7 Methods

7.1 Cell culture methods

7.1.1 Cell line culture

293T cells and HT1080wt cells were cultured in DMEM with 10% FCS, 1% Penicillin/Streptomycin, 10mM HEPES pH7.4 and 4mM L-Glutamine. HeLa cells and IF6 cells were cultured in RPMI with 10% FCS, 1% Penicillin/Streptomycin.
HT29 cells and Sk-ov-3 cells were cultured in McCoy's medium, supplemented with 10% FCS and 1% Penicillin/Streptomycin. CapanI cells were cultured in RPMI with 20% FCS and 1% Penicillin/Streptomycin.
For serum starvation <12h, cells were cultured in the respective medium without FCS, in case of serum starvation >12h, the culture medium was supplemented with 0.5% FCS only.
Cells were splitted every 2-3 days, washed with PBS, detached with Trypsin/EDTA and then diluted in fresh medium to be reseeded in lower density. Cells were cultured at 37°C with 5% CO_2.
For EGF induction, cells were serum starved for 4h to 12h, induced with 25ng/ml EGF for 5min at 37°C, rinsed with PBS and lysed in 2x sample buffer.
Apoptosis was induced with 25ng/ml TNFα in combination with 10 µg/ml cyclohexamid for 4-5h, 60mM cisplatin for 20h or 1.5 mg/ml BrefeldinA for 20h, respectively.

7.1.2 shRNA mediated RNA interference

A common method for the analysis of function of a specific gene product is the knockout of this gene by homologous recombination that results in a complete loss of the protein of interest. As this technique is lengthy and expensive, the last decade saw the development of a method using the endogenous mechanism of RNA interference to knockdown specific mRNAs, thereby reducing the expression of the respective protein. RNA interference can be initiated by transient transfection of small interfering RNAs (siRNAs) or stable expression of small hairpin RNAs (shRNAs). The latter are integrated into the genome and intracellularly processed to siRNAs. siRNA expression by transfection leads to a transient knockdown and, due to limitations in transfection methods, is limited in its

application to a small range of cell lines. Stable expression of shRNAs allows a constitutive mRNA knockdown. Using a lentiviral transfer approach the expression range of shRNAs can be extended to non replicative primary cells and animals. Generated by Rubinson *et al*. the pLL3.7 LentiLox system (Figure 4-1) allows stable shRNA expression under the control of the polymerase III promoter U6 and features as marker protein for a successful infection eGFP under cmv-promoter control[71].

7.1.3 Lentivirus production

Lentiviruses belong to the class of retroviruses, which means that they integrate their genome into the host cell's DNA. Commonly used lentiviral vector systems belong to the 2nd or 3rd generation, ensuring safe application, as these viruses are unable to self-replicate, since the spontaneous self assembly is prevented by distributing the least necessary number of virus elements on three and four plasmids, respectively. Directed virus assembly takes place via transient transfection of the viral plasmids into helper cells, which secrete the virus into the supernatant, from which it can be collected. Commonly available vector systems differ only in their generation and their 'application' plasmid.

7.1.3.1 Large scale production

To produce lentiviral particles, 293T cells were seeded in 15cm^2 plates in a density of 12x10^6 cells 24h before transfection.

For Calcium-Phosphate transfection the DNA was mixed as follows:

3rd generation		2nd generation	
Vector (pLL3.7 constructs)	20 µg	Vector (pLVPT constructs)	20 µg
VSVG	10 µg	VSVG	10 µg
REV	10 µg	Pax2	15 µg
RRE	10 µg		

The DNA was mixed with 400 µl 1.25 M $CaCl_2$ and 1.5 ml ddH_2O. The following steps were done one plate at a time.

2ml 2x HBS were added dropwise to the DNA mixture, bubbling through air flow at the same time, to mix. The transfection mixture was then added dropwise to the plate, while gently swirling the plate back and forward. 3½-4h later the plates were

washed 2x with prewarmed PBS and 20 ml prewarmed 293T culture media was added.

The cells were analyzed via fluorescence microscopy to determine the efficiency of transfection. The percentage of GFP^+ cells should be between 70-100%. The viral supernatant was harvested 36-48 hour later, spun down at 3000g, 7 min at 4°C to collect loose cell debris. The supernatant was then filtered through a 0.45 µm filter. The virus containing supernatant can either be used directly for infection or ultracentrifuged to concentrate the virus for storage and later usage. For concentration the supernatant was ultracentrifuged at 100000 g, 90 min 4°C.

The supernatant was removed and the virus pellet airdried up side down for 10 min. An appropriate volume of PBS (50-200 µl) was added, and the tube was left at 4°C o/n. To resuspend the virus, the tube was held at an angle and the fluid was pipetted over the pellet 20 times, while carefully avoiding touching the pellet. The virus was then aliquoted and either used immediately or flash frozen in liquid nitrogen.

The pLVPT system (2^{nd} generation) is a doxycycline inducible shRNA expression system. To generate virus, doxycycline (1µg/ml) was already added during the transfection approach.

7.1.3.2 Automated production

For the automated production of lentiviral particles, 293T cells were seeded to 20.000 in 100 µl medium in a flat bottom 96 well plate 24h before transfection. For the transfection of one 96 well plate, 8 x 500 µl aliquots of 2x HBS were prepared and a mastermix of packaging plasmids (30 µg psPax2, 10 µg VSVG), 1000 µl 1 M $CaCl_2$ and H_20 to 2 ml total volume was set up. In a round bottom 96 well plate, specific DNA was pipetted to 30 µl à 20 ng/µl in every third column, starting with the first. Virus was produced in triplicates. Set up of the robot and run were according to the program *Transfection for Lentivirus Generation*. 4-6h post transfection the transfection media was carefully replaced with 200 µl fresh 293T culture media After 36h the virus containing supernatant was filtered through a 0.45 µm millipore vacuum filter device directly onto the to be infected cells or onto a round bottom 96 well plate to be stored at -80°C.

7.1.4 Infection and Titration

7.1.4.1 Large scale virus

To titer concentrated virus, the cells of interest were plated to 50,000 per well on a 6 well plate. The infection media was a stock solution of culture media with 10µg/ml polybrene. A 10 fold dilution series of virus was generated, using 1.5 ml /well with 1 µl, 0.1 µl, 0.01 µl, 0.001 µl and 0.0001 µl virus per well.
12h post infection, media was removed and replaced with fresh culture medium.
The cells were kept under growing conditions (37°C, 5% CO_2) for 48h and FACS analyzed for EGFP expression. The Titer corresponds to the amount of cells seeded and is calculated as follows:

$$TU/\mu l = \frac{no.\ seeded\ cells \times (\%\ GFP\ \ pos.\ cells)/100}{volume\ of\ \ virus/\mu l}$$

The value *for % GFP pos. cells* should be between 10%-20% GFP expression, as one can assume a single integration with this infection rate.

For FACS analysis, cells were detached from the culture dish with trypsin and resuspended in culture media or PBS.

7.1.4.2 Automated infection

For infection with virus from the automated production, cells were seeded to 3500 into a flat bottom 96 well plate 24h before infection. To infect with virus, the culture media was removed and 20 µl polybrene/PBS (10 mg/ml) were added. The virus was either directly transferred to the infection plate by filtering it through a Millipore device or pipetted with a multichannel pipette independently of the production time. 12h post infection the infection media was removed and replaced with cell specific culture medium.

7.1.4.3 shRNA induction w/ doxycycline

The pLVPT lentiviral vector system was used to generate cell lines that express shRNAs only upon administration of doxycycline. This tetracycline analogon leads to the release of the KRAB repressor from the tet operon and consequently to the expression of the marker protein GFP and shRNAs. Infected cells were continuously treated with 1µg/ml doxycycline in the cell culture medium and GFP expression was monitored by FACS analysis.

7.1.5 Cell Proliferation assays

7.1.5.1 Cell division

To measure the cell division rate, cells were seed at 50.000 in a 6 well dish. 3 days post seeding, cells were counted in a haemocytometer or with an automated cell counter (Casy1). Again, 50.000 cells were seeded in a new 6 well dish. After repeated performance, gathered data were used to calculate the cell division rate.

$$doubling\ events = \log\left(\frac{new\ cell\ number}{old\ cell\ number}\right) \qquad cell\ division\ rate = 2^{doubling\ events}$$

7.1.5.2 BrdU assay

Besides counting the cells and calculating the cell division rate, cell proliferation was measured by the incorporation and detection of the thymidine analogue BrdU. The instructions provided with the kit, *Cell Proliferation ELISA, BrdU (chemiluminescent)*, Roche were followed. In short, cells were seeded at 3500 in a white rimmed 96 well plate. 24h after seeding, BrdU was added to a final concentration of 10 µM and the cells were incubated for 1h under culture conditions (37°C, 5% CO_2). The cells were washed thrice with PBS and fixed with FixDent solution for 30 min, RT. After removal of the FixDenat solution, the peroxidase conjugated anti-BrdU antibody was added to cells, incubated for 30min-1h at RT, then washed thrice in washing buffer. The reconstituted substrate, containing luminal and 4-iodophenol, was added and the luminescence measured after an incubation of 3-10min using a luminometer.

7.1.5.3 WST-1 assay

The WST-1 assay measures cleavage of the tetrazolium salt WST-1. This reaction involves the mitochondrial succinate-tetrazolium-reductase system and could therefore be used to analyze mitochondrial functionality.
HeLa cells were seeded preconfluent in 96 well plates (ca. $4x10^3$ -$6x10^3$ cells). WST-1 reagent was added in a 1:10 dilution to culture medium and incubated at 37°C for 3h. Absorption was measured at 450-690nm every hour.

7.1.5.4 Propidium iodide staining

Propidium iodide (PI) intercalates stoichiometrically with DNA and can therefore be used to analyze the cell cycle phases. Its absorption maximum is 535 nm when bound to DNA and emission is 617 nm which allows measurement of the fluorescence intensity by FACS (linear scale) at the same time as GFP (logarithmic scale) expression. For the analysis of cell cycle progression cells were analyzed five days post infection with pLL3.7 constructs.

The cells, kept under growing conditions (37°C, 5% CO_2) with 10% FCS in culture medium were harvested and resuspended in 2ml PBS. For fixation, the equivalent amount of 4% PFA was added, while vortexing. Incubation o/n at 4°C ensured the maintenance of GFP fluorescence in the following DNA denaturation procedure with HCl.

Thereto $1x10^6$ cells per construct were permeabilzed for 10 min with 1ml 0.5% Saponin. After washing with PBS cells were resuspended in 200µl 2M HCl and incubated for 20 min at RT. The cells were washed twice in PBS before staining with freshly made PI staining solution for 30 min at RT, protected from light.

7.1.5.5 CFSE staining

The cell divison rate can be analyzed my measuring the distribution of Carboxy fluorescein diacetate succinimidyl ester (CFSE) into the daughter cells with each cell division. This ester couples spontaneously and irreversibly to cellular proteins by reaction with lysine sidechains and other available amines. Consequently, each successive generation in a population of proliferating cells is marked by specific two-fold decrements in cellular fluorescence intensity.

For CFSE staining cells were harvested, washed 2x in PBS and stained with 5µM CFSE for 5 min at RT. Residual CFSE was removed by washing twice with PBS and cells were seeded in 6 well plates and kept under growing conditions. The CFSE fluorescence intensity was gauged by FACS analysis every 24h. For set up, a CFSE unstained but GFP positive cell sample (shLuci induced) was used and the GFP positive sample was set to zero.

7.1.6 Indirect immunofluorescence

Cells grown on glass cover slides were washed with PBS and fixed with 4 % PFA for 30 min at RT or o/n at 4 °C. Cells were washed thrice with PBS and

permabilized with solution A for 5-10min at RT. The cells were blocked successively in solution B and solution C for 15 or 20 min, respectively. Before the solutions were changed, cells were washed again thrice with TBS. Solution B or C were used as antibody dilution solution. 15-20 µl primary antibody solution was pipetted on parafilm, placed in a moistened chamber, on which then the cover slips were placed inverted. After incubation for 1h at 37°C or o/n at 4°C they were lifted from the parafilm by flushing 200 µl TBS underneath. The cover slips were washed by dipping them in five consecutive beakers filled with TBS and placed on 15-20 µl freshly prepared 2^{nd} antibody solution on parafilm. At this step DNA staining with Hoechst or phalloidin staining could take place. After incubation for 1h at 37°C cover slips were again washed as described above in fresh TBS and airdried for 5min. The cover slips were mounted onto slides with Moviol and the preparations were analysed under a fluorescence microscope coupled with a digital camera.

7.1.7 Receptor surface staining

To analyze the expression level of surface receptors and proteins, adherent cells were first detached with accutase. Thereto, cells were washed with PBS and incubated with accutase for 5-10 min at 37°C. The reaction was stopped with cell culture medium containing 10% FCS and cells were again washed with PBS (centrifugation steps at 4°C, 5 min, 500g). Unspecific antibody binding was blocked by incubating the cells w/ 0.5% BSA in PBS for 10 min, on ice. Cells were taken up in a 1:100 antibody dilution (in 0.5% BSA in PBS) and again incubated for 10 min, on ice. Upon removal of residual antibodies by washing 3x w/ PBS, the secondary antibody staining, conjugated to fluorescence dyes like cy3 and cy2 followed. This was again done for 10 min, on ice. Cells were washed 3x w/ PBS and fluorescence intensity was quantified by FACS analysis.

7.1.8 Soft agar assay

To analyze anchorage independent growth, cells were seeded in 0.3% soft agar. First, 1-2 ml of a 1:1 solution of 2% agarose and PBS were plated in a 6 well plate. Cells in a low density (ca. 1000 – 5000, $1/10^{th}$ of a 96 well) were mixed with a 1:2 solution from 1% low melting agarose (melted at 70°C) and cell type specific medium. The agarose should not exceed 40°C when the cells are added. The cell

/agarose mixture was added on top of the 1% agarose layer. After its solidification, 2 ml specific cell medium were added. Formation of colonies was observed within a timeframe of 2-6 weeks, with changing the culture media every three days.

7.1.9 Proliferation in suspension

To analyze proliferation of HeLa cells under suspension conditions, 6 well plates were covered with 1% agarose in PBS. Cells were seeded in normal culture medium and the proliferation rate was assayed by counting the cells once a week.

7.1.10 Adhesion assay

To measure the adhesion capability of cells, microplates were coated with 1mg/ml fibronectin, 2mg/ml collagen or 3mg/ml BSA, respectively. Plates were kept open at RT for 30 min and the residual extracellular matrix was removed by washing 3x with PBS. The cells were seeded in different densities (25.000-75.000/well) and incubated at 37°C, 5% CO_2 for 30 min. Not adherent cells were removed by washing 3x with PBS.

To increase the fluorescence intensity at 488nm, due to a weak GFP expression below the measurable sensitivity of the fluorometer, the cells were, prior to seeding, stained with CFSE, which emits light at the same wavelength as GFP (protocol 7.1.5.5).

7.1.11 Mitochondria isolation

Confluent HeLa cells from a 15cm^2 plate were scraped off with a rubber policeman and washed in PBS (800g 5 min, 4°C). The cell pellet was resuspended in 1ml Buffer B instead of Buffer A, since the cytoplasm was needed for protein precipitation. An additional 3ml of solution B was added and the cell mix was left on ice for >1h to facilitate swelling. The cells were transferred to a 5ml Dounce homogenizer and homogenized with 35 strokes using a drill-fitted pestle. The sample was centrifuged at 800g, 5 min, 4°C and the supernatant, containing mitochondria, microsomes and cytoplasm was collected. To pellet the mitochondria, the supernatant was transferred to microfuge tubes and spun at 10.000g for 10 min at 4°C. The supernatant was discarded or used for precipitation of cytosolic proteins (7.1.12).

Pellets from the same kind were pooled and again centrifuged at 10.000g, 10 min, 4°C. The mitochondria were resuspended in an appropriate volume of Solution B

and the protein content was determined by mixing 1-5 µl (=X µl) mitochondria in 600 µl of 0.1% (w/v) SDS

$$[protein] = \frac{A_{280nm} - A_{310nm}}{1.05} \times \frac{600\mu l}{x\mu l} mg/ml$$

Mitochondria were diluted to 5 µg/µl in sucrose buffer, and stored at -80°C in aliquots to 10-20 µl.

7.1.12 Cytosolic protein preparation

The supernatant from the second centrifugation described in paragraph 7.1.10 was used to prepare cytosolic protein fractions by spinning it at 100.000rpm (TL-100, Beckmann) for 1h at 4°C. Light membrane proteins were pelleted, while cytosolic proteins stayed in suspension. These were concentrated by TCA precipitation. Thereto 1.25% sodiumdesoxycholat was added to 1ml cytoplasm supernatant in a ratio of 1:100 and mixed by vortexing. 250 µl TCA were added (1:5), again vortexed and incubated on ice for ½ h. To precipitate the cytosolic proteins, the mixture was centrifuged at 20.000g for 30min, 4°C in a microfuge. The pellet was washed with 400 µl ice-cold acetone, centrifuged for 10 min at 20.000g, 4°C. The airdried pellet was insoluble and therefore was taken up in 100 µl SDS-sample buffer, the proteins denatured at 60°C, 15 min and the pH adjusted with a drop of 1M Tris pH 8.0.

7.1.13 Membrane potential

The mitochondrial membrane potential was measured using TMRE. This dye interacts minimally with membrane proteins and is only integrated in to the membrane of this organelle, if the membrane potential is stable. The cells were detached from the cell culture dish and washed with PBS. After adding the TMRE dilution (100 nM in culture medium), the cells were kept under growing conditions (37°C, 5% CO_2) for 30 min. After the staining procedure, the cells were washed several times with PBS and analyzed by FACS. As a control, the membrane potential was completely destroyed by adding 1 µM CCCP for 5 min.

The addition of 2 µg/ml oligomycin to the cells 5 min before staining with TMRE blocks the ATP synthase and therefore the maintenance of membrane potential by converting the Proton-pump direction.

7.1.14 ATP measurement

To measure cellular ATP synthesis, directions in the kit *ATP Bioluminescence Assay Kit HSII*, were followed. In short, HeLa cells were diluted to a concentration of 10^5 to 10^8 cells/ml. From this cell dilution 25 µl were pipetted in a white rimmed 96 well plate (Costar 3903). The same volume (25 µl) of cell lysis reagent was added and incubated for 5 min at RT. Addition of 50 µl of the reconstituted luciferase reagent followed by automated injection. The measurement (duration of 2s) started after a 1s delay.

The luciferase from *Photinus pyralis* (American firefly) catalyzes the following reaction:

ATP + D-luciferin + O_2→ oxyluciferin + PP_i + AMP + CO_2 + light. The resulting green light has an emission maximum at 562 nm and can be detected with a luminometer. As the light output is directly proportional to the ATP concentration, the emission intensity can by used to quantify the ATP concentration in the cell.

7.2 Protein methods

7.2.1 SDS-Page and Western Blot

Cells lysed in 2x sample buffer were denatured at 95°C, 10 min and separated on SDS-Page. Preparation of gels took place according to Sambrook *et al*.[72]

Proteins separated on SDS-PAGE were transferred on a Polyvinylidenfluorid- (PVDF) membrane in a wet transfer chamber. The membrane was activated with methanol and equilibrated in transfer buffer. Mounting of the transfer is done in the following order: cathode, foam, two 3 mm Whatman Papers, Gel, PVDF-membrane, two 3mm Whatman Paper, foam, anode. The proteins were transferred at 4°C either for 3½h at 250mA or o/n at 100mA per chamber. The transfer efficiency was controlled by staining the membrane with PonceauS or Coomassie solution. After removal of the staining with TBS-T or 100% methanol, respectively, the membrane was blocked with 10% dry milk in 3% BSA/ TBS-T for 1h at RT, to avoid unspecific binding. After rinsing the membrane with TBS-T, the primary antibody was added in the appropriate dilution in 3% BSA/ TBS-T and incubated o/n at 4°C, rotating or shaking. The membrane was then washed 6 times for 5-10 min with TBS-T, incubated for 1h at RT with the specific horseradish peroxydase-conjugated secondary antibody diluted in 3% BSA/ TBS-T. The

membrane was again washed as described above. Binding of the secondary antibody is detected with the enhanced chemoluminescence (ECL)-system from NEN. The chemoluminescence appeared by conversion of the peroxydase substrate and is revealed with sensitive Kodak film.
Quantification of the protein signal was performed using the AIDA software.

7.2.2 Coomassie staining

Proteins in a SDS-PAGE were stained with Coomassie® by placing them for 30min-1h in Coomassie solution. A surplus of Coomassie was removed by rinsing the gel for 1h - o/n in destainer solution. The gel was dried for 2h at 80°C on whatman paper using a vacuum drier.

7.2.3 In vitro translation

The *in vitro* translation permits expression of proteins. The TNT® T7 Quick coupled Transcription/Translation System provides reagents necessary for transcription and translation at the same time. A T7 Bacteriophage-RNA-Polymerase is used for transcription of pET28c or pcDNA3 plasmid constructs with a rabbit-reticulocyte lysate for translation. The reaction mix for 1 µg DNA plasmid contained 20µl TnT-reticulocyte Lysate, 1 µl S^{35}-Methionine (10 µCi/µl) and H_2O filled up to 25 µl. The expression reaction was performed at 30 °C for 90 min. To control the protein expression 2 µl lysate are mixed with 8 µl 2x Sample buffer, denatured at 95°C, 5 min and separated on a SDS-PAGE. The gel is fixed in 25% MeOH/acetic acid and protein expression detected by exposure to an imaging plate and detection using a PhosphoImager.
The in vitro translated proteins were used in GST-trapping experiments.

7.2.4 Protein expression in E.coli

This protocol permits efficient production of heterologous proteins in *E.coli*. 20 ml of LB culture medium supplemented with antibiotics (34µg/ml chloramphenicol for selection of the pACYC-based plasmid and 100 µg/ml ampicilin for selection of the vector of interest) were inoculated with the clone of interest transformed in BL21 codon plus cells and incubated at 37°C in a shaking incubator at 200 rpm o/n. The next day, this culture was transferred to a 500 ml culture with the appropriate antibiotics and cultured at 37 °C, 200 rpm until the OD_{600} 0.5 was reached. An aliquot of an OD 0.1 was taken as uninduced control. Cultures were then cooled

down to RT, induced with 0.2mM IPTG and incubated at 19°C o/n, 200rpm to avoid formation of inclusion bodies. Again aliquots on an OD 0.1 are taken for expression control. Bacteria are pelleted by centrifugation at 1000g, 10 min, 4°C and stored at – 80 °C. Successful expression was controlled by lyzing aliquots in 2x sample buffer. Samples were separated on a SDS-PAGE and the proteins stained with Coomassie solution.

7.2.5 GST-tagged protein purification from E.coli

The bacteria pellet obtained in paragraph 7.2.4 was lysed in an appropriate volume column buffer, sonicated 4-5 times (puls 10, level 7) and centrifuged at 15000g, 4°C 30 min. The supernatant should contain soluble protein.
In case of inclusion body formation, when no soluble protein could be obtained, the protocol was changed as follows:
The bacteria pellet was resupended in 10ml icecold Buffer1, sonicated 4x15 sec, level 7. 1M MgCl was added to a final concentration of 200 µM as well as lysozyme and incubated for 30 min at RT. 1 µl Benzonase was added for an additional 30 min to digest DNA. The solution was centrifuged with 17000g, 10 min at 4°C. The pellet was resuspended in 10ml ice cold buffer 2, incubated on ice, 30 min, centrifuged for 10 min, 4°C with 17000g. The obtained pellet was again resuspended, this time in 3ml buffer 3 (RT), incubated 10 min at RT and centrifuged with 17000g at RT. To refold the denatured protein a special refolding procedure was included.

7.2.6 Protein refolding

The protein, soluble in buffer 3, was very slowly injected in 50ml fast turning refolding buffer and kept swirling o/n at 4°C. The solution was concentrated by centrifugation using Centrifugal filters with an exclusion size of 30kDa to a final volume of ca. 2ml. The concentrated protein was dialyzed in PBS, o/n, 4°C using a tube with an exclusion size of 15 kDa.
The protein concentration can be estimated by running aliquots on a SDS-PAGE next to defined concentrations of BSA followed by a protein staining with Coomassie®.
The soluble, possibly refolded protein can then be used for binding assays. Per 30 µl prepared glutathione sepharose beads (7.2.8) 100 µl -500 µl soluble purified

protein were added and rotated for 1h at 4°C, All centrifugation steps were performed at 500g, 5min, 4°C. The samples were washed 2-3 times with cold column buffer and were then ready for the binding assay (7.2.8).

7.2.7 GST-tagged protein purification from SF9 cells

A pellet of SF9 cells was lysed in 12ml lysis buffer, rotating at 4°C for 40 min. The lysate was centrifuged at 26900 g, 40 min, 4°C. The soluble protein was then used in the binding assay. Therefore, 6ml to 12ml are mixed with 200 µl prepared glutathione sepharose beads (see 7.2.8) (30 µl per sample) and incubated at 4°C for 2h while rotating. All following centrifugation steps were performed at 500g, 4°C for 5 min. To wash the GST trapped protein, the beads were incubated thrice with 15ml washing buffer, rotating at 4°C for 15 min, interrupted by centrifugation steps. The beads, loaded with GST-tagged protein, were now ready for the binding assay (7.2.8)

7.2.8 Binding assay

Glutathione Sepharose beads were washed 2-3 times with column buffer, centrifuged at 500g, 5 min, 4°C. To obtain a 50% slurry an appropriate amount of column buffer was added.

To the GST-trapped protein *in vitro* translated protein (6 µl) and 100-200 µl binding buffer were added and rotated for 2h at 4°C. This process was followed three times by three subsequent washing steps with washing buffers A to C. Between each step the samples were centrifuged at 500g, 5 min, 4°C and the supernatant discarded. To elute the GST-trapped protein, 100 µl elution buffer was added and the sample shaken at 4°C for 15 min. After a centrifugation of 3min at 20000g the supernatant was collected, 2x sample buffer added, denatured for 5 min, 95°C and the proteins separated on SDS-PAGE followed by a Coomassie staining. Bound, S^{35}-labeled protein was detected by exposure to an imaging plate for 1h to 5d and quantified with the AIDA program.

7.2.9 Immunoprecipitation

To analyze if two or more proteins interact in vivo, an immunoprecitpitation can be performed. Cells from a confluent 10 cm dish were lysed in 1 ml Chaps buffer on ice for 20 min. After sonication (10 sec, level 7), the lysate was centrifuged at 4°C for 30 min and the supernatant was transferred to a fresh microfuge tube. 7µg

antibody of the to be immunoprecipitated protein were added and rotated o/n at 4°C. The next day, 30 µl of a mix of Protein Sepharose A and G beads were added and rotated for 2h at 4°C. A surplus of proteins was removed by washing thrice with Chaps buffer. The beads were taken up in 100 µl 2x sample buffer, boiled for 10 min at 95°C, spun down at 20000g for 5 min and the supernatant transferred to a fresh microfuge tube. Co-immunoprecipitated protein was detected by immunoblotting with a specific antibody.

7.3 Nucleic acid methods

7.3.1 Nucleic acid purification

DNA and RNA were purified using Qiagen kits (DNA Maxi, Midi, Mini kits, DNA endofree-Maxi kit, RNeasy mini kit, PCR purification kit, Gel extraction kit) according to the provided manual.

7.3.2 Generation of cDNA

To clone new genes into expression vectors, RNA had to be transcribed into cDNA using the Thermoscript^{TM} RT-PCR kit from Invitrogen. 2 µg RNA were mixed with 1µl oligo-dT primer in a total volume of 10 µl and incubated at 65°C for 5 min, then placed on ice. A second reaction mix contains 4 µl 5x cDNA buffer, 1 µl 0.1 M DTT, 1 µl RNAse out 2 µl dNTPs (10 mM) and 1 µl thermoscript. This mix was pipetted to the first one, incubated for 1h at 50°C and inactivated at 85°C for 5 min. 1 µl RNAse H was added and incubated at 37°c for 20 min. The sample was put on ice if a PCR reaction follows or stored at -20°C.

7.3.3 Design of shRNAs

To specify a shRNA sequence to target mRNA, the desired mRNA was searched for a sequence with following structure: AAG$(N)_{18}$TT, using the Vector NTI program.

The sequence G$(N)_{18}$ was then checked for ~50%GC content and a terminator sequence with less then 4 A or T. Bizarre looping and homology to other genes should be avoided. To recreate the +1 position of the U6 promoter (in case of cloning into he pLL3.7 vector system), T was added in the beginning of G$(N)_{18}$. The next step was adding the loop TTCAAGAGA as well as the reverse

complement of $G(N)_{18}$ to the end. For the later cloning the anti sense strand was created and the respective restriction enzyme ends were added.
These constructs are termed ‘oligos’ in the following course.

7.3.4 Annealing of shRNA oligos

To clone shRNAs into the corresponding lentiviral vector, a sense and antisense singlestrand with the appropriate restriction sides were ordered and diluted to a concentration of 1mM. The oligos had to be annealed, using 1 µl of each oligo in 48µl 1x annealing buffer in the following process: denaturing at 95°C, 4 min, annealing at 70°C, 10 min. The reaction was cooled down to 4°C with 0.1°C/sec and hold at 4°C for an additional 10 min or longer.

7.3.5 Cloning of shRNA oligos into lentiviral vectors

The cloning of shRNA oligos into lentiviral vectors applies parts of the *Di-/Tri-Sec* method which is described in chapter 7.3.6. These are the Klenow reaction (7.3.6.2) and trimming of the insert (7.3.6.5).

7.3.5.1 Cloning into pLL3.7

The shRNA oligos were cloned into the pLL3.7 vector via HpaI and XhoI restriction sides. For an optimal yield in inserted shRNAs the protocol given below should be followed strictly:

1. step	**2. step**
2 µg pLL3.7 DNA	Klenow reaction: add
10 µl 10x NEBuffer 4	2.5 µl dCTP (20mM)
1 µl 100x BSA	2.5 µl dTTP (“)
2 µl Xho I (NEB)	2.5 µl Buffer H (Roche)
4 µl Hpa I (NEB)	2.5 µl Klenow (Roche)
x µl ddH_2O	50 µl @ RT, 30 min
100 µl @ 37°C o/n	denature 75°C, 15min
PCR purification kit,	
elute in 40µl ddH_2O (Qiagen)	

3.step	**4. step**
Phenol/Chloroform extraction: + 50 µl H_2O 10 µl 3M NaOAc, pH 5.2 110 µl Phenol Chloroform 220 µl, vortex (30''), spin high, 5 min Take top layer, transfer to new tube, +300 µl ice cold ethanol Keep at –20°C, at least 20'- o/n Spin high, 20 min, 4°C, wash pellet w/ 70% ethanol, air dry, resuspend in 30µl H_2O	ligate: 2µl annealed oligos, 6µl prepared DNA 1µl T4 ligase 1µl blunt end ligase buffer 16°C o/n 65°C 15min

2µl-6µl of ligated DNA were transformed into E.coli, plated on Amp+ LB-plates and incubated o/n at 37°C, followed by a colony PCR (7.4.2) to identify successfully inserted shRNAs.

7.3.5.2 Cloning into pLVTHM

The shRNA oligos were cloned into the pLVTHM vector via MluI and ClaI restriction sides.

1. step	**2. step**	**3.step**
5 µg pLVTHM DNA 10 µl 10x NEBuffer 4 1 µl 100x BSA 10 U MluI (NEB) 5 U ClaI (NEB) x µl ddH_2O 100 µl @ 37°C o/n PCR purification kit, elute in 40µl ddH_2O (Qiagen)	T_4 reaction: keep on ice and add 4 µl dGTP (20mM) 0.5 µl 100x BSA (1mg/ml) 0.5 µl 10x NEBuffer 2 5 µl T_4 polymerase 50 µl @ 12°C, 30 min inactivation 75°C, 10min PCR purification kit, elute in 40µl ddH_2O (Qiagen)	ligate: 5µl annealed oligos, 6µl prepared DNA 1µl T4 ligase (800U) 2µl 10x ligase buffer 6µl ddH_2O 16°C o/n 65°C 15min

20µl ligation mix were transformed into E.coli, plated on Amp+ LB-plates and incubated o/n at 37°C, followed by a colony PCR (7.4.2)

7.3.5.3 Subcloning the H1 expression cassette from pLVTHM into pLVPT

1. step	**2. step**	**3.step**
10 µg pLVPT DNA	4 µg pLVTHM DNA	ligate:
10 µl 10x NEBuffer 4	10 µl 10x NEBuffer 4	60 nmol prep. pLVPT
1 µl 100x BSA	1 µl 100x BSA	210 nmol prep. pLVTHM
20 U Xho I (NEB)	20 U Xho I (NEB)	(for calc. 7.3.6.6)
20 U Xba I (NEB)	20 U Xba I (NEB)	1 µl T4 ligase (400 U)
x µl ddH_2O	x µl ddH_2O	2 µl 10x ligase buffer
50 µl @ 37°C o/n	50 µl @ 37°C o/n	x µl ddH_2O
gel purification (0.8% TAE	gel purification	20µl @ 16°C o/n
agarose gel) of the 11,133 bp	(1% TAE agarose gel)	65°C 15min
fragment (QIAquick Gel Extr.	of the 800 bp fragment	
Kit), elute with 50µl ddH_2O	(see 1.step)	

20µl of ligation mix were transformed into E.coli, plated on Amp+ LB-plates and incubated o/n at 37°C. DNA (mini prep) from isolated colonies was subject to a restriction digest with SacI to identify successfully inserted H1-shRNA cassettes.
In case of a display of the expected DNA band pattern of 5840, 2821, 1648, 1483 and 191 bp DNA was send to sequencing.

7.3.6 Cloning of PCR fragments with the Di-/TriSec method

7.3.6.1 Enzyme digest of vector

4 µg vector DNA were digested with the appropriate restriction enzymes in a final volume of 100µl in H_2O o/n at 37°C. In case of EcoRI being a required restriction enzyme, it was added to the reaction mixture after the o/n digest for 1h at 37°C. The reaction mixture consisted of 1x enzyme specific buffer, 1x BSA and the corresponding enzymes in an activity of 10 U. If possible a double digest was performed.

The digested DNA was either purified using the PCR purification kit from Qiagen or in case a fragment had to be discarded the Gel purification kit from Qiagen. Both kits were used according to the manual and the DNA was eluted in 40µl H_2O.

7.3.6.2 Klenow reaction

After digestion of the vector with endonucleases, the 5' overhangs were filled with the respective dNTPs using the Klenow polymerase. The reaction mixture contained 10 U Klenow enzyme, 2.5 µl each dNTP (20mM), 1x specific enzyme buffer, 1x BSA in a total of 50µl and was incubated at RT for 30 min and inactivated at 65°C, 15 min. With providing only selected nucleotides, 5' overhangs could be generated, that fitted perfectly to the T4-trimmed PCR fragments (7.3.6.5).

The in this way treated vector was purified by Phenol/Chloroform extraction.

7.3.6.3 Phenol/Chloroform extraction

To the heat inactivated Klenow reaction mixture 50 µl H_2O were added to a total volume of 100 µl. 10µl 3M sodiumacetate pH 5.2 were added and an equal amount to the total of 110µl of Phenol/Chloroform. The mixture was vortexed for 30sec and centrifuged at 20000g for 5 min. The aqueous top layer containing the DNA was transferred to a fresh microfuge tube, 300 µl ice cold ethanol were added, mixed and incubated at -20°C for 15 min or longer. To pellet the precipitated DNA, the mixture was centrifuged at 20000g, 4°C, 30 min. The supernatant was discarded, the pellet washed with 70% ethanol, airdried and in 30 µl H_2O resuspended.

7.3.6.4 Gradient PCR

A PCR reaction allows the amplification of small amounts of DNA. In a gradient PCR the annealing temperature of the primer varies between two set temperatures and ensures this way the amplification of a fragment with two formerly unused primer. The typical reaction mixtures contained 10 ng vector DNA or 0.5 µl cDNA, 0.5 µl dNTPs (10 µM), 0.1µl of each primer (100 µM) and 0.25 µl long template Expand polymerase filled to a total of 25 µl with H_2O.

The DNA was denatured for 1:30 min at 95 °C, primer were annealed at their specific annealing temperature with a gradient between 50°C and 64°C (8 slots) for 0:30 min and elongation was done at 68 °C for a defined time: 1min for 1 kilo

base (kb). After 30 cycles, a last elongation phase was made at 68 °C for 10 min. The correct amplification of DNA was analyzed on an agarose gel. Positive samples were pooled and purified (eluted in 30µl H_2O) using the PCR purification kit.

7.3.6.5 Trimming of PCR product

To generate sticky ends corresponding to the prepared vector, the PCR product was subject to a T4 -DNA -Polymerase reaction. The large fragment of the T4 polymerase removes 3' ends. Adding specific nucleotides in excess allows a controlled exonuclease activity. The purified PCR fragment was mixed on ice with 1x specific enzyme buffer, 1x BSA, 4 µl of specific dNTPs (20 mM) and H_2O to 48 µl. Finally 2 µl T4-polymerase were added and the mixture was incubated in the cold room at 12°C for 30 min. The reaction was stopped at 75°C, 15 min and the product purified by Phenol/Chloroform extraction (7.3.6.3). The DNA concentration was estimated by running a sample on an agarose gel together with a weight marker.

7.3.6.6 Ligation

Generated and trimmed PCR fragments and the prepared vector were ligated o/n at 16°C. Typically the following formula was used:

$$\frac{8x\mu g\ vector\ x\ size\ insert\ bp}{size\ vector\ bp} = \mu g\ insert$$

The ligation mixture should not exceed 20µl, containing 1-2 µl T4 ligase and 1x specific enzyme buffer. The next day, 5 µl of the ligation mixture was used for the transformation in competent E.coli, Top10F or DH5α (7.4.2).

7.3.7 Vector amplification

To induced deletions of small fragments of a gene / protein, the whole vector had to be amplified. The primer used for the amplification started in opposite direction, leaving the to be deleted fragment open. A new restriction side was induced that was used to ligate the linearized vector.

The PCR reaction is equivalent to the gradient PCR for fragment amplification (7.3.6.4), save for an extended amplification step. The amplified vector was digested with the correspondingly induced restriction side at 37°C, 2h and directly transformed into competent *E.coli* (7.4.2).

7.3.8 Colony screen

To check colonies from the transformation procedure for positive expression of the newly cloned vector, a PCR was done with the bacterial DNA to analyse the presence of the vector/insert of interest. A colony was picked with a yellow tip, transferred to a PCR tube and the rest plated on a fresh LB-plate with the appropriate selection antibiotic. The PCR mix, containing 1 µl of the respective primer (10 µM), 0.3 µl dNTPs (10 µM), 2.5 µl 10x Taq buffer, 0.15 µl Taq polymerase and H_2O to 25µl was added to the tube and the DNA was amplified in the following process: cracking of bacteria at 95°C, 1:30 min, 25 cylces of denaturing DNA at 95°C, 0:30 min, annealing at 55°C, 0:30 min and elongation at 68°C for 1 min (or extended according to length of insert) with a final elongation step at 68°C for 10 min. The DNA was analyzed on an agarose gel.

Another method to screen colonies for positive clones was a restriction digest of prepped DNA, to control the presence of an insert by comparing the size to a control vector. Minipreps of colonies were done according the following protocol: 1.5 ml cells of a 4ml overnight culture from a single colony were harvested by centrifugation and the pellet was resuspended in 700µl STET buffer. 25µl lysozyme stock solution and 1µl RNAse were added. After incubation for 5-10 min on ice, tubes were boiled for 1 min. The cell debris pellet was removed with a toothpick after centrifugation for 10 min at 20000g. To precipitate the DNA, an equal volume of isopropanol was added and the tube was spun for 10-20 min at 20000g. The pellet was washed with 70% ethanol, air dried and resuspended in H_2O. To screen for clones, DNA was digested with appropriate enzymes for 2h, 37°C and run on an agarose gel.

7.3.9 Reverse Transcription and PCR (qRT-PCR)

The RNA knockdown induced through RNA interference was validated by qRT-PCR. Thereto RNA was prepared using the RNeasy kit from Qiagen. For a PCR reaction in 96 well PCR plates 10 µl of RNA (10 ng/µl) and 15 µl PCR mix were needed. Validated primer were provided by the RNAi group. The PCR mix was composed of 1.75 µl H_2O_{depc}, 12.5 µl PCR buffer (contains SYBR Green, dNTPs and Polymerase), 0.5 µl paired primer (mix from 5`and 3`primer, each 20 µM) and 0.25 µl reverse transcriptase. The thermocycler conditions were as follows: transcription of RNA into cDNA at 50°C, 30 min followed by a 90°C denaturation

step for 15 min. Amplification of the specific fragments took place in 45 cycles: 94°C for 20 sec, 60°C for 40 sec, 72°C for 40 sec and finally 95°C, 15°C, 60°C. A dentaturation step of 15 sec, 95°C, 15 sec followed by a slow cooling step, ramp rate 2%, was done to determine the melting temperature. The results were analyzed using the SDS2.2.2 program

7.4 Miscellaneous methods

7.4.1 Preparation of competent E.colis

Competent *E.coli* were cultured in 10 ml LB-medium o/n at 37 °C at 200 rpm in a shaking incubator. This culture was used to inoculate a 200 ml culture, cultured at 37 °C at 200 rpm until the OD_{600} 0.5 was reached. Bacteria were incubated for 10 min on ice and then centrifuged at 4 °C, for 10 min at 4,000 rpm. All further steps were performed at 4 °C. Bacteria were resuspended in 80 ml Tfb1 buffer, incubated 1h on ice and again centrifuged. The pellet was then resuspended in 8 ml Tfb2 buffer and incubated for 30 min on ice. Aliquots of 100 µl were prepared, shock frozen in liquid N_2 and stored at –80 °C.

7.4.2 Transformation of E.coli

50-100 µl of competent *E.coli* cells were thawed on ice and incubated 20 min on ice with a variable amount of DNA. After a 45s heat shock at 42 °C and 2 min incubation on ice, 300 µl prewarmed LB-medium were added and the mixture was incubated at 37 °C, shaking for 30 to 60 min. Bacteria were finally plated on LB-plates with the corresponding antibiotic (Amp, Kan) for the plasmid DNA selection and incubated at 37 °C, o/n.

7.4.3 Quantification of mitochondria fragmentation with ImageJ

ImageJ is a free software in Java to process and analyze images. Mitochondria fragmentation was quantified by first taking confocal images of immunofluorescently labeled mitochondria which were then directly analyzed in ImageJ. Thereto acquired tif.files were converted into 8-bit grayscale and by setting the threshold automatically, changed to a ‘binary’ image in black and white. The scale was set (512 pixels to 159 µm (image size)) and values to analyze particles were adapted. To identify fragmented mitochondria the size to be identified was set to $0\mu m^2$-infinity with a circularity between 0.1 to 1.00.

Filamentous mitochondria were identified by setting the size to 10µm^2-infintiy and the circularity to 0-0.1. The total area for each setting was measured and the percentage filamentous/fragmented mitochondria was evaluated in reference to the overall total area. For each time point of induction ~50 cells were analyzed.

7.4.4 Quantification of protein expression levels with AIDA image analyzer

To be analyzed with the AIDA program, images had to be acquired with the Image Reader (immunoblots) or BasReader and saved as .img files.

To quantify protein expression the 1DE evaluation was chosen. For OPA1 expression, vertical frames for each expression pattern were set and peak determination was set manually by means of the peak profiles as was the background. Total OPA1 expression for one sample was 100% with corresponding distribution within the expression pattern for the single bands a-e,

For quantification of a prohibitin knockdown a horizontal frame was placed across comparative bands and peak determination was set manually according to the display in the peak profiles.