (700 bp long) was treated with *PmI1* restriction enzyme, it was digested and produced two bands (400 bp and 300 bp). (B) When RT-PCR product (700 bp) of C57BL was

treated with *PmI1* restriction enzyme, it was not digested. (C) When RT-PCR product of CD-1/ C57BL was treated with *PmI1* restriction enzyme, it was digested and produced three bands (700 bp paternal and 400bp + 300bp maternal)

3.4.2 Expression study of *Brunol4* transcript of unfertilised oocytes

To study the expression of *Brunol4* transcript of unfertilised oocytes, we collected 150 unfertilised oocytes from CD-1 female mice. Total RNA was isolated from unfertilised oocytes and directly subjected to RT- PCR by using Bru4-Exo-13F and Bru4-3´UTR-R primers, which resulted in a 700 bp long PCR product (Fig 3.26 A). To further prove our strategy (as described in Fig 3.26) PCR product (700 bp) was digested with *PmI*I restriction enzyme and it resulted in expected 400 bp and 300 bp fragments (Fig 3.27 B).

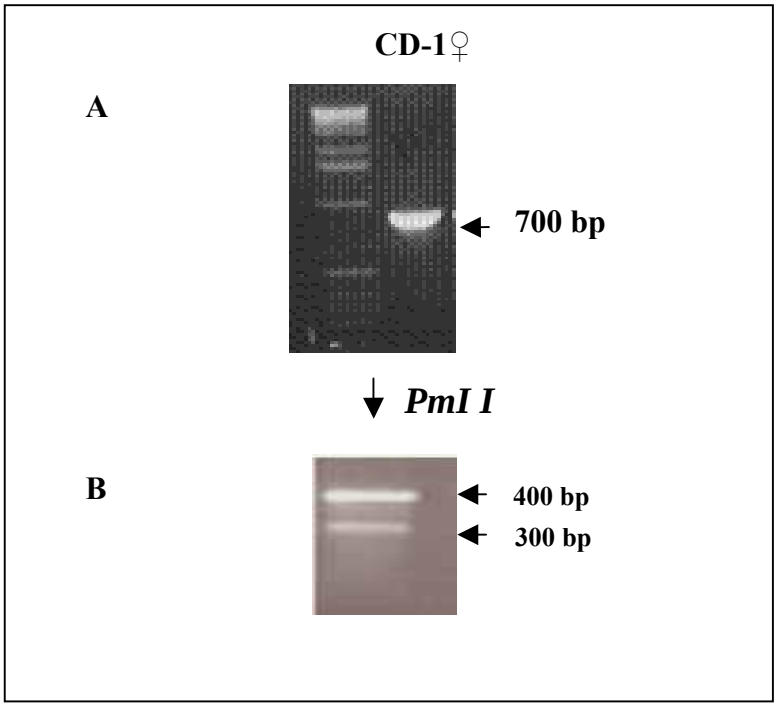


Fig 3.27 Expression study of the *Brunol4* transcript of unfertilised oocytes from CD-1 female mice. (A) RT-PCR product (700 bp) was digested with *PmI*1 restriction enzyme and (B) it resulted in expected 400 bp and 300 bp fragments.

3.4.3 Study of the activation of *Brunol4* gene at two-cell stage

To study the activation of *Brunol4* gene at two-cell stage, CD-1 male mice were mated with C57BL female and the embryos were collected at two-cell stage (E1.5). Total 220 embryos were collected and RNA was isolated. Isolated RNA was subjected to RT-PCR by using Bru4-Exo-13F and Bru4-3´UTR-R primers. PCR product (700 bp) was digested by *PmI1* restriction enzyme to differentiate between maternal and paternal allele. At two-cell stage only maternal allele was found (Fig 3.28). Taken together these observations suggest that at two-cell stage *Brunol4* transcript is only maternal

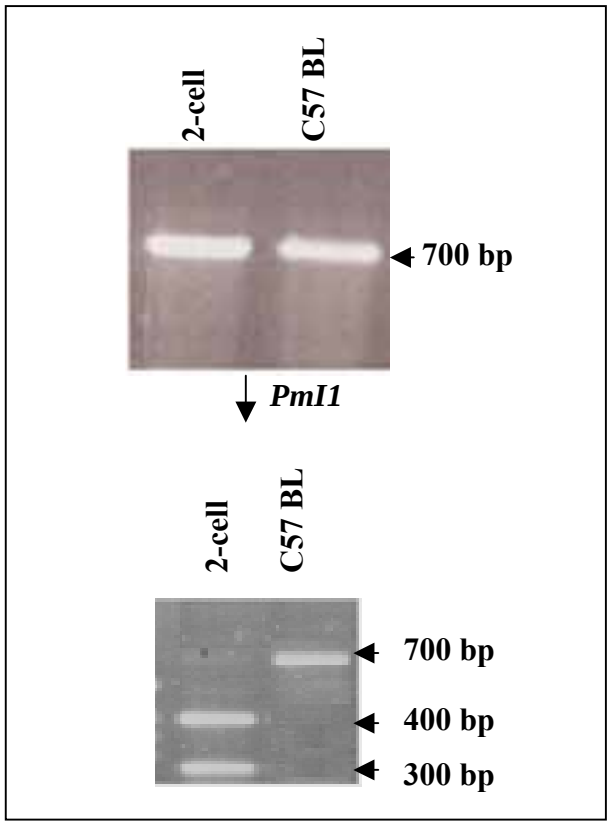


Figure 3.28 Study of the activation of *Brunol4* gene at two-cell stage. (A) To differentiate the maternal and paternal allele at two cell stage of embryonic development, RT-PCR product (700bp) of two cell stage and C57BL adult mouse (as a control) was treated with *PmI1* restriction enzyme (B) When RT-PCR of two cell stage embryos was treated with *PmI1* restriction enzyme it was digested and produced two bands (400bp + 300bp) while the RT-PCR product of C57BL adult mouse (as a control) treated with *PmI1* restriction enzyme was not digested.

3.4.4 Study of the activation of *Brunol4* gene at four- and eight-cell stage

Activation study at two-cell stage showed only the maternally stored transcript of *Brunol4*. To study the activation of *Brunol4* gene at four-cell stage, CD-1 male mice were mated with C57BL female and the embryos were collected at two-cell stage (E1.5). To avoid any maternal contamination, embryos were cultured with the M16 medium (Sigma) to 4 cell (E2.5) and 8-cell (E3.0) stages. Total 190 embryos were collected at 4-cell stage and 140 at 8-cell stage. Total RNA was isolated from 4-cell and 8-cell stages embryos. Isolated RNA was subjected to RT-PCR by using Bru4-Exo-13F and Bru4-3´UTR-R primers. PCR product was digested by *PmI1* restriction enzyme to differentiate between maternal and paternal allele. At four-cell stage only paternal allele was observed and at 8-cell stage both maternal and paternal alleles were observed (Fig 3.29 A). Taken together these observations suggest that at four-cell stage of embryonic development only paternal allele of *Brunol4* is transcribed while at 8-cell stage of embryonic development both maternal and paternal alleles are active.

To further prove this result of *Brunol4* gene activation the breeding strategy was changed from CD-1 ♀ and C57 BL 7♂ to CD-1♂ and C57 BL ♀. Embryos were collected at 4 cell and 8 cell stages. Total RNA was isolated from 4-cell (180) and 8-cell (158) stage embryos. Isolated RNA was subjected to RT-PCR by using Bru4-Exo-13F and Bru4-3´UTR-R primers. PCR products were digested with *PmI1* restriction enzyme to differentiate between maternal and paternal allele. At four-cell and 8-cell stage, both maternal and paternal alleles were observed. Interestingly at 4-cell stage of embryonic development, the intensity of paternal allele was very strong while the maternal allele was very weak (Fig 3.29 B). Result of activation of *Brunol4* gene during early embryonic development is summarized in (Table 3.9). Taken together these observations suggest that the activation of *Brunol4* gene starts at 4-cell stage but at this stage paternal allele is expressed preferentially (Fig 3.29B).

Stages of embryonic development	**Activation of *Brunol4* transcript (maternal or paternal)**
Unfertilized oocytes	Maternal transcript
2-cell stage	Maternal transcript
4-cell stage	Both maternal and paternal transcripts but paternal allele is expressed preferentially
8-cell stage	Both maternal and paternal

Table 3.9 Result of activation of *Brunol4* gene during early embryonic development. At unfertilized oocytes and 2-cell stages only maternal allele is transcribed. At 4-cell stage both maternal and paternal alleles are transcribed but paternal allele is expressed preferentially. At 8-cell stage both maternal and paternal alleles are expressed equally.

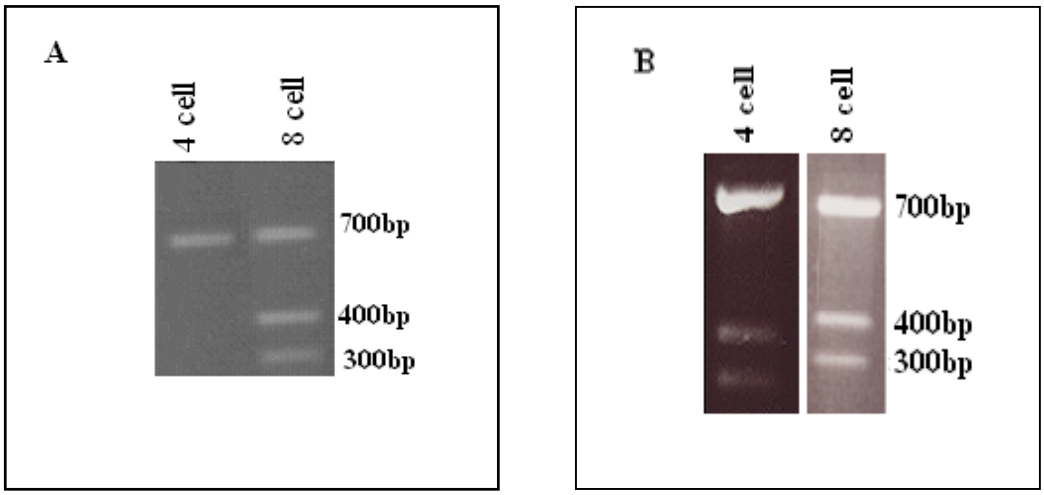


Figure 3.29 Strategy to differentiate between expression of maternal and paternal allele at four-cell and eight-cell stages of embryonic development. (A) RT-PCR product of 4- and 8- cell stages, (CD-1♂ X C57BL♀) was treated with *PmI1* restriction enzyme; at 4-cell stage only a single band (700bp) represents the paternal allele and at 8-cell stage three bands represents both maternal (400bp + 300bp) and paternal allele (700bp). (B) RT-PCR product of 4 and 8-cell stages (CD-1♀ X C57BL♂) was treated with *PmI1* restriction enzyme. At 4-cell stage three bands could be observed, one strong band (700bp) represent the paternal allele, two-week bands (400bp + 300bp) represents the maternal allele and at 8-cell stage three bands represent both maternal and paternal allele.

4. Discussion

RNA-binding proteins are the cog-wheels of molecular machines that regulate gene expression at the post-transcriptional level. As cog-wheels they interact also with other proteins and transmit specific packets of information. However, they do not always fulfil exclusive functions and according to their localization certain can enter into different molecular machineries that ensure different functions. The Elav/Bruno RBPs represent a superfamily of RNA binding proteins. The members of the elav gene family encode RNA binding proteins (RBPs) that contain a highly conserved structure of three RNA recognition motifs (RRMs). The prototype of this gene family, the *elav* gene, was identified in *Drosophila* as a neural specific gene (Yao et al.,1993). In human and mouse, six Bruno-like genes have been identified, whereas other species such as *Drosophila*, *Xenopus laevis*, and *Caenorhabditis elegans* have at least two members of this family, and related genes have also been detected in plants and ascidians. The importance of RBPs in development is underscored by the isolation of mutants with interesting developmental phenotypes where the defective gene encodes an RBP (Yao et al., 1993).

The initial aim of the present study was the elucidation of the mechanistic role of *Brunol1* and *Brunol4* (members of Elav/Bruno family) in cerebral development and spermatogenesis. For this purpose, knockout alleles of *Brunol1* and *Brunol4* were isolated to determine, whether these genes have unique or redundant function.

In the first part of this thesis, Elav/Bruno RBPs family and its evolutionary conservation is discussed. Results concerning about expression analysis of *Brunol1*, its subcellular localisation as well as functional analysis of *Brunol1* by generating knockout mice are discussed. In the second part, expression analysis of *Brunol4* gene, generation of the *Brunol4* knockout mice and its possible duplication are discussed**.** Finally we discuss the results of expression analysis of *Brunol6* and genome activation strategy of *Brunol4*.

4.1 Evolutionary conservation of Elav/ Bruno family

Elav is the superfamily of RBPs that contains a conserved domain, called the RNA recognition motif (RRM). The genes of Elav/Bruno family are conserved through evolution with members present in plants, worms, fruit flies, and vertebrates (Good et al., 2002). The RNA recognition motif (RRM) is highly conserved domain and contain a 80–90 amino acid domain (Siomi and Dreyfuss, 1997) The most highly conserved sequences within the RRM are the ribonucleoprotein motifs (RNP1 and RNP2) that are signature sequences for the

RRM and have been shown to specifically interact with RNA (Nagai et al., 1994). A multiple sequence alignment of the putative mouse, human, and *C. elegans* of full-length Bruno family of proteins to the *Drosophila* Bruno and *Xenopus* BrunoL-1 is presented in figure 4.1. Most of the sequence conservation is within RNP of the RRMs. The amino-terminal and linker regions of the three RRMs are highly variable in different proteins. The third RRM has the most sequence conservation in the different proteins. The sequence of the linker region contains no identifiable motifs. However, several of the Bruno proteins have homopolymeric amino acid tracts such as the stretches of glutamine in mouse Brunol1, *C. elegans* Etr-1 and *Xenopus* BrunoL-1. The possible function of this domain is not known.

All members of the Bruno family share a common domain structure with the Elav family of proteins. A dendrogram based on a pair wise comparison of all members of the Bruno and vertebrate Elav family is presented in figure 4.2. The Elav proteins form a distinct group of proteins. A yeast protein, PUB1, has a domain structure similar to Bruno but is most similar to the Elav family of proteins. The vertebrate Bruno proteins fall into two subfamilies, one containing *Brunol2* and *Bruno3* and the other containing *Brunol1*, *Brunol4, Brunol5*, and *Brunol6*.

Comparison of the amino-terminal and linker regions of proteins within subfamilies reveals some sequence similarity in these regions. Orthologous proteins in *Xenopus* have been identified for BRUNOL1, BRUNOL2 and BRUNOL3; presumably the frog Brunol-4, Brunol-5, and Brunol-6 have not yet been identified. The similarity of the domain structure but divergence of the primary sequence from the Elav proteins that are involved in many different RNA processing events both during development and in normal cell function (Good, 1997; Antic and Keene, 1997) suggests that the Bruno proteins may have related roles in regulating target RNAs but bind to different subsets of mRNAs than the Elav-like proteins.

A

B


```
Xenopusl.ETR-3------IKMFVGQIPRSWEKELKDLFEPYGAVYQINVLRDRSQNPPQSKGCCFVTFYTRKAALE
Similar to ETR-1-----AIKLFIGQIPRNLEKDLKPLFEEFGKIYELTVLKDRFT--MHKGCAFLTYCERESALK
Human BRUNOL4 ------AIKLFIGQIPRNLEKDLKPLFEEFGKIYELTVLKDRFT--MHKGCAFLTYCERESALK
Mouse Brunol4-------AIKLFIGQIPRNLEKDLKPLFEEFGKIYELTVLKDRFT--MHKGCAFLTYCERESALK
Mouse Brunol1-------AIKLFVGQIPRHLEKDLKPIFEQFGRIFELTVIKDKYT-—LHKGCAFLTYCARDSALK
Mouse Brunol6-------AIKLFVGQIPRGLDEQDLKPLFEEFGRIYELTVLKDRLT-LHKGCAFLTYCARDSALK
Xenopusl.ETR-1------AIKLFIGQIPRNLEKDLKPIFEQFGKIYELTVIKDKFT--MHKGCAFLTYCARESALK
Danio rerio ETR-1---AIKLFIGQIPRNLEKDLKPIFEQFGKIYELTVIKDKYT--MHKGCAFLTYCARESALK
Human BRUNOL5-------AIKLFVGQIPRHLEKDLKPLFEQFGRIYELTVLKDPYT--MHKGCAFLTYCARDSAIK
C.elegans ETR-1-----AIKMFVGQIPRQWETDCRRLFEKYGSVFSCNILRDKS---ASKGCCFVTFYHRKDAIE
Consensus sequence      LFVGNL    E  L   F  FG I    I        KGFGFVXF       A
                                  D         V      V

Xenopus1.ETR-3---AQNALHNIKTLPGMHHPIQMKPADSEKSNAVE----------RKLFIGMVSKKCNENDIRV
Similar to ETR-1--AQSALHEQKTLPGMNRPIQVKPADSESRGGSSCLRQPPS--HRKLFVGMLNKQQSEDDVRR
Human BRUNOL4-----AQSALHEQKTLPGMNRPIQVKPADSESRGGSSCLRQPPS--DRKLFVGMLNKQQSEDDVRR
Mouse Brunol4-----AQSALHEQKTLPGMNRPIQVKPADSESRGGSSCLRQPPSH  RKLFVGMLNKQQSEDDVRR
Mouse Brunol1-----AQSALHEQKTLPGMNRPIQVKPADSESRGD-----------KLFVGMLGKQQTDEDVRK
Mouse Brunol6-----AQSALHEEQKTLPGMNRPIQVKPAASEGRGED----------RKLFVGMLGKQQGEEDVR
Xenopus l. ETR-1--AQSALHEQKTLPGMNRPIQVKPADSESRGED-----------LFVGMLGKQQTDEDVRR
Danio rerio ETR-1-AQNALHEQKTLPGMNRPIQVKPADSEGRGD---------- RKLFVGMLGKQLSDADVRK
Human BRUNOL5-----AQTALHEQKTLPGMARPIQVKPADSESRGGR------- --DRKLFVGMLNKQQSEEDVLR
C.elegans ETR-1---AQGALHNIKVIEGMHHPVQMKPADTENRNE-----------KLFIGQLSKKHNEENLRE
Consensus sequence          L G                              LFVGNL    E
                            I  D
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```
Xenopusl.ETR-3-----FSPFGQIEECRILRGPDGLSRGCAFVTFSTRAMAQNAIKAMHQSQTMEGCSSPIVVKFA
Similar to ETR-1---LFEAFGNIEECTILRGPDGNSKGCAFVKYSSHAEAQAAINALHGSQTMPGASSSLVVKFA
Human BRUNOL4-----LFEAFGNIEECTILRGPDGNSKGCAFVKYSSHAEAQAAINALHGSQTMPGASSSLVVKFA
Mouse Brunol4-----LFEAFGNIEECTILRGPDGNSKGCAFVKYSSHAEAQAAINALHGSQTM GASSSLVVKFA
Mouse Brunol1-----MFEPFGTIDECTVLRGPDGTSKGCAFVKFQTHAEAQAAINTLHSSRTLPGASSSLVVKFA
Mouse Brunol6-----MFQPFGHIEECTVLRSPDGTSKGCAFVKFGSGAEAQAAIQGLHGSRTMTGASSSLVVKFA
Xenopusl.ETR-1-----FEPFGNIDECTVLRGPDGTSKGCAFVKFQTHTEAQAAINALHGSRTLPGASSSLVVKFA
Danio rerio ETR-1--MFEPFGSIEECTVLRGPDGASKGCAFVKYQSNAEAQAAISALHGSRTLPGASSSLVVKFA
Human BRUNOL5-----LFQPFGVIDECTVLRGPDGSSKGCAFVKFSSHTEAQAAIHALHGSQTMPGASSSLVVKFA
C.elegans ETR-1—---IFAKFGHIEDCSVLRDQDGKSRGCAFVTFTNRSCAVAATKEMHHSQTMEGCSAPLVVKFA
Consensus sequence     FG I    V     KGFGFVXF        A      L G
                               I                              I
Xenopusl.ETR-3-----DTQKDKEQRRLQQQLAQ----------QMQQLN—ATWGNLTGLGGLTPQYLALLQQ
Similar to ETR-1----DTDKERTMRRMQQMAGQ----------MGMFNP-MAIPFGAYGAYAQA-----LMQQQ
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Human BRUNOL4------DTDKERTMRRMQQMAGQ-----------MGMFNP-MAIPFGAYGAYAQA-----LMQQQ
Mouse Brunol4----DTDKERTMRRMQQMAG----------MGMFNPMAIPFGAYGAYAQA------LMQQQ
Mouse Brunol1------DTEKERGLRRMQQVATQ------------LGMFSP------IALQFGAYSAYTQLMQQQ
Mouse Brunol6------DTDRERALRRMQQMAGQ------------LGAFHPAPLPLGACGAYTT------LMQQQ
Xenopusl.ETR-1-----DTEKERGLRRMQQVANQ------------LGMFSP-IALQFGAYSAYTQAVSDQLMQQQ
Danio rerio ETR-1--DTEKERGIRRMQQVASQ------------LGVISP-MNLHLGAYNAYTQA----LVQQQ
Human BRUNOL5 -----DTDKERTLRRMQQMVGQ------------LGILTPSLTLPFSPYSAYAQA----LMQQQ

Xenopusl. ETR-3----ATTPSNLGAFSGIQQMAGMNALQLQNLATLA A A  A  AAAQTSATT-TNVNPLSTTA
Similar to ETR-1---AALMASVAQGGYLNPMAAFAAAQMQQMAALNMNGLAAAPMTPT------SGGSTPPGITA
Human BRUNOL4------AALMASVAQGGYLNPMAAF AAAQMQQMAALNMNGLAAAPMTPT-----SGGSTPPGITA
Mouse Brunol4------AALMASVAQGGYLNPMAAFAAAQMQQMAALNMNGLAAAPMTPT------SGGSTPPGITA
Mouse Brunol1------AALVAAHSAYLSPMATM AA VHDAAHGCHQ C Q  W PHRHPHHSI—LRNQHPSCHCC
Murine Brunol6-----AALLAAAQGPGLGQVAAVAAQMQHVAAFSLVAA--------PLLPAAANTSPGGNGPGAL
Xenopusl. ETR-1—---AALVAAHSAYLNPMATMAAVQMQQMATINPNGIIATPITQINPITSS--SGTSTPPTLTA
Danio rerio ETR-1--AALV-----AQSAYLSPVATVAAVQMQQLAALNPSSIIATPIASIT  SSGTSTPPTIAA
Human BRUNOL5------TTVLS-------TSGSYLSPGVAFSPCHIQQIGAVSLNGLPATPVAPA-SGLHSPPLLGT
C.elegans ETR-1----QGQQQ---QHQQQQNVLGILGTVLSALGKLTEGGDDASAKSSSEKPRHQALMTSPAPTAT

Xenopusl. ETR-3-----SALGALTSPVAASTANS—-SAGAAMNSLTSLGTLQGLAGATVGLNNINALAGTVNSMAAL
Similar to ETR1-----PAVPSIPSPIGVNGFTG-LPPQANGQPAAEAVFANGIHPYP-------------------
Human BRUNOL4-------PAVPSIPSPIGVNGFTGLPPQANGQPAAEAVFANGIHPYP--------------------
Mouse Brunol4-------PAVPSIPSPIGVNGFTGLPPQANGQPAAEAVFANGIHPYP--------------------
Mouse Brunol1 ------HARLCHPCCLGRQRLQPGAHPAYTGQPAPDALYPNGVHPYPDEALSAERSAGGVPIMSQA
Xenopus l.-ETR-1----- TQVSAIPATLGVNGYSA-VPTQSTVQPSSEAIYTNGLHPYP-----------------
Danio rerio ETR-1---TPVPALPPPIAVNSYPP-VPAAPNGQSASEALYTNGVHPYQ-------------------
Human BRUNOL5-------TAVPGLVAPIT-NGFAG-VVPFPGGHPALETVYANGLVPYP-------------------
C.elegans ETR-1-----SSTSSSASHHHQQHQQQLSQQQQQQQHPQQQGLGNPLLGNPAMAAQNQFDAITMAQIAHQ

Xenopus l. ETR-3---NGGLGAT---GLTNGTAGTMDALTQAYSGIQQYAA-AALP------------------TLY
Similar to ETR-1 --AQSPTAADPLQQAYAGVQQYAA-AAYP----------------------------AAYGQI
Human BRUNOL4 -----AQSPTAADPLQQAYAGVQQYAGPAAYP----------------------------AAYGQI
Mouse Brunol4------AQSPTAADPLQQAYAGVQQYAGPAYP----------------------------AAYGQI
Mouse Brunol1 -----HSWLVMLSAAQSPAAPVDPLQQAYAGMQHYT---AAYP-----------------AAYSLV
Mouse Brunol6 -----GQLGAFHPAPLPLGACGAYTT---------------------------------AAFSLV
Xenopus l. ETR-1 --AQSPVAQLDPLQQAYAGMQHYT—AAYP----------------------------AAYGLV
Danio rerio ETR-1 -AQSPA--LDPLQQAYTGMQHYT--ATYP----------------------------AAYGLV
Human BRUNOL5 -----AQSPTV-AETLHPAFSGVQQYT--AMYPT------------------------ AAITPI
C.elegans ETR-1----QQMLALQGFAVQQGAPSQQQQGLAGGMAGAKTTSPVAASLANHQQIALTPFAGGAAAALDH

Xenopus l. ETR-3---SQSLLQQQSAAGSQKEGL--------------LFISAQGPEGANLFIYHLPQEFGDQDILQ
Similar to ETR-1 --SQAFPQPPPMIPQQ--------------------QREGPEGCNLFIYHLPQEFGDAELMQ
Human BRUNOL4 -----SQAFPQPPPMIPQQ--------------------QREGPEGCNLFIYHLPQEFGDAELMQ
Mouse Brunol4------SQAFPQPPPMIPQQ--------------------QREGPEGCNLLIYHLPQEFGDAELMQ
Mouse Brunol1------- APAFPQPPALVAQQPPPPPQQQQQQQQQQQQQ-QREGPDGCNIFIYHLPQEFTDSEILQ
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Mouse Brunol6--------TLYNNGVSPYPAAYPSAYAPASTAFSQQPSALPQQQREGPEGNLFIYHLPQEFGDAELI

Xenopus l. ETR-1---SPAFTQPPAIIQQQPP-------------QQQQQ-REGPEGCNIFIYHLPQEFTDSEILQ
Danio rerio ETR-1--GQPFPHQPTLVAQQPQQP------------QQLQQ-REGPEGCNIFIYHLPQEFTDSEMLQ
Human BRUNOL5----AHSVPQPPPLLQQQ---------------------Q-REGPEGCNLFIYHLPQEFGDTELTQ
C.elegans ETR-1-----FQAMQQYALLANLQATGGVGVQATTSAAQMVGNGDVKGPDGANLFIYHLPQDFGDSDLIN
Consensus sequence                                              IYIKGM       D
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RRM3

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Xenopus l. ETR-3----MFMPFGNVISAKVFIDKQTNLSKCFGFVSYDNPVSAQAAIQAMNGFQIGMKRLKVQLKRS
Similar to ETR-1----MFPFFGFV -------------------SFDNPASAQTAIQAMNGFQIGMKRLKVQLKRP
Human BRUNOL4 ------MFLPFGFV--------------------SFDNPASAQTAIQAMNGFQIGMKRLKVQLKRP
Mouse Brunol4-------MFLPFGNV--------------------SFDNPASAQTAIQAMNGFQIGMKRLKVQLKRP
Murine Brunol1------MFVPFGHVISAKVFVDRATNQSKCFGFVSFDNPASAQAAIQAMNGFQIGMKRLKVQLKRP
Murine Brunol6------TFLPFGAVVSAKVFVDRATNQSKCFGFVSFDNPTSAQTAIQAMNGFQIGMKRLKVQLKRP
Xenopus l. ETR-1----MFLPFGNVISAKVFVDRATNQSKCFGFVSFDNPGSAQAAIQAMNGFQIGMKRLKVQLKRP
Danio rerio ETR-1---MFLPFGNVISAKVFVDRATNQSKCFGFVSFDNPASAQAAIQAMNGFQIGMKRLKVQLKRP
Human BRUNOL5-------MFLPFGNIISSKVFMDRATNQSKCFGFVSFDNPASAQAAIQAMNGFQIGMKRLKVQLKRP
C.elegans ETR-1-----TFAPFGGILSAKVFIDKVTNLSKCFGFVSYENAQSATNAISAMNGFQIGSKRLKVQLKVD
Consensus sequence   F  FG V           R YA   Y        A      M G
```

Fig. 4.1 The Brunol family members are closely related. (A) All of the Brunol proteins possess the same domain structure: three RRMs and a divergent domain of unknown function between RRM2 and RRM3 (B) Evolutionary relationship of mouse Brunol1, Brunol4 and Brunol6 proteins, sequence alignment of Elav/Brunol family with other species are shown. The sequences of the RNP of motifs are coloured red, the consensus sequence of the motifs are coloured blue (Birney et al., 1993; Burd and Dreyfuss, 1994)

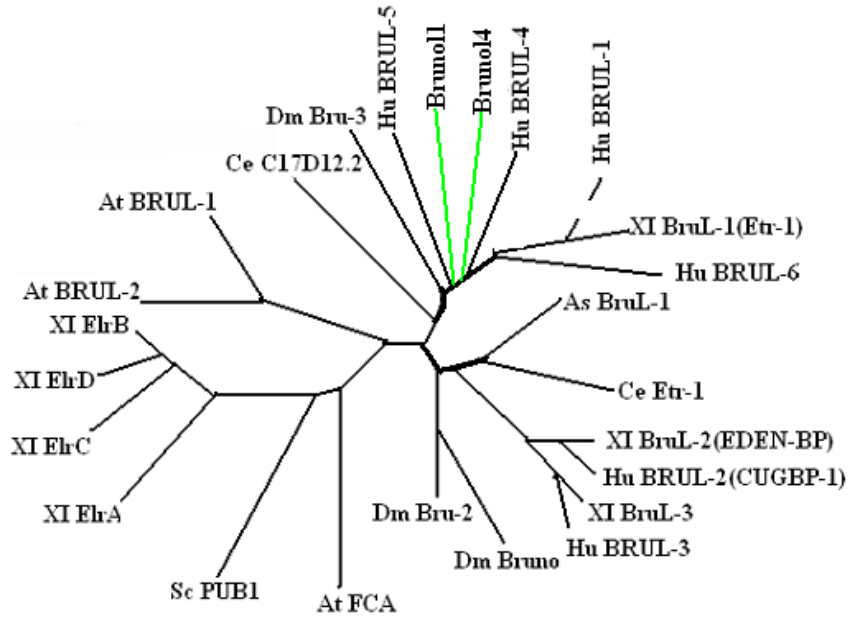

FIG. 4.2 Dendrogram of Bruno family multiple sequence alignments according to Good et al. (2002) extended by mouse protein Brunol1, Brunol4 and Brunol6. The well-known and predicted Bruno proteins (derived from the well-known EST clones) were compared with the program ClustalW and a radial tree displayed using Tree View. The proteins aligned are as follows: Xl EDEN-BP/Xl BruL-2 (AF003923), At (*Arabidopsis*) FCA (Z82989), At BruL-1, encoded by gene F21B7.26 (AC002560), At BruL-2, encoded by gene T4I9.1 (AF069442). Members of the *Xenopus* Elav family: ElrA, ElrB, ElrC, and ElrD and the yeast PUB1 (L20767) are included for comparison to the Bruno proteins. *As,* ascidian sequences from *H. roretzi*.

4.2 Expression analysis of *Brunol1* gene

4.2.1 RT-PCR analysis of *Brunol1* during embryonic development

RT-PCR analysis of *Brunol1* during development shows that, its expression starts at 9.5 dpc. Although we also analysed the expression of *Brunol1* during preimplantation stages of embryos, we could not detect the *Brunol1* expression in preimplantation stages.

Interestingly, *Brunol1* RT-PCR signal was also observed in embryonic stem cells (ES cells). ES cells are derived from inner cell mass (ICM), a cluster of pluripotent stem cells, which exist only temporarily in blastocysts and have the capacity to differentiate into all types of embryonic tissues (Temple, 2003). Because ES cells are obtained from blastocysts, it suggests that *Brunol1* could be expressed at such an early stage of mouse development. Similar situation is, for example, in the case of transcription factor *Oct4*, which is initially expressed in ICM (Schoeler et al., 1990). However, expression of *Oct4* was observed also in preimplantation stages, which was not the case for *Brunol1* (Fig.3.2). This early expression suggests, that *Brunol1* might have a function during very early stages of embryonic development (blastocyst stage).

4.2.2 RT-PCR analysis of *Brunol1* in adult mouse

In RT PCR analysis of *Brunol1* in adult mouse tissues (brain, heart, liver, lung, testis, ovary, muscle and kidney) we have found that *Brunol1* transcript is restricted to brain and testis. Additionally, RT-PCR was performed on RNA isolated from testes of different developmental mutants. Because the RT-PCR product was observed in all of the mutants except W/W^{V} mice and Tfm/y, it proves that expression of *Brunol1* is restricted to germ cells and it is not expressed in Sertoli or Leydig cells of testis (Fig. 3.32B). We also performed RT-PCR on RNA isolated from the testes of males of different postnatal stages. *Brunol1* signal was detected in testes of 20, 25, 30 and 40 days old males (Fig 3.3 C). Appearance of *Brunol1* transcript in day 20 correlates with the appearance of secondary spermatocytes. It is known that post-transcriptional control is a major form of gene regulation during mammalian gametogenesis. In haploid spermatids, proteins required for sperm morphogenesis are synthesized from stores of polyadenylated mRNAs packaged as cytoplasmic ribonucleoprotein particles (RNPs). Unknown signaling events promote unmasking, translational activation, deadenylation and the eventual degradation of translationally repressed mRNAs. Several proteins have been proposed to function as translational regulators in spermatids.

Chromosome condensation in mammalian spermatids involves an initial displacement of the somatic histones with the testis-specific transition proteins TP1 and TP2, and their subsequent replacement by the protamines (Bellve, 1979). The protamine genes *Prm1* and *Prm2* encode small basic proteins first transcribed in haploid round spermatids (Mali et al., 1989). The protamine mRNAs are stored in translationally inert mRNP particles for up to

10 days, until translational activation in elongated spermatids (Balhorn et al., 1984; Kleene et al., 1984; Kleene and Flynn, 1987). Translational regulation of the protamine mRNAs is mediated by sequences in their 3′ untranslated region (3′ UTR) (Braun et al., 1989; Fajardo et al., 1997; Zhong et al., 2001). Translation of the transition protein mRNAs, as well as that of other mRNAs required for spermatid differentiation, is also repressed during spermiogenesis (Balhorn et al., 1984; Mali et al., 1989). Translational repression occurs at a time when other mRNAs are actively being translated, indicating that repression is message specific (Braun, 1998). Several RNA binding proteins have been identified which are expressed in meiotic spermatocytes and postmeiotic spermatids for example like Msy1, Msy2 and Msy4 (Oko et al., 2001). It has been known that Msy2 interacts with Msy4 and binds to the 3′UTR of *Prm1* mRNA and controls its regulation (Giorgini et al., 2001) Another RNA binding protein, Tarbp2, functions as a translational regulator during mouse spermatogenesis by binding to the *Prm1* mRNA (Lee et al., 1996). The meiotic spermatocytes and postmeiotic spermatids specific expression of *Brunol1* shows that it might also control the regulation of *Prm1* or promote unmasking, translational activation, deadenylation and the eventual degradation of translationally repressed mRNAs. In order to know the exact role of Brunol1 protein during spermatogenesis, the target mRNA is yet to be identified.

4.2.3 Expression analysis of *Brunol1* in adult mouse by Northern blot

Expression analysis of *Brunol1* in adult mouse tissues shows that a large transcript of approximately 4.0 kb and a smaller transcript of approximately 2.7 kb are exclusively restricted to brain and testis (Fig 3.4). The differentially spliced region of exon 10 is not enough to explain the difference in size between the two *Brunol1* mRNA isoforms seen by Northern blotting. Size differences are usually attributable to differences in polyadenylation. Indeed, there are numerous mouse *Brunol1* ESTs that extend beyond the polyadenylated end of 3′UTR of *Brunol1* sequence in database suggesting that a down stream polyadenylation site is some time used. Brain and testis specific expression of *Brunol1* is making it a new member of an expanding family of brain/testis protein. Other proteins with expressions restricted to the brain and testis have been shown to have a variety of functions including important role in neurogenesis and spermatogenesis (Noce et al., 1993; Connor et al., 1995; Martin et al., 1996; Josep et al., 1999)

4.2.4 Subcellular localization of Brunol1 protein

We also studied the subcellular localisation of Brunol1 protein. For that we have generated GfpBrunol1 fusion protein by tagging GFP at N and C terminal of Brunol1, respectively (Fig.3.5). Microscopical observation of transiently transfected NIH 3T3 cells emitting a green signal shows that Brunol1 is localised in cytoplasm and nucleus (Fig.3.6). Cytoplasmic and nuclear localization of Brunol1 suggests that it might be involved in multiple functions within the cell affecting mRNA splicing, polyadenylation, transport, localization, stability, and translation. It has been suggested that Bruno-like protein moves between compartments to regulate both nuclear and cytoplasmic mRNA processing (Good et al., 2000; Ladd et al., 2001) Phylogenetic analysis shows that the Bruno family is closely related to the Hu proteins (Ladd et al., 2001), which contain multiple regions that determine its subcellular localization and contribute to its regulatory function of mRNA stability and translation (Knee et al., 1999). It is very likely that like Hu proteins, subcellular localization of Brunol1 is also controlled by a set of elements that collectively drive a balance between a nuclear and cytoplasmic presence.

4.3 *Brunol1* deficient mice are fertile and undergo normal spermatogenesis

To illustrate the function of the *Brunol1* gene in spermatogenesis, we generated knockout mice of *Brunol1* by homologous recombination in ES cells. Through Northern blot and RT-PCR analysis on total RNA isolated from adult testis and brain, we clearly showed that the *Brunol1* transcript was lacking in the *Brunol1* $^{-/-}$ mice (Fig. 3.9). Thus, the *Brunol1* deleted allele is a null mutation and *Brunol1* $^{-/-}$ is a loss of function mouse model system. The homozygous male and female mice are fertile and we detected no abnormalities in the testicular (Fig 3.12) and brain (Fig 3.14) morphology or histology of *Brunol1* deficient mice. To determine whether loss of Brunol1 protein affects the properties of the mature sperm, we analyzed various sperm properties of Brunol1 $^{-/-}$ mice, for example: morphology, acrosome reaction, motility, and total sperm count in the cauda epididymis

A significant reduction in the total sperm count in *Brunol1* $^{-/-}$ mice was observed as compared to Brunol1 $^{+/+}$ mice (Table 3.3). It suggests that *Brunol1* deficiency can affect the normal progression of spermatogenesis thus resulting in reduced number of sperm in *Brunol1*$^{-/-}$ mice. However, *Brunol1*$^{-/-}$ mice are fertile with comparable litter size to that of wild type mice. Therefore, this result indicates that the reduced sperm number is although statistically significant but is not enough to affect the fertility of the *Brunol1*$^{-/-}$ mice.

From the result of the *Brunol1* knockout mice, we can conclude that *Brunol1* is not essential for spermatogenesis in mice. As Brunol1 is a member of the large Elav/Bruno family of RNA binding proteins, and it is known that different RNA binding proteins regulate posttranscriptional regulation during spermatogenesis, it is very likely that function of Brunol1 was compensated by either another member of Elav/Bruno family or by a similar RNA binding protein. Recently, we found another member of the Elav/Bruno family named Brunol6, which has a very high homology with Brunol1. Interestingly, *Brunol6* shows exactly the same pattern of expression like *Brunol1* (Fig 3.24). As the expression of *Brunol1* in the testis is exactly similar to *Brunol6,* therefore, there is high probability that *Brunol1* and *Brunol6* share some function(s) during spermiogenesis. But Northern blot analysis with *Brunol6* as a probe, did not detected any significant increase in *Brunol6* expression in the *Brunol1*$^{-/-}$ mice testis as compared to wild type mice (Fig 3.13). Since *Brunol1* and *Brunol6* belong to the same family and are highly similar, a minor increase in protein level of Brunol6 can significantly influence its function as a mediator in posttranscriptional regulation pathways; therefore, we cannot rule out that *Brunol6* or any other RNA binding protein might compensate the function of the *Brunol1* in *Brunol1* $^{-/-}$ mice.

4.4 Expression analysis of *Brunol4* gene

Brunol4 gene was cloned and characterized for the first time by Meins et al. (2002). They showed by RT-PCR that expression of *Brunol4* starts at 7.5 dpc and continues to later stages of embryogenesis. We confirmed this data and additionally performed RT-PCR on RNA isolated from different stages of embryonic development (unfertilized oocytes, preimplantation and postimplantation stages of embryonic development). By RT-PCR, we could detect the *Brunol4* transcript at all the stages of embryonic development, including preimplanatation and postimplanatation stages. Therefore, *Brunol4* gene expression starts in unfertilised oocytes and continue to all the later stages of embryonic development (Fig. 3.16 A –B). Expression analysis of *Brunol4* in different adult mouse tissues (brain, heart, liver, lung, testis, ovary, muscle and kidney) by Northern blot shows that *Brunol4* expression is restricted to brain. However, by RT-PCR, *Brunol4* transcript could be detected in ovary also, besides brain (Fig. 3.16 C).

It has been known that several RNA binding proteins expressed during early embryonic development, control early posttranscriptional regulation during embryogenesis. One of the best-characterized examples of a RBP that regulates development is provided by the Bruno

protein and its role as a translational repressor of *oskar* mRNA. In *Drosophila*, *oskar* is required for formation of germ cells and positioning of the posterior of the embryo (Lehmann and Nüsslein-Volhard, 1996). Both *oskar* mRNA and the encoded protein must be properly localized to the posterior pole of the oocyte for correct development (Ephrussi and Lehmann, 1992; Kim-Ha et al., 1995). Localized expression of Oskar protein is determined in part by translational silencing of the *oskar* mRNA outside of the posterior of the oocyte. This repression is mediated by 39 nucleotides long, *cis*-acting sequences in the untranslated region (UTR) of *oskar* mRNA called Bruno response elements (BREs), and a corresponding *trans*-acting factor, the Bruno protein (Fig 4.3). By regulating the localized expression of Oskar, Bruno has a key role in germ cell formation and early embryogenesis.

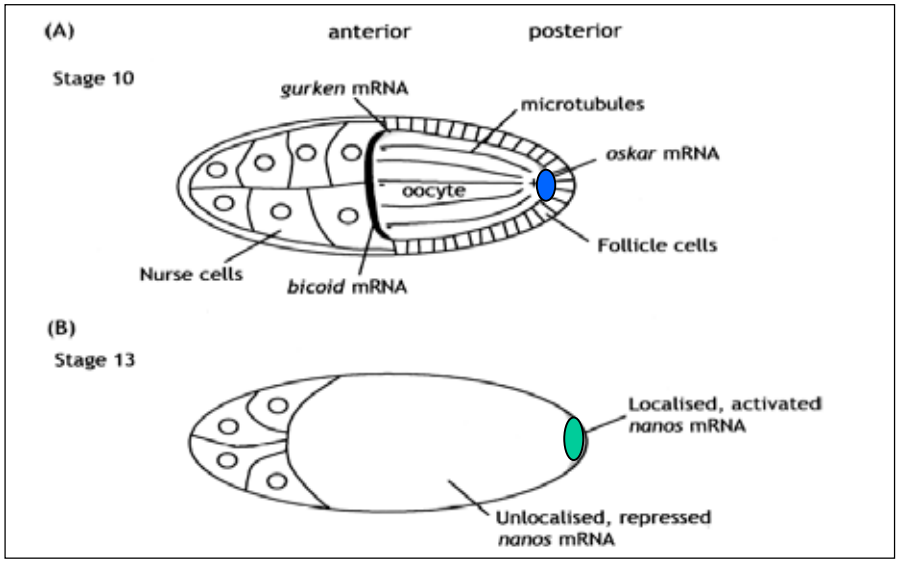

Fig 4.3 Localization of *Drosophila* mRNAs during oogenesis. (A) In stage 10 oocytes, *oskar* mRNA is localized to the posterior pole, *bicoid* to the anterior pole and *gurken* to the anterior-dorsal corner. Microtubules are oriented with their plus ends toward the posterior, allowing directional transport to occur (Theurkauf *et al.*, 1992). (B) In stage 13 oocytes, a proportion of *nanos* mRNA has become localized to the posterior pole, in addition to the previously localized mRNA. Localised *nanos* mRNA is translationally activated while the majority of *nanos* mRNA, which is not localized, remains repressed (Bergsten and Gavis, 1999).

Another example of RNA binding protein, Embryo deadenylation element binding protein (EDBEN), a homolog of Bruno identified in *Xenopus laevis*, has been shown to regulate mRNA deadenylation in early embryonic development (Paillard et al., 1998).

Interestingly, most of the members of Elav/Bruno family show early embryonic expression, which is later on restricted to brain. So, here a question arises that what is common between early embryonic development and brain. Elav is the family of RNA binding proteins and it is well known that RNA binding proteins play a crucial role in posttranscriptional regulation of maternally stored RNA until zygotic genome activation occurs. One of the mechanisms of posttranscriptional regulation is polyadenylation, which is characterized in lower animals. It is known that EDBEN (elav) protein regulates early polyadenylation in early embryo of *Xenopus*. Although cytoplasm polyadenylation is a hallmark of early metazoan development, there is virtually no evidence that it occurs in adult tissues. From a teleological point of view, this would seem very inefficient. That is, a significant genetic load is used to regulate translation by cytoplasmic polyadenylation, so in the last few years, mounting evidence has suggested that the brain might contain dormant mRNAs. In particular, specific mRNAs are present in dendrites (Crino et al., 1996; Steward et al., 1997), and synaptic spines (regions at the bases of synapses) have ribosomes and translation initiation factors (Steward and Levy, 1982). Most importantly, recent studies indicate that translational control in dendrites may be important for long-term changes in synaptic efficacy. Kang et al. (1996) and Wu et al.(1998) determined that CPEB is a factor present in the brain that regulates cytoplasmic polyadenylation. Although CPEB expression is quite restricted in the mouse (Gebauer and Richter, 1996), brain tissue contains readily detectable amounts. Furthermore, CPEB is present in the dendritic layers of the hippocampus, at synapses in cultured hippocampus neurons, and in postsynaptic densities (i.e., large networks of structural and regulatory proteins immediately beneath the postsynaptic membrane) of adult brain (Wu et al., 1998). One mRNA, which is present in dendrites and known to be essential for the long-lasting phase of long-term potentiation (L-LTP) encodes Ca21-calmodulin-dependent protein kinase II (a-CaMKII). The 39 UTR of CaMKII mRNA contains UUUUUAU-type CPEs, which bind CPEB in vitro and drive polyadenylation-induced translation in injected *Xenopus* oocytes. This data demonstrate that this process also occurs in the brain. By assuming the phylogenetic conservation of *Brunol4* with EDBEN of *Xenopus*, it might be possible that *Brunol4* also controls the

crucial regulation of dormant mRNA of neurons. Although to explain the exact mechanistic role of *Brunol4,* it is necessary to identify the target mRNA of *Brunol4*.

It is also known that neurons use various means to regulate posttranscriptional gene expression, including alternative splicing, RNA transport, local translation and RNA editing. Neural RNA-binding proteins are likely to play an essential role in mediating this regulation (Timchenko et al., 1996). The Mammalian ELAV-like neuronal RNA-binding proteins, HuB and HuC, phylogenetically related to Bruno, promote neuronal development in both the central and the peripheral nervous systems (Ladd et al., 2004). Based on phylogenetic relation of Brunol4 with Hu protein and its expression being restricted to brain, it is more likely that *Brunol4* also play important role in brain development.

4.5 Functional analysis of *Brunol4* gene by generation of *Brunol4* deficient mice

4.5.1 Generation of *Brunol4* knockout mice

To elucidate the role of the *Brunol4* gene, we decided to generate knockout mice for the *Brunol4* gene. A region of 800 bp of exon1 including ATG was replaced by GFP/Neo cassette. To generate the knockout construct of *Brunol4*, a 3.8 kb 5′ arm and a 5.3 kb 3′ arm were cloned into pTKNeo targeting vector and the gene was replaced with a GFP/Neo cassette (Fig 3.18). The targeting vector was transfected into the ES cells and out of 400 ES clones only one positive recombinant clone was obtained. The positive clone was injected into blastocysts. Four chimeras: 5%, 2x15% and 25% were obtained. All the four chimeras were bred with C57BL/6J mice, to obtain F1 animals in C57BL/6J background. Germ line transmission was detectable only by one 25% chimera after three and half month of breeding. Only two brown mice were obtained The germ line transmission of the mutant allele was verified by genomic PCR (Fig 3.18 C). While *Brunol4* heterozygous mice were apparently healthy and fertile, no *Brunol4* homozygous mice were identified. This result revealed that homozygous embryos might be lethal. To determine the stage when development of homozygous embryos is arrested, a detailed analysis of embryos at different stages of embryonic development, including up to one-cell stage embryos was done. But *Brunol4* homozygous mutants could not be identified by genotyping (PCR and Southern Blot). These results indicate that there might be two copies/duplication of *Brunol4* gene.

4.5.2 Murine *Brunol4* is present in more than one copy

We could not identify the *Brunol4* homozygous mice by conventional genotyping (PCR and Southern blot). The analysis of *Brunol4* transcript in heterozygous mice by Northern blot also showed that there is no reduction of *Brunol4* transcript as compared to wild type. These results indicated that there might be *Brunol4* homozygous mice among *Brunol4* heterozygous mice but we cannot identify them by genotyping. Then, breeding strategy was used to identify the homozygous mice among *Brunol4* heterozygous mice. To identify the *Brunol4* homozygous mice, fifteen *Brunol4* so called heterozygous mice were bred with wild type and the offspring of all the fifteen breeding pairs were genotyped by PCR. Interestingly, we found four pairs among these fifteen breeding pairs, which gave only heterozygous offspring (Table 3.8). So it is clear that the four parents, which gave all heterozygous offspring after breeding with wild type, are *Brunol4* homozygous mice. Further, these homozygous mice were analysed by Northern Blot analysis but there was no difference in *Brunol4* transcript compared to wild type (Fig 3.22).

To further prove that there are two or more copies of *Brunol4*, we performed Southern blot analysis of 129/Sv mice genomic DNA digested by different restriction enzymes. Southern blot analysis showed only one signal with each restriction enzyme similarly like single copy gene . This result indicates that *Brunol4* is a recently duplicated gene with very similar or even identical sequences.

To investigate whether multiple copies of *Brunol4* gene are transcribed in mice, RT-PCR experiment was performed using adult mouse brain RNA. Resulting PCR products were cloned into pGEM-T Easy vector. Fifty independent clones were sequenced and all of them showed the same sequence. This result suggests that only one copy of *Brunol4* is transcribed or all possible copies of *Brunol4* gene have the same sequence so that we could not differentiate between them by sequencing. Taking all the above observations together suggest that there are more than one copy of *Brunol4* gene and the one, which is not transcribed, was deleted in our homologous recombination

In literature, a number of genes are known for duplication. Bolcun et al. (2003) reported duplication of murine testase2 (ADAM 25). They found two different restriction patterns of subcloned fragments of the gene, indicating the presence of two different testase 2 transcripts. Celera database searches demonstrated that the two transcripts are the products of two testase 2 genes, which are located on mouse chromosome 8 in close

distance of 24 kb. They show high sequence similarity to the published testase 2 gene (87.8 and 95.5%, respectively).

In our case extensive search of NCBI database did not show extra copy of Brunol4. A similar case was reported in case of murine *Tcte3*. Huw et al. (1995) suggested that *Tcte3* exists in multiple copies, which are clustered in tandem array in the mouse genome. However, in the database, the sequence of only one gene is indicated (accession number: NT_039641).

It might be possible that the duplication of *Brunol4* gene occurred in recombinant ES cell clone, which we used for blastocyst injection. Unfortunately, we had only one positive clone so we could not confirm the ES cell derived duplication by using other independent ES cell clones for generating *Brunol4* knockout mice. Although, we again transfected the *Brunol4* knockout construct into ES cell to find other *Brunol4* recombinant ES cell clones and 280 ES cell clones were screened by Southern blot, none of them was recombinant. To answer all the open questions about possible *Brunol4* duplication, further recombinant ES cell clones of *Brunol4* must be identified.

4.6 Activation of *Brunol4* gene in preimplantation embryonic development

Our study of *Brunol4* expression by RT-PCR during early embryonic development shows that *Brunol4* transcript is present at all stages of embryonic development (Fig 3.16A-B), including preimplantation and postimplanatation stages. As we could also detect the *Brunol4* transcript in unfertilised oocytes, so it might be possible that at two-cell stage we amplified the maternally stored transcript of *Brunol4* instead of newly synthesised transcript. It is known that in *Drosophila* and *Xenopus*, mRNAs stockpiled in the oocytes are stable after fertilization and regulate many aspects of embryonic development (Wolpert et al., 2002). Conversely, in mammals, oocyte derived mRNAs are degraded shortly after fertilization and cannot direct more than the first few cell divisions (Thompson et al., 1998). Therefore, zygotic genome activation (ZGA) must occur very early during mammalian development. (Fig 4.4)

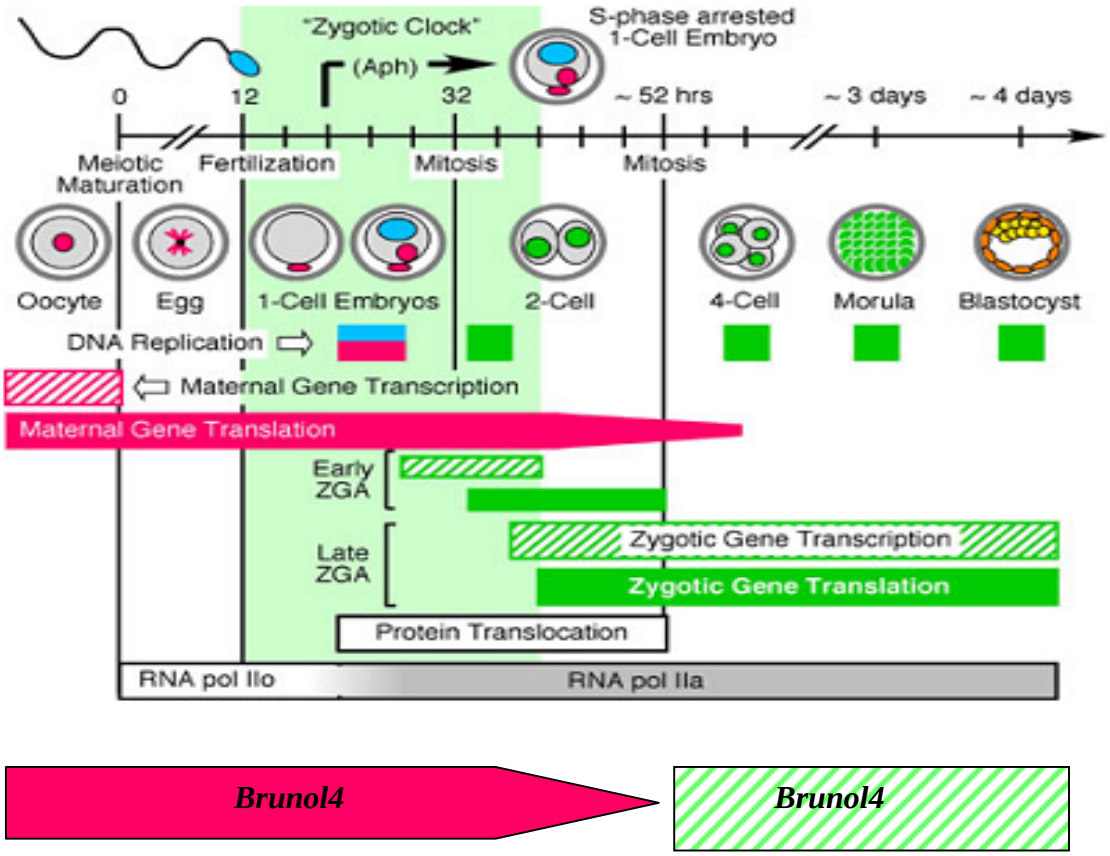

Figure 4.4. Maternal to zygotic gene transition in the mouse. Maternal events are indicated in RED, paternal events in BLUE, and zygotic events in GREEN. Open bars apply to both. Embryonic stem cells ("inner cell mass") are indicated in yellow; trophectodermal cells in orange. Periods of transcription are indicated by hatched bars; translation by solid bars.

To differentiate between the maternally derived transcript and newly synthesised transcript of *Brunol4* at two-cell stage, we exploited the single base polymorphism present at 3´ UTR of *Brunol4* cDNA in different strains of mice (Fig 3.26). Our study for *Brunol4* transcript shows that at two-cell stage only maternal transcript was present (Fig 3.28). At 4-cell stage of embryonic development we observed both maternally and paternally derived transcript (Fig 3.29A). It means the ZGA of *Brunol4* occurs at 4-cell stage. Interestingly at 4-cell stage paternally derived allele was expressed preferentially (Fig 3.29B)

It is known that transcription from the embryonic genome occurs at roughly the 2-cell stage in mice, 4- to 8-cell stage in humans and the 8- to 16- cell stage in cattle (Telford et al., 1990; Memili and First, 2000). In mouse, a minor burst of ZGA towards the end of the one-cell stage is followed by a major burst during the two-cell stage (Latham et al., 1992; Vernet et al., 1992; Aoki et al., 1997; Thompson et al., 1998; Schultz, 2002). ZGA is required for continued development because mRNAs common to the oocyte and embryo (e.g., genes involved in the maternal- to-zygotic transition and housekeeping genes) are replenished and many genes not transcribed in the oocyte are expressed for the first time. The outcome of ZGA is a novel gene expression profile that establishes the totipotent state of each blastomere in the cleavage stage embryo. This step is a prerequisite for future cell lineage commitments and differentiation events that underlie pattern formation and organogenesis. Changes in chromatin structure are thought to play an important role in reprogramming gene expression during ZGA (Schultz and Worrad, 1995; Thompson et al., 1998; Kanka, 2003).

Indeed, in mammals zygotic genome activation (ZGA) occurs synchronously and till now there is no report about asynchronous ZGA. Asynchronous activation of parental alleles was reported only in interspecific hybrids and not in heterozygotes within the same species. Developmental analyses of interspecific hybrids frequently reveal perturbations of gene function. The relatively delayed expression of paternal allelic isozymes has been observed for chicken-quail hybrids (Castro-Sierra and Ohno, 1968; Meyerhof and Haley, 1975) and for interspecific hybrids of trout (Hitzeroth et al., 1968; Goldberg et al., 1969; Schmidtke et al., 1976; Yamauchi and Goldberg, 1974; Wright et al., 1975). Although many interspecific hybrids manifest preferential inhibition of the paternally derived alleles, the analyses of some F1 hybrids have revealed the selective repression of *maternal* allele expression and the expression of the *paternal* alleles (Pipkin and Bremner, 1970; Whitt et al, 1972, 1973a; Vrijenhoek, 1975). These observations suggest that a specific repression of either maternal or paternal alleles may occur in some interspecific hybrids. During the development of hybrids between closely related species, one would expect to detect little or no allelic asynchrony or inhibition (Ohno, 1969). If such is the case, the egg cytoplasm might have a role in recognizing individual gene loci within the zygotic nuclei and activating them at appropriate stages of development. Two alleles within the same species are expected to have a nearly identical base sequence, the difference being a few base substitutions. Thus, the egg cytoplasm would be able to recognize a paternally derived

allele and a maternally derived allele of the heterozygote equally well. In the case of interspecific hybrids, on the other hand, a paternally derived allele from the alien species can have a base sequence considerably different from that of the maternally derived allele. Consequently, the egg cytoplasm might have difficulty in recognizing an alien allele. This phenomenon of preferential activation of maternally derived alleles might fall into the general category of nuclear-cytoplasmic incompatibility.

5. SUMMARY

The subject of this study was the expression and functional analysis of two novel genes of Elav/Bruno RBPs family, namely *Brunol1* and *Brunol4*. *Brunol1* was described for the first time (Wilhelm et al., 2001) in a systematic search for genes responsible for neurodegenerative diseases. Expression analysis of *Brunol1* by RT-PCR during embryonic development shows that its expression starts at 9.5 dpc. In adult mouse, the *Brunol1* expression is restricted to brain and testis. Expression of *Brunol1* was also observed in testes developmental mutants. *Brunol1* transcript was detectable in Leyl-/-, olt/olt and qk/qk mutant but not in W/W^{V} and Tfm/y mutants. This implies that *Brunol1* expression is restricted to the germ cells and not in other cell types of testis and its expression starts at spermatocyte stage. Subcellular localisation of Brunol1 protein tagged with GFP shows that Brunol1 protein is localized in both cytoplasm and nucleus. For the functional analysis, knockout mice for *Brunol1* were generated on C57 BL/6J x 129/Sv and 129/Sv backgrounds. Males from both backgrounds were fertile, but the number of sperms in cauda epididymis was reduced as compared to the wild type. Statistically reduced motility and progressive movement of sperms on both backgrounds was also observed in comparison to wild type. Spermatozoa showed normal acrosome reaction. Histological sections of brain and testes of homozygous mice revealed no abnormalities in cellular type or cellular structure. From these data it can be concluded that *Brunol1* may not be essential for embryonic viability, spermatogenesis and development of neural tissue.

In the second part of this study, expression and function of *Brunol4* was analysed. This gene was described for the first time by Meins et al. (2002), as a brain specific gene. By Northern blot analysis, *Brunol4* expression was found to be restricted to brain only. By RT-PCR, the expression of *Brunol4* was observed in brain, ovary and at all the stages of embryonic development including preimplantation and postimplantation stages. For the functional analysis, knockout mice for *Brunol4* were generated. While *Brunol4* heterozygous mice were apparently healthy and fertile, no *Brunol4* homozygous mice were identified. This result revealed that homozygous embryos might be lethal. To determine the stage when development of homozygous embryos is arrested, a detailed analysis of embryos at different stages of development, including up to one-cell stage embryos was done, but no *Brunol4* homozygous mutant was found. To identify the *Brunol4* homozygous mice among heterozygous mice by breeding strategy, fifteen *Brunol4* so-called heterozygous mice were bred with wild type and the offspring of all the fifteen breeding

pairs were genotyped by PCR. Four pairs among these fifteen breeding pairs gave all heterozygous offspring and it was then clear that they are *Brunol4* homozygous mice. These results clearly indicated that there are in fact *Brunol4* homozygous mice among heterozygous but they could not be identified by genotyping. Further, these homozygous mice were analysed by Northern Blot but there was no difference in *Brunol4* transcript compared to wild type. To investigate whether multiple copies of *Brunol4* gene are transcribed in mice, RT-PCR experiment was performed and sequencing results of 15 independent PCR products showed that only one copy of *Brunol4* is transcribed. So the present study shows that there are more than one copy of *Brunol4* gene and the one, which is not transcribed, was deleted. It might also be possible that the possible duplication of *Brunol4* gene was derived from recombinant ES cell clone, which was used for generation of *Brunol4* knockout mouse. Retransfection of the *Brunol4* knockout construct into ES cells yielded no recombinant ES cell clone. To solve the puzzle about possible *Brunol4* duplication, further recombinant ES cell clones for *Brunol4* must be identified.

In the third part of this study we identified *Brunol6* as a novel member of elav/Bruno family. Expression of *Brunol6* was analysed during embryonic development and in adult tissues. During embryonic development, expression of *Brunol6* starts at 9.5 dpc and continue to the later stages of embryonic development while in adult mouse tissues *Brunol6* expression is restricted to brain and testis. Expression of *Brunol6* was also observed in testes developmental mutants. *Brunol6* transcript was detectable in Leyl-/-, olt/olt and qk/qk mutant but not in W/W^{V} and Tfm/y mutants.

Finally we studied the activation of mouse *Brunol*4 gene during early embryonic stages. Our study shows that at two-cell stage of embryonic development only maternal transcript storage of *Brunol4* is present. Zygotic activation of *Brunol4* gene transcript starts at 4-cell stage of embryonic development. Interestingly it was found that at 4-cell stage the paternal allele of *Brunol4* is expressed preferentially.

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