Expression and functional analyses of murine Pelota (*Pelo*) gene

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CONTENTS

Page

CONTENTS I
ABBREVIATIONS VI

1. INTRODUCTION ...1
1.1 Pelota (*Pelo*) is an evolutionarily conserved gene ... 1
1.2 Expression and function of Pelota gene ... 4
1.3 Objectives of this study ... 6

2. MATERIALS AND METHODS ... 7
2.1 Materials ... 7
2.1.1 Chemicals ... 7
2.1.2 Solutions, buffers and media ... 11
2.1.2.1 Agarose gel electrophoresis ... 11
2.1.2.2 SDS-PAGE ... 11
2.1.2.3 Frequently used buffers and solutions ... 12
2.1.3 Laboratory materials ... 16
2.1.4 Sterilisation of solutions and equipment ... 17
2.1.5 Media, antibiotics and agar-plates ... 17
2.1.5.1 Media for bacteria. ... 17
2.1.5.2 Media for cell and embryo culture ... 18
2.1.5.3 Antibiotics ... 19
2.1.5.4 IPTG/X-Gal plate ... 19
2.1.6 Bacterial strains ... 19
2.1.7 Eukaryotic strains ... 19
2.1.8 Plasmids ... 20
2.1.9 Synthetic oligonucleotide ... 20
2.1.10 Mouse strains ... 23
2.1.11 Antibodies ... 23
2.1.12 Enzymes ... 24

2.1.13 Radioactive substances 25
2.1.14 Kits 25
2.1.15 Equipment 26
2.2 Methods 27
2.2.1 Isolation of nucleic acids 27
2.2.1.1 Isolation of plasmid DNA 27
2.2.1.1.1 Small-scale isolation of plasmid DNA 27
2.2.1.1.2 Large-scale preparation of Endotoxin free plasmid DNA 28
2.2.1.2 Isolation of genomic DNA 29
2.2.1.2.1 Isolation of genomic DNA from tissue samples with isopropanol 29
2.2.1.2.2 Isolation of genomic DNA from cultured cells 29
2.2.1.3 Isolation of total RNA from tissue samples and cultured cells 29
2.2.2 Determination of nucleic acid concentration 30
2.2.3 Gel electrophoresis 30
2.2.3.1 Agarose gel electrophoresis of DNA 31
2.2.3.2 Agarose gel electrophoresis of RNA 31
2.2.3.3 SDS-PAGE for the separation of proteins 32
2.2.4 Purification of DNA fragments from agarose gel 32
2.2.4.1 QIAquick gel extraction method 32
2.2.5 Enzymatic modifications of DNA 33
2.2.5.1 Restriction digestion of DNA 33
2.2.5.2 Ligation of DNA fragments 33
2.2.5.3 TA-Cloning 33
2.2.6 Preparation of competent *E.coli* bacteria 34
2.2.7 Transformation of competent bacteria 34
2.2.8 Polymerase Chain Reaction (PCR) 35
2.2.8.1 PCR amplification of DNA fragments 35
2.2.8.2 Reverse transcription PCR (RT-PCR) 36
2.2.8.3 One-Step RT-PCR 37
2.2.9 Protein methods 37
2.2.9.1 Isolation of total proteins 37
2.2.9.2 Determination of protein concentration 38
2.2.10 Blotting techniques 38

2.2.10.1 Southern blotting of DNA onto nitrocellulose filter 38
2.2.10.2 Northern blotting of RNA onto nitrocellulose filter 39
2.2.10.3 Western blotting of protein onto PVDF membrane 39
2.2.11 "Random Prime" method for generation of ^{32}P labeled DNA 40
2.2.12 Non-radioactive dye terminator cycle sequencing 41
2.2.13 Hybridisation of nucleic acids 41
2.2.14 Histological techniques 42
2.2.14.1 Tissue preparation for paraffin embedding 42
2.2.14.2 Sections of the paraffin block 42
2.2.14.3 Immunofluorescence staining of mouse tissues 43
2.2.14.4 Immunofluorescence staining of cells 43
2.2.14.5 Hematoxylin-eosin (H&E) staining of the histological sections 44
2.2.15 Generation of polyclonal antibody 44
2.2.15.1 Generation of Pelo-GST fusion construct 44
2.2.15.2 Expression of recombinant proteins in the pET vector 45
2.2.15.3 Purification of GST fusion proteins 45
2.2.15.4 Immunisation of rabbits.. … 45
2.2.15.5 Affinity purification of polyclonal antibody 46
2.2.15.6 Protein G purification of antibody 47
2.2.16 Eukaryotic cell culture methods 47
2.2.16.1 Cell culture conditions 47
2.2.16.2 Trypsinisation of eukaryotic cells 48
2.2.16.3 Cryopreservation and thawing of eukaryotic cells 48
2.2.16.4 Isolation and handling of primary mouse embryonic fibroblasts 48
2.2.16.5 Karyotype analysis 49
2.2.16.6 Transfection of mammalian cells 49
2.2.17 Techniques for production of targeted mutant mice 50
2.2.17.1 Production of targeted embryonic stem cell clones 50
2.2.17.1.1 Preparation of MEFs feeder layer 50
2.2.17.1.2 Growth of ES cells on feeder layer 51
2.2.17.1.3 Electroporation of ES cells 51
2.2.17.1.4 Growing ES cells for Southern blot analysis 52
2.2.17.2 Production of chimeras by injection of ES cells into blastocysts 52

2.2.17.3 Detection of chimerism and mice breeding ... 52
2.2.18 Gene silencing by RNA interference ... 53
2.2.18.1 Overview of the RNAi mechanism ... 53
2.2.18.2 Preparation of adaptor oligos ... 54
2.2.19 Generation of transgenic mice ... 54
2.2.19.1 Preparation of DNA for pronuclear microinjection ... 54
2.2.20 Computer analysis ... 55

3. RESULTS ... 56
3.1 Generation of polyclonal antibody against Pelota and expression analysis ... 56
3.1.1 Generation and purification of polyclonal anti-Pelo antibody ... 56
3.1.2 Cellular localisation of Pelo protein in mouse embryonic fibroblasts ... 58
3.1.3 Expression of Pelo protein in different cell lines ... 60
3.1.4 Expression of Pelo protein in mouse tissues ... 61
3.1.5 Expression of Pelo protein during testicular development and in testes of different mutant mice ... 61
3.1.6 Expression of *Pelo* during preimplantation stages ... 63
3.1.7 Expression and localisation of Pelo protein in testicular cell fractions ... 63
3.2 Knockdown of Pelota gene using inducible siRNA system *in vitro* ... 65
3.2.1 Overview of inducible siRNA system ... 65
3.2.2 Selection of siRNA sequences for specific targeting of *Pelo* mRNA ... 67
3.2.3 Efficiency of Pelo-siRNA to knockdown the *Pelo* transcript ... 71
3.3 Pelota conditional knockout mice ... 73
3.3.1.1 Generation of conditional knockout targeting construct ... 73
3.3.1.2 Generation of 5' external probe ... 78
3.3.1.3 Generation of an internal probe ... 78
3.3.1.4 Electroporation and screening of R1 ES cells for homologous recombination ... 81
3.3.1.5 Generation of chimeric mice ... 82
3.3.2 Generation of non-inducible conditional knockout mice ... 84
3.3.2.1 Breeding strategy to generate $Pelo^{flox/-}$ *EIIaCre* double transgenic mice ... 84
3.3.3 Generation of Pelota inducible conditional knockout mice ... 89
3.3.3.1 Breeding strategy to generate $Pelo^{flox/-}CreER^{T}$ double transgenic mice ... 89
3.3.3.2 Fertility test of the TAM-treated mice ... 89

3.3.3.3 Histological analysis of TAM-treated mice 91
3.3.3.4 Immunohistochemical analysis of testes sections of TAM-treated *Pelo*$^{flox/-}$*CreER*^{T} mice 93
3.3.3.5 The efficiency of TAM-induced recombination of *Pelo*^{flox} by *CreER*^{T} 94
3.3.3.6 Protein study in the testes of TAM-treated *Pelo*$^{flox/-}$*CreER*^{T} mice 95
3.3.3.7 The effect of Pelota deficiency on cell proliferation 96
3.3.3.8 Expression analysis of Pelota in OHT-treated fibroblasts at protein level 98
3.3.3.9 Proliferation assay of OHT-treated fibroblasts 98
3.3.3.10 Karyotype analysis of OHT-treated fibroblasts 99
3.4 Pelota transgenic mice 101
3.4.1 Generation of *hEF-Pelo* transgenic construct and purification 101
3.4.1.1 Generation and breeding of transgenic mice 102
3.4.1.2 Expression analysis of *hEF-Pelo* transgenic mice 103
3.4.2 Generation of *hUB-Pelo* transgenic construct and purification 104
3.4.2.1 Generation and breeding of transgenic mice 105
3.4.2.2 Expression studies of *hUB-Pelo* transgenic mice 106

4. DISCUSSION 108
4.1 Expression pattern and subcellular localisation of Pelota 108
4.2 Function of mammalian Pelota 110
4.3 Knockdown of Pelota using inducible siRNAs system 110
4.4 Pelota conditional knockout mice 112
4.5 The effect of Pelota deficiency on cell proliferation 114
4.6 *Drosophila* Pelota controls self renewal of germline stem cells 115
4.6 The effect of overexpression of Pelota gene 117

5. SUMMARY 119
6. REFERENCES 122
7. ACKNOWLEDGEMENTS 133
8. CURRICULUM VITAE 135

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
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
DOX	Doxycycline
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
GSC	Germline Stem Cell
HBSS	Hanks’ Balanced Salt Solution
HE	Heterozygote
HEPES	N-(-hydroxymethyl)piperazin,N’-3-propanesulfoneacid
hr(s)	hour(s)

ICM	Inner Cell Mass
IPTG	Isopropyl-ß-thiogalactopyranoside
kb	kilobase
kDa	Kilodalton
LIF	Leukaemia Inhibitory Factor
MEFs	Mouse Embryonic Fibroblasts
mRNA	messenger Ribonucleic acid
mg	milligram
ml	milliliter
mM	millimolar
µl	microlitre
µm	micrometre
µM	micromolar
min	minute
NaAc	Sodium acetate
NBT	Nitro-blue tetrazolium
NCBI	National Center for Biotechnology Information
Neo	Neomycin
ng	nanogram
NLS	Nuclear Localisation Signal
nm	nanometer
OD	Optimal Density
OHT	4-hydroxytamoxifen
ORF	Open Reading Frame
RT	Room Temperature
TAM	Tamoxifen
Tet	Tetracycline
UV	Ultraviolet Light
WT	Wildtype

Symbols of nucleic acids

A	Adenosine
C	Cystidine
G	Guanosine
T	Thymidine
U	Uridine

Symbols of amino acids

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

1. INTRODUCTION

1.1 Pelota (*Pelo*) is an evolutionarily conserved gene

The *Pelo* gene was originally identified in a mutagenesis screen of male fertility in *Drosophila* (Castrillon *et al.,* 1993). The *Pelo* gene has been then isolated and characterized in archaebacteria *Methanococcus jannaschii* (Bult *et al.,* 1996), *Sulfolobus solfataricus* (Ragan *et al.,* 1996), in yeast *Saccharomyces cerevisiae* (Davis and Engebrecht, 1998), in worm *Caenorhabditis elegans* (Gen Bank, Accession No.Z36238), in plant *Arabidopsis thaliana* (Gen Bank, Accession No.T20628), in human (Shamsadin *et al.,* 2000) and in mouse (Shamsadin *et al.,* 2002). The comparison of the predicted amino acid and nucleotide sequences of *Pelo* from twelve species reveals a high level of sequence conservation (Table 1.1). The protein sequence identity ranges from 55% between mouse and *S.cerevisiae* and 96% between mouse and human (Table 1.1). The encoded protein ranges between 378 and 432 amino acids.

The PROSITE protein motif library fails to reveal major structural motifs indicating a particular biological function of the Pelo protein. However, numerous putative phosphorylation motifs were detected, spread across the length of the predicted protein. This includes motifs for protein kinase C, casein kinase II and tyrosine kinase phosphorylation sites. Furthermore, two N-glycosylation and three N-myristoylation sites were detected in the mammalian protein. It is currently unknown which of these putative motifs are physiologically significant for posttranslational modifications. Davis and Engebrecht (1998) reported that the Pelota ortholog Dom34 of *S.cerevisiae* contains three regions that display similarity to conserved motifs (Fig 1.1): (1) A putative nuclear localization signal (NLS) is located at residues 173-177 of the yeast protein. This sequence PKKKR is similar to PKKKRK of simian virus 40 (SV40) large T antigen. (2) Pelo protein contains three eEF1α-like domains. The eEF1α-like domain is present in several proteins such as the translation elongation factor eEF1α and the translation release factors eRF1 and eRF3 (Frolova *et al.,* 1994). (3) A putative leucine zipper motif is located at the C-terminus of *S.cerevisiae* protein. Leucine zippers have been suggested to mediate protein-protein interactions in a diverse set of functionally unrelated proteins (Busch and Sassone-Corsi, 1990). Alignment of the Pelo protein from

twelve species revealed that all Pelo proteins share the NLS, eRF1 and leucine zipper motifs.

A phylogenetic analysis of Pelo primary amino acid sequences from different species using the TreeFam database is shown in Figure 1.2. The degree of sequence similarity is entirely congruent with the evolutionary relationship of the respective organisms. This is in support of the idea that the *Pelo* genes are all derived from the same ancestral gene.

Symbol	**Species**	**Gene ID**	**Chromosomal location**	**mRNA (bp)**	**Protein (aa)**	Mouse similarity (%)	
						DNA	protein
Pelo	*M.musculus*	105083	13 D2.2	1624	385	**-**	-
PELO	*H.sapiens*	53918	5q11.2	1567	385	**90.3**	96.4
LOC479338	*C.familiaris*	479338	4	1445	432	**89.1**	96.3
Pelo	*R.norvegicus*	294754	2q14	1550	385	**95.0**	98.7
RCJMB04_14b4	*G.gallus*	430689	un	1724	385	**80.1**	92.3
Pelo	*D.melanogaster*	34286	2L30C5	2102	395	**64.1**	66.3
ENSANGG00000005096	*A.gambiae*	1277715	3R	1384	384	**64.6**	66.7
R74.6	*C.elegans*	187888	III	1446	381	**56.0**	59.1
SPCC18B5.06	*S.pombe*	2539371	III	1173	390	**51.7**	48.3
PEL1	*A.thaliana*	828876	4	1168	378	**53.8**	51.5
OSJNBa0011F23.23	*O.sativa*	3066857	un	1137	378	**54.9**	53.1
DOM34	***S.cerevisiae***	**855731**	**XIV**	**2479**	**386**	36.0	55.0

Table 1.1: Identity (%) of the alignment of nucleotide and amino acid sequences for *Pelo* gene between mouse and other species, gene ID and their chromosomal localisation (NCBI).

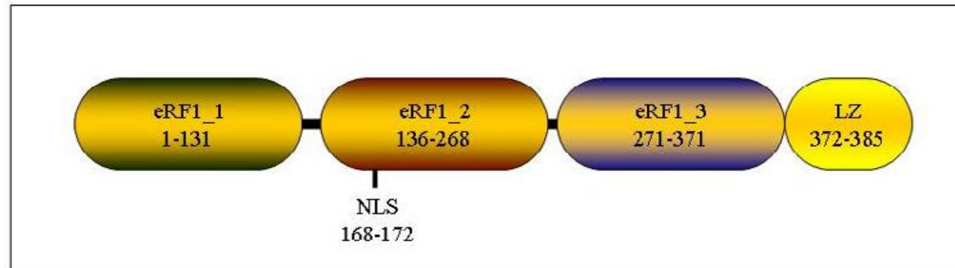


Figure 1.1: Schematic representation of Pelo protein structure

The Pelo protein contains three regions that display similarity to conserved motifs: (1) Residues 1-131, 136-268 and 271-371 are highly similar to a portion of eukaryotic peptide chain release factor subunit 1 (eRF1). The eRF1 family of proteins is involved in the termination step of protein synthesis. (2) A putative nuclear localization signal (NLS) is located at residues 168-172. This sequence is similar to that of simian virus 40 (SV40) large T antigen NLS (Dom34p, PKKKR; SV40, PKKKRKV). (3) A putative leucine zipper motif is located at the C-terminus. Leucine zippers have been suggested to mediate protein-protein interactions in a diverse set of functionally unrelated proteins.

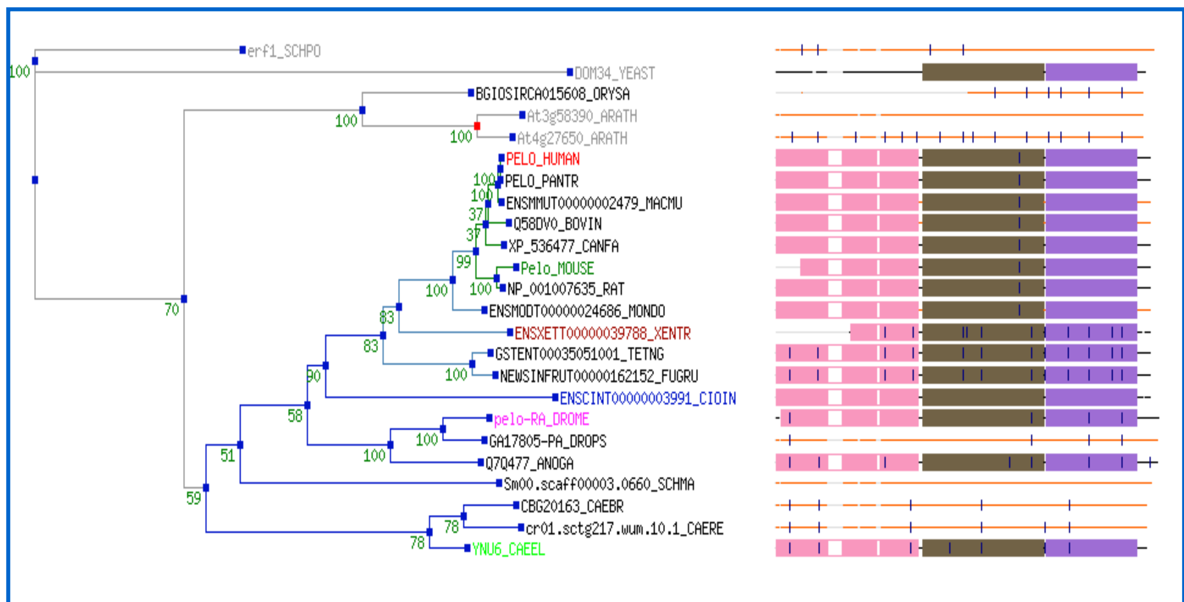


Figure 1.2: A phylogenetic tree of the Pelota gene

Alignment of the amino acid sequences of Pelota from different species using the TreeFam (Tree families) database. It was built by merging several trees together, including Phyml WAG tree, Phyml HKY, NJ dS and NJ dN tree. Branch lengths were estimated from HKY model. The red nodes stand for

duplications and blue for speciations or undefined nodes. The green numbers denote the bootstrap values. The colored boxes correspond to Pfam domains (eRF1_1; eRF1_2 and eRF1_3, respectively), while lines to non-domain regions. Dark lines or boxes show matched parts, while light ones gaps.

Abbreviations for species: **erf1_SCHPO,** *Schizosaccharomyces pombe*; **DOM34_YEAST,** *Saccharomyces cerevisiae*; **BGIOSIRCA015608_ORYSA,** *Oryza sativa*; **At3g58390_ARATH,** *Arabidopsis thaliana*; **At4g27650_ARATH,** *Arabidopsis thaliana;* **PELO_HUMAN,** *Homo Sapiens*; **PELO_PANTR,** *Pan troglodyte*; **ENSMMUT00000002479_MACMU,** *Macaca mulatta*; **Q58DV0_BOVIN,** *Bos taurus*; **XP_536477_CANFA,** *Canis familiaris*; **Pelo_MOUSE,** *Mus musculus*; **NP_001007635_RAT,** *Rattus norvegicus*; **ENSMODT00000024686_MONDO,** *Monodelphis domestica*; **ENSXETT00000039788_XENTR,** *Xenopus tropicalis*; **GSTENT00035051001_TETNG,** *Tetraodon nigroviridis;* **NEWSINFRUT00000162152_FUGRU,** *Takifugu rubripes;* **ENSCINT00000003991_CIOIN,** *Ciona intestinalis;* **pelo-RA_DROME,** *Drosophila melanogaster;* **GA17805-PA_DROPS,** *Drosophila pseudoobscura;* **Q7Q477_ANOGA,** *Anopheles gambiae;* **Sm00.scaff00003.0660_SCHMA,** *Schistosoma mansoni;* **CBG20163_CAEBR,** *Caenorhabditis briggsae;* **cr01.sctg217.wum.10.1_CAERE,** *Caenorhabditis remanei;* **YNU6_CAEEL,** *Caenorhabditis elegans.*

1.2 Expression and function of Pelota gene

The expression and function of *Pelo* has been studied in *Drosophila*, yeast and mouse. Expression analysis of *Pelo* in *Drosophila*, mouse and human revealed that the gene is widely expressed in all adult tissues as well as during embryonic development (Eberhart and Wasserman, 1995; Shamsadin *et al.,* 2000 and 2002).

The function of *Pelo* gene was first studied in *Drosophila.* Male mutants were found to be infertile (Eberhart and Wasserman, 1995). Spermatogenesis in *Pelo* mutants progresses normally during the four mitotic divisions. The 16 spermatocytes undergo a premeiotic S-phase and duplicate their DNA content. However, spermatocytes in the mutant arrested prior to full chromosome condensation, spindle pole organization, and nuclear breakdown. Metaphase and anaphase figures of the meiotic divisions, which are clearly recognised in squashed preparations of wildtype testis, were not observed in testis of the *Pelo* mutant. Although meiotic division arrests in *Pelota* deficient spermatocytes, germ cell differentiation continues, resulting in 4*N* spermatids with head and tail structures. These results indicate that the *Pelo* is required for the meiotic division during the G_2/M transition (Eberhart and Wasserman, 1995). The ovaries of

Pelota homozygous flies are very small. The mitotic zone of ovaries appears disorganised and often contains degenerating cells. Later stages of oogenesis are also affected. The female mutants produce less than 50% eggs. The results suggest that *Pelo* gene is also required for mitotic division in the ovary. Beside the effect of the mutation on spermatogenesis and oogenesis, the eyes of the *Pelo* homozygotes are up to 30% smaller than those of wildtype siblings. These results indicate that *Pelota* is required for *Drosophila* eye development.

Analysis of mitotic and meiotic division in the *dom34* mutant of *Saccharomyces cerevisiae*, which has a mutation in the *Pelota* orthologous gene, reveals that the *dom34* mutant exhibits a G1 delay, while the meiotic division occurs too rapid and fewer spores are produced as compared to wildtype. The yeast mutant also fails to segregate chromosomes properly (Davis and Engebrecht, 1998). Introduction of the *Drosophila* wildtype *pelota* transgene into a *dom34* mutant was found to result in substantial rescue of the *dom34* growth and sporulation defects (Eberhart and Wasserman, 1995).

To analyse the function of *Pelo* in mammalian species, *Pelo* gene was disrupted by homologous recombination in the mouse (Adham *et al.,* 2003). Heterozygous $Pelo^{+/-}$ male and female mice show no apparent abnormalities in development or fertility, indicating that one functional copy of the gene is sufficient for normal development. Genotyping of the progeny of heterozygous intercrosses indicated the absence of $Pelo^{-/-}$ pups and suggests an embryo-lethal phenotype. To assess the consequences of the *Pelo* mutation for embryonic development, embryos were collected from heterozygous intercrosses at different days of gestation and genotyped. The results revealed that $Pelo^{-/-}$ embryos die between E3.5 and E8.5.
To determine the causes and time of embryonic lethality, deciduae at E6.5, E7.5, and E8.5 were dissected and histologically analysed. Mutant embryos at E7.5 and E8.5 were found to be smaller and developmentally retarded as compared to their normal littermates. However, the embryonic germ layers (ectoderm, mesoderm and endoderm) are developed. These results indicate that $Pelo^{-/-}$ embryos have the capacity to generate some of the features of a gastrulated embryos despite impaired growth, and manifest their phenotype between days 6.5 and 7.5 of embryonic development.
In vitro culture of blastocysts revealed the impaired growth and the death of mitotic active inner cell mass (ICM) of $Pelo^{-/-}$ blastocysts, and the survival of mitotic inactive

trophoblast cells of *Pelo*$^{-/-}$ blastocysts. These results demonstrate that *Pelo* is essential for the normal mitotic division and for early embryonic development in the mouse. Analysis of the cellular DNA content revealed the significant increase of aneuploid cells in *Pelo*$^{-/-}$ embryos at E7.5. Therefore, the percentage of aneuploid cells at E7.5 may be directly responsible for the arrested development and suggests that *Pelo* is required for maintenance of genomic stability.

Early developmental arrest of the *Pelo* deficient embryos and subsequently the failure to establish a *Pelo*$^{-/-}$ cell line prevented us to define the cause of aneuploidy observed in cells of the mutant embryos. Even though *Pelo* is required for regulating cell cycle progression from yeast to mammals, it remains unclear how Pelo accomplish this function. Therefore, we have aimed the following objectives to study the distinct function of murine *Pelo* gene.

1.3 Objectives of this study

The first aim of this work was the generation of a polyclonal antibody against Pelo protein. This antibody should be used for the subcellular localisation of Pelo and for analysis of the expression pattern at protein level.

The second goal was to determine the consequence of *Pelo* knockdown on cell proliferation using Tet inducible siRNAs system.

The third purpose was the generation of a *Pelo* conditional knockout mouse utilising the inducible *Cre/loxP* recombination system to circumvent early embryonic lethality and to establish a *Pelo*$^{-/-}$ cell line.

The fourth aim was to generate transgenic *Pelo* mice to study the effect of overexpression of the gene.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals

1 kb DNA Ladder	Invitrogen, Karlsruhe
Acetic acid	Merck, Darmstadt
Acrylamide	Serva, Heidelberg
Agar	Difco, Detroit, USA
Agarose	Invitrogen, Karlsruhe
Ammonium acetate	Fluka, Neu Ulm
Ammonium persulfate	Sigma, Deisenhofen
Ampicillin	Sigma, Deisenhofen
Ampuwa	Fresenius, Bad Homburg
Aprotinin	Sigma, Deisenhofen
Aqua Poly/Mount	Polysciences, Inc, USA
Bacto-tryptone	Difco, Detroit, USA
Bacto-Yeast-Extract	Difco, Detroit, USA
BCIP	Boehringer, Mannheim
Bisacrylamide	Serva, Heidelberg
Blasticidin	Sigma, Deisenhofen
Blocking powder	Boehringer, Mannheim
BSA	Biomol, Hamburg
Cell culture media	PAN-Systems, Nürnberg
Chemiluminescent Substrate	Pierce, Rockford, IL
Chloroform	Baker, Deventer, NL
Colcemid	Sigma, Deisenhofen
Coomassie Blue G-250	Sigma, Deisenhofen
Dextran sulfate	Amersham Pharmalia, Freiburg
Diethyl pyrocarbonate (DEPC)	Sigma, Deisenhofen
Dimethyl sulfoxid (DMSO)	Merck, Darmstadt

Dithiothreitol	Sigma, Deisenhofen
dNTPs	Invitrogen, Karlsruhe
Doxycycline	Sigma, Deisenhofen
Dye Terminator Mix	Applied Biosystems,
EDTA	ICN Biomedicals, Eschwege
EGTA	Applichem, Darmstadt
Ethanol	Baker, Deventer, NL
Ethidium bromide	Sigma, Deisenhofen
Eukitt-quick hardening mounting medium	Fluka, Neu Ulm
FBS	Invitrogen, Karlsruhe
Ficoll 400	Amersham Pharmalia, Freiburg
Formaldehyde	Invitrogen, Karlsruhe
Formamide	Fluka, Neu Ulm
Freund´s adjuvant	Sigma, Deisenhofen
Glutaraldehyde	Sigma, Deisenhofen
Glycerol	Invitrogen, Karlsruhe
Glycine	Biomol, Hamburg
Goat serum	PAN-Systems, Nürnberg
HCl	Merck, Darmstadt
H_2O_2	Merck, Darmstadt
HEPES	Merck, Darmstadt
4-Hydroxytamoxifen	Sigma, Deisenhofen
Horse serum	PAN-Systems, Nürnberg
IPTG	Biomol, Hamburg
Isopropanol	Merck, Darmstadt
KCl	Merck, Darmstadt
KH_2PO_4	Merck, Darmstadt
Lambda DNA	Roche, Penzberg
Leupeptin	Sigma, Deisenhofen
Lipofectamine 2000 TM	Invitrogen, Karlsruhe
Methanol	Merck, Darmstadt
2-Mercaptoethanol	Serva, Heidelberg
Methyl benzoat	Fluka, Neu Ulm
$MgCl_2$	Merck, Darmstadt

Milk powder	Roth, Karlsruhe
Mineral oil	Sigma, Deisenhofen
MOPS	Merck, Darmstadt
NaCl	Merck, Darmstadt
Na_2HPO_4	Merck, Darmstadt
NaH_2PO_4	Merck, Darmstadt
$NaHCO_3$	Merck, Darmstadt
NaN_3	Merck, Darmstadt
NaOH	Merck, Darmstadt
NBT	Roche, Penzberg
Nonidet P40	Fluka, Neu Ulm
Nocodazol	Sigma, Deisenhofen
NuPAGE Novex Bis-Tris 4-12% Gel	Invitrogen, Karlsruhe
NuPAGE MOPS SDS running buffer	Invitrogen, Karlsruhe
NuPAGE SDS sample buffer	Invitrogen, Karlsruhe
Orange G	Sigma, Deisenhofen
OptiMEM I	Invitrogen, Karlsruhe
Paraformaldehyde	Merck, Darmstadt
PBS	PAN-Systems, Nürnberg
Penicillin/Streptomycin	PAN-Systems, Nürnberg
Peptone	Roth, Karlsruhe
Phalloidin	Sigma, Deisenhofen
Phenol	Biomol, Hamburg
Phosphoric acid	Merck, Darmstadt
Picric acid	Fulka, Neu Ulm
Protein A/G PLUS Agarose	Santa Cruz Biotechnology
Proteinase K	Roche, Penzberg
Protein marker	Biorad, Sigma
[α^{32}P]-dCTP	Amersham Pharmalia, Braunschweig
Rediprime™ II	Amersham Pharmacia, Freiburg
RNase A	Qiagen, Hilden
RNase away	Biomol, Hamburg
RNase Inhibitor	Roche, Penzberg
RNA length standard	Invitrogen, Karlsruhe

Salmon sperm DNA	Sigma, Deisenhofen
SDS	Serva, Heidelberg
SeeBlue Plus2 Pre-Stained Standart	Invitrogen, Karlsruhe
Select Peptone	Gibco/BRL, Eggenstein
S.O.C Medium	Invitrogen, Karlsruhe
Sodium acetate	Merck, Darmstadt
Sodium citrate	Merck, Darmstadt
Sun flower oil	Sigma, Deisenhofen
SuperScript II	Invitrogen, Karlsruhe
T4 DNA ligase	Promega, Mannheim
Tamoxifen	Sigma, Deisenhofen
TEMED	Serva, Heidelberg
TRI reagent	Sigma, Deisenhofen
Tris base	Sigma, Deisenhofen
Triton X-100	Serva, Heidelberg
Trypsin	PAN-Systems, Nürnberg
Tween-20	Sigma, Deisenhofen
Vectashield (DAPI)	Vector, Burlingame
X-Gal	Biomol, Hamburg
Xylene	Merck, Darmstadt
Xylencyanol	Bio-Rad, München
Yeast extract	Roth, Karlsruhe
Zeocin	Sigma, Deisenhofen

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

2.1.2 Solutions, buffers and media

2.1.2.1 Agarose gel electrophoresis

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

5 x TBE buffer	450 mM Tris base 450 mM Boric acid 20 mM EDTA (pH 8)
Glycerol loading buffer –I	10 mM Tris/HCl (pH 7.5) 10 mM EDTA (pH 8) 0.025% Bromophenol blue 0.025% Xylenecyanol 30% Glycerol
Glycerol loading buffer –II	10 mM Tris/HCl (pH 7.5) 10 mM EDTA (pH 8) 0.025% Orange G 30% Glycerol

2.1.2.2 SDS-PAGE

40% Acrylamide stock solution	Acrylamide 29.2% (w/w) Bis-acrylamide 0.8% (w/w) 10% Ammonium persulfate solution in H_2O
Sample buffer (2x)	0.5 M Tris/HCl (pH 6.8) 20% Glycerol 4% SDS 10% β-Mercaptoethanol

Running buffer (5x)	25 mM Tris/HCl (pH 8.3)
	192 mM Glycine
	0.1% SDS
Stacking gel buffer (4x)	0.5 M Tris/HCl (pH 6.8)
	0.4% SDS
Separating gel buffer (4x)	1.5 M Tris/HCl (pH 8.3)
	0.4% SDS

2.1.2.3 Frequently used buffers and solutions

Denaturation solution	1.5 M NaCl
	0.5 M NaOH
Denhardt´s solution (50x)	1% BSA
	1% Polyvinylpyrrolidon
	1% Ficoll 400
Depurination solution	250 mM 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
Bouin’s solution	15 volume of Picric acid (in H_2O)
	5 volumes Formaldehyde
	1 volume Acetic acid

Hybridisation solution I	5 x SSPE solution 5 x Denhardt´s solution 0.1% SDS
Hybridisation solution II	5 x SSC 5 x Denhardt´s solution 10% Dextran sulfate 0.1% SDS
Ligation buffer (10x)	600 mM Tris/HCl (pH 7.5) 80 mM $MgCl_2$ 100 mM DTT
Lysis buffer I	100 mM Tris/HCl (pH 8.0) 100 mM NaCl 100 mM EDTA 0.5% SDS
Lysis buffer II	100 mM Tris/HCl (pH 8.0) 5 mM EDTA 200 mM NaCl 0.2% SDS 100 µg/ml Proteinase K
Lysis buffer III (embryo)	50 mM Tris/HCl (pH 8.0) 0.5 mM EDTA (pH 8.0) 0.5% Tween 20 0.2 mg/ml Proteinase K
Lysis buffer IV	1% Tween 20 1% Triton X-100 5 mM EDTA 10 mM Tris pH7.5 2 mM DTT

	20µl/ml Proteinase K
Lysis buffer A	10 mM Tris/HCl pH 8.0
	1 mM EDTA
	2.5% SDS
	1 mM PMFS
Lysis buffer B	50 mM Tris/HCl, pH 7.5
	150 mM NaCl
	1% Nonidet P40
	0.5% Sodium deoxycholate
	1 Protease inhibitor cocktail tablet
10 X MOPS Buffer	41.8 g MOPS
	16.6 ml 3 M Sodium acetate
	20 ml 0.5 M EDTA
	in 1 liter of DEPC water
	adjust pH to 6.75
Neutralisation solution	1.5 M NaCl
	1 M Tris/HCl (pH 7.0)
PBS buffer	130 mM NaCl
	7 mM Na_2HPO_4
	4 mM NaH_2HPO_4
PBT buffer	0.1% Tween-20 in PBS (1x)
SSC (20x)	3 M NaCl
	0.3 M Na_3 citrate (pH 7.0)
SSPE (20x)	0.02 M EDTA
	0.2 M NaH_2PO_4
	3.6 M NaCl (pH 7.0)

Stop-Mix I	95% Formamide 20 mM EDTA 0.05% Bromphenol blue 0.05% Xylene cyanol
Stop-Mix II	15% Ficoll 400 200 mM EDTA 0.1% Orange G
TE buffer	10 mM Tris/HCl (pH 8.0) 1 mM EDTA
Washing solution I	2 x SSC 0.1% SDS
Washing solution II	0.2 x SSC 0.1% SDS

2.1.3 Laboratory materials

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

Cell culture flask	Greiner, Nürtingen
Culture slides	BD Falcon, Heidelberg
Disposable filter Minisart NMI	Sartorius, Göttingen
Filter paper 0858	Schleicher and Schüll, Dassel
Hybond C	Amersham, Braunschweig
Hybond N	Amersham, Braunschweig
HPTLC Aluminum folio	Merck, Darmstadt
Microcentrifuge tubes	Eppendorf, Hamburg
Petri dishes	Greiner, Nürtingen
Pipette tips	Eppendorf, Hamburg
RotiPlast paraffin	Roth, Karlsruhe
Transfection flasks	Lab-Tek/Nalge, Nunc, IL, USA
Superfrost slides	Menzel, Gläser
Whatman blotting paper	Schleicher and Schüll, Dassel (GB 002, GB 003 and GB 004)
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. Glasswares were sterilised overnight in an oven at 220°C.

2.1.5 Media, antibiotics and agar-plates

2.1.5.1 Media for bacteria

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

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

2.1.5.2 Media for cell and embryo culture

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

Embryonic stem (ES) cell medium:

DULBECCO´s Modified Eagles Media (DMEM)

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

Fibroblast cell medium (MEFs):

DULBECCO´s Modified Eagles Media (DMEM)

2 mM L-Glutamine
10% FCS
1% penicillin/streptomycin

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

Freezing medium:	20%	FCS
	10%	DMSO in DMEM

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.

Antibiotics	Stock solution	Working solution
Ampicillin	50 mg/ml	50 µg/ml
Kanamycin	25 mg/ml	50 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**	K-12 strain, F^- Φ80d *lacZ*ΔM15 endA1 *rec*A1 hsdR17 (r_{k-}, m_{k+}) sup E44 *thi*-1 d^- *gyr*A96 Δ(lacZYA-arg) (Invitrogen, Karlsruhe)
***E. coli* BL21 (DE3)**	B strain, F^- *omp*T *hsd*S_B(r_{B^-} m_{B^-}) *gal, Dcm* (Novagen, Darmstadt)

2.1.7 Eukaryotic strains

NIH 3T3	Mouse embryonic fibroblast cell line, ATCC, Rockville, USA
HeLa	Human cervical adenocarcinoma cell line, ATCC, Rockville, USA
LNCaP	Human prostate carcinoma cell line, ATCC, Rockville, USA
PC-3	Human prostate carcinoma cell line, ATCC, Rockville, USA

2.1.8 Plasmids

pBluesript SK (+/-)	(Stratagene, La Jolla, USA)
pcDNA6/TR	(Invitrogen BV, Groningen, The Netherlands)
pGEMTeasy	(Promega, Wisconsin, USA)
pEF-BOS	(BCCM^{TM}/LMBP Plasmid collection, Belgium)
pET 41 (a-b)	(Novagen, Darmstadt, Germany)
pTER^{+}	(Invitrogen, Darmstadt, Germany)
pUB/V5-His A	(Invitrogen, Darmstadt, Germany)

2.1.9 Synthetic oligonucleotides

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

Name	Sequence
PelocKO-F	5’ CTTCCCAGTGCCAGAACTTT 3’
PelocKO-R	5’ AATTCGGAAACACCAAGTCG 3’
PGK-1	5’ TCTGAGCCCAGAAAGCGAAGG 3’
Pelo-F11	5’ TGAGCCCAGACTGTACGTGAC 3’
Pelo-R13	5’ TTCCCGGAACATCCCTGTGTG 3’
PGK3_B	5’ GGATGTGGAATGTGTGCGAGG 3’
Pelo 5’-F1	5’ GCATGTAACAGGAGTTATGATGGA 3’
Pelo 5’-R1	5’ TACTACATTATTTGCCCTTCTGTG 3’
Pelo 5’-F2	5’ AGACTGCAGCTGAGTCAGTACTGA 3’
Pelo 5’-R2	5’ GTTCACTTTGTATAGGAGAGAGAT 3’
Pelo-3’-F1	5’ GCAAGATTTTGACTACATTCAC 3’
Pelo-3’-R1	5’ CATGTGCTACACTGAGGCTTGT 3’
mPelotaF1Wlof	5’ TGAAGGGGACCAATATCCAA 3’
mPelotaR1Wlof	5’ AAGGAATTTGGAGCGGTTTT 3’
Pelo-GFPR1	5’ CCCTCTAGAACTAGAGAATATCCTAACGGTGC 3’
Pelo-GFPR2	5’ CCCTCTAGATTTAGTGTCTGAAAGGCGGCTAG 3’
Pelo:3-F1	5’ GCAAGATTTTGACTACATTCAC 3’
Pelo:3-R1	5’ CATGTGCTACACTGAGGCTTGT 3’
Pelo-Lox.F1	5’ CACGCTAAGGTGCAGAATGAT 3’
Pelo-Lox.R1	5’ CGGTCAGCGCTCTGAAAA 3’
Neo-F1	5’ GCCTTCTATCGCCTTCTTGA 3’
Pelo-Lox.R4	5’ GAGGAAAACCAAGAAAAGTTGG 3’
RosaCreER-F	5’ ACCAGCCAGCTATCAACTC 3’
RosaCreER-R	5’TATACGCGTGCTAGCGAAGATCTCCATCTTCCAGCAG 3’
EIIa-CreF	5’ CCAGGCTAAGTGCCTTCTCTACA 3’
EIIa-CreR	5’ AATGCTTCTGTCCGTTTGCCGGT 3’
mPelo-3ext-F	5’ GTGCGAGAAGGCCAGTTCTA 3’
mPelo-3ext-R	5’ AGGCTGACCATCTGTGGTGT 3’
pET-1g-F	5’ GGGGAAGTCAAAGCCTTGGATG 3’
pET-1g-R	5’ TTCGGGAACAGGGAAGCGGAGA 3’
pET-2a-F	5’ CCCAACCGCCAGTTCACCCTGG 3’

pET-2a-R	5’ AGTGTCTGAAAGGCGGCTAGCC 3’
hPelo-F20	5’ CCCTCTAGACTTCCTTGGCCATGAAGCTCG 3’
hPelo-R20	5’ GGGTCTAGACTTGCAGCTTTCTGTCACAAGG 3’
mPelo.RT-PCR-F1	5’ TCTGGGTTAACTGGATCGTG 3’
mPelo.RT-PCR-R1	5’ GAGGACTCGGTCTGGACCTT 3’
mPelo.RT-PCR-F2	5’ ATCCAGCGCCACATAAACTT 3’
mPelo.RT-PCR-R2	5’ CTCTCCACCTGCTTGAGTCC 3’
mPelo.RT-PCR-F3	5’ CTGCTTAGTCACTCCCAGCA 3’
mPelo.RT-PCR-R3	5’ CGGAAGAGCTCGTCACTGAT 3’
mPelo.RT-PCR-F4	5’ GATGTGGCAGCTGTGGTCAT 3’
mPelo.RT-PCR-R4	5’ CTCCTTCACACTGTCCACCA 3’
mGAPDH-F	5’ CACCACCAACTGCTTAGCC 3’
mGAPDH-R	5’ CGGATACATTGGGGGTAGG 3’
PeloHA-F1	5’ CCCGAATTCAGCTCGTGAGGAAGAACATC 3’
PeloHA-R1	5’ ACCCTCGAGTTAATCCTCTTCAGAACTGGAATC 3’
GFP-Filamin-F1	5’ AGCCAAGCTTGCCCCTCCAAGGTGAAGATGG 3’
GFP-Filamin-R1	5’ GGGAGATCTCAGACTCAGGGCACCACAACG 3’
GFP-Actinin-F1	5’ AGCCAAGCTTGCAACCGGCCTGCCTTCATGC 3’
GFP-Actinin-R1	5’ GGGAGATCTGTGGATTAGAGGTCACTCTCG 3’
hPelo-F20	5’ CCCTCTAGACTTCCTTGGCCATGAAGCTCG 3’
hPelo-R20	5’ GGGTCTAGACTTGCAGCTTTCTGTCACAAGG 3’
Trans.pEF-F	5’ CATTCTCAAGCCTCAGACAGTG 3’
Trans.pUB-F	5’ TCAGTGTTAGACTAGTAAATTG 3’
Trans.hPelo-R1	5’ GAGGACTCTGTCTGTACCTTG 3’
Trans.hPelo-R2	5’ CAGGCTTGAGAGTCGAAGTC 3’
GSF-F1	5’ GAGTATCAGGTCTCCCACGTG 3’
GSF-R1	5’ GGAGCTTCCTTTCACACACAG 3’
GSF-R2	5’ AGGGGTGCAAGGACCCAGATG 3’
GFP-Filamin-R1	5’ GGGAGATCTCAGACTCAGGGCACCACAACG 3’
pUB-F1	5’ CTCCTCGGTCTCGATTCTACG 3’
pUB-R1	5’ AAAGGACAGTGGGAGTGGCAC 3’
pUB-R2	5’ AGGGTTAGGGATAGGCTTACC 3’
hPelo-F21	5’ GACCGGTTTTCCAGGAGCAGT 3’

BC057160-706_S	5' GATCCCCACCGCTCCAAATTCCTTCATTCAAGAG ATGAAGGAATTTGGAGCGGTTTTTA 3'
BC057160-706_As	5'AGCTTAAAAAACCGCTCCAAATTCCTTCATCTCTTGAATGAA TGAAGGAATTTGGAGCGGTGGG 3'
PeloRNAiS1	5'GATCCCCCACAAGTACTCCCTGAAAGTTCAAGAGACTTTCAG GGAGTACTTGTGTTTTTGGAAA 3'
PeloRNAiA1	5'AGCTTTTCCAAAAACACAAGTACTCCCTGAAAGTCTCTTGAA CTTTCAGGGAGTACTTGTGGGG 3'
PeloRNAiS2	5'GATCCCCGTTCACCCTGGCCAAGAAATTCAAGAGATTTCTTG GCCAGGGTGAACTTTTTGGAAA 3'
PeloRNAiA2	5'AGCTTTTCCAAAAAGTTCACCCTGGCCAAGAAATCTCTTGAA TTTCTTGGCCAGGGTGAACGGG 3'
CNXsense	5' (Phosp)CGATGCGGCCGCC 3'
CNXanti	5' (Phosp)TCGAGGCGGCCGCAT 3'
Sense-CNX	5' CCCCCCATCGATGCGGCCGCC 3'
Anti-SNX	5' AAAACCCTCGAGGCGGCCGCA 3'

2.1.10 Mouse strains

Strains C57BL/6J, 129/Sv, CD-1 and NMRI were initially ordered from Charles River Laboratories, Wilmington, USA, and needed in Animal Facility of Institute of Human Genetics, Göttingen. ROSA26CreER^{T} (Berns A, Netherlands) and EIIaCre (Lakso *et al.,* 1996) mice were kindly provided by Prof. Dr. med. H. Hahn, Institute of Human Genetics, Göttingen.

2.1.11 Antibodies

Goat anti-mouse IgG alkaline phosphatase conjugate	Sigma, Deisenhofen
Goat anti-rabbit IgG alkaline phosphatase conjugate	Sigma, Deisenhofen
Goat anti-rabbit IgG Cy3 conjugate	Sigma, Deisenhofen
Goat anti-rabbit GFP antiserum	Sigma, Deisenhofen
Rabbit anti-mouse IgG Cy3 conjugate	Sigma, Deisenhofen
Goat anti-rabbit IgG FITC conjugate	Sigma, Deisenhofen
Rabbit anti-mouse IgG FITC conjugate	Sigma, Deisenhofen
Mouse monoclonal anti α-tubulin	Sigma, Deisenhofen

Mouse monoclonal anti actin	Sigma, Deisenhofen
Mouse monoclonal anti α-actinin	Sigma, Deisenhofen
Goat anti-rabbit IgG horse radish preoxidase conjugate	Sigma, Deisenhofen
Rabbit anti-mouse IgG horse radish preoxidase conjugate	Sigma, Deisenhofen
Rabbit anti Pelota polyclonal antibody	Institute of Human Genetics
Rabbit anti SX polyclonal antibody	Institute of Human Genetics

Polyclonal anti-Pelota and -SX antibodies were generated in the Institute of Human Genetics by immunisation of rabbits with GST-Pelota and GST-SX fusion proteins.

2.1.12 Enzymes

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

2.1.13 Radioactive substances

α-^{32}P-dCTP	(Amersham, Braunschweig)

2.1.14 Kits

BigDye Terminator Cycle	(Applied Biosystems)
Endo Free Plasmid Maxi Kit	(Qiagen, Hilden)
GST-Bind kit	(Novagen, Darmstadt)
HiTrap NHS-activated	(Amersham Biosciences, Sweden)
Megaprime DNA Labeling Kit	(Amersham Pharmacia, Freiburg)
Maxi Plasmid Kit	(Qiagen, Hilden)
Mega Plasmid Kit	(Qiagen, Hilden)
Midi Plasmid Kit	(Invitrogen, Karlsruhe)
Mini Plasmid Kit	(Qiagen, Hilden)
PCR Purification Kit	(Qiagen, Hilden)
Protein Refolding Kit	(Novagen, Darmstadt)
QIAquick Gel Extraction Kit	(Qiagen, Hilden)
RNAeasy Kit	(Qiagen, Hilden)
Rediprime^{TM} II Random Prime	(Amersham Pharmacia, Freiburg)
Labelling System	(Qiagen, Hilden)
One step RT-PCR kit	(Qiagen, Hilden)

2.1.15 Equipment

Autoclave	(Webeco, Bad Schwartau)
Centrifuge 5415D	(Eppendorf, Hamburg)
Centrifuge 5417R	(Eppendorf, Hamburg)
Biophotometer	(Eppendorf, Hamburg)
DNA Sequencer Modell Megabace 1000	(Amersham, Freiburg)
Microscope BX60	(Olympus, München)
GeneAmp PCR System 9600	(Perkin Elmer, Berlin)
Histocentre 2 embedding machine	(Shandon, Frankfurt aM.)
Microtiterplate-Photometer	(BioRad laboratories, München)
Molecular Imager FX	(BioRad laboratories, München)
Phosphoimager Screen	(BioRad laboratories, München)
Semi-Dry-Blot Fast Blot	(Biometra, Göttingen)
Spectrophotometer Ultraspec 3000	(Amersham, Freiburg)
SpeedVac concentrator SVC 100H	(Schütt, Göttingen)
Thermomixer 5436	(Eppendorf, Hamburg)
Turboblotter™	(Schleicher & Schüll, Dassel)
UV Stratalinker™ 1800	(Leica, Nußloch)
X-Ray Automatic Processor Curix 60	(Agfa, München)

2.2 Methods

2.2.1 Isolation of nucleic acids

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

2.2.1.1.1 Small-scale isolation of plasmid DNA (Birnboim and Doly, 1979)

A single *E.coli* colony was inoculated in 5 ml of LB medium with the appropriate antibiotic and incubated in a shaker for 12-16 hrs at 37°C with a speed of 160 rpm. 0.2 ml of this saturated culture was used for making glycerol stocks and rest of the culture was centrifuged at 5000 x g for 10 min. The pellet was resuspended in 100 µl of resuspension solution P1. The bacterial cells were lysed with 200 µl of lysis solution P2, incubated at RT for 5 min and then neutralised with 150 µl of neutralisation solution P3. The precipitated solution was incubated at RT for 5 min and centrifuged at 13000 x g at RT. The supernatant was transferred into a new tube, and 1 ml of 100% ethanol was added to precipitate the DNA. It was then stored at -20°C for 30 min, centrifuged at full speed for 20 min, and finally the pellet was washed with 350 µl of 70% ethanol and after air-drying dissolved in 30 µl of either dH_2O or TE buffer.

P1:	50 mM	Tris/HCl, pH 8.0
	10 mM	EDTA
	100 µg/ ml	RNase A
P2:	200 mM	NaOH
	1%	SDS
P3:	3.0 M	Potassium acetate, pH 5.5

2.2.1.1.2 Large-scale preparation of Endotoxin free plasmid DNA using the Qiagen Maxi Kit

A single clone was inoculated in 5 ml LB medium with appropriate antibiotic as a pre-culture for 6-8 hrs in a 37°C shaker. In 100 ml LB medium with appropriate antibiotic, this pre-culture was added in a dilution of 1/500 fold and incubated overnight at 37°C with shaking. The saturated culture was centrifuged at 6000 x g for 15 min. The pellet was resuspended in 5 ml of solution P1 and cells were lysed with P2 and P3 as described above. The precipitated solution was centrifuged at 20000 x g for 30 min at 4°C. Meanwhile, the column (Qiagen-tip) that was provided with the midi preparation kit was equilibrated with 10 ml of QBT solution. After centrifugation, the lysate was poured into this equilibrated column to allow the DNA to bind with the resin present in the bed of the column. The column was then washed twice with 10 ml of solution QC. Finally, the DNA was eluted with 5 ml of QF solution. To precipitate the DNA, 3.5 ml of isopropanol was added, mixed thoroughly and then centrifuged at 14000 x g for 30 min at 4°C. The DNA pellet was washed with 70% ethanol and dissolved in 100 µl of TE buffer.

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

2.2.1.2 Isolation of genomic DNA

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

The method employed was the same as that of Laird et al. (1991). 1 cm of the tail from a mouse was incubated in 700 µl of lysis buffer I containing 35 µl proteinase K (10µg/µl) at 55°C overnight in Thermomixer 5436. The tissue lysate was centrifuged at 14000 x g for 15 min and the supernatant was transferred into a new e-cup. After transferring, DNA was precipitated by adding an equal volume of isopropanol and mixed by inverting several times and centrifuged at 14000 x g at RT for 15 min. DNA was washed with 1 ml of 70% ethanol, dissolved in 80-100 µl of dH_2O and incubated at 60°C for 10 min.

2.2.1.2.2 Isolation of genomic DNA from cultured cells

To isolate the DNA from cultured cells, cells were washed with PBS and incubated overnight in 500 µl of lysis buffer II at 37°C. Equal volume of isopropanol was added and mixed by inverting several times followed by incubation for 10 min at RT, then centrifuged for 15 min at maximal speed to precipitate the DNA. After washing with 70% ethanol, the DNA was dissolved in 80 µl of dH_2O and incubated at 60°C for 10 min.

2.2.1.3 Isolation of total RNA from tissue samples and cultured cells

Total RNA isolation reagent is an improved version of the single-step method for total RNA isolation. The composition of reagent includes phenol and guanidine thiocyanate in a mono-phase solution. 100-200 mg of tissue sample was homogenised in 1-2 ml of TRI Reagent by using a glass-teflon homogeniser. The sample volume should not exceed 10% of the volume of reagent used for the homogenisation. To isolate total RNA from cultured cells, 350 µl of reagent was added to the Petri dish (6 cm). Cells were homogenised with a rubber stick and the lysate was transferred into a microcentrifuge

tube. The homogenate was incubated at 4°C for 5 min to permit the complete dissociation of nucleoprotein complexes. Then, 0.2 ml of chloroform was added, mixed vigorously, and stored at 4°C for 10 min. After centrifugation at 12000 xg for 15 min at 4°C, the colourless upper aqueous phase was transferred into a new tube. The RNA was precipitated by adding 0.5 ml of isopropanol. Finally, the pellet was washed twice with 75% ethanol and dissolved in 80-100 µl of RNase free water or DEPC-dH_2O.

2.2.2 Determination of nucleic acid concentration

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

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

C = concentration of sample (µg/µl)

E 260= ratio of extinction at 260 nm

E 320= ratio of extinction at 320 nm

f = dilution factor

c = concentration (standard) / absorption (standard)

For double stranded DNA: c = 0.05 µg/µl

for RNA : c = 0.04 µg/µl

for single stranded DNA : c = 0.03 µg/µl

2.2.3 Gel electrophoresis

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

2.2.3.1 Agarose gel electrophoresis of DNA

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

2.2.3.2 Agarose gel electrophoresis of RNA (Hodge, 1994)

Single-stranded RNA molecules often have complementary regions that can form secondary structures. Therefore, RNA was run on a denaturing agarose gel that contained formaldehyde, and before loading, the RNA was pre-treated with formaldehyde and formamide to denature the secondary structure of RNA. 1.25g of agarose was added to 100 ml of 1 x MOPS Buffer and dissolved by heating in a microwave. After cooling it to about 50°C, 25 ml of formaldehyde (37%) was added, stirred and poured into a horisontal gel chamber.
RNA samples were treated as follows:

10 – 20 µg RNA
10 x MOPS Buffer
3 µl Formaldehyde
8 µl Formamide (40%)
1.5 µl Ethidium bromide

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

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

The NuPAGE® Pre-Cast Gel System (Invitrogen) is a polyacrylamide gel system for high performance gel electrophoresis and is based on SDS-PAGE gel chemistry. It consists of NuPAGE® Bis-Tris Pre-Cast Gels and specially optimised buffers which have an operating pH of 7.0, giving the system advantages over existing polyacrylamide gel systems with an operating pH of 8.0. The neutral pH increases the stability of the proteins and provides better electrophoretic results. To 10 µl of whole protein lysate 10 µl of 2 x Protein sample buffer was added. The samples were denaturated by boiling in the water bath for 10 min, cooled at RT for 5 min and loaded in SDS-PAGE (NuPage 4-12% Bis-Tris gel). The gel electrophoresis was run in 1 x MOPS buffer (Invitrogen). To determine the molecular weight of the proteins on the gel, 10 µl of a pre-stained molecular weight standard (See Blue Plus2, Invitrogen) was also loaded. The gel was run at 100 V for 2 hrs at RT.

2.2.4 Purification of DNA fragments from agarose gel

2.2.4.1 QIAquick gel extraction method

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

2.2.5 Enzymatic modifications of DNA

2.2.5.1 Restriction of DNA

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

2.2.5.2 Ligation of DNA fragments

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

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

Blunt-end ligations were carried out at 16°C for overnight, whereas overhang-end ligations were carried out at room temperature for 2-4 hrs.

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

Taq polymerase and other DNA polymerases have a terminal transferase activity that results in the non-template addition of a single nucleotide to the 3' ends of PCR products. In the presence of all 4 dNTPs, dATP is preferentially added. This terminal

transferase activity is the basis of the TA-cloning strategy. For cloning of PCR products, the pGEMT or pGEMTeasy vector systems that have 5‘T overhangs were used.
The following substances were mixed:

50 ng of pGEMTeasy vector
150 ng PCR product
1 µl of T4 DNA Ligase buffer (x10)
1 µl of T4 DNA Ligase
in a total volume of 10 µl

The substances were mixed by pipetting and incubation was done overnight at 16°C.

2.2.6 Preparation of competent *E.coli* bacteria (Dagert and Ehrlich, 1979)

Competent bacterial cells are generated by a physical cell wall modification that facilitates DNA uptake. LB medium (100 ml) was inoculated with a single colony of *E.coli* (strain DH5α) and the culture was grown at 37°C to OD_{600}=0.6. Bacteria were centrifuged (10 min, 4°C, 3000 x g), and the pellet was resuspended in 50 ml of sterile 50 mM $CaCl_2$ solution (4°C) and incubated on ice for 30 min. The suspension of bacteria was centrifuged (10 min, 4°C, 3000 x g) and the pellet was resuspended in 10 ml of sterile 50 mM $CaCl_2$ (4°C) with 15% glycerol. The mixture was dispensed into aliquots of 100 µl and stored at -80°C. Mostly, competent DH5α were purchased from Invitrogen.

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

Transformation of bacteria was done by gently mixing one aliquot of competent bacteria (50 µl) with 5-10 µl of ligation reaction. After incubation for 30 min on ice, bacteria were heat shocked for 45 sec at 37°C or 42°C, cooled down for 2 min on ice. After adding 450-900 µl of LB medium, bacteria were incubated at 37°C, 200 rpm, 1 hr, to allow recovery of heat shocked bacteria which were then plated out on LB-agar plates.

2.2.8 Polymerase Chain Reaction (PCR)

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

2.2.8.1 PCR amplification of DNA fragments

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

10 ng	DNA
1 µl	forward primer (10 pmol)
1 µl	reverse primer (10 pmol)
1 µl	10 mM dNTPs
5 µl	10 x PCR buffer
1.5 µl	50 mM $MgCl_2$
0.5 µl	*Taq* DNA Polymerase (5 U/µl)
Up to 50 µl	dH_2O

The reaction mixture was placed in a 200 µl reaction tube and placed in a thermocycler. Thermal cycling was carried out for 35 cycles with denaturation at 97°C for 30 sec, annealing at 55-60°C for 30 sec and extension at 72°C for 1 min.

2.2.8.2 Reverse transcription PCR (RT-PCR)

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

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

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

2.2.8.3 One-Step RT-PCR

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

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

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

2.2.9 Protein methods

2.2.9.1 Isolation of total protein

100 mg of tissue was homogenised in 500 µl lysis buffer A with a Teflon-glass headed pestle. Then, homogenised samples were handled with ultrasound on ice 2 x 2 min. The samples were centrifuged at 8000 x g for 20 min at 4°C and the supernatant was aliquoted in several microcentrifuge tubes. The tubes were frozen in liquid nitrogen and

stored at -80°C. 5 x 10^6 cells/ml were washed with cold phosphate buffered saline and resuspended in 50 µl of lysis buffer B. The cells were allowed to swell on ice for 30 min, after which the cells were resuspended in 300 µl of lysis buffer B. After homogenisation, samples were handled with ultrasound on ice 2 x 30 sec and centrifuged at 24000 x g for 20 min at 4°C. The protein extract was either used immediately or stored at –80°C for later use.

2.2.9.2 Determination of protein concentration (Bradford, 1976)

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

2.2.10 Blotting techniques

2.2.10.1 Southern blotting of DNA onto nitrocellulose filter (Southern, 1975)

In Southern blotting, the transfer of denaturated DNA from agarose gels onto nitrocellulose membrane is achieved by capillary flow. 20 x SSC buffer, in which nucleic acids are highly soluble, is drawn up through the gel onto nitrocellulose membrane, taking with it the single-stranded DNA that becomes immobilised in the membrane matrix.

After electrophoresis of DNA, the gel was treated with 0.25 M HCl for depurination. It was followed by denaturation solution for 30 min and 45 min in neutralisation solution. The transfer of the DNA to the nitrocellulose membrane was done in a Turbo-Blot-

apparatus (Schleicher & Schuell, Dassel). About 25-28 Whatman filter papers (GB 003) were layered on a Stack Tray, followed by 4 Whatman filter papers (GB 002) and 1 Whatman filter paper (GB 002) soaked with 20 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 top of the blot. The transfer was carried out for overnight. Finally, after disassembling of the blot, the filter was washed briefly in 2 x SSC and the DNA was fixed onto the filter by either baking it at 80°C for at last 2 hrs or by UV-crosslinking in UV Stratalinker 1800.

2.2.10.2 Northern blotting of RNA onto nitrocellulose filter

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

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

Anode buffer I	0.3 M Tris/HCl, pH 10.4 20% Methanol
Anode buffer II	25 mM Tris/HCl, pH 10.4 20% Methanol
Cathode buffer	40 mM ε-Aminocaproic acid 25 mM Tris/HCl, pH 9.4 20% Methanol

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

Membrane staining

Membrane was stained with Coomassie blue for 30 min at RT.

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

Destaining of the membrane

Destaining solution:	40% Methanol
	10% CH_2COOH
	up to H_20

2.2.11 "Random Prime" method for generation of ^{32}P labeled DNA (Denhardt, 1966; Feinberg and Vogelstein, 1984)

Rediprime^{TM} 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 (1984). The reaction mix contained dATP, dGTP, dTTP, Klenow fragment (4-8 U) and random oligodeoxyribonucleotides. Firstly, 25-50 ng of DNA were denaturated in a total volume of 46 µl at boiling water for 10 min and quick chilled on ice for 5 min. After pipetting the denaturated probe in Rediprime^{TM} II Random Prime Labelling System cup, 4 µl of [α-^{32}P] dCTP (3000 Ci/mmol) was added to the reaction mixture. The labelling reaction was carried out at

37°C for 1 hr. The labelled probe was purified from uncorporated [α-^{32}P] dCTP by using microspin columns (Amersham Pharmacia).

2.2.12 Non-radioactive dye terminator cycle sequencing

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

2.2.13 Hybridisation of nucleic acids (Denhardt, 1966)

The membrane to be hybridised was equilibrated in 2 x SSC and transferred to a hybridisation bottle. After adding 10 ml of hybridisation solution and sheared denaturated salmon DNA, the membrane was incubated for 2 hrs in the hybridisation oven at an appropriate temperature, which is usually 65°C. Then, the labelled probe was denaturated at 95°C for 10 min, chilled on ice for 5 min, and then added to the hybridisation solution. The hybridisation was carried out overnight in the oven. Next day, the filter was washed for 10 min with 2 x SSC containing 0.2% SDS at 65°C. Finally, the membrane was washed with 0.2 x SSC containing 0.1 % SDS at the hybridisation temperature. After drying the filter, it was sealed in Saran wrap and exposed to autoradiography overnight at -80°C or to Phosphoimager screen for 1-4 hrs.

The film was developed in X-Ray Automatic Processor Curix 60 or the screen was scanned in Phosphoimager. For quantification of detected bands, the program Quantity One (Bio-Rad) was used.

2.2.14 Histological techniques

2.2.14.1 Tissue preparation for paraffin-embedding

The freshly prepared tissues were fixed in Bouin's solution or 4% (w/v) paraformaldehyde for 24 hrs to prevent alterations in the cellular structure. The tissue to be embedded in paraffin should be free of water. The dehydration process was accomplished by passing the tissue through a series of increasing alcohol concentrations. For this purpose, the tissue was let in 30%, 70%, 90%, and 100% (2x) ethanol for 1hr at RT. Later, the alcohol was removed from the tissue by incubating it in methylbenzoat for overnight. It was then incubated in 5 ml of histoclear (Xylol) for 10-30 min at RT. The second histoclear was not discarded but 5 ml of paraplast were added and the incubation was continued at 60°C for another 30 min. The histoclear and paraffin mixture was discarded and the tissue was further incubated in 5 ml of paraplast at 60°C for overnight. Before embedding, the paraffin was changed at least three times. Finally, the tissue was placed in embedding mold and melted paraffin was poured into the mold to form a block. The block was allowed to cool and was then ready for sectioning.

2.2.14.2 Sections of the paraffin block

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

2.2.14.3 Immunofluorescence staining of mouse tissues

Fixation and subsequent treatment of mouse tissue were performed as described in section 2.2.14.1. Tissue sections were incubated twice for 10 min in Roticlear to remove the paraffin. Then, sections were re-hydrated in a decreasing ethanol series (100%, 96%, 70%, 50%, and 30%) for 2 min each.

For immunofluorescence staining, sections were washed in PBS and then incubated with a blocking solution containing 10% goat serum and 0.02% Tween-20 in PBS for 1 hr at RT. The sections were incubated with primary antibodies for overnight in a humidified chamber at 4°C. Tissue sections were rinsed three times for 5 min in PBS and subsequently incubated with appropriate secondary antibody for 1 hr in a humidified chamber at RT. Finally, sections were washed three times for 5 min in PBS and the nuclei were counterstained with DAPI. Immunostaining of the sections was examined using a fluorescence equipped microscope (BX60; Olympus).

2.2.14.4 Immunofluorescence staining of cells

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

2.2.14.5 Hematoxylin-eosin (H&E) staining of the histological sections

Tissue sections were first incubated three times in xylene for 3 min each, followed by incubation three times in 100% for 3 min, 95% and 80% ethanol for 2 min each. Thereafter slides were washed in dH_2O for 5 min and stained for 3 min in hematoxylin. This staining was followed by rinsing with deionised water and washing in tap water for 5 min. The treated slides were dipped fast in acid ethanol (1ml concentrated HCl in 400 ml 70% ethanol) for 8-12 times to destain, rinsed in tap water for 2 min and in deionised water for 2 min, stained in eosin for 15 sec and then incubated three times in 95% and 100% ethanol for 5 min each. Finally, the stained slides were incubated three times in xylene for 15 min and mounted with Eukitt-quick hardening mounting medium.

2.2.15 Generation of polyclonal antibody

2.2.15.1 Generation of Pelo-GST fusion construct

Pelota cDNA fragment containing the coding sequence was amplified using primers with 5' overhang restriction sites sequences (Pelo pET-F and Pelo pET-R). PCR was performed using *PfuI* DNA polymerase (Promega) with proof-reading activity to avoid mismatches in amplified Pelota sequence.
PCR condition was as follows:

94 °C	5 min	
94°C	1 min	
60°C	1 min	35 cycles
72°C	1 min	
72°C	10 min	

1.1 kb PCR product was digested with *EcoRI* and *XhoI* enzymes, purified from a 1% agarose gel, ligated into pET41a expression vector and then construct was transformed into competent *E.coli* DH5α cells.

2.2.15.2 Expression of recombinant proteins in the pET vector

The recombinant pET41a construct was transformed into the host bacterial strain *E.coli* BL-21 (DE3). The BL-21 strain (Novagen) is lysogenic for a prophage that contains an IPTG inducible T7 RNA polymerase. A single colony was picked from a freshly streaked plate into 50 ml of LB culture medium containing kanamycin and incubated for overnight culture at 37°C. Overnight culture was diluted into 500 ml fresh LB medium and cultured further at 37°C until OD_{600} reached 0.6-0.8. A non-induced sample was collected as a control. Induction was performed by adding IPTG to a final concentration of 1 mM and cultured for 4 hrs. Finally, the bacterial cells were harvested by centrifugation at 5000 x g for 10 min at 4°C and frozen at - 80°C.

2.2.15.3 Purification of GST fusion proteins

The pellet of bacterial cells was resuspended in 10 ml of lysis buffer (pH 8.0) and sonicated on ice. The cell debris from protein extract was removed by centrifugation for 20 min at 10000 x g, 4°C. The supernatant was filtrated through 0.45 µm filter and incubated with preequilibrated glutathione-agarose (Sigma) for 1 hr at 4°C with gentle mixing. The bound resin was washed four times with PBT at 4°C. Thereafter fusion protein was eluted with 3 ml elution buffer (10mM reduced glutathione in 50 mM Tris/HCl, pH 7.5). Purified protein was dialysed overnight at 4°C against PBS. Protein quality and quantity were checked by SDS-PAGE and Western blotting.

2.2.15.4 Immunisation of rabbits

Two rabbits were immunised each with 500 µg of GST-Pelo fusion protein mixed with Freund's complete adjuvant in 1:1 ratio. Before injection, preimmune sera were collected from the animals. First booster immunisation was performed 14 days after first injection with 1:1 ratio of antigen with Freund's incomplete adjuvant. Second booster was given 28 days and a third booster 56 days after the first immunisation. Final

bleeding was done 70 days after the first immunisation. The antiserum was aliquoted and stored at -80°C.

2.2.15.5 Affinity purification of polyclonal antibody

For affinity purification of the antibody, HiTrap NHS-activated 1 ml columns (Amersham) were used. The columns are made of medical grade polypropylene, which is biocompatible and non interactive with biomolecules. NHS-Activated Sepharose is designed for the covalent coupling of ligands containing primary amino group. Non-specific adsorption of proteins to HiTrap columns is negligible due to the hydrophilic properties of the base matrix. The activated gel is supplied in 100% isopropanol to preserve the stability of the activated gel prior to coupling.

Ligand coupling

Coupling buffer: 0.2 M NaHCO3
0.5 M NaCl (pH 8.3)

Pelo-GST fusion protein (1 mg) was dialysed overnight against coupling buffer. Isopropanol present in column was washed with 1 M HCl (ice cold). The flow rate during the pumping was adjusted to about 1 ml/min. Immediately after washing, 1 ml of the ligand solution was injected onto the column, then the column was sealed and let it stand for 4 hrs at 4°C.

Washing and deactivation

Buffer A: 0.5 M Ethanolamine, 0.5 M NaCl (pH 8.3)

Buffer B: 0.1 M Acetate, 0.5 M NaCl (pH 4.0)

A series of alternate washings (3 x 2 ml) with buffer A and buffer B was done. Finally, the column was washed with 2 ml of PBS.

Affinity purification of antiserum

The column was equilibrated with 10 column volumes of start buffer (PBS). The antiserum was diluted two times with PBS and filtered through a 0.45 µm filter and then applied onto the column. During pumping, a constant flow rate of 0.5 ml/min was maintained. The column was washed with 10 column volumes of PBS. Elution was done with three volumes of 100 mM glycin/HCl (pH 2.7). Eluted antibody fractions were neutralised by adding 3.5 µl of 1 M Tris/HCl (pH 7.5). The purified antibody fraction was desalted by dialysis against PBS. The column was re-equilibrated with 10 volumes of PBS. The antiserum solution was recovered from the dialysis tubing and concentrated using Centrisart columns to about 0.5 ml.

2.2.15.6 Protein G purification of antibody

Protein G sepharose beads (Santa Cruz) were equilibrated by washing five times in binding buffer (20 mM sodium phosphate buffer, pH 7.0, 0.15 M NaCl). Antiserum diluted in ratio 1:1 with binding buffer was mixed with protein G sepharose and incubated at RT for 1.5 hrs. After brief centrifugation step, the supernatant was removed and protein G was washed four times with binding buffer. Elution was done with four protein G volumes of elution buffer (100 mM glycin/HCl, pH 2.7). Eluted antibody fractions were neutralised by adding 3.5 µl of 1 M Tris/HCl (pH 7.5). Finally, antibody was dialysed overnight against PBS.

2.2.16 Eukaryotic cell culture methods

2.2.16.1 Cell culture conditions

NIH 3T3 and HeLa cells were grown in DMEM medium with L-glutamin containing 10% FBS and 1% penicillin/streptomycin solution. The cells were cultured at 37°C in a humidified incubator with 5% CO_2.

2.2.16.2 Trypsinisation of eukaryotic cells

Cells were washed twice with sterile PBS and incubated in minimal amount trypsin-EDTA (0.5 g/l trypsin, 0.2 g/l EDTA) at 37°C until they had detached from the dish. The process was controlled under an inverted microscope. Trypsin was inhibited by addition of growth medium in which the cells were subsequently resuspended. The trypsin was removed by centrifugation at 2000 x g for 3 min. Cells were resuspended in an appropriate volume of cell culture medium and transferred into a new flask with medium.

2.2.16.3 Cryopreservation and thawing of eukaryotic cells

Trypsinised cells were spun down (1000 x g for 5 min) in 4 ml of growth medium. The supernatant was aspirated and the cells were resuspended in freezing medium (DMEM, 20% FCS, 10% DMSO). Aliquots of the cells were kept for 2 days at -80°C and then stored in liquid nitrogen. For revitalisation, frozen cells were quickly thawed and cells were inoculated in a suitable amount of growth medium.

2.2.16.4 Isolation and handling of primary mouse embryonic fibroblasts (MEFs)

In order to isolate MEFs, pregnant female mice were sacrificed at 13-14 p.c. by cervical dislocation. Dissect out the uterine horns, briefly rinse them in 70% (v/v) ethanol and place into a Petri dish containing PBS. Then separate each embryo from its placenta and surrounding membranes. Cut away brain for genotyping and remove dark red organs and as much blood as possible, wash with fresh PBS and finely mince the embryos until they become “pipettable”. Suspend cells/tissue in several ml of trypsin-EDTA (1-2ml per embryo) and incubate with gentle shaking at 37°C for 5-10 min together with a few 5 mm glass beads. Ideally, the resulting cell suspension should be essentianly free of any larger pieces of tissue and should not be too viscous (genomic DNA-lysed cells). Transfer the suspension to 10 ml falcon tube and add about 2 volumes of fresh medium,

then resuspend it followed by cenrifugation at 1000 x g for 5 min and plate it out at 1 embryo equivalent per 10 cm dish. Change medium on the following day.

2.2.16.5 Karyotype analysis

After culturing of blastocyst for 48-72 hrs, 3 µl Nocodazol (1 µg/ml) was added to 30 µl of culture medium and cells were further incubated for 6 hrs at 37°C in 5% CO_2. Cells were washed twice with PBS, trypsinised with 0.5 g/l trypsin and incubated at 37°C, 5% CO_2 for 5 min. Trypsin was inactivated with 150 µl of ES cell medium. Cells were resuspended two times with a micropipette, transferred in an Eppendorf tube and centrifuged at 250 x g for 5 min. After resuspension in 100 µl of 75 mM KCl (0.563%) cells were incubated for 20 min at 37°C and then centrifuged at 500 x g for 5 min. After removing of the KCl, cells were fixed in 100 µl ice cold methanol/acetic acid 3:1 (Vol/Vol) for 10 min at 4°C and centrifuged at 650 x g for 5 min. Fixation step was repeated. Finally, cells were resuspended in 15 µl of fixative solution and dropped onto prewarmed (37°C) glass slides. Slides were stained with Giemsa (5% Giemsa in PBS buffer) for 10 min and washed under running tap water for 3 min.

2.2.16.6 Transfection of mammalian cells

The transfection involves the introduction of foreign DNA into mammalian cells for its expression. The reagent used in this study was "Lipofectamine 2000 TM" (Invitrogen, Karlsruhe, Germany). For transfection, approximately, 0.5 x 10^6 HeLa cells were plated in small flasks with 5 ml of complete DMEM and incubated overnight at 37°C, 5% CO_2. 12 µl of Lipofectamine 2000 TM reagent and 4 µg of the DNA of interest were diluted each in a total volume of 100 µl, respectively, with OptiMEM I reduced serum medium (Invitrogen, Karlsruhe, Germany) and incubated at RT for 10 min. Subsequently mixed them together in a reaction tube and then incubated at RT for 30 min. The DMEM containing cells were washed with PBS and the medium was replaced with 1 ml of OptiMEM I medium without antibiotics/FCS.

The reaction mixture was added in the flask drop-by-drop and incubated for 2.5-3 hrs at 37°C, followed by adding 1ml of OptiMEM I medium with 20% FCS and incubated for

next 3 hrs, then replaced transfection reagent with DMEM medium containing antibiotics/FCS. The cells were then incubated at 37°C for 24-48 hrs.

2.2.17 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 revolutionised our ability to study gene function *in vitro* and *in vivo*. In theory, this technique will allow us to generate any type of mutation in any cloned gene. Over twenty years ago, pluripotent mouse embryonic stem cells (ES) derived from inner cell mass (ICM) 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 germline by producing chimera mice. 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*.

2.2.17.1 Production of targeted embryonic stem cell clones

2.2.17.1.1 Preparation of MEFs feeder layers

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

2.2.17.1.2 Growth of ES cells on feeder layer

One vial of frozen ES cells was quickly thawed at 37°C 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 6 cm dishes containing MEFs 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 (6 cm) or to 2 dishes (10 cm) containing feeder layers. The cells were passaged every second day as described above.

2.2.17.1.3 Electroporation of ES cells

ES cells, which have grown for two days in 10 cm dishes, were trypsinised. The cell pellet was resuspended in 20 ml PBS and centrifuged. The cell pellet was then resuspended in 1 ml PBS. The 0.8 ml of cell suspension was mixed with 40 µg of linearised DNA-construct and transferred into an electroporation cuvette. The electroporation was performed at 240V, 500µF with the Bio-Rad gene pulser™ apparatus. 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 10 cm dishes containing feeder layers. The medium was changed every next day. Two days after the electroporation, the drugs for selection were added (active G418 at 400µg/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.

2.2.17.1.4 Growing ES cells for Southern blot analysis

The drug resistant colonies that were formed after about eight days of selection were picked with a drawn-out Pasteur pipette under a dissecting microscope. Each colony was transferred into a 24-well plate containing feeders and ES cell medium. After 2 days, the ES cells were trypsinised with 100 µl trypsin 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.

2.2.17.2 Production of chimeras by injection of ES cells into blastocysts

The standard procedure is to inject 10-20 ES cells from 129/Sv, which are recombinant for the targeted locus, into the blastocoel cavity of recently cavitated blastocysts that have been recovered by flushing the uteri of day 4 pregnant mice (C57BL/6J). After injection, embryos are cultured for a short period (2-3 hrs) to allow re-expansion of the blastocoel cavity and then transferred to the uterine horns of day three pseudopregnant mice. Pseudopregnant females are obtained by mating 6-8 weeks old oestrous females with vasectomised males.

2.2.17.3 Detection of chimerism and mice breeding

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

2.2.18 Gene silencing by RNA interference

The term RNA interference (RNAi) describes the use of double-stranded RNA to target specific mRNA for degradation, thereby silencing their expression. RNAi is one manifestation of a broad class of RNA silencing phenomena that are found in plants, animals and fungi (Cogoni and Macino, 2000). The discovery of RNAi has changed the understanding of how cells guard their genomes, led to the development of new strategies for blocking gene function and may yet yield RNA-based drugs to treat human diseases.

2.2.18.1 Overview of the RNAi mechanism

In the initiation step, input dsRNA is digested into 21-23 nucleotide small interfering RNAs (siRNAs), which have also been named "guide RNAs" (Elbashir *et al.,* 2001). The siRNAs are produced when the enzyme Dicer, a member of the RNase III family of dsRNA-specific ribonucleases, processively cleaves dsRNA (introduced directly or via a transgene or virus) in an ATP-dependent. The dsRNA successive cleavage events degrade the RNA to 19-21 bp duplexes (siRNAs), each with 2-nucleotide 3' overhangs (Hutvagner *et al.,* 2002). In the effector step, the siRNA duplexes bind to a nuclease complex to form what is known as the RNA induced silencing complex, or RISC. An ATP-depending unwinding of the siRNA duplex is required for activation of the RISC. The active RISC then targets the homologous transcript by base pairing interactions and cleaves the mRNA ~12 nucleotides from the 3' terminus of the siRNA (Elbashir *et al.,* 2001). Consensus RNase III active site in the second RNase III domain inactivates the central catalytic sites, resulting in cleavage at 22-nucleotide intervals.

2.2.18.2 Preparation of adaptor oligos

The 21 bp single stranded sense and anti-sense oligonucleotides containing *ClaI*, *NotI* and *XhoI* restriciton enzymes (CNX-sense: 5'-CCCCCCATCGATGCGGCCGCC-3'; CNX-anti sense: 5'-AAAACCCTCGAGGCGGCCGCA-3') were diluted to a final concentration of 100 pmol/µl. Then for each of the above oligonucleotides 2.5 µl was added to 45 µl of annealing buffer (100 mM NaCl and 50 mM HEPES). After denaturation for 4 min at 95°C, the oligonucleotides were incubated for 10 min at 85°C, 80°C and 75°C, then for 4 min at 70°C, 65°C, 60°C, 55°C and 53°C respectively, followed by for 10-15 min at 37°C, and then cooled down on ice.

2.2.19 Generation of transgenic mice

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

2.2.19.1 Preparation of DNA for pronuclear microinjection

Transgenic constructs were released from cloning vector by restriction digestion. Digested fragments were separated by agarose gel electrophoresis (without EtBr) in the way that 25 µg of digested plasmid was loaded to many slots of the gel. After separation, outer lanes were cut out and stained with EtBr. After staining, gel was reconstructed and appropriate gel slices were cut out from the rest of the gel under UV light. DNA was then eluted from gel with QIAquick extraction kit and filtered through 0.45 µm microfilter (Milipore). Concentration of DNA was estimated by EtBr electrophoresis of DNA aliquots in comparison with Smart ladder marker (defined DNA amounts in each band). For microinjection, DNA was diluted to 4 ng/µl in microinjection buffer (10 mM Tris, pH 7.5; 0.1 mM EDTA, pH 8.0).

2.20 Computer analysis

For the analysis of the nucleotide sequences, programs like BLAST, BLAST2, MEGABLAST and other programs from National Center for Biotechnology Information (NCBI) were used (www.ncbi.nlm.nih.gov). Information about mouse alleles, phenotypes and strains were used from Jackson Laboratory (www.informatics.jax.org). For protein studies ExPASy tools (www.expasy.ch) were used. Mouse genome sequence and other analysis on mouse genes, transcripts and putative proteins were downloaded from Celera discovery system (www.celera.com).

3. RESULTS

3.1 Generation of polyclonal antibody against Pelota and expression analysis

3.1.1 Generation and purification of polyclonal anti-Pelo antibody

To study the expression of Pelo protein and determine the subcellular localisation of Pelo, anti-Pelo specific antibody was generated against Pelo-GST fusion protein. Pelo-GST fusion protein was purified as described in methods section (2.2.15.3) and used for the immunisation of rabbits. To remove other proteins from polyclonal serum, IgG purified antibody was isolated as described in methods section (2.2.15.6) and checked for specificity by Western blot analysis. Total protein extracts from mouse ES-, mouse NIH 3T3- and human LNCaP cells were separated on SDS-PAGE gel and transferred onto nitrocellulose membrane. As shown in Figure 3.1A, the IgG polyclonal antibody did not recognise a specific protein in any protein extracts. In order to purify anti-Pelo antibody from serum, Pelo-GST fusion protein was coupled to NHS-Hi Trap column and used for the purification of anti-Pelo antibody (2.2.15.5). The purified Pelo-GST antibody was checked by Western blot (Fig 3.1B) and immunostaining analysis (Fig 3.4D). The affinity purified antibody detects approximately 44 kDa protein in all extracts and further high molecular weight proteins. Because the polyclonal antibody was raised against Pelo-GST fusion protein and purified with same protein, the affinity purified antibody should contain anti-Pelo and anti-GST antibodies. The specificity of anti-Pelo polyclonal antibody to recognise the 44 kDa protein was determined by competition assay.

Western blot with protein extracted from testes was incubated with anti-Pelo antibody (Fig 3.2A) and the other blot was probed with anti-Pelo antibody which was preincubated with GST protein to remove other unspecific antibodies (Fig 3.2B). In the competition assay only a specific band of 44 kDa was recognised. These results demonstrate that purified anti-Pelo antibody specifically detects the 44 kDa Pelo protein.

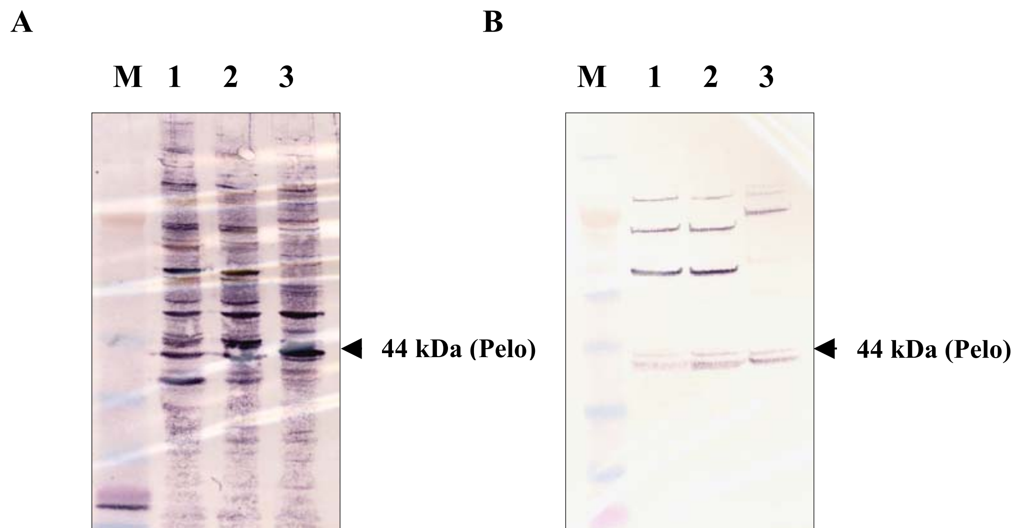


Figure 3.1: Western blot analysis using polyclonal anti-Pelo antibody

Western blot with total protein extracts from ES- (1), NIH 3T3- (2) and LNCaP (3) cells was probed with IgG anti-Pelo antibody (A) and GST-Pelo purified anti-Pelo antibody (B).

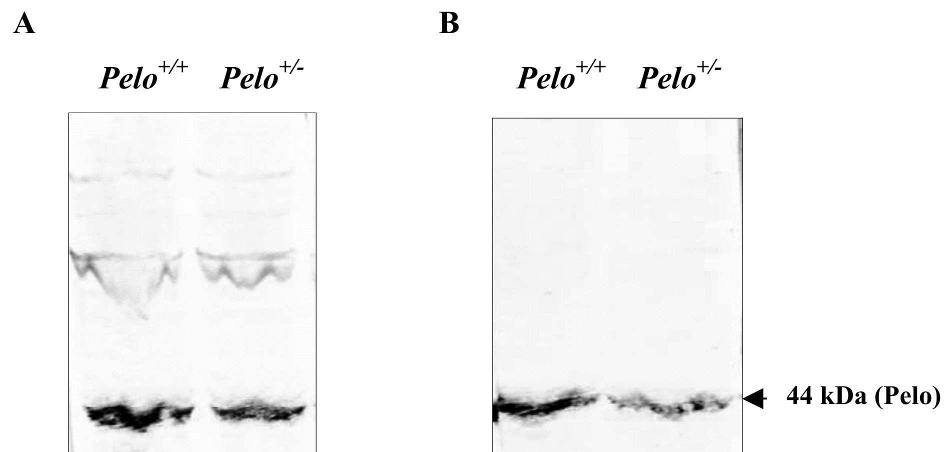


Figure 3.2: Competition assay to verify the specificity of polyclonal anti-Pelo antibody

Western blots with testes protein extracts from *Pelo*$^{+/+}$ and *Pelo*$^{+/-}$ animals were probed with Pelo-GST purified anti-Pelo antibody (A) and with GST-preincubated anti-Pelo antibody (B). Dilutions of antibodies were 1:100.

3.1.2 Cellular localisation of Pelo protein in mouse embryonic fibroblasts

Affinity purified polyclonal anti-Pelo antibody was used to identify the subcellular localisation of Pelo protein. In parallel, GFP-fluorescence was determined in GFP-Pelo transgenic fibroblasts which were isolated and cultured from GFP-Pelo transgenic embryos. As shown Figure 3.3, GFP-fluorescence is restricted to the cytoskeleton and cytoplasm. GFP-Pelo transgenic fibroblasts were then probed with anti-GFP (Fig 3.4A) and anti-Pelo (Fig 3.4D) antibodies. After incubation of fibroblasts with secondary antibodies, slides were then probed with FITC-labeled phalloidin which is specifically associated with the stress actin filaments (Fig 3.4B; E). As shown in Figure 3.4A and 3.4D, anti-Pelo and anti-GFP antibodies recognise a similar subcellular structure in GFP-Pelo transgenic fibroblasts. Colocalisation of actin filaments with the phalloidin staining demonstrates that the Pelo protein is associated to cytoskeleton.

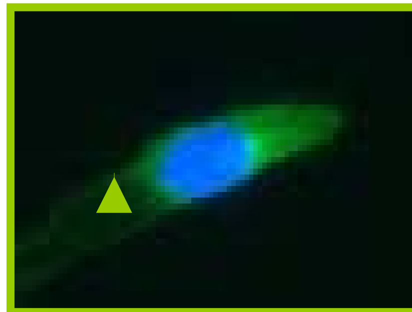


Figure 3.3: Pelo protein is localised at cytoskeleton

GFP-fluorescence was detected at cytoskeleton (arrow) of fibroblasts which were isolated and cultured from GFP-Pelo transgenic embryos.

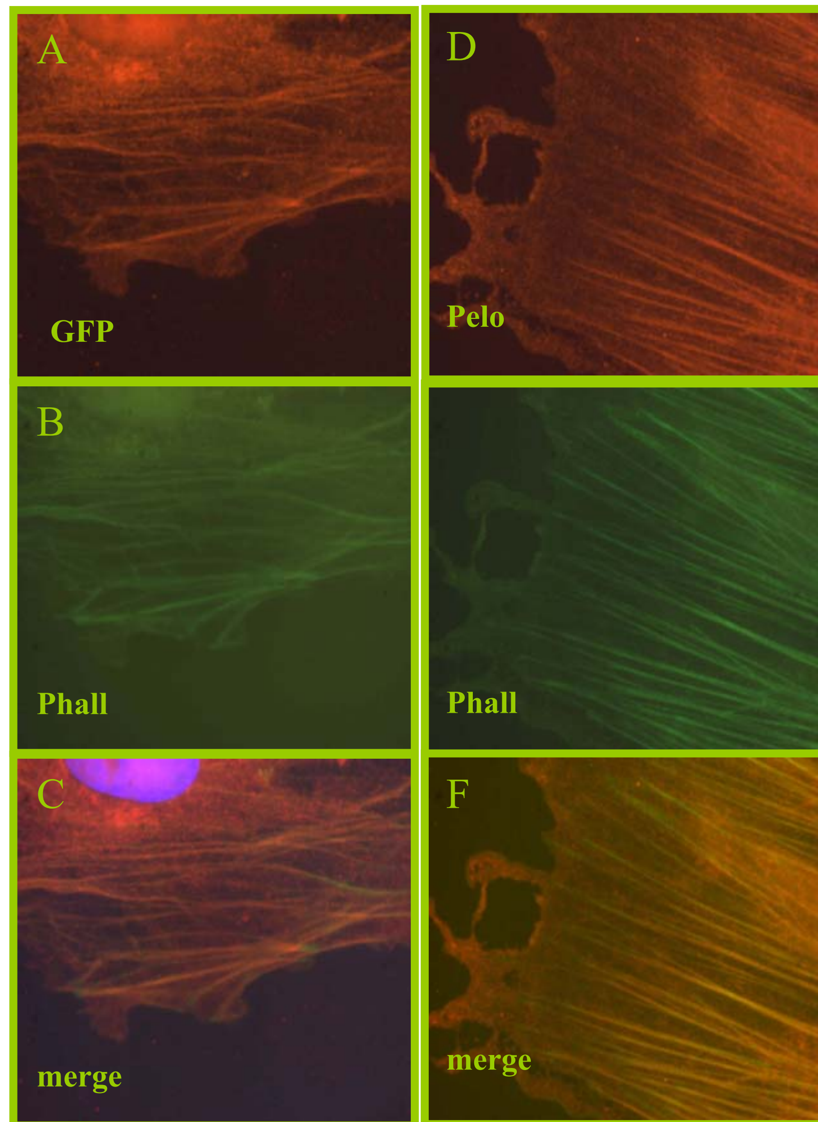


Figure 3.4: Association of Pelo protein to cytoskeleton of GFP-Pelo transgenic fibroblasts

GFP-Pelo transgenic fibroblasts were fixed in 4% paraformaldehyde and probed with anti-GFP (A) and anti-Pelo (D) antibodies. The slides were incubated with Cy3-labelled goat anti-rabbit IgG as a secondary antibody. Fibroblasts in A and D were then incubated with FITC-labelled phalloidin. Colocalisation of the Pelo and GFP stained actin filaments in red (A, D) and phalloidin stained actin filaments in green (B, E) appears orange in the merged image (C, F).

3.1.3 Expression of Pelo protein in different cell lines

To validate the expression of Pelo protein *in vitro*, Western blot analysis with total protein extracted from mouse and human cell lines was performed with the polyclonal anti-Pelo antibody. As shown in Figure 3.5, Pelo protein is present in all studied cell lines.

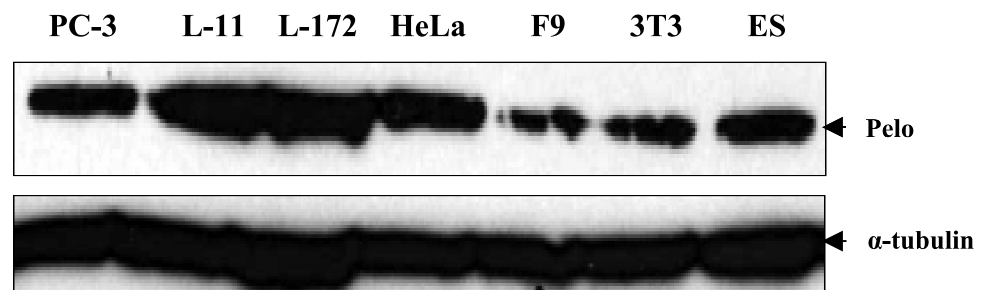


Figure 3.5: Expression of Pelo protein in different cell lines

The 44 kDa Pelo protein was detected in all studied cell lines: PC-3, human prostate carcinoma cell; L-11, LNCaP-11 human prostate carcinoma cell androgen dependent; L-172, LNCaP-172 human prostate carcinoma cell androgen independent; HeLa, human cervical cancer cell; F9, murine teratocarcinoma cell; 3T3, NIH 3T3 mouse embryonic fibroblasts; ES, mouse embryonic stem cell. 50 kDa α-tubulin protein served as a loading control.

3.1.4 Expression of Pelo protein in mouse tissues

To determine the expression pattern of the Pelo protein in different tissues, Western blot analysis was performed. Total protein was extracted from different tissues of adult mice, including testis, lung, heart, spleen, thymus, stomach, kidney and liver, electrophoresed on SDS-PAGE and transferred onto a nitrocellulose membrane. The polyclonal anti-Pelo antibody recognised the 44 kDa Pelo protein in all studied tissues (Fig 3.6).

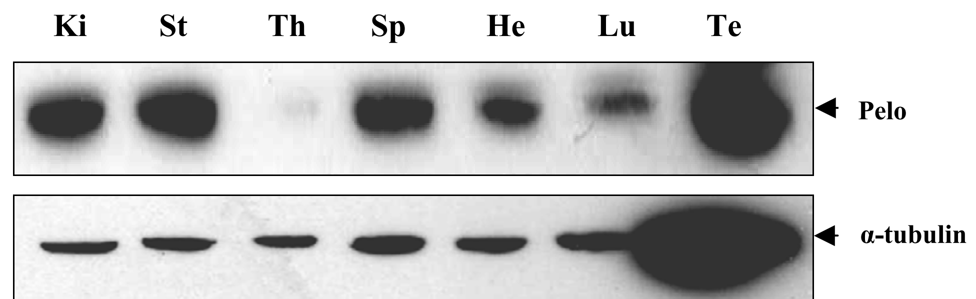


Figure 3.6: Expression of Pelo protein in different tissues of mouse

A polyclonal antibody raised against Pelo-GST fusion protein was used to probe Western blot with protein extracted from different tissues. The 44 kDa band was detected in all studied tissues. Ki, kidney; St, stomach; Th, thymus; Sp, spleen; He, heart; Lu, lung; Te, testis. The membrane was stripped and subsequently probed with a monoclonal anti α-tubulin antibody to monitor the loading.

3.1.5 Expression of Pelo protein during testicular development and in testes of different mutant mice

To evaluate the expression of the *Pelo* gene during postnatal development of testis at the protein level, total protein extracts from testes of mice at different developmental stages were analysed by Western blot. The 44 kDa Pelo protein was found in lysates of all testicular stages studied (Fig 3.7A).

The presence of the Pelo protein was also examined in the testes of mutant mice, in which spermatogenesis is arrested at different stages: W/W^{V}, Insl3$^{-/-}$, olt/olt and qk/qk. W/W^{V} mice are characterised by lack of all germ cells (de Rooij and Boer, 2003), in Insl3$^{-/-}$ mutants spermatogenesis is arrested at pachytene spermatocyte stage (Zimmermann *et al.,* 1999). In olt/olt, spermatogenesis is arrested at round spermatid stage and in qk/qk at elongated spermatid stage (Bennett *et al.,* 1971, Moutier, 1976). The 44 kDa Pelo protein was detectable in testes of all these mutants (Fig 3.7B). Detection of 44 kDa Pelo protein in W/W^{V} mutants demonstrates that Pelo is expressed in somatic testicular cells.

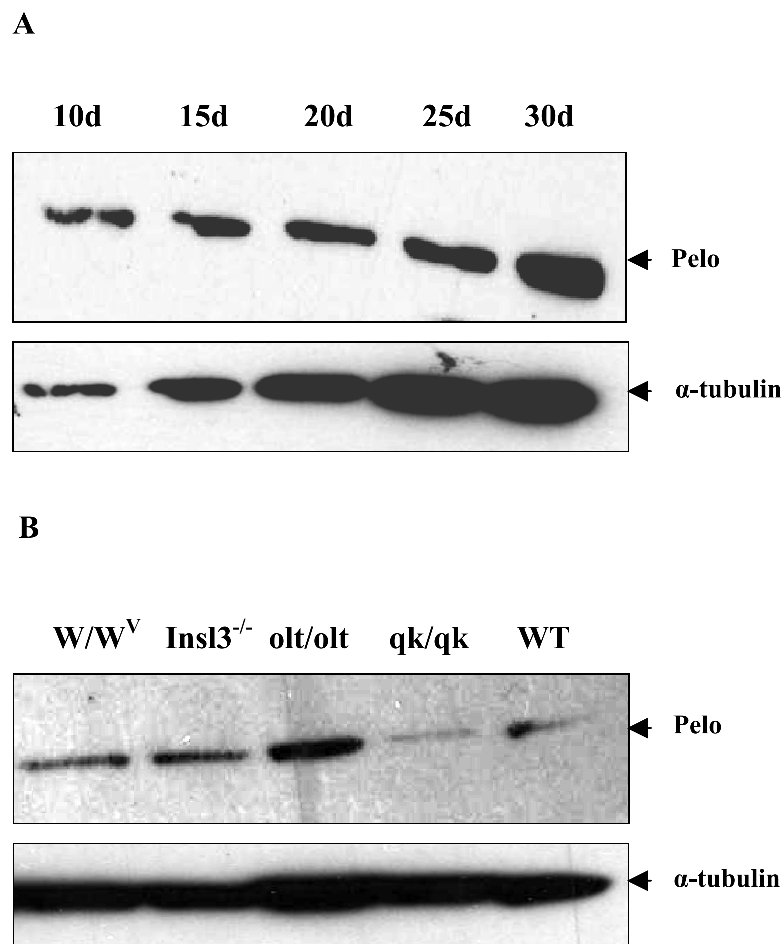


Figure 3.7: Expression of Pelo protein during testicular development and in testes of different mutant mice

Polyclonal anti-Pelo antibody recognises the 44 kDa protein in testicular lysates from all postnatal developmental stages (A) and mutant mice. 50 kDa α-tubulin protein is shown as a control for protein loading.

3.1.6 Expression of *Pelo* during preimplantation stages

To determine the profile of preimplantation expression of *Pelo* gene, RT-PCR was performed with total RNA prepared from 1-, 2-, 4-cell, morula and blastocyst stages. To control the relative abundance of Pelota transcript, we included RT-PCR analysis for the *Gapdh* gene which is ubiquitously expressed throughout preimplantation. The expression level of *Pelo* was high in unfertilized eggs, then decreased in 2- and 4-cell stages and increased again after 8-cell stage (Fig 3.8). These results suggest that maternal *Pelo* transcript is decreased from 1- to 4-cell stages whereas the expression of embryonic *Pelo* transcript starts at 8-cell stage.

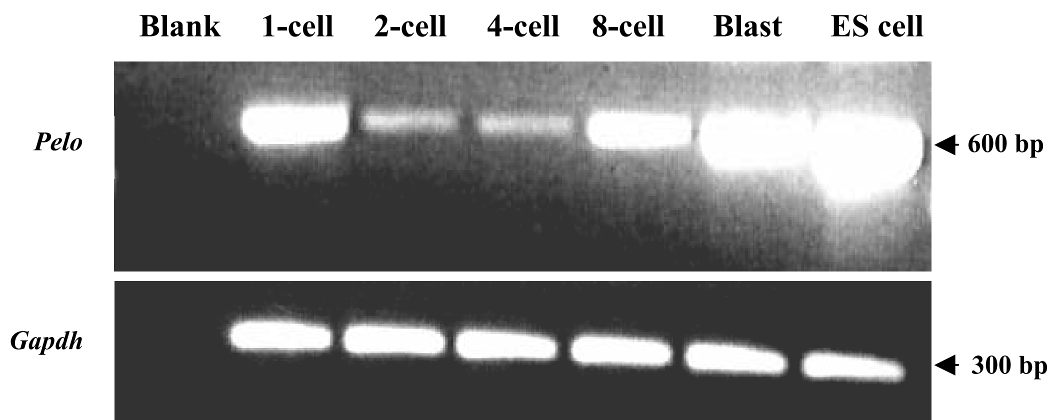


Figure 3.8: RT-PCR expression analysis of *Pelo* gene during preimplantation stages

RT-PCR was performed to detect *Pelo* and *Gapdh* transcripts in total RNA preparation from 1-, 2-, 4-cell, morula, blastocysts stages and ES cells.

3.1.7 Expression and localisation of Pelo protein in testicular cell fractions

In order to confirm the results of immunocytochemistry analyses, we performed Western blot analyses with protein extracted from different cellular fractions of testis, namely cytoplasmic, nuclear, cytoskeleton and membrane protein fractions. The blot was subsequently probed with different antibodies. The polyclonal anti-Pelo antibody

was found to recognise a 44 kDa protein in cytoskeleton and membrane fractions. The restricted localisation of α-actinin and H3 histone in cytoskeleton and nuclear fractions, respectively, suggests the proper separation of different fractions. These results confirm the subcellular localisation of Pelo is at cytoskeleton. The presence of Pelo in the membrane fraction, as shown in Western blot, might be due to association of cytoskeleton to the membrane. The results clearly demonstrate that expression and subcellular localisation of Pelo protein is at cytoskeleton as well as in the membrane (Fig 3.9).

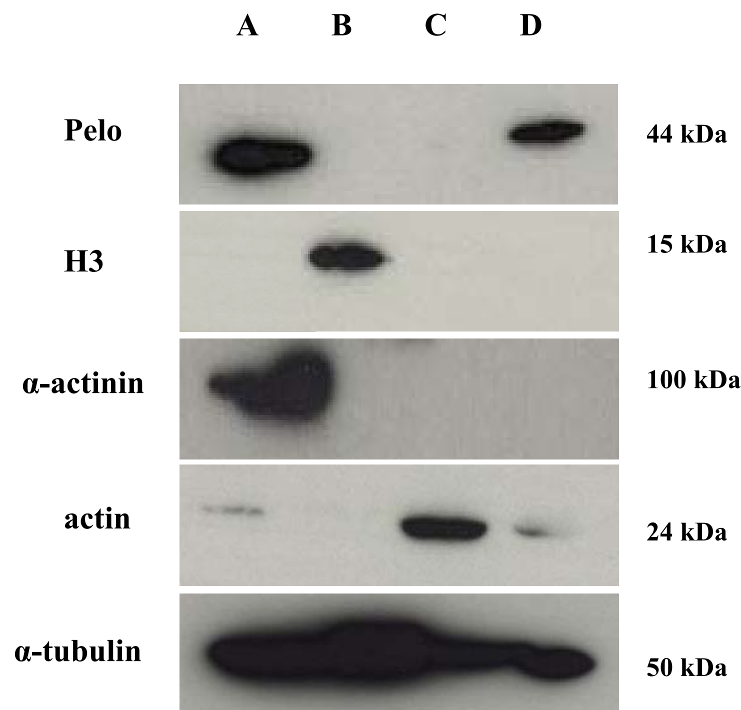


Figure 3.9: Expression and localisation of Pelo and other protein in testicular cell fractions

Western blot analysis with protein extracted from different cellular fractions of adult mouse testis. A, cytoskeleton; B, nuclear; C, cytoplasmic and D, membrane fraction. The blot was subsequently probed with antibodies against Pelo, H3 histone, α-actinin, actin and α-tubulin. The restricted localisation of H3 histone and α-actinin to nuclear and cytoskeleton fractions, respectively, suggests the proper separation of different fractions.

3.2 Knockdown of Pelota gene using inducible siRNAs system *in vitro*

3.2.1 Overview of inducible siRNAs system

RNA interference (RNAi) is a process of sequence-specific posttranscriptional gene silencing mediated by double-stranded RNA and is a powerful genetic approach to analyse gene function in many organisms. The endogenous mediators of sequence-specific mRNA degradation are 21- and 22-nt short interfering RNAs (siRNAs) generated from longer double-stranded RNAs by the ribonuclease III activity of the evolutionary conserved dicer enzyme. The functional siRNA silencing mechanism by dsRNA was first shown in *C. elegans* (Fire *et al.,* 1998). Recently, it was demonstrated that RNAi-mediated gene silencing can be reached in cultured mammalian cells by delivery of chemically synthesised short (<30 nt) double-stranded siRNA molecules or by endogenous expression of short hairpin RNAs (shRNAs) bearing a fold-back stem-loop structure.

Plasmid- and viral vector-based constitutive expression of shRNAs by RNA polymerase III U6 and H1 small nuclear RNA promoters (U6 or H1) often result in stable and efficient suppression of target genes. However, the inability to adjust levels of suppression has imposed limitations in the analysis of genes essential for cell survival, cell cycle regulation, and cell development. Besides, gross suppression of a gene for longer periods may result in nonphysiological responses. This problem can be circumvented by generating inducible regulation of RNAi in mammalian cells. The two most widely used inducible mammalian systems use tetracycline- or ecdysone-responsive transcriptional elements.

Using a tet on/off strategy it has been shown that the exogenous control of shRNAs expression in tissue culture is possible (van de Wetering *et al.*, 2003). The Tet on/off strategy relies on two components: a Tet-repressor (TetR) and a Tet-responsive promoter, the activity of which is regulated by binding or release of the TetR. In the absence of the tetracycline repressor (TetR), the *tetO* sites in the tetracycline responsive promoter are bound by the TetR and transcription of the shRNA is repressed (Fig 3.10).

Conversely, in the presence of Dox, TetR is released from the *tetO* sites, allowing transcription of shRNA and thus, the mRNA-specific knockdown to proceed.

The essential role of *Pelota* for cell viability prevented us to establish a cell line from Pelo deficient embryos. Therefore, the tetracycline inducible siRNA system was suggested to be a convenient strategy to establish a stable cell line and conditional induction of Pelo-shRNA. The conditional induction of Pelo-shRNA should allow for time-controlled loss-of-function and thus circumventing the lethal phenotype.

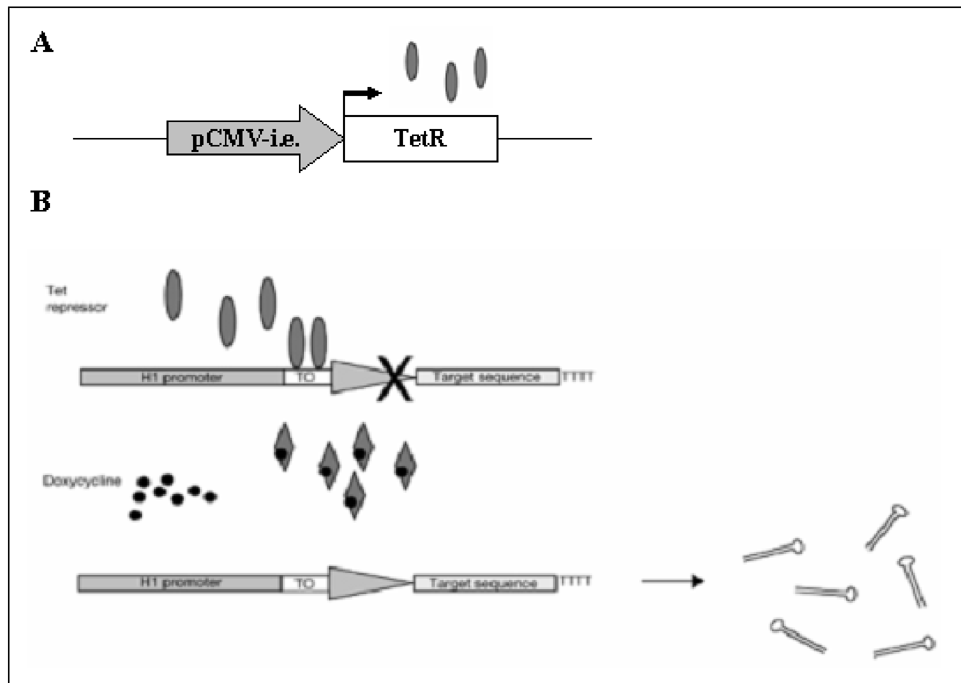


Figure 3.10: Overview of the tetracycline inducible siRNA system

(A) Schematic representation of the vector containing the TetR gene, which is controlled by CMV promoter that is highly transactivated in different tissues. (B) Schematic representation of the proposed pTER 'roadblock'. Transcription of the H1 promoter is blocked in cells expressing the Tet repressor (upper panel). Addition of doxycycline to the medium inhibits the binding of the Tet repressor and transcription is derepressed (lower panel).

3.2.2 Selection of siRNA sequences for specific targeting of *Pelo* mRNA

To facilitate the knockdown of the murine *Pelo* transcripts by the RNAi system, three different RNAi target sequences and their complementary oligonucleotides were designed (Fig 3.11A, B). The RNAi forward oligonucleotide was annealed with its complementary oligonucleotide and then cloned into the pTER vector, which was linearised with *BglII* and *HindIII* restriction enzymes (Fig 3.11C).
The *Pelo* gene has 3 exons which code for a 1624 bp mRNA. First 19 bp targeted sequences are located at 1009-1025 nucleotides (nt), whereas a second sequence is located at 1045-1064 nt and a third targeted sequence is located at 627-646 nt of the murine *Pelo* mRNA sequence. To ensure unique targeting of the murine *Pelo* mRNA, BLAST search (www.ncbi.nlm.gov/BLAST) was performed to prevent unspecific knockdown of unrelated mRNA sequences.

A. RNAi forward oligonucleotide:

sense orientation **anti-sense orientation**

5'GATCCCC**CACAAGTACTCCCTGAAAG**ttcaagaga**CTTTCAGGGAGTACTTGTG**TTTTTGGAAA3'

B. RNAi reverse oligonucleotide:

sense orientation **anti-sense orientation**

5'AGCTTTTCCAAAAA**CACAAGTACTCCCTGAAAG**tctcttgaa**CTTTCAGGGAGTACTTGTG**GGG 3'

C. Annealing of oligonucleotides to generate a siRNA template:

Loop **Stop signal**

5'- GATCCCC**CACAAGTACTCCCTGAAAG**ttcaagaga**CTTTCAGGGAGTACTTGTG**TTTTTGGAAA -3'

GGG**GTGTTCATGAGGGACTTTC**aagttctct**GAAAGTCCCTCATGAACAC**AAAAACCTTTTCGA

Bgl II **Loop** **Hind III**

Figure 3.11: Design of the Pelo-siRNA vector

A. RNAi forward oligonucleotide: The left *BglII* cloning overhang (capitals, underlined) is follwed a 19 bp Pelota specific knockdown sense sequence (bold), a 9 bp loop (lower case, underlined), the anti-sense Pelo-specific knockdown sequence (bold), followed by the TTTTT stop signal and the *HindIII* specific overhang (capitals, underlined).

B. The RNAi reverse primer oligonucleotide contains the reverse complementary sequence for the forward oligonucleotide therefore producing an siRNA sequence which is then transcribed into a short hairpin loop (shRNA) by transcription with the *PolIII* enzyme. The cloning of annealed oligonucleotides (C) into pTER expression vector results in the Pelo-siRNA knockdown vector.
The pTER backbone is shown in black and the RNAi-oligonucleotide cloning region is indicated in blue. H1 *T*O promoter, histone 1 promoter containing a tetracycline operator sequence; CMV-i.e., human cytomegalovirus immediate early promoter; BGH pA, bovine growth hormone polyadenylation signal.

To confirm the successful integration of three different siRNA templates into pTER vector, a test digestion was performed with *EcoRI* restriction enzyme. The vector contains two *EcoRI* sites which are flanking the promoter region and the cloning site (Fig 3.11). DNA from positive clones was extracted as described in methods section (2.2.1.1.1) and an integration of the insert was confirmed by sequencing. The result of the sequencing analysis demonstrated that all three Pelo-siRNA vectors did harbour the selected Pelo-siRNA templates. The three different Pelo-siRNA vectors (Pelo1-siRNA, Pelo2-siRNA and Pelo3-siRNA) were further analysed for knockdown efficiency in cell culture.

The R1 ES cells were used to determine the efficiency of Pelo-siRNA vectors to knockdown of *Pelo* transcript. The ES cells were transfected with *Bst1107* digested pcDNA6/TR vector, which contains tetracycline repressor under the control of CMV promoter (Fig 3.12). Transfected cells were cultured in standard ES cell medium containing Blasticidin. After 10 days, Blasticidin resistant-clones were genotyped for the integration.

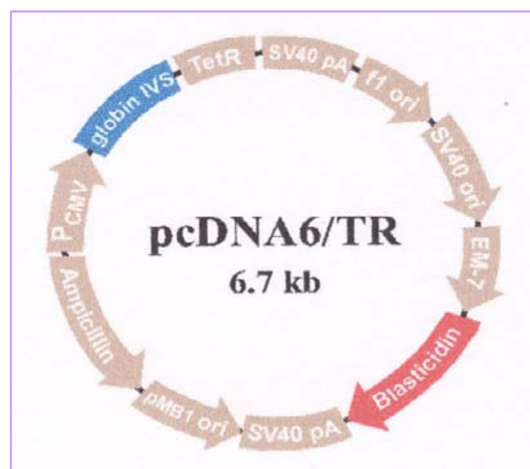
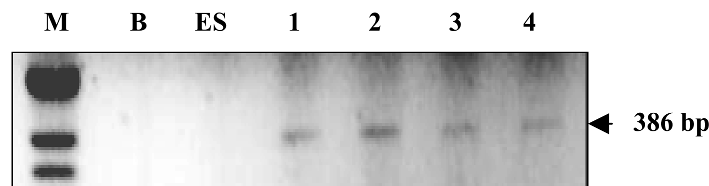


Figure 3.12: Schematic representation of the pcDNA6/TR vector

pcDNA6/TR is a 6662 bp vector that sustains high level of the TetR gene expression under the control of human CMV promoter. The T-REx coding region shown in blue and brown (IVS, T-REx intron and T-REx cDNA) is followed by a SV40 pA. Blasticidin resistance gene is shown in red for mammalian cell selection.

The pcDNA6/TR vector was detected in the genome of ES cells by PCR assay using primers Blast-F and Blast-R, which contain sequences of Blasticidin gene (Fig 3.13A). One Blasticidin-resistant ES clone was then separately transfected with Pelo1-siRNA, Pelo2-siRNA and Pelo3-siRNA vectors. Transfected cells were cultured in ES cell medium with 200 ng/ml of Zeocin. After 10 days culture, four Zeocin-resistant clones were selected from each transfection experiment and genotyped for insertion of Pelo-siRNA vectors in the genome by PCR analysis (Fig 3.13B). The used primers Zeo-F and Zeo-R contain sequences located in Zeocin gene.

A. PCR with Blasticidin primers



B. PCR with Zeocin primers

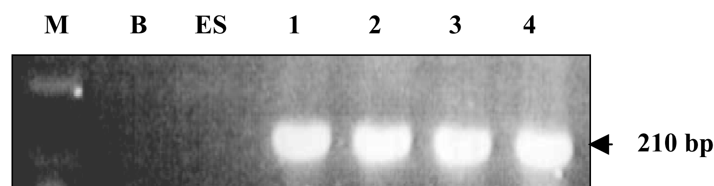


Figure 3.13: PCR screening of Pelo-siRNA positive clones

Four different Pelo-siRNA specific clones (1-4) were generated using the pTER vector. To identify successfully transfected plasmids, PCR approach was established as described in methods section (2.2.8). Resulting products were run on a 2% agarose gel from individual bacterial colonies with individual clone numbers indicated above the lanes. B, blank control, no DNA template; ES, negative control (untransfected); (A) 386 bp, PCR fragment of transfected pcDNA6/TR vector. (B) 210 bp, the ligated oligonucleotides in the pTER vector indicating the positive clones.

3.2.3 Efficiency of Pelo-siRNAs to knockdown the *Pelo* transcript

To determine the knockdown efficiency of the three different individual RNAi constructs (Pelo1-3-siRNA), three selected clones from each transfection assay were cultured for 3 days with Doxycycline (Dox) at a concentration of 2 µg/ml. Total RNA and protein were extracted from Dox-treated ES cells. The Northern blot analysis with total RNA isolated from Dox-treated ES cell clones which are transfected with Pelo1-siRNA constructs revealed that there is no significant reduction of the *Pelo* mRNA. Rehybridisation of the blot with β-actin probe shows that the observed reduction in intensity of Pelo band in clone 1 and 2 compared to control is due to unequal amount of RNA loading (Fig 3.14).

Expression of Pelo in Dox-treated clones was further confirmed by Western blot at the protein level. As shown in Figure 3.15, no apparent reduction in the level of Pelo protein of Pelo1-siRNA transfected clones was found. Similar results were also obtained by analyses of Pelo2-siRNA and Pelo3-siRNA transfected vectors. These results suggest that the Pelo-shRNAs are not able to knockdown the *Pelo* mRNA.

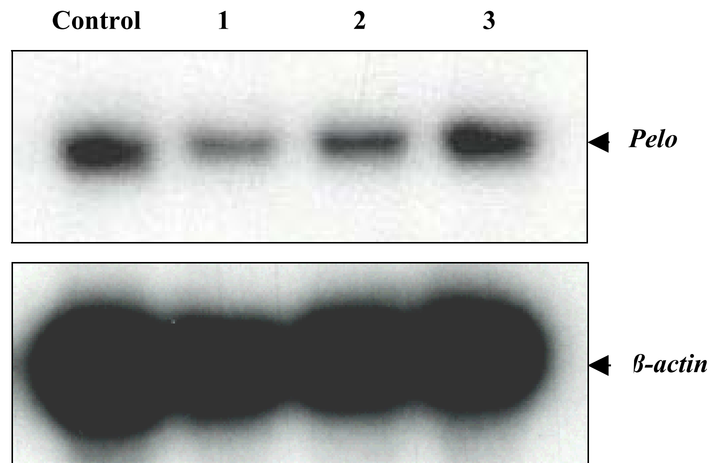


Figure 3.14: Northern blot analysis showing *Pelo* expression in different Pelo1-siRNAi recombinant clones

Total RNA (20μg) was extracted from Pelo1-siRNA recombinant clones (1-3) and subjected to Northern blot hybridisation using murine Pelota cDNA as a probe. Control lane contains RNA isolating from untransfected ES cells. The probe hybridised with 1.6 kb murine Pelota cDNA. Equal loading of the RNA samples were confirmed by rehybridisation of blot with 1.7 kb β-actin probe.

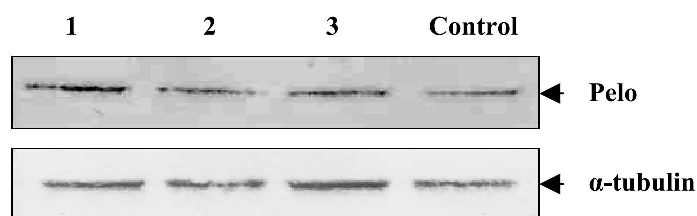


Figure 3.15: Western blot analysis of Pelo-siRNA knockdown efficiency

Protein was extracted from Pelo1-siRNA recombinant clones (1-3) and subjected to Western blot analysis using anti-Pelo antibody. Control lane contains protein isolating from untransfected ES cells. A monoclonal antibody against α-tubulin was used as a loading control.

3.3 Pelota conditional knockout mice

3.3.1.1 Generation of conditional knockout targeting construct

In order to conditionally disrupt the *Pelota* gene in the mouse, *Pelota* conditional allele was designed in pKS*LoxP*NT vector. In the floxed Pelo construct, two *loxP* sites were integrated in intron 1 and 3' flanking region of the gene. The neomycin phosphotransferase (*Neo*) gene under the control of the phosphoglycerate kinase promoter was inserted upstream of the second *loxP* site. Introduction of the Herpes simplex virus thymidine kinase (*TK*) gene at the 3' end of the construct enabled us to use negative selection (Fig 3.18).

DNA of clone 16-kb, which contains the entire *Pelo* gene (Fig 3.16A) was digested with the restriction enzyme *SpeI*. The 9 kb and 6 kb *SpeI* fragments were purified from agarose gel. The 9-kb *SpeI* fragment was self-ligated to give the clone 9-kb *SpeI* (Fig 3.16B). The 6-kb *SpeI* genomic fragment was subcloned into the *SpeI* digested pBlueScript vector to produce the clone 6-kb *SpeI* (Fig 3.16C). The clones were generated by Dr. M. Sallam (Sallam, 2001) and used to construct the floxed Pelo targeting vector.

pKS*LoxP*NT vector (Fig 3.17A) was digested with *HindIII* and both 2 kb and 5 kb fragments were purified from agarose gel. The 2-kb *HindIII* fragment containing *Neo/loxP* fragment was cloned into *HindIII* digested clone 4H/S (Fig 3.16D) to obtain clone *Neo*-4H/S (Fig 3.17C).

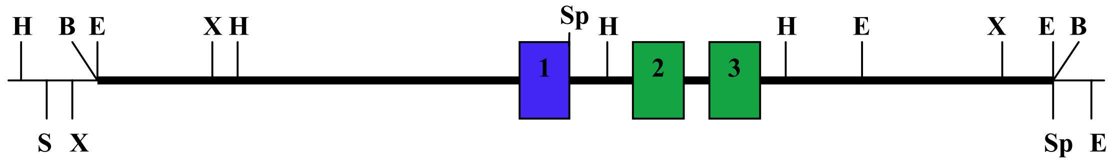
The 5-kb *HindIII TK/loxP* fragment of the vector was self-ligated to give the clone 2. The 2 kb *HindIII* fragment containing exons 2 and 3 of *Pelota* gene was isolated from clone 6-kb *SpeI* and cloned into *HindIII* digested clone 2 to produce clone 3 (Fig 3.17D).

150 bp fragment containing a sequence of intron 1 was amplified with forward primer containing *SpeI* site and reverse primer containing *KpnI* site. The 150-bp PCR fragment was subcloned into pGEMTeasy vector, then *SpeI* fragment was isolated and subcloned into clone 9-kb to obtain clone 4 (Fig 3.17B). The 2-kb *KpnI* fragment was isolated from clone 3 and subcloned into *KpnI* digested clone 4 to give clone 9 (Fig 3.17E).

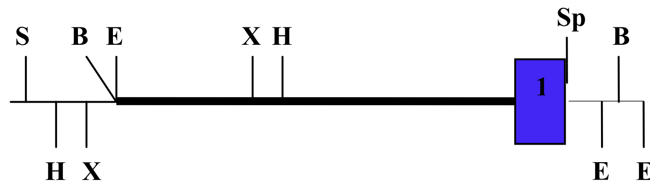
To be able to introduce the *Neo* cassette in clone 9, oligonucleotide adaptor (pink line in Fig 3.17E) containing the sequence of *ClaI-NotI-XhoI* restriction sites was generated, annealed and ligated with *ClaI/XhoI* digested clone 9 to yield clone 10 (Fig 3.17F). The 6-kb *HindIII/SpeI* fragment was isolated from clone *Neo*-4H/S and ligated with *HindIII/SpeI* digested clone 10 to produce clone 11 (Fig 3.17G).

Finally, the *NotI/XhoI* fragment containing the *TK* cassette was inserted in *NotI/XhoI* digested clone 11 to complete the final targeting vector, clone 12, *loxP-Pelo-Neo-loxP-TK* (Fig 3.17I). The DNA of the final targeting vector was digested with different restriction enzymes to verify the presence and orientation of the cloned fragments. The targeting vector *loxP-Pelo-Neo-loxP-TK* was linearised by *XhoI* enzyme before transfection into R1 embryonic stem cells.

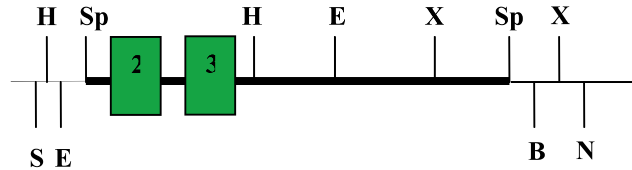
A. Clone 16 kb



B. Clone 9 kb SpeI



C. Clone 6 kb SpeI

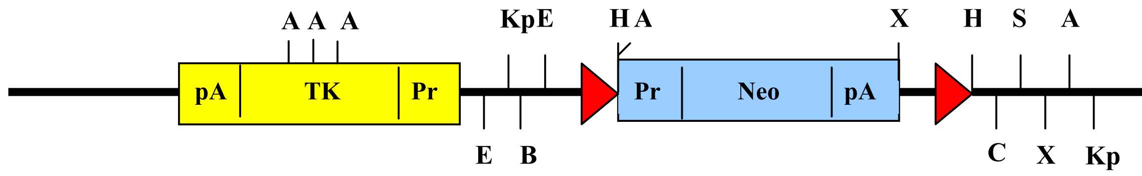


D. Clone 4 H/S

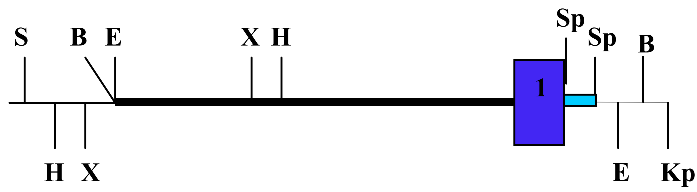
Figure 3.16: A restriction map of Pelo genomic fragment and subclones

A genomic clone (A) containing the 16 kb genomic fragment of *Pelo* gene was cloned in pGEM3 vector. A 9 kb *BamHI/SpeI* and 6 kb *SpeI* fragment containing the 5' (B) and 3' region of *Pelo* gene was subcloned in pBluescript and pGEM3 vectors, respectively. The 4 kb *HindIII/SpeI* fragment containing the 3' flanking region was subcloned in pBluescript vector (D). Restriction sites for following enzymes are shown: B, *BamHI*; E, *EcorI*, H, *HindIII*; S, *SalI*; Sp, *SpeI*; X, *XbaI*; Xh, *XhoI*; N, *NotI;* Kp*, KpnI*. The exons of *Pelota* gene are shown as blue (E1) and green color (E2 and E3) boxes.

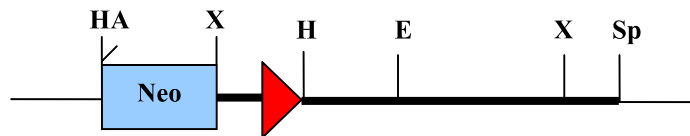
A. pKS*LoxP*NT



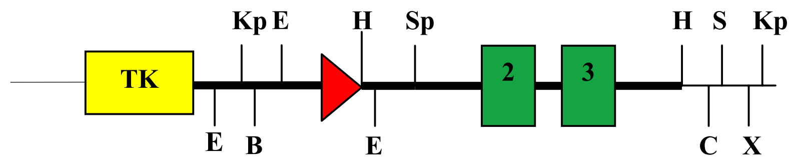
B. Clone 4



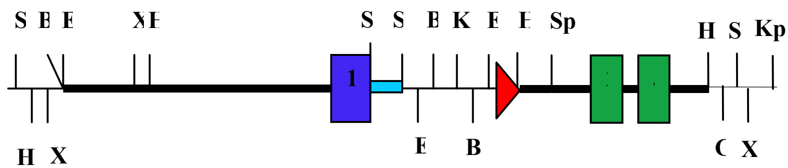
C. Clone *Neo*-4H/S



D. Clone 3



E. Clone 9



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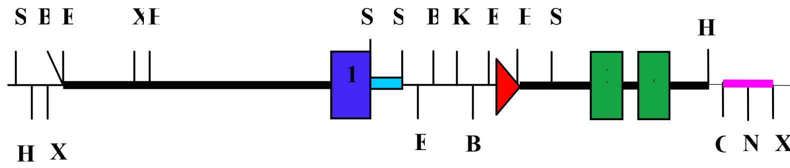
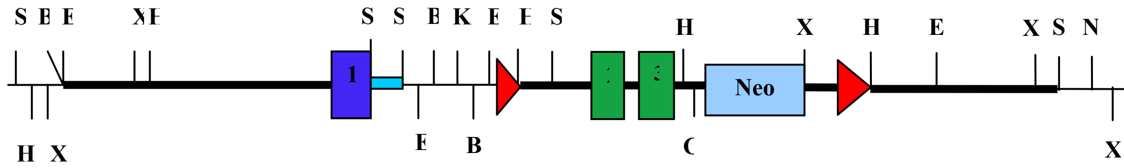
F. Clone 10



G. Clone 11



I. Clone 12

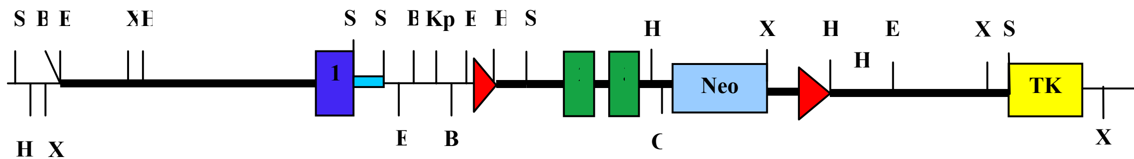


Figure 3.17: Cloning strategy to generate the conditional Pelo targeting allele

A backbone of the pKS*LoxP*NT vector containing the *Neo*, *TK* and *loxP* cassettes was used for cloning the floxed Pelo construct (A). The 150 bp fragment of the intron 1 was cloned into clone 9 kb *SpeI* (Fig 3.16) to generate the clone 4 (B). The 2 kb *HindIII* fragment of *Neo* cassette was isolated from the vector and cloned into clone 4H/S (Fig 3.16D) to generate the clone *Neo*-4H/S (C). The 2 kb *HindIII* fragment containing the exon 2 and 3 of Pelo was cloned upstream of the *TK* cassette, clone 3 (D). The 2 kb *KpnI* fragment was isolated from clone 3 and subcloned in *KpnI* digested clone 4 to give clone 9 (E). An adaptor containing the restriction sites *ClaI-NotI-XhoI* was cloned in *ClaI/XhoI* digested clone 9 to give clone 10 (F). The 6 kb *HindIII/SpeI* fragment was isolated from clone *Neo*-4H/S was inserted in *HindIII/SpeI* digested clone 10 to produce clone 11 (G). Finally, the *Neo/XhoI* fragment containing *TK* cassette was cloned into *NotI/XhoI* digested clone 11 to produce the final targeting construct, clone 12 (I). Restriction sites are shown as letters, *loxP* sites are in red, 150-bp fragment is in light blue, *ClaI-NotI-XhoI* oligonucleotide adaptor is in pink. The cloned fragments are shown as bold bars.

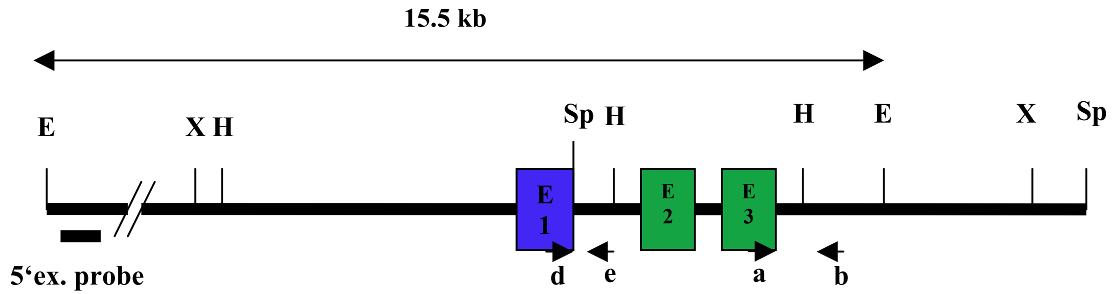
3.3.1.2 Generation of 5' external probe

A 5' external probe was generated and used as a hybridisation probe for Southern blot analysis to distinguish between wildtype (*Pelo*$^{+}$) and floxed (*Pelo*^{flox}) alleles (Fig 3.18I; III; V). For this purpose, 568 bp fragment located in the 5' flanking region of the *Pelota* gene was amplified by PCR assay using Pelo.5'-F1 and Pelo.5'-R1 primers and genomic DNA as a template. The PCR products were subcloned in pGEMTeasy vector and subsequently sequenced. The 568 bp *EcoRI* fragment was purified and used as a 5' external probe for Southern blots with genomic DNA extracting from recombinant ES-clones.

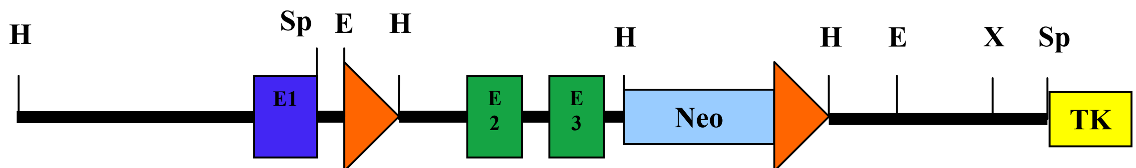
3.3.1.3 Generation of an internal probe

The 1.0 kb *EcoRI/HindIII* fragment was isolated from clone 6-kb *SpeI* (Fig 3.16C) and inserted into the *EcoRI/HindIII* site of pBlueScript vector to produce clone 1.0 kb E/H probe (Fig 3.18III; IV). The DNA of the clone 1.0 kb E/H was then double digested with *EcoRI/HindIII*, the 1.0 kb fragment was extracted from the agarose gel and used as an 3' internal probe for Southern blot hybridisation with genomic DNA extracted from tail biopsies, tissues and mouse embryonic fibroblasts.

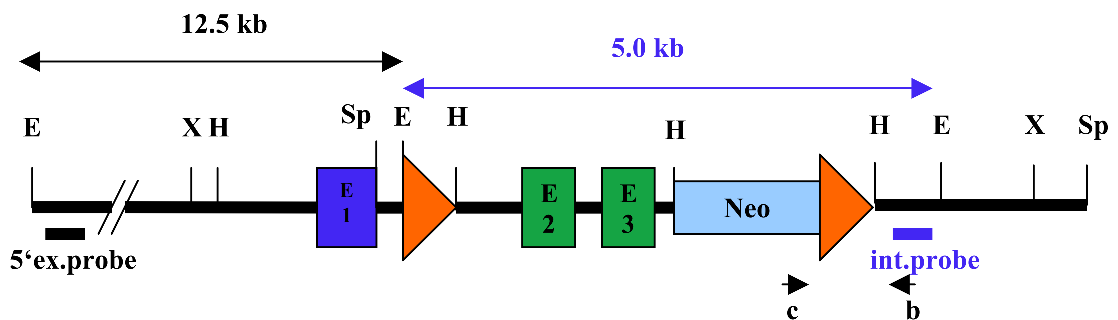
I. Wildtype allele *(Pelo$^+$)*



II. Targeting vector



III. Floxed allele *(Pelo^{flox})*



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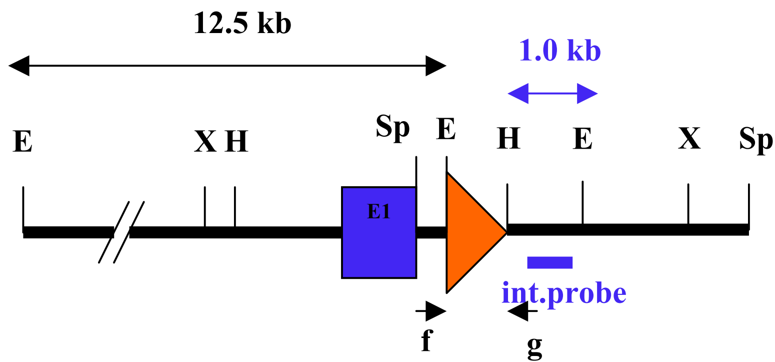
IV. Deleted allele (*Pelo*$^{\Delta}$)



V. Mutant allele *(Pelo*$^{-}$*)*

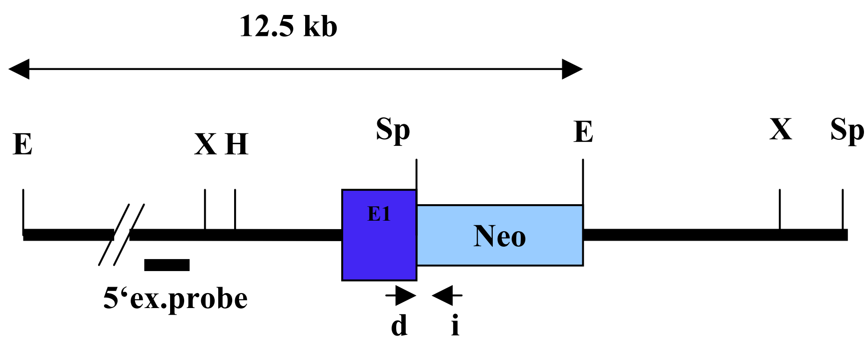


Figure 3.18: Schematic representation of the different *Pelo* alleles

A restriction map of the wildtype allele (I), targeting vector (II), *Pelo*^{flox} allele (III), deleted allele (IV) and conventional *Pelo* knockout allele (V) are shown. The primers **a**, **b** and **c** used to amplify the wildtype and floxed alleles are shown in I and III. Primers **d**, **e** and **i** used to amplify the wildtype and *Pelo*$^{-}$ alleles are shown in I and IV. Primers **f** and **g** used to amplify the *Pelo*$^{\Delta}$ allele are shown in IV. The 5' external and internal probes used to detect the predicted length of *EcoRI* restriction fragment in Southern blot hybridisation are shown in I, III, IV and V.

3.3.1.4 Electroporation and screening of R1 ES cells for homologous recombination

ES cell line R1 was cultured in culture medium as described in materials section (2.2.17.1.1-3). Confluent plates were washed in PBS, trypsinised and the cells were suspended in the same buffer at $2x10^7$ cells/ml. Aliquots of ES cell suspension were mixed with 40 µg of linearised targeting vector *loxP-Pelo-Neo-loxP-TK* and electroporated at 240V and 500 µF using a Bio-Rad Gene Pulser apparatus. The cells were plated onto nonselective medium in the presence of G418-resistant mouse embryonic fibroblasts. After 36 hrs, selection was applied using medium containing G418 at 400 µg/ml and gancyclovir at 2 µM. After 10 days of selection, totally ~800 individual drug-resistant clones from all transfections were picked into 24-well plates for freezing and isolation of DNA.

Targeted integration into the *Pelota* gene was verified by Southern blot analyses using the hybridisation probe located 5' to the targeting construct (Fig 3.18). As expected for homologous recombination event (Fig 3.18), the 5' external probe recognized 15.5 kb wildtype ($Pelo^{+}$) and 12.5 kb floxed ($Pelo^{flox}$) alleles. Of the approximately 800 ES clones screened, 3 clones had undergone correct homologous recombination (Fig 3.19).

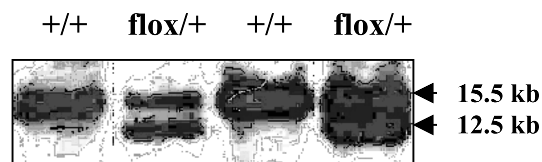


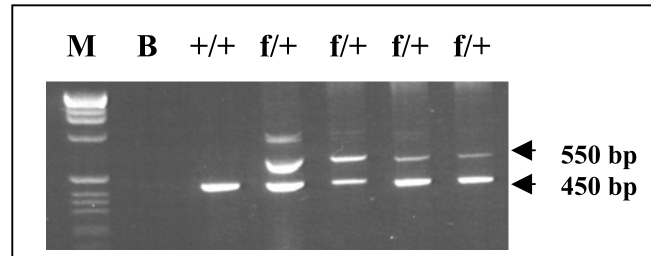
Figure: 3.19: The screening of ES cells for homologous recombination

Southern blot analyses was performed using 5' external probe. ES cell DNA was digested with *EcoRI*. The 0.5 kb external probe detects 15.5 kb wildtype and 12.5 kb floxed alleles.

3.3.1.5 Generation of chimeric mice

The homologous recombinant ES cells were injected into 3.5 dpc blastocysts derived from C57BL/6J mice. The blastocysts were transferred into pseudopregnant CD-1 mice to generate chimeric mice. This work was performed in the MPI for experimental Medicine, Göttingen. ES cells of clones No123 and 127 yielded 7 male and 3 female chimeras, in which the chimerism ranged from 10-85%. None of these male and female chimeras transmitted the targeted gene through their germline. Therefore, ES cells of clone No29 were used to generate chimeric mice. Two high percent male chimeras (90% and 95%) were generated and bred with C57BL/6J female mice to obtain F1 animals. Both chimeric males transmitted the targeted gene through their germline producing heterozygous animals. To determine the genotype of F1 generation, we performed PCR assay with primers a, b and c (Fig 3.18), which amplify 550 bp fragment of floxed allele $Pelo^{flox}$ and 450 bp fragment of wildtype allele $Pelo^{+}$ (Fig 3.20A). Heterozygous $Pelo^{flox/+}$ are viable, fertile and show no abnormalities. Breeding of heterozygous animals yielded homozygous $Pelo^{flox/flox}$ mice. These animals are normal and fertile. These results demonstrate that the insertion of the *loxP* sites and neomycin cassette (*Neo*) in intron 1 and 3' flanking region does not affect the function of *Pelota* gene.

A



B

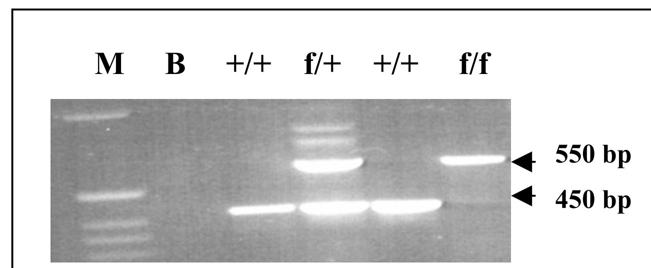


Figure 3.20: PCR genotyping for floxed *Pelo* allele

The wildtype allele for *Pelo* is amplified by PelocKO-F and PelocKO-R primers resulting in a PCR product of about 450 bp. The floxed *Pelo* allele generated a fragment of 550 bp by using the PGK1 and PelocKO-R primers. The PCR products were separated on 1.5% agarose gel and stained with EtBr. B, blank control, no DNA template; +, wildtype allele; f, floxed allele.

3.3.2 Generation of non-inducible conditional knockout mice

3.3.2.1 Breeding strategy to generate *Pelo*$^{flox/-}$*EIIaCre* double transgenic mice

Several *Cre*-fusion alleles were used to catalyse the excision of DNA flanked by *loxP* sequences in cell culture or *in vivo* (Jonkers and Berns, 2002; Branda and Dymecki, 2004). Tissue specific promoters were used to restrict *Cre* activity to the cell type of interest. Inducible *Cre* expression and expression of ligand-dependent *Cre* recombinases were utilised to induce time-controlled specific DNA arrangements (Brocard *et al.,* 1997; Metzger and Chambon, 2001).
In 1989, Dooley et al. found that the adenovirus *EIIa* promoter is active in the oocyte prior to and for a short time after fertilisation. In 1996, Lakso et al. utilised this information to generate a transgenic mouse in which the adenovirus *EIIa* promoter directed the action of *Cre* recombinase gene to the single-cell zygote. This is a practical approach towards deleting DNA sequences between *loxP* sites from all cells of a developing embryo, provided that *Cre* indeed excises the target DNA sequence when the embryo is still at the one-cell zygote stage. If so, then all cells of the developing animal will lack the specified DNA sequence, including germ line cells that give rise to progeny of these mice.
To determine whether DNA sequences between *loxP* sites can be excised by *Cre* recombinase, *EIIaCr*e transgenic allele was introduced in the genome of mice that harbour the floxed Pelo (*Pelo*^{flox}) and the conventional targeted allele (*Pelo*$^{-}$) (Adham *et al.,* 2003). The reason to introduce *EIIaCre* allele in the genome of *Pelo*$^{flox/-}$ but not in that of *Pelo*$^{flox/flox}$ is due to results which have shown that Cre activity in some cells can only excise DNA sequences between *loxP* sites of one floxed allele and yield heterozygous *Pelo*$^{flox/\Delta}$ cells.

To generate the *Pelo*$^{flox/-}$*EIIaCre* mice, we did two intercrosses between *Pelo*$^{flox/+}$ males with *Pel*o$^{+/+}$*EIIaCre* females and between *Pelo*$^{+/-}$ males with *Pelo*$^{+/+}$*EIIaCre* females (Fig 3.21). Genotyping of the offsprings was performed by PCR assay (Fig 3.22).
According to transactivation of the adenovirus *EIIa* promoter in the oocyte (Dooley *et al.,* 1989), we have expected that the recombination of the floxed allele in the

Pelo$^{flox/+}$*EIIaCre* occurs in F1 generation. However, PCR analysis revealed that 20 out 60 analysed animals were chimeras for *Pelo*$^{flox/+}$*EIIaCre* and *Pelo*$^{\Delta/+}$*EIIaCre* genotypes.

To determine the efficiency of recombination, Southern blot with *EcoRI* digested DNA was hybridised with the internal probe (Fig 3.18) which recognises the 15.5 kb *Pelo*$^{+}$, 5.0 kb *Pelo*^{flox} and 1.0 kb *Pelo*$^{\Delta}$ deleted alleles. As shown in Figure 3.23A, the efficiency of deletion of *Pelo*^{flox} allele in *Pelo*$^{flox/+}$ animal No1 is higher than in animal No2. These results demonstrate that the *Pelo*$^{flox/+}$*EIIaCre* double transgenic mice show variable degrees of mosaicism, which is due to partial Cre-mediated recombination of floxed allele. These results suggest that Cre-mediated recombination of floxed allele did not completely occur in the 1-cell stage.

The *Pelo*$^{flox/+}$*EIIaCre* females and *Pelo*$^{+/-}$*EIIaCre* males from F1 generation were bred to produce *Pelo*$^{flox/-}$*EIIaCre* in F2 generation. Eight different genotypes were expected in F2 generation: *Pelo*$^{flox/+}$*EIIaCre*, *Pelo*$^{flox/-}$*EIIaCre*, *Pelo*$^{+/+}$*EIIaCre*, *Pelo*$^{+/-}$*EIIaCre*, *Pelo*$^{flox/+}$, *Pelo*$^{flox/-}$, *Pelo*$^{+/+}$ and *Pelo*$^{+/-}$. Except the genotypes *Pelo*$^{flox/+}$*EIIaCre* and *Pelo*$^{flox/-}$*EIIaCre*, all six other genotypes were found in 70 analysed mice in F2 generation. Genotyping of those mice using PCR assay with primers f and g (Fig 3.18), which amplified deleted allele *Pelo*$^{\Delta}$, resulted in 8 *Pelo*$^{\Delta/+}$*EIIaCre* mice. In contrast, Pelo$^{\Delta/-}$*EIIaCre* could be identified among 70 analysed animals of F2 generation. To verify the targeted deletion of *Pelota* in all cells of *Pelo*$^{\Delta/+}$*EIIaCre* animals, we performed Southern blot analysis using internal probe. As shown in Figure 3.23B, the probe only recognises the 15.5 kb *Pelo*$^{+}$ wildtype and the 1.0 kb *Pelo*$^{\Delta}$ deleted fragments.

Taken together, these results demonstrate the *EIIaCre* completely recombined floxed allele in F2 generation and this excision might have occurred during gametogenesis and 1-cell stage. Furthermore, the absence of *Pelo* in F2 generation is due to the embryonic lethality similar to that shown for the conventional knockout mouse (Adham *et al.,* 2003). The complete excision of the floxed Pelo (*Pelo*^{flox}) allele in *Pelo*$^{flox/+}$*EIIaCre* double transgenic mice and embryonic lethality of *Pelo*$^{\Delta/-}$*EIIaCre* clearly showed that the generated *Pelo*^{flox} allele can successfully recombine with *Cre* recombinase in an *in vivo* system.

F0 ♂ ***Pelo***$^{flox/+}$ **X** ♀ ***Pelo***$^{+/+}$***EIIaCre*** ♂ ***Pelo***$^{+/-}$ **X** ♀ ***Pelo***$^{+/+}$***EIIaCre***

↓ ↓

F1 ♀ ***Pelo***$^{flox/+}$***EIIaCre*** **X** ♂ ***Pelo***$^{+/-}$***EIIaCre***

↓

F2

Pelo$^{flox/+}$***EIIaCre***	***Pelo***$^{flox/+}$
Pelo$^{flox/-}$***EIIaCre***	***Pelo***$^{flox/-}$
Pelo$^{+/+}$***EIIaCre***	***Pelo***$^{+/+}$
Pelo$^{+/-}$***EIIaCre***	***Pelo***$^{+/-}$

Figure 3.21: Breeding strategy to produce double transgenic mice

Offspring from female *Pelo*$^{+/+}$*EIIaCre* and male floxed mice (*Pelo*$^{flox/+}$) were screened for *Pelo*$^{flox/+}$*EIIaCre* double transgenic mice, while offspring from female *Pelo*$^{+/+}$*EIIaCre* and male *Pelo*$^{+/-}$ were screened for *Pelo*$^{+/-}$*EIIaCre* in F1 generation. Then, breeding between double transgenic *Pelo*$^{flox/+}$*EIIaCre* female and *Pelo*$^{+/-}$*EIIaCre* male (F1) was performed in order to obtain *Pelo*$^{flox/-}$*EIIaCre* mice in F2 generation.

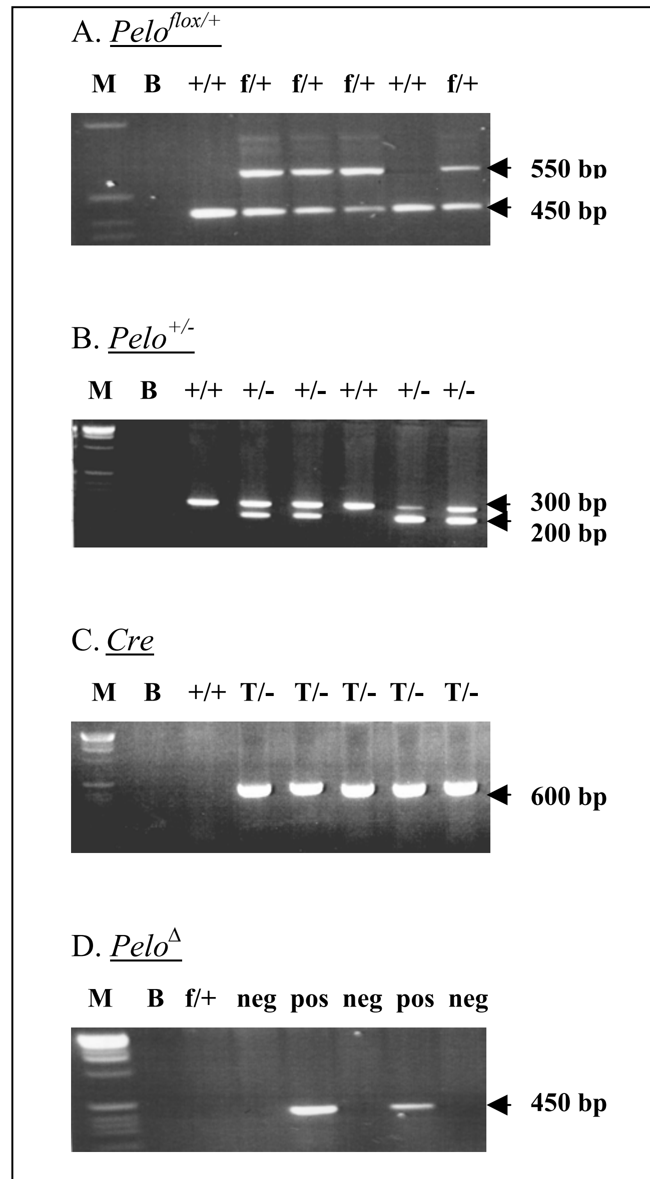


Figure 3.22: Genotyping PCR to identify different alleles of *Pelo*

The genotypes of mice were determined by PCR amplification of tail DNA. Primers were designed to amplify different alleles of *Pelo*, particularly, $Pelo^{+}$, $Pelo^{flox}$, $Pelo^{\Delta}$ and C*re*. (A) **a** and **b** for the $Pelo^{+}$ (450 bp), **c** and **b** for $Pelo^{flox}$ (550 bp); (B) **d** and **e** for $Pelo^{+}$ (300 bp), **d** and **i** for $Pelo^{-}$ (200 bp); (D) **f** and **g** for $Pelo^{\Delta}$ (450 bp) alleles (Fig 3.18) as well as C*re* (600 bp) transgenic allele (C).

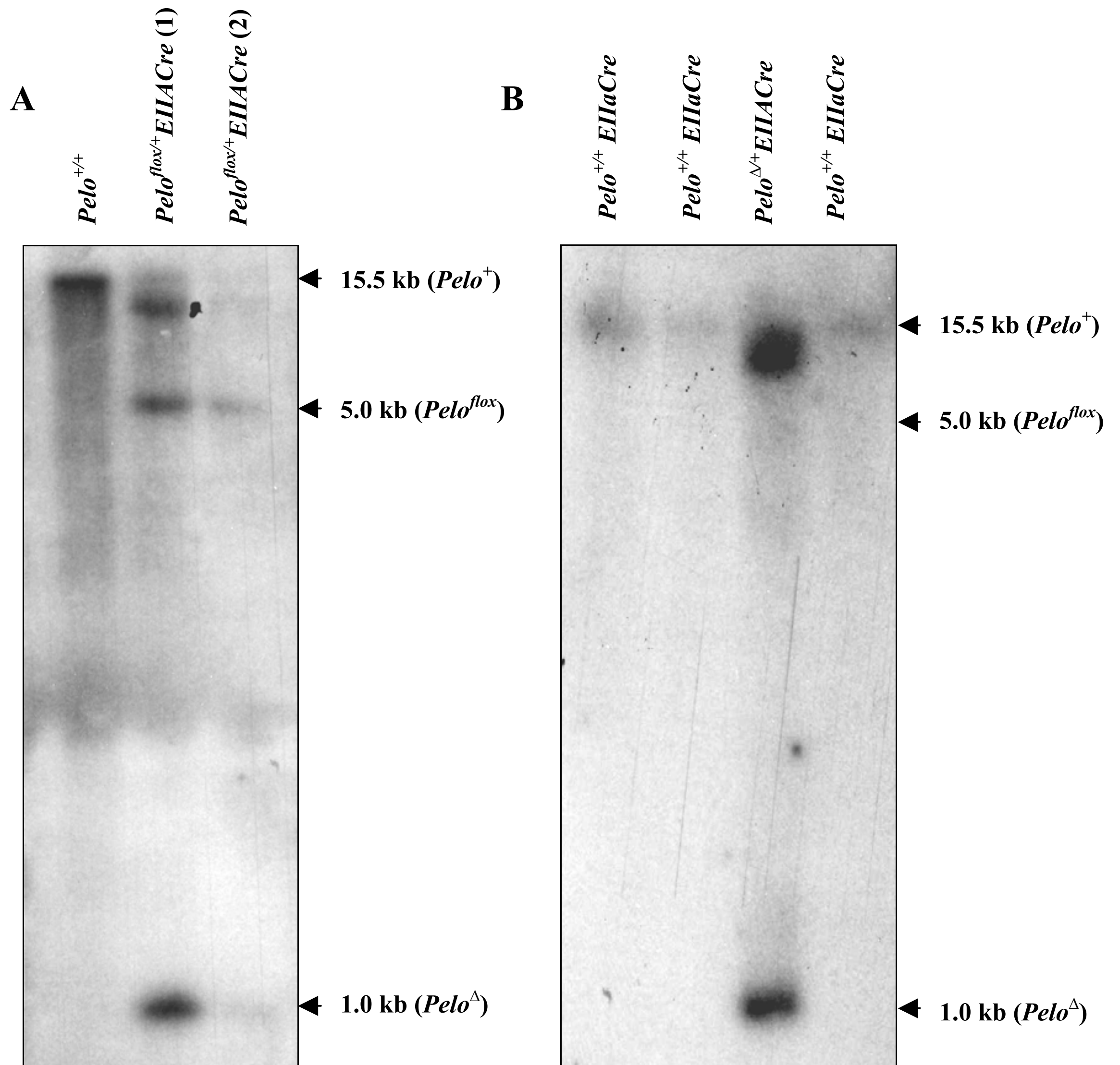


Figure 3.23: Southern blot analysis to evaluate the efficiency of Cre-mediated recombination

A. Lane 1, wildtype as a control. Lanes 2 and 3, $Pelo^{flox/+}$*EIIaCre* mice in which Cre-mediated recombination has partially occurred producing the genotype $Pelo^{\Delta/+}$ in F1 generation.

B. Lanes 1, 2 and 4, wildtype as a control. Lane 3, $Pelo^{flox/+}$*EIIaCre* mice in which Cre-mediated recombination has completely occurred producing the genotype $Pelo^{\Delta/+}$ in F2 generation.

3.3.3 Generation of Pelota inducible conditional knockout mice

3.3.3.1 Breeding strategy to generate *Pelo*$^{flox/-}$Cre*ER*^{T} double transgenic mice

To achieve temporal inactivation of floxed Pelo allele *in vivo*, *Cre-ER*^{T} fusion allele was introduced in the genome of *Pelo*$^{flox/-}$ mice. In this fusion allele, the *Cre* recombinase gene is fused with the mutated ligand binding domain of the human estrogen receptor (ER^{T}). The fusion protein become active upon administration of the synthetic estrogen antagonist 4-hydroxytamoxifen (OHT), but not in the presence of the natural ligand 17β-estradiol (Feil *et al.,* 1996). To avoid the random integration of the *CreER*^{T} transgenic allele into the mouse genome which often results in mosaic gene expression (Garrick *et al.,* 1998; Henikoff 1998), the *CreER*^{T} fusion gene was inserted into the ubiquitously expressed ROSA26 (R26) locus by gene targeting (Voojis *et al.,* 2001). The R26CreER^{T} is expressed throughout mouse development and in adult life, but *Cre* recombinase only become active after administration of the OHT or tamoxifen (TAM).

To determine the efficiency of the ligand-dependent *Cre* recombinase (CreER^{T}) for excision of *Pelo*^{flox} after administration of the ligand, 9 males and 1 female *Pelo*$^{flox/-}$ *CreER*^{T} mice were injected intraperitoneally (i.p) for 5 consecutive days with 2 mg of TAM. The controls, 3 males and 2 females *Pelo*$^{+/+}$*CreER*^{T} animals were also treated.

3.3.3.2 Fertility test of the TAM-treated mice

To check the fertility, we crossed the animals with wildtype CD-1 mice one month after TAM administration. As shown in Table 3-1, 6 *Pelo*$^{flox/-}$*CreER*^{T} male mice were subfertile and 3 *Pelo*$^{flox/-}$*CreER*^{T} male mice were fertile. The wildtype TAM-treated control animals (*Pelo*$^{+/+}$*CreER*^{T}) were fertile. One treated female died 10 days after treatment, while the other *Pelo*$^{flox/-}$*CreER*^{T} and wildtype females are fertile.

No. of mice	Sex	Genotype	Littersize
26	♂	*Pelo*$^{flox/-}$*CreER*^{T}	4
51	♂	*Pelo*$^{flox/-}$*CreER*^{T}	2
79	♂	*Pelo*$^{flox/-}$*CreER*^{T}	3
78	♂	*Pelo*$^{flox/-}$*CreER*^{T}	3
68A	♂	*Pelo*$^{flox/-}$*CreER*^{T}	3
73A	♂	*Pelo*$^{flox/-}$*CreER*^{T}	3
40	♂	*Pelo*$^{flox/-}$*CreER*^{T}	13
68	♂	*Pelo*$^{flox/-}$*CreER*^{T}	17
45	♂	*Pelo*$^{flox/-}$*CreER*^{T}	13
43	♂	*Pelo*$^{+/+}$*CreER*^{T}	13
76	♂	*Pelo*$^{+/+}$*CreER*^{T}	fertile
69	♂	*Pelo*$^{+/+}$*CreER*^{T}	14
54	♀	*Pelo*$^{flox/-}$*CreER*^{T}	died
37	♀	*Pelo*$^{+/-}$*CreER*^{T}	fertile
39	♀	*Pelo*$^{+/+}$*CreER*^{T}	fertile

Table 3-1: Fertility test of TAM-treated mice bred with wildtype animals

To investigate the cause of infertility and to determine the efficiency of deletion of floxed allele in some tissues *in vivo,* including testis, spleen, stomach and kidney were isolated from three subfertile *Pelo*$^{flox/-}$*CreER*^{T} males (No26, 51 and 79) and from two control *Pelo*$^{+/+}$*CreER*^{T} males.

One month after administration of TAM, testis size of *Pelo*$^{flox/-}$*CreER*^{T} mice was drastically reduced as compared to TAM-treated *Pelo*$^{+/+}$*CreER*^{T} control mice (Fig 3.24).

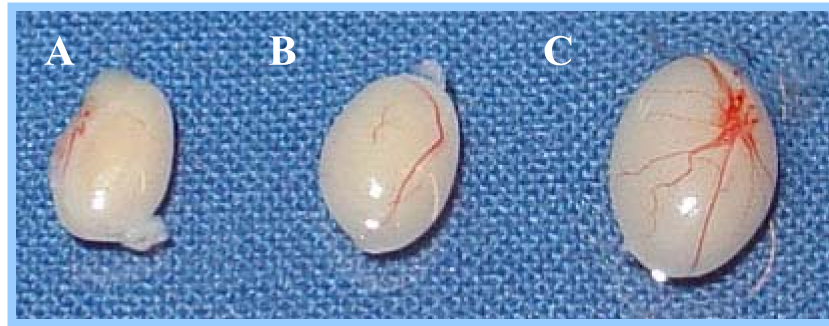


Figure 3.24: The phenotypic alteration of testis from TAM-treated *Pelo$^{flox/-}$CreER^{T}* animals

The testis size is severely reduced in TAM-treated *Pelo$^{flox/-}$CreER^{T}* No26 and 79 mice (A and B) as compared with TAM-treated *Pelo$^{+/-}$CreER^{T}* control mouse (C) at 3 months of age.

3.3.3.3 Histological analysis of TAM-treated mice

Histological analysis of *Pelo$^{flox/-}$CreER^{T}* testes from TAM-treated mice revealed extensive degeneration of a large fraction (20-60%) of seminiferous tubules of mutant animals No26 and 51, while depletion of germ cells in testes of mutant animals No79 was less apparent (Fig 3.25). Whereas testes of TAM-treated *Pelo$^{+/+}$CreER^{T}* control mice demonstrated robust spermatogenesis, the affected fractions of seminiferous tubules of mutant testes from animals No26 and 51 showed markedly reduced late meiotic (i.e late pachytene and diplotene spermatocytes) and postmeiotic (i.e spermatids and spermatozoa) germ cells and contained significant vacuolisation. This vacuolisation is caused by severe germ cell depletion through endocytosis.

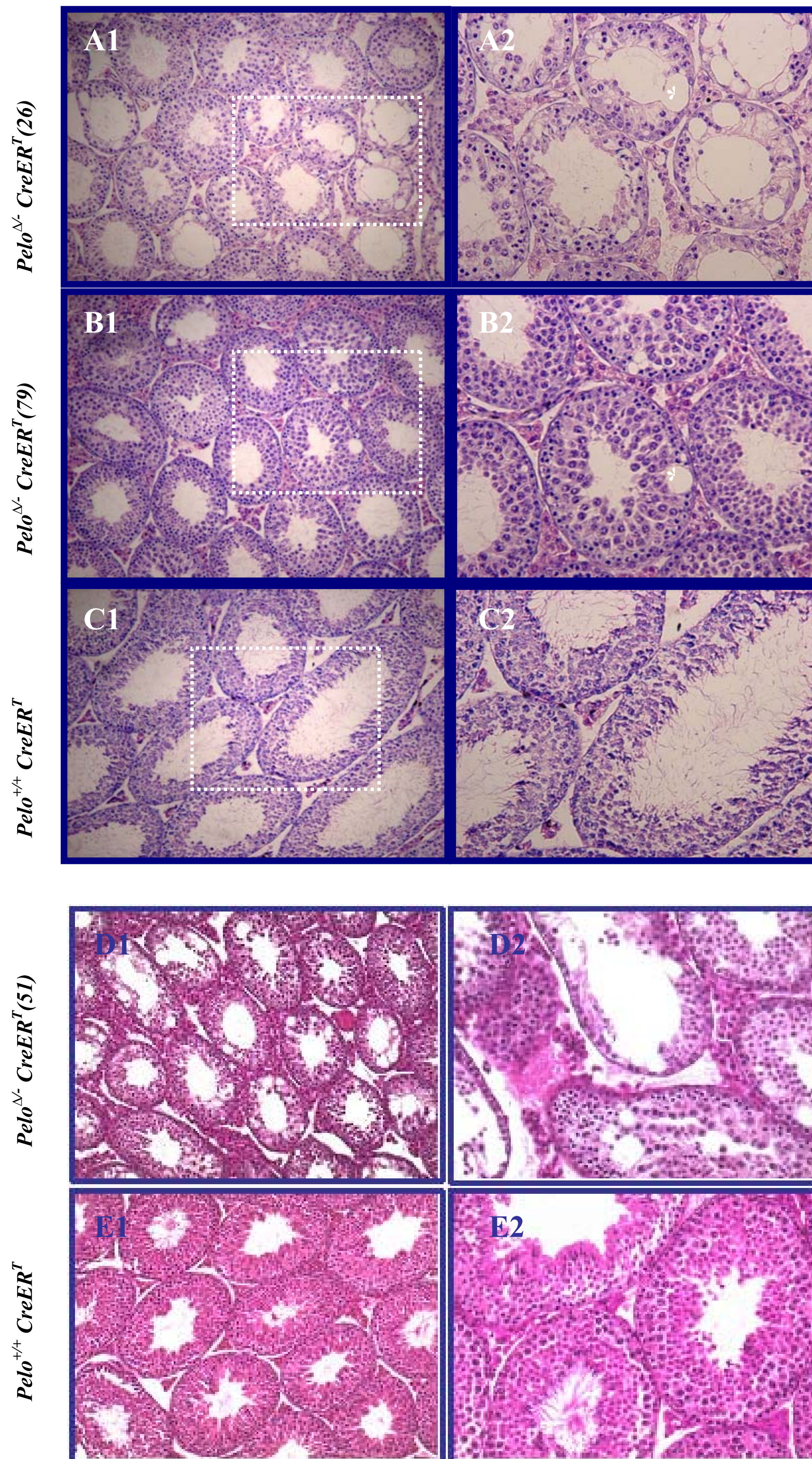

A1
A2
B1
B2
C1
C2
D1
D2
E1
E2
Pelo$^{Δ/-}$ CreER^{T}(26)
Pelo$^{Δ/-}$ CreER^{T}(79)
Pelo$^{+/+}$ CreER^{T}
Pelo$^{Δ/-}$ CreER^{T}(51)
Pelo$^{+/+}$ CreER^{T}

Figure 3.25: Hematoxylin-eosin (H&E) staining of sections of TAM-treated *Pelo*$^{flox/-}$*CreER*^{T} testes

Sections through testis demonstrate tubules of smaller diameter, dilated vacuoles (asterisk), and drastic reduction of spermatogenic cells in the *Pelo*$^{\Delta/-}$*CreER*^{T} mutant mice No: 26, 79 and 51 (A1, B1 and D1) in contrast to *Pelo*$^{+/+}$*CreER*^{T} males as control (C1 and E1). A2, B2, C2, D2 and E2: Higher-magnification images corresponding to the boxed areas in A1, B1, C1, D1 and E1, respectively.

3.3.3.4 Immunohistochemical analysis of testis sections of TAM-treated *Pelo*$^{flox/-}$*CreER*^{T} mice

To further confirm our histological findings, we investigated whether the depletion of germ cells in testes of *Pelo*$^{\Delta/-}$*CreER*^{T} mice is due to the defect in spermatogonia or due to the arrest of germ cells in later stages. For these experiments, we analysed the expression of Apg2 which is a marker for spermatogonia. By immunohistochemical analysis, we detected numerous Apg2 positive spermatogonia in TAM-treated *Pelo*$^{flox/-}$*CreER*^{T} testis (Fig 3.26) whereas the number of Apg2 positive spermatogonia varied among the seminiferous tubules of mutant testes.

Taken together, these analyses show that the depletion of germ cells in TAM-treated *Pelo*$^{flox/-}$*CreER*^{T} animals is not uniform in all seminiferous tubules and the degree of germ cell damage varies between TAM-treated mutants. This observed variability may be due to the fact that the ligand-dependent *Cre* recombinase (CreER^{T}) does not efficiently delete the floxed allele in all germ cells. This means that TAM-treated *Pelo*$^{flox/-}$*CreER*^{T} mice are chimera for *Pelo*$^{\Delta/-}$ and *Pelo*$^{flox/-}$ cells.

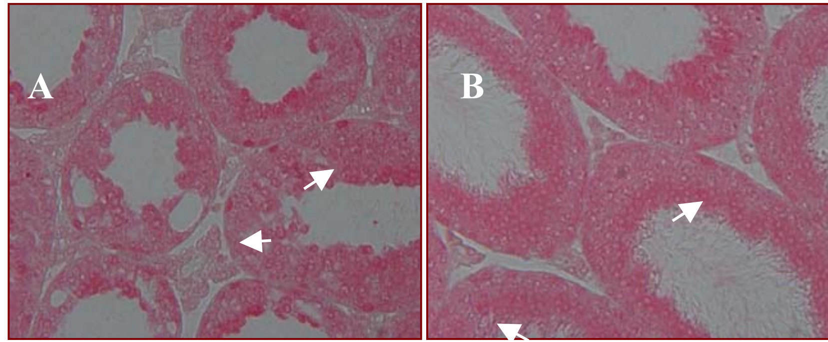


Figure 3.26: Apg2 expression in TAM-treated *Pelo*$^{flox/-}$*CreER*^{T} mice

Immunohistochemical analysis on sections through testes of *Pelo*$^{\Delta/-}$*CreER*^{T} (A) and *Pelo*$^{+/+}$*CreER*^{T} (B) was performed with anti-Apg2 antibody show tubules of smaller diameter, dilated vacuoles and germ cell damage. Arrows indicate spermatogonia.

3.3.3.5 The efficiency of TAM-induced recombination of *Pelo*^{flox} by *CreER*^{T}

To address whether TAM-treated *Pelo*$^{flox/-}$*CreER*^{T} animals are mosaics, Southern blot was performed with *EcoRI* digested DNA extracted from spleen, kidney and tail of three treated mutant animals No51, 79 and 26. The blots were hybridised with the internal probe that recognises the floxed (*Pelo*^{flox}) and the deleted (*Pelo*$^{\Delta}$) alleles. As shown in Figure 3.27, the efficiency of deletion in spleen was higher than that in kidney and tail of mice.

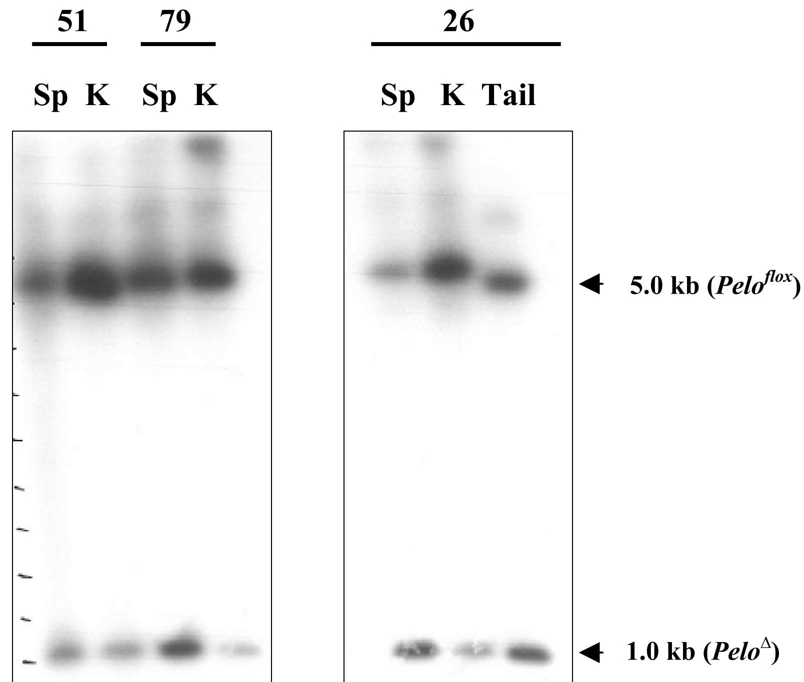


Figure 3.27: TAM-induced recombination of *Pelo*^{flox} allele in adult organs

Southern blot analysis of *EcoRI* digested genomic DNA isolated from spleen and kidney of *Pelo*$^{flox/-}$*CreER*^{T} mice (No51, 79 and 26) treated with 2mg/day TAM for 5 consecutive days. According to Southern blot strategy (Fig 3.18) the expected sizes of fragments that hybridise to the ^{32}P-labeled DNA fragment of 900 bp internal probe are indicated the corresponding floxed allele (*Pelo*^{flox}) and deleted allele (*Pelo*$^{\Delta}$). Sp, spleen; K, kidney.

3.3.3.6 Protein study in the testes of TAM-treated *Pelo*$^{flox/-}$*CreER*^{T} mice

Western blot analysis was performed with the proteins extracted from testes of TAM-treated Pelo$^{flox/-}$CreER^{T} No26 and 79 as well as from Pelo$^{+/+}$CreER^{T} control animals. This blot was probed with anti-Pelo antibody. As shown in Figure 3.28, the level of Pelo protein in testis of animal No26 is markedly reduced (80%) as compared to control animal. The level of Pelo protein in testis of animal No79 is slightly reduced. The reduction of Pelo protein in testes of animals No26 and 79 correlates with the observed damage of germ cells in testes of animals (Fig 3.25), where the depletion of germ cells in testis of animal No26 was significantly higher than in testis of animal No79.

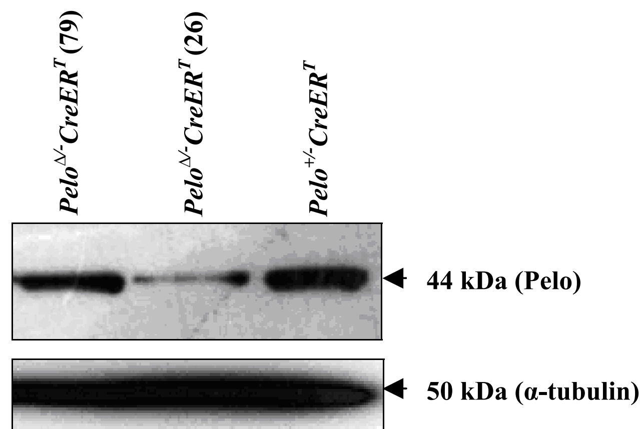


Figure 3.28: Expression of Pelo in testes of TAM-treated animals

Western blot with protein extracting from testes of TAM-treated *Pelo*$^{\Delta/-}$*CreER*^{T} mice (No79 and No26) and *Pelo*$^{+/-}$*CreER*^{T} mice (control) was probed with anti-Pelo antibody. The level of Pelo protein in testes of *Pelo*$^{\Delta/-}$*CreER*^{T} (No26) is markedly reduced (80%) as compared to control, while the level of Pelo in animal No79 is slightly reduced. A monoclonal antibody against α-tubulin was used as a loading control.

3.3.3.7 The effect of Pelota deficiency on cell proliferation

To study the consequences of Pelota deficiency on cell proliferation, mouse embryonic fibroblasts (MEFs) were isolated from *Pelo*$^{flox/-}$*CreER*^{T} and *Pelo*$^{+/+}$*CreER*^{T} embryos. *Pelo*$^{flox/+}$*CreER*^{T} male and *Pelo*$^{+/-}$ female animals were crossed and embryos were isolated at E13.5. Fibroblasts were cultured from each embryo as described in methods section (2.2.16.4), DNA was extracted from head of each embryo and used for genotyping. During the fibroblasts culture, we genotyped the embryos and found two *Pelo*$^{flox/-}$*CreER*^{T} embryos. Fibroblasts of *Pelo*$^{flox/-}$*CreER*^{T} and *Pelo*$^{+/+}$*CreER*^{T} (control) were frozen and used for further experiments.

We tested firstly whether conditional floxed allele (*Pelo*^{flox}) could be efficiently switched to *Pelo*$^{\Delta}$ in cultured *Pelo*$^{flox/-}$*CreER*^{T} fibroblasts in the presence of 0.2 and 1.0 µM hydroxytamoxifen (OHT). 0.6x10^{6} cells were plated in 6 cm dish and after 24 hrs of the culture, OHT was added to the medium. After 3 days of culture in the presence of OHT, DNA and protein were extracted. To determine which concentration of OHT is able to promote the recombination of *Pelo*^{flox} to *Pelo*$^{\Delta}$ allele in culture, we performed

firstly PCR analysis. Using primers f and g (Fig 3.18) that amplify the deleted fragment, we found that recombination of *Pelo*^{flox} allele was more efficiently in culture with 1 µM of OHT.

To investigate the frequency of recombination in *Pelo*$^{flox/-}$*CreER*^{T} fibroblasts cultured in the presence of 0.2 and 1 µM OHT, Southern blot with *EcoRI* digested DNA was hybridised with internal probe (Fig 3.18) that recognises *Pelo*^{flox} and *Pelo*$^{\Delta}$ genomic fragments. As shown in Figure 3.29, the intensity of the 5.0-kb *Pelo*^{flox} was higher than that of 1.0-kb *Pelo*$^{\Delta}$ deleted fragment in DNA isolated from cultured *Pelo*$^{flox/-}$*CreER*^{T} fibroblasts in the presence of 1 µM OHT.

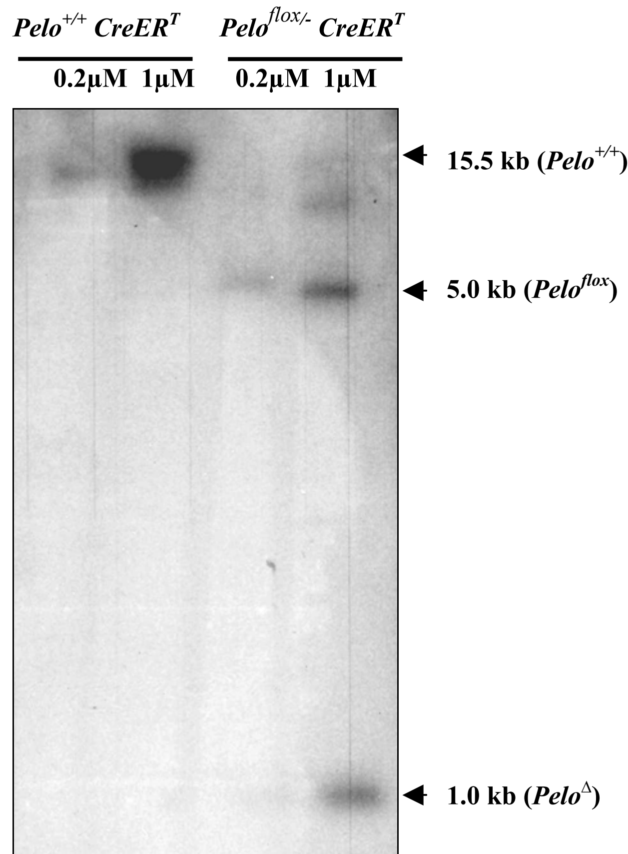


Figure 3.29: Southern blot analysis to evaluate the efficiency of Cre-mediated recombination

Southern blot analysis with *EcoRI* digested genomic DNA isolated from *Pelo*$^{flox/-}$*CreER*^{T} fibroblasts treated with 1 µM/day of OHT for 3 consecutive days. Southern blot was hybridised with the internal probe, which recognises the 15.5 kb fragment of wildtype allele (*Pelo*$^{+}$), 5.0 kb fragment of floxed *Pelo* allele (*Pelo*^{flox}) and 1.0 kb fragment of deleted allele (*Pelo*$^{\Delta}$).

3.3.3.8 Expression analysis of Pelota in OHT-treated fibroblasts at protein level

To confirm the results of Southern blot at protein level, Western blot analysis was performed using total protein extracted from cells which were grown in the presence of 0.2 and 1 µM OHT ($Pelo^{flox/-}CreER^{T}$ and $Pelo^{+/+}CreER^{T}$) and probed with anti-Pelo antibody. The Western blot analysis showed that the expression of Pelo protein was apparently reduced in cells of $Pelo^{flox/-}CreER^{T}$ in comparison to $Pelo^{+/+}CreER^{T}$ control. This result demonstrates that the $Pelo^{flox}$ allele is not recombined in all $Pelo^{flox/-}CreER^{T}$ cells and suggest that Cre-mediated deletion occurred in less than 50% of $Pelo^{flox/-}CreER^{T}$ cells.

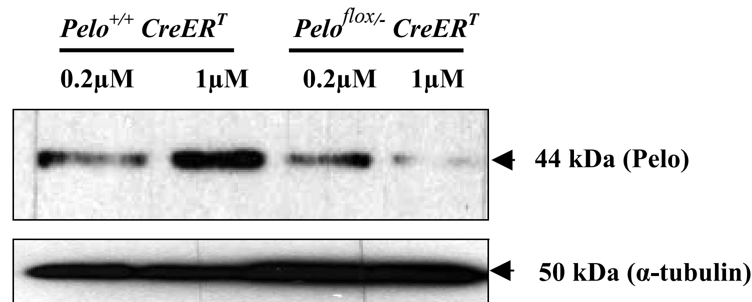


Figure 3.30: Expression of Pelo in OHT-treated $Pelo^{flox/-}CreER^{T}$ and $Pelo^{+/+}CreER^{T}$ fibroblasts

Western blot with protein extracting from OHT-treated MEFs was probed with anti-Pelo antibody. Equal protein loading was determined by reprobing the blot with anti-α tubulin antibody.

3.3.3.9 Proliferation assay of OHT-treated fibroblasts

To determine the proliferation of the $Pelo^{flox/-}CreER^{T}$ in culture in the presence of 1 µM OHT, $0.4x10^{6}$ cells were plated in 6 cm dish and cultured for 2 days in the presence of 1 µM OHT. Cells were then trypsinised, counted and replated in four 6 cm dishes and cultured in the presence of 1 µM OHT. The number of cells was counted after 2, 4 and 6 days. Results of two independent experiments show that the proliferation of $Pelo^{flox/-}CreER^{T}$ cells in the presence of 1 µM OHT is not significantly different from that of control cells (Fig 3.31). This result suggests that either *Pelota* deficiency does not

influence cell proliferation or that the *Pelo*$^{\Delta/-}$*CreER*^{T} cells might be rapidly diluted in culture by fast growth of the nondeleted cells (*Pelo*$^{flox/-}$*CreER*^{T}).

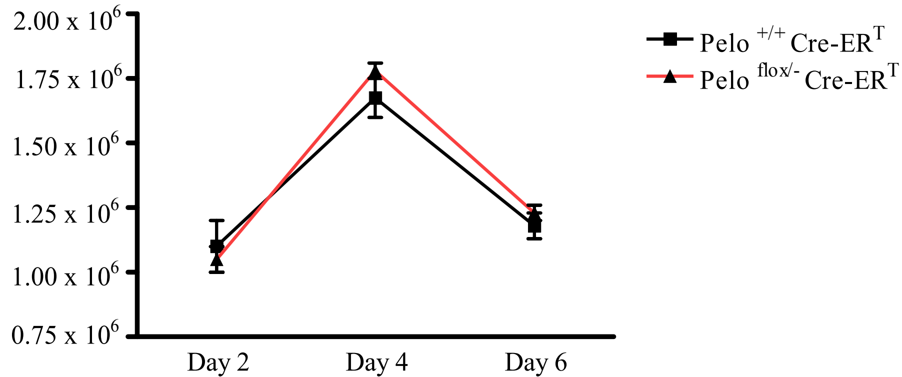


Figure 3.31: Proliferation assay of OHT-treated fibroblasts

Pelo$^{flox/-}$*CreER*^{T} and *Pelo*$^{+/+}$*CreER*^{T} fibroblasts were cultured in the presence of 1 µM OHT. Cell number was determined at day 2, 4 and 6. The number of cells was not significantly different between *Pelo*$^{\Delta/-}$*CreER*^{T} and control fibroblasts.

3.3.3.10 Karyotype analysis of OHT-treated fibroblasts

Analysis of the conventional *Pelota* knockout mice revealed the significant increase of aneuploid cells in Pelo$^{-/-}$ embryos at E7.5. To count the number of aneuploid cells, *Pelo*$^{flox/-}$*CreER*^{T} MEFs were cultured in the presence of 1 µM OHT for 48 hrs and then treated with 100 ng/ml colcemid for 4 hrs to arrest proliferating cells at metaphase. Cells were fixed and stained with Giemsa as described in methods section (2.2.16.5). Individual metaphase cells were photographed. Photographic files were shuffled and scored blind for the number of chromosomes. The result revealed that 6 out of 21 *Pelo*$^{+/+}$*CreER*^{T} and 25 out of 40 *Pelo*$^{flox/-}$*CreER*^{T} metaphases were aneuploid (Fig 3.32).

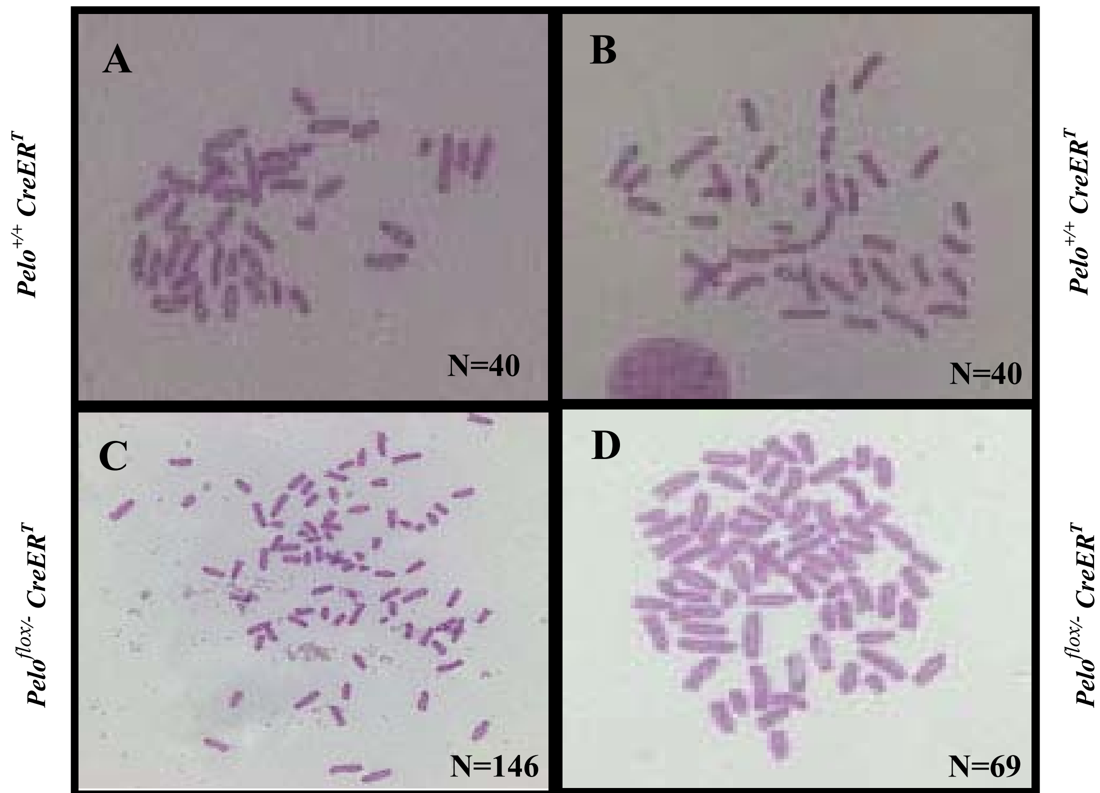


Figure 3.32: Metaphase spreads of OHT-treated fibroblasts

More than 60% of MEFs cells derived from OHT-treated *Pelo*$^{flox/-}$*CreER*^{T} (C and D) were found to contain an aneuploid number of chromosomes, whereas ~30% of control *Pelo*$^{+/+}$*CreER*^{T} cells (A and B) were aneuploid.

3.4 Pelota transgenic mice

3.4.1 Generation of *hEF-Pelo* transgenic construct and purification

To study the effect of *Pelota* overexpression on progression of mitotic and meiotic division, we generated a transgenic construct containing the human *Pelota* cDNA under the promoter of *human elongation factor-1α* (*hEF-1α*). The 3' untranslated region of the human granulocyte macrophage colony stimulating factor, which contains polyadenylation signal (*GM-GSF polyA*) was fused downstream of *Pelota* cDNA. *hEF-1α* promoter is ubiquitously transactivated and is highly active in testis.

To generate the transgenic construct, a 1.2 kb fragment of human *Pelota* cDNA was amplified using specific primers hPelo-F20 and hPelo-R20. The 1.2-kb *Pelota* cDNA fragment containing *XbaI* restriction site was subcloned into pGEMTeasy vector and sequenced. Subsequently, the 1.2 kb *Xba I* fragment was isolated and cloned into *XbaI* digested pEF-BOS vector. Positive clones were detected by colony hybridisation. To check right orientation of the insert, test digestion was performed by *SstI* enzyme. The *hEF-Pelo* fusion fragment was released from the vector backbone by *PvuI* and *HindIII* enzymes, purified and used for microinjection of oocytes. The oocytes were isolated from superovulated FVB/N females, injected with the *hEF-Pelo* transgenic construct and then retransferred into oviducts of pseudopregnant females. Transgenic mice were generated in MPI for experimental Medicine, Göttingen.

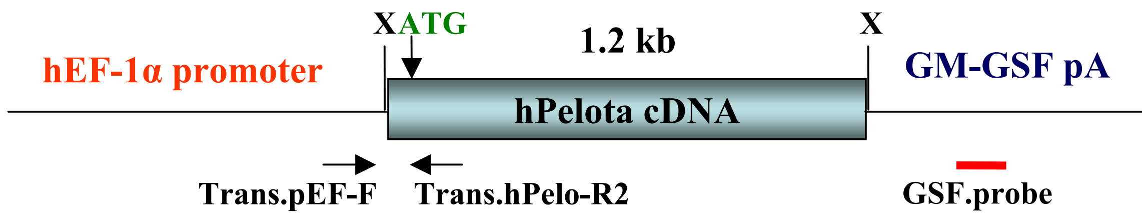


Figure 3.33: Schematic representation of *hEF-Pelo* transgenic construct

The construct contains the *human elongation factor-1α* promoter (1.2kb) fused to the human *Pelota* cDNA (1.2kb) and human granulocyte macrophage colony stimulating factor polyadenylation signal (*GM-CSF polyA*). The primers (arrows) were used for genotyping PCR and *GSF*-probe was designed for Northern blot hybridisation. X, *Xba I* cloning site.

3.4.1.1 Generation and breeding of transgenic mice

Transgenic mice harbouring *hEF-Pelo* transgenic allele were identified by PCR with genomic DNA extracted from tail samples of founder mice (Fig 3.34).
Two males out of nine founders were transgenic. From these founders, two transgenic lines were established (Eva #1 and #6). Founder mice were crossed with FVB/N females to establish transgenic Eva #1 and Eva #6. PCR analysis revealed that the transgenic *hEF-Pelo* allele is transmitted to offspring of male founders #1 and #6.

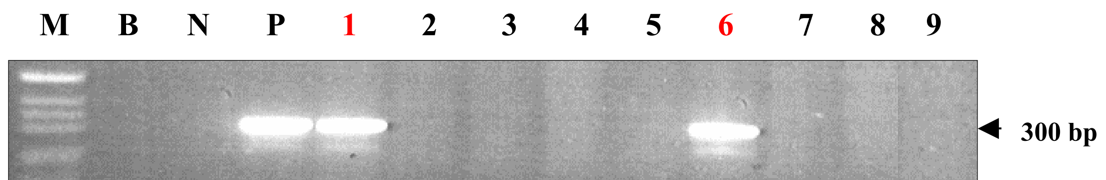
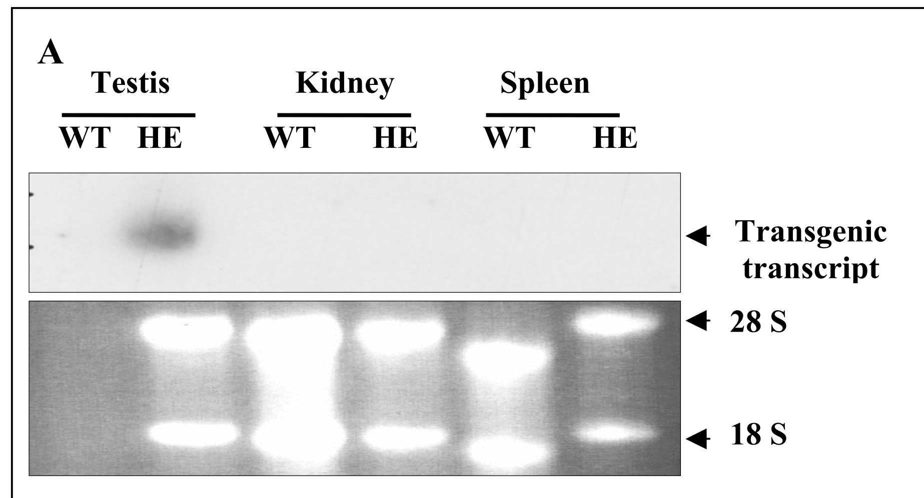


Figure 3.34: Genotyping PCR of *hEF-Pelo* founder mice.

B, blank control, no DNA template; N, negative control (wildtype DNA); P, positive control (transgenic construct DNA); the primers amplified a 300 bp transgenic allele. Mice No1 and 6 (in red) were found to harbour the transgenic allele.

3.4.1.2 Expression studies of *hEF-Pelo* transgenic mice

To examine the expression of *hEF-Pelo* allele in transgenic mice, total RNA was extracted from testis, kidney and spleen and subjected to Northern blot hybridisation. The hybridisation probe, *GSF*, was generated by amplification of the *GM-GSF polyA* cassette of transgenic construct. The probe recognised the fusion transcript only in testis of Eva #1, whereas no expression of transgenic allele could be detected in kidney and spleen (Fig 3.35A). Expression analysis of Eva #6 revealed high expression in all studied tissues (Fig 3.35B). To determine the consequence of the Pelota overexpression, transgenic males and females were crossed to wildtype mice. All studied F1 transgenic animals were fertile. Histological analysis of transgenic testis of Eva #1 displays normal structure of seminiferous tubules (Fig 3.36).



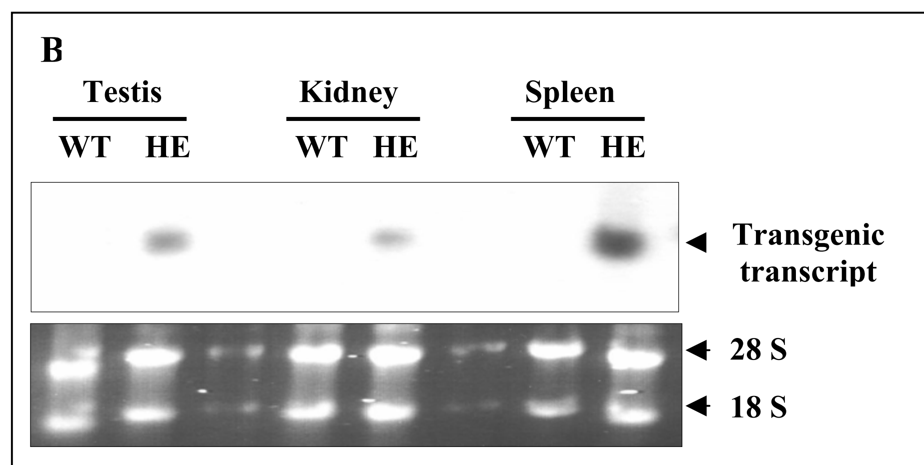


Figure 3.35: Expression analysis by Northern blot

Total RNA was isolated from testis, kidney and spleen of each transgenic line and hybridised with *GSF* specific probe. A, Eva #1; B, Eva #6. WT, wildtype animal; HE, transgenic mice from F1 generation. Integrity of RNA was shown by 28S and 18S RNA signals on Northern gel picture.

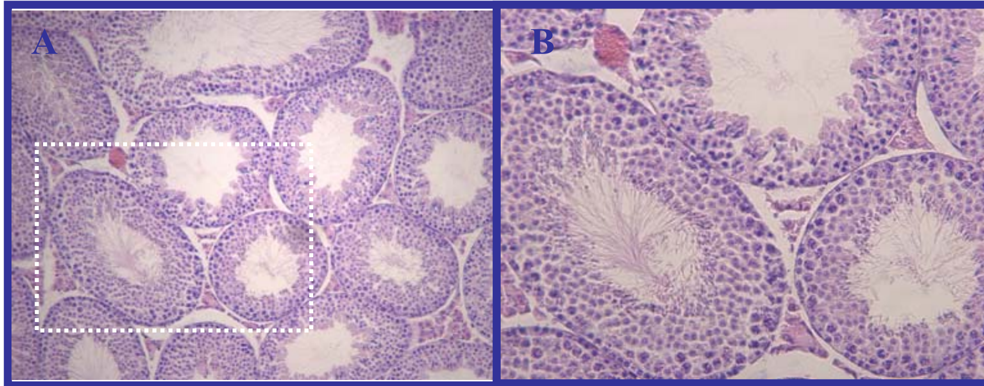


Figure 3.36: Hematoxylin-eosin (H&E) staining of section of *hEF-Pelo* testis

Section through testis of Eva #1 demonstrates normal tubules (A); higher magnification images corresponding to the boxed areas (B).

3.4.2 Generation of *hUB-Pelo* transgenic construct and purification

In addition to *hEF-Pelo* transgenic mice, we also generated a transgenic construct containing the human *Pelota* cDNA under the control of the ubiquitously expressed *human ubiquitin* (*hUbC*) promoter. The 3' untranslated region of bovine growth hormone, which contains the polyadenylation signal (*BGH polyA*) was fused downstream of *Pelota* cDNA (Fig 3.37).

To generate the transgenic construct, a 1.2 kb fragment of human *Pelota* cDNA was amplified using specific primers hPelo-F20 and hPelo-R20. The 1.2-kb *Pelota* cDNA fragment containing *XbaI* restriction site was subcloned into pGEMTeasy vector and sequenced. Subsequently, the 1.2 kb *EcoRI* fragment was isolated and cloned into *EcoRI* digested pUB6/V5-His A vector. Positive clones were detected by colony

hybridisation. To check right orientation of the insert, test digestion was performed by *SstI* enzyme. The *hUB-Pelo* fusion fragment was released from the vector backbone by *BglII* and *BglI* enzymes, purified and used for microinjection of oocytes. The oocytes were isolated from superovulated FVB/N females, injected with the *hUB-Pelo* transgenic construct and then retransferred into oviducts of pseudopregnant females. Transgenic mice were generated in MPI for experimental Medicine, Göttingen.

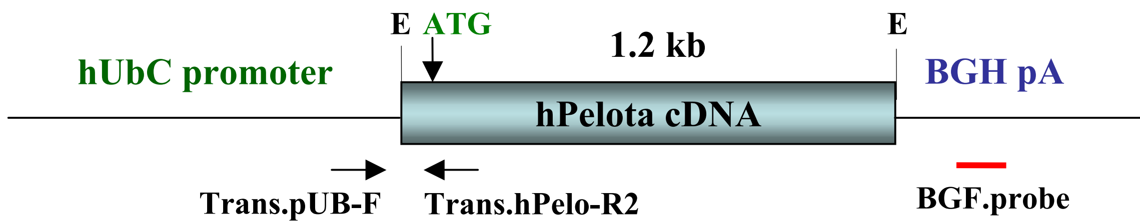


Figure 3.37: Schematic representation of *hUB-Pelo* transgenic construct

The construct contains the *human ubiquitin C* promoter (1.2kb) fused to the human *Pelota* cDNA (1.2kb) and bovine growth hormone polyadenylation signal (*BGH polyA*). The primers (arrows) were used for genotyping PCR and *BGH-probe* was designed for Northern blot hybridisation. E, *EcoRI* cloning site.

3.4.2.1 Generation and breeding of transgenic mice

Transgenic mice harbouring *hUB-Pelo* transgenic allele were identified by PCR with genomic DNA extracted from tail samples of founder mice (Fig 3.38).

Five males out of fifteen founders were transgenic. From these founders, four transgenic lines were established (Adam #1, #9, #10 and #13). Founder mice were crossed with FVB/N females to establish transgenic Adam #1, #9, #10 and #13. PCR analysis revealed that the transgenic *hUB-Pelo* allele is transmitted to offspring of male founders #1, #9, #10 and #13. Transgenic mice harbouring *hUB-Pelo* transgenic allele were identified by PCR with genomic DNA extracted from tail samples.

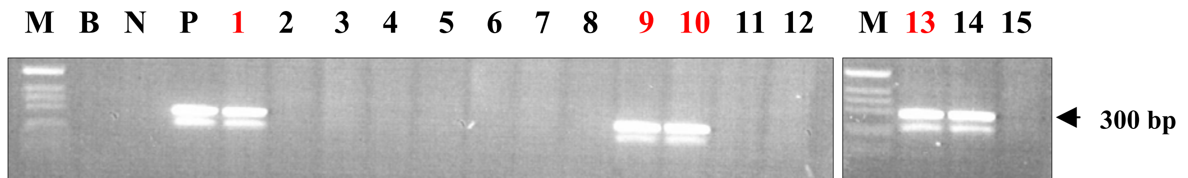
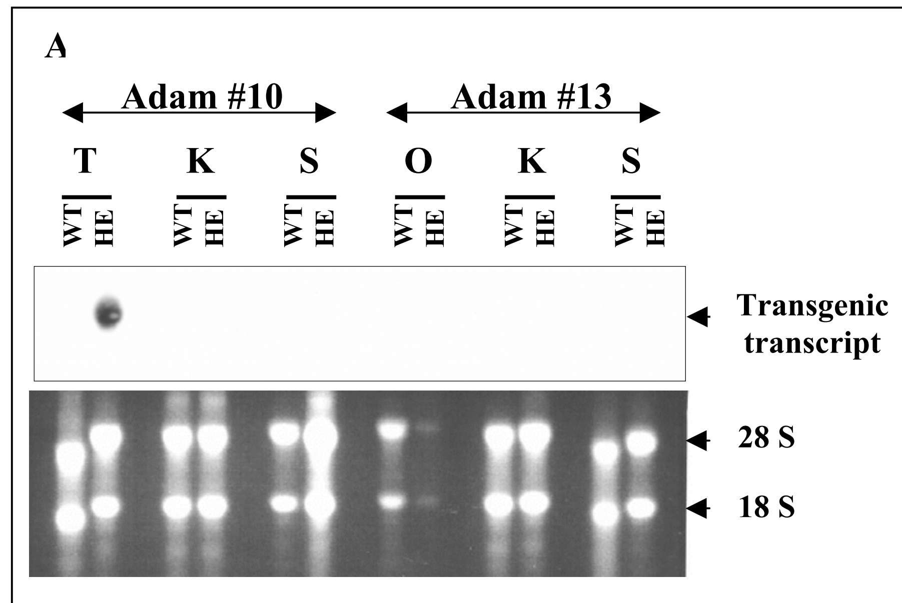


Figure 3.38: Genotyping of *hUB-Pelo* founder mice.

B, blank control, no DNA template; N, negative control (wildtype DNA); P, positive control (transgenic construct DNA); the primers amplified a 300 bp transgenic allele. Mice No1, 9, 10 and 13 (in red) were found to harbour the transgenic allele.

3.4.2.2 Expression studies of *hUB-Pelo* transgenic mice

To examine the expression of *hUB-Pelo* allele in transgenic mice, total RNA was extracted from testis, kidney and spleen and subjected to Northern blot hybridisation. The hybridisation probe, *BGH*, was generated by amplification of the *BGH polyA* cassette of transgenic construct. The probe recognised the fusion transcript only in testis of Adam #1, #9 and #10, whereas no expression of transgenic allele could be detected in kidney and spleen (Fig 3.39A; B). In Adam #13, there was no expression detected in all examined tissues including ovary (Fig 3.39).



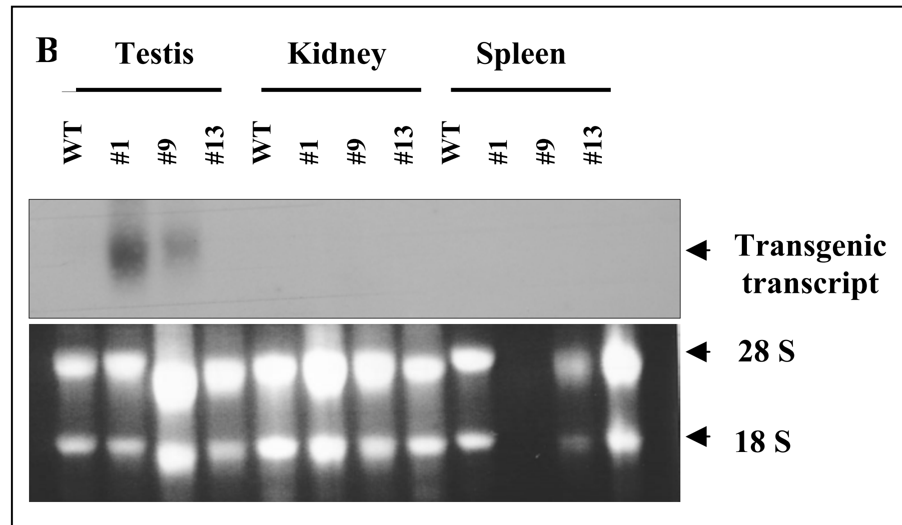


Figure 3.39: Expression analysis by Northern blot

Total RNA was isolated from testis, kidney, and spleen of each transgenic line, also from ovary from Adam #13, and hybridised with *BGH* specific probe. A, Adam #10 and #13; B, Adam #1, #9 and #13. WT, wildtype animal; HE, transgenic mice from F1 generation. Integrity of RNA was shown by 28S and 18S RNA signals on Northern gel picture.

To determine the consequence of the Pelota overexpression, transgenic males and females were crossed to wildtype mice. All studied F1 transgenic animals were fertile. Histological analysis of transgenic testis of Adam #13 revealed extensive degeneration of germ cells in all seminiferous tubules (Fig 3.40).

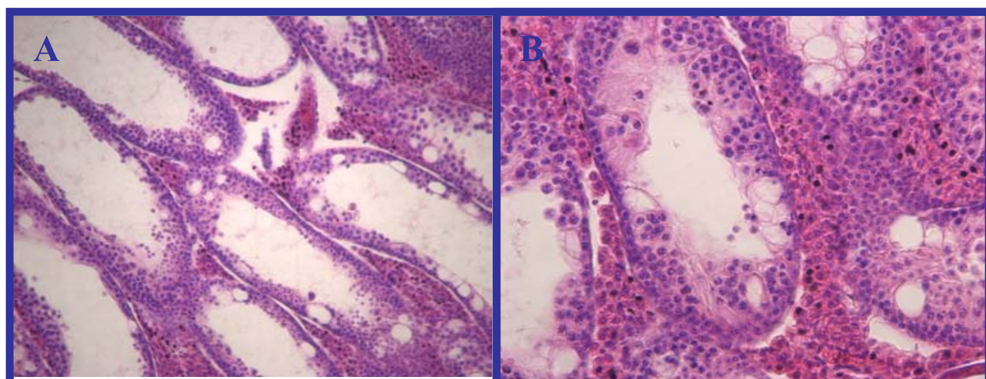


Figure 3.40: Hematoxylin-eosin (H&E) staining on section of *hUB-Pelo* testis

Section through testis of Adam #13 demonstrates drastic reduction of spermatogenic cells in testis of transgenic *hUB-Pelo* mice (A); higher magnification images of same testis sections (B).

4. DISCUSSION

4.1 Expression pattern and subcellular localisation of Pelota

The expression pattern of *Pelo* has been studied in *Drosophila,* mouse and human at the RNA-level (Eberhart and Wassermann, 1995; Shamsadin *et al.,* 2000; 2002). In mouse and human, the *Pelo* is ubiquitously expressed in all adult tissues as well as during fetal development. Expression pattern of *Pelo* in testes at different developmental stages and in testis of W/W^V mutant mice that lack germ cells revealed that *Pelo* is expressed in both somatic and germ cells. Similar expression pattern of *Pelo* was also found in *Drosophila*. Analysis of the expression of *Pelo* gene in *Drosophila* revealed that Pelo transcript is present in all developmental stages, but is most abundant in 0-2 hrs old embryos and in adults (Eberhart and Wassermann, 1995). The presence of the Pelo transcript in the germlineless progeny of oskar mutant flies and in *iab* mutant flies that lack a gonad demonstrated that the *Pelo* expression is not restricted to the germline.
In this study, we have determined the expression of *Pelo* in preimplantation stages of mouse. The RT-PCR analysis revealed that the level of Pelo transcript is high in unfertilised oocytes, sharply decreases in 2-, 4-cell stages and then increases again after 8-cell stage (Fig 3.8). This expression pattern demonstrates maternal Pelo-dosage in oocyte which is transferred and used by early embryos during the first rounds of division. The increase of Pelo transcript in morula may be due to the start of expression of zygotic *Pelo*. To prove the expression pattern of maternal and zygotic Pelo transcript, we have to distinguish between the maternal and paternal transcript by identification of polymorphism in the Pelo transcript. This work is in progress.
In order to study the expression of Pelo at the protein level and to determine the subcellular localisation of Pelo, we have generated polyclonal anti-Pelo antibody that was raised against the Pelo-GST fusion protein. The affinity purified anti-Pelo antibody specifically detects a protein of 44-kDa in Western blot analysis, which is similar in length to the predicted molecular weight of Pelo protein (Fig 3.2). Western blot analyses revealed the presence of the 44-kDa Pelo protein in all studied cell lines and adult mouse tissues (Fig 3.5; Fig 3.6). Like the expression pattern of Pelo transcript, Pelo

protein was also recognised in testes of mice at different developmental stages and in testis of W/W^V mutant mice, which lack germ cells (Fig 3.7A; B).

Immunofluorescence staining of mouse embryonic fibroblasts with the affinity purified anti-Pelo antibody revealed that the Pelo is localised with the cytoskeleton and the perinuclear region (Fig 3.4D). Coimmunofluorescence staining of fibroblasts with anti-Pelo antibody and phalloidin (Fig 3.4D; E), which specifically binds to stress actin filament, revealed the association of Pelo to the stress actin filament. Localisation of Pelo to cytoskeleton was consistent with the localisation of GFP in transgenic GFP-Pelo fibroblasts (Adham, unpublished data). In *Drosophila*, Xi et al. (2005) have shown that the subcellular localisation of Pelo is restricted to the cytoplasm. Analysis of amino acid sequences of *Drosophila* Pelo revealed the presence of a putative nuclear localisation signal sequence (NLS) at its N-terminus (Eberhart and Wassermann, 1995; Nair *et al.,* 2003). The sequence of nuclear localisation signal (NLS) is conserved in mammalian *Pelo* (Shamsadin *et al.,* 2002). The high fluorescence signal that was found in perinuclear region of fibroblasts using anti-Pelo antibody led us to address the question whether the Pelo is localised to the perinuclear region or in nuclear compartment. Western blot analysis with protein extracted from different cellular fractions of testis revealed the only presence of the 44-kDa Pelo protein in cytoskeleton and membrane fractions (Fig 3.9). The absence of Pelo in nuclear fraction suggests that Pelo is localised in cytoskeleton and perinuclear region. The presence of Pelo in the membrane fraction as shown in Western blot analysis might be due to association of cytoskeleton to the membrane. Further evidence for subcellular localisation of Pelo comes from the identification of the putative interacting partners of Pelo protein (Ebermann 2004; Kirchenmeyer, unpublished results). Most of proteins that specifically interact with Pelo are cytoskeleton-associated proteins.

The role of NLS for Pelo function must be addressed in future experiments. This NLS might be responsible for transport of Pelo and its interacting proteins to the nucleus at specific stage of cell cycle.

4.2 Function of mammalian Pelota

The physiological role of *Pelo* was determined and analysed by generation of a conventional knockout mouse (Adham *et al.,* 2003). These analyses revealed that Pelo null embryos die after the initiation of gastrulation (E6.5), a particularly active period of cell division characterised by a very short cell cycle (as short as 2 hrs) (Snow, 1977; Hogan *et al.,* 1994). The role of *Pelo* for control of cellular proliferation was obtained from the results of *in vitro* culture of blastocysts. While the inner cell mass (ICM) of *Pelo*$^{+/+}$ and *Pelo*$^{+/-}$ blastocysts continued to expand throughout the 7-day culture period, Pelo$^{-/-}$ ICM cells failed to proliferate. In contrast, *Pelo*$^{-/-}$ trophoblast cells continued to grow in size through 7 days of culture. These results demonstrate that the death of Pelo deficient cells is restricted to the rapidly dividing cells of the ICM. The survival of mitotically inactive *Pelo*$^{-/-}$ trophoblast cells further argues that *Pelo* is required selectively in cells undergoing mitosis. To determine the role of *Pelo* in cell proliferation, we tried to establish *Pelo*$^{-/-}$ cell line by culture of *Pelo*$^{+/-}$ ES cells in medium with increasing G418 concentration. Our approach was unsuccessful. Therefore, we started to establish *Pelo*$^{-/-}$ cell line by using the tetracycline inducible siRNA knockdown system and generation of conditional knockout mice using *Cre/loxP* recombination system, respectively.

4.3 Knockdown of Pelota using inducible siRNAs system

RNA interference (RNAi), a well established method for gene knockdown in model organisms (Sharp, 2001), can also be used for gene knockdown in mammalian cells (Elbashir *et al.,* 2001). So-called small interfering RNA (siRNA) has been introduced into mammalian cells by the transient transfection of the synthetic double-stranded RNA. Alternatively, promoters of genes transcribed by RNA polymerase III have been used to drive the expression of hairpin RNAs, which are very similar to siRNAs (Brummelkamp *et al.,* 2002; McManus *et al.,* 2002; Paul *et al.,* 2002; Sui *et al.,* 2002; Yu *et al.,* 2002; Paddison *et al.,* 2002). These siRNA expression vectors have two advantages: they can be stably introduced into cells as selectable plasmids and they are relatively cheap to generate. However, as with conventional knockout strategies, stably

introduced siRNA vectors cannot be used when the target, as is the case for *Pelo*, is essential for cellular survival and proliferation. Therefore, the tetracycline inducible siRNA system (van der Wetering *et al.,* 2003) was suggested to be a convenient strategy to establish a stable cell line and conditional induction of Pelo-shRNA (3.2.1). The conditional induction of Pelo-shRNA should allow for time-controlled loss-of-function and the circumventing of the lethal phenotype. Clever's group was the first to report a stable system for inducible expression of shRNAs. In this report, a tet-regulated (tet repressor (TetR)-responsive) variant of the RNA polymerase III-dependent H1 promoter was used for doxycycline (Dox)-induced shRNA expression and knockdown of β-catenin in stable transfected colorectal cancer cell clones (van de Wetering *et al.*, 2003). Tetracycline based methods in the mouse with an inducible siRNA expression system have also been used to knockdown several genes (Chen *et al.*, 2003; Czauderna *et al.*, 2003; Dickins *et al.*, 2005). To knockdown the *Pelo* gene using the Tet-inducible system, we synthesised three different double-stranded oligonucleotides and inserted them in pTER vector. When these oligonucleotides are transcribed, they form a hairpin consisting of a 19-bp stem and a 9-bp loop. Criteria to select the optimal siRNA target sequences were designed according to Reynold et al. (2004). We have expected that one of these transcribed siRNAs is efficient to RNAi-mediated silencing of *Pelo*. However, RNA analysis of all stable cell lines that transcribed these three different Pelo-siRNAs revealed that the three Pelo-siRNAs failed to RNAi-mediated gene silencing of *Pelo*. One of the possible reasons for failure of Pelo-siRNA to silence *Pelo* expression might be due to the designed siRNAs. It is reported that the most effective siRNA can reduce target gene expression by over 90%, but some have minimal or no effect (Bonetta, 2004). Despite the great success of RNAi mediated approaches, the design of highly efficient siRNA still remains a hurdle that has to be overcome (Kurreck, 2006).

4.4 Pelota conditional knockout mice

The role of a particular gene product *in vivo* is being studied increasingly by either deletion of the gene (gene targeting or knockout) or the gain of its function by transgenic overexpression. Gene targeting is the introduction of a defined modification at a specific location in the genome by homologous recombination. This powerful technique was introduced by Thomas and Capecchi in 1987. Since then, numerous mouse models with defined genomic mutations have been developed.

Although, the conventional gene knockout approach revealed a potential role of *Pelo* for cell proliferation and/or viability (Adham *et al.,* 2003), the early embryonic lethality prevents us to establish a Pelo-deficient cell line that can be used to study the exact role of *Pelo* gene. Such a cell line would help us to determine the molecular pathway, in which *Pelo* is involved. To overcome the early embryonic lethality and to allow us to dissect the role of *Pelo* in individual cell types (spatial) or during development (temporal), we have used the *Cre/loxP* system to generate conditional knockout mice. Cre-mediated recombination is achieved by the introduction of the *loxP* sites within the genomic DNA and expression of the *Cre* recombinase. *Cre* is a 38-kDa recombinase that recognises the *loxP* (locus of crossover P1) site, a 34-bp sequence consisting of two 13-bp inverted repeats, separated by an 8-bp directional spacer. Intramolecular recombination results in either excision of intervening DNA (if the two *loxP* sites are tandem repeats) or DNA inversion (if the sites are positioned in opposite orientations).

Using homologous recombination, we have established a mouse line in which the coding region of the *Pelo* gene is flanked by two *loxP* sites (Fig 3.18). We successfully generated several high chimeric male mice. These chimeric mice were bred to C57BL/C6 females to generate heterozygous $Pelo^{flox/+}$ mice in F1 generation. The $Pelo^{flox/+}$ mice were then intercrossed with $Pelo^{flox/+}$ or with $Pelo^{+/-}$ mice to generate $Pelo^{flox/flox}$ and $Pelo^{flox/-}$ animals in F2 generation. All $Pelo^{flox/flox}$ and $Pelo^{flox/-}$ mice are viable and fertile indicating that the insertion of *loxP* and neomycin cassette (*Neo*) in intron 1 and 3' flanking region of the gene did not disrupt the $Pelo^{flox}$ allele.

To determine whether the floxed Pelo allele can be deleted *in vivo*, Pelo floxed mice were mated with *EIIaCre* transgenic mice, in which the *Cre* gene is under the control of the adenovirus *EIIa* promoter. The transactivation of the *EIIa* promoter is restricted to oocytes and preimplantation stages of the embryo (Lakso *et al.,* 1996). Southern blot

analyses showed that the efficiency of Cre-mediated recombination in F1 generation was variable and resulted in different degree of mosaicism (Fig 3.23A), whereas *EIIaCre* completely recombined floxed allele in F2 generation (Fig 3.23B). The complete excision of the floxed Pelo ($Pelo^{\Delta}$) allele in $Pelo^{flox/+}$*EIIaCre* double transgenic mice clearly showed that the generated $Pelo^{flox}$ allele can successfully recombine with *Cre* recombinase in an *in vivo* system. A similar mechanism of *Cre* expression during preimplantation stages for partial excision was postulated for *EIIaCre* mediated recombination (Holzenberger *et al.*, 2000). As expected, we did not find $Pelo^{\Delta/-}$*EIIaCre* mice in F2 generation suggesting that these mice died during embryonic development like the $Pelo^{-/-}$ mice.

Subsequently, to achieve temporal inactivation of floxed Pelo allele *in vivo* and to study the distinct function of *Pelo* gene, $Cre\text{-}ER^{T}$ fusion allele was introduced in the genome of $Pelo^{flox/-}$ mice. TAM-treated $Pelo^{\Delta/-}CreER^{T}$ mice were subfertile. In contrast, all treated control $Pelo^{+/+}CreER^{T}$ were fertile. Histological analysis of testes from TAM-treated $Pelo^{flox/-}CreER^{T}$ and $Pelo^{+/+}CreER^{T}$ control animals revealed extensive degeneration of a large fraction of seminiferous tubules in mutant mice (Fig 3.25). However, the observed germ cell depletion varied between TAM-treated $Pelo^{flox/-}CreER^{T}$ mice. Western blot analysis revealed the significant decrease of protein level of Pelo in testis of TAM-treated $Pelo^{flox/-}CreER^{T}$ mice with severe germ cell depletion (Fig 3.28), while mice with less germ cell depletion did not show a significant downregulation of Pelo protein in testis. These results demonstrate that the inducible deletion of floxed Pelo allele by $CreER^{T}$ did not occur in all testicular cells resulting in mosaic of $Pelo^{flox/-}$ and $Pelo^{\Delta/-}$ cells. Southern blot analysis of different tissues of TAM-treated $Pelo^{flox/-}CreER^{T}$ mice revealed that the efficiency of $Pelo^{flox}$ deletion was high in spleen (<50%) and less efficient in kidney and tail (~25%). Such variability in the efficient deletion of floxed allele in different tissues was also described in several published reports (Vooijs *et al.,* 2001; Alonzi *et al.,* 2001). The reduced efficiency of deletion by $CreER^{T}$ can be explained by a lower or less uniform expression of R26-$CreER^{T}$ or less efficient activation of $CreER^{T}$ due to a lower local concentration of OHT (Robinson *et al.,* 1991). Nevertheless, the increase of the germ cell depletion in testes of TAM-treated mice suggests that *Pelo* is essential for spermatogenesis.

4.5 The effect of Pelota deficiency on cell proliferation

To determine the consequence of *Pelo* deficiency on cell proliferation, mouse embryonic fibroblasts (MEFs) were isolated from $Pelo^{flox/-}CreER^{T}$ and $Pelo^{+/+}CreER^{T}$ at embryonic day 13.5. Primary MEFs were cultured for two days in the presence of 100 nM and 1 µM of 4-hydrotamoxifen (OHT), respectively, and the proliferation rate of both cell lines was then determined. These experiments did not show significant differences in the proliferation rate between $Pelo^{flox/-}CreER^{T}$ and control cells. We could not show differences between both cell lines in cell cycle profile, which is determined by flow cytometry (data not shown). Recombination of a floxed Pelo allele was determined after culturing cells with 100 nM and 1 µM OHT for 4 consecutive days. No Cre-mediated recombination of the floxed Pelo allele has occurred in the presence of 0.1 µM OHT, while the recombination of $Pelo^{flox}$ allele to $Pelo^{\Delta}$ allele was more efficient (50%) in culture with 1 µM OHT. These results suggest that either *Pelo*-deficiency does not influence cell proliferation or that the $Pelo^{\Delta/-}CreER^{T}$ cells might be rapidly diluted in culture by fast growth of the nonrecombined cells ($Pelo^{flox/-}CreER^{T}$). Other published reports have shown that after growth of $Brca2^{flox/flox}$ fibroblasts in the presence of 1µM OHT for 24 hrs and a subsequent period of 24 hrs to allow for clearance of the OHT-bound CreERT protein, ~90% of the MEFs had undergone recombination of a floxed target gene (Vooijs *et al.,* 2001). Therefore, it is recommended to determine the OHT concentration and OHT exposure times, which result in high recombination efficiency of floxed allele and without toxic effect on cells (Loonstra *et al.,* 2001). Furthermore, a marked difference in recombination efficiency between different primary cell types was found. For example, culture of primary $R26CreER^{T}$ keratinocytes at low dose of OHT (100 nM) is already sufficient for ≥90% recombination of the floxed $Brca2^{flox}$ allele within 48 hrs (Vooijs *et al.,* 2001).

Analysis of the conventional *Pelota* knockout mice revealed a significant increase of aneuploid cells in $Pelo^{-/-}$ embryos at E7.5. Karyotype analysis of OHT-treated MEFs revealed an increased rate of aneuploid cells in analysed metaphases of $Pelo^{flox/-}CreER^{T}$ MEFs, while the rate of aneuploid cells was 30% in control metaphases ($Pelo^{+/+}CreER^{T}$). Like the observed results in $Pelo^{-/-}$ embryos, most of aneuploid $Pelo^{flox/-}CreER^{T}$ cells were polyploid. The polyploidy can arise by exit of a cell from mitosis following failures of spindle assembly, chromosome segregation or cytokinesis

(Andreassen *et al.,* 1996). Actin assembly is required for cytokinesis (Aubin *et al.,* 1981; Martineau *et al.,* 1995). Deficiency of *Pelo*, which we found to be associated with stress actin, may affect of the actin assembly during the cytokinesis. The resulting tetraploid cells become arrested in the cell cycle G1 by activation of the tetraploidy checkpoint. Inactivation of this checkpoint would uniformly result in aneuploidisation following passage of tetraploid cells through the next cell cycle and consequent induction of multipole spindles. Polyploid embryos of survivin-deficient mice fail to survive past E4.5 (Cutts *et al.,* 1999; Uren *et al*., 2000). Studies of human embryos revealed that 70% of all preimplantation embryos exhibit a mosaic aneuploid karyotype (Sandalinas *et al.,* 2001).

Three different types of mosaicism have been described within preimplantation embryos diploid polyploid, mitotic non-disjunction and chaotic aneuploidy (Delhanty *et al.,* 1997). It has been suggested that 90% of all human mosaic aneuploid embryos are lost during the first trimester (Munne *et al.,* 2004). In mouse, the mosaic aneuploid embryos can develop and implant into the female uterine tissue and initiate the gastrulation process (E6.5), but then quickly degrade and succumb by E8.0 (Lightfoot *et al.,* 2006). The time point of death of mosaic aneuploid embryos coincides with the death of $Pelo^{-/-}$ embryos, which display a mosaic aneuploidy (Adham *et al.,* 2003).

4.6 Drosophila Pelota controls self renewal of germline stem cells

Recently, an unexpected role for *Pelota* in control of self-renewal of germline stem cells (GSC) has been reported in *Drosophila* (Xi *et al.,* 2005). In the *Drosophila* ovary, germline stem cell (GSC) self-renewal is controlled by both extrinsic and intrinsic factors. In the *Drosophila* ovary, GSCs reside in a structure called the germarium, which is at the anterior end of an ovariole (Lin, 2002). At the anterior tip of the germarium, three types of somatic cells, cap cells and inner sheath cells, constitute a niche that supports two or three GSCs. One GSC divides to generate two daughter cells: the daughter cell maintaining contact with the cap cells renews itself as a stem cell, while the daughter cell moving away from the cap cells differentiates into a cystoblast. The cystoblast divides four times with incomplete cytokinesis to form a 16-cell cyst, in which one cell becomes an oocyte and the rest becomes nurse cells in Figure 4.1 (Xi *et al.,* 2005).

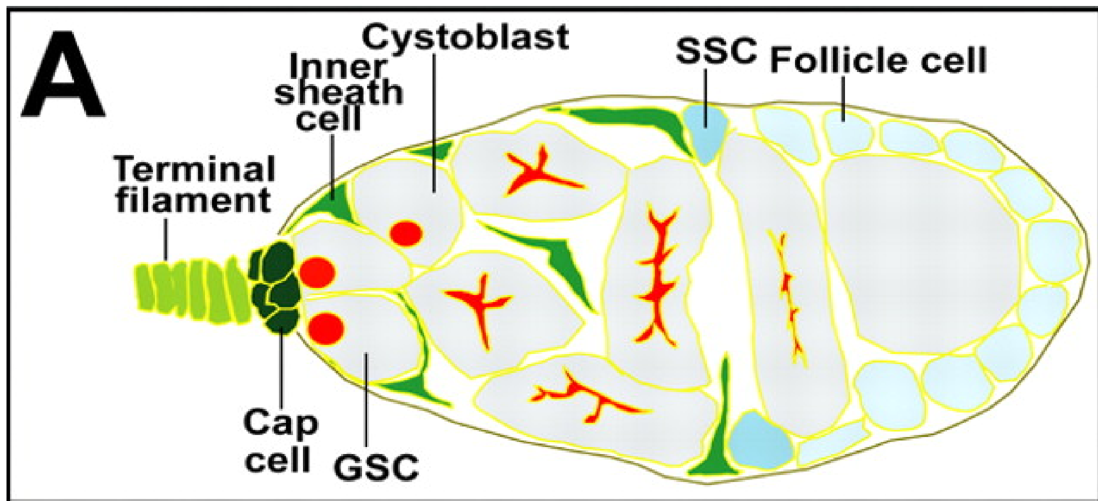


Figure 4.1 A schematic diagram of the germarium. Red circles indicate spectrosomes (GSCs and cystoblasts) and branched red structures indicate fusomes (germ cell cysts) (Xi *et al.,* 2005).

Bone marrow protein (BMP)/dpp produced from cap cells function as short-range signals that directly repress the transcription of differentiation-promoting gene *bam* in GSCs to maintain their self-renewal, and also allow cystoblasts lying one cell diameter away to differentiation (Chen and McKearin, 2003a; Song *et al.,* 2004). Overexpression of *bmp/dpp* in the inner sheath cells of germarium of transgenic flies leads to repression of the *bam* expression in all GSCs daughter cells. The maintenance of self-renewal and blocking of germ cell differentiation in all GSCs daughter cells results in the formation of GSC-like tumors and consequently in female sterility (Song *et al.,* 2004). In genetic screen to identify genes which are potentially involved in *Bmp* signalling in GSCs, Xi et al. (2005) has identified *Pelo* as a dominant suppressor of the *bmp/dpp* overexpression-induced GSC tumor phenotype. Mutation of *Pelo* gene in transgenic ovary, which overexpresses *bmp/dpp* signals, induces the expression of differentiation-promoting genes in GSCs and thereby rescues *Bmp/dpp* overexpression-induced female infertility. These results indicate that *Pelo* is required intrinsically for controlling GSC self-renewal by repression of differentiation-promoting genes. These results also support that expression of *Pelo* is regulated by *Bmp* signalling. Furthermore, this report found that *Pelo* downregulates the expression of *Dad* gene, which is one of *Bmp*-target genes in GSC. The *Dad* is the orthologous gene of mammalian *Smad* gene. The identified role of *Pelo* for repressing differentiation pathways in germline stem cells might explain the

proliferation arrest of embryonic stem cells in murine *Pelo*$^{-/-}$ blastocysts. The absence of *Pelo* in mammalian ES cells may result to induce the expression of differentiation-promoting genes that block the self-renewal of embryonic stem cells during gastrulation.

To identify the role of mammalian *Pelo* in control of self-renewal and to determine its role in the *Bmp*-signal pathway, we have to establish a *Pelo*$^{flox/-}$*CreER*^{T} ES-cell line and *Pelo*$^{flox/-}$*CreER*^{T} spermatogonia stem cells, and then to determine proliferation and pluripotency of these cell lines after deletion of Pelo floxed allele (*Pelo*$^{\Delta/-}$*CreER*^{T}). Analysis of expression and activity of different members of *Bmp*-pathway in the *Pelo*$^{\Delta/-}$*CreER*^{T} stem cells will give us information about the role of *Pelo* in the *Bmp* signalling.

In this study, we have determined the consequence of *Pelo* deletion in adult mice and found that spermatogenesis is affected. To determine the role of Pelo *in vivo* on the self-renewal of fetal and adult stem cells, we have to treat the *Pelo*$^{flox/-}$*CreER*^{T} mice with Tamoxifen at different stages of pre and postnatal development.

4.7 The effect of overexpression of Pelota gene

In the present study, the effect of *Pelo* overexpression was investigated by generation of transgenic mice (*hEF-Pelo*) containing the human *Pelota* cDNA under the promoter of *human elongation factor-1α* (*hEF-1α*). The expression analysis revealed a high expression of the transgenic allele in testis of one transgenic line (Eva #1), but not in other tissues. In the other transgenic line (Eva #6), expression of the transgenic allele was detectable in all tissues. Histological analysis of testis of transgenic line Eva #1 (F1) displays normal structure of seminiferous tubules (Fig 3.36). Despite high levels of exogenous *Pelo* expression in multiple tissues, the male *Eva* #6 mice displayed normal development, indicating that overexpression of *Pelo* does not affect embryogenesis and early development. The *hEF-1α* gene is normally expressed in most tissues (Uetsuki *et al.,* 1989; Mizushima *et al.,* 1990) and the regulatory region of the gene which was used for our transgene construct is known to direct strong expression in various cultured cells (Mizushima *et al.,* 1990). The truncated regulatory region apparently activates transcription preferentially in germ cells in a physiological context. Nevertheless, the parallel increase of the endogenous *Pelo* in transgenic mice raises the possibility that the exogenous Pelo protein is not active in mice and could antagonise the effect of the

endogenous protein. Thus, it might be the lack rather than the excess of *Pelo* expression that is responsible for the interruption of spermatogenesis.

In our study, we have also generated a second transgenic mouse (*hUB-Pelo*), in which the human *Pelota* cDNA is under the control of the ubiquitously expressed *human ubiquitin C* (*hUbC*) promoter. The *human ubiquitin C* promoter was chosen because it provides a strong cellular promoter capable of driving expression of our transgene *in vivo*, as previously described (Schaefer *et al.,* 2001). We have detected the fusion transcript only in testis of three transgenic lines (Adam #1, #9 and #10), whereas no expression of transgenic allele could be detected in kidney and spleen (Fig 3.39) by Northern blot analyses. Interestingly, in transgenic line (Adam #13) the expression of the transgenic allele was not detected in all examined tissues. However, histological analysis of transgenic testis of Adam #13 revealed depletion of germ cells in all seminiferous tubules (Fig 3.40). There are two explanations for this phenotype. Firstly, the overexpression of *Pelo* in transgenic testis leads to the depletion of Pelo-overexpressing germ cells. Therefore, we were not able to detect the expression of the transgenic allele in the line Adam #13 by Northern blot analyses. However, the overexpression of the transgenic allele does not affect the spermatogenesis in other transgenic line (Eva #1) which does not support the hypothesis that overexpression of *Pelo* disrupts spermatogenesis. Therefore, germ cell depletion shown in testis of transgenic line Adam #13 may be due to the insertion of the transgenic allele in a genomic locus, which is involved in the germ cell development. Further analyses of transgenic *Pelo* mice will provide us with information about the effect *Pelo* overexpression in germ cell development.

5. SUMMARY

The aims of this study were to determine expression pattern and subcellular localisation of Pelo protein, and generation and analysis of *Pelo* conditional knockout mice.

In order to study the expression of Pelo at the protein level, we have generated polyclonal anti-Pelo antibody that was raised against the Pelo-GST fusion protein. The affinity purified anti-Pelo antibody specifically detects a protein of 44-kDa in Western blot analysis, which is similar in length to the predicted molecular weight of Pelo protein. Western blot analyses revealed the presence of the 44-kDa Pelo protein in all studied cell lines and adult mouse tissues.

Immunofluorescence staining of mouse embryonic fibroblasts with the affinity purified anti-Pelo antibody revealed that the Pelo is localised to the cytoskeleton and perinuclear region. Coimmunofluorescence staining of fibroblasts with anti-Pelo antibody and phalloidin, which specifically binds to stress actin filament, revealed the association of Pelo to the stress actin filament. Localisation of Pelo to cytoskeleton was consistent with the localisation of GFP in transgenic GFP-Pelo fibroblasts. Western blot analysis with protein extracted from different cellular fractions of testis revealed the only presence of the 44-kDa Pelo protein in cytoskeleton and membrane fractions. The absence of Pelo in nuclear fraction suggests that Pelo is localised in cytoskeleton and perinuclear region.

To investigate the consequence of *Pelo* knockdown on cell proliferation, Tet inducible siRNAs system was performed. Three siRNA sequences targeting different regions within the Pelo mRNA were tested for their efficiency to specifically knockdown *Pelo* in cell culture. The Western blot and Northern blot analyses revealed the failure of the three Pelo-siRNAs to RNAi-mediated gene silencing of *Pelo*.

To circumvent early embryonic lethality of conventional *Pelo* null mice being able to investigate the *in vivo* roles of murine *Pelo* gene during later stages of embryonic development and adult, we generated *Pelo* conditional knockout mice using inducible *Cre/loxP* recombination system. We successfully generated several high chimeric male mice, which transmitted the $Pelo^{flox}$ allele to their offspring. The $Pelo^{flox/+}$ mice were then intercrossed with $Pelo^{flox/+}$ or with $Pelo^{+/-}$ mice to generate $Pelo^{flox/flox}$ and $Pelo^{flox/-}$ animals in F2 generation. All $Pelo^{flox/flox}$ and $Pelo^{flox/-}$ mice are viable and fertile

indicating that the insertion of *loxP* and neomycin cassette (*Neo*) in intron 1 and 3' flanking region of the gene did not disrupt the *Pelo* allele.

To determine whether the floxed Pelo allele can be deleted *in vivo*, Pelo floxed mice were mated with *EIIaCre* transgenic mice in which the adenovirus *EIIa* promoter directed the action of *Cre* recombinase gene to the single-cell zygote. The complete excision of the floxed Pelo ($Pelo^{flox}$) allele in $Pelo^{flox/+}$*EIIaCre* double transgenic mice clearly showed that the generated $Pelo^{flox}$ allele can successfully recombine with *Cre* recombinase *in vivo* system. As expected, we did not find $Pelo^{\Delta/-}$*EIIaCre* mice in F2 generation suggesting that these mice died during embryonic development like the conventional *Pelo* deficient mice.

To achieve temporal inactivation of floxed Pelo allele *in vivo* and to study distinct function of *Pelo* gene, $Cre\text{-}ER^T$ fusion allele was introduced in the genome of $Pelo^{flox/-}$ mice. Tamoxifen (TAM)-treated $Pelo^{flox/-}CreER^T$ mice are subfertile. Histological analysis of testes from TAM-treated $Pelo^{flox/-}CreER^T$ mice revealed severe germ cell depletion. The increase of the germ cell depletion in testes of TAM-treated mice suggests that *Pelo* is essential for spermatogenesis.

Moreover, Southern blot analysis of different tissues of TAM-treated $Pelo^{flox/-}CreER^T$ mice revealed that the efficiency of $Pelo^{flox}$ deletion was high in spleen (<50%) and less efficient in kidney and tail (~25%). Western blot analysis revealed that the significant decrease of protein level of Pelo in testes of TAM-treated $Pelo^{flox/-}CreER^T$ mice with severe germ cell depletion, while that with less germ cell depletion did not show a significant downregulation of the Pelo in testis. These results revealed the inducible deletion of floxed Pelo allele by $CreER^T$ did not occur in all testicular cells.

To study the consequences of *Pelo* deficiency on cell proliferation, mouse embryonic fibroblasts (MEFs) were isolated from $Pelo^{flox/-}CreER^T$ and $Pelo^{+/+}CreER^T$ embryos. Southern blot anaylsis showed that the Cre-mediated recombination occurred partially in OHT-treated fibroblasts of $Pelo^{flox/-}CreER^T$ mice. The proliferation assay of OHT-treated $Pelo^{flox/-}CreER^T$ fibroblasts suggests that either *Pelo* deficiency does not influence cell proliferation or that the $Pelo^{\Delta/-}CreER^T$ cells might be rapidly diluted in culture by fast growth of the nondeleted cells ($Pelo^{flox/-}CreER^T$). Karyotype analysis of OHT-treated MEFs revealed an increased rate of aneuploid cells in analysed metaphases of $Pelo^{flox/-}CreER^T$ MEFs.

To investigate the effect of *Pelo* overexpression, we generated two transgenic constructs and mice. In the first transgenic construct, the human *Pelota* cDNA is under the control

of *human elongation factor-1α* promoter (*hEF-Pelo*). Expression analysis revealed a high expression of transgenic allele only in testis of one transgenic line. In the other transgenic line, expression of transgenic allele was detectable in all studied tissues.
The second transgenic mice (*hUB-Pelo*) contain the human *Pelota* cDNA under the control of *human ubiquitin C* (*hUbC*) promoter. The expression analysis revealed that the expression of the transgenic transcript is restricted in testis of three generated lines.

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