2 Materials and Methods

2.1 Materials

2.1.1 General reagents

Reagent	Manufaturer
β-mercaptoethanol	Merck
Acetic acid 100%	Merck
Acetone	Merck
Agarose	Invitrogen
Amino acids	Sigma
Ammonium persulfate	BioRad
Ampicillin	Sigma
Aqua ad inectabilia	Baxter
BACTO-Yeast extract	BDSciences
BACTO-Agar	Difco
BACTO-Triptone	Difco
Biotin	Sigma
Biotin-16-UTP	Roche
Boric acid	Merck
Bovine Serum Albumin	Serva / Sigma
Bradford reagent	Sigma
Bromphenolblue	Serva
Chlorampenicol	Fluka
Chloroform	Merck
Complete/Complete mini protease inhibitors	Roche
Coomassie G250/Coomassie R250	Serva
DABCO (1,4-diazobicyclo-2,2,2-octane)	Sigma
DAPI (4,6-diamino-2-phenylindole-2HCl·H_2O)	Serva
d-Biotine	Sigma
Deoxycholic acid sodium salt (DOC)	Fluka
Diethylpyrocarbonat	Sigma
DMEM (Dulbecco´s Modified Eagle Medium)	Cambrex
DMSO	Sigma
dNTPs	Fermentas
DTT	Promega
EDTA	Merck
EGTA	Merck
Ethanol	Merck
Ethidium bromide	Serva
Fetal calf serum (FCS)	Biochrom
Ficoll	Pharmacia
Fixing solution	Agfa
Formaldehyde	Merck
Glucose monohydrate/Sucrose	Merck
Glutamine	Cambrex
Glycerol	merck

Glycin	Merck
Glycogen	Roche
GTP	Sigma
H_3PO_4	Merck
Heparin	Sigma
HCl	Merck
Imidazole	Fluka
Immersion oil immersol 518F	Zeiss
IPTG	Fermentas
Isopropanol	Merck
K_2PO_4	Merck
Kanamycin	Sigma
KCl	Sigma
LiAc	Sigma
LiCl	Sigma
Lipofectamine 2000 transfection reagent	Invitrogen
Lysozyme	Boehringer-Ingelheim
M280 streptavidine coated magnetic beads	DYNAL
Methanol	Merck
$MgCl_2$	Merck/ PerkinElmer
$MgSO_4$	Merck
Milk powder	Nutricia-Zoetermeer
Mineral oil	Sigma
Na_2HPO_4	Merck
NaAc/NaCl/NaOH	Merck
$NaHPO_4$	Merck
NBT/BCIP	Roche
Ni-NTA agarose	Qiagen
NP40	Fluka
Oligofectamine	Invitrogen
OPTIMEM	GIBCO
Paraformaldehyde	Sigma
pdN_6	Pharmacia
PEG	Sigma
Penicillin/streptomycin	Cambrex
Phenol	Roth
PIPES	Sigma
Polyfect	Qiagen
Polyhomoribopolymers agarose (poly-rG, poly-rU, poly-rC, poly-rA)	Sigma
Prime RNAase inhibitor	Eppendorf
Protein agarose A/G	Roche
RNA guard	Amersham Pharmacia biotech
RNA loading dye	Fermentas
Rothiphorese gel 30	Roth
Roty-Nylon Plus membrane	Roth
SDS (sodium dodecylsulfate)	BioRad/ Serva
siRNA oligos	Qiagen/Dharmacon
Streptavidine-AP	Roche
Taxol (Paclitaxel)	Sigma

Taxol (Paclitaxel)	Sigma
TEMED	GIBCO BRL
Trichloro acetic acid (TCA)	Sigma
Tris-(hydroxymethyl)-aminomethane	Merck
Triton X-100	Serva
Trizol	Merck
Tween20	Sigma
Urea	Merck/BioRad
Vivaspin 500	Vivascience
X-Gal	Appligene
Xylencianolblue	Sigma
Yeast base without amino acids	Difco
Zeocin	Invitrogen

Table 2.1. General reagents

2.1.2 Enzymes

Enzyme	Concentration	Manufacturer
Cloned Pfu DNA polymerase	2,5 U/μl	Stratagene
DNAse I	1 U/μl	Promega
Proteinase K	10 mg/ml	Roche
Restriction endonucleases	10-20 U/μl	New England biolabs / Fermentas
RNAse A	10 mg/ml	Roche
Superscript II reverse transcriptase	200 U/μl	Invitrogen
T4 DNA ligase	400 U/μl	Promega
Tag DNA polymerase	5 U/μl	House made
Thrombin, restriction grade	1,58 U/μl	Novagen

Table 2.2. Enzymes

2.1.3 Kits

Kit	Manufacturer
Advantage 2 PCR Kit	Clontech
Endofree Plasmid Maxi Kit	Qiagen
Profound Pull-Down Biotinylated Protein: Protein Interaction Kit	Pierce
Qiaprep Spin Miniprep Kit	Qiagen
Qiaquick Gel Extraction Kit	Qiagen
Quick Change Directed Mutagenesis Kit	Stratagene
RiboMAX Large Scale RNA Production System-T7	Promega
RNeasy Mini Kit	Qiagen
Termination Ready Reaction Mix	PerkinElmer

Table 2.3. Kits

2.1.4 Instruments and Disposables

Instrument/disposable	Manufacturer
0,025 μm filters	Millipore
Centrifuge Rotanta 46R/Rotina 4R	Hettich zentrifugen
Centriplus/Centricon	Millipore
Concentrator 5301	Eppendorf

Cover slips	Menzel Glaser
Electrophoresis power supply 2	House made
Extra thick blot paper	BioRad
Filter paper	Schleicher and schuell
Fixogum	Marabu
Gene Pulser cuvettes/ Gene pulser I-Pulser chamber	BioRad
Handee Mini Spin Columns	Pierce
Horizontal gel apparatus Horizon® 11.14 and 20.25	Life technologies
Hypercassete	Amersham biosciences
Inverted microscope Elipse TS100	Nikon
L8-70M ultracentrifuge	Beckmann
Microscope slides	Roth
Phase lock gel light	Eppendorf
pH-meter	Knick
Photometer Ultrospec II	LKB biochrom
Pipett boy	Integra biosciences
Pipettes	Gilson
Potter	B. Braun Melsungen
Power Pac 300 electrophoresis power supply	BioRad
PVDF membrane	Roche
QIAshredder	Qiagen
RNA tips	Biozym
Rnase ZapWipes	Ambion
Rotors TLA120.1, TLS-55, SW40	Beckmann
Single use filter unit Minisart	Sartorius
Slide-A-Lyzer mini dyalisis unit	Pierce
Sonicator, SONOPLUS Homogenisator HD2070	Bandolin electronics
Sorvall RC-5B refrigerated superspeed centrifuge	Sorvall
Steril plastic disposables for cell culture	TRP
Table centrifuge 5415C	Eppendorf
TL100 ultracentrifuge	Beckmann
UV stratalinker 1800	Stratagene
UV trasiluminator	UVPinc
UVette	Eppendorf

Table 2.4. Instruments and disposables

Note: Instruments and disposables not included in table 4 are indicated in the corresponding sections. All reusable lab ware was autoclaved before use.

2.1.5 Antibodies

Antibody	WB	IF	IP	Animal	Purchased from
Anti-α4	1:200			Rabbit	Self-produced (Trockenbacher et al., 2001)
Anti-MID1	1:1000			Rabbit	Self-produced (Winter et al., 2004)
Anti-Actin	1:500			Rabbit	Sigma
Anti-Tubulin MCA 77s	1:1000	1:3000		Rat	Serotec
Anti-Tubulin MCA 78s	1:1000	1:3000		Rat	Serotec
Anti-c-myc monoclonal	1:500	1:500		Mouse	Clontech
Anti-c-myc polyclonal	1:500	1:800	1,5 μg	Rabbit	Santa Cruz

Anti-V5	1:3000	1:800		Mouse	Invitrogen
Anti-FLAG-polyclonal	1:400		1,6 μg	Rabbit	Sigma
Anti-FLAG-monoclonal	1:1000	1:500		Mouse	Stratagene
Anti-Nucleophosmin	1:1000			Mouse	Zymed
Anti-EF-1α	1:1000			Mouse	Upstate
Anti-Lamin A+C	1:350			Mouse	Chemicon
Anti-Annexin II	1:1000			Mouse	BD-transduccion laboratories
Anti-Hsp90	1:2000			Rat	Stressgene
Anti-Hcs70	1:10000			Rat	Stressgene
Anti-RACK1	1:2500			Mouse-IgM	BD-transduccion laboratories
Anti-HuR	1:1000			Mouse	Santa Cruz
Anti-GFP	1:500			Mouse	Roche
Ribosomal antibodies	1:1000			rabbit	Gift from J. Stahl (Lutsch et al., 1990)
Anti-Rabbit HRP	1:2000			Donkey	Amersham
Anti-Rabbit HRP non-reduced	1:25000			mouse	Sigma
Anti-Mouse HRP	1.5000			Goat	Dianova
Anti-Mouse IgM -HRP	1:2500			Donkey	Dianova
Anti-Rat HRP	1:1000			Rabbit	Serotec
Anti-Rat HRP	1:1000			Goat	Santa Cruz
Anti-Rabbit Cy3		1:1000		Goat	Dianova
Anti-Mouse Cy3		1:1000		Donkey	Dianova
Anti-Rat Cy3		1:1000		Donkey	Dianova
Anti-Rabbit FITC		1:250		Goat	Dianova
Anti-Mouse FITC		1:1000		Goat	Dianova
Normal mouse IgG					Santa Cruz

Table 2.5. Primary and secondary antibodies

2.1.6 Vectors

Vector	Host	Tag	Resistance/ Selection	Use	Manufacturer
pCMV Tag 2A-C	Mammalian	Flag	Kanamycin	IF, IP	Stratagene
pCMV Tag 3A-C	Mammalian	Myc	Kanamycin	IF, IP	Stratagene
pBUD CE4	Mammalian	Myc / V5	Zeocin	IF, IP	Invitrogen
pEGFP	Mammalian	GFP	Kanamycin	IF	Clontech
PinPoint Xa	*E. coli*	Biotin/HIS (incorporated)	Ampicillin	Protein expression	Promega
pET-32a	*E.coli*	His-tag, S·Tag, Trx·tag	Ampicillin	Protein expression	Novagen
pBMT116a	*E.coli* /*S.cerevisae*		Ampicillin/ Tryptophane	Yeast two-hybrid	Clontech
PGAD1o	*E.coli* /*S.cerevisae*		Ampicillin/ Leucine	Yeast two-hybrid	Clontech

Table 2.6. Vectors

2.1.7 Buffers and Media

Buffer/Media	Composition
APS 10%	10% w/v APS in water, aliquoted and stored at -20°C
Annealing buffer	100 mM KAc, 30mM Hepes-KOH pH 7,4, 2 mM $MgAc_2$

Blocking buffer	5% milk powder in PBST
Blotting buffer 1 x	5x blotting buffer: MeOH: bidest H_2O 1:1:3
Blotting buffer 5 x	29,11g Tris; 14,65g Glycin; 18,75ml SDS in 1l bidest water
Colloidal coomassie staining	1g CBB G-250 in 100 ml bidest H_2O; 100 g $(NH_4)_2SO_4$ and 20 g H_3PO_4 (85%) in 800 ml bidest H_2O. Mix both solutions and adjust the volume to 1 l bidest H_2O.
Coomassie	1 g Coomassie blue R250, 500 ml H_2O, 100 ml HAc, and 400 ml methanol. Mix and filter through a filter paper
Destaining Coomassie	500ml H_2O, 100ml HAc, and 400 ml methanol.
DPEC H_2O	0,1% DEPC was overnight stirred in water and afterwards autoclaved.
Dropout solution 10x	200 mg/l L-Adenine Hemisulfat salt, L-Arginine HCl; L-Histidine HCl Monohydrate, L-Methionine, L-Tryptophane, L-Uracil; 300 mg/l L-Isoleucine, L-Lysine HCl, L-Tyrosine; 500 mg/l L-Phenylalanine; 1000 mg/l L-Leucine; 1500 mg/l L-Valine; 2000 mg/l L-Threonine; in 1l Bidest; autoclave
-TL/-HTL Dropout solution	Dropout solution without leucine and threonine/ and histidine
Elution buffer (HIS)	50 mM NaH_2PO_4; 300 mM NaCl; 250 mM imidazole. Adjust to pH 8,0 using NaOH
Ethidium bromide	10 mg/ml EtBr in bidest H_2O
HS buffer	4 mM Hepes, 0,32 M Sucrose in bidest H_2O, filter sterilized, stored at –20°C.
HSNM buffer	4 mM buffer, 0,32 M Sucrose, 100 mM NaCl, 5 mM $MgCl_2$ in DEPC-H_2O
IP1 buffer	150 mM NaCl; 10 mM Tris pH 7,5; 1% NP40; autoclaved
IPTG	100 mM in bidest, filter sterilized and stored at -20°C
Laemmli buffer	25 mM TRIS p.a, 190 mM Glycin, 0,1% SDS in bidest H_2O
LB (**L**uria **B**ertani) medium	15 g Agar; 10g Tryptone; 5g yeast extract in 1l bidest water; autoclaved
DNA-Loading buffer (LX, LB)	15% Ficoll, 0,25% Bromphenolblue or Xylenecyanol in bidest H_2O
Lysis buffer (QIAexpressionist)	50 mM NaH_2PO_4; 300 mM NaCl; 10 mM imidazole adjust to pH 8,0 using NaOH
Magic mix 2x	48% Urea (BioRad), 15mM Tris-HCl pH 7,5, 8,7% Glycerin, 1%SDS, 0,004% Bromphenolblue, 143 mM β-mercaptoethanol (add fresh)
Microtubule assembly buffer	0,1 M PIPES ph 6,8; 1 mM $MgSO_4$; 2 mM EGTA; 2 mM DTT; 0,1 mM GTP
MOPS 10 x	0,4 M MOPS; 0,1 M NaAc; 10 mM EDTA pH 7,0
Mounting medium	90% glycerol; 0,1 M Tris-HCl pH 8,0; 2,3 % DABCO
Paraformaldehyde	3,7% formaldehyde in 1,2 PEM Buffer
Paraformaldehyde 3,7%	In 1,2 x PEM. Dissolve at 60°C. Store at -20°C
PBS 1 x	137 mM NaCl; 2,7 mM KCl; 10,1 mM Na_2HPO_4; 1,8 mM KH_2PO_4
PBST	1 x PBS; 1:1000 Tween 20
PEM 10X	1 M PIPES, 0,5 M EGTA, 0,02 M $MgCl_2$ in bidest H_2O, adjust pH 7 with 10N NaOH and autoclave
RNA loading buffer	1 µl MOPS, 5 µl loading dye, 1,5 µl 37% formaldehyde, 10 µl DEPC.water
SDS-PAGE buffer 5x	15% ß-Mercaptoethanol, 15% SDS, 1,5% Bromphenolblue, 50%glycerol
Separating gel buffer	1,5 M Tris-HCl, 0,4 % SDS pH 8,8
SSC 10x	3M NaCl, 0,3M Na-citrate in bidest H_2O, adjust pH 7 with 1M HCl, filter
Stacking gel buffer	0,5 M Tris-HCl, 0,4 % SDS pH 8,8
Sucrose cushion	0,2 g sucrose; were dissolved by shaking at 37°C in 280 µl MT buffer, afterwards 4µl dGTP (100 mM) and 1µ taxol (5 mg/ml) were added

TAE buffer 50 x	50 mM EDTA, 5,71% v/v acetic acid, 2M Tris-HCl
TBS	10 mM Tris-HCl (pH 8,0); 150 mM NaCl
TBST buffer	10 mM Tris-HCl (pH 8,0); 150 mM NaCl; 0,05% tween 20
TE	10 mM Tris-HCl pH 7,5; 1 mM EDTA
TES	10 mM Tris-HCl pH 7,5; 5 mM EDTA; 0,2 % SDS
TKM buffer	20 mM Tris, 100 mM KCl, 5 mM $MgCl_2$ in DEPC-H_2O
Wash buffer (QIAexpressionist)	50 mM NaH_2PO_4; 300 mM NaCl; 20 mM imidazole adjust to pH 8,0 using NaOH
Washing buffer for column	HS buffer with 250 mM NaCl, 0.5% Tween
YPD buffer	20 g/L BACTO-tryptone, 10 g/L yeast extract, 20 g/L Bacto-Agar (only for plates)
Z-buffer	1,61 g/L Na_2HPO_4*7H2O, 5,5 g/L $NaH_2PO_4 \cdot H_2O$, 0,75 g/L KCl,0,246 g/L $MgSO_4 \cdot 7H_2O$,pH 7,0

Table 2.7. Buffers and media

2.1.8 Cell lines

Cell line	Description	Medium
HeLa (**He**nrietta **La**cks)	Human epithelial cells from cervical carcinoma	DMEM, 10% FCS, 2mM L-glutamine, 100 μg/ml streptomycin, 100 units/l penicillin
COS-7	Transformed African Green Monkey Kidney Fibroblast Cells	
855/02	Human fibroblasts from OS patient	
756/01	Human fibroblast (man)	
18/98	Embryonic human fibroblasts	

Table 2.8. Cell lines

2.1.9 Bioinformatic tools and Databases

The bioinformatic databases and tools used during this thesis are indicated in Table 2.9 including their online resource:

Tool name	Type	URL/Programm
BLAST at NCBI	Blast	http://www.ncbi.nlm.nih.gov/BLAST/
ENSEMBL	Sequence retrieval and analysis	http://www.ensembl.org
ExPaSy	Proteomics Server	http://www.expasy.org/
GCG package	DNA sequences analysis	http://www.accelrys.com/products/gcg_wisconsin_package
STADEN package	DNA sequences analysis	http://www.hgmp.mrc.ac.uk/Registered/Option/staden.html
GenBank	Genome database	http://www.ncbi.nlm.nih.gov/Genbank/
Online Mendelian Inheritance in Man (OMIM)	Database of human genes and genetic disorders	http://www.ncbi.nlm.nih.gov/Omim
Primer3	Primer design	http://frodo.wi.mit.edu/cgi
PubMed	Medline biomedical articles	http://www.pubmed.org
RZPD (Resource zentrum Primary Database)	Clone collections	http://www.rzpd.de
STADEN package	DNA sequences analysis	Version 1999.0

Swiss-Prot	Protein knowledgebase	http://www.expasy.org/sprot/
UCSC Human Genome Working Draft	Genome Browser	http://genome.ucsc.edu/

Table 2.9. Bioinformatic tools and databases

2.1.10 Patients

The first patient was born to consanguineous parents and presented with hypertelorism, broad nasal bridge, strabismus, cleft lip and palate, hypospadias and small ears with a right pre-auricular pit. He was found to have a de novo 388G>A mutation in MID1, predicting an A130T change in the Bbox1 domain.

The second patient had hypertelorism, down-slanting palpebral fissures, broad nasal bridge, posteriorly rotated ears, cleft lip and palate, and hypospadias. He was found to harbour a de novo 433T>A MID1 mutation, predicting a C145S change in the Bbox1 domain.

2.2 Nucleic acid methods

2.2.1 Polymerase chain reaction (PCR)

PCR was used for the amplification of DNA fragments of known sequence using either genomic DNA or cDNA as template. If not otherwise stated, each PCR reaction was prepared as follows:

Component	Amount
DNA/cDNA	100 ng /3 μl
PCR buffer,	5 μl
$MgCl_2$ (25 mM)	x μl
dNTP mix (each dNTP 2,5 mM)	2μl
Forward + reverse primer (each 10 pmol/μl)	2μl
Taq polymerase	1μl
Aqua ad inectabilia.	50 μl

Table 2.10 Components of PCR reactions

The following thermal profile was used for the amplification:

Step	Temperature	Time	Cycles
Initial denaturation	95°C	4 min	1x
Denaturation	95°C	30 sec	29x
Annealing	Primer specific	30 sec	
Elongation	68°C/72°C	1 min/kb amplified DNA 2 min/kb for Pfu polymerase	
Final elongation	68°C/72°C	10 min	1x
Storage	4°C	Infinite	

Table 2.11. Thermal cycle reactions for PCR reactions

The amount of $MgCl_2$, Taq polymerase used and annealing temperature are indicated for each pair of primers in the corresponding sections.

Touchdown PCR was performed for fragments with sequences difficult for simple amplification.

Step	Temperature	Time	Cycles
Initial denaturation	95°C	4 min	1x
Denaturation	95°C	30 sec	
Annealing	(T_A+10°C)-1°C cycle	30 sec	10x
Elongation	68°C/72°C	1 min/kb amplified DNA	
Denaturation	95°C	30 sec	
Annealing	Primer specific (table)	30 sec	29x
Elongation	68°C/72°C	1 min/kb amplified DNA	
Final elongation	68°C/72°C	10 min	1x
Storage	4°C	infinite	

Table 2.12. Touch down PCR thermal cycle

PCR reactions were carried out in a PTC200 Peltier Thermal cycler (Biozym).

All PCR products were evaluated by DNA electrophoresis. When required, PCR products were purified either directly with the QIAquick PCR purification kit, or run on an agarose gel and subsequently extracted with the Gel Extraction Kit. DNA was eluted with 30-50 μl EB buffer (Qiagen) diluted 1:10 in bidest water.

2.2.2 Agarose gel electrophoresis

1% - 2% w/v ultrapure agarose gels in TAE buffer were used to separate RNA or DNA samples. 0,5 μg/μl EtBr was added to the gels to visualise DNA or RNA via UV light. 1 μl of loading buffer* was added per each 9 μl of DNA solution before loading the samples on the gel. Samples were run at 80-150 V in an electrophoretic chamber for 30-60 min depending on the fragment size. Different DNA/RNA molecular weight markers were used according to the size of the analysed products. Fragments were visualised, and pictures were taken, with the E.A.S.Y Win32 gel documentation system (Herolab). The following DNA/RNA ladders were used, the sizes of the fragments characterised is indicated:

DNA/RNA ladders	Fragment size	Manufacturer
Hyperladder I	300 - 7000 bp	Bioline
Hyperladder V /100bp DNA Ladder	50 –500bp	Bioline/GIBCO
0,24-9,5 KB RNA Ladder	all	Invitrogen

Table 2.13. Nucleic acids ladders

*Note: LX was used for DNA fragments bigger than 1 kb and LB for fragments shorter that 1 kb. RNA samples were run in RNA loading buffer and heated for 10 min at 70°C prior to loading. 1 μl of RNA ladder was treated in the same way.

2.2.3 RNA preparation and cDNA synthesis

RNA was prepared with the RNeasy kit according to manufacturer´s instructions. For subsequent removal con contaminant DNA in the sample, 1 μg of RNA was digested with 1 μl

DNAse I in 2 µl DNAse buffer and bidest H_2O up to 20 µl. The reaction took place for 30 min at 37°C. For purification, samples were subjected to phenol:chloroform extraction and EtOH precipitation. RNA samples were kept at –20°C.

For cDNA synthesis, 2 µg of RNA were mixed with 2 µl dNTPs (2,5 mM each), 6 µl $pd(N)_6$ (100 ng/µl) and DEPC-H_20 up to 31µl of total volume. The reaction was incubated for 5 min at 70°C and quickly chilled on ice. Next, 10 µl of 5x 1st strand buffer, 5 µl of 0.1 M DTT and 1 µl of RNA guard were added, and the mixture was incubated for 2 min at 42°C. The samples were divided into two aliquots of 23,5 µl. To one of the aliquots 2 µl of Superscript II were added. The second aliquot was kept as negative control for the reaction. RNA was reverse-transcribed by incubation for 1 h at 42°C and the enzyme was subsequently inactivated at 70°C for 15 min. cDNAs were stored at –20°C.

2.2.4 Phenol:Chloroform extraction

To DNA or RNA containing solutions one volume of phenol:chloroform (1:1) (TE pH:7.5 saturated phenol for DNA, H_2O saturated phenol pH:4,5 for RNA) in a Phase Lock Gel (PLG) light expender was added. The mixture was rotated for 10 min and centrifuged at 16000 *x g* for 5 min. To the aqueous upper phase, one volume of chloroform was added, and the phases were again mixed for 10 min and centrifuged for 5 min at 16000 *x g*. The upper phase was transferred to a new eppendorf tube and DNA or RNA was precipitated with EtOH.

2.2.5 EtOH precipitation of nucleic acids

For EtOH precipitation of nucleic acids, 2,5 volumes of 95% EtOH, 1:10 LiCl for RNA or NaAc for DNA, and 1:100 glycogen were added to the samples. The mixture was placed at –20°C for > 30min and subsequently centrifuged for 20 min at 16000 x *g* at 4°C. Afterwards, the pellet was washed with 200 µl 70% EtOH and centrifuged for 10 min at 16000 x *g* at 4°C. The pellet was air dried and resuspended in TE buffer pH 75.

2.2.6 Cloning

2.2.6.1 Cloning of pEGFP constructs and mutagenesis

5´-gctaagcttcgatggaaacactggagtcag-3´ and 5´-tcgaattctcggcagctgc-tctgtgca-3´ primers, containing HindIII and EcoRI sites respectively, were used to amplify MID1 cDNA from either control or patient fibroblast carrying the 388G>A (A130T) mutation RNA. 1 mM $MgCl_2$, Pfu Taq polymerase and 54°C annealing temperature were used. PCR products and pEGFP-C1 vector were digested with the corresponding restrictions enzymes for 1 h at 37°C, run in an agarose gel, excised and purified with the Gel Extraction Kit (Qiagen). The products were ligated overnight at 16°C with T4 DNA ligase.

For the ligation an insert-vector ratio of 3:1 was used as follows:

$$ng\ insert = \frac{ng\ vector\ x\ Kb\ fragment}{Kb\ vector}\ x3$$

The MID1 splice variant, Ex2d.7, was generated in pEGFP-C2 from a pBUDCE4-Ex2d.7 clone available in the laboratory (Winter et al., 2004). The insert was extracted with SalI and HindIII restriction enzymes and ligated into pEGFP-C2 (which provides the correct ORF) as described above.

The constructs lacking the RING and the RING plus the Bboxes were amplified with the primers 5´-gctcaagcttgggaaagcatcagtgagcggg-3´ and 5´-gctcaagcttgggccttgt-gtaaactggttgg-3´as forward primers respectively, both containing HindIII restriction site, and 5´-tcgaattcttcacggcagctgcacagt-3´as reverse primer for both, which contained EcoRI restriction site. 1 mM $MgCl_2$, Pfu polymerase and 57°C annealing temperature were used. PCR products were ligated into pEGFP-C1 vector as described above.

Mutagenesis experiments were performed to produce the remaining mutated constructs according to the instruction manual provided with the QuickChange® Site Directed Mutagenesis Kit. In brief, 50 ng of template were amplified in 5 µl of 10x reaction buffer with 1,25 µl (125 ng) of each primer, 1 µl of dNTP mix and 1 µl *Pfu Turbo* polymerase in a 50 µl reaction in bidest H_2O.

The following thermal profile was used for the amplification

Step	Temperature	Time	Cycles
Initial denaturation	95°C	4 min	1x
Denaturation	95°C	30 sec	12-18x*
Annealing	55°C	30 sec	
Elongation	68°C	2 min/kb plasmid length	
Storage	4°C	Infinite	

Table 2.14. Thermal profile for mutagenesis reactions

*Note: 12 cycles for point mutations, 16 cycles for single amino acid changes and 18 cycles for multiple amino acids deletions or insertions.

Non-mutated plasmids DNA were restricted with *Dpn* I (10 U/µl) for 1 h at 37°C and subsequently, 1 µl of the reaction was chemically transformed into XL1-Blue supercompetent cells. Plasmid DNA from the mutated constructs was prepared as described in section 2.2.7. Controls for the transformation and for the amplification reaction were included for every reaction.

Primer	Nucleotide	Sequence (5´→ 3´)	Cycles
403-411delfor	403_411del9bp	gacgctgtgaagacctgtgaagtatcctactgtgacgag	18
403-411delrev		ctcgtcacagtaggatacttcacaggtcttcacagcgtc	
H178Yfor	532C>T	ggctgatgtgcttggagtatgaggatgagaaggtg	12
H178Yrev		caccttctcatcctcatactccaagcacatcagcc	

C145Sfor C145Srev	434T>A	cctactgtgacgagagcctgaaagccactc gagtggctttcaggctctcgtcacagtagg	12
ΔBbox2for Δbbox2rev	Deletion of Bbox2	cacatccgggggctgatggtggcagctttgagtgag cacactcaaagctgtcaccatcagcccccggatgag	18
Δbbox1for Δbbox1rev	Deletion of Bbox1	ctccgccgagaaggtcctccgtctgattgagccaattc gaattggctcaatcagacggaggaccttctcggcggag	18
V183Tfor V183Trev	547GT>AC	gagcatgcggatgagaagacgaatatgtactgtgtgacc ggtcacacagtacatattcgtcttctcatcctcatgctc	12
C198Afor C198Arev	592TG>GC	gttaatctgtgccttggctaaactggttgggcggc gccgcccaagtttagccaaggcacagattaac	12
C195Ffor C195Frev	584G>T	ccgatgaccagttaatctttgccttgtgtaaactgg ccagtttacacaaggcaaagattaactggtcatcgg	12
C175Afor C175Arev	523TG>GC	cacatccgggggctgatggccttggagcatgaggatgag ctccatcctcatgctccaaggccatcagcccccggatgt	12
ΔBbox2+7aafor ΔBbox2+7aarev	Deletion of Bbox2 +7aa	gcttggagcatgaggatgaggtggcagcttgacgtgagc gctcactcaaagctgccacctcatcctcatgctccaagc	18
ΔBbox2+7aa-C175Afor ΔBbox2+7aa-C175Arev	Deletion of Bbox2+ 7aa+523TG>GC	ccgggggctgatggccttggagcatgagg ctcatgctccaaggccatcagccccgg	18
ΔBbox2+7aa-H178Afor ΔBbox2+7aa-H178Arev	Deletion of Bbox2+ 7aa+532CA>GC	ctgatgtgcttggaggctgaggatgaggtggc gccacctcatcctcagcctccaagcacacag	18

Table 2.15. Oligos used for mutagenesis

2.2.6.2 Preparation of the pCMVTag2C and pBMT116 constructs

The MID1 (wild-type and mutated forms) cDNA from the different constructs in the pEGFP-C1 vector was cut out with HindIII and SalI and ligated into the multiple cloning site of pCMVTag2C.

For the cloning in the pBMT116 vector, the different MID1 inserts from the pEGFP-C1 clones were amplified with the 5´-tggctggaattcgaaaacactggagtcagaactg-3´ and 5´-aggtcgacggattcacggcagctgctctgtgc-3´ primers, containing EcoRI and SalI restriction sites respectively. PCR products and pBMT116 vector were digested, purified and ligated as described above.

A pBMT116 clone including MID1 splice variant Ex2d.7 was already available in the laboratory (Winter et al., 2004)

2.2.6.3 α4/44aa peptide clones

pGAD10-α4, pBudCE4-α4 and pCMVTag3a-α4 were already available in our laboratory (Trockenbacher et al., 2001; Winter et al., 2004).

For the cloning of the 44aa peptide into the PET32a vector, the 44aa peptide sequence was amplified with 5´-atctgggtaccaggcctccagtgaaaccc-3´ and 5´-gcaagcttgtt-atgctccatatttccgatgttg-3´primers, which contained restriction sites for KpnI and HindIII respectively. 1,5 mM $MgCl_2$, Pfu polymerase and 55°C annealing temperature were used.

PCR product was digested and cloned into the multiple cloning site pET32a vector as described above.

For cloning the 44aa peptide into the Pinpoint™–Xa vector, the primers 5´-gctgggatccggaggcctccagtgaaaccc-3´ and 5´- ggagatctgttatccatatttccgatgttg-3´, containing BamHI and BglII restriction sites respectively, were used for amplification of the insert. 1 mM of $MgCl_2$, Pfu polymerase and 58°C annealing temperature were used. Both reverse primers contained a stop codon.

To incorporate the His-Tag, 1 µl (100 picomoles) of the following oligos: 5´-agtttcaagaggatcgcatcaccatcaccatcacgg-3´ and 5´-gatcccgtgatggtgatggtgat-gcgatcctcttga-3´ containing the BamHI and HindIII restriction sites respectively were annealed in 48 µl annealing buffer. The following thermal reaction was performed:

Step	Temperature	Time
Initial denaturation	95°C	4 min
Annealing	70°C	10 min
Cooling	- 0.1°C / sec	down to 4°C
Final step	4°C	10 min
Storage	4°C	infinite

Table 2.16. thermal profile to anneal oligos

Annealed oligos were digested with BamHI and HindIII and ligated into the multiple cloning site of the Pinpoint vector already containing the 44aa peptide (N-terminally located with respect to the peptide).

2.2.7 Plasmid DNA isolation and transformation

2.2.7.1 Plasmid DNA isolation

Single bacterial colonies carrying the plasmid of interest were grown overnight at 37°C with vigorous shaking in 5 ml LB medium with the appropriate antibiotic (Table 2.6). Next day, plasmids were prepared using the Miniprep Kit (Qiagen) according to the manufacturer instructions.

When larger amounts of DNA were required, 5 ml of LB/antibiotic were inoculated with a single colony, and incubated during 8 hours. 0,5 ml of this culture were used to inoculate 100 ml of LB/specific antibiotic, which was incubated overnight at 37°C with vigorous shaking. Next day, plasmid DNA was prepared using either the Maxiprep Kit or the Endofree Maxiprep Kit (for plasmids required for transfection of eukaryotic cells) according to the manufacturer´s instructions.

To verify whether the obtained plasmids contained the expected fragments, the constructs were digested with corresponding restriction enzymes and checked on agarose gels. Clones carrying inserts of the right size were sequenced.

2.2.7.2 Chemical transformation

Plasmid DNA (10 ng of supercoiled or 100 ng of ligation product) was incubated with 100 µl of chemically competent cells (DH 5^{α}, XL-1 blue) for 30 min on ice, heat shocked at 42°C for 1 min 30 sec and chilled on ice for 2 min. Cells were incubated for 1 h in 1 ml LB with vigorous shaking at 37°C. Afterwards, cells were plated onto selective agar plates containing the appropriate antibiotic and incubated overnight at 37°C.

2.2.7.3 Electroporation

40 µl of competent cells (BL21, JM109) were added to plasmid DNA that had been previously dialysed, and the mixture was placed in a 1 mm electroporation cuvette. Electroporation took place at 25 µF capacity, 1,7 V voltage and 200 Ω resistance with a time constant of about 4,5 msec. Immediately, 1 ml LB was added to the transformed cells. The solution was incubated at 37°C with vigorous shaking for 1 h and afterwards, cells were plated onto selective agar plates containing the appropriate antibiotic and incubated overnight at 37°C.

2.2.8 Transfection

2.2.8.1 siRNA transfection

$1x10^6$ cells in 75 cm^2 culture flasks were seeded the previous day to transfection. A solution "A" containing 40 µl of siRNA oligo and 1,3 ml of OptiMEM, and a solution "B" containing 40 µl of Oligofectamine and 375 µl of OptiMEM were prepared. After 8 min incubation at RT, both solutions were mixed and incubated for 20 min at RT. In the meanwhile, cells were washed with PBS, and 2 ml of fresh medium without antibiotics were added to the cells. The transfection mix was slowly added onto the cells, and the cells were incubated at 37°C in a humidified incubator with 5% CO_2 v/v for 48h.

2.2.8.2 Polyfect transfection of plasmid DNA

$1x10^5$ COS-7 cells in a 6 well plate with cover slips for immunofluorescence experiments ($1,5x10^6$ in a 75 cm^2 flask or $3x10^6$ in a 150 cm^2 flask for immunoprecipitation experiments) were seeded the day previous to transfection. Transfection was performed according to manufacturer´s instructions. Briefly, a transfection mix containing 1,5 µg of plasmid DNA (20 µg, 30µg), 100 µl OptiMEM, (750 µl, 1,2 ml) and 10 µl polyfect (75 µl, 100 µl) was prepared and incubated for 10 min at RT. In the meanwhile, cells were washed with PBS and 1,5 ml of fresh medium without antibiotics (12 ml, 16 ml) were added to the cells. After 10 min, 0,6 ml of fresh medium (4,8 ml, 6 ml) were added to the transfection mixture and after mixing, the

solution was slowly placed onto the cells. Cells were incubated at 37°C in a humidified incubator with 5% CO_2 v/v for 24h.

Blue indicates the amounts used for 75 cm^2 cell culture flasks and orange for 150 cm^2 cell culture flasks.

2.2.8.3 Lipofectamine transfection of plasmid DNA

$3x10^6$ in a 150 cm^2 flask were seeded the day previous to transfection. A solution "A" containing 15 μg of plasmid DNA and 2,5 ml OptiMEM, and a solution "B" with 50 μl lipofectamine and 2,5 ml OptiMEM were prepared and kept for 5 min at RT. Subsequently, solutions were mixed and incubated at RT for 20 min. In the meanwhile, cells were washed with PBS and 18 ml of fresh medium without antibiotics were added. Transfection mixture was slowly added to the cells, which then were incubated at 37°C in a humidified incubator with 5% CO_2 v/v for 24h.

2.2.9 Sequencing

2 ng DNA per 100 bp DNA length for PCR products, or 100 ng of plasmid DNA, were mixed with 6 μl H_2O, 1 μl sequencing primer (10 pmol/μl) and 3 μl "Terminator ready reaction mix".

Thermal profile used for amplification and labelling of probesin indicated in Table 2.17:

Step	Temperature	Time	Cycles
Pre-denaturation	96°C	1 min	1x
Denaturation	96°C	10 sec	25x
Annealing	Primer specific (table)	5 sec	
Elongation	60°C	4 min	
Storage	4°C	Infinite	

Table 2.17. Thermal cycle for sequencing reactions

Sequencing reactions were purified with EtOH. To a 10 μl reaction, 25 μl of absolute EtOH were added and thoroughly mixed by inversion of the tube. After 10 min incubation, samples were centrifuged for 45 min at 4000 rpm at RT. Supernatants were discarded and 100 μl of 70% EtOH were added to the samples. After inverting the tubes a few times, samples were centrifuged for 15 min at 4000 rpm at RT. The ethanol was removed and the samples were air-dried. The samples were analysed in an ABI377 DNA sequencer.

Primers used for sequencing are indicated in Table 2.18

Primer	Sequence (5´→ 3´)	T[a]	Vector
Sp6	atttaggtgacactatag	47	Pinpoint
Pinpoint	cgtgacgcggtgcagggcg	67	Pinpoint
T7	gtaatacgactcactatagggc	58°C	pCMV
T3	aattaaccctcactaaaggg	56°C	pCMV
S*Tag	cggttctggttctggccata	58°C	PET32a
T7terminator	gctagttattgctcagcggt	58°C	PET32a
GFPfor	gacaaccactacctgagcac	58°C	pEGFP

GFPrev	tgtttcaggttcaggggggag	58°C	pEGFP
BMT116r	gttggggttattcgcaac	52°C	PBMT116
BMT116r	cataagaaattcgcccg	52°C	PBMT116

Table 2.18. Sequencing primers

The following primers were used for the precise sequencing of MID1 mutations : Ex2r-1r , 2RT-8, 2RT-9, 2RT-10A, 2RT-4, Ex2f1, 3´-RACE1, Ex3-r1, 2RT-19, 2RT-11, 2RT-13, 2RT-17, 2RT-6, 2RT-18, 2RT-12. They were already available in the laboratory and have been described previously (So et al., 2005; Winter, 2003; Winter et al., 2004).

Sequences were analysed by using the databases indicated in section 2.1.9

2.3 Protein methods

2.3.1 Bradford assay

A starting 10 μg/ml BSA solution was prepared by diluting a 5 mg/ml BSA stock solution 1:500 in the working buffer (specific for each experiment), which had been previously diluted 1:1000 in bidest water (2 μl 5 mg/ml BSA + 998 μl 1:1000 buffer).

BSA standard solutions were prepared by diluting the 10 μg/ml BSA solution in 1:1000 working buffer as indicated in Table 2.19:

BSA standard curve (Name-μg/ml)	**BSA 10 μg/ml** (μl)	**1:1000 buffer** (μl)
S6-10	----	----
S5-7.5	150	50
S4-5	100	100
S3-4	80	120
S2-2	40	160
S1-1	20	180

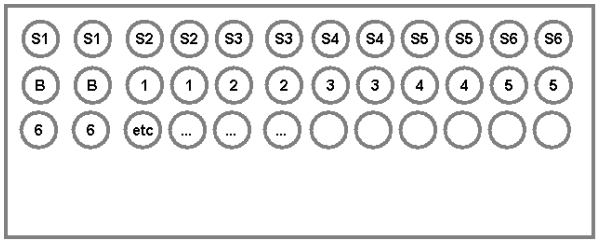

Table 2.19. Standard BSA curve preparation **Figure 2.1. Disposition of samples in Falcon plate**

2 x 80 μl of each sample, a blank (only 1:1000 buffer) and the standards (S) were placed into a Falcon microtitre plate as shown in Figure 2.1, 20 μl of Bradford reagent were added with a multichannel pipette and the samples were extensively mixed. The reaction proceeded for 1-5 min. Bubbles were carefully removed and the absorbance at 595 nm of each probe was measured in an anthos 2020 spectrophotometer (anthos), which also calculated the protein concentration by correlation with the BSA standard curve.

2.3.2 SDS-PAGE Gel

For the separation of proteins according to their molecular weights, samples were run in SDS-PAGE gels. 5 ml of separating gel and 1 ml of stacking gel were prepared per SDS-PAGE gel as showed in Table 2.20:

Component volumes in ml per 5ml of separating gel/1ml stacking gel				
Separating gel / Components	10%	12%	15%	stacking gel
Bidest H_2O	1,9	1,6	1,2	0,68
Rothiphorese gel 30	1,7	2	2,5	0,17
Separating gel buffer	1,3	1,3	1,3	0,13 stacking buffer
Ammonium persulfate	0,05	0,05	0,05	0,01
TEMED	0,002	0,002	0,002	0,001

Table 2.20. SDS gels composition

Separating gels were poured in between a short plate and a spacer plate using the Protean III system, covered with isopropanol and allowed to polymerise for 45 min at RT. Afterwards, the isopropanol was removed and the gels were washed with bidest H_2O. The stacking gel was then added on top of the separating gel, and a comb was incorporated in between the 2 glass plates to form the wells. They polymerised for 30 min.

2.3.3 Western blot

Protein samples were mixed with either 5x SDS-PAGE buffer or 2x magic mix and denatured for 5 min at 95°C before loading. The separation of the samples was performed in 1x Laemmli buffer at 200 V for 50-70 min using a Mini-PROTEAN 3 electrophoresis system (Bio-Rad). After running, gels were equilibrated for 15 min in 1x blotting buffer.

Different protein markers were run with the samples, according to the size of the proteins in study. Table 2.21 indicates the sizes covered by each marker and the study in which they were used:

Protein ladders	Size	Study	Manufacturer
Kaleidoscope	10-250 KDa	MID1 complex	BioRad
Rainbow	14,3-220 KDa	MID1 complex	Amershan Biosciences
See Blue Plus2	4-250 KDa	Peptide expression	Invitrogen

Table 2.21. Protein markers

The blotting PVDF membrane was equilibrated for 3 sec in MeOH, 2 min in bidest H_2O and 15 min in 1x blotting buffer. Proteins were then blotted onto the membrane in a Trans-Blot SD Semi-dry Transfer Cell (Bio-Rad) at 15 V for 30 min. To saturate the unbound regions on the blot, it was incubated in PBST/5% milk powder for 30 min at RT or overnight at 4°C. Then, it was incubated with the primary antibody diluted in PBST/1%BSA for 1 hour at RT or overnight at 4°C followed by three wash steps of 5 min each in PBST. The corresponding HRP conjugated secondary antibody was diluted in PBST and, subsequently, added to the blot for 30 min, followed by three washing steps as before. Protein signals were detected by incubating the blots with the Western Lightning Chemiluminescence Reagent Plus

(PerkinElmer) for 1 min and subsequent exposure to Fuji Medical X-Ray films in a hypercassette. Blots were developed in a Curix 60 automatic film processor (Agfa).

2.3.4 Silver staining

Silver staining was performed using the SilverQuest Silver Staining Kit, following manufacturer´s instructions. Shortly, gels were fixed for 20 min to overnight in 40 ml EtOH/10 ml HAc/50 ml H_2O, washed in 30 ml EtOH/70 ml H_2O, sensitised in 30 ml EtOH/10 ml sensitizer, 60ml H_2O again washed in 30 ml EtOH/70ml H_2O and for a second time in water, stained with 1 ml stainer solution, 100 ml H_2O, washed in water, developed in 10 ml developer solution/1 drop developer enhancer solution/90 ml H_2O and stopped with 10 ml stopper solution directly added to the developing solution when the expected signals were detected. Finally, gels were washed with H_2O. The whole procedure was performed at RT.

2.3.5 Coomassie/Colloidal coomassie staining

For colloidal Coomassie staining (CBB), gels were fixed overnight in 2% H_3PO_4/50% MeOH on a shaking platform.

Next day, the CBB stock solution was throughfully mixed and diluted with methanol 4:1 (CBB:MeOH) while stirring. Gels were allowed stain until bands were observed (12 hours to 4-5 days). Finally, gels were washed with bidest H_2O. The whole procedure was performed at RT.

For standard Coomassie staining, gels were incubated for >1 h in Coomassie staining solution and subsequently destained for 1 h in destaining solution.

2.3.6 Immunofluorescence

10^5 cells COS-7 per well were seeded onto cover slips in six well plates. Next day, they were transfected with the corresponding endofree prepared plasmid DNA using polyfect (section 2.2.8.2) and allowed to grow for 24 or 48 hours. Afterwards, the medium was removed and cells were washed in 1.2 x PEM, fixed in 3,7% paraformaldehyde for 10 min at RT and shortly washed in 1x PBS. Next, they were permeabilized with 0,2 % Triton-100 in 1x PBS for 10 min at RT and washed 3 times in 1x PBS. To block unspecific interactions, cover slips were incubated for 20 min at RT in 1x PBS-0,5% BSA. Primary antibodies were diluted in PBS-0,5% BSA, added onto the covers slips and incubated for 1 h in a humid chamber, followed by three washings in 1x PBS for 5 min. The corresponding Cy3-labelled secondary antibodies were diluted in 1x PBS and added to the cover slips for 30 min, followed by 3 washes as before. Finally, cover slips were placed onto a 12 µl drop of 0,5 µg/ml DAPI in mounting medium previously added onto a slide, dried by pressing the slide between two pieces of Whattman paper and fixed to the slide with Fixogum.

Cells were visualised by fluorescence microscopy with a Zeiss Axioskop epifluorescence microscope equipped with single band pass filters for excitation of green, red and blue fluorescence (Chroma Technologies), and 63x or 100x plan-neofluolar lenses. Digital black and white images were recorded with a cooled CCD camera (Hamamatsu Photonics) and merged into RGB-images by the ISIS immunofluorescence image analysis system (MetaSystems).

2.3.7 Microtubule assembly experiment

Microtubules were polymerised *in vitro* from HeLa cell extracts according to previously reported protocols (Kimble et al., 1992; Vallee, 1982). $3x10^7$ HeLa cells were incubated in ice cold PBS for 10 min at –20°C and for 30 min at 4°C to depolymerise the microtubules. After washing twice with ice-cold PBS, cells were disrupted with 1,8 ml of ice-cold microtubule assembly buffer (plus a complete mini tablet each 10ml of buffer) and centrifuged at 55000 rpm for 1 h at 4°C in a TL100 centrifuge. The pellet was discarded, to the supernatant 18 μl of dGTP (100 mmol) and 7,2 μl taxol (5 mg/ml) were added, and the microtubules were allowed to polymerise for 30 min at 37°C. In the meanwhile, a 50% sucrose solution (0,2 g sucrose, 1 μl Taxol, 4 μl dGTP in 280 μl microtubule assembly buffer) was prepared. Polymerised microtubules were added on top of 180 μl of the sucrose solution, and centrifuged at 25000 rpm for 30 min at 37°C in a TL100 centrifuge. The supernatant was discarded, and polymerised microtubules were washed by resususpension in microtubule assembly buffer with taxol and pelleted again at 25000 rpm for 30 min at 37°C in a TL100 centrifuge. The pellet was finally resuspended in 50 μl microtubules assembly buffer, 0,5 μl dGTP and 0,25 μl Taxol and left for some minutes in the fridge. Polymerised microtubules were stored at –80°C.

2.3.8 Immunoprecipitation

During the study of the Bboxes, for the immunoprecipitation of MID1-FLAG or α4-myc polyclonal anti-FLAG or anti-myc antibodies were used as follows:

COS-7 cells overexpressing MID1-FLAG were homogenised by sonication in IP1 buffer, supplemented with proteinase inhibitors. Nuclei and cellular debris were pelleted for 15 min at 12000 *x g* at 4°C. 1 mg of cytosolic protein extract in 1 ml IP1 buffer was placed in a new eppendorf tube. A preclearing step to avoid unspecific absorption of the agarose beads was performed by incubation the solution with 50 μl of 50% protein A agarose suspension (previously washed for 3 times in IP1) for > 2 h at 4°C with rotation. Beads were pelleted by centrifugation for 1 min at 3000 rpm in a table centrifuge at 4°C and the supernatant was transferred to a new eppendorf tube. 1,5 μg of anti-myc or 1,6 μg of anti-FLAG (Table 2.5), were added to the sample which was incubated overnight at 4°C on a rocking platform. Next day, 75 μl of 50% protein A agarose suspension were added and the mixture was incubated

for > 2h at 4°C on a rocking platform. Agarose beads were pelleted by centrifugation for 1 min at 3000 rpm in a table centrifuge, followed by 3 washings with 500 µl of IP1 buffer. Beads were boiled for 5 min at 95°C in freshly prepared SDS buffer (20 µl IP1, 3,8 µl 1M DTT, 2 µl 10x Laemmli buffer and 5 µL 5x SDS-PAGE buffer per sample). Immunoprecipitated proteins were analysed on 10 % SDS gels and Western blots with anti-FLAG or anti-myc monoclonal antibodies.

For the immunoprecipitation of MID1-FLAG for the verification of the novel members of the MID1 complex, the following protocol was used:

HeLa cells overexpressing MID1-FLAG were lysed in TKM buffer, supplemented with proteinase inhibitors and 1% NP40, incubated for 15 min on ice and passed 5 times through a 27¾ Gauge needle. Nuclei were pelleted for 15 min at 12000 *x g* at 4°C. 4 mg of cytosolic extract were precleared with 25 µl of protein-A/G agarose and 10 µg of mouse IgG for 1,5 h at 4°C on a rocking platform. Beads were pelleted by centrifugation for 1 min at 3000 rpm in a table centrifuge at 4°C and the supernatant was transferred to a new eppendorf tube. MID1-FLAG immunoprecipitation took place overnight with 75 µl of anti-FLAG M2 coated beads in 1 ml TKM buffer. Anti-FLAG M2 agarose matrix had been previously equilibrated in TKM buffer, blocked with 1 mg/ml BSA for 30 min and washed again with TKM buffer. Next day, agarose beads were pelleted by centrifugation for 1 min at 3000 rpm in a table centrifuge and washed 3 times with 500 µl TKM buffer supplemented with 0,2 % NP40 for 10 min at 4°C rotating. Bound proteins were finally eluted for 45 min with 200 µl of 3 x FLAG (400 µg/ml). 40 µl were directly analysed by Western blotting, and the remaining 160 µl of the elution fraction were concentrated in vivaspin 500 disposable with a cut-off membrane of 5 kDa for 20 min at 15000 *x g* at 4°C. Concentrated fractions were analysed on 10% SDS page and Western blotting with the panel of antibodies for the respectivecomplex members. Note: Western blots shown in the result section correspond to the concentrated fractions.

2.3.9 Yeast two-hybrid experiments

2.3.9.1 Yeast transformation

Several colonies of the L40 yeast strain were inoculated in 500 µl of YPD medium, vigorously vortexed for 3 min, transferred to flasks containing 50 ml YPD medium and was incubated for 16 h at 30°C with shaking (225 rpm).

Next day, 19 ml of the overnight cultures were diluted in 300 ml of YPD medium in a 2 l flask to produce a OD_{600} of 0,2 to 0,3 and incubated until OD_{600} had reached 0,4-0,6 (approximately 3,5 h). Cells were then placed in 50 ml tubes and centrifuged for 5 min at 1000 x g. The supernatant was discarded and pellets were washed with 50 ml of TE buffer and centrifuged again. The supernatant was discarded and cells were resuspended in 1,5 ml of

freshly prepared 1x TE/1x LiAc. Then 0,1 μg of plasmid DNA and 0,1 mg of herring testes carrier DNA (previously denaturated for 20 min at 95°C) were mixed in an eppendorf tube and mixed by vortexing with 0,1 ml of the freshly prepared yeast competent cells. 600 μl of PEG/LiAc were added, and the solution was vortexed for 10 sec before being incubated for 30 min at 30°C. Afterwards, 70 μl of DMSO were added and the solution was mixed by inversion. Cells were heat shocked for 15 min at 42°C and consequently chilled on ice for 1-2 min. Cells were then centrifuged for 5 sec at 14000 rpm in a table centrifuge, the supernatant was discarded and the cells were resuspended in 500 μl of TE buffer. Finally, transformed yeast were placed on plates with -Leu/-Trp selections medium and incubated for 3-4 days at 30°C. For the selection of colonies presenting interaction of both plasmids, several colonies were placed on plates with -His/-Leu/-Trp selective medium and incubated again 3-4 days at 30°C.

For each construct, each experiment was repeated at least twice. As controls, empty pGAD10 was cotransformed with pBMT116-MID1, empty pBMT116 with pGAD10-α4 and both empty vectors together.

2.3.9.2 β-Galactosidase activity

β-galactosidase activity was assayed by the *o*-nitrophenyl-β-D-galactopyranoside (ONPG) method (Fields and Sternglanz, 1994). A colony from the selective medium containing plates was inoculated into 5 ml cultures containing the corresponding selective medium (-His/-Leu/-Trp or -Leu/-Trp), well mixed by vortexing and incubated for 16-18 h at 30°C with shaking (225 rpm).

Next day, ONPG was diluted in Z buffer to a concentration of 4 mg/ml by stirrig for 1-2 hours. Cell clumps in the overnight culture were dissolved by vortexing and diluted in YPD-medium until the OD_{600} was 0,2-0,3 (approximately 10 ml). The culture was incubated for 3-5 h at 30°C with shaking (225 rpm). After 2 h, the OD_{600} was measured every 30 min until the cultures had reached the mid-log phase (OD_{600} 0,5-0,8).

1,5 ml of the cultures were split into three tubes and centrifuged at 10000 x *g*. Supernatants were discarded and cell pellets were resuspended in 300 μl of Z buffer. 0,1 ml of each probe was transferred to a new eppendorf tube, hold for 1 min in N_2 and then placed for 1 more minute in a water bath at 37°C. Freezing/thawing cycles were repeated twice.

To the probes 700 μl of Z buffer+ β-mercaptoethanol (0,27 ml β-mercaptoethanol in 100 ml Z buffer) were added and subsequently 160 μl of ONPG in Z buffer. A blank probe was performed in parallel with only 100 μl of Z buffer. When the probes turned yellow, Na_2CO_3 was added to stop the reaction. Time passed between ONPG addition and stop of the reaction was measured.

The Photometer was calibrated with the blank sample at a wavelength of 420 nm and subsequently the OD_{420} of the samples were measured.

For calculating the β-galactosidase units the following equation was used:

$$\beta\text{-galactosidase units} = \frac{1000 \times OD_{420}}{t \times V \times OD_{600}}$$

t = incubation time (ONPG-addition-stop)
V = o,1 ml x concentration factor (5 in this case)

2.3.10 Cell culturing and trypisnation

HeLa or COS-7 cells were grown in the previously described media. For seeding cells, they were trypsinised and counted with the cell counter and analysis system CASY1 (Schärffe system).

Trypsination

1 ml per each well in a six well plate (4 ml for a 75cm^2 flask and 8 ml for a 150cm^2 flask) of medium from the cells was kept. After removing the remaining medium, cells were washed with sterile PBS. Next 0,5 ml (2 ml, 4 ml) of trypsin (prewarmed at 37°C) were added to the cells and incubated for 3 min at 37°C. When cells were loosened, the trypsine was inactivated by adding medium. Cells were then centrifuged at 1200 *x g* for 10 min at 4°C, the supernatant was discarded, and the pellet was washed with PBS and pelleted again. When not used immediately, cell pellets were frozen with liquid N_2 and kept at –80°C.

For cell seeding, cells were counted after trypsination and the necessary amount indicated for each experiment was mixed with fresh medium and place in the cell culture flasks or plates.

2.4 Preparation of the 44aa peptides

Two different 44aa peptides of α4 were prepared:

Biotinylated 44aa peptide: created in the PinPoint vector system. Used for the production of the column.

44aa peptide: created in the PET32 vector system, containing a Trx, His and S-Tag. After tags removal peptide was called ***free 44aa peptide***. The last was used for elution of the column.

2.4.1 Growth of *E.Coli* cultures

Pinpoint vector constructs were expressed in JM109 *E.coli* cells (Promega) and PET32a constructs in BL-21Codon-Plus (DE3)-RIL (Novagen) *E.coli* cells.

20 ml of LB medium with antibiotic (PET32 vector system: 50 μg/ml ampicillin and 34 μg/ml chloramphenicol; PinPoint vector system: 50 μg/ml ampicillin, 2 μM biotin) were inoculated with a colony and grown overnight with vigorous shaking. Next day, 1 l culture with antibiotic was inoculated 1:50 with the overnight culture. The culture was grown until OD_{600} was 0,5-0,7 and then induced with 0,1 mM IPTG during 2 h for the PET32 vector system and 4 h for the PinPoint vector system. An aliquot of each culture was resuspended in magic mix and directly analysed on a Coomassie stained 15 % SDS gel. Cells were harvested by centrifugation for 20 min at 4000 x *g*. When not directly used, cell pellets were frozen in liquid N2 and kept at –80°C.

For the establishment of optimal conditions for protein expression 5 ml cultures were performed as described above. Different conditions tested are indicated in the result section.

2.4.2 Preparation of cleared *E.Coli* lysates

Cell pellets were resuspended in native condition lysis buffer (QiaExpressionist) at 3 ml/gram wet weight. Lysates were 2x frozen in liquid N_2/thawed on ice and sonicated 5x at 50% power for 10 sec with 10 sec cooling period between each burst. Lysates were centrifuged at 10000 *x g* for 20 min at 4°C. An aliquot of the pellet was kept for analysis and the rest was discarded. The supernatant was kept at –80°C.

2.4.3 Purification of 6xHis tagged proteins from *E.Coli*

1 ml of 50% Ni-NTA slurry was added to each 4 ml of cleared lysate, and it was gently mixed for 1h on a rotary platform at 4°C. The lysate Ni-NTA mixture was loaded into a propylene column with the bottom outlet capped. The bottom cap was removed and the flow through collected; afterwards, the column was washed twice with 8 ml of washing buffer (QiaExpressionist) per each ml of packed column volume. Finally, peptides were eluted with 1 ml elution buffer (QiaExpressionist) per each ml of packed column volume. For future use, protein samples were dialysed (or concentrated with a centricon) into the required buffer.

2.4.4 Thrombin digestion

For thrombin digestion the peptide was previously dialysed in HS buffer.

Optimal conditions were established after testing different enzyme concentrations and reaction times (see result section) following manufacture's instructions (Novagen). Final conditions were used as follows: 10 μg of protein were digested with 1 μl of Thrombin (0,02 U/μl) in a 50 μl reaction in bidest water from 4 h to overnight at 4°C. For bigger amounts of proteins, the reactions were scaled up with the same ratio of components. When necessary, thrombin was removed by dialysis or centricon (Millipore).

To cleave the free 44aa peptide from the tags, Ni-NTA agarose was used. 1 mg of digested peptide was incubated with 300 μl of Ni-NTA slurry and equilibrated in HS buffer for 1 h at 4°C in a rocking platform. The flow-through was collected and free peptide was concentrated in a centricon device with a 3 kDa cut-off membrane.

2.5 Chromatography column and Mass spectrometry

For generating the column, 50 μg of biotinylated 44aa peptide in TBS were incubated with 250 μl of washed streptavidin coated agarose slurry (ProFound pull down kit) for 1 h at 4°C rotating in a 500 μl final volume reaction. A control column without peptide was performed in the same way. Beads were blocked with free biotin for 5 min, washed with HS buffer and again blocked with 1 mg/ml BSA overnight at 4°C. Beads were place in a Handee-mini spin tube to create the columns, followed by washing with 100 volumes of HS and 2 volumes of washing buffer (250 mM NaCl/0,05% Tween), and eluted with an excess of free peptide after thrombin digestion (section 2.4.4). Columns were again washed as before and equilibrated in HS buffer.

Cytoplasmic HeLa extracts from $1x10^7$ cells, previously subjected to α4 knock down (section 2.2.8.1), were obtained by lysis in HS buffer for 15 min on ice, potter disruption for 10 times and centrifugation for 10 min at 3000 rpm at 4°C in a table centrifuge. Approximately 4 mg of cytoplasmic extracts were incubated with the columns overnight at 4°C on a rocking platform. Next day, columns were washed as before and eluted 3x 15 min with excess of free peptide. The eluted fractions were dialysed against 20 mM TRIS pH 8, run in a 10% SDS gel and stained with colloidal Coomassie. Differential bands (1-12, indicated in the results section 3.2.3) were excised, trypsinised and analysed by mass spectrometry.

Mass spectrometry was performed by the service group in the SFB 577. Briefly, the peptide mixture in the cut bands was identified by chromatographic separation on a LC Packings 75 μm PepMap C18 column (Dionex) using a capillary liquid chromatography (CapLC) system delivering a gradient to formic acid (0.1%) and acetonitrile (80 %). The eluting peptides were ionised by electrospray ionisation on a Q-TOF hybrid mass spectrometer (Micromass). The instrument, in automated switching mode, selects precursor ions based on intensity for peptide sequencing by collision induced fragmentation tandem MS. The MS/MS analyses were conducted using collision energy profiles that were chosen based on the m/z value of the precursor. The mass spectral data were processed into peak lists containing m/z value, charge state of the parent ion, fragment ion masses and intensities, and correlated with the SwissProt database using Mascot software (Perkins et al., 1999).

2.6 Sucrose gradients

2.6.1 Discontinuous gradients

HeLa cells, with and without overexpressed MID1-FLAG, were lysed for 15 min on ice in TKM buffer supplemented with 1 mM DTT, 0.5 % NP40 and 1 U/μl sample of Prime RNase inhibitor and passed 10 times through a 27¾ Gauge needle. Cell debris and nuclei were discarded by centrifugation 10 min at 12000 *x g* at 4°C. Approximately 2 mg of protein dissolved in 900 μl of buffer were added on a sucrose discontinuous gradient, formed by 500 μl of a 50% sucrose solution in TKM and 600 μl of a 20% sucrose solution of TKM. Centrifugation was performed in a TLA 100.2 rotor at 55000 rpm for 270 min at 4°C (for overexpressing cells) or 15 hours at 30000 at 4°C in a TLS55 rotor (for non-overexpressing cells) in a TL100 ultracentrifuge.

Ribosome studies:

0.5% DOC, 0.5 M KCl, 30mM EDTA treatment: to 2 mg of concentrated cytoplasmic extracts, the necessary amounts of 10% DOC, 1 M KCl, or 0,5 M Na_2EDTA were added to have a final concentration of protein of 2 mg/ml in TKM buffer (supplemented with 1 mM DTT, 0.5 NP40 % and Prime RNase inhibitor) with 0,5% DOC, 0,5 M KCl or 30 mM EDTA. Ribosomes were run through a TKM sucrose gradient, as previously described, for 15 hours at 30000 at 4°C in a TLS55 rotor.

RNase A/DNAse I treatments: cytoplasmic extracts were obtained as previously described, excpt that Prime RNase inhibitor was not included. To 2 mg of cytoplasmic extracts either 500 μg/ml of RNAseA, 50 units of DNAse I or 12 μl of RNA guard were added and the volumes of the reactions were brought up to 1 ml in TKM buffer (supplemented with 1 mM DTT, 0.5 NP40 %). Ribosomes were run through a TKM sucrose gradient, as previously described, for 15 hours at 30000 rpm at 4°C in a TLS55 rotor.

Puromycin/cycloheximide: HeLa cells in culture were treated either 0,1 mg/ml puromycin for 6 hours or with 0,35 mM cycloheximide for 20 minutes at 37°C in a humidified incubator with 5% CO_2 v/v for 48h. Cell extracts were prepared, and sucrose gradient run, as previously described.

2.6.2 Linear sucrose gradient

$3x10^7$ HeLa cells were lysed for 15 min on ice in TKM buffer supplemented with 1 mM DTT, 0.5 % NP40 and 1 U/μl sample of Prime RNase inhibitor, passed 10 times through a 27¾ Gauge needle and centrifuged for 15 min at 12000 *x g* to eliminate the nuclei and cellular debris. The cytoplasmic homogenate was sedimented in a 15%-45% (w/v) sucrose gradient by centrifugation for 120 min at 38000 rpm at 4°C in a Beckmann SW40 rotor. 1 ml fractions were collected starting from the bottom of the centrifuge tube with a 2132 Microperpex peristaltic

pump a 2111 multirac system (LKB Bromma). The absorbance of the fractions was measured with a 2138 UVI-CORD (LKB Bromma). Fractions were analysed by Western blotting either directly, or after being precipitated overnight with trichloroacetic acid and 5 μg BSA as carrier.

15%-45% linear sucrose gradients were prepared in TKM buffer in a 14 x 95 mm Ultra-Clear centrifuge tube (Beckmann) with a gradient master (Biocom).

2.7 RNA-Protein binding experiments

2.7.1 Homopolymer binding assay

MID1-FLAG overexpressing HeLa cells were homogenised in HSMN buffer, supplemented with proteinase inhibitors and 1 U/μl Prime RNase inhibitor, in a potter-elvehjem. Binding of overexpressed MID1 to homoribopolymers was performed as previously described (Brown, 1998; Siomi et al., 1993b; kiledjian and dreyfuss, 1992; Swanson and Dreyfuss, 1988). Briefly, 100 μg of cytosolic extract were incubated with 5 μg of homoribopolymer coated beads in 500 μl of HSMN buffer during 30 min at 4°C. Beads were pelleted and washed 3 times for 5 min at 4°C with 500 μl HSMN buffer before being resuspended in magic mix. Bound proteins were eluted by boiling the sample at 95°C for 5 min and analysed by Western blotting with anti-FLAG antibody.

For the different treatments performed the standard procedure was the same, except that this time 2 μl of poly-rG agarose coated beads were used. After lysis, cytosolic extracts were modified according to each experimental condition as follows:

For the different salt treatments the concentration of NaCl in the cytosolic extracts was adjusted to 250 mM and 500 mM with 1 M NaCl. For heparin treatments, heparin from a 100 mg/ml stock solution in HSMN buffer was added to the samples in order to have a final concentration of 1 or 2 mg/ml. For the competition experiments with free homopolymer, 5 or 50 μl of a 10 mg/ml solution of the corresponding free homopolymer (poly-rU or poly-rG) was added to create a 1:1 or 1:10 bound:free homopolymer solution.

2.7.2 Ephrin mRNAs-MID1 binding experiments

2.7.2.1 Amplification of G-quartets and EFNB1 mRNA

All forward primers contained the T7 promotor sequence (5´-ccaagcttctaatacgac-tcactatagggaga-3´) to allow subsequent *in vitro* transcription of the PCR product. PCRs were performed with advantage polymerase. PCR conditions and primers for the different amplification reactions are given in Table 2.22. TD indicates PCRs that were performed with a touchdown thermal programm.

Primer	Sequence (5´→3´)	Annealing T[a] (°C)
EFNB1a	T7cccatgctcttgtgccttcc	65

	gtcctgggaaggtggcaag3	
EFNB1b	T7ggtcagccaggaagcatagg tgggacaccttgcccagtg	64
EFNB1c	T7cttaattggctgttgcctgg gtaaggagagaacagggtgg	64
EFNB1d	T7cctagcacaggtgggtaac agccgtgctggcaaggaactg	64
EFNB1bAS	T7tgggacaccttgcccagtg ggtcagccaggaagcatagg	64
EFNB2a	T7ggtgccctttagccagatg cctttccaactttctgtttcag	66-55 TD
EFNB2b	T7cgatgtgcaggaagaaaagcc gtaccacaacagtcctgcc	64
EPBH1	T7gtcagtcaccaacggcaatgg tcagctgagaagccagtctct	66-55 TD
EPBH2a	T7ctaatggactccactacagc caaacacgttgcacacctgg	66-55 TD
EPBH2b	T7gttgatcctgcatctgggtttg gtcaaagtgtcacttcattgtc	55
EPBH3a	T7ctcatggacacaaaatgggtaac gcacattacacacctggtatg	66-55 TD
EPBH3b	T7cttcctgctctccagcag ctgctctccgcctacctg	66-55 TD
EPBH3c	T7gatttgttctgggggctgag cacatcccatctctggctctg	65
EPBH4a	T7gatgagagcgagggctgg ctgcttctctcccattgctc	66-55 TD
EPBH6a	T7gcagcagcttcttaaccagc gtttctgggtcaatcttaccct	66-55 TD
EPBH6b	T7ggaagcaagcttagctgtacac tgggcagcccttcagtag	64
EFNB1-3´UTR	T7gaagatggctcggcctggg gtgccgggcactcagaccttg	74-64 TD
EFNB1+1Gq	T7gaagatggctcggcctggg gtcctgggaaggtggcaag	74-64 TD
EFNB1+2Gq	T7gaagatggctcggcctggg tgggacaccttgcccagtg	74-64 TD
EFNB1+3Gq	T7gaagatggctcggcctggg gtaaggagagaacagggtgg	74-64 TD
EFNB1+4Gq	T7gaagatggctcggcctggg agccgtgctggcaaggaactg	74-64 TD
EFNB1+3Gq rev	T7agccgtgctggcaaggaactg gaagatggctcggcctggg	74-64 TD
EFNB1+4Gq rev	T7gtaaggagagaactgggtgg gaagatggctcggcctggg	74-64 TD

Table 2.22. Primers for amplification of G-quartets and ephrin mRNAs

As template for the PCR reactions, the IRATp970C0237D6 clone containing EFNB1 coding sequence or cDNA from embryonic human fibroblasts were used.

PCRs were checked on agarose gels and purified by gel extraction before RNA transcription was performed. Longer PCRs fragments were additionally digested with Proteinase K by incubation with 100 μg/ml proteinase K, 0.5 % SDS in 50 mM TRIS-HCl (pH 7,5) and 5 mM $CaCl_2$ for 30 min at 37°C. Subsequently, they were purified by phenol:chloroform extraction.

2.7.2.2 *In vitro* transcription of biotinylated RNA

Amplified transcripts were in vitro transcribed with the RiboMAX™ Large scale RNA production system-T7 from Promega, following the manufacture´s instructions with some modifications. Briefly, 2μg of purified PCR product were transcribed for 4 h at 37°C in the following reaction mixture:

Reaction components	Sample reaction
T7 transcription buffer	4 μl
rNTPs (25 mM rATP, rGTP, rCTP, 1,6 mM biotin-rUTP, 2,5 mM UTP)	6 μl
PCR template	2 μg
RNA polymerase enzyme mix	2 μl
DEPC-H_2O	Up to 20 μl

Table 2.23. *In vitro* transcription

Transcribed RNA was purified by phenol:chloroform extraction and precipitation with ethanol. Products were kept in nuclease free TE.

RNA concentration was measured with a NanoDrop ND-1000 Spectrophotometer (PEQLAB) or with a Gene Quant RNA/DNA calculator (Pharmacia). The efficiency of the biotinylation was tested by Dot Blotting.

2.7.2.3 Biotinylation efficiency dot blot

6 x 10 μl of 6x SSC buffer were pipetted onto a piece of parafilm. To the first dot, 1 μl RNA was added, then, 1 μl of this first dot was taken and applied to the next dot, and successively like that until the sixth dot, which had a final dilution of RNA of $1:10^6$.

1 μl of each dot was transferred to a Roty-Nylon Plus membrane. The RNA was cross-linked to the membrane with a UV linker. The membrane was blocked in PBS-5% BSA for 15 min, followed by incubation with 1:1000 streptavidin-AP in PBS and 5% BSA for another 15 min. The blot was then washed and the AP activity was developed by incubation with freshly prepared NBT-BCIP in the dark until signals appeared. Membranes were finally washed with H_2O and kept in a plastic bag.

Note: NBT-BCIP was prepared by dissolving a tablet in 10 ml DEPC water.

2.7.2.4 RNA-PROTEIN binding assay

1 or 2 μg of RNA (G-quartets or longer EFNB1 fragments) were incubated with 150 μg of cytosolic protein extract from HeLa cells (cells were lysed as described in section 2.6.1) containing overexpressed MID1-FLAG in 450 μl of TKM buffer with proteinase inhibitors and 30 μl of Prime RNase Inhibitor for 1 hour at 4°C. Subsequently, the mixture was incubated for 2 h at 4°C with 40 μl of 50% slurry of M280 streptavidin coated magnetic beads. Beads were washed 3 times with TKM buffer for 10 min at 4°C and bound proteins were eluted by boiling

the beads in magic mix for 10 min at 95°C. Bound proteins were tested by Western blotting with the respective antibodies.

Note: for the experiment with the cDNA containing varying numbers of G-quartets, the concentration of KCl in TKM buffer had to be adjusted to 50 mM to observe specific binding.

2.7.3 Biofinformatics

Bioinformatic approaches were performed by the bioinformatics department at the Max Planck Institute for Molecular Genetics (Berlin). Briefly, Bioperl and Ensembl-API (both available from CVS-concurrent version systems) were used to create a programm to screen all the gene entries of the ENSEMBL database (Version v37. human) for the presence and number of G-quartets.