2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Chemicals and enzymes

Chemicals used in this thesis were purchased from Roth (Karlsruhe, Germany) and Sigma (Deisenhofen, Germany) unless indicated otherwise.

2.1.2. Bacterial strains

Escherichia coli DH-5-α (Invitrogen)
Escherichia coli XL1-Gold (Invitrogen)

2.1.3. Vectors/plasmids

pGEM-Teasy (Promega)
pTV.flox (gift of C. Birchmeier)

2.1.4. Cell lines

ES cell line:

Embryonic stem (ES) cells from the line E14.1, derived from the 129/Ola mouse strain (Hooper et al., 1987; Kuhn et al., 1991) were a gift of C. Birchmeier and they were used for introducing targeted mutations into the mouse.

Feeder cells:

Primary mouse fibroblast (Feeder) cells were prepared from embryos derived from mouse strains homozygous for a transgene containing a *neomycin* resistance cassette (*neoR*) and were a gift of C. Birchmeier.

MEFs:

Mouse embryonic fibroblasts (MEFs) were prepared from $OPA1^{+/+}$ and $OPA1^{-/-}$ E8.5 embryos in the laboratory of D. C. Chan. $OPA1^{+/-}$ mice were first crossed to the Black

Swiss outbred strain, litters were dissected and embryonic fibroblasts were cultured in DMEM containing 15% fetal calf serum, 1 mM L-glutamine, 1 mM pyruvate, and 50 µg/ml uridine. After one passage, cells were immortalized with SV40 large T antigen, as described (Chen et al., 2003).

2.1.5. Mouse strains

C57BL/6	Charles River
129/Ola	C. Birchmeier

2.1.6. Bacterial and cell culture media

Mouse Fibroblast Medium:

500 ml Dulbecco's MEM (DMEM) with Glutamax-I, 4500 mg/l Glucose, with Pyridoxin, Natriumpyruvat (Gibco BRL)

60 ml fetal calf serum (FCS; heat inactivated at 55°C for 30 min, Sigma)

5.7 ml 100x non-essential aminoacids (Gibco BRL)

5.7 ml Penicillin/Streptomycin-solution (10000 U/ml (units/ml) Penicillin G/10000 µg/ml Streptomycin; Gibco BRL)

1.2 ml 50 mM ß-Mercaptoethanol (Gibco BRL)

ES Cell Medium:

500 ml DMEM/Glutamax (see above, Gibco BRL)

90 ml FCS (heat inactivated at 55°C for 30 min, Sigma)

6 ml 100x non-essential amino acids (Gibco BRL)

6 ml Penicillin/Streptomycin-solution (Gibco BRL)

1.2 ml 50 mM ß-Mercaptoethanol (Gibco BRL)

60 µl leukaemia inhibitory factor (LIF) (500-1000 U/ml)

Mouse Embryonic Fibroblasts (MEFs) Medium:

500 ml Dulbecco's MEM (DMEM) with Glutamax-I, 4500 mg/l Glucose, with Pyridoxin, Natriumpyruvat (Gibco BRL)

60 ml FCS (Sigma)

6 ml 100x non-essential aminoacids (Gibco BRL)

5.7 ml Penicillin/Streptomycin-solution (10000 U/ml Penicillin G/10000µg/ml Streptomycin; Gibco BRL)
1.2 ml 50 mM ß-Mercaptoethanol (Gibco BRL)
LB (Luria broth) Medium:
10 g/l bacto-tryptone
5 g/l yeast extract
10 g/l NaCl

2.1.7. Antibodies

a) Primary

Monoclonal anti-mouse OPA1 (V.R. Akepati); dilution 1:5000
Monoclonal anti-mouse HSP60 (Sigma); 1:500
Monoclonal anti- mouse β actin (Sigma); 1:5000
Monoclonal anti- mouse cytochrome c (Beckston Dickinson); 1:800
Polyclonal anti-rabbit cytochrome c (Santa Cruz Biotechnology); 1:800
Antibodies specific for oxidative phosphorylation (Molecular Probes):
Monoclonal anti- mouse NADH (Complex I; 39 kDa subunit (SU)); 1:1000
Monoclonal anti- mouse succinate dehydrogenase, SDH (Complex II; 70 kDa SU); 1:2000
Monoclonal anti- mouse cytochrome c oxidase, COX (Complex IV; SU I); 1:500
Monoclonal anti- mouse ATPase (Complex V; SU ß); 1:2000

b) Secondary

Anti-mouse IgG, HRP (horsereddish peroxidase) coupled (Sigma); dilution 1:10.000
AlexaFluo-594 anti-mouse IgG (Molecular Probes); 1:800
Anti-rabbit, HRP coupled (Sigma); dilution 1:30.000

2.2. Methods

Besides the techniques detailed in the following section, standard protocols for various procedures in molecular biology, like molecular cloning, vector constructions, Southern and northern blot analyses were performed according to “Molecular Cloning” (Sambrook,

2001), Current Protocols in Molecular Biology (Ausubel et al., 2000) or manufacturers' instructions in the case of kits.

2.2.1. Bioinformatics

Computer analysis of DNA sequences was accomplished using the following web sites:
http://www.ncbi.nlm.nih.gov/gquery/gquery.fcgi (information on *OPA1*)
http://www.celera.com/ (full genomic sequence of *OPA1*)
http://www.ncbi.nlm.nih.gov/BLAST/ (cDNA sequence search)
Programs used to analyse (EditSeq) and align (SeqMan) sequences and draw restriction maps (MapDraw) were from a software package: Lasergene- DNASTAR.
http://www.ncbi.nlm.nih.gov/entrez/ (EST (Expressed Short Tags) search)
ESTs are cDNAs most often generated by reverse transcription of polyA$^+$-RNA initiated by an oligodeoxythymidylate (oligo(dT)) primer that binds to the poly(A) tail of the transcripts. All ESTs in these alignments were examined for the presence of residual poly(A) tails. Mispriming of the oligo(dT) primer to genomically occurring poly(A) stretches can result in the creation of false poly(A) tails upon first-strand synthesis of the cDNAs. Therefore, each polyadenylated EST was checked for the presence of polyadenylation signals and probable sites of mispriming in vicinity to the putative cleavage site. Sequences fulfilling these criteria and containing at least 7-10 adenines within their final 15 bases were accepted as properly processed mRNA ends.

2.2.2. Molecular Biology

2.2.2.1. Plasmid DNA isolation

Isolation of plasmid DNA was done using ``Plasmid Mini Kit``, ``Plasmid Midi Kit`` and ``Plasmid Maxi Kit`` (Quiagen) according to manufacturer's protocols.

2.2.2.2. Isolation of genomic DNA from embryonic stem (ES) cells

ES cells were screened for homologous recombination events by Southern hybridisation of genomic DNA. For the genomic DNA isolation, ES cells were grown in gelatinised 96 well plates up to a confluent layer. Cells were rinsed twice with phosphate buffered saline

(PBS; Gibco) and lysed in 50 µl ES cell lysis buffer per well (10 mM Tris-HCl, pH 7.5, 10 mM EDTA, 10 mM NaCl, 0.5% N-Lauroylsarcosin, 200 µg/ml proteinase K) at 60°C in a humid chamber over night. The DNA was precipitated for 30 min at room temperature with 100 µl of 100% ethanol containing 0.15 M sodium acetate, pH 5.2. Following precipitation, DNA was washed twice with 70% ethanol, air-dried for 20 min and directly digested with the corresponding restriction enzyme (MfeI). Restriction digests were performed at 37°C over night in 50 µl reaction mix (1x restriction buffer, 100 µg/ml bovine serum albumin (BSA), 50 µg/ml RNAse, 10-15 U of MfeI restriction enzyme).

2.2.2.3. Isolation of genomic DNA from mouse tissue

Genomic DNA was isolated from the tissues with the help of Dneasy Tissue Kit (Qiagen) according to manufacturer's protocol. From the tails DNA was isolated using 200 µl of the lysis buffer (50 mM KCl, 10 mM Tris-HCl pH 8.3, 2.5 mM $MgCl_2.6H_2O$, 0.45% NP40, 0.45% Tween^{20} and 60 µg/ml proteinase K final) at 55°C (Thermomixer comfort, Eppendorf) for 3 hours. Proteinase K was deactivated 5 min at 95°C and 1-5 µl of this mixture was used for PCR-genotyping.

2.2.2.4. Isolation of genomic DNA from the embryos

For embryo genotyping DNA was not extracted as usual, because of the very small amount of cells. Embryos were lysed over night at 55°C in 10-15 µl of the lysis buffer (1x PCR buffer, 0.2 µg/µl of proteinase K). Proteinase K was deactivated 5 min at 95°C and this mixture was used directly for PCR genotyping.

2.2.2.5. Isolation of total RNA from mouse tissues

Most important for all RNA experiments is to guarantee the isolation of intact RNA, which is difficult due to chemical instability of RNA and the ubiquitous presence of RNases. Thus, it is important to optimise the isolation of RNA from a given biological source and to prevent introduction of RNases and inhibitors of reverse transcriptase such as guanidinium salts, SDS (sodium dodecyl sulphate) and EDTA (ethylene-diaminetetraacetic acid). To avoid RNase contamination all solutions should be treated with diethylpyrocarbonate (DEPC; Roth), apart from solutions containing amino groups (e.g., Tris (Tris-(hydroxymethyl) aminoethane), MOPS (3-(N-morpholino)propanesulfonic

acid), EDTA, HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), etc.) because they inactivate DEPC. Those should be prepared in DEPC-treated water.

RNA from various mouse tissues was isolated using TRIzol method. Tissues were placed in 1 ml of TRIzol Reagent (Invitrogen) and homogenized using glass-teflon homogenisers (Th.Geyer). The volume of the tissue should not exceed 10% of the volume of the TRIzol reagent. The samples were incubated 5 min at room temperature and then 0.2 ml of chloroform per 1 ml of TRIzol Reagent was added. The samples were shaken vigorously for 15 s, incubated at room temperature for 2–3 min and centrifuged at 12000 g (Centrifuge 5415 R, Eppendorf) at 4°C. Following centrifugation the upper aqueous phase was transferred to a fresh tube with 500 µl of isopropanol per 1 ml of TRIzol. After 10 min incubation at room temperature, the samples were centrifuged with 12000 g at 4°C for 15 min. The pelleted RNA was washed once with 1 ml of 75% EtOH in DEPC-water and recentrifuged at 7500 g for 5 min at 4°C. The supernatant was discarded, the pellet dried, but not completely. The pellet was then resuspended in DEPC-water and incubated for 10 min at 55- 60°C to dissolve the RNA completely. After determination of the concentration, the RNA samples were stored at -70°C.

2.2.2.6. Measuring of Nucleic Acid Concentration by UV-Spectrophotometry

By UV-spectrophotometry (SmartSpec^{TM} 3000, BIORAD) the concentration and the purity of a sample is determined. The nucleic acids absorb UV light at 260 nm. The OD_{260} = 1.0 is equal to 50 µg/ml of double-stranded DNA (dsDNA), 33 µg/ml of single-stranded DNA (ssDNA) and 40 µg/ml of RNA. To calculate a protein contamination of a sample the quotient OD_{260}/OD_{280} is used. A value of 1.8 for a sample ensures a good DNA quality.

2.2.2.7. PCR

Polymerase Chain Reaction is a cyclic, enzymatic amplification by Taq-polymerase of a specific segment of DNA defined by single stranded oligonucleotides - the primers. Optimal PCR conditions were established according to general rules (Innis, 1989). For all PCR reactions, if not indicated differently, same cycling conditions were used with the changes in annealing temperature (AT) and elongation time (ET). Those conditions were: **94°C for 2 min (1x); 94°C for 10 sec, AT°C for 30 sec, 72°C for ET (35x); 72°C for 7 min**. For amplification of fragments up to 3 kb, AmpliTaq DNA Polymerase (Applied Biosystems) was used, and for fragments longer than 3 kb Expand Long Template PCR

System (Roche), according to manufacturer's protocol. PCR genotyping of tails and embryos was done using FastStart Taq DNA Polymerase (Roche). All PCR reactions were performed using PCR Termo-Cycler (DNA Machine, BIORAD). In order to visualise PCR products 8-12 µl of 25 µl PCR reaction were mixed with gel loading buffer (1x final; 6x stock: 15% Ficoll-400, 0.25% xylene cyanol FF, 0.25% bromophenol blue) and loaded on agarose gels. Agarose (0.7 – 3.0%) was boiled in 1x TAE buffer (1 l of 50x stock: 242 g Tris, 100 ml 0.5 M Na_2EDTA (pH 8.0), 57.1 ml glacial acetic acid) and finally ethidium bromide solution was added (0.5 µg/ml). DNA was visualized with UV-light (312 nm) and directly photographed (Chemi Doc, Biorad). For cloning PCR products were purified using ``PCR Purification Kit`` (Quiagen). Primers were purchased from Biotez. A list of all primers and reaction conditions used for each PCR is presented below.

Cloning the probes for Northern blots

Human:

Mitochondrial leader probe:

hML.Forward: ATGTGGCGACTACGTCGGGCCG

hML.Reverse: GGTGCTAACTTTACAAGGTC

Expected product: 500 bp

Conditions:AT=55°C; ET=30 s

Acidic domain probe:

hAD.Forward: GGTGGCTACACAGCCAAAAAGAC

hAD.Reverse: CACTTTCAGATCCACGATCTG

Expected product: 330 bp

Conditions: AT=55°C; ET=30 s

CC1 domain probe:

hCC1.Forward: CAGACAAAGAGAAAATTGACCA

hCC1.Reverse: TGACCTGAGGTAAAAAAGTCCT

Expected product: 230 bp

Conditions: AT=55°C; ET=30 s

GTPase domain:

hGTP.Forward: TGGAACGATTAGAAAAGGAGAACAAAG

hGTP.Reverse: GCTTCAATGCTTTCAGAGCTG

Expected product: 850 bp

Conditions: AT=55°C; ET=1 min

Middle domain probe:

MD.Forward: CGAGAGTCTGTTGAACAACAG

MD:Reverse: TAATCAAGCTTGGATCTACTTCT

Expected product: 0.9 kb

Conditions: AT=55°C; ET=1 min

CC2 domain probe:

CC2.Forward: CGTATACAGCGCATGCTTGC

CC2.Reverse: TTATTTCTCCTGATGAAGAG

Expected product: 250 bp

Conditions: AT=58°C; ET=30 s

Probe 1-3'UTR:

Tr4hOPA1F: 5'-CCAGCCTCTTTTTCTTCTGC-3'

Tr4hOPA1R: 5'-AACACCTAGCATTGTATTTCTGGA

Expected product: 300 bp

Conditions: AT=51°C; ET=30 s

Probe 2-3'UTR:

Tr3hOPA1F: 5'-TCACCACATGAAAGAAAAATGG

Tr3hOPA1R: 5'-TGAAAAGGCAGCAAATGAGA

Expected product: 600 bp

Conditions: AT=51°C; ET=30 s

Probe 3-3'UTR:

Tr2hOPA1F: 5'-AATGCATGCTTCGTTGTGAT

Tr2hOPA1R: 5'-TTTTATTTTCTTTCAATAACATCTGC

Expected product: 800 bp

Conditions: AT=50°C; ET=1 min

Probe 4-3'UTR:

Tr1hOPA1F: 5'-TTCGAACTAGCCACAGAATCA

Tr1hOPA1R: 5'-TTGATCAAGCACAGGCAAAA

Expected product: 800 bp

Conditions: AT=55°C; ET=1 min

Mouse:

Probes for confirmation of mouse 3'UTR:

m3'UTRforward: TCCTTTCCTCCCGTTCTACC

m3'UTRreverse:CACTTCAGTATCCTTTAATTAC

Expected product: 2.8 kb

Conditions: AT=55°C; ET=2.5 min

Mitochondrial leader probe:

mML.Forward: CTGTGTGTGGTCTGCTCTGG

mML.Reverse: TCCCATATAAAGTCAGGCACAATCC

Expected product: 450 bp

Conditions: AT=58°C; ET=30 s

Acidic domain probe:

hAD.Forward: GGTGGCTACACAGCCAAAAAGAC

hAD.Reverse: CACTTTCAGATCCACGATCTG

Expected product: 330 bp

Conditions: AT=55°C; ET=30 s

CC1 domain probe:

mCC1.Forward: GGAGAAAATTAGAAAAGCCCTTCC

mCC1.Reverse: TTCTGCAGCACCAGCTTCCT

Expected product: 300 bp

Conditions: AT=55°C; ET=30 s

GTPase domain:

hGTP.Forward: TGGAACGATTAGAAAAGGAGAACAAAG

hGTP.Reverse: GCTTCAATGCTTTCAGAGCTG

Expected products: 850 bp

Conditions: AT=55°C; ET=1 min

Middle domain probe:

mMD.Forward: GCTGTCGTAACAGGAAAAGG

mMD.Reverse: TCAGCATCTTCTCCAACTCG

Expected product: 900 bp

Conditions: AT=55°C; ET=1 min

CC2 domain probe:

mCC2.Forward: GAGTGGAAGTCGATCCAAGC

mCC2.Reverse: CGTTTGCCAGTGAGCAATTTAACC

Expected product: 332 bp

Conditions: AT=58°C; ET=30 s

Probe for shorter 3'UTR:

3'UTR.F1: GTGTTCTGTATTCAGGAACGC

3'UTR.R1: CTTTAAAATAGTGTTTGAGGGC

Expected product: 450 bp

Conditions: AT=60°C; ET=45 s

Probe for longer 3'UTR:

3'UTR.F2: GACTTTAGGCTCAGAAAGCAA

3'UTR.R2: CACTCCAGTATCCTTTAATTAC

Expected products: 400 bp

Conditions: AT=60°C; ET=40 s

OPA1 knockout construct creation

Short arm cloning:

Short arm forward (XhoI site introduced):

GGGCTCGAGCAGCAAGGTTAGCTGCAAGACTC

Short arm reverse (Not I site introduced):

CCGGCGGCCGCTCTCCTTGTCAAGGAGGAATACG

Expected product: 2.2 kb

Conditions: AT=63°C; ET=1 min 30 s

Long arm cloning:

Long arm forward (BamHI site introduced):

CCGGATCCGGGTTGACCCATCACTACTTTCC

Long arm reverse (AscI site introduced):

CCCGGCGCGCCGAGAACTGCTGAAATGGTGTCC

Expected product: 5.8 kb

Conditions: 94°C for 2 min (1x); 94°C for 10 s, 63°C for 30 s, 68°C for 4 min (10x); 94°C for 15 s, 63°C for 30 s, 68°C for 7 min, with prolongations in each cycle for 20 s (25x); 68°C for 7 min

Southern blot probe cloning

E2-KO-south.pr.F: CTGTGTTAGGCAAACTTATATCC

E2-KO-south-pr.R: GCCCAAGCTAATTCCAATTAC

Expected product: 0.6 kb

Conditions: AT = 58°C; ET=45 s

Genotyping

ES cells genotyping:

forward 1: TTTGATTCCCATCACCCATT

reverse 1: TGTTACGGTCAGCAGAAACG

forward 2 (*neomycin*): CTCGTCCTGCAGTTCATTCA

Primers were used in 2 separate reactions, each forward primer with reverse in combination.

Expected products: forward 1/reverse 1: wild-type cells: 3.5 kb and positive cells: 3.5 kb and 4.6 kb; forward 2/reverse 1: wild-type cells: no product and positive cells: 3.1 kb.

Conditions: 94°C for 2 min (1x); 94°C for 10 s, 55°C for 30 s, 68°C for 3.5 min (10x); 94°C for 15 s, 55°C for 30 s, 68°C for 3.5 min, with prolongations in each cycle for 20 s (25x); 68°C for 7 min.

Mice and embryos genotyping:

mOPA1-forward: GCAGTTCTCTTCTCTAACTC

neomycin forward: GTTCGAATTCGCCAATGACA

mOPA1-reverse: AATGTAAGTGTTCTTACTCCC

These primers were used in one mutiplex PCR.

Expected products: wild-type: 0.4 kb; heterozygous: 0.4 kb and 0.55 kb; knockout: 0.55 kb.

Conditions: AT= 58°C; ET= 45 s

2.2.2.8. RT-PCR reaction

Reverse transcriptase (RT) has the capacity to use a messenger RNA (mRNA) as template and generate complementary DNA in 3' to 5' direction (Retroscript, Ambion). The reactions were primed with oligo(dT) primer targeting the 3' poly(A) tail.

RT-PCR is a PCR amplification of a product after the reverse transcription reaction. Usually, max. 5 µl of the RT product was used. Primers used to amplify products from cDNA created in RT reaction are listed above.

2.2.2.9. Real-time PCR

Quantitative PCR was performed using Quanti Tect SYBR Green PCR kit (Qiagen) according to (Wittwer et al., 1997a; Wittwer et al., 1997b). The PCR was monitored in real-time with an iCyler (BIORAD). For wild-type and mutant embryos and tissues, PCR reactions were performed as previously described (Hance et al., 2005) applying following primers and contidions:

Cytochrome c oxidase subunit I to detect mitochonrial DNA:

Cox I forward primer: GCCCCAGATATAGCATTCCC

Cox I reverse primer: GTTCATCCTGTTCCTGCTCC

Primers were used in one multiplex PCR reaction using cycle conditions described below.

β-actin to detect chromosomal DNA:

ß-actin forward primer: TGTTCCCTTCCACAGGGTGT

ß-actin reverse primer: TCCCAGTTGGTAACAATGCCA

Primers were used in one multiplex PCR reaction using following cycle conditions.

Conditions:

95°C for 15 min (1x); 94°C for 15 s, 60°C for 45 s, 72°C for 30 s (40x); 95°C for 1 min (1x); 55°C for 10 s (80x; melt curve).

In order to obtain a normalized value, the copy number obtained for mtDNA was divided by the copy number for chromosomal DNA. The values were presented as percentage of the mean levels of the relative mtDNA amounts in wild-type embryos or tissues.

2.2.2.10. Southern blotting

DNA fragments of digested genomic DNA from ES clones (see 2.2.1.4.) were separated on a 0.7% agarose gel, stained with ethidium bromide and photographed with a ruler, in order to be able to corelate the proportion of separation between a size-marker and the DNA fragments. The fragments were depurinated using 0.2 M HCl and transferred from the gel to a solid support (positively charged nylon membrane, Amersham) following the protocol for transfer to positively charged nylon membranes from Molecular Cloning.

2.2.2.11. Northern blotting

In order to separate RNA formaldehyde-agarose gels were used, prepared with DEPC water and 10x MOPS buffer (0.4 M MOPS; 50 mM sodium acetate; 10 mM EDTA in

DEPC H_2O; pH 7.0). To visualise RNA, 2 µg of total RNA was loaded on a gel and ran in 1 x MOPS buffer. For a northern blot 5 µg of total RNA was loaded on a formaldehyde gel and ran 60 V for 3 hours. Blotting was done according to the Molecular Cloning protocol for charged nylon membranes. Commercially available blots for various mouse and human tissues were also used (Clontech).

2.2.2.12. Hybridisation with radioactively labelled DNA probes to Southern and Northern blots

Probes used for hybridisation were created by PCR as described above (2.2.1.8.). Before hybridisation blots were prehybridised in a hybridisation oven (Mytron) for at least 30 min. For this Expess Hyb Solution (Clontech) was used at 68°C for ~0.5 kb probe. Probes for hybridisation were synthesised from sequenced RT-PCR products applying Ready-To-Go-DNA Labelling Beads -dCTP (Amersham) according to the manufacturers protocol. 2 to 10 ng of DNA per 5 ml of hybridisation solution was used for labelling. After 30 min labelling with radioactive α-^{32}P-dCTP (Amersham) at 37°C, access of unincorporated nucleotides was removed using MicroSpin Column S-200HR (Amersham) according to manufacturer's protocol. Purified labelled probes were denatured for 3 min at 95°C, cooled 2 min on ice and added to the hybridisation solution. Blots were than hybridised for 1 h at 68°C. After hybridisation, membranes were washed 3x 10 min in 2x saline sodium citrate (SSC: 3 M NaCl, 0.3 M sodium citrate pH 7.0), 0.05% SDS solution at room temperature and 2x 20 min in 0.1% SSC, 0.1% SDS. Washed membranes were slightly air-dried, wrapped into fresh plastic wrap and exposed to an X-Ray film in an exposure cassette. The cassette contained 2 screens that intensified the signal each 2 times when used at –80°C. Exposure time depended on intensity of the signal, ranging from 3 hours to over night.

2.2.2.13. Cloning

2.2.2.13.1. Restriction digestion

The complete digestion of 1 µg plasmid DNA with or without an insert is already achieved with 1 unit (U) of a restriction endonuclease in one hour. The volume of the enzyme should not exceed 1/10 of the total volume of the reaction. Restriction enzymes used in this work were obtained from Amersham and NEB (New England Biolabs). Restriction

digested DNA fragments were separated on agarose gels and gel pieces containing fragments of wanted size were excised from the gel. DNA fragments were extracted using ``DNA Gel Extraction Kit`` (Quiagen).

2.2.2.13.2. Ligation

In order to clone PCR products into the pGEM-Teasy vector (Promega), which has T-overhangs, A-overhangs have to be created. This is accomplished using Ampli-Taq polymerase in an incubation reaction for 15 min at 72°C at the end of the PCR reaction, if this enzyme is used for PCR. If proofreading enzymes are used, like in Expand Long Template PCR System, this step has to proceed with the addition of dATPs (0.125 mM final). A ligation is followed using T4 ligase (Amersham) according to the given protocol. The molar ratio between vector and the insert (PCR product or restriction digested DNA fragment) should be 1:3.

2.2.2.13.3. Transformation of chemi-competent bacteria

After transformation of *E. coli* strains using the given protocols, aliquots were plated on selective agar-plates, and grown over night at 37°C. All plasmid constructs made for this thesis were ampicillin resistant. Constructs cloned into pGEM-Teasy vector were screened using blue-white selection, only if propagated into DH-5-α strain. Cells containing plasmid DNA were cultured in sterilized LB-medium with ampicillin (100 µg/ml). All constructs were confirmed by automatised DNA sequencing (Invitek, Berlin).

2.2.2.14. Preparation of total protein extracts from cells and tissues

To obtain total protein extract from different mouse tissues and cells, material was homogenised in glass-teflon homogenisers with 4-6 strokes using 1 ml ice-cold protein-lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 10 mM EGTA and protease inhibitors- Roche) per 1 mg of tissue. Homogenised tissue was adjusted to 1% NP40 and shaken for 30 min at 4°C and before sonicating (3 x 40% pulse for 20 s, one 50% pulse for 20 s and one 30% pulse for 20 s). Sonicated homogenisates were centrifuged for 20 min at 16000 g at 4°C. Finally, the supernatant was transferred to a fresh tube and stored at –20°C. The protein concentration was estimated using Bradford

essay. Serial dilutions of standard protein (IgG, 1.4 mg/ml stock solution) were prepared (5, 10, 20, 40 µg/ ml) together with two dilutions of test solution in 1 ml of Bradford reagent (BIORAD). Samples were incubated 5 min at room temperature and measured at OD_{595} on UV-spectrophotometar (SmartSpec™ 3000, BIORAD).

2.2.2.15. SDS-PAGE

20-100 µg of protein samples was diluted 1:4 with 5x SDS/sample buffer (1x final; stock 5x: 1.875 mM Tris/HCl (pH 6.8), 0.75 mM EDTA, pH6.8, 1.5% SDS, 10% ß-mercaptoethanol, 0.005% bromphenolblue) and denatured for 5 min at 95°C. Cooled protein samples were then loaded on 10% SDS gels (5 ml of stacking gel: 650 µl of 30% acrylamide/0,8% bisacrylamide, 625 µl of 1M Tris.HCl, pH 6.8, 50 µl of 10% SDS, 3.7 ml H_2O, 25 µl of 10% APS and 5 µl of TEMED; 10 ml of separating gel: 3.3 ml of 30% acrylamide/0,8% bisacrylamide, 3.75 ml of 1 M Tris.HCl, pH 8.8, 100 µl of 10% SDS, 2.8 ml H_2O, 50 µl of 10% APS and 10 µl of TEMED) and ran in 1x protein running buffer (25 mM Tris, 192 mM Glycin, 0.1% SDS) at 200 V on Mini-PROTEAN 3 System (BIORAD) for one to two hours.

2.2.2.16. Western blot

After separation of the proteins the SDS-PAGE gel was rinsed for 5 min in PBS to reduce SDS. After soaking the gel for 5 min in ice-cold transfer buffer (25 mM Tris, 192 mM Glycin, 15% Methanol), proteins were blotted using wet-blotting Mini Trans-Blot System (BIORAD) according to manufacturer's instructions.

After a successful transfer the membrane was blocked for non-specific binding of antibody with 50 ml of blocking buffer (5% dry, low fat milk in 1x TBS (Tris-Buffered Saline)) for 1 hour, shaking at room temperature and than washed (2 x rinse in 1 x TBS, 2 x 10 min in 1 x TBS at room temperature). The primary, specific antibody was diluted in blocking buffer and incubated with the membrane for an additional 1 hour. After intensive washing (see above), the secondary peroxidase conjugated antibody was applied for 1 hour. The membrane was then washed again and developed with ECL™ (enchanced chemiluminescence) Western Blotting Reagent (Amersham). Signals were detected after exposure of blots to ECL films (Amersham).

2.2.3. Cell culture

All cell culture procedures were based on protocols according to “Gene Targeting: A Practical Approach” (Joyner, 1999). They were carried out under sterile conditions using a laminar flow cabinet, sterile plastic ware and detergent-free glassware. The media and solutions were prepared from directly sterile, autoclaved or sterile filtrated components. Before the cells were placed in the media, solutions had to be warmed to 37°C. Mouse feeder and embryonic stem (ES) cells used in this work were a kind gift of C. Birchmeier.

2.2.3.1. Embryonic Feeder cells

Feeder cells derive from embryonic fibroblasts and are used as a layer for ES cells to grow on and as inhibition of ES cell differentiation. They are primary fibroblast cells routinely prepared from embryos derived from mouse strains homozygous for a transgene containing a *neomycin* resistance cassette (*neoR*). *neoR* feeder cells survive during positive selection of ES cells with G418 (geneticin). Frozen vials with Feeder cells were quickly warmed up at 37°C and then the cells were transferred to 10 ml of warm medium. After centrifugation (Eppendorf, 1100 rpm, 3 min) the cells were resuspended in fresh medium and platted on the gelatinised cell culture dish. They were grown on gelatinised plates (0.1% gelatine, Sigma) in feeder medium. When they reached confluency, they were washed twice with PBS and split using 3 ml 1x Trypsin/EDTA, several minutes on 37°C. Feeder medium was added to stop trypsinisation and cells were resuspended, centrifuged at 1100 rpm for 2 min and plated. Feeder cells were passaged up to three times, and treated with mitomycin C for growth arrest prior to culture with ES cells.

2.2.3.2. Growth arrest of Feeder cells by Mitomycin C

Feeder cell mitosis has to be inactivated before they are used to support ES cell growth. A confluent plate of embryonic fibroblasts was washed with PBS and incubated for 2 h with 100 µl of mitomycin C solution (1 mg/ml in PBS, 5% DMSO, Sigma) in 10 ml feeder medium. Then the cells were washed two times with PBS, and the fresh medium was added or they were seeded onto new gelatinised plates. Medium was changed every three days. Mitomycin treated cells are usable for up to two weeks.

2.2.3.3. Cell culture of ES cells

Undifferentiated ES-cells under optimal cell culturing conditions give colonies with clear-border definition. The individual cells in a colony are hard to distinguish from each other; they are recognised through a characteristically large, pigmented nucleus.

ES cells were cultivated at 37°C and 7.5% CO_2 on gelatinised plates with a layer of growth-arrested feeder cells for two to three days. To maintain pluripotency, beside feeder layer, ES cells grew in a medium containing murine leukaemia inhibitory factor (LIF, a gift of M. Schäfer) (Smith et al., 1988), and it had to be changed daily or even twice a day if culturing at higher concentration. The density had to be regulated by frequent cell splitting (every 2 to 3 days) to avoid cell differentiation. Frozen ES cells were thawn rapidly and the DMSO-containing medium was immediately replaced with warm (37°C) ES medium.

For splitting, the cells were treated the same way as feeder cells but using 900 rpm for centrifugation. When trypsinising ES cells it is important to fully disintegrate cell aggregates, because larger aggregates tend to start differentiating. The maximum ES cell concentration is 1.5×10^7 cell/ 10 cm plate. The supernatant was removed, the cell-pellet resuspended in ES medium and plated. ES cells should not be passaged more often than absolutely necessary because longer maintenance in cell culture might cause mutations preventing germline transmission. Also, ES cells represent a heterogeneous population, and the fraction of cells having lost pluripotency may increase with the passage number. Any cells treated under suboptimal conditions have to be discarded.

2.2.3.4. Electroporation of ES cells and selection for G418-resistant clones

For electroporation one dense 10 cm Ø plate of ES cells is needed where single colonies are still visible. ES cells were cultivated for two to three days as long as the culture contained sufficient cells for electroporation but still was in the optimal growth phase. A few hours before transfection, ES medium was exchanged to increase the number of cells in S-phase in order to enhance efficiency of gene targeting. 20 µg of linearised targeting vector (NotI) in 0.8 ml PBS was electroporated into 10^7 ES cells (240 V, 500 µF, BioRad Gene Pulser) using established procedures (Chester et al., 1998). The cells were plated on five gelatinised plates with a feeder-layer in ES medium at a 1:3 dilution.

After electroporation the cells grew undisturbed for 48 hours until the medium was changed with ES medium containing G418 (400 µg/ml of active G418). Fresh selection

medium was added daily to the ES cells. After additional 5-7 days of culture with selective medium, single, undifferentiated ES cell colonies were picked and cultured for additional 1-2 days in 96-well plates on feeder cells. Then, the ES cell colonies were trypsinised and split into two 96-well plates; one plate without feeder cells for screening (replica plate – see below) and one plate with feeder cells for freezing. To freeze ES cells down, 1 volume of ice-cold 2x freezing medium (ES medium with 13.3% DMSO) was added to confluent, trypsinised 96 well plates that contained 1 volume of trypsin in them. The plates were carefully wrapped in Parafilm (American National can^{TM}) and gradually frozen to –80°C in Styrofoam boxes. To screen the cells for homologous recombination by Southern analysis, a replica plate was made of each 96-well plate. The replica plate was coated with 0.1% gelatine (Sigma) before seeding of ES cells. Such plates were grown to confluence and used to extract DNA for Southern blotting to screen for targeted clones as described above (2.2.2.1.).

2.2.3.5. Manipulation of blastocyst and transfer into pseudopregnant mice

Isolation of blastocysts (C57BL/6 background), injection of ES cells (129/Ola background) into their inner cavity and their transfer into uteri of foster mothers were done in RCC, Berlin, in order to create chimeric mice.

2.2.3.6. Mouse embryonic fibroblasts (MEFs)

Mouse embryonic fibroblasts (MEFs) were prepared from OPA1$^{+/+}$ and OPA1$^{-/-}$ E8.5 embryos on the Black Swiss outbred strain (Silver, 1995) in the laboratory of D. C. Chan, as described (Chen et al., 2003). The cells were immortalized with SV40 large T antigen as described previously (Chen et al., 2003). Once established, OPA1$^{-/-}$ MEFs were maintained in growth medium described above.

2.2.3.7. Apoptosis assay

1x 10^6 MEFs of the indicated genotypes were grown in 12-well plates and treated with 2 µM staurosporine (Sigma), with or without previous caspase inhibition with 40 µM z-VAD-fmk (Sigma), or with 10 ng/ml of TNF-α (Tebu-bio) for indicated times. Cells were stained using Annexin-V-FLUOS Staining Kit (Roche), according to the manufacturer's protocol. In the early stages of apoptosis phophatidylserine from the inner part of the plasma membrane translocates to the outer layer, becoming exposed at the external surface of

the cell (Vermes et al., 1995). Annexin V has a high affinity for binding phophatidylserine and is therefore suited for the detection of apoptotic cells. Since necrotic cells lose their membrane integrity exposing phophatidylserine, they have to be excluded from the measurement. This is accomplished by using a DNA stain, like propidium iodide (PI), simultaneously with Annexin V. PI binds to the ends of degraded DNA, but has to first enter the nuclear membrane. In contrast to apoptotic cells, necrotic cells have destroyed nuclear membrane and PI can easily bind to their DNA. Apoptosis was measured by flow cytometry using FACScalibur (Becton Dickinson) and CellQuest Pro analysis software. Cells that turned out to be both Annexin V and PI positive were taken for necrotic cells and were excluded from the calculations.

Cells used for immunostaining with cytochrome c were grown on poly-D-lysine coated glass coverslips (Roth), and were treated first with broad caspase inhibitor z-VAD-fmk (100 µM) in order to avoid detaching of apoptotic cells from the coverslips. After one hour of treatment, both wild-type and knockout MEFs were treated with 2 µM staurosporine for 3 h, control MEFs were left untreated. Cells were further stained with MitoTracker Green^{R} and cytochrome c, as described below.

2.2.3.8. Membrane potential measurement

Mitochondrial membrane potential was determined using the Mitochondrial Staining Kit (Sigma). 1x 10^6 cell/ml were loaded with JC-1 (5,5',6,6'-tetrachloro-1,1',3,3' tetraethylbenzimidazolocarbocyanine iodide) (2 µM) and incubated for 20 min at 37ºC, 5% CO_2. JC-1 is a mitochondrial dye that accumulates in the matrix of intact mitochondria and forms red fluorescent aggregates. When membrane potential decreases, JC-1 dissociates into green fluorescent monomers. Cells were washed once, resuspended in 1x PBS and immediately analysed by flow cytometry using FACScalibur (Becton Dickinson) and CellQuest Pro analysis software.

2.2.4. Histology and cell staining

For phenotypic analysis, OPA1$^{+/-}$ mice of a mixed background (50% 129/Ola and 50% C57BL/6) were crossed. To obtain these mice, chimeras were bread with inbred C57BL/6 mice. All animal studies were performed in accordance with the guidelines for the human

use of laboratory animals by the Max-Delbrück-Center for Molecular Medicine and were approved by the local ethics committee.

2.2.4.1. Histological analysis of mouse retinae, optic nerves and embryos

Mice of desired age were anaesthetised using ether and perfused with 4% paraformaldehyde (PFA) in 1x PBS. Eyes were isolated and fixed in 4% PFA over night, washed in 1x PBS and dehydrated with increasing concentration of ethanol (30, 50, 70, 80, 90, 2x 98.9%, each step for 1-2 h). After dehydration, samples were embedded with Technovit (Kulzer), following the manufacturer's protocol. Embedded eyes were cut at 5 µm (Leica Microtom) and processed for cresyl-violet staining: soaked in staining solution (for 100 ml: 0.5 g cresyl-violet, 60 ml water, 6 ml 1M Na-acetate and 35 ml 1M acetic-acid) for 6 min at room temperature and incubated at 60°C for 15 min. Stained sections were destained in 96% ethanol as long as all background staining was removed, washed shortly with distilled water and air dried.

OPA1 heterozygous females, which were mated with OPA1 heterozygous males, were dissected with the help of a dissecting microscope (Zeiss Stemi 2000-C). Deciduae and embryos were isolated at different embryonic stages according to a vaginal plug referred to as 0.5 days post-coitum (dpc, E). Embryos were photographed using a digital camera (Zeiss AxioCam MRc).

2.2.4.2. Electron microscopy (EM) of mouse tissues and cells

Optic nerves and embryos were dissected rapidly in 3% formaldehyde in 0.2 M HEPES and fixed in 6% formaldehyde/0.1% glutaraldehyde in 0.2 M HEPES for 8 days at 4°C. Following postfixation with 1% osmium tetroxide, they were dehydrated in a graded ethanol series and propylene oxide, and embedded in Poly/Bed ^{812} (Polysciences Inc., Eppelheim). Ultrathin transverse sections were contrasted with uranyl acetate and lead citrate and examined with a Zeiss 910 electron microscope equipped with a high speed slow scan CCD camera (Proscan). Mouse embryonic fibroblasts (MEFs) were first washed with PBS, immediately fixed with 2.5% glutaraldehyde in sodium carcodylate-hydrochloric buffer and then centrifuged for 5 min at 800 g. After fixing for one to three days at 4°C, tissues and cells were processed for EM as described before (Cerritelli et al., 2003). Ultra-thin sections (60-70 nm) were produced on an UltraCutS from Leica,

contrasted with 5% uranyl-acetate, washed, stained with Pb-cytrate (Reynolds, 1963). Sections were analysed and photographed using an EM 906 (Zeiss).

2.2.4.3. Whole-mount *in situ* TUNEL assay in embryos

TUNEL assay in embryos was done using *In Situ* Cell Death Detection Kit, Fluorescein from Roche. TUNEL (TdT-mediated dUTP nick end labelling) is based on incorporation of fluorescently labelled nucleotides at DNA strand brakes by TdT (Terminal-deoxynucleotidyl transferase). In apoptotic cells nuclear DNA is cleaved into oligonucleosome-sized fragments, which are detected by the TUNEL reaction.

Embryos were fixed in 4% PFA over night at 4°C, and then washed for 30 min with 1x PBS. Embryos were permeabilised for 5 min in proteinase K solution (4.5 µg/ml in 10 mM Tris-HCl, pH 7.4-8) at 37°C. Finally; embryos were washed twice with PBS before labelling. After labelling and one more PBS washing, embryos were immediately observed using inverse fluorescence microscope Axiovert 135 (Zeiss) and photographed with AxiocamHRc (Zeiss).

2.2.4.4. Staining of mitochondria

For visualization of mitochondria, MEFs, grown on coverslips, or freshly isolated embryos were stained with MitoTracker^{R} Green CMXRos (200 nM) and MitoTracker^{R} Red CMXRos (250 nM) (Molecular Probes) for 45 min in MEFs medium, and washed in the same medium without MitoTracker for 60 min. Embryos were mounted in Prolong Gold Antifade Reagent (Molecular Probes) and analysed with a confocal microscopy (Zeiss LSM-510Meta). MEFs were fixed and pearmeabilised with 4% PFA/0.01% Triton for 10 minutes at room temperature and observed or used further for immunocytochemistry.

2.2.4.5. Immunocytochemistry

After fixation and permeabilisation (see 2.2.4.4.) cells were washed 3 times in 1x PBS and blocked for 45 min (2% bovine serum albumin (BSA) in 1x PBS). Washing steps were repeated 3 times after each step. Cells were treated with 2 drops per well of Image-iT^{TM} FX Signal Enhancer (Molecular Probes) and incubated with anti-mouse cytochrome c antibody in blocking solution over night at 4°C or 2 h at room temperature. After incubation with secondary antibody (AlexaFluor-594, anti-mouse IgG) for 90 min at room temperature and last washing, cells were mounted with Vectashield Mounting Medium for

Fluorescence with DAPI (Vector Laborotories, Inc.). Successfully stained mitochondria were observed under fluorescence microscope, Axioplan (Zeiss) and recorded with the camera (AxiocamHRc, Zeiss).

2.2.4.6. Staining for activity of respiratory chain complexes

MEFs were grown to confluency in one 75 ml tissue culture flask, trypsinised and pelleted 600 rpm, 5 min. Cell pellets were instantly frozen in Tissue-Tack (Sakura) on dry ice and cut in 7 µm thin sections on a Cryostat LEICA LM3050 S. Sections were than shortly air dried and immediately used for staining. All solutions used were freshly prepared and reactions were performed at 37°C.

For activity of respiratory complex II (succinate dehydrogenase, SDH) activity, sections were incubated in 0.2 M sodium succinate and 1 mg/ml 4-nitrobluetetrazoliumchlorid (NBT) in 0.2 M phosphate buffer (22.75 g KH_2PO_4 and 5.4 g NaH_2PO_4; pH= 7.4) for 2 h.

Complex IV (cytochrome c oxidase, COX) activity was determined by incubating sections in 1 mg/ml cytochrome c from horse heart (Sigma) and 1 mg/ml 3,3' diaminobenzidine (DAB; Sigma) in 0.1 M phosphate buffer, pH=7.0, for 1 h.

After successful staining sections were washed 3 times with distilled water, mounted (Vectashield Mouning Medium, Vector Laborotories, Inc.), covered, observed under fluorescence microscope, Axioplan (Zeiss) and recorded by camera (AxiocamHRc, Zeiss).