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Production and characterization of a recombinant UCP2 tagged with a new tool for its subcellular visualization

Bachelor thesis

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1. Introduction and goals:

1.1. Introduction

1.1.1. Mitochondria

Mitochondria are known as “the powerhouse of the cell” due to their prominent role in the producing energy through respiration and regulation of the cellular metabolism. Energy is essential for fundamental processes such as cell movement, biosynthesis and ion transport and is provided by mitochondria in the form of adenosine triphosphate (ATP). Mitochondria consist of an outer and an inner membrane, separated by the intermembrane space. Unlike the outer membrane, the inner membrane has a more restricted permeability and is populated with proteins involved in ATP synthesis.

1.1.2. ATP generation in mitochondria (coupling)

The main source of ATP synthesis is oxidative phosphorylation (Figure 1.1) where electrons are transferred from electron donors to electron acceptors. This redox reaction is facilitated by the electron transport chain (ETC) located in the inner membrane of the mitochondria. The ETC consists of 5 different protein complexes (I – V*).* During glycolysis and the citric acid cycle, the coenzymes NAD^+ and FAD^+ are reduced and their electrons are later released to ETC. Complex I receives the electrons from NADH and Complex II receives electrons from $FADH_2$, these electrons reduce ubiquinone to ubiquinol. Ubiquinol is then oxidized in complex III and its electrons are accepted by cytochrome C. The reduced cytochrome C is oxidized by complex IV and consequently a water molecule is formed. Alongside with the electron transport, the complexes I, III and IV pump protons from the mitochondrial matrix into the intermembrane space. This creates a proton gradient which is used by the F0F1-ATP synthase (complex V) to produce ATP via oxidative phosphorylation (Mitchell 1961).

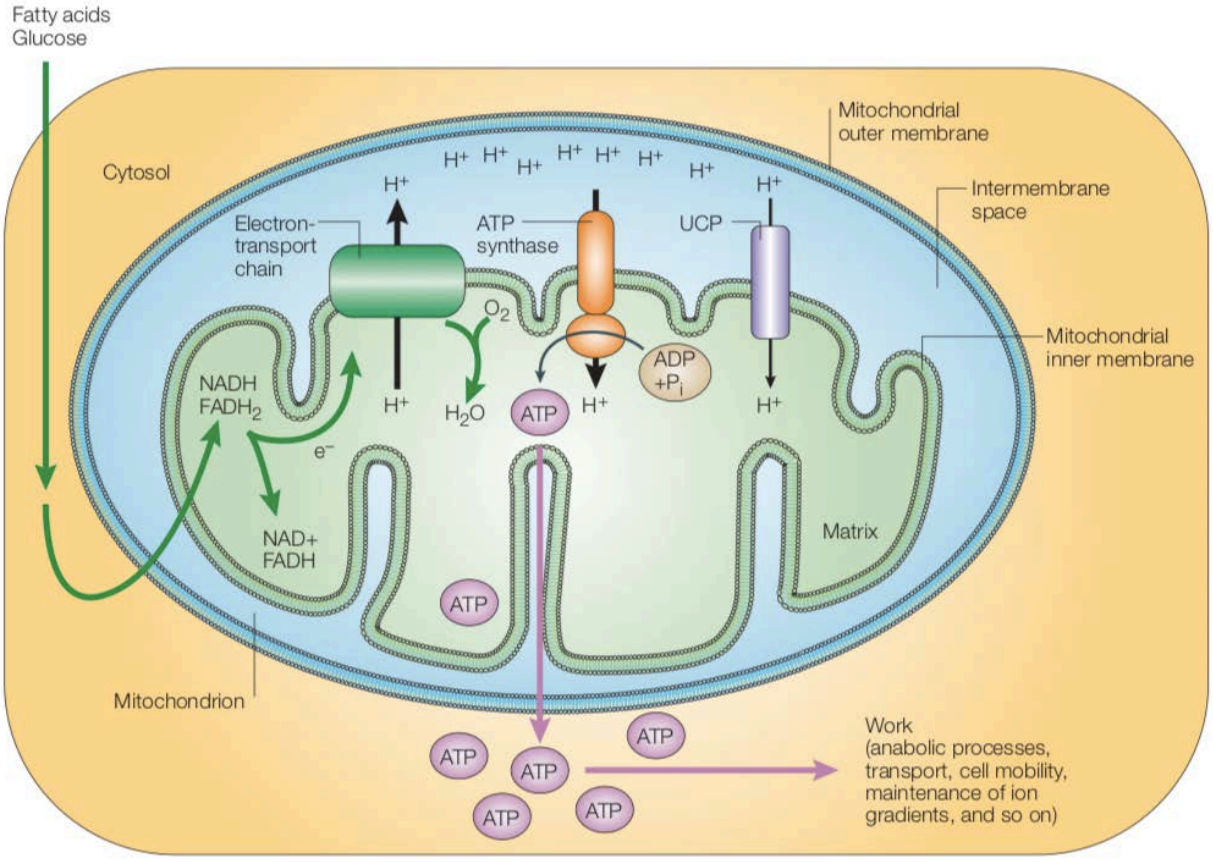

Figure 1.1 ***Oxidative phosphorylation in mammalian cells (Krauss, Zhang et al. 2005)***

1.1.3. Uncoupling in mitochondria

Protons can return to the matrix independently of ATP synthase, resulting in lower efficiency of oxidative phosphorylation and heat production which, is known as “proton leak” and has been observed in mitochondria of various tissues and individuals at different degrees(Brand 1990). Proton leak can be induced through the adenine nucleotide translocase (ANT) and uncoupling proteins (UCPs)

1.1.4. Family of uncoupling proteins

The uncoupling protein subfamily belongs to the mitochondrial carrier (MC) superfamily (SLC25). The members of this superfamily are mainly anion carriers and are claimed to shuttle metabolic substrate across the mitochondrial inner membrane (Palmieri and Pierri 2010).

The UCP subfamily consists of five known members (UCP1 - UCP5). UCP1 was first discovered in 1976 (Nicholls 1976)It is, so far, the most investigated member of the UCP family. UCP1 is expressed in brown adipose tissue (BAT), where it mediates proton leak and produces heat (Shabalina, Petrovic et al. 2013). This dissipates the proton gradient across the inner mitochondrial membrane and reduces ATP generation. Therefore, UCP1 plays an important role in non-shivering thermogenesis and burning calories. Unlike UCP1, which is expressed only in BAT, the tissue distribution of other UCPs are more diverse. For example UCP2 is highly abundant in fast proliferating cells, which rely on an aerobic glycolysis, such as cancer cells, stem cells and cells of the immune system (Rupprecht, Sittner et al. 2014), while UCP3 has been found in heart and skeletal muscle and is very abundant in BAT (Hilse, Kalinovich et al. 2016).There is a constant confusion about the distribution of UCPs in different tissues, which can be explained by the lack of specificity of the commercial antibodies for UCPs, as well as the proteins short lifetime. It hinders the investigation of their function. Therefore, the exact functions for UCP2-UCP5 remain unknown. There are different theories about the role of UCPs. A popular hypothesis is the regulation of reactive oxygen species (ROS) in mitochondria through UCPs (for review (Krauss, Zhang et al. 2005)). Figure 1.2 shows the suggested concept for the expression of different uncoupling proteins from (Pohl, Rupprecht et al. 2019).

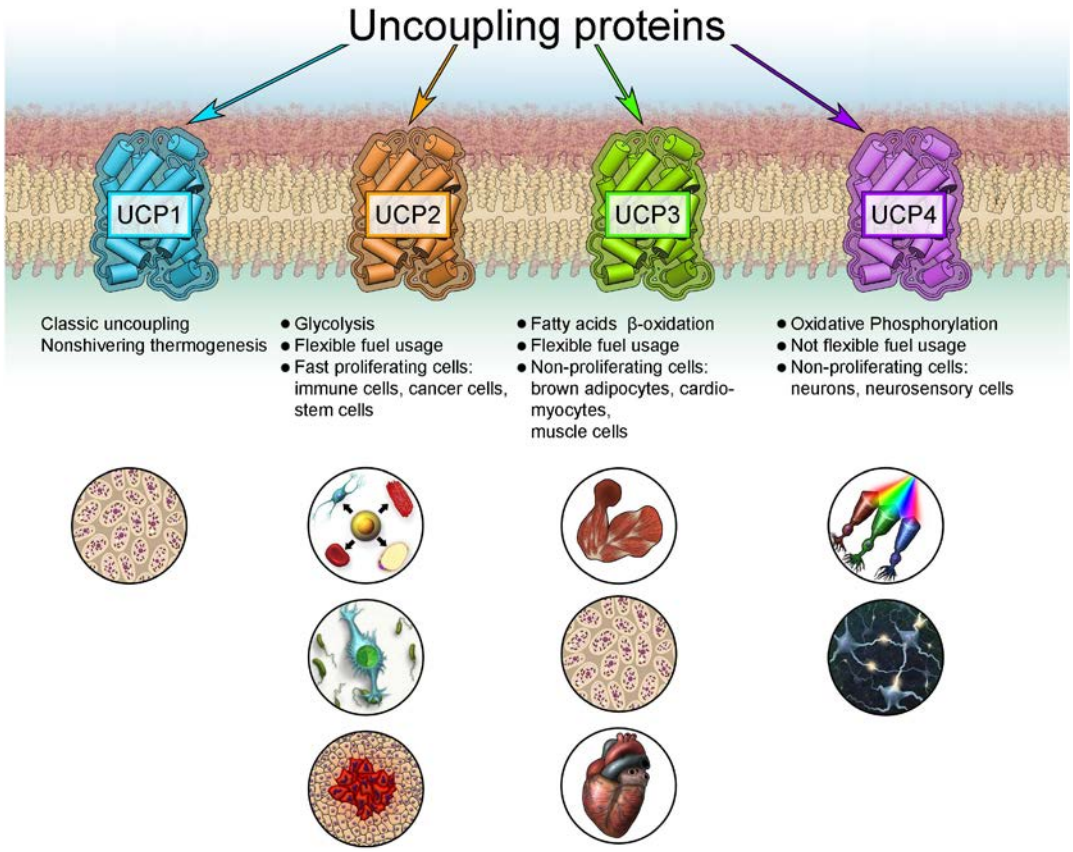

Figure 1.2 ***UCP family tissue expression adapted from (Pohl, Rupprecht et al. 2019)***

1.1.5. Mitochondrial uncoupling protein 2

UCP2 was first discovered in 1997. It consists of 309 amino acids and has a molecular weight of 33 kDa. UCP2's amino acid sequence has 59% homology to UCP1's (Fleury, Neverova et al. 1997). UCP2 is very unstable with a half-life of only 30 minutes (Rousset, Mozo et al. 2007). The transmembrane arrangement of UCP2 is shown in Figure 1.3.

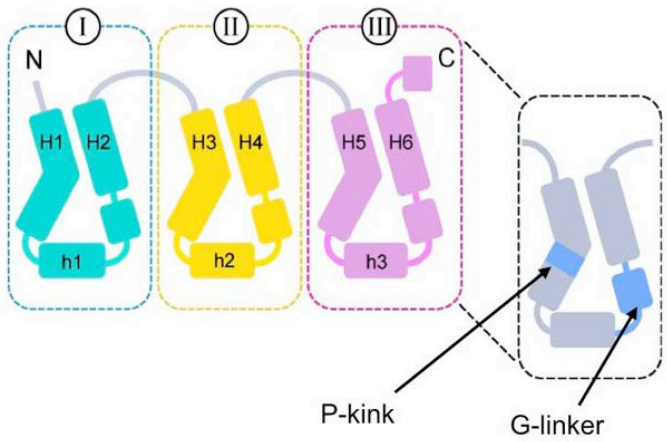

Figure 1.3 ***The transmembrane arrangement of UCP2 adapted from (Berardi and Chou 2014):*** *The transmembrane arrangement of UCP2 consists of 6 alpha helical regions spanning the lipid bilayer, N and C represent the respective termini.*

1.1.6. Activation and proton transport function of UCP2

It was shown that despite their different physiological function, both UCP2 and UCP1 have a similar protonophoric function (Beck, Jaburek et al. 2007). Free long chain fatty acid (FA) is known to activate UCP2, therefore mediating proton leak (Jezek, Jaburek et al. 2019). The exact mechanism of FA-mediated protonophoric activity of UCP2 is still not clear. However, all the suggested models can be categorized into two groups.

The first group hypothesizes the FA cycling mechanism (section 1.1.6.1) introduced by (Garlid, Jaburek et al. 1998) and (Skulachev 1991), while the second group identifies UCP as a proton transporter in the presence of FA (section 1.1.6.2).

1.1.6.1. Fatty acid cycling model

This model proposes that UCP2 acts as an anion channel, transporting fatty acid anions, which are normally unable to cross bio-membranes from the mitochondrial matrix to the intermembrane space. This model suggests that a FA anion will get protonated and flip-flop across the membrane

to the matrix side (Kamp and Hamilton 1992), where it becomes deprotonated again, continuing the cycle (Figure 1.4.A) (Garlid, Jaburek et al. 1998),(Skulachev 1991).

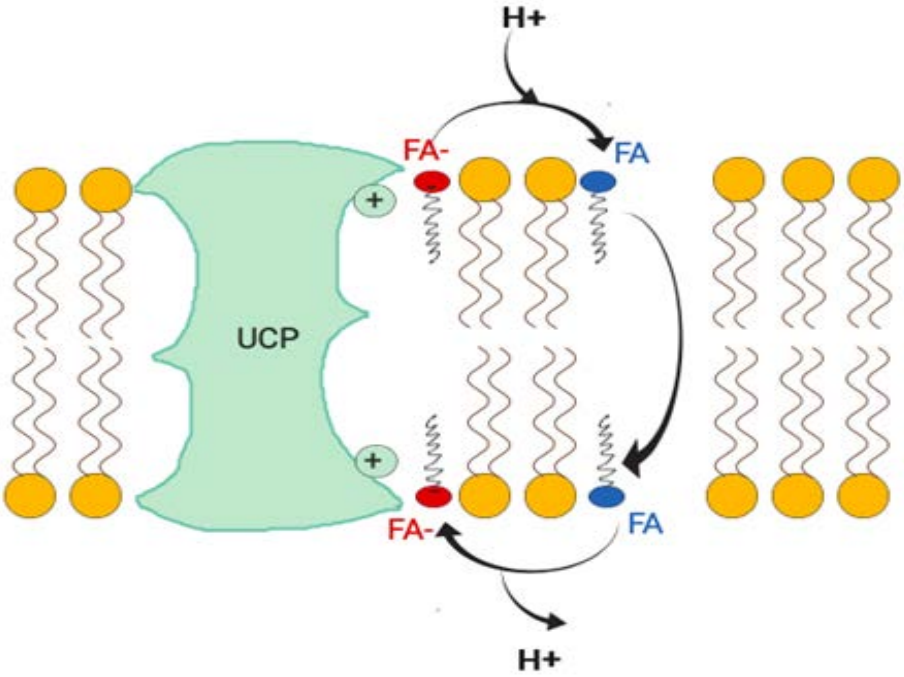

Figure 1.4.A ***Fatty acid cycling model (modified from (Garlid, Jaburek et al. 2001)):*** *The fatty acid anions (red) are protonated (blue) in the intermembrane space, enabling them to diffuse to the matrix side, through the membrane. At the matrix side the fatty acids become deprotonated (red) and are transported back to the intermembrane space with the help of UCPs.*

1.1.6.2. Fatty acid buffering and fatty acid shuttle model

In the FA buffering model, UCP2 is a proton channel, while FA acts as its co-factor. FA anion binds inside the UCP2 cavity and its carboxyl group enables protons to translocate through the channel, from the cytosolic side to the matrix side (Klingenberg and Huang, 1999; Klingenberg, 2010). FAs are bound to UCP2 through hydrophobic interactions (Figure 1.4.B).

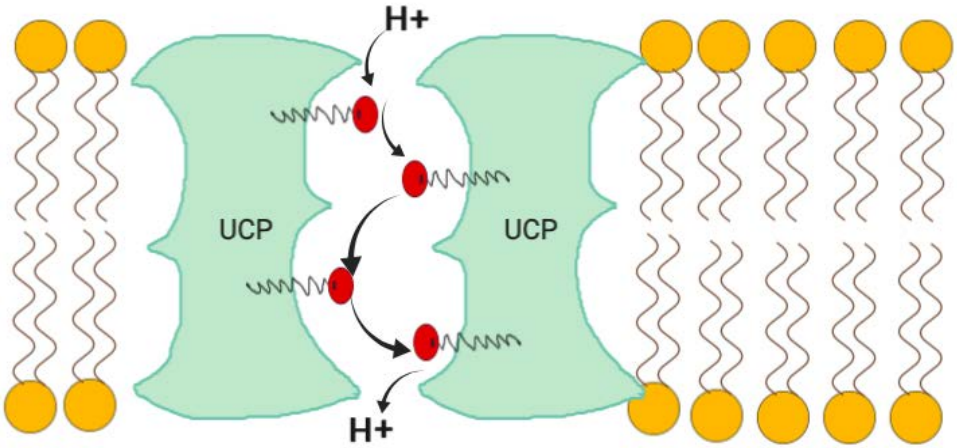

Figure 1.4.B ***Fatty acid buffering model:*** *The fatty acid anions (red) are bound to the protein (UCP). The bound fatty acids transport the proton by buffering it from the intermembrane space side to the matrix side.*

Recently a modification to the FA buffering model was purposed: the "FA shuttle model", which suggests that FA anion binds inside the pore of UCP2 (intermembrane space site) and transfers protons by shuttling from them to the matrix side.

1.1.7. Inhibition of UCP2

Purine nucleotides, ATP, ADP, GTP and GDP, are known to bind to UCP2 with high affinity and inhibit its activity (Jaburek, Varecha et al. 1999).

1.1.8. Biological function of UCP2

UCP2 is known to be abundant in fast proliferating cells such as stem cells, cancer cells or cells of the immune system, which undergo an aerobic glycolysis. It has been suggested that UCP2 facilitates metabolic adaptability, allowing the cell to survive during specific nutrient deficiencies (Rupprecht, Moldzio et al. 2019). However, the exact function of UCP2 is under debate, due to its strict regulation and its short half-life (30 minutes). Knowing the correct localization of UCP2 is

crucial for studying its function. However, the low specificity of available antibodies hinders investigating the exact localization of UCP2 and as a result causes confusion about its specific function.

Therefore, our goal was to introduce a small peptide tag (Spot-Tag®) into the primary sequence of UCP2 to target UCP2 specifically.

1.1.9. Spot-Tag

The Spot-Tag® is an affinity optimized, inert and short peptide tag consisting of 12 amino acids with the sequence of: "PDRVRAVSHWSS". Its tag sequence can be added to the N- or C-terminus of the protein of the interest. It has a molecular weight of 1.4 kDa and is bound strongly to Spot Nanobody. Comparing to other tags such as FLAG®-, Myc-, or HA-tags, Spot-Tag is less negatively charged. Additionally, Spot-Tag comprises no Lysine and therefore, is suitable for ubiquitination assays.

The Spot Nanobody, also termed Spot VHH, is derived from single-domain alpaca antibody fragments. It recognizes and binds to the sequence motif of spot-tag (Figure 1.5). In comparison to other antibodies, Spot Nanobody is more robust and stable and also has a higher affinity of 6nM.

The Spot Nanobody can be coupled to beads, enabling immunoprecipitation and affinity purification, or it can be coupled to fluorescent dyes for immunofluorescence and Western blotting.

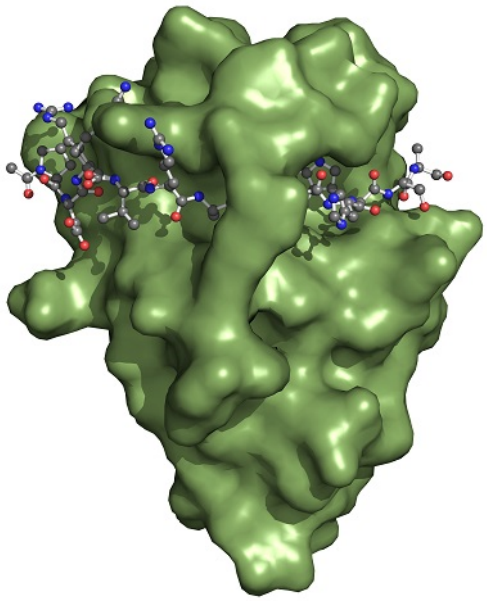

Figure 1.5 ***The interaction of Spot Nanobody with Spot-Tag:*** *The soluble accessible surface of the Spot Nanobody is shown in green.*

1.2. Goals

Since not much about UCP2 is known, due to its short half-life and the low specificity of the available antibodies, an extra tool is needed to target UCP2 specifically and to study it in depth. In this study we aimed to (i) insert an inert short peptide tag (Spot-Tag®) into the sequence of murine recombinant UCP2, and verify the expression of the tag on UCP2 using a specific Spot Nanobody and (ii) examine whether the insertion of the tag affects the protein's integrity and function by performing electrophysiological measurements using planar bilayer membranes reconstituted with recombinant tagged UCP2.

2. Materials and methods

2.1. Materials

2.1.1. Kits

The Assay kits used for site-directed mutagenesis, isolation of plasmids, DNA purification and protein concentration determinations are listed in Table 2.1.

Table 2.1 ***Assay kits***

Kits	Company
Q5® Site-Directed Mutagenesis Kit	New England Biolabs, Germany
Monarch® Plasmid Miniprep Kit	New England Biolabs, Germany
NucleoSpin® Gel and PCR Clean-up	Machery & Nagel, Germany
Micro BCATM Protein Assay Kit	Thermo Fisher Scientific, Austria

2.1.2. Primers

The designed primers (Table 2.2) were produced by Biomers using standard purification conditions. The primers had a concentration of 100µM and were pre-diluted with nuclease-free water to 10 µM to perform site-directed mutagenesis.

Table 2.2 ***Primers designed for the insertion of the spot-tag in C- or N-terminus***

Designated insertion	Direction of the primer	Sequence 5´- 3´	Length	% GC	Tm	Ta
Spot-tag in C-terminus	forward	gtctctcactggagcagcGTTGGTTTCAAGGCCACA	36	56	63 °C	59 °C
	reverse	tgcgcgcacgcgatccggCATTATATCTCCTTCTTAAAGTTAAAC	45	47	58 °C	
Spot-tag in N-terminus	forward	gtctctcactggagcagcTGAGAGCTCCGTCGACAAG	37	59	66 °C	67 °C
	reverse	tgcgcgcacgcgatccggGAAAGGTGCCTCCCGAGA	36	69	67°C	

2.1.3. Expression plasmid

The open reading frame (ORF) of mUCP2 was previously cloned into NdeI and EcoRI sites of PET24a vector (Hilse et al. 2016).

For expression of the target protein in bacteria, the pET24a plasmid (Novagen, Schwalbach, Germany) was selected. This plasmid carries an expression region under control of the T7 promotor and has a kanamycin resistance gene.

2.1.4. E. Coli expression strain

The E. coli expression strain Rosetta (DE3) pLys (Novagen, Germany) was used to express mUCP2 and the modified variant of mUCP2. This strain contains the gene coding for the T7-Polymerase, which allows the expression of proteins under control of the T7 promotor. The T7-polymerase is under control of a lactose-inducible promotor. To ensure that the expression of the target protein does not start before the induction, there is a plasmid with chloramphenicol resistance in Rosetta strain. This chloramphenicol resistant plasmid continuously expresses T7-lysozyme, inhibiting the basal expression of T7-Polymerase. Additionally, the Rosetta strain also expresses tRNAs, which are rarely used to produce bacterial proteins but yet are important for the production of eukaryotic proteins.

2.1.5. Antibiotics

The used antibiotics are shown in Table 2.3.

Table 2.3 ***The antibiotics used for the bacterial culture media***

Antibiotics	Stock concentration	Company	End concentration in culture media
Kanamycin	12.5 mg/ml (diluted in water)	VWR, Germany	25 mg/l
Chloramphenicol	34 mg/ml (diluted in isopropanol)	Lactan, Austria	34 mg/l

2.1.6. Cultivation media for bacteria

Table 2.4. shows the components of the used cultivation media for bacteria (DYT media).

Table 2.4 ***Composition of media used for the cultivation of bacteria***

Substance	Yeast extract and tryptone media (DYT) for plates	DYT liquid media
Peptone from casein	10 g/l	16 g/l
Yeast extract	5 g/l	10 g/l
Sodium chloride	10 g/l	5 g/l
Agar	20 g/l	-

2.1.7. Restriction enzyme

The restriction enzyme used, and its cutting site is shown in Table 2.5.

Table 2.5 ***Restriction enzyme***

Enzyme	Cutting site	Company
Sph-I	5'...GCATG/C...3'	New England Biolabs, Germany

2.1.8. Antibodies

The primary and secondary antibodies used for the western blot analysis are shown in Table 2.6.

Table 2.6 ***Antibodies used for the western blot analysis***

Primary antibody	Company	Secondary antibody	Company
UCP2	*	Anti-Rabbit IgG	Cell signaling technology
Spot VHH recombinant binding protein	Chromotek	Anti-Alpaca	Chromotek
		6x-His Tag Monoclonal Antibody, HRP	Thermofisher

*UCP2 was designed and validated in the laboratory of Prof. Pohl (Rupprecht, Plos One 2012)

2.2. Methods

2.2.1. Site directed mutagenesis

The Insertion of the spot-tag sequence (CCGGATCGCGTGCGCGCAGTCTCTCACTGGAGCAGC) at the C terminus of mUCP2 was achieved using Q5 site directed mutagenesis kit, NEB Biolabs. The construct was later verified by sequencing (microsynth).

2.2.2. Designing the primers

The NEBaseChanger online design software was used to design primers. The sequence of UCP2 as well as the sequence of the spot-tag to be inserted, was given to the software and the start and

stop codons were marked. Forward and the reverse primers for spot-tag insertion in N-terminus (UCP2-spot-tag-N) and C-terminus (UCP2-spot-tag-C) of UCP2 were designed.

2.2.3. DNA concentration determination

To determine the DNA concentration as well as the purity of the isolated plasmid, the absorbance of the samples was measured at 260 nm and 280 nm using NanoDropTM 2000/2000c Spectrometer. With the absorbance at 260 nm, the DNA concentration was calculated. With the ratio of the absorbance at 260 nm to 280 nm (A260/A280) the purity of the samples was determined.

2.2.4. Polymerase chain reaction

PCR was performed according to the manufacturer's protocol (Q5 site directed mutagenesis kit, NEB Biolabs). The previously cloned mUCP2 into pET vector was used as a template.

A total volume of 25 µl of the master mix, containing the primers and the DNA template, was added to PCR tubes (Table 2.7).

The PCR ran with different cycling conditions for UCP2-spot-tag-C and UCP2-spot-tag-N. The annealing temperatures were chosen in accordance with the primer design online software NEBaseChanger. The cycling conditions are shown in Table 2.8.

Table 2.7 ***The PCR approach***

Name	Volume in µl	Final concentration
Nuclease-free water	9	-
2 x Q5 Hot Start High-Fidelity Master Mix	12.5	1 x
10 µM Forward Primer	1.25	0.5 µM
10 µM Reverse Primer	1.25	0.5 µM
Template DNA (1-25 ng/µl)	1	18.2 ng

Table 2.8 ***The PCR cycling conditions for UCP2-spot-tag-C or -N***

Steps for UCP2-spot-tag-C	Temperature	Time in seconds
Initial Denaturation	98 °C	30
25 Cycles	98 °C	10
	55 °C	20
	72 °C	155
Final Extension	72 °C	120
Hold	10 °C	∞
Steps for UCP2-spot-tag-N	Temperature	Time in seconds
Initial Denaturation	98 °C	30
25 Cycles	98 °C	10
	63 °C	20
	72 °C	155
Final Extension	72 °C	120
Hold	10 °C	∞

2.2.5. DNA digestion

The PCR product was digested with kinase, ligase and DpnI to remove the template DNA. 1 µl of the PCR product was added into a PCR tube containing reagents (Table 2.9). The mixture was homogenized by pipetting up and down and then incubated at room temperature for 5 minutes.

Table 2.9 ***DNA digestion***

Substance	Volume in µl	Final concentration
Nuclease-free buffer	3	-
2 x KLD Reaction Buffer	5	1 x
10 x KLD Enzyme Mix	1	1 x
PCR Product	1	-

2.2.6. Plasmid transformation in E. Coli

To transform the modified plasmids into E. coli, the digested PCR product was transferred into E.coli strain *NEB 5-alpha* (New England Biolabs). 5 µl of the digested PCR product was added and mixed with heat competent *NEB 5-alpha* E.coli cells. The bacterial cells were incubated for 30 minutes on ice, followed by a heat-shock at 42 °C for 30 seconds. The cells were then placed back on ice for 5 minutes and 950 µl of SOC medium (New England Biolabs) was added followed by 1 hour of incubation and shaking at 250 rpm at 37 °C.

The cell suspension in 4 different volumes (25 µl, 50 µl, 75 µl and 100 µl) was spread on kanamycin-containing DYT-media plates and the plates were incubated overnight at 37 °C to obtain colonies. For plasmid isolation (mini prep), a modified protocol was used (Birnboim and Doly 1979). 5 ml of liquid DYT media containing kanamycin was inoculated with one colony of

the NEB 5-alpha cells containing transformed pET24a-mUCP2 mutant plasmid. Bacteria were grown in an incubator (3033, GFL, Germany) overnight at 37 °C, while shaking at 185 rpm. 6 mini prep approaches were prepared for each mutant.

2.2.7. Preparation of glycostocks

To preserve the obtained clones, 1 ml of the clone overnight culture was added to 1 ml of DYT liquid medium containing 50% glycerin. The mixture was incubated over night at room temperature and then stored at -80 °C.

2.2.8. Plasmid isolation

Bacteria were pelleted using 4 ml of the overnight culture by centrifuging at 50 rpm, 5 minutes. The pellet was resuspended in 200 µl E1 buffer (Table 2.10) followed by addition of 200 µl of E2 buffer (Table 2.11). To lyse the cells, the solution was gently mixed. After incubation 3 to max. 5 minutes, 200 µl of E3 buffer (Table 2.12) was added and gently mixed. The mixture was centrifuged at 22 °C at 14000 x g for 20 minutes. To precipitate the plasmid DNA, 900 µl of isopropanol were added to the supernatant. The sample was then mixed thoroughly and then centrifuged at 22 °C at 16000 x g for 20 minutes. The supernatant was discarded and the pellet containing the plasmid DNA was washed with 1 ml of 70% ethanol and centrifuged again at 22 °C at 14000 x g for 5 minutes. The obtained supernatant was discarded, and the pellets were left to dry for 15 minutes. The DNA pellet was solved in 30 µl pre-warmed to 30 °C nuclease-free water. Finally, the obtained plasmid DNA was stored at -20 °C.

Table 2.10 ***Buffer E1***

Substance for buffer E1	Concentration	Amount for 50 ml
EDTA	10 mM	0.186 g
TRIS	25 mM	0.151 g
RNase	10 ng/ml	500 µl
Lysozyme	10 ng/ml	500 µl

Table 2.11 ***Buffer E2***

Substance for buffer E2	Concentration	Amount for 50 ml
SDS	1%	0.5 g
NaOH	0.2 M	0.4 g

Table 2.12 ***Buffer E3***

Substance for buffer E3	Concentration	Amount for 100 ml
Potasium acetate	1M	9.815 g
Acetic acid 100%	23%	23 ml
Distilled water	-	57 ml

2.2.9. Restriction digestion of isolated plasmids

To control that the plasmid DNA holds the target gene, a restriction digestion was performed.

The restriction enzyme Sph-I was used for which expected fragments in lengths of 5559 bp and 644 bp are expected. 2 µl of plasmid DNA was used for a final volume of 20 µl (Table 2.13). The final solution was incubated for 60 minutes at 37 °C, while shaking at 300 rpm.

Table 2.13 ***Restriction digestion approach***

Substance	Volume in µl
Nuclease-free water	15
Restriction enzyme buffer	2
Restriction enzyme (Sph-I)	1
Plasmid DNA (Mini)	2

2.2.10. Agarose gel electrophoresis

To analyze visualize the restriction digestion of the plasmid DNA, agarose gel electrophoresis with SYBR green staining was performed. 1% agarose gel containing SYBR green (1:10000) was prepared in the electrophoresis chamber (OwlTM EasyCastTM B2 Mini Gel Electrophoresis Systems, Thermo Scientific). 5 µl of a 4x loading dye containing Bromphenol Blue were added to 20 µl of digested product and mixed. 20 µl from the mixture was loaded on the gel. 5 µl of marker (HyperLadder I, BIOLINE) was loaded in one slot as a size marker. The running buffer TBE was used (Table 2.14) The electrophoresis conditions were 90 V for 10 minutes and then 120 V for 2 hours (EC-105 Power Supply, E-C Apparatus Corporation). A picture from the gel was captured with UV-light using Quantum Vilber Lourmat and visualized with the program Vision Capt.

Table 2.14 ***TBE buffer solved in 1L distilled water***

Substances for 1L TBE buffer	Concentration mM	Used mass in grams
Tris	89	10.5
Boric acid	89	5.5
EDTA	2	0.744

2.2.11. Isolated plasmid purification

To purify the isolated plasmid DNA, the Nucleo Spin Gel and PCR Clean-up Kit (Machery & Nagel, Germany) was used.

300 µl NT1 binding buffer were added to 30 µl of the plasmid DNA and mixed. The solution was transferred to a spin column. The column was centrifuged at 11000 x g for 30 seconds and the flow-through was discarded. The plasmid DNA was washed by 700 µl of NT3 washing buffer added to the spin column and then centrifuged at 11000 x g for 30 seconds. The flow-through was discarded and the spin column was centrifuged again at 11000 x g for 1 minute to dry the column. The column was then transferred into a clean 1.5 ml-tube. 40 µl of pre-warmed water (50 °C) was added to the column. The column was centrifuged again at 11000 x g for 1 minute and its flow-through containing the purified plasmid DNA, was stored at -20 °C.

2.2.12. DNA Sequencing

To verify the insertion of the spot-tag into the sequence of UCP2, the obtained purified plasmid DNAs were sent to Microsynth Austria GmbH. The plasmids were sequenced from both directions using T7- and T7term primers.

2.2.13. Transformation of plasmids into expression strain

The obtained plasmids were transformed the E. coli expression strain *Rosetta.* 1 µl of the purified plasmid DNA was added to 300 µl of thawed *Rosetta* cells followed by an incubation of 10 minutes on ice. Bacteria were heat-shocked at 42 °C for 30 seconds, followed by addition of 1 ml DYT liquid medium, and were shaking at 1100 rpm at 37 °C for one hour.

100 µl and 200 µl of the *Rosetta* suspension were spread on agar plates containing kanamycin and chloramphenicol incubated overnight at 37 °C. Single colonies were obtained and used to prepare overnight cultures for the protein expression as well as glycostocks preparation.

2.2.14. Protein expression in E. Coli

For the expression of the protein, an overnight culture was prepared with 40 ml of DYT medium with the appropriate antibiotics kanamycin and chloramphenicol. One colony of Rosetta containing the desired plasmid was introduced to the flask. The flask was incubated at 37 °C overnight while shaking 185 rpm. To test the protein expression 2 ml of the mentioned overnight cultures were added to 300 ml Erlenmeyer flasks containing 100 ml DYT medium and kanamycin as well as chloramphenicol.

For large scale protein expression, 2x 250 ml of liquid DYT medium including kanamycin and chloramphenicol was inoculated each with 6 ml of the overnight culture. As the flasks were incubated at 37 °C (185 rpm), the optical density (OD) of the samples in flasks, were measured using a spectrometer at the wavelength of 600 nm (OD 600) and DYT medium was used as the blank value. An OD 600 of 1 corresponds to 10^9 bacteria in 1 ml. The flasks were incubated until an OD 600 of 0.3-0.5 was reached, which indicate the exponential growth phase of the culture. The protein expression was then induced by adding 1 mM of IPTG. Before the induction and then every hour after the induction up to 4 hours, samples of 1 ml from the culture were taken and bacteria pellets were collected by centrifuging 2 minutes at 8000 x g. The supernatant was discarded, and the pelleted bacteria were stored at -20 °C to be used in the Coomassie and silver staining analyses.

To induce the protein expression 250 µl IPTG was then added. The cultures were incubated for 3 hours while the OD was measured, and the bacteria were harvested by centrifugation at 3000 x g in 15 °C for 15 minutes. The supernatant was discarded, and the pelleted bacteria were stored at -20 °C for the isolation of inclusion bodies.

2.2.15. Inclusion body isolation

Bacteria pellets of 500 ml E. coli culture were resuspended on ice in 25 ml TE buffer (Table 2.15) followed by 1 mM DTT and 0.5 ml bacterial protease inhibitor cocktail (Sigma-Aldrich, Austria). A French Press (One Shot, Constant Systems Ltd., United Kingdom) with the pressure of 1 kbar, was used to destroy the bacteria. The obtained lysate was then centrifuged at 15000 x g in 4 °C for 30 minutes. The pellets contained the inclusion bodies. A 20G syringe and a 22G syringe were used to resuspend the inclusion body containing pellets in 50 ml PA buffer (Table 2.16) supplemented with 2% Triton X-100, 1 mM DTT and 0.5 ml bacterial protease inhibitor cocktail. The resuspended pellets were then centrifuged at 1000 x g in 4 °C for 3 minutes. The supernatant was aliquoted in 1.5 ml tubes and centrifuged at 14000 x g in 4 °C for 20 minutes. The supernatant was again discarded, and the pellets which were the final inclusion bodies were frozen in liquid nitrogen and stored at -80 °C.

Table 2.15 ***TE buffer in 500 ml of distilled water***

Substance for TE buffer	Concentration	Amount for 500 ml
Tris	100 mM	6.06 g
EDTA	5 mM	0.93 g
HCL to get pH 7.5	-	-

Table 2.16 ***PA buffer in 250 ml of distilled water***

Substance for PA buffer	Concentration	Amount for 250 ml
NaH_2PO_4	150 mM	6.06 g
EDTA	25 mM	0.93 g
Ethylene glycol	5%	12.5 ml
5 M NaOH to get pH 7.9	-	-

2.2.16. Reconstitution of the expressed protein into liposomes

Reconstitution of spot-tag containing UCP2 (UCP2-spot-tag-C) as well as wild-type of UCP2 (UCP2-wt) was performed as described before for mUCP2 (Hilse *et al.* 2016). The scheme of reconstitution is shown in Figure 2.1.

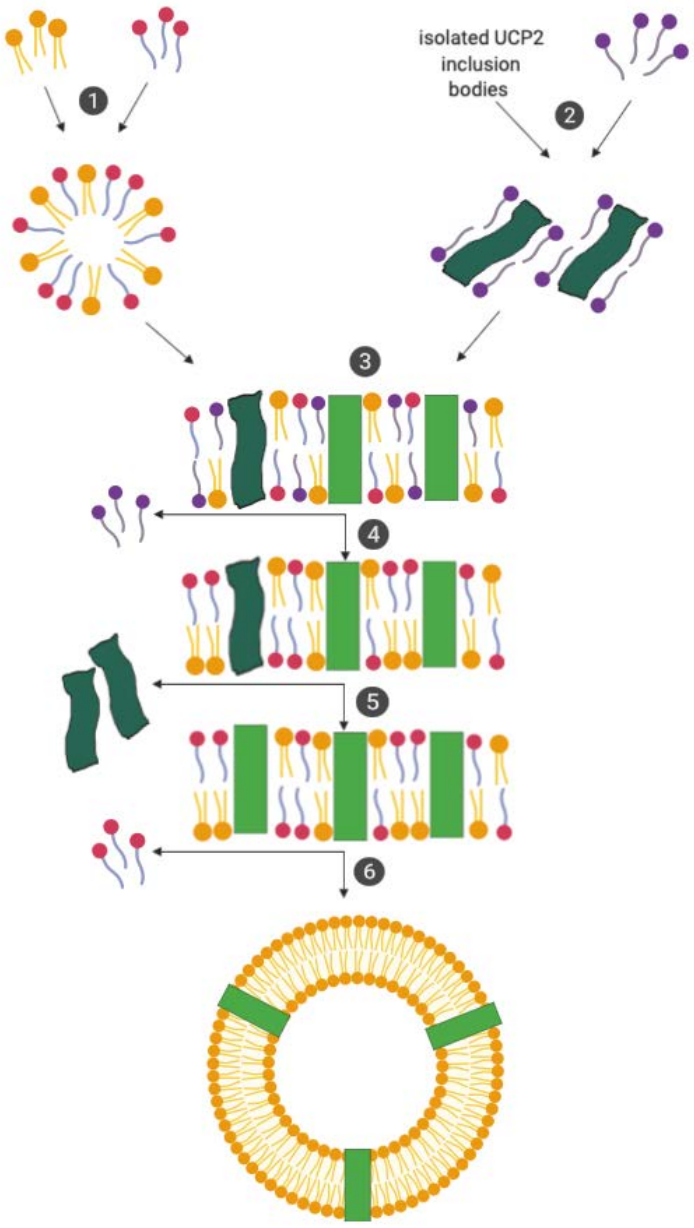

Figure 2.1 ***Reconstitution of UCP2-spot-tag-C or UCP2-wt into liposomes: 1:*** *Dried E. coli lipids (orange) were solved with non-ionic detergents (red).* ***2****: UCP2-spot-tag-C or UCP2-wt were solubilized with anionic detergent (purple).* ***3:*** *solubilized UCP2 and solubilized lipids were mixed together.* ***4:*** *the mixture of UCP2, detergent and liposomes were dialyzed to remove the anionic detergent.* ***5:*** *Falsely folded proteins (dark green) were removed from the mixture using HTP column.* ***6:*** *non-ionic detergent was removed using bio-beads forming pure proteoliposomes.*

2.2.16.1. Lipid preparation

31.8 mM DOPC (1,2-Dioleoyl-sn-glycero-3-phosphocholine), 31.8 mM DOPE (1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine) and 12.5 mM CL (Cardiolipin) were used to create a lipid mixture. The lipids were obtained in a solved form and contained chloroform to enable pipetting. After mixing, the chloroform was evaporated and the lipid was dried under vacuum for one hour, using a vacuum pump (LABOPORT®, KNF LAB). 200 µl of Triton X-114, 50 µl of n-Octylpolyoxyethylene (C8En) (sigma) and 2.25 ml of TE/G buffer were added to the dried lipid. The lipids mixtures were incubated at 4 °C overnight by shaking overhead.

2.2.16.2. Solubilization of protein

An Inclusion body aliquot was used for the solubilization of each UCP2-spot-tag-C and UCP2-wt. The inclusion bodies were washed by adding 10 ml TE/G buffer and 2% Triton X-114 to the inclusion bodies in a 50 ml falcon tube. The tube was then centrifuged at 14000x g at 4 °C for 10 minutes. The obtained pellet was then re-suspended with 10 ml TE/G buffer and 0.1% SLS and centrifuged again at 14000 x g at 4 °C. The pellet was then solubilized by re-suspending in 10 ml solubilization buffer (TE/G buffer + 2% SLS + 1 mM DTT) and vortexed. The obtained solution was then pressed through a 20 1½ G and then a 26 5/8 G needle. To solubilize the protein, the solution was then incubated at room temperature for one hour while shaking 45 rpm. To remove the insolubilized proteins, the solution was centrifuged at 14000 x g for 10 minutes at room temperature. To obtain a final protein concentration of 1 mg/10 ml, the obtained supernatant from the centrifugation was diluted with solubilization buffer. The solution was transferred into a small cooled beaker with a magnet stirrer, stirring at 4 °C.

2.2.16.3. Lipid solution preparation

For a final volume of 25 ml (15 ml lipid and 10 ml solubilized protein); 2.5 ml of the dissolved lipids (from section x) were added to 7.5 ml of TE/G buffer. In addition, 1 mM DTT (0.00225 g) and 2 mM GTP (0.0262 g) in 5 ml TE/G buffer were added to the lipids. After gently mixing, lipid solutions were kept on ice.

2.2.16.4. Protein refolding

This step took place in a 4 °C laboratory. 5 ml of the lipid/ GTP, DTT- solution was added to the solubilized protein solution (in the beaker) and mixed gently for 15 minutes. This step was repeated twice after mixing 15 minutes with additional 5 ml from the lipid/ GTP, DTT- solution. The final solution containing 25 ml of lipid and solubilized protein was incubated stirring for 3 hours.

2.2.16.5. Concentrating the detergent-containing proteoliposomes

The aim was to concentrate the refolding solution from 25 ml to 5 ml. To concentrate the solution an ultrafree-15 centrifugal filter device with 30 kDa pore size (Amicon® Ultra-15, Merck) was used. The centrifugal filters were equilibrated with 5 ml distilled water. 7 ml of the refolding solution was pipetted in the filter tube and centrifuged at 3500 x g at 4 °C for 10 minutes. After the centrifugation the flow-through was discarded. These steps were repeated until the refolding solution was concentrated to 5 ml.

2.2.16.6. Dialysis in TEG/BSA

A dialysis against BSA was performed in order to remove the N-Lauroylsarcosine sodium salt. A dialysis bag (ZelluTrans 6.0, ROTH, molecular weight cut-off: 6000-8000 Da) was washed for 30 minutes in 500 ml water at room temperature. The concentrated refolding solution was transferred into the dialysis bag. All dialysis buffers (Table 2.17) had a volume of 900 ml. The first dialysis buffer contained 1 mg/ml BSA and 1 mM DTT in TE/G buffer (Table 2.18).

The dialysis was performed at 4 °C. After two hours of stirring, the dialysis bag was transferred into a second beaker containing 1 mg/ml BSA without DTT and incubated overnight.

The third dialysis containing 1 mg/ml BSA without DTT was then performed by 3 hours.

Table 2.17 ***Composition of all three dialysis buffers***

1st Dialysis buffer	900 ml TE/G buffer + 1 mg/ml BSA + 1 mM DTT
2nd Dialysis buffer	900 ml TE/G buffer + 1 mg/ml BSA
3rd Dialysis buffer	900 ml TE/G buffer + 1 mg/ml BSA

Table 2.18 ***Composition of TE/G buffer***

Substance	Concentration	Amount of 2 l
Tris	100 mM	24.2 g
EDTA	5 mM	3.7 g
Glycerine	10%	200 ml
pH 7.5 (HCI)	-	-

2.2.16.7. Protein purification and refolding in liposomes

After the third dialysis, the dialysis bag was transferred to a new beaker containing 900 ml assay buffer (Table 2.19). Total of four dialyses in the assay buffer with the following incubation times were performed: 2 hours, 4 hours, overnight and lastly 3 hours.

Table 2.19 ***Composition of Assay buffer***

Substance	Concentration	Amount of 2 l
Sodium sulfate	50 mM	14.2 g
Tris	10 mM	2.42 g
MES	10 mM	3.90 g
EGTA	0.6 mM	0.456 g
pH 7.34 (NaOH)	-	-

2.2.16.8. Protein purification and refolding in liposomes

To remove the aggregated proteins in the proteoliposome solution, the obtained proteoliposomes, after the dialysis, were transferred into a 50 ml Falcon tube and centrifuged at 14000 x g for 10 minutes at 4 °C.

To remove the falsely folded proteins, the supernatant was transferred to an HTP column (0.5 g, Hydroxyapatite Bio-Gel® HTP Gel, Biorad) and the flow-through was collected. 5 ml of Bio-Beads (Bio-BeadsTM SM-2 Adsorbent Media, Biorad) were equilibrated in assay buffer. In order to remove the non-ionic detergent, the proteoliposomes were added to the Bio-Beads and incubated at 4 °C, while shaking overhead, for 2 hours at 4 °C. The Bio-Beads were then removed from the solution using a column. 5 ml of newly equilibrated Bio-Beads were added again to the proteoliposomes and incubated at 4 °C while shaking for 30 minutes overhead at 4 °C.

The Bio-beads were finally removed and the final proteoliposomes were aliquoted, frozen in liquid nitrogen and then stored at -80 °C.

2.2.17. Protein concentration determination

For the determination of protein concentration of both the inclusion bodies and the proteoliposomes, a Micro BCAT Protein Assay Kit, Thermo Scientific, was used according to its protocol. For the assay, 2 ml total volume of the samples and BSA standards and blank was obtained. An aliquot of inclusion bodies was solubilized in 1 ml of 10% SDS solution and diluted 1:20 with 19 ml of water. 50 µl and 100 µl of the protein solution were filled up with water to 1 ml. For the proteoliposomes protein concentration determination, 50 µl and 100 µl of the proteoliposomes were mixed with 200 µl 10% SDS solution (as suggested by the protocol for the determination of membrane proteins). Finally, 1 ml of the reagent was added, and the samples were incubated at 60 °C for one hour. The absorbance of each sample at the wavelength of 562 nm was measured and the protein concentration was calculated according to the Lambert-Beer law, using appropriate BSA standards.

2.2.18. SDS-PAGE

SDS-PAGE was used to separate proteins based on their molecular weight. The gel consisted of a stacking gel and a separating gel both made of polyacrylamide (Table 2.20). The stacking gel had a percentage of 3.75% of the polyacrylamide while the separating gel had a percentage of 15% or 10%. The gels were made using Mini- PROTEAN® Tetra handcast system (Biorad). The thickness of the gels, their length and their width were respectively, 0.75 mm, 7 cm and 8 cm. The gels made for the Coomassie staining and silver staining analysis had 10 slots while the gels for the western blot had 15 slots.

Table 2.20 ***Composition of polyacrylamide gels***

Separating gel		Stacking gel	
Substance	Amount per gel	Substance	Amount per gel
Tris 2	1.5 ml	Tris 1	750 µl
30% Acrylamide solution (gel A)	3.0 ml	30% Acrylamide solution (gel A)	375 µl
2% Bisacylamid solution (gel B)	1.3 ml	2% Bisacrylamid solution (gel B)	150 µl
dH_2O	200 µl	dH_2O	1.725 µl
Temed	8 µl	Temed	3.9 µl
10% APS	60 µl	10% APS	30 µl

Table 2.21 ***Composition of Tris buffer solutions***

Tris 1		Tris 2	
Substance	Amount for 1 l	Substance	Amount for 1L
Tris	60.6 g	Tris	181.6 g
SDS	4 g	SDS	4 g
pH 6.8 (+ HCl)	-	pH 8.8 (+ HCl)	-

Bacterial samples were prepared according to their OD 600 values. A bacteria pellet sample with OD 600 of 0.5 was re-suspended in 100 µl RIPA buffer (Table 2.22) containing 25% of 4x sample buffer (Table 2.23). The samples used for the silver staining analysis were prepared by diluting the samples according to their protein concentrations. The final volume of 20 µl (containing 5 µl sample buffer) was loaded per slot in a 10-slot gel. For 15-slot gels the final volume of 14 µl (containing 3.5 µl sample buffer) was loaded per slot.

The samples were prepared on ice and then were incubated at 95 °C for 10 minutes.

The gels were placed in the electrophoresis chamber Mini-PROTEAN® Tetra handcast system, Biorad) The chamber was filled with electrophoresis buffer (Table 2.24).

5 µl of the protein size marker (Precision Plus ProteinTM Standards, Biorad) was loaded on one slot and diluted 1:10 for silver staining. The electrophoresis conditions were 30 minutes at 80 V to collect the samples through the stacking gel and 80 minutes at 130 V to separate the proteins through the separating gel (Consort EV231, Roth).

Table 2.22 ***Composition of RIPA buffer***

Substance	Concentration	Amount for 250 ml
Tris	50 mM	1.51 g
NaCl	150 mM	2.19 g
Desoxycholic acid sodium salt	1%	2.5 g
Triton X-100	1%	2.5 ml
EDTA	1 mM	0.093 g
SDS	0.1%	0.25 g
pH 7.4 (+ HCL)	-	-

Table 2.23 ***Composition of sample buffer***

Substance	Amount for 100 ml
Tris	1.21 g
SDS	4 g
Glycerine	10 ml
B-mercaptoethanol	4 ml
Bromphenol blau	Point of a knife

Table 2.24 ***Composition of electrophoresis buffer***

Substance	Amount for 2 l 10x
Tris	60.58 g
Glycin	288.24 g
SDS	20 g

2.2.18.1. Coomassie blue staining

Coomassie-staining was used to visualize the proteins from the bacterial samples in the SDS gels.

The proteins are visualized by binding of the Coomassie blue to the basic side chain of the amino acids. Coomassie staining has a detection limit of 0.1 µg per band.

The separation gels were placed into the Coomassie solution (Table 2.25). and heated until the solution was boiling. The gels were then re heated with water to remove the excessive Coomassie.

Table 2.25 ***Composition of Coomassie blue solution***

Substance	Amount for 1 l
Brilliant blue R-250	2.5 g
Isopropanol	454 ml
Acetic acid 100%	92 ml
dH_2O	454 ml

2.2.18.2. Silver staining

Silver staining analysis were performed to visualize the different steps of the inclusion body isolation and purification as well as the proteoliposomes of both wild-type and spot-tagged-UCP2. During each step of protein refolding, a sample was taken, which was later loaded on the SDS gels and stained afterwards by silver staining (Table 2.26) (according to Blum et al. 1987). Silver staining has a detection limit between 0.1 and 1 ng protein per band.

Table 2.26 ***Composition of solutions and different steps for silver staining***

Solution	Composition	1000 ml	Time
Fix	40% Ethanol 10% Acetic acid 50% mQ H_2O	400 ml Ethanol 100 ml Acetic acid 500 ml mQ H_2O	30 minutes
Wash	mQ H_2O	0.2 g Sodiumthiosulfate 1000 ml mQ H_2O	3 x 10 minutes
Sensitizer	0.0% Sodiumthiosulfate	-	60 s
Wash	mQ H_2O	2 g Silver nitrate	3 x 20 s
Silver	0.2% Silver nitrate 0.02% 37% Formaldehyde	0.2 ml 37% Formaldehyde	20 minutes (dark)
Rinse	mQ H_2O	1000 ml mQ H_2O	3 x 20 s
Develop	3% Sodiumcarbonate 0.05% 37% Formaldehyde 0.0005% Sodiumthiosulfate	30 g Sodiumcarbonate 500 µl 37% Formaldehyde 0.5 mg Sodiumthiosulfate Fill up to 1000 ml with mQ H_2O	Incubation till bands are visible
Stop	5% Acetic acid	50 ml Acetic acid 950 ml mQ H_2O	Some minutes
Wash	mQ H_2O	-	Some minutes

2.2.18.3. Western blot

To detect UCP2-wt and UCP2-spot-tag-C specifically, western blot analysis with the recombinant UCP2s and tissue samples for controls were performed.

The gels were blotted on nitrocellulose membranes with the size of 6 cm in 9 cm (Protean Nitrocellulose BA 85 Schleicher & Schüll).

To blot a semi dry blotter (Peq lab) was used. The membrane was soaked in blot buffer (Table 2.27) for a few minutes. Meanwhile two filter papers (Biorad) per gel, were also soaked in blot buffer. To obtain the blotting sandwich, the first filter paper was placed on the blot chamber (peq lab) and the membrane was placed on top of it and few drops of blot buffer were added to the membrane. Using a pencil, the membrane was labeled with the date and a number. The separating gel was then dislodged carefully from its glass discs and placed carefully on top of the soaked membrane. The air bubbles between the membrane and the gel were removed carefully and the second filter paper was placed on their top. Finally, the chamber was closed with the chamber lid which was connected to the voltage source with 14 V, for one hour.

Table 2.27 ***Composition of Blot buffer***

Substances for 2L Blot buffer	Amount
Glycin	28.84 g
Tris	6.06 g
Methanol	400 ml

To control the blotting, the membranes were stained with Ponceau S (Table 2.28) solution and then washed shortly with distilled water.

Table 2.28 ***Composition of Ponceau S solution***

Substances for 1 L Ponceau S solution	Mass in grams
Ponceau S	0.2
TCA	3

The membranes were then blocked with 2% BSA blocking solution for 90 minutes at room temperature, while shaking. After the blocking, the membranes were washed three times with wash buffer (TBST) (Table 2.29), each 15 minutes. After the washing steps, the membrane was ready to be incubated with the primary antibody.

Table 2.29 ***Composition of TBST (wash buffer) and TBS***

Substances for 1L TBST	Amount
TBS	100 ml
Tween 20	1 ml
Distilled water	900 ml
Substances for TBS	Mass in grams
Tris 0.5M (pH 7.4)	60.57
NaCl 1.5M	87.66

To specifically detect UCP2 (both wild-type and spot-tagged), UCP2 antibody was used as the primary antibody with the incubation time of 2 days in 4 °C on a shaker. For the analyses in which only UCP2-spot-tag-C had to be detected, spot-VHH antibody was used as the primary antibody, with the incubation time of 1 day also shaking in 4 °C.

After the incubation with the primary antibody the membranes were washed three times, each 10 minutes with wash buffer and then incubated with the secondary antibody.

A rabbit secondary antibody was used to bind to UCP2 primary antibody. While for the spot-VHH primary antibody, either Alpaca or 6x His-tag secondary antibodies were used.

The antibodies were diluted in BSA solution in appropriate concentrations.

After the incubation with the secondary antibody, the membranes were again washed (3 x 10 minutes) and 2 ml of ECL western blotting substrate (Bio-Rad) was prepared per each membrane. The reagent solution consisted of two reagents (A and B) with the ratio of one to one. The reagent solution was dropped on the membrane and the membrane was incubated for 2 minutes in dark conditions. The reagent was then removed, and the marker was labeled. The membrane was finally ready for the detection.

2.2.19. Electrophysiological measurements

To test and compare the functionality of the UCP2-spot-tag-C and the UCP2-wt, electrophysiological measurements, as described by Beck and colleagues (Beck et. al. 2006), were performed.

2.2.19.1. Preparation of proteoliposomes

For the first electrophysiological measurements of the proteoliposomes with UCP2-wt-Ch24 and UCP2-spot-tag-C-Ch1, the final concentration of lipid was adjusted to 1.5 mg/ml and the protein concentration to 4 µg/ml.

For the electrophysiological measurements with proteoliposomes UCP2-wt-Ch25 and UCP2-spot-tag-C-Ch2, the final concentration of lipid was adjusted to 1.5 mg/ml and the protein concentration to 4, or 4.5 or 5 µg/ml. Charge x (Chx) stands for the refolding number.

To adjust the lipid concentration, additional lipids, dissolved in chloroform, were added and the chloroform was evaporated and the lipids were dried using a vacuumpump for 30 minutes.

Lipids used in the experiment were:

- 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) 45%
- 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) 45%
- Cardiolipin 10%

The dried lipids were then dissolved in assay buffer and the mixture of lipids and assay buffer was vortexed for 1 minute. The required volume of proteoliposomes was lastly added to the mixture. The volume of added assay buffer was calculated complementary to the volume of required proteoliposomes, to maintain final volume of 1 ml.

750 µl of this 1 ml solution was then pipetted into a plastic container with the width of 12 mm. The container containing the solution was maintained at 32 °C during the whole experiment. For the protein activation of arachidonic acid (to the final concentration of 15 mol%) was added to the extra lipids and vortexed. The mixture of lipids and fatty acid was then dried using the pump. For the inhibition of the protein, a 4 mM ATP solution was added post measurement to the solution and after 15 minutes incubation the measurements of the inhibition were performed.

2.2.19.2. Experimental setup

The electrophysiological setup consisted of two silver/silver chloride electrodes connected to an amplifier. The reference electrode was placed into a plastic container, containing the solution for the measurements. In Figure 2.2 the measurement electrode inside a cut and bended pipette tip filled with solution are depicted. The setup was placed on a vibration-free table inside a Faradays cage in order to minimize the effect of surrounding electrical and magnetic fields.

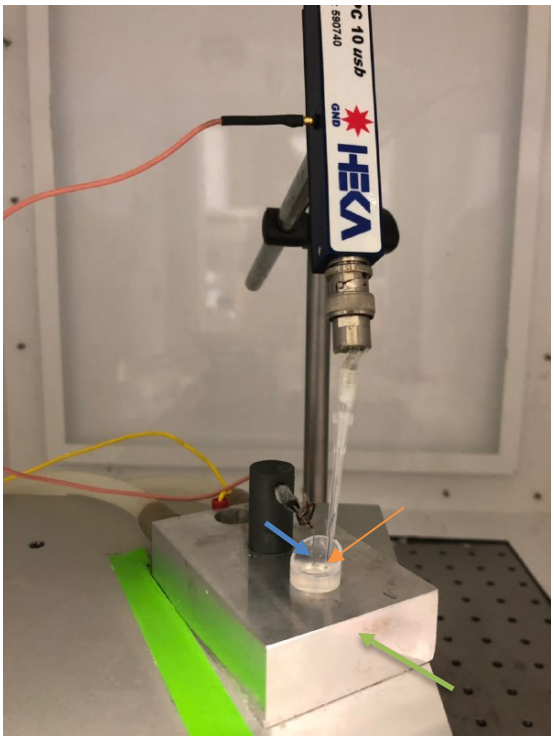

Figure 2.2 ***The experimental setup for electrophysiological measurements:*** *The two electrodes are shown in blue and orange arrows. Orange is the electrode inside the bended tip and blue is the reference electrode. The green arrow points to the thermostatic unit. Under the stand there is a screw (not shown in the figure) which facilitates raising the tip in and out of the solution.*

2.2.19.3. Lipid bilayer formation

Pipette tip was cut, bended and then coated by a 1:20 hexadecane: hexane solution (Beck, Jaburek et al. 2006)). The tip was then filled with 3.5 µl of the solution from the vessel. The first electrode was placed in the tip and the second in the buffer solution outside the tip. (The diameter of used pipette tips was in the range of 225 µm-250 µm.)

To form a lipid bilayer, it was ensured to place the tip into the container, inside the solution, in a way that the angle between the surface of the solution and the tip of the pipette tip was greater than 90 °.

After 30-60 minutes, the tip was raised up slowly out of the solution and a lipid monolayer was formed inside the tip. The tip was then lowered slowly back to the solution, forming a lipid bilayer inside the tip (Figure 2.3).

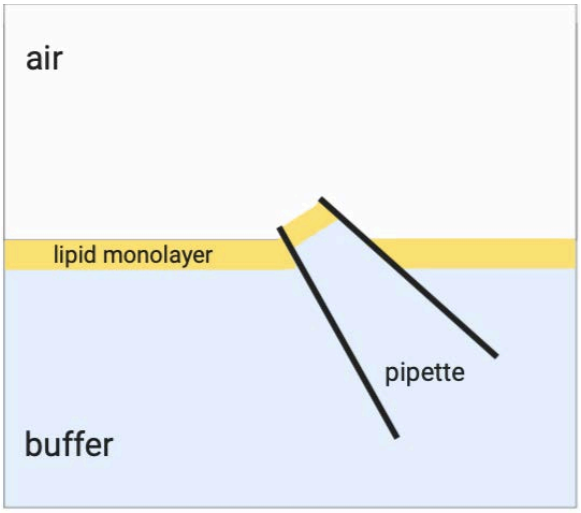

***Figure 2.3* Formation of a lipid bilayer, from the lipid monolayer, at the tip of the pipette (Adapted from(Beck, Jaburek et al. 2006)):** *Moving the pipette tip up and down (from air to buffer and vice versa) will form a lipid bilayer at the opening of the pipette tip, as long as the angle between the solutions surface and the pipette tip is greater than 90 degrees.*

2.2.19.4. Measurement procedure

Measurements were monitored using the "Patch-clamp-amplifier EPC-10" (HEKA electronics, Germany) and visualized by the "Pulse" software.

To verify the formation of the lipid bilayer, the membrane capacity was monitored. A triangle voltage signal with an amplitude of 100 mV and a frequency of 5 Hz was applied. A capacity between 0.67 µF/cm2 and 0.8 µF/cm2 was expected to indicate the correct formation of lipid bilayer membranes in this setup. Therefore, only the membranes were measured, which showed a

capacity in this range. After the confirmation of the lipid bilayer formation, the electrical conductance of the membrane was measured.

The membrane conductance, G, was calculated from a linear fit in an interval, U (applied voltage), between -50 mV and 50 mV. The conductance was measured in the absence or presence of fatty acid and/or protein, depending on the experiment.

The inhibition took place only after the conductance measurement. Whereas, 4 mM of ATP was added as an inhibitor to the solution inside the plastic container and the conductance was measured again after a few minutes of waiting.

3. Results

3.1. Insertion and validation of spot-tag in UCP2

3.1.1. Transformation of the target sequence to E. coli

After designing the required primers for the insertion of the spot-tag into N- or C-terminus of mUCP2 sequence, the PCR products were transferred in NEB5a E. Coli and the plasmid DNA was isolated. To control the existence of the target gene in all plasmid DNAs, they were digested using Sph-I restriction enzyme. Figure 3.1 shows the agarose gel electrophoresis image of the isolated plasmids. The presence of two bands in all samples (UCP2 and plasmid) not only indicates successful restriction digestion, but also confirms that all the plasmids contain the mUCP2 sequence (644bp).

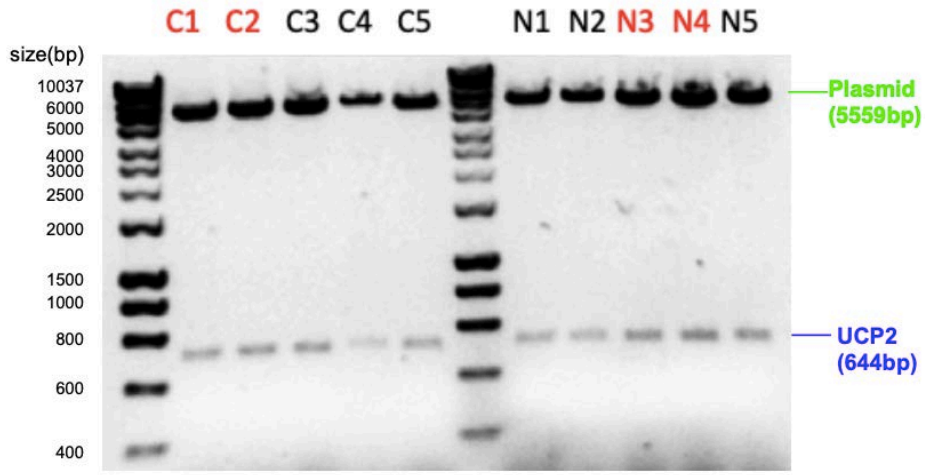

Figure 3.1 ***Agarose-gel electrophoresis analysis of isolated plasmid DNAs:*** *After restriction digestion, the isolated plasmid DNAs were loaded on the gel. C1-C5 are plasmids with UCP2 containing the spot-tag in the C-terminus, N1-N5 are plasmids with UCP2 containing the spot-tag in the N-terminus. Red color indicates the samples chosen for sequencing.*

To verify the insertion of the spot-tag into N- or C-Terminus of UCP2, the isolated plasmids were sequenced. The insertion of Spot-Tag in N- or C-terminus of UCP2 is sketched and showed in Figure 3.2.A.

Figure 3.2.B shows the multiple sequence alignment of UCP2 with the assumed inserted spot-tag in C- (UCP2-spot-tag-C) (Figure 3.2.B) or N-terminus (UCP2-spot-tag-N) (Figure 3.2.C), against the wild-type of UCP2 (UCP2-wt).

The results demonstrate the successful integration of the spot-tag in both N- and C-terminus of UCP2.

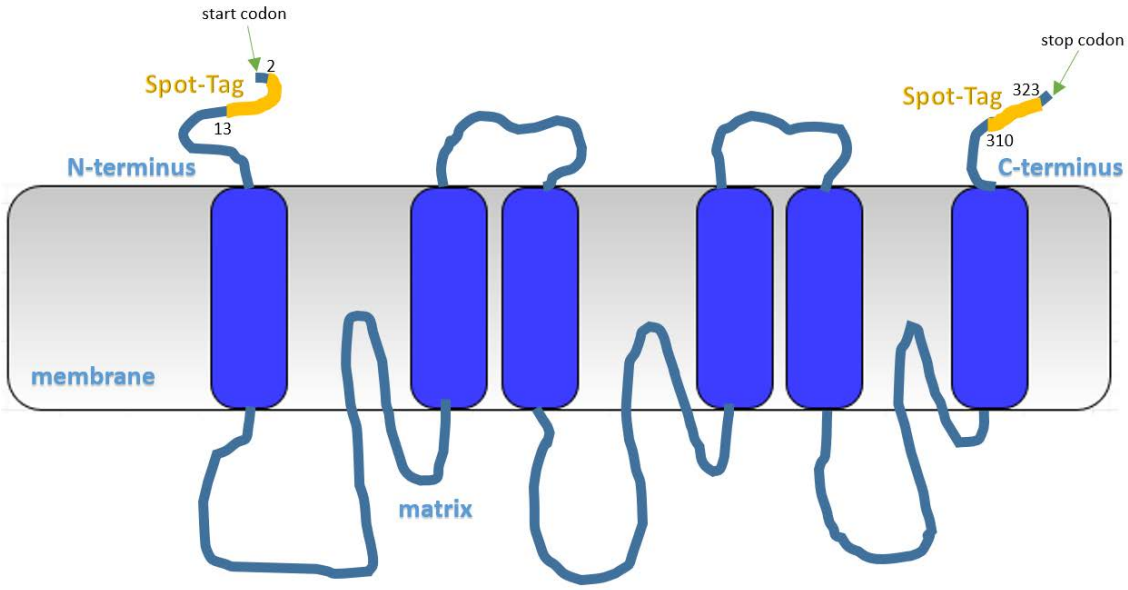

***Figure 3.2.A* Sketch of the transmembrane arrangement of UCP2 with the Spot-Tag inserted in N- or C-terminus:** *The blue cylinders represent the six transmembrane domains. Color yellow represents the Spot-Tag inserted either after the start codon, in the N-terminus or before the stop codon, in the C-terminus.*

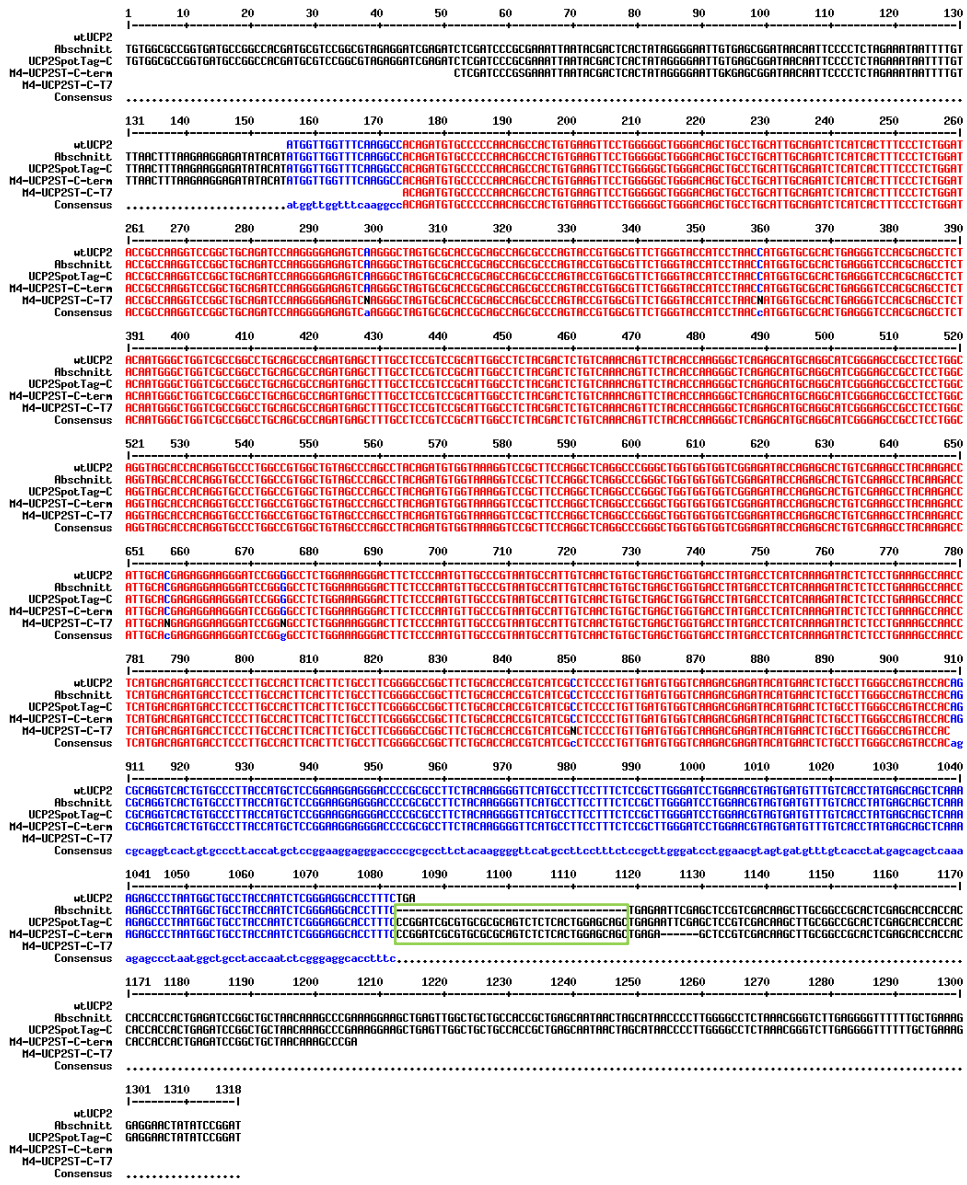

Figure 3.2.B ***Multiple sequence alignment of wild-type UCP2 and the modified UCP2 with the tag in the C-terminus:*** *The protein containing the tag in its C-terminus (UCP2-spot-tag-C) was sequenced from both directions using T7 and T7-term primers. Both nucleotide sequences were aligned with the wild-type of UCP2 (UCP2-wt). Red color represents highly conserved residues, the blue color represents weakly conserved residues. A position with no conserved residue is represented by a dot in the consensus line. The sequence of the spot-tag is highlighted inside the green box.*

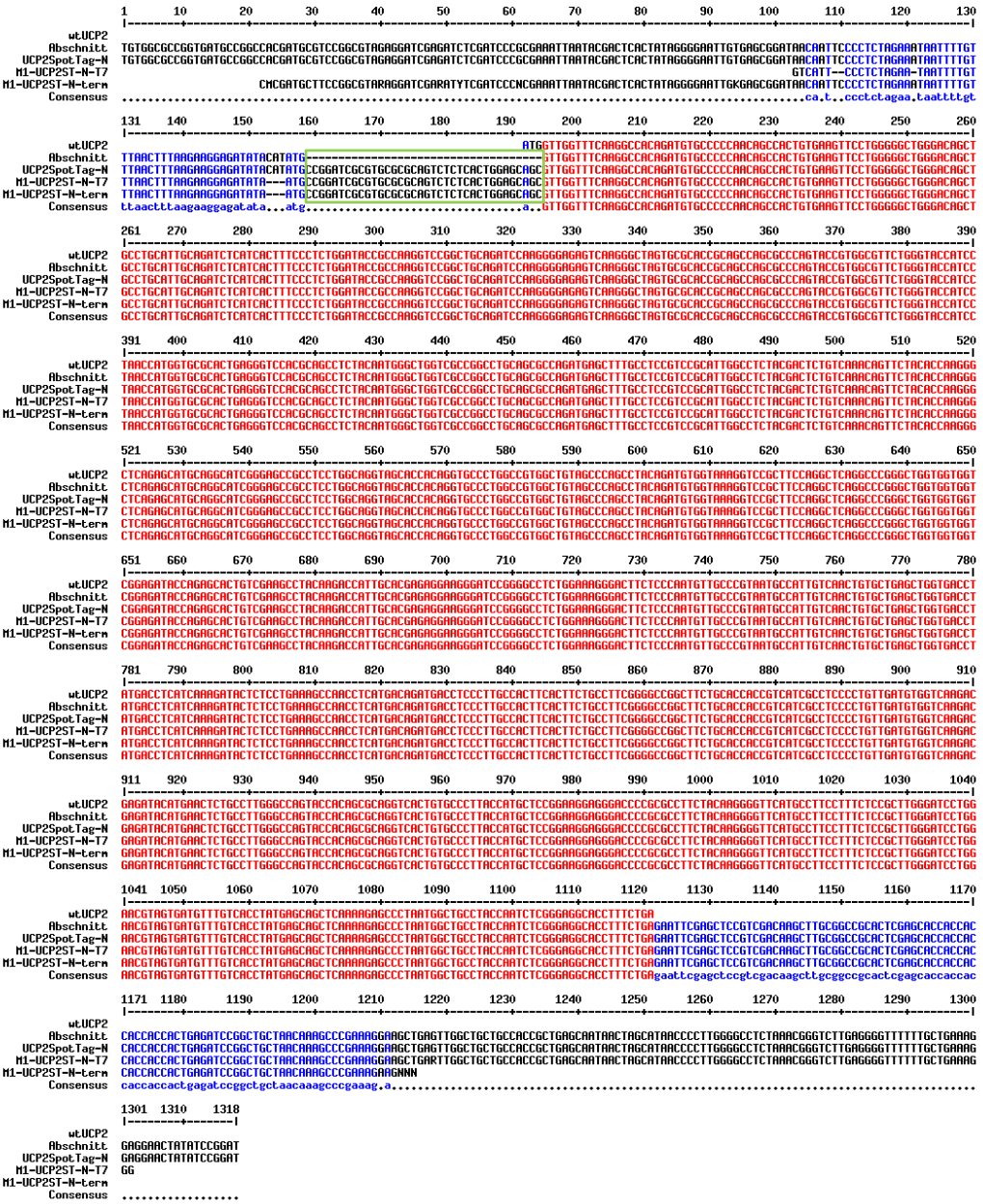

Figure 3.2.C ***Multiple sequence alignment of UCP2-wt and the modified UCP2 with the tag in the N-terminus:*** *The protein containing the tag in its N-terminus (UCP2-spot-tag-N) was sequenced from both directions using T7 and T7-term primers. Both nucleotide sequences were aligned with the wild-type of UCP2 (UCP2-wt). Red color represents highly conserved residues, the blue color represents weakly conserved residues. A position with no conserved residue is represented by a dot in the consensus line. The green box shows the sequence of the spot-tag.*

3.1.2. Expression of the spot-tag-containing UCP2

After the confirmation of spot-tag's insertion in UCP2, the next step was to express the modified proteins, (UCP2-spot-tag-C and UCP2-spot-tag-N), in Rosetta-competent E. coli. An E. coli sample was taken before the induction of protein expression. The expression was carried out in a period of 4 hours and every hour an E. coli sample was collected. All samples were then compared to each other, using Coomassie staining analysis. Figure 3.3 shows the expression of UCP2-spot-tag-C and UCP2-spot-tag-N, over time. The expression of UCP2-spot-tag-C is clearly visible 2 hours after the expression induction, due to the presence of thick bands, which have the approximate molecular weight of UCP2 (33 kDA + 1.4 kDa). However, this pattern does not appear for the expression of UCP2-spot-tag-N, suggesting that only a small amount of this modified protein is present in the cells.

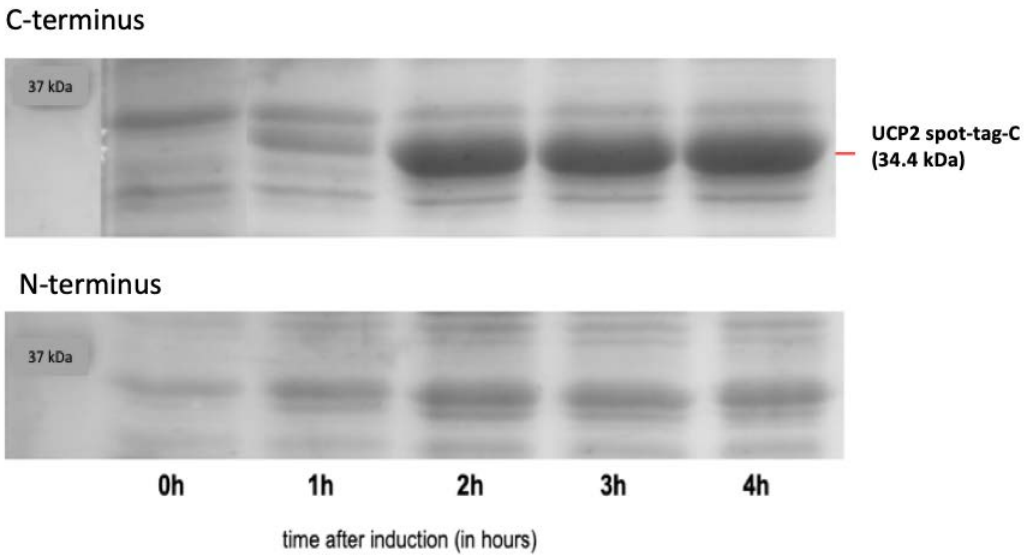

Figure 3.3. ***Coomassie staining analysis of spot-tag-containing UCP2 expression:*** *The expression of UCP2-spot-tag in Rosetta competent cells were compared for both N- and C-terminus before (0 hour) and every hour after induction of protein expression (1-4 hours).*

3.1.3. Inclusion body isolation

After the protein expression, the Rosetta cells were destroyed, and their lysate was centrifuged to obtain the so called “inclusion bodies”. Inclusion bodies are dense, granular structures that usually arise as a result of the heterologous expression of a foreign protein in E. Coli.

A sample from the obtained lysate was taken before the centrifugation. Post centrifugation, the supernatant was collected additionally, and the pellets were dissolved and isolated as inclusion bodies. Figure 3.4 shows the centrifuge tubes with the pellets from UCP2-spot-tag-N and UCP2-spot-tag-C. The pellets from UCP2-spot-tag-N appear less dense in comparison to the pellets from UCP2-spot-tag-C. We conclude that the protein expression is decreased when the tag is inserted in the N-terminus (Figure 3.4). Therefore, for the future steps, only the inclusion bodies of UCP2-spot-tag-C were used.

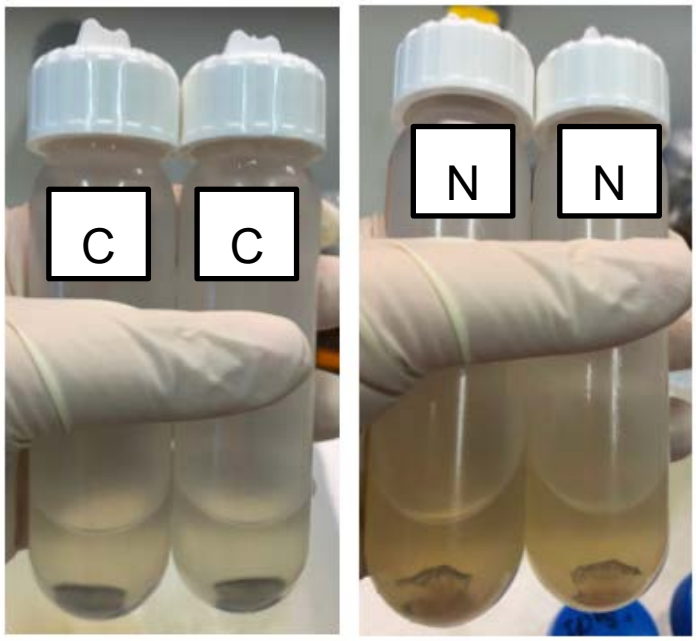

Figure 3.4 ***Isolated inclusion bodies containing the UCP2-spot-tag in N- or C-terminus:*** *The lysate of destroyed Rosetta cells were centrifuged to obtain inclusion bodies of UCP2-spot-tag for both C- (left) and N-terminus (right).*

3.1.4. Purification and reconstitution of the proteins into liposomes

As the proteins in the inclusion bodies are not folded correctly, UCP2-spot-tag-C as well as UCP2-wt, had to be refolded in a proper way. This was achieved by isolation and purification of proteins from inclusion bodies followed by their reconstitution into a lipid bilayer membrane.

The protein concentration was measured using Pierce BCA protein assay kit. Lipid concentration in the proteoliposomes and the protein to lipid ratio (µg/mg) were calculated. Table 3.1 shows that the UCP2-spot-tag-C has a lower protein and higher lipid concentration compared to the UCP2-wt proteoliposome.

Table 3.1 ***Protein concentration of proteoliposomes***

Name (charge*)	Protein concentration in µg/ml	Lipid concentration in mg/ml	Protein concentration in µg per mg lipid (protein to lipid ration)
UCP2-wt-Ch24	32.8	5.68	5.78
UCP2-spot-tag-C-Ch1	27.7	6.6	4.20

**charge x (Chx) indicates the refolding attempt*

To verify the purification degree of proteins from the inclusion bodies, samples were collected after each purification and refolding step and a comparison with the final proteoliposomes was made using silver staining analysis (Figure 3.5). The comparison of the samples after each inclusion body purification and protein refolding steps for UCP2-spot-tag-C is shown in Figure 3.5.A. Lane 1 shows the lysate before centrifugation, lanes 2 and 3 show the supernatant after each centrifugation step. Lane 4 shows the isolated inclusion bodies obtained from the pellet.

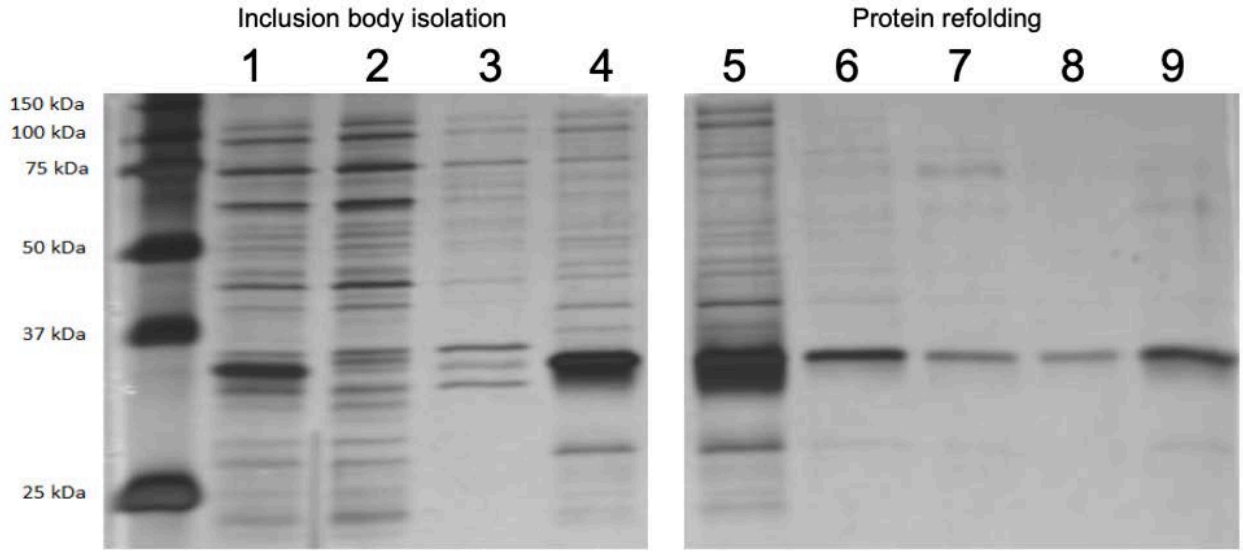

Figure 3.5.A ***Silver staining analysis of different inclusion body isolations and protein refolding steps for UCP2-spot-tag-C:*** *Inclusion bodies and proteoliposomes from different working steps were separated by SDS PAGE and visualized with silver staining. The first 4 lanes show the purification steps of the inclusion body isolation from bacterial pellets. The 5th – 8th lanes show the solubilization and purification steps of the reconstitution of UCP2-spot-tag-C into liposomes. The 9th lane shows the purified proteoliposomes containing UCP2-spot-tag-C.*

Since the inclusion bodies were isolated from the pellets after the centrifugation (Figure 3.4), the supernatant (lane 2 and 3) was expected to contain no UCP2. That was verified by the absence of a band at around 34.4 kDa. Lane 5 (Figure 3.5.A) shows the first step of the refolding: the solubilization with detergent. In this lane, UCP2, as well as many other bands, are visible. Lanes 6, 7 and 8 respectively, are samples after dialysis, HTTP column, and bio-beads. In lanes 6-8, only the UCP2 band maintains its intensity as the other bands start to fade. This indicates that the protein is purified after each step. Lane 9 shows the purified UCP2-spot-tag-C proteoliposomes.

Figure 3.5.B shows the same refolding steps (lane 1-4) for UCP2-wt, however the inclusion body isolation steps are excluded. Just like UCP2-spot-tag-C, the non-UCP2 bands begin to fade after the dialysis. In lane 4, additionally to UCP2 another band is observed. However, this band is not

visible after the final purification step (lane5). This implies that the obtained proteoliposomes are purified. Lanes 5-8 compare proteoliposomes of both UCP2-spot-tag-C (lane 6 and 8) and wild-type UCP2 (lanes 5 and 7) at different concentrations. The bands in the UCP2-spot-tag-C (lanes 6 and 8) are higher than the bands in the UCP2-wt (lanes 5 and 7). This is expected, as the weight of the Spot-Tag® (1.4 kDa) adds up to the molecular weight of UCP2 (33kDa).

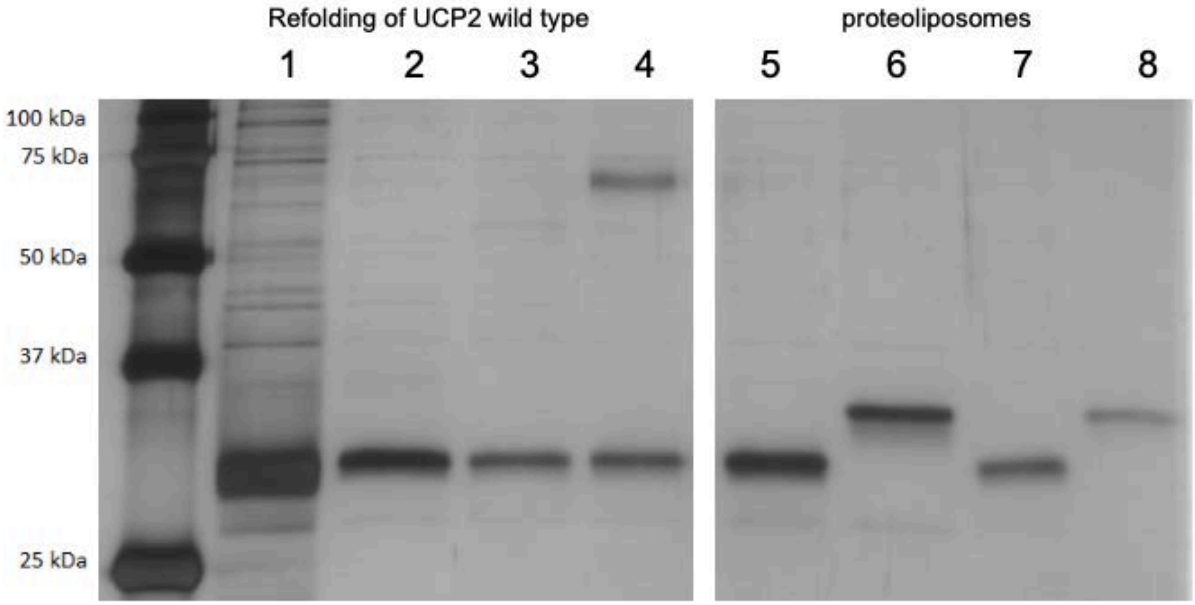

Figure 3.5.B ***Silver staining analysis of different protein refolding steps for UCP2-wt and the purified proteoliposomes of both UCP2-wt and UCP2-spot-tag-C:*** *Proteoliposomes during different working steps were separated by SDS PAGE and visualized with silver staining. The first 4 lanes show the solubilization and purification steps of the reconstitution of UCP2 wild type into liposomes. The 5th and 7th lanes show the purified proteoliposomes of UCP2 wild type with 400 and 200 ng respectively. The 6th and the 8th lanes show the purified proteoliposome of UCP2-spot-tag-C with 400 and 200 ng respectively.*

To verify that both the higher (34.4 kDa) and the lower bands (33 kDa) are due to the presence of UCP2, western blot analysis was performed using an antibody against UCP2 (Figure 3.6). In to additionally ensure that the modified recombinant proteins don't aggregate with other proteins, different samples, prior to loading on the gel, were pre-mixed with UCP2-spot-tag-C and then

loaded (lanes 1-4). For example, lane 1 is the mixture of total-protein of spleen tissue of a UCP2 knock-out mouse mixed with UCP2-spot-tag-C while lane 2 is the mixture of UCP2-wt and UCP2-spot-tag-C. In lane 1 there is only one strong band detected. This is expected, since the spleen tissue is derived from a UCP2 knock-out mouse and therefore, the only detectable band would be the premixed UCP2-spot-tag-C. In lane 2 however, two bands are detected, meaning that the antibody detected both the UCP2-wt and UCP2 spot-tag-C, which was also expected, as the target epitope should not change. Lanes 3 and 4 are similar to lane 2, with the exception that other wild-type variants (different charges (refolding attempts)) of UCP2-wt are used as a control. The bands in lanes 2-4 share the same intensity and height, indicating the presence of UCP2. The wild-type controls (different charges) were loaded separately in lanes 5 and 6 for extra comparison. Lanes 12 and 13 show the UCP-wt and UCP2-spot-tag-C respectively. The height difference is again visible in lanes 12 and 13 due to the addition of spot-tag (1.4 kDa) , which increases the proteins final molecular weight to 34.4 kDa.

The total protein of heart, spleen, and mouse embryonic stem cells were used as positive controls for UCP2 (Rupprecht, Moldzio et al. 2019) and the total protein of spleen of a UCP2 knock-out mouse was used as the negative control.

The band for UCP2 (33 kDa) is visible in mouse embryonic stem cells (lane 11) and faintly visible in heart of a 7-day old mouse (lane 7). The same band however is not visible in heart of a 30-day old mouse (lane 8). In the spleen of a UCP2 knock-out mouse (lane 10), the UCP2 band is, as expected, clearly absent. In the spleen of normal mouse however, this band is clearly visible. All these results indicate that the antibody detects UCP2. However, the presence of many non-UCP2 bands in the tissue samples indicates that the antibody is unspecific in tissue samples.

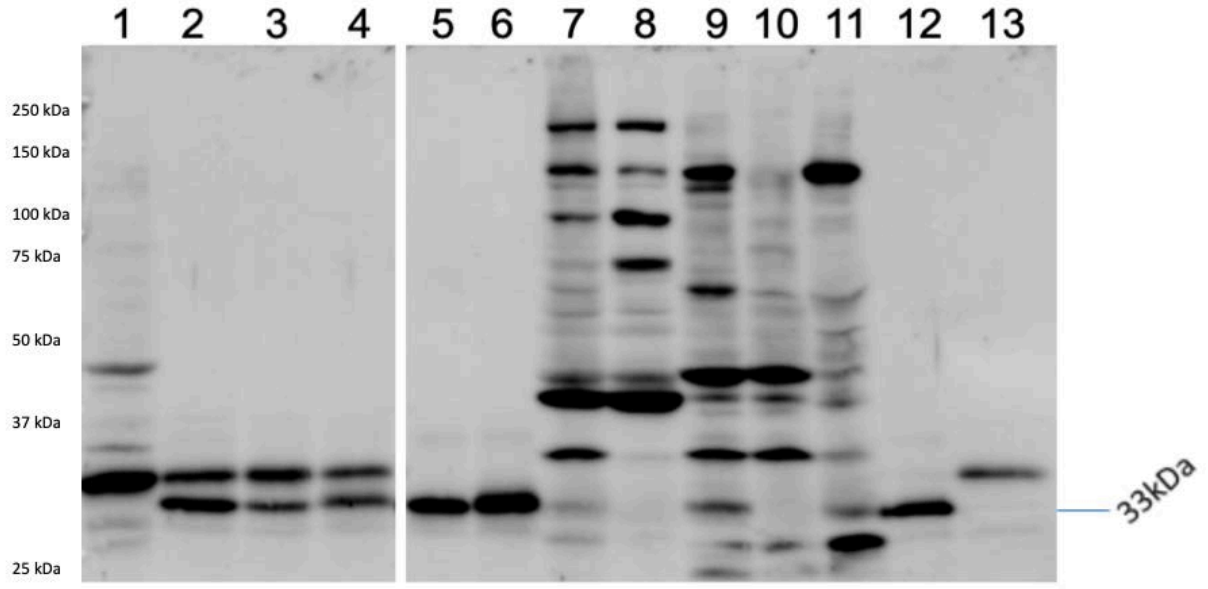

Figure 3.6 ***Western Blot Analysis of wild type recombinant UCP2, UCP2-spot-tag-C and different mouse organs using UCP2-antibody****: Lanes 1-4 show 5 µg of different tissue or UCP2 recombinant samples premixed with 5 µg of UCP2-spot-tag-C. Lanes 5-6 show two different charges of recombinant wild-type UCP2 (5 ng protein). Lanes 7-11 show total-protein of different mouse tissue samples, as well as mouse embryonic stem cells. Lane 12 and 13 respectively show recombinant wild-type of UCP2 and UCP2-spot-tag-C each in 5 ng (the detailed description of all lanes is shown in Table 3.2)*

Table 3.2 ***Samples loaded in the lanes of*** *Figure 3.6*

Lane	Samples
1	UCP2 Knockout spleen + UCP2-spot-tag-C
2	Wild type recombinant UCP2-wt-Ch24 + UCP2-spot-tag-C
3	Wild type recombinant UCP2-wt-Ch17 + UCP2-spot-tag-C
4	Wild type recombinant UCP2-wt-Ch19 + UCP2-spot-tag-C
5	Wild type recombinant UCP2-wt-Ch17
6	Wild type recombinant UCP2-wt-Ch19
7	Total protein of a 7-day-old mouse
8	Total protein of a 30-day-old mouse
9	Total protein of spleen of a mouse
10	Total protein of a UCP2 knockout mouse spleen
11	Mouse embryonic stem cells
12	Wild type recombinant UCP2-wt-Ch24
13	UCP2-spot-tag-C

In order to ensure that the Spot-Tag® is expressed properly, a similar western blot analysis was performed, in which a membrane was incubated with spot-VHH recombinant binding primary antibody, which binds to the Spot-Tag® sequence. Figure 3.7.A shows the result of the western blot analysis using spot-VHH primary antibody and anti-Alpaca secondary antibody. Although all samples of UCP2-spot-tag-C are detected, a band for a UCP2-wt as well as some unspecific bands in the tissue samples are also detected (lane 5). This is unexpected, since this wild-type variant has no spot-tag inserted. This raised the question of whether the detected bands are due to unspecific staining of the secondary or primary antibody. To answer this question, an identical membrane with the identical samples was incubated with only the secondary antibody (Figure 3.7.B). Indeed, nearly identical results were obtained for both membranes. This confirmed that the observed bands appear due to the unspecific binding of the anti-Alpaca secondary antibody.

To overcome the aforementioned issue, we took advantage of the presence of a 6x-His-tag on the primary spot-VHH antibody and therefore used a secondary anti-His Tag antibody instead.

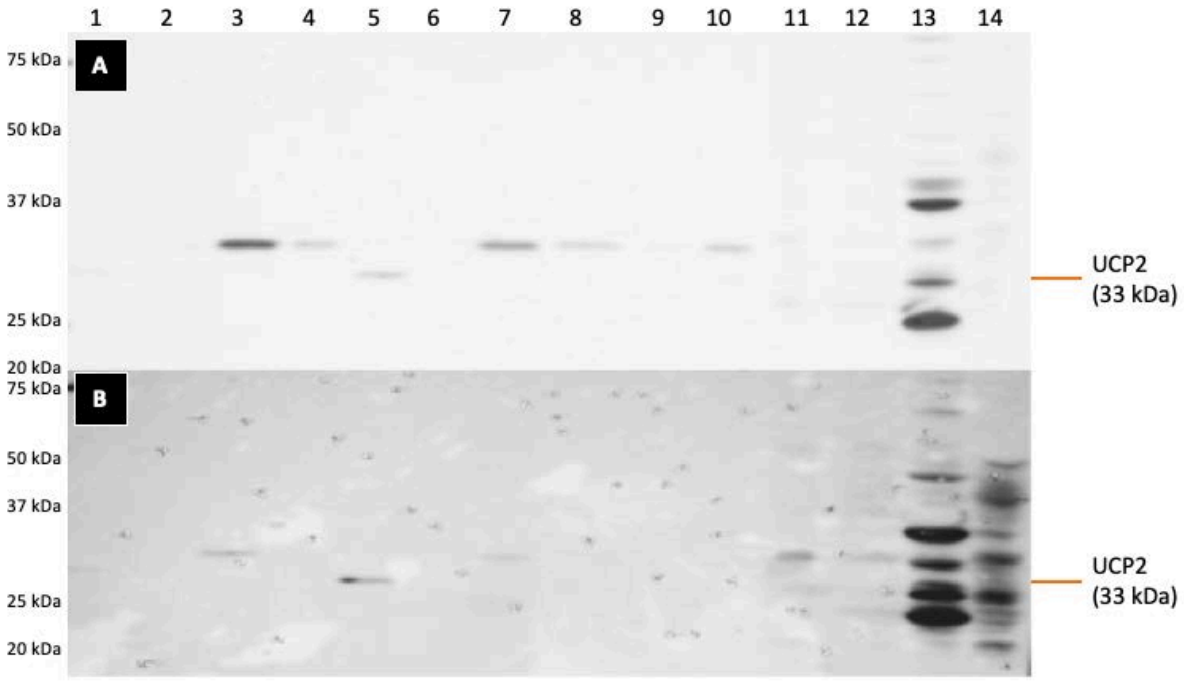

***Figure 3.7* Representative western Blot Analysis of UCP2-wt, UCP2-spot-tag-C and total protein of different mouse organs using Spot-VHH antibody or only anti-Alpaca (secondary) antibody:** *To check the cross-reactivity of the secondary antibody (anti-Alpaca), two membranes with identical samples were incubated with different antibodies. Membrane A was incubated with both primary (spot VHH) and secondary (Alpaca) antibody, while membrane B was incubated with secondary antibody only. Lanes 1,2,5,6 show different concentrations of UCP2-wt and lanes 3 and 4 show different concentrations of UCP2-spot-tag-C. In lanes 7-10 different samples were premixed with UCP2-spot-tag-C and then loaded on the gel. Lanes 11-14 show different mouse organs (20 µg) as well as mouse embryonic stem cells (10 µg). (the detailed description of all lanes is shown in Table 3.2)*

Table 3.3 ***Samples loaded in the lanes on*** *Figure 3.7*

Lane	Samples
1	Wild type recombinant UCP2-wt-Ch24 (291 ng)
2	Wild type recombinant UCP2-wt-Ch24 (100 ng)
3	UCP2-spot-tag-C (344 ng)
4	UCP2-spot-tag-C (100 ng)
5	Wild type recombinant UCP2-wt-Ch17 (291 ng)
6	Wild type recombinant UCP2-wt-Ch19 (100 ng)
7	UCP2 Knockout spleen + UCP2-spot-tag-C (20 µg + 291 ng)
8	Wild type recombinant UCP2-wt-Ch24 + UCP2-spot-tag-C (344 ng + 291 ng)
9	Wild type recombinant UCP2-wt-Ch24 + UCP2-spot-tag-C (100 ng + 100 ng)
10	Wild type recombinant UCP2-wt-Ch17 + UCP2-spot-tag-C (291 ng + 291 ng)
11	Total protein of spleen of a mouse
12	Total protein of a UCP2 knockout mouse spleen
13	Total protein of a mouse heart
14	Mouse embryonic stem cells

3.1.5. Production and analysis of new proteoliposomes

To obtain better results for electrophysiology measurements, new refolding for both UCP2-wt (UCP2-wt-Ch25) and UCP2-spot-tag-C (UCP2-spot-tag-C-Ch2), were made. Table 3.4 shows the protein concentration, the lipid concentration and the protein to lipid ratio of the new proteoliposomes.

Unlike the older proteoliposomes (Table 3.1), the protein concentration of the new proteoliposomes is more than two times larger. According to the experiments from the past years, a concentration of this magnitude is expected. This could suggest that the previously made proteoliposomes (UCP2-wt-Ch24 and UCP2-spot-tag-C-Ch1) were not successfully refolded.

Table 3.4 ***Concentration of the new proteoliposomes***

Name (charge)	**Protein concentration in µg/ml**	**Lipid concentration in mg/ml**	**Protein concentration in µg per every mg lipid**
UCP2-wt-Ch25	67.6	8.33	8.12
UCP2-spot-tag-C-Ch2	77.7	6.6	11.78

To ensure that UCP2 is reconstituted into liposomes and to compare both old and new wild-type and the modified variant of UCP2, the proteoliposomes were loaded on a gel and analyzed by SDS-page which was then stained by silver staining.

Figure 3.8 verifies the presence of UCP2 (33 kDa), as a single appearing in all probes. The proteoliposomes from the previous refolding attempt (lanes 5-8) look similar to the proteoliposomes from the recent refolding attempt (lane 1-4). Just like the previous silver staining and western blot analysis, a visible difference in the height of the bands, due to the molecular weight of spot-tag (1.4 kDa) is noticeable.

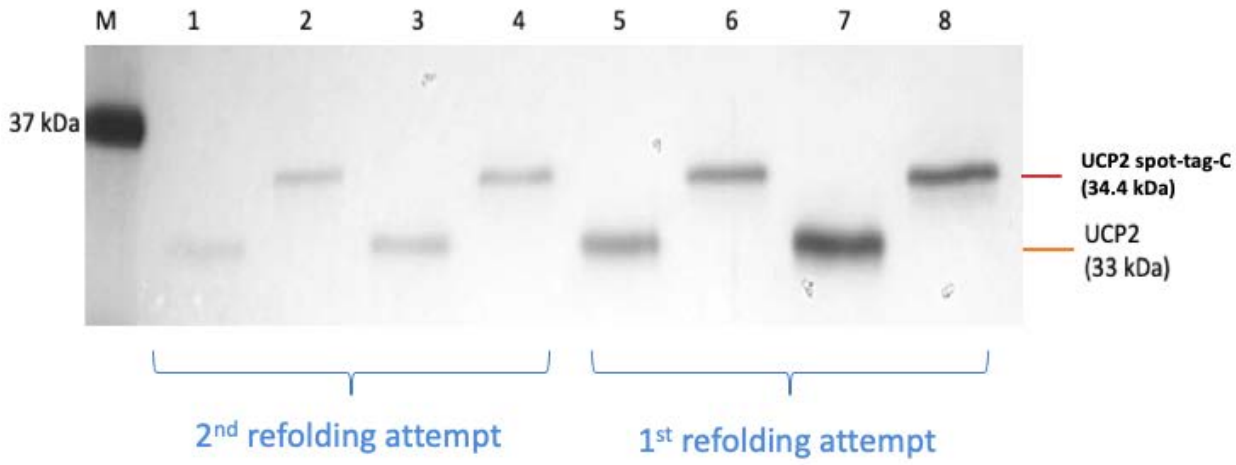

Figure 3.8 ***Silver staining analysis of different proteoliposomes****: M is the protein marker at 37 kDa. Lanes 1 and 3 show the recent proteoliposomes of the wild-type UCP2 (charge 25) at 200 and 400 ng respectively. Lanes 2 and 4 show the recent proteoliposomes of UCP2-spot-tag-C (charge 2) also at 200 and 400 ng. Lanes 5 and 7 represent the previous proteoliposomes of wild-type UCP2 (charge 24) at 200 and 400 ng. Lanes 6 and 8 represent the previous UCP2-spot-tag-C (charge 1) at 200 and 400 ng.*

Both proteoliposomes from the first and second refolding attempt, were compared and analyzed by western blot analysis. Tissue samples of spleen and mouse embryonic stem cells were taken as the positive control, while spleen of a UCP2-knock-out mouse was used as the negative control for UCP2.

To verify the presence of UCP2 in the new proteoliposomes, UCP2 primary antibody was used (Figure 3.9). No difference between the detected bands of proteoliposomes from the first and second refolding attempt are visible. The 1.4 kDa height difference from the Spot-Tag®, is also evident in both previous and recent UCP2-spot-tag-C proteoliposomes. The UCP2 band at 33 kDa is also detected in the samples from the spleen tissue (lane 10) and mouse embryonic stem cells (lane 11). As expected, no UCP2 band (33 kDa) is detected in the sample of UCP2-knock-out spleen tissue (lane 9). Faint bands are visible above and below all the

proteoliposome samples (lane 1- lane 8). This faint bands are potentially present due to the impurities of the proteoliposomes. Ideally, the impurities should not be detected by the UCP2 antibody, however some unspecific staining of the UCP2 antibody is already known and evident specially in the tissue samples.

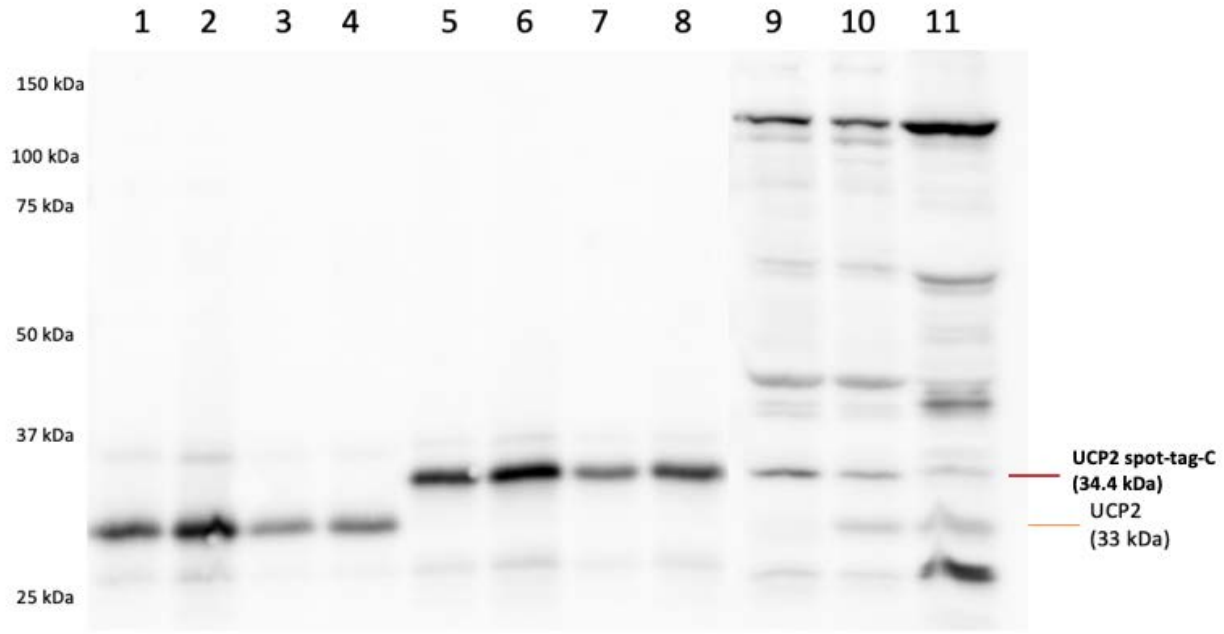

Figure 3.9 ***Western blot analysis of the previous and the recent proteoliposomes and tissue samples using UCP2 antibody****: In lanes 1 and 2, UCP2-wt-Ch24 (old proteoliposomes) are loaded in 5 and 10 ng respectively. Lanes 3 and 4 contain UCP2-wt-Ch25 (new proteoliposome) also in 5 and 10 ng respectively. In Lanes 5 and 6 UCP2-spot-tag-C-Ch1 (old proteoliposome) is loaded in 5 and 10 ng. Lanes 7 and 8 contain UCP2-spot-tag-C-Ch2 (new proteoliposome) in 5 and 10 ng respectively. Lane 9 contains spleen tissue of a UCP2-knock-out mouse, while lane 10 contains a spleen tissue of a normal mouse. Lane 11 contains mouse embryonic stem cells.*

To analyze and specifically detected UCP2-spot-tag-C only, western blot analysis using a primary Spot-Tag-binding antibody (spot-VHH) and a secondary anti-His-tag antibody, were performed (Figure 3.10.A). As expected only the previous and the recent proteoliposomes of UCP2-spot-tag-C are detected. However, some minor unspecific staining is visible in the tissue samples. The

unspecific bands appear most-likely due to the cross reactivity between the secondary antibody (anti-His-tag) and the tissue samples as well as the marker. To test that, another western blot analysis was performed, in which the membrane was only incubated with the secondary antibody (Figure 3.10.B). As expected, the same unspecific bands were detected again. Therefore, it can be concluded that, the primary antibody binds specifically to the UCP2-spot-tag-C and can be used for immunohistochemistry in the future studies.

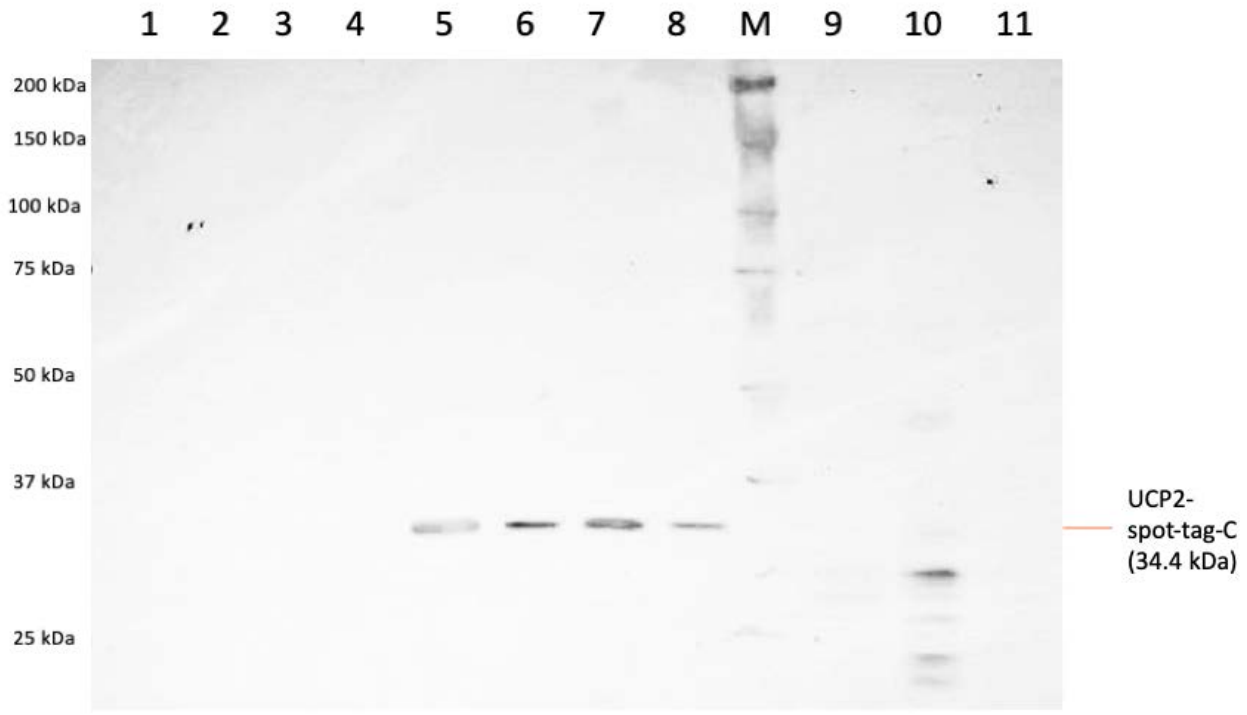

Figure 3.10.A ***Western blot analysis of the previous and the recent proteoliposomes and tissue samples using Spot-Tag-binding antibody:*** *In lanes 1 and 2, UCP2-wt-Ch24 (old proteoliposomes) is loaded in 100 ng and 250 ng respectively. Lanes 3 and 4 contain UCP2-wt-Ch25 (new proteoliposomes) also in 100 and 250 ng respectively. In Lanes 5 and 6 UCP2-spot-tag-C-Ch1 (old proteoliposomes) is loaded in 100 and 250 ng. Lanes 7 and 8 contain UCP2-spot-tag-C-Ch2 (new proteoliposomes) in 100 and 250 ng respectively. Lane 9 contains spleen tissue of a UCP2-knock-out mouse, while lane 11 contains spleen tissue of a normal mouse. Lane 10 contains mouse embryonic stem cells.*

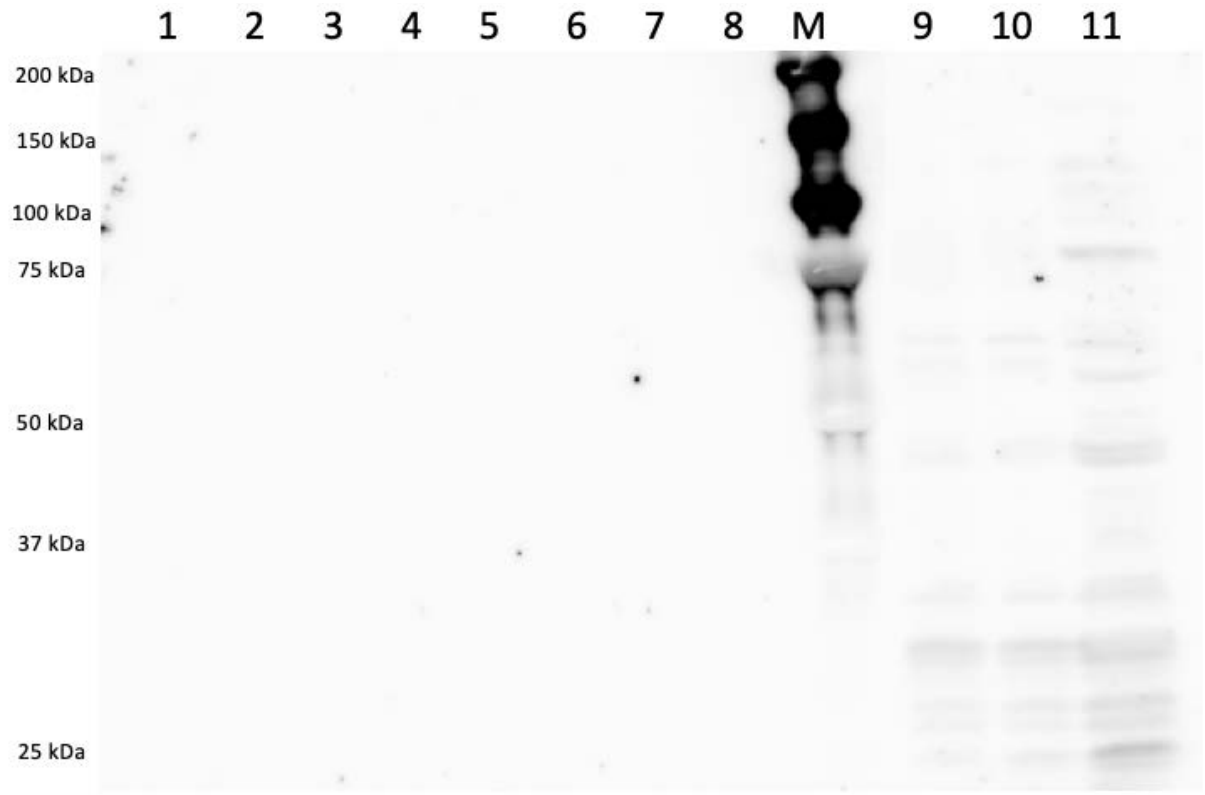

Figure 3.10.B ***Western blot analysis of the previous and the recent proteoliposomes and tissue samples using only the secondary antibody:*** *In lanes 1 and 2, UCP2-wt-Ch24 (old proteoliposomes) is loaded in 5 ng and 10 ng respectively. Lanes 3 and 4 contain UCP2-wt-Ch25 (new proteoliposomes) also in 5 and 10 ng respectively. In Lanes 5 and 6 UCP2-spot-tag-C-Ch1 (old proteoliposomes) is loaded in 5 and 10 ng. Lanes 7 and 8 contain UCP2-spot-tag-C-Ch2 (new proteoliposomes) in 5 and 10 ng respectively. Lane 9 contains spleen tissue of a UCP2-knock-out mouse, while lane 10 contains spleen tissue of a normal mouse. Lane 11 contains mouse embryonic stem cells.*

3.2. Electrophysiological measurements

3.2.1. Measurement of the membrane conductance

To test whether the insertion of the Spot-Tag® affects the functionality of UCP2, lipid membranes were constructed, and their electrical conductance was measured in absence and presence of FA (arachidonic acid (AA)), as well as in the absence are presence of the proteoliposomes (UCP2-spot-tag and UCP2-wt).

The basal conductance of a constituted lipid membrane alone as well as another membrane with the same lipid compositions with fatty acid or with UCP2-wt was measured (Figure 3.11). No stable membranes were achieved in the presence of UCP2-spot-tag-C and therefore no results were obtained. A possible explanation for that could be, that UCP2-spot-tag-C-Ch1 was refolded unsuccessfully. Therefore, a new protein refolding was made and as a result new proteoliposomes were produced (UCP2-wt-Ch25 and UCP2-spot-tag-C-Ch2).

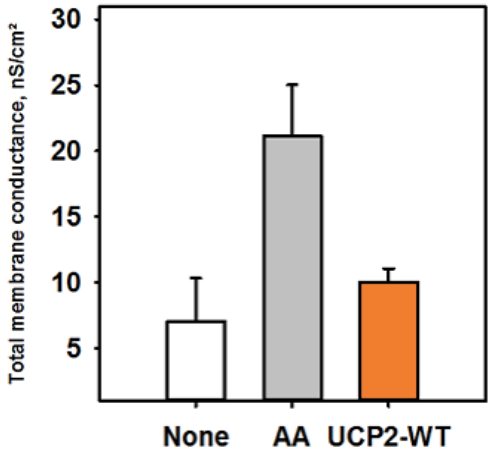

Figure 3.11 ***Electrophysiological measurement of total membrane conductance:*** *Total membrane conductance in nS/cm2 of different membranes were measured in absence or presence of 15 mol% arachidonic acid (AA) as well as Lipid membrane by itself (None) and Lipid membrane containing the wild-type of UCP2 (UCP2-wt).*

After the production and validation of new proteoliposomes, new electrophysiological measurements were performed. Unlike the previous proteoliposomes, stable membranes were achieved in the presence of Spot-Tag®, enabling to obtain results for the conductance measurements. Figure 3.12 shows the total membrane conductance of new proteoliposomes (UCP2-wt and UCP2-spot-tag-C) in different concentrations and in absence or presence of fatty acid. The membrane conductance after the inhibition through 4 mM ATP is shown in dashed bars. The measurements suggest a slight decrease of the membrane conductance in UCP2-spot-tag-C compared to UCP2-wt, in the presence of FA. However, the measurements do not show a decrease in the inhibition of UCP2-spot-tag-C. Therefore, the decrease of the conductance alone, does not prove any functional alterations caused by the Spot-Tag®.

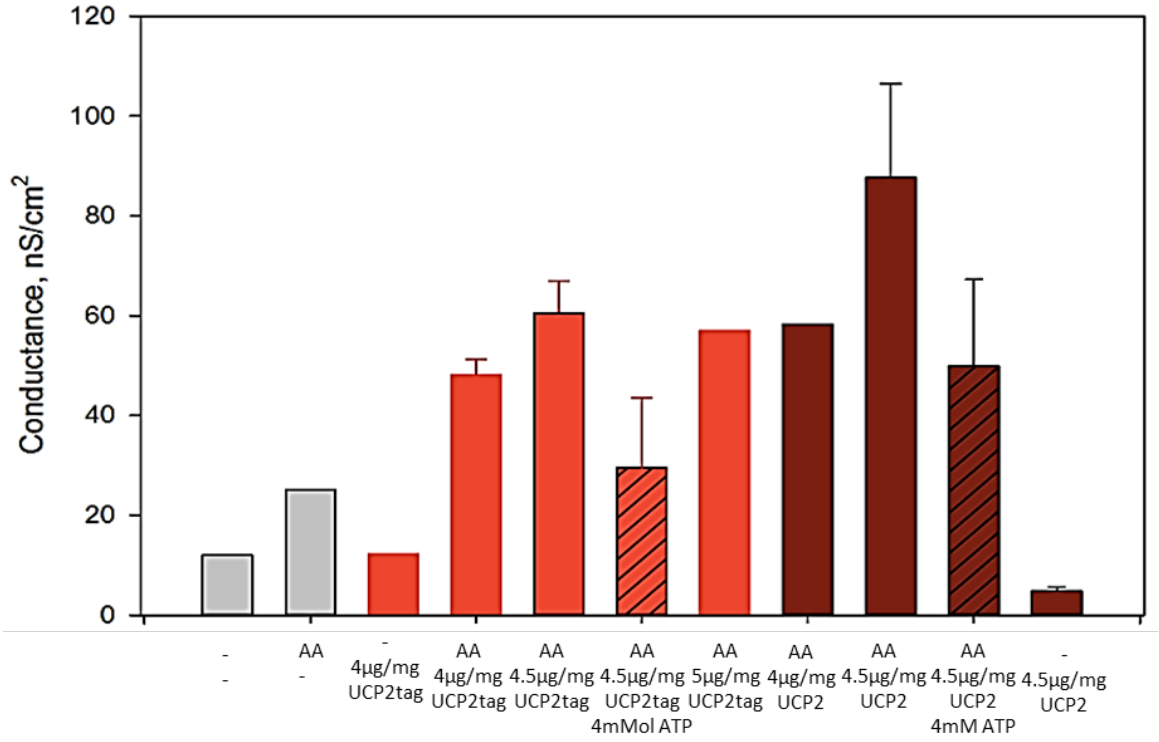

Figure 3.12 ***Conductance measurements of new proteoliposomes:*** *Gray bars represent the measurements without proteins, red bars represent the measurements with UCP2-spot-tag-C-Ch2 and maroon bars represent measurements with UCP2-wt-Ch25. Measurements after inhibition with 4 mM ATP is represented through dashed bars. AA stands for arachidonic acid (fatty acidarchi). The protein concentration of each measurement is written below the bar in µg/mg. Values are depicted as mean ±SEM, n = XY independent experiments.*

4. Discussion and Outlook

4.1. Discussion

We inserted Spot-Tag® in the primary sequence of mUCP2 and verified its successful insertion in the N- or C-terminus by sequencing. We tested the expression of UCP2-spot-tag in both N- and C-terminus and concluded that UCP2-spot-tag is successfully expressed only if inserted in the C-terminus. Moreover, we confirmed that UCP2-spot-tag is detected specifically by Spot Nanobody enabling subcellular visualization of UCP2 for the future studies.

Additionally, we tested the functionality of UCP2 with inserted Spot-Tag® and showed that the inhibition of UCP2 by ATP, is not affected by the presence of Spot-Tag®.

4.1.1. The unspecificity of secondary antibodies for Spot Nanobody

The Spot-Tag-binding primary antibody (Spot Nanobody) was used to detect UCP2-spot-tag-C specifically. However, as shown in Figure 3.7 and Figure 3.10, unspecific bands were detected due to cross-reactivity between the samples and the secondary antibodies.

The main goal of introducing Spot-Tag® into UCP2 is to enable studying UCP2, by immunohistochemistry or other subcellular visualization techniques. Using Atto-conjugated anti-spot antibodies, UCP2-spot-tag can be directly detected *in vivo,* without any need for a secondary antibody. Therefore, the specificity of the secondary antibodies, used for Spot Nanobody, is irrelevant for further studies.

4.1.2. The effect of Spot-Tag® on formation of a stable membrane

Protein refolding is crucial for recovering the biological activity of recombinant proteins from inclusion bodies. Therefore, a successful refolding is very important for proper electrophysiological measurements of UCP2.

The conductance measurements of proteoliposomes from the first refolding attempt (UCP2-spot-tag-C-Ch1 and UCP2-wt-Ch24) were not successful, since no stable membranes for the electrophysiological measurements of UCP2-spot-tag-C-Ch1 were formed. However, after the second refolding, stable membranes were easily formed. The protein concentration of the proteoliposomes from the first refolding (Table 3.1) were unexpectedly low, while the protein concentration of proteoliposomes from the second refolding attempt were in the expected range (Table 3.4). The low measured protein concentration suggests that the inability to form stable membranes was due to the unsuccessful refolding, not due to the presence of Spot-Tag.

4.1.3. The effect of Spot-Tag® on function of UCP2

The electrophysiological measurements were performed to test the functionality of UCP2-spot-tag by measuring the electrical conductance, caused by its proton flux. Figure 3.12 shows that, when activated (presence of FA), UCP2-spot-tag-C has a slightly lower conductance than UCP2-wt. However, when inhibited (addition of ATP), both proteins are inhibited with a similar ratio. This can suggest that the presence of spot-tag may potentially have an effect on the activation of UCP2 but has no effect on its inhibition. However, the slight decrease of conductance alone, specially that the inhibition is unaffected, does not prove that Spot-Tag® affects UCP2's functionality. Therefore, more experiments with different charges (different refoldings) of protein are required to prove any significant effect of Spot-Tag® on the functionality of UCP2.

4.2. Outlook

To confirm that the insertion of Spot-Tag® does not significantly affect the integrity and functionality of UCP2, the production of UCP2-spot-tag should be improved and more extensive functional testing should be performed.

The next steps would be (i) to endogenously express UCP2-spot-tag and (ii) to target UCP2-spot-tag with Spot Nanobody in vivo, which consequently enables (ii) the sub-cellular visualization of UCP2-spot-tag using single molecule fluorescence microscopy.

5. Summary

Uncoupling protein 2 (UCP2) is a member of the mitochondrial uncoupling protein family. It is known to be highly abundant in fast proliferating cells which have an aerobic glycolysis metabolism, such as cancer cells, stem cells and cells of the immune system. UCP2 was shown to transport protons, contributing to the uncoupling of mitochondria and to regulate reactive oxygen species. Recent studies suggest that UCP2 facilitates metabolic adaptability, allowing the cell to survive during specific nutrient deficiencies. However, the absence of specific antibodies suitable for immunohistochemistry hinders further investigation of UCP2's localization and its function in cells. In order to target UCP2 specifically, a small inert peptide tag was introduced at the N-terminus into the primary sequence of UCP2 and the modified construct was verified by sequencing. Protein expression was then induced in E. Coli following the isolation of protein containing the tag. After proper refolding of the protein into proteoliposomes, the presence of the tag was verified by means of western blot analysis. To check the integrity of the protein after genetic manipulation, electrophysiological measurements were performed, in which proton transport function of the modified protein was tested against a wild-type UCP2. The labeling of UCP2, which does not affect the protein's integrity and function, serves as a tool that enables the visualization of UCP2 in vivo. This tool ultimately enables the targeting of UCP2 using nanobody-based antibodies, which facilitates super-resolution microscopy in the follow-up studies.

Uncoupling Protein 2 (UCP2) ist ein Mitglied der Familie der mitochondrialen Entkopplungsproteine. Es ist bekannt, dass es in schnell proliferierenden Zellen, die einen aeroben Glykolyse-Stoffwechsel haben (wie z. B. Krebszellen, Stammzellen und Zellen des Immunsystems), sehr häufig vorkommt. Es konnte schon gezeigt werden, dass UCP2 Protonen transportiert, zur Entkopplung der Mitochondrien beiträgt und reaktive Sauerstoffspezies reguliert. Jüngste Studien legen nahe, dass UCP2 die metabolische Anpassungsfähigkeit erleichtert und es der Zelle ermöglicht, bei spezifischen Nährstoffmängeln zu überleben. Das Fehlen spezifischer, für die Immunhistochemie geeigneter Antikörper erschwert jedoch die weitere Untersuchung der Lokalisierung von UCP2 und seiner Funktion in Zellen. Um UCP2 spezifisch anzusprechen, wurde ein kleiner inerter Peptid-Tag am N-Terminus in die Primärsequenz von UCP2 eingeführt und das modifizierte Konstrukt durch Sequenzierung verifiziert. Die Proteinexpression wurde dann in E. Coli nach der Isolierung des Proteins, das den Tag enthält, induziert. Nach ordnungsgemäßer Rückfaltung des Proteins in Proteoliposomen wurde die Anwesenheit des Tags mittels Western-Blot-Analyse überprüft. Um die Integrität des Proteins nach der genetischen Manipulation zu überprüfen, wurden elektrophysiologische Messungen durchgeführt, bei denen die Protonentransportfunktion des modifizierten Proteins gegen einen Wildtyp UCP2 getestet wurde. Die Markierung von UCP2, die die Integrität und Funktion des Proteins nicht beeinträchtigt, dient als ein Werkzeug, das die Visualisierung von UCP2 in vivo ermöglicht. Dieses Werkzeug ermöglicht schließlich die gezielte Ansprache von UCP2 mit Hilfe von Nanokörper-basierten Antikörpern, was die superauflösende Mikroskopie in den Folgestudien erleichtert.

6. References

Beck, V., et al. (2006). "A new automated technique for the reconstitution of hydrophobic proteins into planar bilayer membranes. Studies of human recombinant uncoupling protein 1." Biochim Biophys Acta **1757**(5-6): 474-479.

Beck, V., et al. (2007). "Polyunsaturated fatty acids activate human uncoupling proteins 1 and 2 in planar lipid bilayers." FASEB J **21**(4): 1137-1144.

Berardi, M. J. and J. J. Chou (2014). "Fatty acid flippase activity of UCP2 is essential for its proton transport in mitochondria." Cell Metab **20**(3): 541-552.

Brand, M. D. (1990). "The proton leak across the mitochondrial inner membrane." Biochim Biophys Acta **1018**(2-3): 128-133.

Fleury, C., et al. (1997). "Uncoupling protein-2: a novel gene linked to obesity and hyperinsulinemia." Nat Genet **15**(3): 269-272.

Garlid, K. D., et al. (1998). "The mechanism of proton transport mediated by mitochondrial uncoupling proteins." Febs Letters **438**(1-2): 10-14.

Garlid, K. D., et al. (2001). "Mechanism of uncoupling protein action." Biochem Soc Trans **29**(Pt 6): 803-806.

Hilse, K. E., et al. (2016). "The expression of UCP3 directly correlates to UCP1 abundance in brown adipose tissue." Biochim Biophys Acta **1857**(1): 72-78.

Jaburek, M., et al. (1999). "Transport function and regulation of mitochondrial uncoupling proteins 2 and 3." J Biol Chem **274**(37): 26003-26007.

Jezek, P., et al. (2019). "Uncoupling mechanism and redox regulation of mitochondrial uncoupling protein 1 (UCP1)." Biochim Biophys Acta Bioenerg **1860**(3): 259-269.

Kamp, F. and J. A. Hamilton (1992). "pH gradients across phospholipid membranes caused by fast flip-flop of un-ionized fatty acids." Proc Natl Acad Sci U S A **89**(23): 11367-11370.

Krauss, S., et al. (2005). "The mitochondrial uncoupling-protein homologues." Nat Rev Mol Cell Biol **6**(3): 248-261.

Mitchell, P. (1961). "Coupling of phosphorylation to electron and hydrogen transfer by a chemi-osmotic type of mechanism." Nature **191**: 144-148.

Nicholls, D. G. (1976). "Hamster brown-adipose-tissue mitochondria. Purine nucleotide control of the ion conductance of the inner membrane, the nature of the nucleotide binding site." Eur J Biochem **62**(2): 223-228.

Palmieri, F. and C. L. Pierri (2010). "Mitochondrial metabolite transport." Essays Biochem **47**: 37-52.

Pohl, E. E., et al. (2019). "Important Trends in UCP3 Investigation." Front Physiol **10**: 470.

Rousset, S., et al. (2007). "UCP2 is a mitochondrial transporter with an unusual very short half-life." Febs Letters **581**(3): 479-482.

Rupprecht, A., et al. (2019). "Glutamine regulates mitochondrial uncoupling protein 2 to promote glutaminolysis in neuroblastoma cells." Biochim Biophys Acta Bioenerg **1860**(5): 391-401.

Rupprecht, A., et al. (2014). "Uncoupling protein 2 and 4 expression pattern during stem cell differentiation provides new insight into their putative function." PLoS One **9**(2): e88474.

Shabalina, I. G., et al. (2013). "UCP1 in brite/beige adipose tissue mitochondria is functionally thermogenic." Cell Rep **5**(5): 1196-1203

Skulachev, V. P. (1991). "Fatty acid circuit as a physiological mechanism of uncoupling of oxidative phosphorylation." Febs Letters **294**(3): 158-162.

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