

5 METHODS

5.1 MOLECULAR BIOLOGY

5.1.1 RNA isolation

All the reagents and all glass- and plasticware used for RNA experiments, were either purchased RNase-free, or made RNase-free.

Avoiding RNase contamination

Glassware: bake over night 200 °C.

Plasticware: rinse with 0.1N NaOH/1 mM EDTA and diethyl pyrocarbonate (DEPC) treated water.

Solutions: add 0.05 % DEPC and stir overnight at room temperature; autoclave. This does not apply to Tris buffers.

Total RNA from 5×10^6 N2a cells was isolated using the SV Total RNA Isolation System (Promega) according to the manufacturer`s manual. Briefly: the cells were lysed in a high – salt RNA lysis buffer containing guanidine isothiocyanate in order to deactivate RNases, followed by centrifugation to remove the cell debris. After addition of ethanol to adjust the binding conditions, the cleared lysate was transferred to a spin column, where another centrifugation causes the DNA and RNA to bind to the silica matrix. DNase was applied on the column to degrade the DNA. The RNA bound to the silica matrix was eluted with water. The average yield was 10 µg RNA/ 1×10^6 N2a cells. The obtained RNA was stored in aliquots at –80 °C.

Yield determination

Concentration: 1 Absorbance unit measured at 260 nm corresponds to 40 µg RNA/ml H₂O

Purity: Ratio of absorbances measured at 260 and 280 nm (A_{260}/A_{280}) should be 1.9–2.1 in 10 mM Tris·Cl

Integrity of the sample: agarose gel electrophoresis (see 5.1.4.3) with denatured sample; the ribosomal bands (18S ⇔ 1.9kb and 28S ⇔ 4.7kb) should appear as sharp bands on the stained gel and the 28S band should have twice the intensity of the 18S band.

Sample denaturation: mix 1-2 μ l of RNA with 18-20 μ l of RNA sample buffer and 2-5 μ l RNA loading buffer. Heat for 5-10 min at 65-70 °C prior to loading.

RNA sample buffer:

10 ml deionized formamide
3.5 ml 37 % formaldehyde
2.0 ml MOPS buffer
store at -20 °C up to 6 months
do not freeze-thaw

RNA loading buffer:

50 % glycerol
1 mM EDTA
0.4 % bromphenol blue
1 mg/ml ethidium bromide
store at -20 °C

MOPS buffer:

0.2 M MOPS (pH 7.0)
50 mM sodium acetate
5 mM EDTA (pH 8.0)

5.1.2 cDNA synthesis

Total RNA (5 μ g) was incubated with 200ng oligo(dT)₁₇ primer for 10 min at 65 °C. The Reverse Transcriptase Buffer, 15nmol of each dNTP, 0.3 μ mol DTT, 1 μ l RNaseOut (RNase inhibitor) and 300U of Reverse Transcriptase (Gibco) were added to a total volume of 30 μ l. After 1 h incubation at 37 °C, the reaction was supplemented with 30 μ l H₂O, 6 μ l of Second Strand Buffer and 5U of RNaseH (Gibco) and incubated for another 30 min at 37 °C. The resulting cDNA was stored in aliquots at -80 °C. Reverse Transcriptase Buffer and Second Strand Buffer were purchased from Gibco.

5.1.3 PCR

The needed DNA fragments were amplified *in vitro* by the method of polymerase chain reaction (PCR). For a typical amplification, following ingredients were mixed: 10ng template, Pfu polymerase buffer, 10nmol of each dNTP, 50pmol of each primer, 1U Taq polymerase, 0.03U Pfu polymerase and H₂O to a total volume of 50 µl. The reaction mixture was kept on ice until placed in a preheated (94 °C) cycler.

Typical program for amplification: see Tab. 5-1.

Denaturation	96 °C 60 s	
Denaturation	94 °C 15 s	← 25x
Annealing	70 °C* 60 s	
Extension	70 °C 60 s [#]	
Final extension	72 °C 10 min.	* 5 °C below the melting temperature of the primers # 60 s/1kb target sequence

Tab. 5-1 Typical program for DNA amplification by PCR.

For primer and template combinations used to amplify target DNA sequences see Tab. 5-2. The size and integrity of the amplified DNA was checked on an agarose gel (see 5.1.4.3).

TARGET DNA	TEMPLATE	FORWARD PRIMER	REVERSE PRIMER
KIAA0810	HK05647 ¹ (human)	5'GCCACAATGCGGCGG CGCGAGGCAGTATGG	5'CTTGACAGGTTTCGCC ATGAACTCTGAACCG
KIAA0668	HK02329 ¹ (human)	5'GCCACAATGCTAAGAG GAGTCCCTGTG	5'GTGGGCGGGCTCCCCA TGCAC
Lamin B receptor	LBR cDNA ² (human)	5'ATGCCAAGTAGGAAAT TTGCCG	5'GTAGATGTATGGAAA TATACGGTAG
LAP 2 β	LAP 2 β cDNA ³ (human)	5'GTCTCAGCATATGCCG GAGTTCCTAGAGG	5'CTGCAATTGGCGTTGGA TATTTTAGTATCTTGAAG
LAP 2 β short bact. expr.	LAP 2 β cDNA ³ (human)	5'GTCTCAGCATATGCCG GAGTTCCTAGAGG	5'CTGCAATTGGCAACGGA GCGTCTCTTCTTTG
Lamin B2	N2a cDNA	5'GATGGCGTCTCTGCCG	5'CATCAGTCGGCAGCCC
LUMA	N2a cDNA	5'GCCACAATGGCCGCGA ATTATTCCAGTACC	5'CTCCAACTTTTTGGCT GGCACCCGTGTCCG
LUMA Δ 201-400	N2a cDNA	5'ATTCCTCTAGAATGGC AGCGAACTATTCCAG	5'ATTCCAAGCTTCAATCT TGTCATGAGGCCTG
LUMA Δ 309-400	N2a cDNA	5'ATTCCTCTAGAATGGC AGCGAACTATTCCAG	5'ATTCCAAGCTTCCCAT GTCTTCATGGAATTGC
LUMA Δ 1-200, 346-400	N2a cDNA	5'ATTCCTCTAGAATGGAC AACTTCAAGGCCCTG	5'ATTCCAAGCTTCGTTGA CAAGGTCTCGGAAG
LUMA Δ 1-200	N2a cDNA	5'ATTCCTCTAGAATGGAC AACTTCAAGGCCCTG	5'ATTCCAAGCTTCTCCA GCTTTTTGGCTGG
LUMA Δ 1-200, 309-400	N2a cDNA	5'ATTCCTCTAGAATGGAC AACTTCAAGGCCCTG	5'ATTCCAAGCTTCCCAT GTCTTCATGGAATTGC
LUMA Δ 1-52, 309-400	N2a cDNA	5'ATTCCTCTAGAATGAAC GAGGGCCGAGCACTG	5'ATTCCAAGCTTCCCAT GTCTTCATGGAATTGC
LUMA Δ 1-52, 309-400, bact. expr.	N2a cDNA	5'ATTCCGGTACCATGAAC GAGGGCCGAGCACTG	5'ATTCCGAATTCCCCCA TGTCTTCATGGAATTGC

¹ kindly provided by T.Nagase, Kazusa DNA Research Institute, Japan

² kindly provided by Howard J. Worman Columbia University, New York, NY, USA (Ye Q. and Worman HJ., 1994)

³ kindly provided by Amos J. Simon Tel-Aviv University, Israel

Tab. 5-2 Primer and template combinations used to amplify target DNA sequences

5.1.4 Vector construction

The amplified DNA fragments were cloned into appropriate vectors, either using restriction sites or through TOPO cloning. When restriction sites were utilized for cloning, the following strategy was applied: generate a DNA fragment framed with appropriate restriction sites (5.1.3); purify the fragment through elution from agarose gel or using a spin column (5.1.4.4); digest the fragment and the vector with the same restriction enzymes (5.1.4.1); if not preparing for directional cloning – dephosphorylate the vector (5.1.4.2); purify the digested vector and the digested DNA fragment through elution from agarose gel or using a spin column (5.1.4.4); ligate the purified DNA fragment with the purified vector (5.1.4.5). For TOPO cloning see 5.1.4.6. For list of vector/DNA fragment combinations and the cloning strategy applied see Tab. 5-3.

DNA FRAGMENT	VECTOR	RESTRICTION ENZYMES/ TOPO CLONING
KIAA0810 KIAA0668 Lamin B receptor Lamin B2 LUMA	pcDNA3.1/V5-His (Invitrogen)	TOPO cloning
All LUMA deletion mutants	pcDNA3.1(-)myc/His C (Invitrogen)	XbaI - HindIII
LUMA Δ1-52, 309-400 LAP 2βshort, bacterial expression	pHO4c (kindly provided by H. Otto)*	KpnI - EcoRI

*pHO4c was generated from pHO2c (Fasshauer et al., 1997) by inserting DNA coding for a C-terminal His6-tag followed by a c-myc epitope and a stop codon in between the EcoRI and the BamHI site of pHO2c. The insert encompasses the following sequence: 5' gcaattcgggccacatcaccaccatcacggcggacagaaactgatcagcgaagaagatctgaacta ggatccg 3'.

Tab. 5-2 Vector/DNA fragment combinations and the cloning strategy applied.

5.1.4.1 Restriction digest

5 µg of the DNA was mixed with suitable restriction digest buffer (provided by the supplier with each restriction enzyme) and 10U of each restriction enzyme in a total volume of 20 µl.

The reaction mixture is incubated for 1 h at 37 °C. The size and integrity of the digested DNA is checked on an agarose gel (5.1.4.3).

5.1.4.2 Dephosphorylation

To prevent the religation of the cohesive ends of the vector, a dephosphorylation of 5'-ends was performed. After the completed restriction digest, 1U of Calf Intestinal Phosphatase was simply added to the reaction mixture, which was then incubated for another 1hr at 37 °C, followed by 10 min at 65 °C to heat-inactivate the enzyme.

5.1.4.3 Agarose gel electrophoresis

Agarose gel electrophoresis was used for separation of DNA fragments of different sizes. A typical gel would be made of 0.8 % agarose in 1xTAE supplemented with 0.5 µg/ml ethidium bromide. This gel strength is sufficient for separation of fragments of 0.6–10kb. Dependent on the size of the gel, the electrophoresis was performed at 50–150V and the DNA was visualized through UV illumination (302nm, ethidium bromide fluorescence). Prior to loading, the sample was supplemented with DNA gel loading buffer.

1xTAE

40 mM Tris/acetate pH 8.5

2 mM EDTA

6x DNA gel loading buffer

0.25 % Bromophenol blue

0.25 % Xylene cyanol FF

15 % Ficoll Type 4000

120 mM EDTA

5.1.4.4 Purification of DNA fragments

DNA fragments were purified using various Gel Extraction- or Mini Prep-kits from different suppliers (Qiagen, Clontech, Peqlab). No differences in performance could be observed.

The procedure: enzymatic reactions or solubilized gel slices were mixed with the appropriate high-salt binding buffer and then applied to the spin column where DNA binds to the silica-gel membrane. Impurities were washed away, and pure DNA was eluted in a small volume of water or of the low-salt elution buffer provided with the kit.

5.1.4.5 Ligation

T4 DNA ligase catalyzes the formation of a phosphodiester bond between juxtaposed 5'-phosphate and 3'-hydroxyl termini in duplex DNA or RNA.

Digested and optionally dephosphorylated and purified vector DNA (50-400ng) and foreign DNA to be inserted were mixed in a microcentrifuge tube. Equal or up to 3-fold molar excess of insert DNA over vector DNA was used. The reaction mixture was then supplemented with 10X ligation buffer, deionized water to a total volume of 20 µl and 2U of T4 DNA ligase. The mixture was incubated for 1 hour at 22 °C and then either used directly for transformation or stored at -20 °C.

10X Ligation Buffer

400 mM Tris-HCl

100 mM MgCl₂

100 mM DTT

5 mM ATP (pH 7.8 at 25 °C).

5.1.4.6 TOPO cloning

The principle of TOPO Cloning: the plasmid vector (pcDNA3.1/V5-His) is linearized, has single thymidine (T) overhangs at both 3' ends and a topoisomerase molecule covalently bound to it.

Because of Taq polymerase's nontemplate-dependent terminal transferase activity, the 3' ends of PCR products get a single deoxyadenosine (A) overhang. This allows PCR inserts to ligate efficiently with the vector. Topoisomerase I from *Vaccinia* virus binds to duplex DNA at specific sites and cleaves the phosphodiester backbone after 5'-CCCTT in one strand. The energy from the broken phosphodiester backbone is conserved by formation of a covalent bond between the 3'-phosphate of the cleaved strand and a tyrosyl residue (Tyr-274) of topoisomerase I. The phospho-tyrosyl bond between the DNA and enzyme can subsequently be attacked by the 5'-hydroxyl of the original cleaved strand, reversing the reaction and releasing topoisomerase.

Adding 3' adenines: direct cloning of DNA amplified by proofreading polymerases like Pfu into TOPO TA Cloning vectors is difficult and has very low cloning efficiency because their proofreading activity removes the 3' A-overhangs necessary for cloning.

Procedure: after amplification with the Pfu polymerase and gel purification of the PCR product, Taq polymerase buffer, 10nmol dATP and 1U of Taq polymerase were added and the mixture was incubated 15 minutes at 72 °C; then used as it was in the TOPOCloning reaction.

TOPO Cloning reaction: 4 µl of fresh PCR product, 1 µl of Salt Solution and 1 µl of TOPO vector were mixed in a microcentrifuge tube and incubated for 30 minutes at room temperature (22-23 °C). The reaction was then placed on ice and immediately used for transformation or stored at -20 °C.

Salt Solution

1.2M NaCl

0.06M MgCl₂

5.1.5 Preparation of competent *E.coli*

100 ml of SOB medium in 500 ml flask was inoculated with a few colonies picked from a LB plate w/o antibiotics and grown at room temperature with vigorous shaking. When the OD₆₀₀ reached 0.6 (takes > 30 hours), the cells were placed on ice for 10 min and then pelleted at 2000 g for 10 minutes. The supernatant was removed and the cells resuspended gently in 30 ml of ice-cold TB followed by an incubation on ice for 10 min. The cells were pelleted again at 1000 g and resuspended gently in 8 ml TB. DMSO was added to a final concentration of 7 % and the cells were incubated on ice for another 10 min. Finally, the cells were aliquoted and frozen immediately in liquid N₂.

TB

10 mM Pipes

15 mM CaCl₂

250 mM KCl

pH 6.7 with KOH

add MnCl₂ to 55 mM final conc.

filter sterilize

SOB per litre:

20 g tryptone

5 g yeast extract

0.5 g NaCl

autoclave, then add:

10 ml 0.25 M KCl

5 ml 2 M MgSO₄

100 µl 5 M NaOH

The procedure was adapted from Inoue et al., 1996.

5.1.6 Transformation into *E.coli*

2 µl of either the ligation or the TOPO Cloning reaction were added to 50 µl of competent DH5α and mixed gently. The bacteria were incubated for 30 minutes on ice, followed by a 45 seconds heat-shock (42 °C). The tubes were then transferred to ice and 250 µl of room temperature SOC medium were added. The tubes were then shaken horizontally (200rpm) at 37 °C for 1 hour. Thereafter, 150 µl from each transformation were spread on a prewarmed LB plate containing an appropriate antibiotic for selection and incubated overnight at 37 °C.

<u>SOC medium</u>	<u>LB medium</u>	<u>LB agar plates</u>
2 % Tryptone	1.0 % Tryptone	LB medium + 15 g/L agar
0.5 % Yeast Extract	0.5 % Yeast Extract	
10 mM NaCl	1.0 % NaCl	Autoclave
2.5 mM KCl	pH 7.0	Cool to 55 °C before adding
10 mM MgCl ₂	Autoclave	antibiotic (ampicillin:
10 mM MgSO ₄	Cool to 55 °C before adding	100 µg/ml; kanamycin:
20 mM glucose	antibiotic (ampicillin:	50 µg/ml)
Autoclave;	100 µg/ml; kanamycin:	
Add the sterile filtered glucose	50 µg/ml)	

5.1.7 Analytical and preparative plasmid preparation

5.1.7.1 Analytical plasmid preparation

To analyze the plasmids for inserts by restriction analysis or by PCR screening or for use as templates in sequencing reaction, the plasmid DNA was isolated on a small scale using various mini prep-kits from commercial sources (Qiagen, Clontech, Peqlab).

Typically, 10 colonies would be picked and cultured overnight in 3 ml of LB medium (5.1.6) containing 100 µg/ml ampicillin or 50 µg/ml kanamycin.

The plasmid DNA isolation procedure: 2 ml of bacteria was pelleted, lysed, neutralized and the cell debris was separated by centrifugation. The supernatant contained plasmid DNA in an appropriate high-salt binding buffer and was applied to a spin column where the DNA bound to the silica-gel membrane. Impurities were washed away, and pure DNA was eluted in a small volume of a low-salt elution buffer or water. Typical yield: 20 µg.

The remaining 1 ml of bacteria was used for a glycerol stock (900 μ l of bacteria + 100 μ l 80 % sterile glycerol), which was then stored at 4 °C.

5.1.7.2 Preparative plasmid preparation

To obtain high-purity and high-concentration plasmid DNA for transfection experiments, the DNA was isolated on a larger scale using either midi or maxi prep. scale. For that purpose, 50–100 ml of LB medium (see 5.1.6) containing 100 μ g/ml ampicillin or 50 μ g/ml kanamycin was inoculated with the bacteria harbouring the appropriate plasmid and cultured overnight.

The midi and maxi prep. kits (Qiagen, Clontech) work according to the same principle as the mini prep. kits, except that instead of silica-gel matrix the columns contain an anion exchanger matrix and the eluted DNA is precipitated with isopropanol. Typical yield: 0.5-1 mg.

DNA concentration and purity determination

Concentration: 1 Absorbance unit measured at 260 nm corresponds to 50 μ g DNA/ml H₂O

Purity: Ratio of absorbances measured at 260 and 280 nm (A_{260}/A_{280}) should be 1.6–2.0 in H₂O

5.1.8 Sequence analysis of the DNA

The correct sequences of all PCR-derived were confirmed by sequencing. Therefore, a special sequencing service from GATC AG (Konstanz), ‘Sequence Yourself’ was employed. ‘Sequence Yourself’ means that the sequencing reaction was prepared here, using the ABI PRISM™ Big Dye Terminator Cycle Sequencing Ready Reaction Kit and applied to an ABI 377 HT by GATC AG.

Sequencing reaction: 500ng plasmid DNA was mixed with 3.2pmol primer and 4 μ l of Ready Reaction Mix in a total volume of 10 μ l and was cycled as follows (repeated for 25 cycles):

Rapid thermal ramp* to 96 °C * = 1 °C/s

96 °C for 30 s

Rapid thermal ramp to 50 °C

50 °C for 15 s

Rapid thermal ramp to 60 °C

60 °C for 4 min

The Ready Reaction Mix contains the dye terminators, deoxynucleoside triphosphates (dITP,dUTP,dATP,dCTP), AmpliTaq DNA polymerase FS, *rTh* pyrophosphatase, magnesiumchloride and buffer, all premixed in optimal concentration for fluorescence-based cycle sequencing reactions.

5.1.9 Protein expression in *E.coli*

The plasmids were transformed into competent BL21(DE3) *E.coli* cells. Some colonies were picked and analyzed for positive clones. To find the best expressing clone and to determine the optimal time scale for expression, a mini-induction experiment was performed.

Mini-induction: the BL21(DE3) positive clone was cultured overnight in 10 ml TB medium supplemented with the selecting antibiotic at 30 °C. On the next day, the culture was supplemented again with the same amount antibiotic, induced with 1 mM IPTG and left in the shaker for another 4 h. An 500 µl aliquot was taken every hour, the cells were pelleted and resuspended in 80 µl of SDS-PAGE sample buffer (5.3.3). 10 µl were then loaded on SDS-PAGE, the gels were blotted and probed with appropriate antibody (see 5.3.4). The clones were compared in respect to greatest amount of protein expressed. The best expressing clone was chosen and used for maxi-induction.

Maxi-induction: the best expressing clone was cultured overnight in 500 ml TB medium supplemented with the selecting antibiotic at 30 °C. On the next day, the culture was supplemented again with the same amount of selective antibiotic, induced with 1 mM IPTG and left in the shaker for the optimal expression time (typically 3-4 h). Thereafter, the cells were pelleted and the pellet was stored at -20 °C until needed.

TB (Terrific Broth) Medium

<i>Solution I</i>	<i>Solution II</i>
12 g tryptone	2.3 g KH ₂ PO ₄
24 g yeast extract	12.5 g K ₂ HPO ₄
4 ml glycerol	in 100 ml water
in 900 ml water	

Autoclave both solutions, cool and mix together.

5.1.10 *In vitro* transcription

Anti-sense oligonucleotides (18-mers) were designed to match the LUMA cDNA approximately every 100bases. To find the oligonucleotide which would pair with the mRNA most efficiently, all the oligonucleotides were tested *in vitro* first. For that purpose, the cDNA had to be translated *in vitro* into mRNA.

Procedure: 10 µg of LUMA in pcDNA3.1/V5-His was digested with XhoI and purified through elution from a gel (5.1.4.4). 40 µl of purified DNA, 20 µl of Transcription Buffer (Promega), 30 µl of rNTPs (Promega), 1.5 µl RNasin (RNase inhibitor) and 10 µl of T7 polymerase were mixed and incubated for 4h at 37 °C. The DNA was removed through DNase treatment, the proteins through phenol:chloroform extraction.

DNase treatment: add 5 µl of RQ DNase, incubate for 15 min and then add 5 µl of EDTA (0.5 M, pH 8.0).

Phenol:Chloroform extraction: add 110 µl of Phenol:Chloroform:Isoamylalcohol mixture (25:24:1), vortex 30 s, centrifugation for 2 min, transfer the aqueous phase into a new tube. Add 110 µl of Chloroform, vortex 30 s, centrifugation for 2 min, transfer the aqueous phase into a new tube. Vortex 30 s, centrifugation 2 min and remove any organic phase. Add 10 µl of 3M Sodium Acetate (pH 5.2) and 250 µl of 95 % EtOH, cooled to -20 °C. Incubate at -20 °C for 30 min, spin at top speed in a microcentrifuge for 20 min. and wash the pellet once with 70 % EtOH. Resuspend the pellet in 100 µl of RNase free water. Store at -70 °C. Typical yield: 10 mg/ml.

5.1.11 Messenger primer walking

RNase H cleaves the RNA in a RNA/DNA duplex. If an oligonucleotide binds efficiently to the mRNA, a treatment with RNase H results in degradation of the mRNA.

Procedure: 1pmol of LUMA mRNA was mixed with RNase H buffer, 5pmol oligonucleotide and 0.4U of RNase H in a total volume of 10 μ l and incubated at 37 °C. The reaction was stopped after 7.5 min by addition of 1.5 μ l EDTA (0.5 M, pH 8.0). The sample was immediately loaded on an agarose gel (for sample preparation – see 5.1.1).

The oligonucleotides which performed best were then ordered as phosphothiorates and used for the transfection of cells (5.2.2).

5.2 CELL BIOLOGY

5.2.1 Cell culture (N2a, COS-7, 3T3, CHO)

N2a cells (mouse neuroblastoma), COS-7 cells (African green monkey kidney) and NIH/3T3 (mouse embryo fibroblasts) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10 % fetal bovine serum, 100 µg/ml streptomycin and 100 µg/ml penicillin at 37 °C in a humidified atmosphere with 5 % CO₂. CHO cells (Chinese hamster ovary) were cultured in αMEM with the same supplements plus 2 mM L-glutamine and under the same conditions. Typically, the cells were subcultured once a week. Medium renewal: twice a week.

5.2.2 Transfection of mammalian cells

COS-7 cells were used for all overexpression experiments, 3T3 cells for knock-down experiments. The cells were cultured on 12-mm glass coverslips. Monolayer cultures at 50 % confluency were transfected using SuperFect (Qiagen) according to the manufacturer's manual.

Procedure: 1 µg of plasmid DNA was mixed with 5 µl of SuperFect in serum-free medium in a total volume of 65 µl. The mixture was incubated for 10-15 min at room temperature to allow the complex formation between DNA and the activated dendrimer, supplemented with 350 µl of complete medium and then added to the PBS-washed cells. The cells were then incubated at 37 °C in a humidified atmosphere with 5 % CO₂ for 2-3h. The medium was replaced and another 19 h incubation followed to allow for gene expression (12 h for anti-sense oligonucleotides).

PBS (phosphate buffered saline)

137 mM NaCl

2.7 mM KCl

4.3 mM NaH₂PO₄

1.47 mM KH₂PO₄

adjust to a final pH of 7.4

5.2.3 Immunofluorescence staining of cells

Immunofluorescence studies were carried out to examine the subcellular distribution of the recombinant proteins.

Procedure: 19 h post transfection, cells were fixed with 3 % paraformaldehyde in PBS for 15 min, washed 4x with PBS, permeabilized with 0.5 % TritonX-100 in PBS for 10 min, washed 4x with PBS, blocked with 5 % NGS (normal goat serum) in PBS for 30 min and probed with a primary antibody diluted in 5 % NGS in PBS for 1hr. The cells were then washed again 4x with PBS and then incubated for 1hr with a Cy2- or TexasRed-conjugated secondary antibody, diluted 1:500 in 5 % NGS in PBS. After washing 4x with PBS, the cells were either stained with 0.00004 % DAPI (4,6-diamino-2-phenolindol2HCl) or mounted directly on slides with Fluoromount G (Serva).

Antibodies used:

Construct	Primary antibody	Dilution	Secondary antibody
TOPO cloned constructs	mouse monoclonal anti-V5	1:500	anti mouse Texas Red
LUMA deletion mutants	mouse monoclonal anti-myc	1:1500	anti mouse Texas Red
LAP 2 β	rabbit polyclonal anti-LAP 2 β/ϵ	1:800	anti rabbit Cy2

5.2.4 Fluorescence microscopy

Fluorescence images were obtained either with an inverse fluorescence microscope (Leitz, DMIRB) or with a confocal laser scanning microscope (LSM 510, Carl Zeiss Jena GmbH, Jena, Germany).

5.2.5 Preparation of metaphase chromosomes from CHO cells

CHO-cells were arrested at the metaphase of mitosis and lysed. The chromosomes (mostly intact equatorial plates) were centrifuged in a sucrose gradient, where they enrich in the middle of the gradient at phase borders between 40 and 50 % sucrose.

Procedure (adapted from Dechat et al., 1998): 20 dishes with densely grown CHO cells were incubated in full medium supplemented with 1x non essential amino acids and 2 mM thymidine in order to arrest the cells in the S-phase. After 16 h incubation, the cells were washed with PBS and incubated in full medium for another 4 h. Thereafter Nocodazole was

added to a final concentration of 0.2 µg/ml, followed by 4-6 h incubation. The cells were then collected by rinsing off the loosely attached mitotic cells and centrifugation at 500 g for 5 min. The pellet was resuspended in full medium containing 20 µM Cytochalasin B and 0.2 µg/ml nocodazole and incubated for 30 min at 37 °C. The cells were pelleted again and resuspended in 4 ml chromosome buffer (CHB) supplemented with 0.1 % (v/v) TritonX-100. The cells were homogenized with 40 complete strokes in a Dounce homogenizer. The homogenate was mixed with 2 ml 60 % sucrose in CHB and following gradient was set up:

- 3 ml homogenate
- 3 ml 30 % sucrose in CHB
- 3 ml 40 % sucrose in CHB
- 3 ml 50 % sucrose in CHB
- 3 ml 60 % sucrose in CHB

and centrifuged for 10 min at 500 g. The fractions at the 30/40 % sucrose (chromosomes, some cellular fragments) and at the 40/50 % sucrose (purest chromosome fraction) were collected. The DNA content was checked by measuring A_{260} (5.1.7) and the protein concentrations were determined according to Bradford (5.3.1). A typical yield was: twice as much protein and half the DNA amount in 30/40 fraction as compared to 40/50 fraction.

CHB (chromosome buffer)

- 5 mM Hepes-NaOH pH 7.4
- 5 mM NaCl
- 5 mM MgCl₂
- 0.5 mM EDTA
- 1 mM DTT
- protease inhibitors

5.2.6 Preparation of nuclei from N2a cells

20 large culture dishes with densely grown N2a cells were washed with PBS (5.2.3) and the cells were scraped from the surface with a rubber spatula. Cells from up to 5 dishes were resuspended in 2x 10 ml PBS and pelleted at 500 g. The pellet was resuspended in 15 ml ice cold SHM 0.25, then 0.05 % (w/v) Nonidet P-40, 1 mM DTT and protease inhibitors were added, followed by homogenization with 30 complete strokes in a 30 ml Glas Dounce

Homogenizer (Type S). After homogenization, SHM 0.25 was added to a total volume of 16.5 ml. The homogenate was then supplemented with 33.5 ml SHM 2.1 and the following sucrose gradient was set up:

SHM 0.25	5 ml
SHM 1.5/Homogenate	20 ml
SHM 2.1	2 ml

The nuclei were pelleted for 75 min at 100.000 g, 4 °C. The pellet was washed once with SHM 0.25 and resuspended in a small volume of SHM 0.25. Protein concentrations were determined according to Bradford (5.3.1). Typical yield: 30 mg protein/20 dishes N2a cells.

SHM 0.25 buffer

20 mM Hepes-NaOH pH 7.4
0.25 M Sucrose
5 mM MgCl₂
protease inhibitors

SHM 2.1 buffer

20 mM Hepes-NaOH pH 7.4
2.1 M Sucrose
5 mM MgCl₂
protease inhibitors

5.2.7 Preparation of nuclear envelopes from N2a nuclei

The N2a nuclei (5-8 mg of protein) were resuspended in 40 ml of ice cold TP buffer containing 250 µg/ml Heparin, 400 units of Benzon Nuclease (Merck) and protease inhibitors (1 tablet protease inhibitor cocktail mini from Roche). After stirring for 1 h at 4 °C and additional 20 min at room temperature, the nuclear envelopes were sedimented by centrifugation (10.000×g) for 30 min at 4 °C and resuspended in STM 0.25 buffer. Protein concentrations were determined according to Bradford (5.3.1). Typical yields were 8 % for pure and 20 % for crude preparation. Crude preparation was achieved through using half of the volume of TP and half of the amount of Heparin as compared to a pure preparation described above.

TP buffer

10 mM Tris/HCl pH 7.4
10 mM NaH₂PO₄/Na₂HPO₄ pH 7.4

STM 0.25 buffer

20 mM Tris/HCl pH 7.4
0.25 M Sucrose
5 mM MgSO₄
protease inhibitors

5.3 BIOCHEMISTRY

5.3.1 Protein concentration assay (Bradford)

The sample containing no more than 20 µg protein was dissolved in water in a final volume of 500 µl and supplemented with 500 µl Bradford reagent. For calibration curve a solution of BSA was used. The samples were then measured spectrophotometrically at 595 nm (Bradford, 1976).

Bradford reagent

0.06 % (w/v) Coomassie Brilliant Blue G250

3 % (v/v) HClO₄

5.3.2 Solubilization of nuclear envelopes

5.3.2.1 TX-100 and urea/carbonate

The NE preparation (200 µg protein per sample) in STM 0.25 (5.2.7) was supplemented either with TX-100 (final concentration 0.5 % (w/v)), or with urea and Na₂CO₃ (final concentration 4 M and 0.1 M, respectively). The samples were incubated for 15 min in a shaker at 4°C. TX-100 resistant material was pelleted at 13.000rpm in a Biofuge (Heraeus). Chaotrope-resistant material was pelleted at 50.000rpm in a Beckman tabletop ultracentrifuge. The pellets were resuspended in BAC PAGE Sample Buffer (5.3.5).

5.3.2.2 n-Dodecyl-β-maltoside, Tween 80

The NE preparation in phosphate buffer (200 µg protein per sample) was supplemented with n-Dodecyl-β-maltoside (final concentration 0.5 % to 1 % (w/v)), or with Tween 80 (final concentration 0.5 % to 1 % (v/v)). Additionally, non-detergent sulfo-betaine was added in a final concentration of 0.5 to 1 M. The samples were incubated for 30 min in a shaker at 37 °C followed by centrifugation for 10 min at 13.000rpm in a Biofuge (Heraeus).

Phosphate Buffer

0.05 M NaH₂PO₄/NaH₂PO₄ pH 7.4

0.15 M NaCl

5 mM MgCl₂

protease inhibitors

5.3.3 SDS PAGE

The 1D – SDS PAGE (Laemmli, 1970) was run on 3 % stacking gels and 10 % separating polyacrylamide gels in a Mini-Protean II Chamber (BioRad) in a 80 x 55 x 1.5mm format. The samples were mixed with SDS PAGE Sample Buffer and incubated for 3 min at 95 °C prior to loading. Running conditions: 30mA/1.5mm thick gel.

After completed electrophoresis the gels were either stained (1hr in Staining Solution, overnight in Destaining Solution) or blotted.

Stacking Gel (3 %)

2.5 ml 500 mM Tris·HCl pH 6.8

1.0 ml AA/Bis (30 % acrylamide,
0.8 % bis-acrylamide)

6.5 ml H₂O

15 µl TEMED

200 µl 10 % (w/v) ammonium persulfate

Separating Gel (10 %)

2.5 ml 1.5 M Tris·HCl pH 8.8

3.3 ml AA/Bis (30 % acrylamide,
0.8 % bis-acrylamide)

4.2 ml H₂O

12.5 µl TEMED

50 µl 10 % (w/v) ammonium persulfate

SDS PAGE Sample Buffer (2x)

130 mM Tris·Cl, pH6.8

20 % (v/v) Glycerol

4.6 % (w/v) SDS

0.02 % Bromophenol blue

2 % DTT

Running Buffer

0.3 % Trisbase

1.44 % Glycine

0.1 % SDS

Staining Solution

0.1 % Coomassie blue R 250

10 % acetic acid

40 % ethanol.

Destaining Solution

10 % acetic acid

40 % ethanol.

5.3.4 Western Blot

5.3.4.1 Semi dry Blot

In order to transfer the proteins from a polyacrylamide gel matrix to a blot membrane, a semi-dry electroblotting procedure was employed. In this procedure, a sandwich is build of three sheets of Whatman 3MM filter paper, a nitrocellulose membrane, the gel and again three sheets of Whatman 3MM filter paper. The filter paper is equilibrated in Blot Buffer and the nitrocellulose membrane in H₂O prior to transfer. Blotting conditions: 1hr at 1mA/cm² of gel. After completed transfer, the membranes were stained with Ponceau S (5 min in Ponceau S Solution, destaining in water until protein bands are visible).

Blot Buffer

48 mM Tris·HCl

39 mM glycine

20 % methanol

0.0375 % SDS

pH 9.2

Ponceau S Solution

0.2 % (w/v) Ponceau S

3 % acetic acid

5.3.4.2 Immunodetection

After staining with Ponceau S, the proteins of interest were detected using specific antibodies. Procedure: the nitrocellulose membrane was incubated for 1hr in Blocking Solution at room temperature. The primary antibody was diluted 1:1000 in the Blocking Solution and another 1hr incubation followed. Thereafter, the membrane was washed 3x 10 min with TBST and incubated 1hr with the horseradish peroxidase (HRP) conjugated secondary antibody diluted 1:3000 in Blocking Solution. The membrane was then washed 3x 10 min with TBST and developed with ECL (Pharmacia).

Antibodies used:

<u>Primary:</u>	<u>Secondary:</u>
anti-LAP 2 β / ϵ , anti-Sun2	anti-rabbit HRP
anti-myc, anti-V5, anti-lamin A/C	anti-mouse HRP
anti-laminB	anti-goat HRP

Blocking Solution

20 mM Tris.HCl pH 7.4

150 mM NaCl

0.1 % Tween-20

5 % non-fat dry milk

TBST

20 mM Tris.HCl pH 7.4

150 mM NaCl

0.1 % Tween-20

5.3.5 2D-BAC/SDS-PAGE

The BAC (Benzyldimethyl hexadecyl AmmoniumChloride) polyacryl amide gel electrophoresis in combination with conventional SDS-PAGE is the first dimension of a two-dimensional gel system. Proteins are isolated here in the presence of the cationic detergent 16-BAC using an acidic buffer system in reverse polarity compared to the SDS PAGE. Since ionic detergents are used in both dimensions, the system is particularly suitable for samples containing membrane proteins (Hartinger et al., 1996).

Procedure: for the first dimension, a 6 %-10 % (w/v) acrylamide gradient gel was used. The separating gel was buffered to pH 2.1, the stacking gel (4 % w/v) to pH 4.1. The samples were resuspended in 2x BAC-PAGE Sample Buffer and incubated for 5 min at 60 °C. Electrophoresis took place with 10mA through the stacking gel, and with 25mA in the separating gel. For the second dimension a SDS-PAGE was used. Gradient separating gels from 7.5 % to 15 % were used and a 3 % stacking gel (5.3.3). After the Coomassie staining/destaining procedure, the gel from the first dimension was equilibrated overnight in 62.5 mM Tris:HCl pH 6.8 to the conditions of the stacking gel of the second dimension. In each case, one gel lane from the first dimension was put horizontally on the stacking gel of the second dimension and overlaid with 2x SDS-PAGE Sample Buffer. After 10 min incubation, the electrophoresis (10mA in the stacking gel, 30mA in the separating gel) was started. After completed electrophoresis, the gels were treated like a normal SDS-PAGE.

Separating gel (10 ml)

6 %

3.2 ml H₂O

2 ml acrylamide/bis-acrylamide (30 %/0.8 %)

10 %

1.9 ml H₂O bidest

3.3 ml acrylamide/bis-acrylamide (30 %/0.8 %)

2.5 ml phosphatpuffer pH 2.1 (0.3 M)

1.8g urea

280 µl Bis-acrylamide (10 % w/v)

100 µl 16-BAC (10 % w/v)

500 µl ascorbic acid (14.1 mg/ml)

16 µl FeSO₄ (1.4 mg/ml)

200 µl H₂O₂ (1:500 diluted from 30 % stock)

Stacking gel 10 ml

4 %

3.2 ml H₂O bidest

1.35 ml acrylamide/bis-acrylamide
(30 %/0.8 %)

2.5 ml phosphatpuffer pH 4.1 (0.3 M)

1 g urea

1.38 µl Bis-acrylamide (10 % w/v)

70 µl 16-BAC (10 % w/v)

520 µl ascorbic acid (14.1 mg/ml)

11 µl FeSO₄ (1.4 mg/ml)

350 µl H₂O₂ (1:500 diluted from 30 % stock)

BAC PAGE Sample Buffer

250 mM 16-BAC

8.3 M urea

10 % (w/v) glycerol

75 mM DTT

0.01 % (w/v) Pyronin Y

Running Buffer

2.5 mM 16-BAC

150 mM glycine

50 mM phosphoric acid

5.3.6 Tryptic in gel digest

After staining/destaining a gel, spots on the gel which contained proteins of interest were cut out with a scalpel, chopped in cubes of approximately 1mm³ and transferred into a 0.5 ml tube. An incubation for 15 min in Digest Buffer followed.

Removal of Coomassie Blue and SDS: after 15 min shaking, acetonitrile was added to the Digest Buffer (1:1 v/v) and the tube was incubated again for 15 min on a shaker. With this step the gel pieces shrink and the Coomassie dye is removed completely. The supernatant was removed and replaced by 100 % acetonitrile. The incubation continued, until the gel pieces had become white. Afterwards the supernatant was removed again, the Digest Buffer was

added as above and the procedure was repeated again, until the gel pieces were destained (and neutralized).

Reduction/Carbamidomethylation: the gel pieces were lyophilised after the last acetonitrile step. Subsequently, Reduction Solution was added and the samples were incubated for 30 min at 56 °C. The supernatant was removed and the gel pieces were shrunk again by addition of acetonitrile. After removal of the acetonitrile the gel pieces were incubated for 20 min at room temperature in the dark with Iodoacetamide Solution. The solution was then removed and replaced by Digest Buffer. 1:1 (v/v) acetonitrile was added after further 15 min. Finally, the gel pieces were shrunk in pure acetonitrile and then lyophilised.

Tryptic cleavage: the gel pieces were rehydrated for 30 min on ice in Trypsin Solution. Surplus liquid was then removed and replaced with so much Digest Buffer that the gel pieces were just covered. The digest reaction was incubated for 24 h under mild shaking at 37 °C. After 12 h, a 1 µl aliquot was taken from the supernatant and was measured with MALDI MS using fast evaporation/nitrocellulose (FENC) matrix preparation (5.3.7.1).

Elution of the peptides: a fivefold volume of Digest Buffer was added to the samples. After 15 min shaking, an equal volume of acetonitrile was added. The supernatant was removed 15 min later and the gel pieces were shrunk again with acetonitrile. All supernatants were saved and pooled. The gel pieces were rehydrated again in 5 % formic acid for 15 min, before the same volume of acetonitrile was added again. These supernatants were removed again after 15 min and pooled with the supernatants of the respective samples from the preceding elution steps. The pooled supernatants were then lyophilised and stored at -20 °C until needed.

Digest Buffer

100 mM NH₄HCO₃

Reduction solution

10 mM DTT

100 mM NH₄HCO₃

Iodoacetamide Solution

55 mM Iodoacetamide

100 mM NH₄HCO₃

Trypsin Solution

12.5 µg/ml Trypsin (bovine, sequencing grade)

25 mM NH₄HCO₃

5.3.7 MALDI-TOF mass spectrometry

The matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS, Karas and Hillenkamp, 1988) is at the present one of the most important massspectrometric methods in protein analytics. In combination with suitable computer programs, MALDI-MS allows the identification of proteins at the subpicomol level.

Principle of the MALDI MS: the sample is co-cristallized with an light absorbing matrix and the ionization of the molecules is accomplished by laser irradiation of the sample (laser desorption). For mass analysis, usually the flying time of ions between source and the detector ("Time Of Flight", TOF) is measured.

Identification of proteins: the measured peptide masses are aligned with theoretically predicted peptide masses of proteins in protein data bases (e.g. SwissProt, EMBL data base, NCBI non-redundant data base). The alignment takes place with help of analysis programs accessible on the Internet. This programs evaluate those proteins as best hits, which show the largest number of agreement between measured and predicted sets of peptide masses. The stringency of the search (e.g. the tolerated deviation of the measured masses from the theoretical masses) is chosen by the user.

MALDI-MS and database search: The mass spectrometric measurements were performed on a Bruker Reflex MALDI-TOF-mass spectrometer (Bruker Daltonik, Bremen) equipped with an ion gate and pulsed ion extraction. Post source decay fragment ion spectra were obtained using the FAST method (Bruker Daltonik). The peptide mass fingerprint spectra were matched to the NCBI non-redundant database entries using the program ProFound at <http://www.proteometrics.com>. The mass tolerance was set to 50 ppm, one missed cleavage site was tolerated and the search was restricted to mammalian proteins. A size cut-off was set to about 200 % of the apparent molecular weight of the protein as estimated from the gels. The proteins were regarded as identified according to the significance criteria of the search program.

5.3.7.1 Sample preparation

The aliquots from the digest supernatants (5.3.6) were generally prepared for the measurement by a variation of the matrix thin layer preparation (fast evaporation/nitrocellulose matrix, FENC (Shevchenko et al., 1996)).

Procedure: a saturated solution of α -Cyano-4-hydroxy-cinnamoyl acid (CCA) in acetone in the ratio 4:1 (v/v) was mixed with nitrocellulose (10 mg/ml in acetone:acetonitrile 1:1 (v/v)). 0.4 μ l of the FENC solution was applied on the MALDI target. After fast evaporation of the solvent, a thin matrix film formed. 0.6 μ l 5 % (v/v) formic acid were then applied on the same spot and 0.4 μ l of the sample were added. When dry, the target was washed once with 5 μ l 5 % (v/v) formic acid and once with 5 μ l H₂O.

5.3.7.2 ZipTip purification

The eluates of the gel pieces were dried and desalted using C₁₈ ZipTips (Millipore).

Procedure (Watty et al., 2000): the peptides were resuspended in 0.5 μ l 50 % methanol/5 % formic acid (v/v). 4.5 μ l 5 % formic acid was added and the tubes were sonicated for 5 s. The ZipTips were equilibrated with 3x 10 μ l 70 % methanol/5 % formic acid, 3x 10 μ l 5 % formic acid and 3x 10 μ l 5 % methanol/5 % formic acid. The peptides were bound to the ZipTip through aspiration of the peptide solution (20x), then washed with 3x 10 μ l 5 % formic acid and 3x 10 μ l 5 % methanol/5 % formic acid. The elution was carried out stepwise with 4 μ l of each 30 %, 50 %, and 70 % (v/v) methanol/5 % formic acid. These samples were prepared for MALDI-MS using a sandwich matrix preparation.

Sandwich matrix preparation: the sample was mixed 1:1 (total volume 1 μ l) with CCA and applied on top of the FENC matrix (5.3.7.1). When dry, the target was washed once with 5 μ l H₂O.

5.3.7.3 Identification of proteins

The peptide mass fingerprint spectra were calibrated internally with the trypsin self-digest peptides and matched to the NCBI non-redundant database entries using the program ProFound at <http://www.proteometrics.com>.

5.3.8 TCA protein precipitation

To concentrate the samples before electrophoresis, the proteins were precipitated with trichloro acetic acid (TCA).

Procedure: 0.1x volumes of 0.15 % deoxycholate were added to the sample and incubated for 15 min at room temperature. Then 0.1x volumes of 100 % TCA were added and the sample

was incubated on ice for 30 min, followed by 15 min centrifugation (10.000 g at 4 °C). The pellet was washed with ice-cold acetone, centrifuged again and resuspended in a suitable amount of H₂O.

5.3.9 Gel filtration

To isolate native complexes of LAP 2 β , gel filtration was performed on a Superdex 200 PC 3.2/30 SMART column and, for scale up, on a Superose 12 FPLC column (both from Pharmacia). Typically, nuclear envelopes prepared from N2a cells (5.2.7) and resuspended in phosphate buffer (300 μ g NE for SMART, 3 mg for FPLC) were solubilized with 0.5 % n-dodecyl- β -maltoside (5.3.2.2), the supernatant was filtered through 0.45 μ filter device and loaded on the equilibrated column. Equilibration and elution were carried out with phosphate buffer. Flow rates: SMART 30 μ l/min, FPLC 300 μ l/min. To calibrate the columns, 50 μ g of calibration mix was loaded on the SMART-, 1 mg on FPLC-system. Fractions of 90 and 600 μ l (SMART and FPLC, respectively) were collected, TCA-precipitated (5.3.8) and separated by SDS-PAGE (10 % separating gel, 5.3.3). To locate the LAP 2 β , an immunoblot was performed (5.3.4.2).

Phosphate Buffer

0.05 M NaH₂PO₄/NaH₂PO₄ pH 7.4

0.15 M NaCl

5 mM MgCl₂

0.5 % n-dodecyl- β -maltoside

protease inhibitors

Calibration Mix

2.5 mg/ml Thyroglobulin

3 mg/ml Human IgG

2 mg/ml BSA

2 mg/ml Ovalbumin

2 mg/ml Cytochrome C

1 mg/ml Blue Dextran

5.3.10 Glycerol gradient centrifugation

Another method used for isolation of native LAP 2 β complexes was the centrifugation in a glycerol gradient.

Procedure: the sample was prepared just as described for gel filtration (see 5.3.9), but without filtration through the 0.45 μ filter device. A continuous glycerol gradient 10-50 %, 13 ml total volume, was poured in Hepes Buffer, the sample was loaded and then centrifuged for 8-10 h,

35.000 rpm (TST41 rotor) at 4 °C. As a size marker served cross-linked glutamate dehydrogenase (GluDH). After completion of the run, 1 ml samples were collected and separated by SDS-PAGE (10 % separating gel; GluDH on 4-12 % gradient gel).

Hepes Buffer

0.02 M HepesNaOH pH 7.4

0.15 M NaCl

5 mM MgCl₂

1 mM DTT

0.5 % n-dodecyl-β-maltoside

protease inhibitors

5.3.11 Cross-linking GluDH

GluDH was cross-linked with dimethyladipimidate (DMA).

Procedure: 0.5 mg of GluDH was mixed with 0.2 ml 0.5 M triethanolamine pH 8.5 and 134 μl 0.1 M Na₂HPO₄ pH 8.0 in a final volume of 0.5 ml. 1 mg of DMA was added, and the sample was incubated for 1hr at room temperature.

5.3.12 Blue Native Electrophoresis

Blue Native Electrophoresis (Schägger and Jagow, 1991) is another method used for separation of native protein complexes. In contrast to SDS-PAGE, no denaturing ionic detergents are used in this electrophoretic system. The migration of protein complexes towards the anode is achieved by addition of Coomassie Brilliant Blue G-250. This dye is negatively charged and binds to the protein complexes without disrupting the protein-protein interactions.

Procedure: a 4 to 16 % gradient gel was cast and left to polymerize at room temperature. No stacking gel was used, the comb was inserted directly into the separating gel. Nuclear envelope prepared as described (5.2.7), typically 200 μg, was supplemented with 1 % (w/v) n-dodecyl-β-maltoside and 25 % (v/v) of Blue Native Sample Buffer. The sample was constantly shaken for 30 min at room temperature and was then centrifuged for 15 min, 13.000rpm at 4 °C. The pellet was resuspended in 40 μl of 4x SDS-PAGE Sample Buffer

(5.3.3) and supplemented with 1 % (w/v) of n-dodecyl- β -maltoside. The following samples were loaded: 30 μ l of the supernatant, 10 μ l of the pellet and 5 μ l of the cross-linked GluDH (5.3.11), which was used as a size marker. The gel was run at 130mV/1.5mm thick gel.

<u>Separating Gel</u>	<u>16 %</u>	<u>4 %</u>
3x BNEB	3.8 ml	3.8 ml
AMBA	6.2 ml	1.6 ml
H ₂ O	-	6.6 ml
Glycerol	2 ml	-
TEMED	8 μ l	8 μ l
10 % APS	40 μ l	40 μ l

3x BNEB

1.5 M amino-caproic acid
150 mM Bis-Tris·HCl pH 7.0

Blue Native Sample Buffer

750 mM amino-caproic acid
5 % Coomassie Brilliant Blue G-250
50 % glycerol

Kathode Buffer

15 mM Bis-Tris·HCl pH 7.0
50 mM tricine
0.002 % ServaBlueG

Anode Buffer

50 mM bis-tris·HCl pH 7.0

5.3.12.1 2D BNE/SDS PAGE

After the first dimension, whole lanes of the 1st dimension gel were cut out, incubated with 4x SDS-PAGE Sample Buffer (5.3.3) for 15 min and loaded on either a 10 % or a gradient SDS-PAGE gel, analogous to 2D-BAC/SDS-PAGE (5.3.5).

5.3.13 Purification of proteins expressed in *E.coli*

Proteins expressed in *E.coli* are purified using their recombinantly attached 6x histidine tag, which specifically binds to nickel containing agarose beads (NiNTA columns).

Procedure: bacterial pellets containing the protein of interest (5.1.9) were resuspended in 20 ml of lysis buffer. Protease inhibitors and 2.5 μ l β -mercapto-ethanol were added and the

bacteria were sonicated 2x 30 s (setting 7, 50 % duty). After sonication, 10 µl of benzonase and more protease inhibitors were added, followed by 15 min, 12.500rpm centrifugation at 4 °C. The supernatant was centrifuged again to make sure that all the cell debris was separated. 0.5-1 ml Ni-NTA beads (Qiagen) were equilibrated with 20 ml of wash buffer and then incubated 30 min on a end-over shaker at 4 °C. The beads were washed 3x with 20 ml wash buffer, loaded on the column and stepwise eluted with 2x 1 ml 20, 50, 100 and 400 mM imidazole in wash buffer. To check the yield, a SDS PAGE with following immunoblot were performed. Typically, the highest amount and purity of the expressed protein was found in the 2nd elution of 50 mM fraction.

<u>Lysis Buffer</u>	<u>Wash Buffer</u>	<u>2x HHL</u>
1x HHL	0.5x HHL	10 M Hepes NaOH pH 7.8
1.5 M NaCl	0.75 M NaCl	1 M NaCl

5.3.14 Chromosome binding assay

This chromosome binding assay makes use of the fact that dense chromosomes are able to pellet through dense sucrose layer. Any protein bound to them will coprecipitate, while non-binding proteins stay in the supernatant.

Procedure: 63 µl of 4x CBB were mixed with chromosomes (preparation see 5.2.5) and recombinant protein in a total volume of 250 µl. The sample was incubated for 30 min at room temperature, underlaid with 35 % sucrose in CHB (5.2.5) and then centrifuged for 10 min, 4000rpm at 4 °C in a Biofuge (Heraeus). Pellet and supernatant were checked by immunoblotting for the presence of the recombinant protein.

4x CBB
 20 mM Hepes pH 7.6
 150 mM NaCl
 5 mM MgCl₂
 2 mM EGTA
 0.2 % TritonX-100
 1 mM DTT
 protease inhibitors

5.4 BIOINFORMATICS

To obtain bioinformatic information about novel proteins, like LUMA, KIAA0810 and KIAA0668, different computer programs freely available in internet were employed. All the programs used for this work were accessed either trough ExPASy Proteomics tools for protein identification and characterization (<http://www.expasy.ch/tools/>) or through PubMed (<http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=PubMed>). Details of search or alignment criteria are specified in the Results section.