2 MATERIALS

2.1 Laboratory equipment

Balance	Mettler-Toledo GmbH, Giessen
Centrifuges	RC-M150 GX, RC-5B, Ultra Pro80, Sorvall GmbH, Bad Homburg
	Biofuge Fresco, Kendro Bioproducts, Germany
	5415C, Mini spin plus, Eppendorf AG, Hamburg
Electroporator	Easy Jet, Equi Bio
Filmprocessor	Curix 60, Agfa-Gevaert, NV, Mortsel, Belgium
Freezer, -80°C	Forma, ThermoQuest Analytische Systeme GmbH, Egelsbach
-20°C, 4°C	Liebhere premium, Germany
FPLC system and Superose 6 (Gel filtration) column	Amersham Pharmacia Biotech Europe GmbH, Freiburg
Gel documentation system for ethidiumbromide-stained agarose gels	Bio-Rad Chemidoc, BioRad Laboratories GmbH, München
Gel electrophoresis equipment	Bio-Rad Laboratories GmbH, München
GeneAmp PCR system 9600	PE Biosystems, The Perkin Elmer Corporation, CA, USA
Heating block	Grant Instruments, Cambridge, UK
Hot air oven	Heraeus Instruments GmbH, Wiesloch
Incubator	Heraeus Instruments GmbH, Wiesloch
Incubator shaker	New Brunswick scientific GmbH, Nürtingen
Mincer PT3100	Polytron, Switzerland
Microscope	Carl Zeiss, Germany
Pipettes, adjustable	Eppendorf AG, Hamburg
pH meter	Mettler-Toledo GmbH, Giessen
Power supply	Bio-Rad Laboratories GmbH, München
Semi dry western blotting apparatus	Bio-Rad Transblot SD apparatus
Sequence Detection system ABI Prism 7700	PE Biosystems, The Perkin Elmer Corporation, CA, USA
Shaker	Rocky, Fröbel Labortechnik, Wasserburg
Sonifier UP200 S (Ultraschall Desintegrator)	Geprüft Sieherheit, Berlin, Germany
Spectrophotometer	Bio-Rad Smart spec^{TM} 3000, BioRad Laboratories GmbH, München
Thermocycler	PTC100, MJ Research, Inc.; Watertown, USA
	Biometra, Biometra GmbH, Germany
	Bio-Rad Laboratories GmbH, München
Thermomixer	Eppendorf AG, Hamburg
Vertical gel electrophoresis system	Bio-Rad Mini Protean II
Vortex	Vortex Genie 2, Bender und Hobein AG, Zürich, Switzerland
Water bath	GFL, Germany

2.2 Chemicals and enzymes

Agarose	Gibco Life Technologies, Karlsruhe
Agarose, low melting point	FMC SeaPlaque GTG, Biozym, Hessisch Ohlendorf
Ampicillin	Sigma, Deisenhofen
APES	Sigma-Aldrich
Bacto Agar	Difco, Becton Dickinson, Sparks, MD; USA
Bacto trypton	Difco, Becton Dickinson, Sparks, MD; USA
Bacto yeast extract	Difco, Becton Dickinson, Sparks, MD; USA
Bromophenol blue	Sigma
Chloramphinicol	Sigma
dATP, dCTP, dGTP, dTTP sodium salt	Amersham Pharmacia
DNA molecular weight standards	MBI Fermentas
DNA Pfu-Polymerase	Promega
DNA Taq-Polymerase	PE Applied Biosystems
DNase (RQ1 DNase)	Promega
DTT	Serva
EDTA (Titriplex® III)	Merck
Ethidium Bromide, 1% solution	Fluka
Formaldehyde	Sigma-Aldrich
Formamide	Sigma-Aldrich
Glucose	Merck
Glycerol	Merck
HEPES	Sigma
Isopropanol	Merck
IPTG	Amersham Biosciences
Kanamycin	Sigma
Lysozyme	Boehringer Mannheim GmbH
β-Mercaptoethanol	Sigma
Nonidet P-40(NP-40)	Sigma
Paraformaldehyde	Sigma-Aldrich
Phenol	Carl Roth
PMSF	Sigma
Restriction Enzymes	New England Biolabs, Amersham Pharmacia
Reverse Transcriptase (Superscript II)	Gibco Life Technologies
SeaPlaque GTG low melt agarose	FMC Bioproducts, Rockland, ME
Sigmacote	Sigma
TEMED	Sigma-Aldrich
Tris	Merck
Triton X-100	Sigma-Aldrich
TRIzol	Invitrogen
Tween-20	Pierce Chemical, P/N 28320

Salts, acids, bases and solvents not mentioned in the table were *pro analysis* quality from Merck (Darmstadt) or Sigma (Deisenhofen).

2.3 Kits

ABI PRISM BigDye Terminator Cycle Sequencing Ready reaction kit	PE Applied Biosystems, Foster City, CA, USA
Plasmid Mini- und Maxipreparation	Qiagen, Hilden
Gel purification kit	Qiagen, Hilden
PCR purification kit	Qiagen, Hilden
RNeasy Kit	Qiagen, Hilden
RT-PCR	RETROScript, Ambion.
TA-cloning Kit (pGEM-T Easy)	Promega, Mannheim

2.4 Consumables

3 MM Blotting paper	Whatman GmbH, Göttingen
Agar plates Bio Assay Dish	Nunc GmbH & Co. KG, Wiesbaden
ECL- films	Amersham biosciences
Eppendorf tubes	Eppendorf
Hybond-N	Amersham biosciences
IgG-Bovine Plasma Gamma Globulin	Bio-Rad
Imperial Protein Stain	Pierce, Rockford, USA
Lambda ladder PFG marker	New England Biolabs, Schwalbach/Taunus
PCR plates, 96-well	Abgene, Surrey, UK
Polypropylene tubes 15 ml and 50 ml, sterile	Greiner Labortechnik GmbH, Frickenhausen
Size standard, DNA marker PhiX174 DNA/BsuRI	MBI Fermentas, Germany
Sterile filters	Cellulose nitrate membrane, pore size 0.2 µm, Nalgene, Hamburg

2.5 Cells, Vectors and antibodies

DH5α chemocompetent cells	Promega, Mannheim
XL-Gold chemocompetent cells	Stratagene, CA, USA
BL-21(RIL) bacterial expression cells	Stratagene, CA, USA
pGEX-2T vector	Amersham biosciences, Germany
pET vector	Merck Biosciences
Primary antibodies	Molecular Probes, Invitrogen
	Santa Cruz Biotechnology Inc., CA, USA
Secondary antibodies	Molecular Probes, Invitrogen
	Sigma, USA

2.6 Solutions, buffers and media

Antibiotics (1000x)	50 mg/ml Ampicillin; 30 mg/ml Kanamycin
	34 mg/ml Chloramphenicol

LB medium	Bacto-tryptone 10 g Bacto-yeast extract 5g NaCl 10 g pH adjusted to 7.2; autoclaved
LB agar	LB medium 15 g/l Bacto agar
2x YT	16 g Bacto-tryptone 10 g Bacto-yeast extract 5 g NaCl H_2O added to 1 l; autoclaved
6x DNA loading buffer	0.2% Bromophenol blue 60% Glycerol 60 mM EDTA
5x RNA loading buffer	50% Glycerol 1 mM EDTA 0.25% Bromophenol blue
50x TAE buffer (1 litre)	242 g Tris base 57.1 ml Glacial acetic acid 100 ml 0.5 M EDTA pH 8.0
10x TBE buffer	108 g Tris base 55 g Boric acid 40 ml 0.5 M EDTA pH 8.0
TE buffer	10 mM Tris-HCl 1 mM EDTA; pH 8.0
10x TBS	100 mM Tris 9% NaCl pH adjusted to 7.4 with HCl
PBS	instamed PBS Dulbecco w/o Mg^{2+} , Ca^{2+}
Blocking buffer	1x TBS 3% BSA
4x Laemmli buffer 100ml	8g SDS 40 ml Glycerin 40 ml 0.6M Tris pH 6.8 80 mg bromophenol blue H_2O added to 80ml 20 ml β-Mercapto ethanol

SDS-PAGE running buffer	25 mM Tris-HCl pH 8.3 190 mM Glycine 0.1% SDS
Western blotting running buffer	20 mM Tris 150 mM Glycin 20% methanol 0.08% SDS
Ponceau-S staining solution	0.2% Ponceau 3% Trichloroacetic acid
Gel Filtration Buffer	0.1% CHAPS 10 mM Tris-HCl pH 7.5 5 mM EDTA 100 mM NaCl
Mitochondrial lysis buffer	1% Digitonin 100 mM NaCl 10 mM Tris-HCl pH 7.5 5 mM EDTA Complete Protease inhibitor
Protein elution buffer	25 mM Tris 192 mM Glycine 0.1% SDS

Glycerol stocks plasmids clones were prepared in 25% glycerol and stored at –80°C.

2.6.1 Solutions for mitochondrial isolation

Solution A	100 mM Saccharose 1 mM EDTA 20 mM MOPS pH 7.4 1 g/L BSA
Medium B	840 mL Solution A 10 mM Triethanolamine 1 mM PMFS 5% (w/v) Percoll Complete Protease inhibitor
Percoll gradient	30% (w/v) Percoll 250 mM Saccharose 0.1 mM EDTA
Wash solution	250 mM Saccharose 0.1 mM EDTA

Medium B (Brain)	85 ml Solution A 10 mM Triethanolamine 1 mM PMFS 5 % (w/v) Percoll Complete Protease inhibitor 0.1 g/L Digitonin

2.6.2 Solutions for silver staining

Fixer solution	50% methanol 12% acetic acid
Staining solution	0.2% silver nitrate 75 µl Formaldehyde for 100 ml solution
Developing solution	95 ml 6% Potassium carbonate 5 ml 0.02% Sodium thiosulphate 50µl Formaldehyde for 100 ml solution
Stop solution	30% acetic acid

2.7 Software

• BLAST	NCBI, Bethesda, USA
• Aida Image analyser v 4.015	Raytest GmbH, Germany
• Axio vision 3.1	Carl Zeiss Vision GmbH, Germany
• 3730 sequence Analysis software	Applied Biosystems, Weiterstdt
• DNAStar	Madison, Wisconsin, USA
• OLIGO 6.0	MBI, USA

3 METHODS

3.1 Molecular biology

Standard molecular biology methods were used according to Sambrook and colleagues (1989) and Asubel and colleagues (1997) and are not described here in detail. All centrifugations were performed at 4°C unless otherwise mentioned.

3.1.1 Isolation of plasmid DNA

3 ml LB media supplemented with appropriate selection antibiotics were inoculated with a single colony and grown overnight at 37°C on a shaker. Cells were centrifuged at 3,000 rpm for 10 min. Plasmid DNA was isolated using the QIAGEN Plasmid Mini Kit, which is based on a modified alkaline lysis procedure, followed by binding of plasmid DNA to an anion-exchange resin under appropriate low salt and pH conditions, according to the manufacturer's protocol. The DNA pellet was washed two times with 70% ethanol and dissolved in 30 µl 1x TE buffer.

3.1.2 DNA cloning

All the sub-clones generated in this study contain fragments that were PCR-amplified from clone 3F-1R-pCDNA (generated by cloning an ORF obtained from human OPA1 splice form-1 KIAA0567 in the pCDNA vector - gift by C. Alexander). The *Pfu* polymerase enzyme has 3'-5' exonuclease proof reading activity that reduces the errors in nucleotide incorporation during PCR amplification and hence was used in generating all fragments for DNA cloning. All PCR products were first cloned into the pGEMT-easy vector (Promega) by TA-cloning and thereafter the sequence of the clones were confirmed by sequencing using T7 and SP6 vector primers. The pGEMT clones confirmed for the insert were subcloned into bacterial expression vectors in the right reading frame using restriction enzymes incorporated into the PCR fragments through primers and/or those that are present in pGEMT vector. The details about the primers used and annealing temperatures are provided in the Chapter 7.2 and 7.3.

PCR

DNA was amplified by PCR using the high fidelity *Pfu* polymerase enzyme. The reaction volume was 50µl.

Template DNA	50 ng
10x buffer	5 µl
dNTP's (10mM)	1 ml

Forward Primer (5 pmol)	1 ml
Reverse Primer (5 pmol)	1 ml
Pfu polymerase (3 U/ml)	0.5 ml
H_2O to	50 ml

The following PCR profile was used:

1.	Initial denaturation	95°C	2 min
2.	Denaturation	95°C	30 s
3.	Annealing	55°C	30 s
4.	Extension	72°C	6 min (2 min for every 1000 bp)
5.	Cycle from step 2 to step 4		25x
6.	Final extension	72°C	10 min
7.	Storage	4°C	hold

The PCR products were agarose gel extracted.

Gel extraction and PCR purification

The gel extraction kit from Qiagen was used according to the manufacturer's instructions for PCR product purification and to extract DNA fragments from agarose gels.

Generating 'A-tailing' to blunt-ended PCR fragments

Pfu polymerase enzyme has 3'-5' exonuclease activity hence generates blunt-ended PCR fragments. dATP was added at the 3' end of the gel purified PCR product in order to enable TA-cloning of the PCR product into the pGEMT-easy vector.

Template	16 µl
10x buffer	2 µl
AmpliTaq polymerase	0.3 µl (2 U/µl)
dATP's	2 µl (0.2 mM final concentration)

After incubating the reaction mixture for 30 min at 72°C, the polymerase enzyme was inactivated by freezing the reaction mixture.

Ligation

The final reaction volume for ligation was 10 µl. 100 ng of vector was used with the molar ratio of vector to insert being set at 1: 3.

Vector	100 ng
3x insert	1 µl
10x buffer	1 µl

T_4-DNA-ligase	1 µl
H_2O to	10 µl

The reaction mixture was incubated at 24°C for 1 h. 3 to 5µl of ligated mixture was used for transformation into competent bacteria.

3.1.3 Chemotransformation

Chemocompetent bacteria were thawn on ice for 5-10 min. For a single transformation 1 aliquot (50 µl) of bacterial suspension was mixed with 3-5 µl of ligation product in an Eppendorf tube, equalling approximately 1-10 ng of circular plasmid DNA. Immediately after heat-shock (37°C for 45 s for DH5α; 42°C for 30s for XL-gold cells) the tube was placed on ice for 2 min. 950 µl of LB-medium was added and this suspension was incubated with shaking at 37°C for 1 h. After incubation, 100 µl were plated on an agar plate containing the appropriate selection antibiotic for the plasmid and incubated at 37°C over night.

3.1.4 Preparation of electro-competent bacteria

BL21(DE3)RIL cells were grown over night in 20 ml of LB-medium at 37°C. Further 200 ml of LB-medium was added and the bacteria were grown to an OD_{600nm} = 0.5–0.6. Cells were cooled on ice for 20 min then centrifuged at 4000g for 15 min. The pellet was washed two times with 200 ml of ice-cold distilled water. After the final centrifugation the pellet was resuspended in 20 ml 10% glycerol. Cells were aliquoted on dry ice into 50 µl aliquots and stored at –80°C until use.

Electroporation

Electrocompetent bacteria were thawn on ice for 5–10 min. For a single transformation 1 aliquot (50 µl) of bacterial suspension was mixed in a cold transformation cuvette with 3-5 µl of the ligation product, equalling approximately 1–10 ng of circular plasmid DNA. Immediately after the electroporation (U = 2.5 kV; C = 25 µF; R < 200 Ω; time pulse = 5 ms) 900 µl of LB-medium was added and this suspension was transferred to an Eppendorf tube. After incubating the cells at 37°C for 1 h, 100 µl were plated on an agar plate containing the appropriate selection antibiotic for the plasmid and incubated at 37°C over night.

3.1.5 DNA Sequencing

DNA sequencing was carried out using the dideoxy nucleotide chain-termination method (Sanger et al., 1977) with dye terminator labelling of purified plasmids or PCR products. Specific forward and reverse primer for PCR products and universal M13 forward and reverse

primer for purified plasmids were used. The DNA samples were precipitated using ethanol and the pellets were washed twice with 70% ethanol. The purified templates were dissolved in sterile water and the ABI PRISM Big Dye Terminator Cycle Sequencing Ready reaction kit was used to set up the sequencing reactions. About 300 to 400 ng of plasmid DNA (10% of the product size) and 50 to 100 ng of PCR fragments (10 ng per 100 bp) were used in one reaction. Sequences were analysed on ABI 377 and 3700 automatic sequencers.

3.2 RNA preparation

To avoid RNAse contamination, disposable plastic ware and RNAse-free water was used whenever possible. RNAse-free water was prepared by stirring double distilled water with 0.01% (v/v) DEPC overnight, followed by autoclaving to remove the DEPC.

3.2.1 Isolation of total RNA

Total RNA from mouse tissue was isolated according to Chomczynski and Sacchi (1987). With this method, cell homogenates are directly added to a monophasic acidic phenol guanidine isothiocyanate solution (TRIzol Reagent, Invitrogen). Upon adding chloroform, the RNA remains in the aqueous phase, while proteins and high molecular weight DNA enter the organic phase and the interphase, respectively. Total RNA is recovered by isopropanol precipitation.

50 to 100 mg of tissue was homogenised by grinding under liquid nitrogen and directly transferred into 1 ml of TRIzol, which maintains the integrity of the RNA during lysis. The mixture was homogenised in a dounce homogeniser and passed through a 29½ G needle. After incubation of the homogenized samples for 5 min at room temperature to permit the complete dissociation of nucleoprotein complexes, 0.2 ml chloroform per ml of TRIzol was added. The samples were mixed vigorously and centrifuged at 10,000 rpm for 15 min at 4°C to separate the aqueous and inorganic phases. The aqueous, upper phase was recovered, mixed with 0.5 ml of isopropanol, incubated for 10 min at room temperature and centrifuged at 12,000 rpm for 10 min at 4°C. The pellet was washed once with 75% ethanol, centrifuged again for 5 min, air-dried and dissolved in 50 µl DEPC treated water. Total RNA was stored at -80ºC.

3.2.2 Measurement of quantity and purity of total RNA

The concentration of RNA was determined by measuring the absorbance at 260 nm (A_{260}) in a spectrophotometer. RNA samples were diluted in RNAse-free water. Cuvettes were cleaned with 0.1 M NaOH, 1 mM EDTA followed by RNAse-free water. At A_{260} 1 unit corresponds

to 40 µg of RNA per ml. This relation is valid only for measurements in water. The concentration in µg/ml can be calculated from the absorbance value:

A_{260} x dilution factor x 40 µg/ml

The ratio of the absorbance values at 260 nm and 280 nm (A_{260}/A_{280}) provides an estimate of the purity of RNA with respect to contaminants that absorb in the UV spectrum, such as protein. Pure RNA has an A_{260}/A_{280} ratio of 1.8 to 2.1, ideally, in 10 mM Tris-HCl pH 7.5. Since all RNA samples were diluted in RNAse-free water for the measuring the concentration, the A_{260}/A_{280} ratio was determined in water. However, the A_{260}/A_{280} ratio is influenced by pH and since water is not buffered, the pH and the resulting A_{260}/A_{280} ratio can vary. RNA samples with an A_{260}/A_{280} ratio between 1.5 and 2 were considered to be sufficiently pure for further experiments.

3.2.3 DNAse treatment of total RNA sample and RNA cleanup

DNAse treatment was used to remove DNA from total RNA preparation. To the total RNA sample (~ 2 µg/µl diluted in RNAse-free water) 5 µl 10x RQ1 DNAse buffer, 5 µl 100 µM DTT and 2 units RQ1 RNAse-free DNAse (Promega) was added to a final volume of 50 µl. The mix was incubated for 30 min at 37°C. After the enzymatic reaction the volume of the sample was adjusted to 100 µl and the RNAeasy kit (QIAGEN) was used to clean up the total RNA. The RNA was eluted twice in the same tube with RNAse-free water to obtain a higher total RNA amount.

3.3 Reverse transcription

Total RNA was reverse transcribed by Superscript II Reverse Transcriptase (RT), an enzyme engineered from Moloney Murine Leukaemia Virus RT. Depending on further experimental analysis oligo (dT) primers, which bind to the polyA tail of the mRNA, or random hexamers were used.

3.3.1 First strand cDNA synthesis from total RNA

5-10 µg total RNA of each sample was mixed with 1 µl oligo dT_{12-18} (0.5 µg/µl), or 3 µl random hexamers (50 ng/µl). DEPC-treated water was added up to the final volume of 12 µl. Each sample was incubated at 70°C for 10 min for primer hybridisation and chilled on ice for at least 1 min. Samples were briefly centrifuged and 8 µl of the reaction mix, containing 4 µl 5x first strand buffer (Gibco), 2 µl DTT (0.1 M), 1 µl dNTP mix (10 mM), 1 µl RNAse inhibitor (Rnasin 40 U), were added. Contents of the tubes were mixed and incubated at 42°C (using oligo dT_{12-18}) or at 25°C (using random hexamers) for 5 min. 1 µl (200 U) of

Superscript II RT was added before incubating the samples at 42°C for 50 min. cDNA synthesis reaction samples containing random hexamers were first incubated at 25°C for 10 min, followed by 50 min at 42°C. The reaction was inactivated by incubating at 70°C for 15 min. 1 µl (2 U) of RNAse H was added to remove RNA followed by incubating the tubes at 37°C for 20 min. The cDNA was stored at -20°C. The cDNA synthesis was verified by amplification of the actin gene via PCR with control primers provided by the manufacturer (RETROScript, Ambion).

3.3.2 PCR amplification of cDNA

2.5 µl of reverse transcribed cDNA was used for PCR amplification with specific primer pairs. The reaction volume was 25µl.

Forward Primer (5 pmol)	1 µl
Reverse Primer (5 pmol)	1 µl
dNTP (10 mM)	2.5 µl
10x Buffer	2.5 µl
Taq polymerase (5U/µl)	0.15 µl
H_2O	15.35 µl
cDNA	2.5µl

The following PCR profile was used:

Initial denaturation	95°C	1 min	
Denaturation	94°C	30 s	
Annealing	55°C – 64°C	30-60 s	
Extension	72°C	30-60 s	35 cycles
Final extension	72°C	10 min	
Storage	4°C	hold	

The PCR product was applied to an agarose gel and bands of the expected size were gel-extracted and sequenced. The PCR products were also analysed on a 5% polyacrylamide gel followed by silver staining.

3.4 Gel electrophoresis

Electrophoresis through agarose or polyacrylamide gels lies near the heart of molecular cloning and is used to separate, identify and purify nucleic acid fragments and proteins. The technique is simple, rapid to perform and capable of resolving fragments of DNA and/ RNA.

3.4.1 Formaldehyde gels (RNA)

RNA quality and quantity was analyzed via formaldehyde-agarose gel electrophoresis. RNA retains much of its secondary structure during electrophoresis unless it is first denatured. The addition of formaldehyde to the agarose gel maintains the RNA in its linear (denatured) form. Electrophoresis equipment was soaked in DEPC treated water. A 1.25% agarose gel was prepared by dissolving 1.25 g agarose in 85 ml sterile water in the microwave. After cooling down to 65˚C, 10 ml pre-warmed 10x MOPS buffer and 5 ml pre-warmed (55°C) formaldehyde were added and the gel was poured. The gel was placed into the electrophoresis tank and equilibrated in running buffer (1x MOPS buffer, 12.3 M formaldehyde) for 30 min. 4.7 µl of the RNA sample (~ 10-20 µg) was mixed with 2 µl of 5x MOPS buffer, 3.3 µl of formaldehyde and 10 µl of formamide. The samples were incubated at 65°C for 10 min and cooled on ice. 2 µl of RNA loading buffer was added to each sample and samples were loaded. The gel was run at 5 V/cm, measured as the distance between the electrodes. The gel was stained in 0.5 µg/ml ethidium bromide in 1x MOPS buffer.

3.4.2 Agarose gel electrophoresis (DNA)

Conventional agarose gel electrophoresis was used to analyze DNA fragments. The electrophoretic mobility of DNA fragments mainly depends on the fragment size and to a lesser extent on the conformation of the DNA, type and concentration of agarose used as well as applied voltage and electrophoresis buffer used. Agarose gels have greater range of separation and can resolve DNAs from 50 bp to 20 kbp in length. For separating smaller sized PCR fragments 2% and 3% gels were used. Low melting agarose (LMP) gels were used for the purification of PCR fragments. All agarose gels contained 1 µg/ml ethidium bromide for visualization of the DNA bands and were run in 1x TAE buffer.

3.4.3 Neutral polyacrylamide gel electrophoresis (DNA)

Polyacrylamide gel electrophoresis (PAGE) is used as electrically neutral matrix to separate double-stranded DNA fragments according to size and single-stranded DNA according to size and conformation. Polyacrylamide gels are more effective, than agarose gels in separating small fragments of DNA (5-500 bp) and molecules of DNA whose lengths differ by as little as 0.1% (i.e., 1 bp in 1000 bp).

Native polyacrylamide gels

Native or nondenaturing polyacrylamide gels are used for separation and purification of fragments of double-stranded DNA molecules according to their size. However, the mobility

is also affected by the base composition and the sequence, so that duplex DNA fragments of exactly the same size can differ in mobility by up to 10%. For analysing products up to a size of 500 bp, a 5% native polyacrylamide gel is sufficient to achieve a resolution of up to 1 bp in size difference.

Gel plates of 18 x 16 cm^2 were washed thoroughly and rinsed with double distilled water, followed by ethanol and air-dried. One of the plates facing the gel matrix was treated with siliconizing fluid (Sigmacote) to ease the removal of the gel in-between the glass plates without tearing the gel. The Sigmacote was completely wiped dry and the plates were assembled and secured by clamps. 100 ml of 5% gel solution was prepared with the following components:

Acryl amide: Bis solution (29:1)	16.6 ml
5x TBE	20 ml
Distilled water	62.7 ml
10% APS	0.7 ml
TEMED	35 µl

After polymerisation, the glass plates were secured into the electrophoresis system and samples were loaded. 2 µl of 6x loading buffer was added to 8 µl of the sample and loaded into the wells. Gels were run at 5 V/cm, measured as the distance between the electrodes, in 1x TBE buffer. After the run was completed the gel was carefully transferred into a staining chamber and silver stained.

Urea-denaturing polyacrylamide gel

Denaturing polyacrylamide gels are used for the separation of single-stranded DNA. Such gels are polymerised in the presence of 7 M urea that suppresses base pairing in nucleic acids. Denatured DNA migrates through these gels at a rate that is almost completely independent of its base composition and sequence. The gel was prepared in a similar manner to that of native polyacrylamide gels except for the 7 M urea that was dissolved in the polyacrylamide solution before pouring the gel. 6 µl formamide was added to 6 µl of the samples and the samples were incubated at 99°C for 10 min and snap cooled on ice. 3 µl of 6x loading buffer was added to each sample and loaded into the wells. The gel was stained with silver for visualising the DNA fragments.

3.4.4 SDS-polyacrylamide-gel electrophoresis (Protein)

Protein samples (prepared in Laemmli buffer followed by boiling at 95°C for 5 min) were separated on 12% mini SDS-PAGE gels (Mini-protein II Dual Slab Cell, Bio-Rad; Laemmli

1970). In this system, proteins denatured in the presence of SDS and 2-mercaptoethanol as thiol reducing agent acquired a rod-like shape and a uniform charge-to-mass ratio proportional to their molecular weights. The gels were stained with colloidal Coomassie stain and the protein sizes were determined by comparing the migration of the protein band to a molecular mass standard (PageRuler prestained Marker, Fermentas).

3.4.5 Silver staining

The PAGE gels were first fixed in fixer solution for 2 h with changing the solution once in between. After fixing, the gel was washed once with 20% ethanol and once with distilled water for 10 min each. The gel was then sensitized with 0.02% sodium thiosulphate for 1 min. Following sensitisation the gel was washed three times with distilled water for 20 s and then stained with staining solution for 30 min. Before developing, the gel was washed two times with distilled water for 20 s. The bands were developed with developing solution. The development was stopped with stop solution once the bands were clearly visible. The gel was then washed once with 20% ethanol before drying the gels.

3.4.6 Electro-elution of proteins

The *OPA1: 111-960* GST fusion protein formed inclusion bodies during expression that remained insoluble. Hence after pelleting, the bacterial cells were solubilised in Laemmli buffer, boiled for 5 min at 95°C and separated on 8% SDS-PAGE. The overexpressed protein was excised and eluted out from the gel according to the instructions provided by the manufacturer of Model 422 Electro-Eluter assembly (Bio-Rad). In brief, the membrane caps were soaked in protein elution buffer for 1 h at 60°C. The gel slice was loaded into the Electro-Eluter and elution was performed at 8-10 mA/ glass tube constant current for 3-5 h. The sample in the membrane cap was transferred into a microfuge tube and dialysed against PBS to remove SDS bound to the protein. The precipitated protein, during dialysis, was separated from the soluble fraction after a brief centrifugation at 10,000 rpm for 10 min. The soluble protein fraction was used for immunisation.

3.5 Western blotting

Proteins were transferred from the SDS gel onto a nitrocellulose membrane for 90 min at 200 mA using SD Semi-dry Transblot Apparatus (Bio-Rad). Then, the membrane was quickly rinsed with distilled H_2O and protein bands detected with a Ponceau-S stain. After washing twice with H_2O, the membrane was incubated with gentle agitation throughout all steps. After bathing in blocking buffer for 1 h at RT to reduce unspecific binding, the membrane was

incubated with primary antibody, diluted in blocking buffer for 2 h at room temperature. Next, the membranes were washed (two quick washings followed by two washings with 5 min incubation) with TBS-Tween (0.5%) and incubated with appropriate secondary antibodies diluted in blocking buffer for 2 h at RT. The membranes were washed as previously and the protein bands were detected using chemiluminescent substrate from Amersham biosciences and visualized on Amersham ECL films after automatic development in Curix 60 film processor.

3.6 Prokaryotic protein expression

3.6.1 Expression of fusion proteins in bacteria

For prokaryotic expression, a bacterial clone carrying the desired plasmid was revived in 3 ml LB broth containing ampicillin (100 μg/ml) and chloramphenicol (34 μg/ml) overnight at 37°C with constant shaking. The next day, 10 ml of fresh broth including antibiotics was inoculated with 100 μl of overnight culture and left to grow to an OD_{600} of 0.6-0.7 at 37°C. Then, the expression of recombinant protein was induced by adding IPTG to a final concentration of 1 mM. After an additional 3 h of growth, the bacterial cells were pelleted by centrifugation at 10,000 rpm for 5 min. After washing the pellets once with ice cold PBS, the pellets were stored at -80°C until use. Overexpression of the fusion proteins was confirmed by SDS-PAGE analysis.

3.6.2 Generation of hybridoma lines and production of ascites fluid

Antibodies were prepared by INSERM institute (Strasburg, France). In brief, six-week-old female BALB/c mice were injected intraperitoneally with 200 μg of purified recombinant protein and 200 μg of polyI/C as adjuvant. Three injections were performed at 2-week intervals. Four days prior to hybridoma fusion, mice with positively reacting sera were reinjected. Spleen cells were fused with Sp2/0.Agl4 myeloma cells as described by St. Groth (1980). Hybridoma culture supernatants were tested at day 10 by ELISA, for cross-reaction with OPA1 protein. Positive supernatants were then tested by immunocytochemistry and western blot of COS-1 cells. Specific cultures were cloned twice on soft agar. Six specific hybridomas lines were established. For ascites fluid production, 2 x 10^6 cells of the fastest growing hybridoma with the greatest antibody production (clone-1 OPA 1A8) were injected into pristane primed BALB/c mice. The specificity of this antibody was confirmed using western blotting, immunocytochemistry and Mass-spectrometry.

3.7 Protein preparation

3.7.1 Isolation of mitochondria from tissue

Two to three months old C57/Bl-6 mice were used for the isolation of mitochondrial proteins. Mice were killed by head dislocation and the tissues were dissected and washed with ice-cold PBS. All the subsequent steps were performed on ice. These tissues were put in appropriate ice-cold Medium B and minced with tissue mincer (Polytron PT-3100). The lysate was centrifuged for 10 min at 600 g and the pellet containing the cell debris and nuclei was discarded. The supernatant was transferred it into a fresh tube and centrifuged at 10,000 g for 10 min to pellet the mitochondria and lysosomes. The supernatant that contained the cytoplasmic fraction of the cell was discarded. The pellet was washed once by resuspending in 25 ml wash solution and centrifuging at 8,000 g for 10 min. The pellet was resuspended in 1 ml wash solution and overlaid on a 30% Percoll gradient (1 ml resuspension on every 8 ml gradient) followed by centrifugation at 50,000 g for 1 h 30 min. Mitochondria free of lysosomes concentrated in a dark brown band, which was carefully aspirated with a needle into a fresh tube and washed once with wash solution (every 1 ml mitochondrial band with 8 ml wash solution). The mitochondria were pelleted down at 10,000 g for 10 min and then transferred into a fresh Eppendrof tube after resuspending the pellet in 1 ml wash solution. This pellet was washed again once with wash solution as described before. The purified mitochondrial pellet was stored at –80°C till used, after estimating its concentration by Bradford reagent.

3.7.2 Alkaline extraction of proteins

The basic principle underlying carbonate extraction is to release peripheral membrane proteins from within membrane bound compartments by converting the limiting membranes into flat sheets (Fujiki et al., 1982). Following centrifugation, integral membrane proteins are pelleted, while peripheral membrane proteins are released into the supernatant. This preparation typically separates proteins that are integrated into the membrane with transmembrane loops versus soluble cytoplasmic and membrane associated proteins. In brief, 1 mg of isolated mitochondria was mixed to 900 µl of ice cold 0.2 M Na_2CO_3, adjusted to pH 13 with 1 N NaOH. After incubation on ice for 30 min and mixing the sample intermittently by gently inverting the tube, the membranes were pelleted by centrifugation for 1 h at 100,000 g. Proteins in the supernatant were precipitated with TCA (Chapter 3.7.4) and pellet was dissolved in 140 µl Laemmli buffer. The membrane pellet also was dissolved in 140 µl of Laemmli buffer and 20 µl of the samples were analysed by western blotting.

3.7.3 Immunoprecipitation of OPA1 protein

Protein G Sepharose CL-4B beads (Amersham) were prepared by washing five times with five column volumes of ice-cold PBS buffer containing complete protease inhibitor. After each wash, the beads were spun briefly at 3,000 rpm. Appropriate amount of antibody was bound to the prepared beads by mixing gently at 4°C for 2 h. The beads were washed two times with five column volumes of ice-cold PBS buffer containing complete protease inhibitor to remove unbound antibodies. Next, the antibody bound beads were equilibrated by washing two times with five column volumes of mitochondrial lysis buffer.

Soluble mitochondrial lysates were prepared by adding the isolated mitochondria (Chapter 3.7.1) to mitochondrial lysis buffer at a final concentration of 2 mg/ml, and incubating them on ice for 40 min with gentle intermittent mixing, followed by centrifugation at 100,000 g for 1 h to remove the insoluble membrane fraction. The soluble mitochondrial lysates were incubated with the antibody-bound Sepharose beads at 4°C. After 4 h of gentle mixing, the beads were washed four times with five column volumes of mitochondrial lysis buffer. After washing the beads, Laemmli buffer was added and boiled for 5 min at 95°C. All of the liquid supernatant containing the proteins was carefully eluted using a Hamilton syringe avoiding any contamination with beads. The eluates were stored at –80°C until further use.

3.7.4 Trichloroacetic acid precipitation of proteins

The proteins present in very low concentrations, in samples, were precipitated with trichloroacetic acid (TCA) and deoxycholate (DOC). In brief, to one volume of protein solution 1/100 volume of 2% DOC was added, vortexed and incubated on ice for 30 min. After incubation one-tenth volume of 100% TCA was added, vortexed and incubated for over night at 4°C. The precipitated proteins were pelleted by centrifuging at 16,000 g for 15 min. The pellets were then washed two times with one volume of ice-cold acetone. After washing the pellets were air dried and resuspended in required volume of 1x Laemmli buffer. In case the sample appeared acidic yellow due to the presence of TCA traces, titrate it with 1 M Tris-HCl pH 8.5 to restore the blue colour.

3.7.5 Protein assay of Bradford

A colorimetric protein assay, based on the Bradford method, was used for the measurement of protein concentration. This assay is based on a shift in the absorbance maximum when Coomassie® Brilliant Blue G-250 dye associates with proteins. The Lambert-Beer's Law is applied for quantification of protein by selecting an appropriate ratio of dye volume to sample concentration. At the assay pH, the dye molecules are doubly protonated and are present as

the red cationic dye form. Binding of the dye to protein stabilizes the blue anionic dye form, detected at 595 nm. Dye binding requires a protein containing active basic or aromatic residues. 1 ml of Bradford reagent (Bio-Rad) was mixed with 57 µl of the sample, incubated for 5 min at room temperature and absorbance was determined at 595 nm. A calibration curve was established each time a protein assay was performed with bovine IgG dilutions of known concentrations. Using the standard curve, the concentration of each sample was determined according to its absorbance by interpolation (Bradford, 1976).

3.8 Protein analysis

3.8.1 Mass spectrometry

Mass spectral analysis was done in collaboration with Albrecht Otto and Eva-Christina Müller (Department of Neuroproteomics, MDC, Berlin). In brief, the proteins were immunoprecipitated with OPA1 antibodies, separated on an extra long (28 cm) 8% SDS-PAGE gel and stained with the new Imperial Protein Stain (Pierce, Rockford, USA). Protein bands of interest were excised from the gel and digested with trypsin or Glu-C. Resulting fragments were identified after chromatographic separation of the peptide mixture on an LC Packings 75-µm PepMap C18 column (Dionex, Idstein, Germany) by using a capillary liquid chromatography (CapLC) system delivering a gradient from 5.0 to 80% acetonitrile and 0.1% formic acid at a flow rate of 200 nl/ml. The eluting peptides were ionized by electro spray ionization using a Q-TOF hybrid mass spectrometer (Micromass, Manchester, United Kingdom). This instrument, in automated switching mode, selected precursor ions based on intensity for peptide sequencing by collision-induced fragmentation tandem MS. The MS/MS analyses were conducted by using collision energy profiles that were chosen based on the m/z value, the charge state of the parent ion, and the fragment ion masses and intensities. The results were correlated with the protein databases by using the Mascot software (www.matrixscience.com). The results were validated manually.

3.8.2 N-terminal microsequencing

N-terminal microsequencing, using Edman degradation, for OPA1 proteins was done at WITA GmBH (Wittmann Institute of technology and Analysis of Biomolecules). Proteins were immunoprecipitated with OPA1 monoclonal antibody and separated on a 28 cm long 8% SDS-PAGE gel (run at constant 15 mA) overnight. The proteins were blotted on to sequi-blot polyvinyl difluoride membrane (PVDF) for 90 min at 200 mA using SD Semi-dry Transblot Apparatus (Bio-Rad), after equilibrating the membrane for 5 min in 100% methanol.

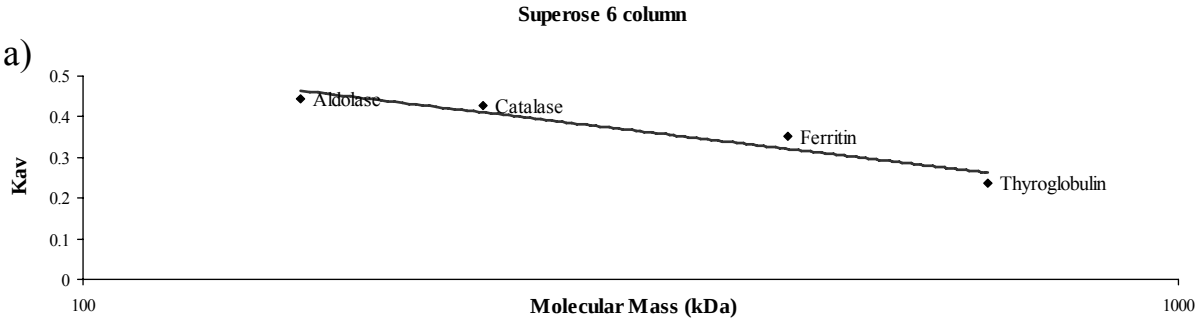
Subsequently, the membrane was stained with Imperial Protein Stain (Pierce, Rockford, USA) according to the manufacturer. The bands of interest were excised from the blot and processed for microsequencing by Edman degradation.

3.8.3 Size exclusion chromatography

The use of gel filtration chromatography or size exclusion chromatography for the determination of the molecular weight of proteins is well documented. The technique is based on the ability of the gel filtration media such as Superose to separate the molecules according to size. Molecular weight determinations by gel filtration are carried out by comparing average elution volume parameter, such as K_{av} of the protein of interest, with values obtained for several known calibration standards. In principle for homologous series of compounds a sigmoid relationship exists between their various elution parameters and the logarithm of their molecular weights. A calibration curve was prepared by measuring the elution volumes of several standard proteins, calculating their corresponding K_{av} values and plotting their K_{av} values versus the logarithm of their molecular weight. The molecular weight of an unknown protein can be determined from the calibration curve once its K_{av} value is calculated from the measured elution volume.

$$K_{av} = \frac{\text{Elution volume - Void volume}}{\text{Total volume - Void volume}}$$

a)



b) $\mathbf{x} = e^{[(\mathbf{y}-1.1708)/(-0.1396)]}$; $\mathbf{x}$ = Molecular mass and $\mathbf{y} = K_{av}$

Figure 3.1: Calibration of Superose-6 column.

a) Calibration curve using various known protein standards.

b) Formulae generated from the curve for calculating the molecular mass of unknown proteins.

For this study, Superose 6 column (HiLoad 16/60 Superose 6, Pharmacia Biotech) was used to efficiently resolve the protein complexes ranging from 100 kDa to 1000 kDa. The column was calibrated with different standard proteins as instructed by the manufacturer using gel

filtration running buffer. The K_{av} was calculated using the formulae given below. The total volume of the column was taken as 24 ml (as mentioned by the manufacturer) and the void volume is the elution volume of Blue Dextran 2000, which was calibrated as 7.625 ml. The calibration curve was plotted from the calculated K_{av} to logarithm of the molecular mass for the various standards (Fig. 3.1a). To calculate the molecular mass of the unknown protein from K_{av} a formulae was deduced from the calibration curve using the computer program (Fig. 3.1b).

Table 3.1: Recalculated molecular mass of the standard proteins.

Protein standards	Molecular weight (kDa)	Elution volume (ml)	K_{av}	Recalculated Mass (kDa)
Thyroglobulin	669	11.5	0.2366	806 (+20%)
Ferritin	440	13.375	0.3511	355 (-19%)
Catalase	232	14.625	0.4275	205 (-11%)
Aldolase	158	14.875	0.4427	184 (+16%)

Mitochondria isolated from mouse tissues were lysed in mitochondrial lysis buffer for 45 min on ice, occasionally mixing the sample by inverting the tube. The suspensions were centrifuged at 100,000 g for 45 min. The soluble fraction was filtered and injected in to the column equilibrated with running buffer. 30 fractions were collected from 5 ml to 20 ml elution volumes and the proteins in these fractions were precipitated with TCA (Chapter 3.7.4). The pellets were dissolved in Laemmli buffer and analysed by western blotting.

3.9 Immunocytochemistry

Mitochondrial live staining was achieved using MitoTracker (Molecular Probes). 50 µg of MitoTracker red (M-7513) was dissolved in 200 µl DMSO. Growth media on the cultured cells was replaced with dye solution (33 µl of the dye in 5 ml HBSS) and the cells were incubated at 37°C. After 45 min the dye was replaced with HBSS for 30 min at 37°C in order to wash out excess of MitoTracker from the cytoplasm. Following three washes with PBS, the cells were fixed with 4% paraformaldehyde in PBS for 10 min and then washed three times with PBS. After permeabilisation with 0.05% Triton X-100 in PBS for 5 min and washing with PBS, non-specific binding was blocked by incubating the cells in 3% normal goat serum in PBS with 0.05% Triton X-100 for 30 min. Then the cells were incubated with the primary antibody (diluted 1:500 in 3% goat serum) over night at 4°C. The unbound antibody was removed by three washes with PBS followed by incubation with Alexa Fluor 488 and/or Alexa Fluor 594-coupled secondary antibodies (dilution 1:250) for 1 h at RT. After subsequent three PBS washes, the cells were mounted with Citifluor and examined under a

fluorescent microscope (Carl Zeiss) Alexa Fluor 488 light emission was captured with the XF22 filter (excitation 495 nm, emission 519 nm) and Alexa Fluor 594 were detected using the XF33 filter (excitation 590 nm, emission 617 nm, Omega Optical).