2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Materials, substances and solutions

Table 1: Materials, substances and solutions.

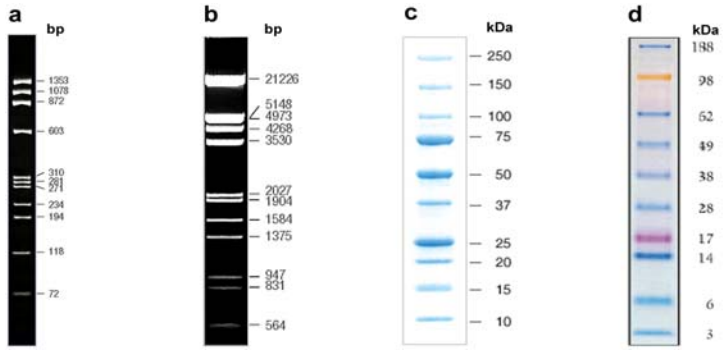
Chemicals	Producer and Location
Acetic Acid	Roth, Karlsruhe
Acrylamid/Bisacrylamid 19:1	Roth, Karlsruhe
Acrylamid-/Bisacrylamid 37,5:1	Roth, Karlsruhe
Ampicillin	Serva, Heidelberg
APS	Sigma, Steinheim
Bactoagar	Difco Microbiology
Bactotrypton	Difco Microbiology
Boric Acid	Sigma, Steinheim
Bradford Reagent	Sigma, Steinheim
Bromphenolblue	Sigma, Steinheim
BSA	Sigma, Steinheim
Chlorophorm	Roth, Karlsruhe
Chloramphenicol	Merck, Darmstadt
Complete Protease Inhibitor Cocktail Tablets	Roche, Mannheim
DAPI	Sigma, Steinheim
DEPC	Serva, Heidelberg
DMEM	Gibco, Paisley, Scotland, UK
DMSO	Sigma, Steinheim
DNaseI	Boehringer Mannheim, Mannheim
dNTPs	Amersham Bioscience, NJ, USA
Doxycycline hyclate	Sigma, Steinheim
DTT	Sigma, Steinheim
EDTA	Sigma, Steinheim
EGTA	Sigma, Steinheim
Ethanol	Roth, Karlsruhe
Ethidiumbromide	Sigma, Steinheim
Fe(NH4)2(SO4)2	Sigma, Steinheim
FBS	Gibco, Paisley, Scotland, UK
G-418	Sigma, Steinheim
Glucose	Sigma, Steinheim
Glycerol	Roth, Karlruhe
Glycine	Sigma, Steinheim
hCG	Sigma, Steinheim
Hyaluronidase	Sigma, Steinheim
H2O2	Sigma, Steinheim
HCl	Roth, Karlruhe
Humalog Insulin	Lilly, USA
IFN-α2a	Jena Bioscience GmbH, Jena Germany
IPTG	Fermentas, Burlington, CDN
Kanamycin	Sigma, Steinheim
KCl	Sigma, Steinheim
Ketavet	Pharmazeutische Handelsgeselschaft GmbH,

	Garbsen
KH2PO4	Merck, Darmstadt
L-arabinose	Sigma, Steinheim
L-Glutamin	Gibco, Paisley, Scotland, UK
Lipofectamine™2000	Invitrogen, Carlsbad, CA, USA
Methanol	Roth, Karlsruhe
MgCl2	Sigma, Steinheim
Milk Powder	Roth, Karlruhe
M16 medium	Sigma, Steinheim
M2 culture medium	Sigma, Steinheim
Na2HPO4	Roth, Karlruhe
NaCl	Roth, Karlruhe
NaOH	Roth, Karlruhe
Natriumacetat	Sigma, Steinheim
NP-40	Sigma, Steinheim
NTPs	Promega, Madison, WI, USA
Paraffin	Roth, Karlruhe
Penicillin/Streptomycin	Gibco, Paisley, Scotland, UK
Phosphatase Inhibitor Cocktail I + II	Roche, Mannheim
PMSG	Pharmazeutische Handelsgeselschaft GmbH, Garbsen
PMSF	Sigma, Steinheim
Potassium Acetat	Sigma, Steinheim
Rompun	Bayer AG, Leverkusen
RNasin	Promega, Madison, WI, USA
Roti®-Block	Roth, Karlruhe
Roti®-Load	Roth, Karlruhe
Saccharose	Merck, Darmstadt
SDS	Serva, Heidelberg
Tamoxyfen	Sigma, Steinheim
TEMED	Sigma, Steinheim
Tetracycline	Sigma, Steinheim
Tris	Roth, Karlruhe
Triton X-100	Sigma, Steinheim
TRIZOL	Invitrogen, Carlsbad, CA, USA
Trypsin/EDTA	Gibco, Paisley, Scotland, UK
Tween-20	Sigma, Steinheim
Urea	Sigma, Steinheim
[α-32P] UTP	PerkinElmer, Meriden, CT, USA
Xylol	Roth, Karlsruhe
Yeast Extract	Merck, Darmstadt
β-Mercaptoethanol	Sigma, Steinheim
[γ-32P] ATP	PerkinElmer, Meriden, CT, USA

2.1.2. Kits, Enzymes and Markers

Table 2: Kits, Enzymes and Markers.

Components	Producer and Location
Expand™ Long Template PCR System	Boehringer Mannheim, Mannheim
JetStar Plasmid Purification MAXI Kit 2.0	Genomed GmbH, Löhne
RestoreTM Plus Western Blot Stripping Buffer	Pierce, Rockford, IL, USA
QIAquick® Gel Extraction Kit	Qiagen, Hilden
QuickSpin Columns for radiolabeled RNA	Roche, Mannheim
DekadeTM Marker System Kit	Ambion, Austin, TX, USA
RPA II Kit	Ambion, Austin, TX, USA
SuperSignaling® West Dura Extended Duration	Pierce, Rockford, IL, USA
ECLTM Western Blotting Analysis System	GE Healthcare, Buckinghamshire,
Wizard® SV Gel and PCR Clean-up System	Promega, Madison, WI, USA
Rat/Mouse Insulin ELISA kit	LINCO Research, Missouri, USA
DNaseI	Roche, Mannheim
M-MLV Reverse Transkriptase	Promega, Madison, WI, USA
Proteinase K	Merck, Darmstadt
Restrixction enzymes	New England Biolabs, Ipswich, MA,
RNaseA	Boehringer Mannheim, Mannheim
SP6-RNA-Polymerase	Promega, Madison, WI, USA
T4-DNA-Ligase	Promega, Madison, WI, USA
T7-RNA-Polymerase	Promega, Madison, WI, USA
T3-RNA-Polymerase	Promega, Madison, WI, USA
TaqDNA Polymerase	Invitrogen, Carlsbad, CA, USA
Precision Blue Protein™ Standard All Blue	BioRad Laboratories Richmond, USA
SeeBlue®Plus2 Pre-stained Protein Standard	Invitrogen, Carlsbad, CA, USA
λ DNA/EcoRI + HindIII Marker, 3	Fermentas, Burlington, CDN
Φ174 DNA/BsuRI (HaeIII) Marker, 9	Fermentas, Burlington, CDN
Quick and Easy BAC Modification Kit	Gene Bridges, Dresden
BAC RP24-85L15	RZPD, Berlin



Marker. a: λ DNA/EcoRI + HindIII Marker, 3; b: Φ174 DNA/BsuRI (HaeIII) Marker, 9; c: Precision Blue Protein™ Standard; d: All Blue SeeBlue®Plus2 Pre-stained Protein Standard

2.1.3. Lab instruments, machines and other material

Table 3: Lab instruments, machines and other material.

Instrument, machine, material	Producer and Location
8-chanal-Pipette M300	Biohit, Rosbach v. d. Höhe
96-Well-Photometer Anthos htII	Anthos Labtech Instruments, Salzburg, AT
Saldo Gel Dryer Model SE 1160	Hoefer Scientific Instruments, San Francisco, USA
Automatic Pipette Witoped XP	Witeg Labortechnik GmbH, Wertheim
Shaker Certomat®H	B.Braun, Melsungen
Cryo 1°C Freezing Container	Nalgene®Nunc, Rochester, NY, USA
Dialysis Membranes	Millipore, Billerica, MA, USA
One-way Pipettes Cellstar® 1, 2, 5, 10, 25 mL	Greiner bio-one, Frickenhausen
Electroporator 2510	Eppendorf, Hamburg
Falcon Tubes	TPP®, Trasadingen, Schweiz
Digital Balance	Sartorius Research, Göttingen
GenePulse® Kuvetts	BioRad Laboratories Richmond, USA
Horizontal Agarose Gel Electrophoresis Chamber	Biometra, Göttingen
Hybridisation oven 3032	GFL, Hannover
Cell Culture Incubator Heracell	Heraeus Instuments GmbH, Düsseldorf
Cryotubes Cryo.S	Greiner bio-one, Frickenhausen
Centrifuge Sigma 3K12	Sigma, Osterode am Harz
Centrifuge Sorvall®PC5C Plus	Kendro, Hanau
Centrifuge Megafuge 1.0R	Heraeus Instuments GmbH, Düsseldorf
Liquid Scintillation Analyzer Tri-Carb 1900 TR	PerkinElmer, Meriden, CT, USA
Magnetfish MR3001	Heidolph, Schwabach
Microwave 8020	Privileg, Fürth
Microskop CKX31	Olympus Deutschland GmbH, Hamburg
Pasteur Pipettes	Roth, Karlsruhe
PCR Tubes	Biozym Scientific GmbH, Oldendorf
pH-Meter pH Level 1	WTW, Weilheim
Phosphoimager Fujix BAS2000	Fuji, Tokyo, J
Phosphoimagerplatte BAS-III	Fuji, Tokyo, J
Photometer GeneQuant pro	Amersham Bioscience, Little Chalfon, UK
Pipettes	Gilson, Langenfeld
PVDF Membrane	Amersham Bioscience, Little Chalfon, UK
Roller Mixer SRT1	Snijders, Tilburg, NL
X-ray Retina Film	Fotochemische Werke GmbH, Berlin
Save-Lock Tubes	Eppendorf, Hamburg
SDS-PAGE Gel Electrophoresis Chamber	BioRad Laboratories Richmond, USA
Power Spply PowerPac™HC	BioRad Laboratories Richmond, USA
SpeedVac SVC100	Savant Instruments, Farmingdale, NY, USA
Laminair®HB2448	Heraeus Instuments GmbH, Düsseldorf
Tankblotter	BioRad Laboratories Richmond, USA
Thermocycler PTC-200	BioRad Laboratories Richmond, USA
Thermomixer 5436	Eppendorf, Hamburg
Centrifuge Biofuge pico	Heraeus Instuments GmbH, Düsseldorf
Centrifuge 5415D	Eppendorf, Hamburg
Centrifuge Labofuge 400e	Heraeus Instuments GmbH, Düsseldorf
Transilluminator MultiImage™Light Cabinet	Alpha Innotech Corporation, CA, USA
Sonificator Sonoplus	Bandelin electronic, Berlin
Ultra-Turrax T25 basic	IKA® Labortechnik, Staufen

Membran Vacuumpump	Vacumbrand GMBH+CO, Wertheim
Vertical Gel Electrophoresis Chamber	Biometra, Göttingen
Vortex Genic 2	Bender & Hobein AG, Zürich, SUI
Balance 440-43N	Kern & Sohn GmbH, Baldingen-Frommern
Watter Bath	GFL, Burgwedel
Whatman Paper	Whatman® International, Maidstone, UK
Cell Culture Plates	TPP®, Trasadingen, SUI
Accu Check Sensor Comfort	Roche, Mannheim
Accu Check Sensor Comfort Stripes	Roche, Mannheim
0,2 µm Celluloseacetat Filter	Whatman® GmbH Schleicher & Schuel, Dassel
Electroporator Gene Pulser™	BioRad Laboratories Richmond, USA
Capacitance Extender	BioRad Laboratories Richmond, USA
x-Rax Film Processor AGFA CURIX 60	AGFA GEVAERT N.V., Mortsel, Belgium

2.1.4. Antibodies

Table 4: Antibodies.

Primary Antibody	Dilution	Blocking	Producer and Location
Rabbit anti-InsR	1:200	2% MP in TBS	Santa Cruz Biotechnology, Santa Cruz, California, USA
Mouse anti-TetR	1:8000	2% MP in TBS	Mo Bi Tec GmbH, Göttingen
Rabbit anti-βActin	1:1000	5% BSA in TBST	Cell Signaling Technology, Danvers, MA, USA
Rabbit anti-PKR	1:5000	5% MP in TBST	AbCam, Cambridge, UK
Rabbit anti-GFP	1:5000	5% MP in TBST	RDI Research Diagnostics, INC Wien, Austria
Mouse anti-Penta-His	1:1000	3% BSA in TBS	Qiagen GmbH, Düsseldorf

Secondary Antibody	Dilution	Blocking	Producer and Location
Goat anti-mouse	1:1000	see primary antibodies	Pierce, Rockford, IL, USA
Goat anti-rabbit	1:2000	see primary antibodies	Pierce, Rockford, IL, USA

2.1.5. Commercial Cloning Vectors

- pcDNA^{TM} 3.1/Hygro (+) (Invitogen, Carlsbad, CA, USA)
- pGEM®-T (Promega, Madison, WI, USA)
- pGEM®-T_{easy} (Promega, Madison, WI, USA)
- pBluescript II SK (+) (Stratagene, La Jolla, CA, USA)
- pEGFP-N3 (Clontech, Heidelberg, Germany)

2.1.6. Oligonucleotides

Oligonucleotides for cloning

siGFP3a
5‘- ACCGCCCAACCACTACCTGAGCACCCAGTTCAAGAGACTGGGTGCTCAGGTAGTGGTTTTTGG -3'

siGFP3b
5‘– CGCGCCAAAAACCACTACCTGAGCACCCAGTCTCTTGAACTGGGTGCTCAGGTAGTGGTTGGG -3'

GFPSI1
5'- TAATACGACTCACTATAGGGAGATGCAAGCTGACCCTGAAGTTATTCAAGAA -3'

GFPSI2
5'- TTCTTGAATAACTTCAGGGTCAGCTTGCATCTCCCTATAGTGAGTCGTATTA -3'

RosaAmpUp new
5’- CAATGTCAGCGCTAGAAGCACTTTGCACACCTCTGTTGCTGACTTAGCCCTAGGGAT AACAGGGTAATTGCA ACGGAAATTG CTCA -3'

RosaAmpRew
5’-
TGGTGTAGGTAAGACAGACTCGTTTAGAGTTGGAAGCCAGCCTGGGCTTCATTACCCTGTTATCCCTACGCGCTAG CGGAGTG -3'

Oligonucleotides for RPA probes

SenseInsR
5’- CGACCAGACCCGAAGATTTCTTCAAGAGA -3

AntisenseInsR
5’– CTAGTCTCTTGAAGAAATCTTCGGGTCTGGTCGGTAC -3’

rlet7a1
5'- GTAATACGACTCACTATAGGGACTATACAACCTACTACCTCATCCCACAGTG -3'

rlet7a2
5'- CACTGTGGGATGAGGTAGTAGGTTGTATAGTCCCTATAGTGAGTCGTATTAC -3'

rmir122a
5'- GTAATACGACTCACTATAGGGAAACACCATTGTCACACTCCAGAGCTCTGCTAAGG -3'

rmir122b
5'- CCTTAGCAGAGCTCTGGAGTGTGACAATGGTGTTTCCCTATAGTGAGTCGTATTAC -3'

PCR primers

Table 5: PCR primers.

Primer	Sequence 5'→3'
For Amp	GAATGACACC GGTAAACCG
Rew BAC	CCATAGTGGGACCTGGTCAA
Amp5	TTTG AGCGTCAGAT TTCGTG
BAC3	CCAGCTCCCAAAAGATTGTC
Amp5X	TGGCATCT TCCAGGAAATCT
BAC3X	GTTCAATTCCCAGCAACCAC
NeoRew1	TCGCCTTCTTGACGAGTTCT
NeoRew2	TGCTCCTGCCGAGAAAGTAT
RosaFw1	AAAGCAGCACAGCCTCTCTC
AmpRW1	TGCCACCTGACGTCTAAGAA
RosaFw2	AGGCTGCTTGTCTTGCACTT
AmpRw2	CATTTCCCCGAAAAGTGC
CassRew	CACCTTGTACACCAGGCAGT
NeoFor	AGCAGCCGATTGTCTGTTGT
Rosa55	TCGTCGTCTGATTGGCTCTC
CassNeoRew	CATCCAGGTCGTGTGATTC
Pola51	GCATTCTAGTTGTGGTTTGTCC
RosaShortFW	GAAATGTGGCTCCAATGTCA
NeoTho	TGCCTGCTTGCCGAATATCATGG
Ub_fv	TAAATTCTGGCCGTTTTTGG
TBP_rv	CAGGCTGTTGTTCTGATCCA
FRG1fv	TGTTTGGGGTGAAAAATGGT
FRG1rev	CCCTTGAGGGAGTCTAGCAG
FRG2fv	CTGTGGGCACAGAAACACAC
FRG2rev	CCCAGGACCTTGCATGTATT
FRG3fv	CCAGCCACTAGAGAAACGAA
FRG3rev	GGAGGCCTGACTAGTGGTGA
FRG4fv	TTCTGCCTGCTTTTGTCTCC
FRG4fv	TTGTGTGCTCAGCAAGTCCT
FRG5fv	TGGTTCTGTGATCACCTTGG
FRG5rev	GGCACAGACAATCCTTCCTT
FRG6fv	AGATCCATCTGCCTCTGGAA
FRG6rev	CCCCAGTGCTTTGTGGTATC
PGKneo	CTGTCCATCTGCACGAGACT
tetOfv1	CCTCCCCGAGTTGCTGAG
TetOrev	TCGCACGATTACCATAAAAGG
tetOfv2	CTAGACCGGTTCGAGATCCA
NeoBACK	ATACTTTCTCGGCAGGAGCA
Neo1	ACAACAGACAATCGGCTGCT
RTcaggsFor primer	TGACCGCGTTACTCCCACAG
RTtetRrew primer	TTCATTCAGGAGCTCCAAGGC

Genotyping primers

Table 6: Genotyping primers.

Primer	Sequence 5'→3'
U6for U6rev	GGATGGAGTGGGACAGAGAA- TCTTTCCCCTGCACTGTACC
U65 U63	ATCCAGTGGAAAGACGCGCAG GTATAGGAACTTCGGTTACCG
TetRfor TetRback	CAAGTTGCCAAGGAGGAGAG AACCGGTCTAGAATCGATGG
Pola51 Rosa32	GCATTCTAGTTGTGGTTTGTCC TTGATCCTTTGCCTTGATCC
NeoRew1 Rosa55	TCGCCTTCTTGACGAGTTCT TCGTCGTCTGATTGGCTCTC

2.2. Methods

2.2.1. DNA

2.2.1.1. Cloning of DNA

2.2.1.1.1. Polymerase Chain Reaction (PCR)

Optimal PCR conditions were established according to general rules (Innis, 1989).
For all PCR reactions, if not indicated differently, the same cycling conditions were used with changes in annealing temperature (AT) and elongation time (ET). Those conditions were:

Denaturation	95° C for 3 min
Denaturation	94° C for 30 sec (35x)
Annealing	AT° C for 30 sec (35x)
Elongation	72° C for ET (35x)

For amplification of fragments up to 1 kb, the Taq DNA polymerase was used. All PCR reactions were carried out in the PCR Thermo-Cycler (DNA Machine BIORAD).
In order to visualize PCR products 8-12 µL of 50 µL PCR reaction were mixed with DNA gel loading buffer and loaded on agarose gel. Agarose (0.7-3%) was boiled in 1x TAE buffer and finally ethidium bromide solution was added (0.5 µg/mL).

2.2.1.1.2. Reverse transcription

Reverse transcription is very sensitive technique for mRNA detection and quantitation or synthesis of cDNA for further cloning. 2-5 µg of total isolated RNA was mixed with 5 µL of random hexamer primers (20 µM) in a volume of 15 µL DEPC H_2O. To avoid formation of RNA secondary structures, which might interfere with the synthesis, the mixture was heated to 80°C for 3 min, and then quickly chilled on ice. After a brief centrifugation, the following was added to the mixture:

6 µL 5x First strand buffer
3 µL 0.1 M DTT
3 µL 5 mM dNTPs
1 µL RNasin (10 U/µL)
2 µL M-MLV (200 U/µL)

The content of the tube was mixed gently and incubated at 42° C for 1h. The reaction was inactivated by heating at 80° C for 10 min. After the complete cDNA synthesis 5 to 10 µL of reaction was used as a template for a PCR.

2.2.1.1.3. Restriction of DNA

An enzymatic digestion was performed by incubating double-stranded DNA molecules with an appropriate amount of restriction enzymes in their respective buffer as recommended by the supplier at optimal reaction temperature (usually 37°C). Typical digestions included 2 to 10 U of enzyme per microgram of DNA. The reactions for the plasmid DNA were incubated for 1-3 hrs or over night to ensure complete digestion. The visualization of the digested DNA was performed with agarose electrophoresis.

2.2.1.1.4. Filling-up reaction

Digested DNA (5-10 µg) was mixed with dNTPs (end concentration 0.05 mM), BSA (end concentration 0,1mg/mL) and 1-5 U of T4 Polymerase. The reaction at 12° C for 20 min and stopped by heating at 75° C for 10 min were preformed in the PCR Thermo-Cycler (DNA Machine BIORAD).

2.2.1.1.5. Annealing of oligonucleotides

Oligonucleotides were purchased from BIOTEZ. Lyophilized oligonucleotides were dissolved in TE buffer to the final molarity of 50 µM. 5 µL of each complementary oligonucleotides were mixed with 40 µL of sterile distillated water, heated at 95° C for 5 min and incubated at 37° C for 1h. Annealed oligonucleotides were assayed with 2-3 % agarose gel electrophoresis and isolated from agarose gel for further cloning.

TE-Buffer *10 mM Tris-HCl pH 8.0*
1 mM EDTA

2.2.1.1.6. Ligation of DNA fragments

The ligation of an insert into a vector was carried out in the following reaction mix:
30 ng vector DNA, 50-100 ng insert DNA, 1 µL ligation buffer (10x), 1 µL T4 DNA ligase (5 U/µl) in a total volume of 10 µl, filled up with H_2O bidest.
The ligation reactions were running at 16° C overnight or at RT for 2h.
Alternatively a quick ligation using 2x ligation buffer was preformed at RT for a half an hour up to 2h.

2.2.1.1.7. TA-cloning

Taq and other polymerases have a terminal transferase activity that results in the non-templated addition of a single nucleotide to the 3'-ends of PCR products. In the presence of all 4 dNTPs, dA is preferentially added. This terminal transferase activity is the basis of the TA- cloning strategy. For the cloning of PCR products, pGEM-T Vector or pGEM-T Easy Vector systems with 5'T overhangs were used. To perform the ligation (see above) 50 ng of pGEM-T Vector or pGEM-T Easy Vector and 100-300 ng of PCR product were taken.

2.2.1.1.8. DNA extraction from agarose gel

PCR fragments, which were used for cloning, labeling, and microinjections were gel extracted using QIAquick Gel Extract Kit (Qiagen). The extraction procedures recommended by the supplier were followed.

2.2.1.2. DNA transformation in bacteria

2.2.1.2.1. Preparation of electro competent E.coli bacteria

LB medium (100 mL) was inoculated with a single colony of *E.coli* (strain DH5α) and the culture was grown at 37° C over night. Next day 1 mL of overnight culture was inoculated into 250 mL LB medium and grown at 37° C up to OD 0.6. Cells were cooled down on ice for 15 min, than transferred to a pre-chilled centrifuge bottle and centrifuged at 2 600 g at 2° C for 15 min. The pellet was re-suspended in 250 mL ice-cold 10% glycerol. The centrifugation and re-suspension steps were repeated three times more while the pellet was re-suspended in 250 mL firstly, than in 10 mL and finally in 1 mL of ice-cold 10% glycerol. Aliquots (30 µL per Eppendorf tube) were stored at -80° C.

2.2.1.2.2. Transformation of electro competent bacteria

Electroporation of the bacteria was done by gently mixing one aliquot of competent bacteria (30 µL) with 2 µL of ligation reaction or with 50 ng of pure plasmid DNA.
Cells mixed with DNA were transferred to a pre-chilled cuvette (0.1 cm electrode gap) of which the outside surface must be dry before placing it into the sample chamber of the Electroporator 2510. The electroporation was carried out at 1350 V and time constant 5 ms. Immediately 1 mL of fresh LB medium was added and cells were transferred to a new sterile Eppendorf tube. After shaking at 37° C for 60 min an aliquot of 150 µL was spread on LB-ampicillin plate with desired supplements (X-Gal, IPTG).
The rest of cells was shortly centrifuged, re-dissolved in 100 µL LB medium and spread on another LB-ampicillin plate with desired supplements (X-Gal, IPTG). Plates were incubated at 37°C overnight. The selection for the presence of the lacZ gene was carried out by the usual blue-white screening method (Sambrook et al., 1989).

LB Plates
10-15 % Bactoagar
100 µg/Ll X-Gal
80 µg/mL IPTG
100 µg/mL Ampicillin in LB Medium
Alternatively Kanamycin 15 µg/mL or Chloramphenicol 15 µg/mL or Tetracycline 3 µg/mL

LB Medium
5 g Yeast extract
10 g Bactotrypton
10 g NaCl
100 µg/mL Ampicillin ad. 1000 mL H2O
Alternatively Kanamycin 15 µg/mL or Chloramphenicol 15 µg/mL or Tetracycline 3 µg/mL.

2.2.1.2.3. DNA amplification in bacteria

Mini preparation

Three mL of LB medium with 50 µg/mL Ampicillin were inoculated with a single *E.coli* colony and incubated at 37° C with shaking overnight. 2 mL of this culture was centrifuged at 13 000 g for 1 min. The pellet was resuspended in 300 µL of solution E1. Cells were lysed by adding 300 µL of E2 solution. The same amount of E3 solution was added to the tube, and immediately mixed by inverting. Cell debris and chromosomal DNA were pelleted by centrifugation at 13 000 g, RT for 5 minutes. The supernatant was transferred into a new tube and 0.6 mL of isopropanol was added to precipitate the DNA. After centrifugation (13 000 g, RT, 10 min) and washing with 70% ethanol, air-dried pellet was resuspended in 50 µL TE buffer. 2-5 µL of DNA was taken for the control digestion. DNA was kept at -20° C.

E1 solution	*50 mM Glucose*
(GTE-buffer)	*10 mM EDTA pH 8.0*
	25mM Tris-HCl pH 8.0
	0.4 mg/mL RNaseA
E2 solution	*100 mM NaOH*
(Lysis buffer)	*0.5 % SDS*
E3 solution	*3.1 M Potassium Actetate*

Maxi preparation

Plasmid DNA was isolated from 200 mL of night cell culture using JetStar Plasmide Purification MAXI Kit 2.0 according to the manufacturer's instruction. The DNA was usually dissolved in 100-400 µL of TE buffer and kept at -20° C.

2.2.1.2.4. Measurement of nucleic acids concentration

The quality and quantity of DNA were measured by comparison of band-intensity on ethidium bromide stained agarose gels. The DNA molecular weight marker was used as a standard, or, alternatively, photometrically (Gene-Quant-Pro, Amersham Bioscience) by measuring absorption of the samples at 260 nm. DNA quality (i.e. contamination with salt or proteins) was checked by the measurements at 260, 280, and 320 nm. The concentration was calculated according to the formula:

$C = (E\ 260 - E\ 320) \times f \times c$

C = concentration of sample (µg/µL)
E 260 = absorption at 260 nm
E 320 = absorption at 320 nm
f = dilution factor
c = 0.05 µg/µL for double stranded DNA
c = 0.04 µg/µL for RNA
c = 0.03 µg/µL for single stranded DNA

2.2.1.2.5. DNA sequencing

The DNA samples were submitted to automatic sequencing using the thermo sequenase fluorescent-labeled primer reaction. The sequencing was performed by the company Invitek (Berlin-Buch, Germany).

2.2.1.3. DNA recombineering in bacteria

2.2.1.3.1. BAC (Bacterial Artificial Chromosome) recombineering

Conventional DNA cloning for modification of large DNA molecules has been limited until recently. To permit an alteration of any size of DNA molecules homologous recombination *in vivo* in *E. coli* using BAC recombineering has now become available.

The BAC RP24-85L15 was purchased from RZPD (Berlin, Germany). The whole modification procedure was carried out using the Gene Bridge Kit according to the protocol described in the guide Quick and Easy BAC modification (Gene Bridge, Dresden Germany).

A DNA construct containing an shRNA cassette was designed to carry BAC arms required for homologous recombination with the BAC genome. Homologous recombination was carried out by the recombinase protein pairs encoded by the Red®ET® plasmid that carries a Tetracycline (Tet) resistance gene. The expression of genes mediating Red®ET® proteins is activated with L-arabinose and temperature shift from 30° C to 37° C.

To reduce the size of the DNA, BAC shaving was performed. 1 kb DNA fragment holding BAC homologous arms and an ampicilln (Amp) resistance gene was used for this.

Finally, to screen for BAC positive clones double antibiotic selection was employed and further analysis such as PCR and enzymatic digestions of isolated DNA were performed.

2.2.2. RNA

2.2.2.1. RNA isolation

Total tissue RNA was isolated using TRIzol reagent according to the manufacturer's instruction. 100-200 mg tissue sample was homogenized in 1 mL of TRIzol reagent using a glass-teflon homogenizer. To isolate total RNA from cultured cells, 1 mL of TRIzol reagent was added to the dish (10 cm diameter), and the lysate was transferred into an eppendorf tube. Cell or tissue homogenate was incubated at RT for 5 min to permit the complete dissociation of nucleoprotein complexes, and then 0.2 mL of chloroform was added. After vigorous vortexing, the homogenate was incubated at RT for another 15 min. After centrifugation of samples at 13 000 g and 4° C for 15 min, the colorless 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 with 75% ethanol, dissolved in 80-100 µL of DEPC H_2O. The concentration of extracted RNA was measured as described in Measurement of nucleic acids concentration (2.2.1.2.4). Isolated RNA was kept at -80° C.

DEPC Water *0.1 % Diethylpyrocarbonat (DEPC)*
Stir over night and autoclaved 2x

2.2.2.2. RNAse Protection Assay

2.2.2.2.1. DNA probe design and digestion

The DNA probe was designed by cloning of a partial sequence for the gene target or annealed oligonucleotides into a multicloning site of a commercial plasmid. Alternatively, oligonucleotides containing the T7 promoter sequence including a partial sequence for the gene target were purchased from BIOTEZ and annealed as described in 2.2.1.1.5.

For RPA analysis 10 µg of DNA probe was digested with an appropriate enzyme in volume of 50 µL over night. The enzymatic reaction was assayed with electrophoresis (1% agarose gel) before probe labeling.

2.2.2.2.2. Probe labeling

The labeled antisense RNA probe was synthesized by T7 or T3 or SP6 RNA polymerase in the presence of [α-^{32}P] UTP using an RNA transcription kit (Stratagene, USA). To perform in vitro transcription 200 ng of DNA was mixed with:

1 µL 10 mM rATP
1 µL 10 mM rCTP
1 µL 10 mM rGTP
5 µL 5x Transcription buffer
1 µL RNasin
1 µL 0.2 M DTT
3 µL [α-^{32}P] UTP (800 Ci/mM)
1 µL of T7, or T3 or Sp6- polymerase
in a final volume of 25 µL (DEPC H_2O)

After incubation at 37° C for 1 h, 1µL of RNase-free DNase I was added to digest the DNA template at 37° C for 15 min. To clean the probe from free unincorporated nucleotides, 25 µL of DEPC were added to the total reaction mixture of 25 µL and all together was purified with QuickSpin Columns for radiolabeled RNA purification according to the protocol of manufacturer. The activity of the probe was measured by the scintillation counter LS600SC (Beckman, Minnesota, USA).

2.2.2.2.3. RNA marker labeling

The Decade^{TM} Marker System kit (Ambion) was used to prepare radiolabeled RNA marker with radioactive dATP [γ-^{32}P] (300 Ci/mmol) as prescribed in the given protocol of the manufacturer.

2.2.2.2.4. RNA-RNA hybridization

RNA expression was analyzed by RPA using commercially available Ambion RPA II kits (AMS Biotechnology, Whitney, United Kingdom), according to the protocol of the manufacturer. 20 µg total RNA of different organs or cell culture, and 20 µg RNA of yeast as a control were used for RPA. RNA samples were hybridized with 80.000 cpm of the radio-labeled RNA antisense probe at 42° C for 16 h.
Once hybridized fragments were protected from RNase A+T1 digestion and precipitated by RNAse inactivation solution, they were separated by electrophoresis on a denaturing gel and analyzed using a FUJIX BAS 2000 Phospho-Imager system.
For small fragments, like siRNAs (less than 50 bases) 200 µL of EtOH was added into the RNase inactivation solution for better precipitation and 15% polyacrylamide gel (8 M urea) is recommended for separating small frements.

Urea-polyacryl gel	*7 M Urea*
	10x TBE
	15 % (v/v) Acrylamid-/Bis-Acrylamid 19:1
	0.0008 % (v/v) APS
	0.001 % (v/v) TEMED
TBE Buffer	*130 mM Tris*
	45 mM Boric acid
	2.5 mM EDTA

2.2.3. Protein

2.2.3.1. Protein isolation

With a glass-teflon homogenizer 100-200 mg of rat tissue was homogenized in 300-500 µL of RIPA buffer containing protease and phosphatase inhibitors.
Cultured cells (10 cm dish or 6 wells plate) were rinsed with PBS for two times and scraped in 5 mL of cold (4° C) PBS. After centrifugation at 2 500 g for 2 min the pellet was resuspended in 30 – 50 µL of lysis buffer containing protease inhibitor.
Rat or cell tissue homogenate samples were sonicated for 30 sec and centrifugated at 13 000 g, 4° C for 10 min. The upper layer with the protein was stored at -20° C.

PBS	*137 mM NaCl*
	2.7 mM KCl
	8.0 mM Na_2HPO_4
	1.5 mM KH_2PO_4
	pH 7.5
Protein Lysis Buffer	*20 mM Tris-HCl pH 7.4*
	150 mM NaCl
	1 mM EDTA
	1 mM EGTA
	0.5 % Triton X-100
	1 mM PMSF
	Complete Protease Inhibitor Cocktail (1 Tablet/10 mL)

RIPA Buffer	*150 mM NaCl* *50 mM Tris pH 8.0* *0.5 % Na-deoxycholate* *0.1 % SDS* *1 % Nonidet P-40* *Complete Protease Inhibitor Cocktail, Mini, EDTA-free (1 Tablet/10 mL)* *Phosphatase Inhibitor Cocktail I (100 µL/10 mL)* *Phosphatase Inhibitor Cocktail II (100 µL/10 mL)*

2.2.3.2. Measurement of protein concentration

The quantity of protein was estimated by Bradford method in 96-wells plate. Protein samples were diluted (usually 1:5) in lysis or RIPA buffer and 5 µL of each sample was mixed with 250 µL of the Bradford reagent. In parallel, for the standard curve BSA standards in 10 known concentrations (0.1 – 2.5 µg BSA/µL) were prepared and mixed with the Bradford reagent as well. After incubation at RT for 10 min all the samples including the standards were measured for the protein concentration using photometer at 595 nm. The protein concentration was graphically calculated regarding the protein standard curve.

2.2.3.3. Western blot

10 µg - 30 µg of protein samples were adjusted with lysis or RIPA buffer to an equal final volume and mixed with 4x SDS loading buffer (Roti®-Load). After boiling at 95° C for 5 min samples were loaded into the SDS-polyacrylamide gel, 10–15 %.
Proteins were separated in the gel, running at 120 V for approximately 2h and than transferred to a PVDF membrane (Amersham Bioscience) by blotting at 250 mA and 4° C for 2h. After protein transfer the membrane was blocked at RT for 1h. The blocking solution was set in accordance with specific antibody requirements. The blocked membrane was washed with TBS or TBST or PBST (depending on the

specific antibody) at RT two times for 10 min. First antibody was incubated with rolling at 4° C over night. On the next day the membrane was washed with TBS or TBST or PBST at RT four times for 10 min. Than the second antibody was applied at RT for 1h. Finally, after the last washing (four times 10 min) the enzymatic reaction was performed using SuperSignaling® West Dura Extended Duration Substrate (Pierce) or ECL Western Blotting Analysis System (GE Healthcare) according to the protocol of the manufacturers.

Protein expression was assayed using x-ray film that was exposed to the membrane treated with luminescence substrate (1 sec up to 2 h) and than developed.

To detect different proteins of similar molecular weight from the same membrane, the membrane was stripped with Restore™ Plus Western Blot Stripping Buffer (Pierce) as prescribed by the manufacturer and used again for first and second antibodies.

SDS-PAGE	*30 % Bis/Acrylamide 37,5:1*
Acrylamide Gel	*1M Tris pH 8.8 (for resolving gel)*
	1M Tris pH 6.8 (for stacking gel)
	10 % SDS
	10 % APS
	TEMED
SDS-PAGE	*25 mM Tris*
Running buffer	*200 mM Glycin*
	0.1 % SDS
Transfer buffer	*25 mM Tris*
	200 mM Glycin
	20 % Methanol
TBS	*50 mM Tris*
	150 mM NaCl
	pH 7.5

TBST	*0.5 %Tween20 in TBS*
PBST	*0.5 %Tween20 in PBS*

2.2.4. Cell culture

2.2.4.1. Culturing and transfection

Mammalian cells were cultured at 37° C and 5% CO_2 in a humidified atmosphere in a cell-line specific medium containing Penicillin/Streptomycin (Gibco) and FBS (fetal bovine serum; Gibco). Cells were subcultured every 3-4 days by detaching them with trypsin/EDTA (Gibco) and seeding in new plates at a dilution of 1:3. For transfection with Lipofectamine™ 2000 (Invitrogen), cells were plated in a concentration of 2 x 10^5 cells/mL in 6-well plates one day before. Next day, each well with cells was transfected with 1 µg - 5 µg of DNA plasmid mixed with Lipofectamine™ 2000 according to the given protocol.

Alternatively, cells (~70 % confluent) in 10 cm dish were detached with trypsin/EDTA and collected by centrifugation at 800 g, RT, 2 min, than taken in 250 µL medium and mixed with 50 µg of DNA. Cells were electro-porated at 360 mV and 500 µF using 0,4 cm electrode gap cuvette.

For a long term preservation cells were frozen in FBS (Fetal bovine Serum) containing 10 % DMSO and kept at -80° C in an Cryo 1° C Freezing Container over night and than frozen in liquid nitrogen (-276° C).

CELL LINE: (organism, organ)	**MEDIUM:**
COS7 Cercopithecus aethiops, monkey, African green Kidney	**Dulbecco's modified Eagle's medium (DMEM)** 4 mM L-Glutamin 1.5 g/l Natriumbicarbonat 4.5 g/l Glucose 10 % FBS 1000 u/µL Penicillin/Streptomycin
HEK293 Homo sapiens Kidney	**Dulbecco's modified Eagle's medium (DMEM)** 4 mM L-Glutamin 1.5 g/l Natriumbicarbonat 4.5 g/l Glucose 10 % FBS 1000 u/µL Penicillin/Streptomycin

2.2.4.2. Drug treatment of cells

Based on experimental purposes, transfected or non-transfected cells were incubated with appropriate drugs in corresponding concentrations or molarities for 24 h up to 72 h. The medium containing a drug was prepared freshly and changed daily.

2.2.5. Animals

Rats were maintained in individually ventilated cages (Techniplast) under standardized conditions (at a temperature of 21±2° C, with a humidity of 65±5 %) with an artificial 12 h light/dark cycle, with free access to standard chow (0.25% sodium; SSNIFF) and drinking water *ad libitum*. Sprague-Dawley (SD) rats were obtained from a commercial animal breeder (Taconic). All experimental protocols were performed in accordance with the guidelines for the humane use of laboratory animals by the Max-Delbrück Center for Molecular Medicine and were approved by local German authorities with standards corresponding to those prescribed by the American Physiological Society.

2.2.5.1. Generation of transgenic rats

2.2.5.1.1. Superovulation and isolation of embryos

SD (Sprague-Dawley) or GFP (Green fluorescent protein) transgenic rats were induced to superovulate by intraperitoneal injection of PMSG (Intervet) followed by the injection of hCG (Sigma) (Popova, Krivokharchenko et al. 2002). Briefly, rats received PMSG (15 IU) by i.p. injection between 11 a.m. - 1 p.m. of day -2. Approximately 48 h after the beginning of gonadotropin treatment, rats were given hCG (30 IU) by i.p. injection and mated with fertile males on the afternoon of day 0. Rats were sacrificed at 12 a.m. - 2 p.m. on day 1 to collect fertilized eggs. Criterion for mating was the presence of a vaginal plug.

2.2.5.1.2. Embryo Culture

One-cell embryos were recovered on day 1 at 2 p.m. from excised oviducts into M2 culture medium (Sigma). Zygotes were transferred into the same medium containing 0.1% (w/v) hyaluronidase (Sigma) to remove cumulus cells. Then, the embryos were

washed in M16 medium. After microinjection, eggs were cultured two hours in M16 medium to estimate the survival and then transplanted into pseudopregnant recipients. For *in vitro* development to the blastocyst stage the ova (10-20 embryos) were cultured overnight in M16 medium and transferred into 700 µL mR1ECM medium (Miyoshi, Kono et al. 1997) in 4-well culture dishes (Nunc) and cultured under 5% CO_2 in air at 37° C (Krivokharchenko, Galat et al. 2002). Two-cell stage embryos were recovered from the excised oviducts 45–48 h after hCG and immediately transferred into mR1ECM medium. Previously, the culture medium was equilibrated with the gas phase and temperature in a CO_2 incubator for 2-3 h.

2.2.5.1.3. Preparation of DNA for microinjection

Linearised DNA fragments for microinjection were isolated from agarose gels using a QIAquick Gel Extract Kit. The DNA was dissolved in microinjection buffer, adjusted to the concentration of 3 ng/µL and filtrated through a 0.22 µm filter. The BAC DNA was re-suspended in a special BAC microinjection buffer, always freshly prepared.

Tris-EDTA	*8 mM Tris-HCl pH 7.4*
Microinjection Buffer	*0.15 mM EDTA*
BAC	*10 mM Tris-HCl pH 7.5*
Microinjection Buffer	*0.1 mM EDTA pH 8.0*
	100 mM NaCl
	1x Polyamines

2.2.5.1.4. Microinjection of foreign DNA into the pronucleus

The solution of linearized DNA fragment (3 ng/µL) was microinjected into the male or female pronucleus of zygotes in M2 medium (Sigma) with the aid of a pair of manipulators (Eppendorf) (Popova, Krivokharchenko et al. 2004). Integration of the transgene was determined by DNA genotyping of genomic DNA isolated from the tails of the offspring.

2.2.5.1.5. Embryo transfer

Pseudopregnancy of females was induced by mating with vasectomized males with proven sterility. For embryo transfer, 4- to 5-month-old rats (200-280 g) were anaesthetized with a mixture of 0.25 mL Ketavet (100 mg/mL; Pharmacia & Upjohn GmbH) and 0.05 mL Rompun (2%; Bayer AG, Leverkusen) per animal (Popova, Bader et al. 2005; Popova, Bader et al. 2005). Intact zygotes were transferred into SD foster mothers (7-10 zygotes per recipient) after short-time incubation in M16 medium. Two hours after microinjection the surviving DNA-injected zygotes in M16 medium (Sigma) were transferred into the oviducts of day 1 (the day the vaginal plug was detected) pseudopregnant SD recipients (approximately 20 embryos per female).

2.2.5.1.6. Fluorescence detection of expressed GFP and RFP in preimplantation embryos

The expression of the GFP gene in embryos of pre-and postimplantation stages was assessed under UV light (489-nm; fluorescent microscope (Leica)). RFP fluorescence excitation was detected using a 590-nm emission filter.

2.2.5.2. Genotyping of transgenic rats

2.2.5.2.1. Isolation of genomic DNA from rats' tails

To isolate the genomic DNA 1 cm of a rat tail was digested in 89 µL of Ear Buffer containing Proteinase K at 55° C overnight with shaking. Next morning, the Proteinase K was denaturated by incubation at 95° C for 10 min and than hold on ice for 5 min. 750 µL of TE-Buffer with RNase A was added to samples. After 15 min at RT DNA samples were ready for PCR genotyping or until that kept at -20° C.

Ear Buffer with	*100 mM Tris-HCl pH 8.0*
Proteinase K	*5 mM EDTA*
	200 mM NaCl
	0.2 % SDS
	1 mg/mL Proteinase K
TE-Buffer with	*10 mM Tris-HCl pH 8.0*
RNase A	*1 mM EDTA*
	20 µg/mL RNase A

2.2.5.2.2. Genotyping

The genotyping of offspring was performed with a PCR reaction using sequence-specific primers. PCR conditions are described in 2.2.1.1.1.

PCR mix:

Genomic DNA	4 µL
Primer 1	1 µL
Primer 2	1 µL
dNTPs (5 mM)	2 µL
$MgCl_2$ (50 mM)	2 µL
DMSO	1 µL
PCR-Buffer (10x)	5 µL
Taq Polymerase (5u/ µL)	0.2 µL
H_2O	Add 50 µL

2.2.5.3. Animal treatment and phenotyping

2.2.5.3.1. Doxycycline treatment

To induce expression of shRNA, animals were treated with varying concentrations of doxycycline (DOX; Sigma) in the drinking solution. The DOX solution was daily prepared fresh and kept dark due to the light sensitivity of DOX. The drinking solution contained various percentages of sucrose depending on the DOX concentrations and was also given to the control animals.

To check the functionality of the system animals were treated with 2 mg/mL DOX in the drinking water containing 10% sucrose for 4 days.

In the reversibility tests animals received different doses of DOX per day (20, 2 and 0.5 mg/kg body weight). To this end rats were offered their daily dose of DOX in about 20 ml of 1% sucrose. After this volume was consumed they got normal water ad libitum. Once plasma glucose levels reached 250 to 300 mg/dL in the treated transgenic rats, DOX was withdrawn from their drinking solution.

To establish a chronic model of diabetes mellitus type II a group of rats was treated daily with 5 µg/mL of DOX solution containing 1% sucrose. When blood glucose reached 300 mg/dL (after 8 days of treatment) the concentration was changed to 1 (or 2 and than to 1) µg/mL DOX solution (in 1% sucrose) for in total 40 days. After treatment animals were killed by decapitation. Organs were rapidly isolated and first frozen in liquid nitrogen (-276° C) and than kept in -80° C freezer until use for molecular analysis (Western blot, Ribonuclease protection assay).

2.2.5.3.2. Blood glucose test

The blood glucose concentration was analysed taking a drop of tail-vein blood using Accu Chek Sensor (Roche, Mannheim, Germany).

2.2.5.3.3. Insulin test

Concentration of plasma or serum insulin, isolated from 200-300 µL of tail-vein blood, was estimated using Rat/Mouse Insulin ELISA kit (LINCO Research) according to the manufacturer's protocol. Tail-vein blood was centrifuged at 2 000 g for at 4° C for 15 min. The colorless supernatant containing proteins was stored at -20° C until ELISA was done.

2.2.5.3.4. Urine and albumin analysis

To collect urine for validation of urine volume and albuminuria experimental animals were kept in metabolic cages under standardized conditions for one day (24h) per week during a period of 3 weeks. After 24 h, the volume of collected urine was determined. For quantification of albumin the urine was centrifuged (600 g, 10 min, 4° C) and stored at -20° C until sent for analysis to CellTrend GmbH (Luckenwalde, Germany).