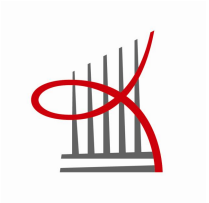

TESTING AND SELECTING CUSTOM ANTIBODIES FOR TWO SUBUNITS OF THE *DROSOPHILA MELANOGASTER* MITOCHONDRIAL RESPIRATORY CHAIN COMPLEX I

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Professional specialization studies
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ABSTRACT

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TUOMELA TEA:
Testing and selecting custom antibodies for two subunits of the *Drosophila melanogaster* mitochondrial respiratory chain complex I.

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The mitochondrial respiratory chain produces energy for the whole organism. It is divided into five different complexes named I to V. Disorders caused by mutations in the subunits of the mitochondrial complexes are severe and often lead to lethality. *Drosophila melanogaster* RNAi knockdown lines are used to study mitochondrial disorders, reproducing in flies symptoms observed in patients and to find out the possible gene therapy for the diseases. The RNAi knockdown lines of two subunits of complex I (CG3683 and CG6020) are used in the search of possible gene therapy. The aim of this development project is to generate custom made antibodies against these two subunits, because there are no commercial antibodies available.

The custom made polyclonal antiserums were tested and selected for affinity purification by dot blotting and western blotting. The preselection of the antibodies was done by the dot blot assay using designed peptides as antigens. The final selection of the antibodies was done by western blotting using mitochondrial proteins from *Drosophila melanogaster*.

In this development project high quality antibodies for subunits CG3683 and CG6020 were found. These antibodies will be used in further research of the RNAi knockdown lines. The level of the knockdown of these proteins will be determined by western blotting from whole flies and from the testes of sterile males.

Keywords: Antibody, *Drosophila melanogaster*, mitochondrion, dot blot and western blot

LIST OF CONTENTS

1 INTRODUCTION

This development project was done in the Institute of Medical Technology (IMT) at the Finnish research unit of mitochondrial biogenesis and disease (FinMIT), where I work as a research assistant, headed by professor Howard Jacobs. This project was done under the supervision of Eric Dufour.

The research of Mitochondrial Gene Expression and Disease Group is aimed at understanding how mutations that affect the mitochondria cause defects in cellular energy production and why they result in specific disorders. To study this, the team uses different kinds of model systems, such as mammalian cell and fruit fly models, to analyse mitochondrial genetic functions. Research interests are mitochondrial DNA, mitochondrial gene expression, mitochondrial biogenesis models, mechanisms of mitochondrial diseases (especially deafness and male infertility) and the mitochondrial theory of ageing.

Our molecular biology research group uses RNAi knockdown fly lines for two subunits (CG3638 and CG6020) of the mitochondrial respiratory chain complex I and we want to confirm the knockdown of these proteins by western blotting. The aim of the developing project was to get good antibodies for these subunits in *Drosophila melanogaster*, since there are no commercial ones available. Purpose of the developing project was to test and select two custom designed antibodies by dot blots and western blots. The dot blot assay was used for the initial selection of the antibodies using the designed peptides as antigens. The final selection was done based on the results of the western blot assay.

2 REVIEW OF THE LITERATURE

2.1 Production of the antibodies

Natural adaptive immune responses are normally induced by antigens that are produced by pathogenic microorganisms to protect animal from infection (Janeway et al. 2001). This normal immune response can be used to produce antibodies. An antibody (also called an immunoglobulin, Ig) is a protein synthesised by an animal in response to a foreign substance (an antigen). Antibodies that are produced by a certain antigen have specific and high affinity for that antigen. The antibodies recognize a cluster or specific group of amino acids on a large molecule called an epitope (an antigenic determinant). (Berg et al. 2002.)

Synthetic peptides can also be used to produce antibodies. The small synthetic molecule presents a recognised epitope and is attached covalently to a macromolecular carrier (a hapten). The haptens are small organic molecules that have a simple structure. They don’t produce antibodies when injected themselves. (Berg et al. 2002; Janeway et al. 2001.)

Any substance that can start an immune response is immunogenic and is called an immunogen. The difference between immunogen and antigen is a clear operational distinction. Any substance that can bind to a specific antibody is defined as an antigen. All antigens have potential to produce specific antibodies, but some of them are not immunogenic and they need to be mixed with an adjuvant. The adjuvants are substances that enhance the immunogenicity of a mixture. The difference between haptens and adjuvants is that an adjuvant does not form a stable linkage with an antigen. (Janeway et al. 2001.)

Usual methods for producing antibodies involve immunisation with a purified or partially purified antigen substance. Most commonly proteins or peptides are used as antigens, but carbohydrates, nucleic acids, cells and cell and tissue extracts can be used. When producing antibodies the first consideration is

whether monoclonal or polyclonal antibodies are needed. Polyclonal antibodies are good for immunoprecipitation or immunoblotting. Monoclonal antibodies are more specific and can be used for almost any purpose. (Cooper & Paterson 2009.)

2.1.1 Monoclonal antibodies

Monoclonal antibodies are produced by cells (or animals injected with cells) that produce only a single antibody (figure 1). At first a mouse is injected with an antigen. After several weeks the mouse spleen is removed. A mixture of plasma cells from the spleen are fused in vitro with myeloma cells. Myeloma cells are immortal cells derived from a cancer, multiple myeloma. In this cancer a single cell divides uncontrolled and generates a large number of cells of a single kind (clones). Plasma cells from the spleen and the myeloma cells are fused to hybrid cells called hybridoma cells. (Berg et al. 2002.) In the hybridoma the spleen cells provide the ability to produce the specific antibody and the myeloma cells provide the ability to grow indefinitely in culture and secrete the antibody continuously. (Janeway et al. 2001.)

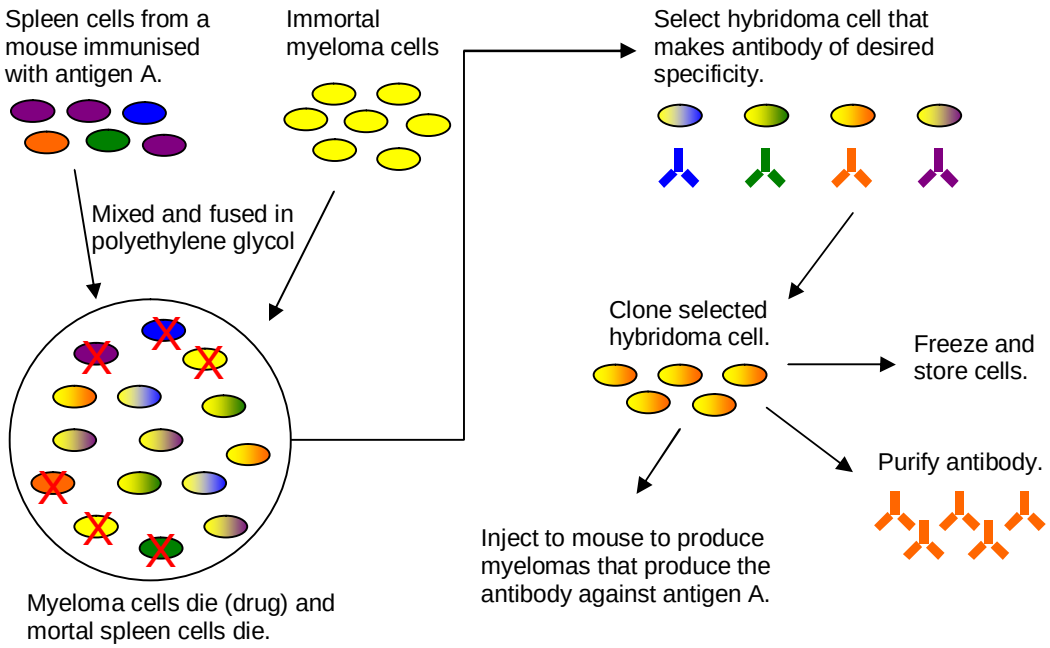

Figure 1. Preparation of the monoclonal antibodies (figure modified from Berg et al. 2002; Janeway et al. 2001).

After fusion hybridoma cells are selected using drugs that kill the parental myeloma cells and the mortal parental spleen cells are not long living cells. Hybridoma cells are screened to find out which cells produces the antibody with desired specificity. Those cells are cloned by regrowing the cultures from single cells. Hybridoma cells can be grown in culture medium and the antigen can be purified from medium. Hydridomas can be injected into mice to induce myelomas and the antibody can be purified from the serum of the mice. The hybridoma cells can be frozen and stored for long periods. (Berg et al. 2002; Janeway et al. 2001.)

2.1.2 Polyclonal antibodies

Producing polyclonal antibodies is faster than producing monoclonal antibodies. Polyclonal antibodies can be used for immunoprecipitation, immunoblotting, and enzyme-linked immunosorbent assays (ELISA). When choosing an animal for the production of antibodies there are a few things to keep in mind: the amount of antibody wanted and the evolutionary distance between the species. Rabbits are usually used for production of antibodies because they are genetically different from human and mouse, which are the proteins that are most often studied. Rabbits also provide a reasonable volume of serum, as much as 25 ml from each bleed. Inbred mouse strains can be used for production of antibodies for smaller scale experiments. (Cooper & Paterson 2009.)

The animal is bled prior to immunisation. Preimmune bleeding is a critical control to make sure that detected antibody activity in later bleeds is due to the immunisation. The antigen with the adjuvant is injected intramuscularly, intradermally, or subcutaneously into an animal of the chosen species. Naïve B cells are stimulated to differentiate into antibody secreting plasma cells. After 5 to 7 days the specific antibody begins to appear in the serum. The concentration of antibody (titer) rises and peaks around day 12, it then starts to decrease. Some of the antibody stimulated B cells turn into memory B cells, which are activated quickly after boost injections that started 4 to 8 weeks after the primary immunisation and are continued at 2 to 3 week intervals. Animals

are bled between the boosts, the serum (called an antiserum) is prepared from whole blood and the titer is checked. (Cooper & Paterson 2009.) The antiserum contains antibodies to all antigens to which the animal has been exposed. Only some of the antibodies are the antibodies specific to the injected protein. They are not a single molecular species. They are polyclonal (heterogenous). (Berg et al. 2002.)

2.1.3 Antibody purification by affinity chromatography

Affinity chromatography can be used for isolating specific antibody from an antiserum (figure 2). In this process molecules are separated on the basis of their affinity for one another. Affinity means the strength of binding of the antibody to its antigen. (Janeway et al. 2001.)

In this method antibody binds to an antigen that is held on a solid matrix. An antiserum is added to a column that is filled with beads to which the antigen is covalently bound. The specific antibody binds to the antigen beads while all other proteins in the serum can be washed away. After several rinses the column is eluted to retrieve the purified antibody. (Janeway et al. 2001.)

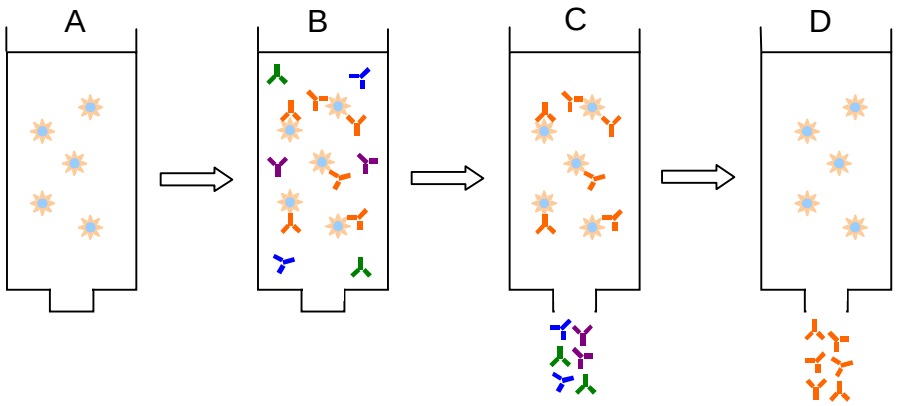

Figure 2. Purification of the antibody by affinity chromatography: A) antigen A is bound to beads, B) mixture of antiserum is added to column, C) wash away unbound molecules and D) elute of the specific antibody (figure modified from Janeway et al. 2001.)

2.1.4 Antibody testing by western blotting

Polyclonal antiserum contains a mixture of antibodies that react against multiple sites on the immunizing antigen. Antibodies can be used in a variety of ways to detect proteins in cell extracts. Western blotting (immunoblotting) is one method to test antibodies against proteins from cell extracts. (Cooper 2000.)

A mixture of proteins is run on SDS-polyacrylamide gel electrophoresis (SDS-PAGE) where they are separated only by their size (figure 3). Proteins are dissolved in a solution that contains negatively charged detergent molecules which denature the proteins and gives them an overall negative charge. In the electrophoresis all proteins migrate toward the positive electrode. After the electrophoresis proteins are transferred to a membrane. The membrane is incubated with the solution of primary antibody, specific to the protein of interest. The antibody binds to the band that contains this protein. The membrane is then incubated with a secondary antibody that binds to the bound primary antibody. The secondary antibody is enzyme linked and when substrate is added the bound antibody can be detected by various methods: radioactivity, fluorescence or chemiluminescent detection. (Cooper 2000; Lodish H et al. 2000.)

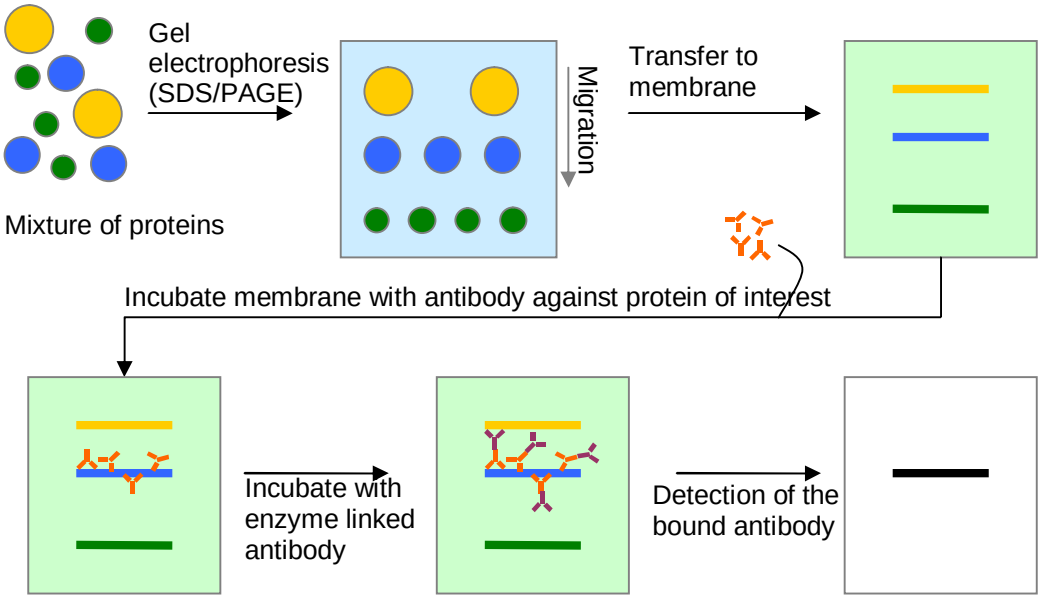

Figure 3. Western blotting (figure modified from Cooper 2000; Lodish et al. 2000).

2.2 Mitochondria

Mitochondria are cell organelles that have a double membrane: a mitochondrial outer membrane and a mitochondrial inner membrane. Mitochondria exist in a budding and fusing network in the cells. Mitochondrial proteins are encoded by mitochondrial DNA (mtDNA) and nuclear DNA (nDNA). mtDNA is a circular double stranded DNA molecule that encodes some of the respiratory chain polypeptides and the nucleic acids (rRNA and tRNA) needed in intramitochondrial protein synthesis. (Chinnery & Schon 2003.)

The majority of the mitochondrial respiratory chain polypeptides are encoded by nuclear genes. These proteins are synthesised in the cytoplasm; they contain a mitochondrial targeting sequence, which is cleaved before the subunit is assembled in the inner mitochondrial membrane. (Chinnery & Schon 2003.)

Mitochondria are the power plants of the cells. They produce energy from the intermediary metabolites of carbohydrates, proteins, and fats via fatty acid beta oxidation, the urea cycle and the respiratory chain – the final pathway for ATP production. (Chinnery & Schon 2003.)

2.2.1 Mitochondrial respiratory chain

Within the inner mitochondrial membrane is located the mitochondrial respiratory chain which consist of five enzyme complexes (figure 4). Each complex is build from multiple subunits. Reduced cofactors (NADH and $FADH_2$), that are generated from the metabolism of carbohydrates, proteins and fats, donate electrons to complex I (NADH) and complex II ($FADH_2$). These electrons pass to ubiquinone pool, complex III, cytochrome c and complex IV to be finally delivered to oxygen to produce water. (Chinnery & Schon 2003.)

This electron flow creates an electrochemical gradient which is used by complexes I, III and IV to pump protons (H^+) from the mitochondrial matrix to the intermembrane space. Complex V uses this proton gradient to synthesise

adenosine triphosphate (ATP) from adenosine diphosphate (ADP) and inorganic phosphate (Pi). This process is called oxidative phosphorylation (OXPHOS). The high energy source ATP produced by OXPHOS is used for all active metabolic processes within the cell. ATP needs to be transported out of the mitochondrion into the cytosol, and cytosolic ADP needs to be transported into the mitochondria. Adenine nucleotide translocator (ANT) performs this process. (Chinnery & Schon 2003; Lu & Cao 2008.)

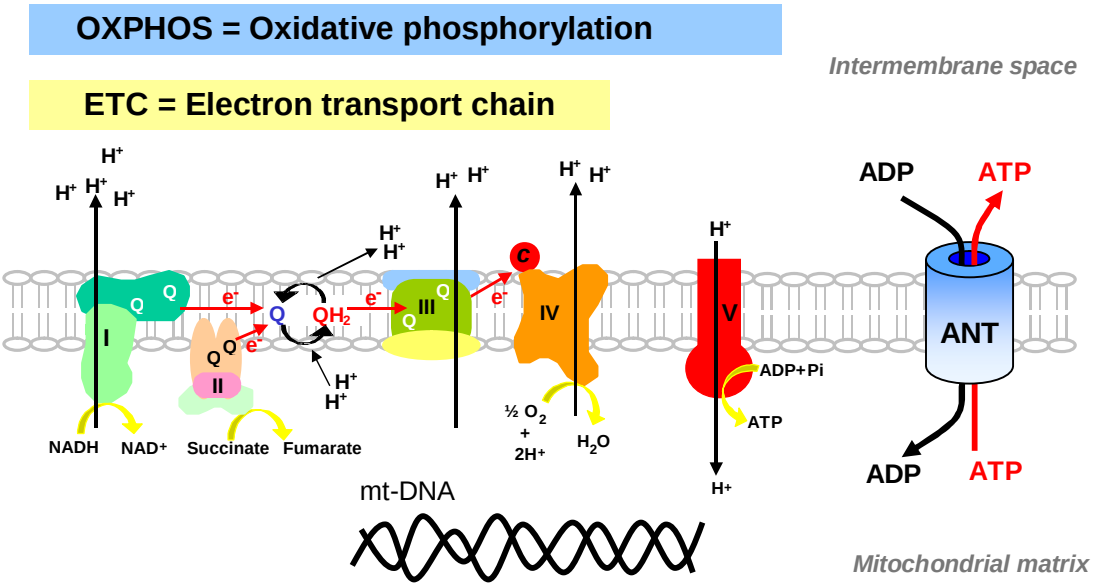

Figure 4. Complexes I-IV belong to the electron transport chain and with complex V they produce the oxidative phosphorylation (OXPHOS). Adenine nucleotide translocator (ANT) is transferring ATP and ADP across the inner mitochondrial membrane. (Figure modified from Chinnery & Schon 2003.)

2.2.2 Complex I of the mitochondrial respiratory chain

Complex I (reduced nicotinamide adenine dinucleotide (NADH):ubiquinone oxidoreductase) is the largest of the mitochondrial respiratory chain complexes and is composed of over 40 polypeptide subunits (Chinnery & Schon 2003; Benit et al. 2001).

Complex I catalyses electron transfer from NADH to ubiquinone. It is embedded in the inner mitochondrial membrane and a part of it is in the mitochondrial

matrix. Complex I can be divided into three parts: 1) the flavoprotein fraction, 2) the iron protein fraction and 3) the hydrophobic fraction (figure 5). The flavoprotein fraction contains the binding sites for NADH – flavin mononucleotide (FMN), and iron-sulfur (Fe-S) clusters. The iron protein fraction has several Fe-S clusters and the hydrophobic fraction binds quinone in the inner membrane. (Benit et al. 2001.)

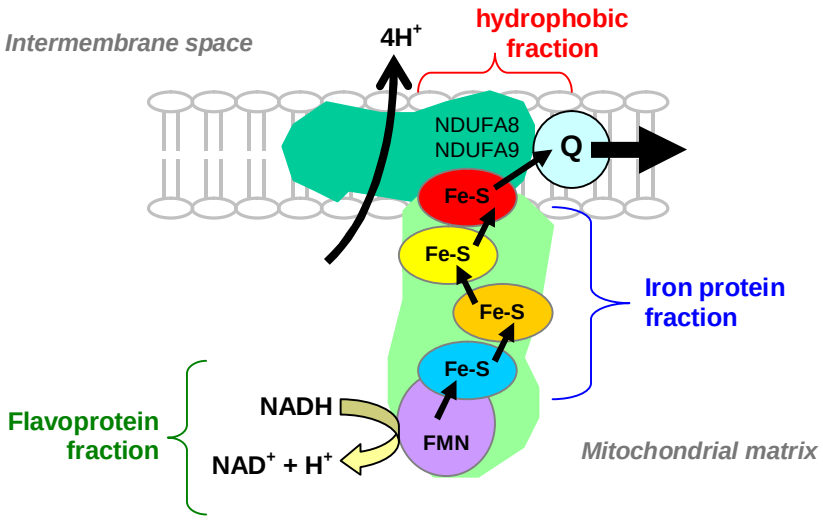

Figure 5. L-shaped mammalian complex I and showing the location of NDUFA8 and NDUFA9 (figure modified from Koene et al. 2010; Leshinsky-Silver et al. 2005).

In humans most of the 45 complex I subunits are encoded by nuclear genes and only seven are mitochondrially encoded (table 1). The most common cause of mitochondrial disorders is the complex I deficiency. Cellular energy production is decreased in these disorders. (Benit et al. 2001, Koene et al. 2010.)

Table 1. Seven of complex I subunits are encoded by the mtDNA and 38 subunits are encoded by the nuclear genome (nDNA). (Koene et al. 2010.)

	Core subunits		
nDNA	NDUFV1	mtDNA	ND1
	NDUFV2		ND2
	NDUFS1		ND3
	NDUFS2		ND4
	NDUFS3		ND4L
	NDUFS7		ND5
	NDUFS8		ND6

	Accessory subunits			
nDNA	NDUFA1	NDUFA8	NDUFV3	NDUFB7
	NDUFA2	NDUFA9	NDUFAB1	NDUFB8
	NDUFA3	NDUFA10	NDUFB1	NDUFB9
	NDUFA4	NDUFA11	NDUFB2	NDUFB10
	NDUFA5	NDUFS4	NDUFB3	NDUFB11
	NDUFA6	NDUFS5	NDUFB5	NDUFC1
	NDUFA7	NDUFS6	NDUFB6	NDUFC2
	DAB13	GRIM		

The complex I deficiencies are severe pathologies like Parkinson's disease and Leigh syndrome, which are often lethal. Mutations have been found in both mtDNA and nDNA encoded subunits. (Leshinsky-Silver et al. 2005; Marella et al. 2008; Koene et al. 2010.)

2.3 *Drosophila melanogaster*

The fruit fly, *Drosophila melanogaster*, is a 3 mm long insect. This insect is one of the most valuable of organisms in biological research. *Drosophila* has been used as a model organism for research for a century and today several thousand scientists are working with *Drosophila melanogaster*. (Leister & Herrmann 2007, 33.)

One of the reasons why *Drosophila* is favoured in the research is that it is easy to handle. It requires little technical skill to take care of the flies. Since flies are tiny animals it is very practical to use them because it requires only a small amount of space to store a large number of flies. (Greenspan 1997, 17.)

Drosophila's life cycle is short (~10 days in +25°C) and it includes the following stages: egg, three larval (instar) stages, pupal stage and the adult fly. The eggs are 0,5 mm long. After the fertilisation of the eggs it takes about 24 hours to become a larva. The larvae eat and grow continuously. They pass several stages: the first, second and third instar before changing into an immobile pupa which is attached to the walls of the food vials. The pupal stage lasts for 4-6 days during which time metamorphosis occurs. (Ashburner 2005, 122; Leister & Herrmann 2007, 34-35.)

When the metamorphosis is completed, the adult fly ecloses from the puparium. The newly emerged fly looks pale and puffy before its cuticle has hardened and darkened. When crossing flies of different genotypes, it is important to use virgin females. Virgins can be recognised by their pale colour, and virgin females do not mate before 10-12 hours post-eclosion. (Ashburner 2005, 122; Leister & Herrmann 2007, 38.)

2.3.1 UAS/GAL4 system

UAS-GAL4 system allows the control of expression of a gene of interest in different tissues or cell types. It requires co expression of two elements in the same fly: 1) The yeast transcriptional activator protein GAL4, which is inserted into the *Drosophila* genome to create transgenic flies expressing GAL4 from a specific genomic enhancer. This means that the activator protein is present only in specific tissue or cells of these flies but there is no target to activate. (Brand & Perrimon 1993.)

2) A GAL4-dependent target gene (UAS-*Gene X*) can be made by cloning any sequence downstream of GAL4 binding sites (UAS). The target gene is silent in the absence of the activator. When crossing enhancer trap GAL4 flies with UAS-*Gene X* flies, expression of the target gene X is activated only where and when the genomic enhancer driving the GAL4 expression is active (figure 6). (Brand & Perrimon 1993.)

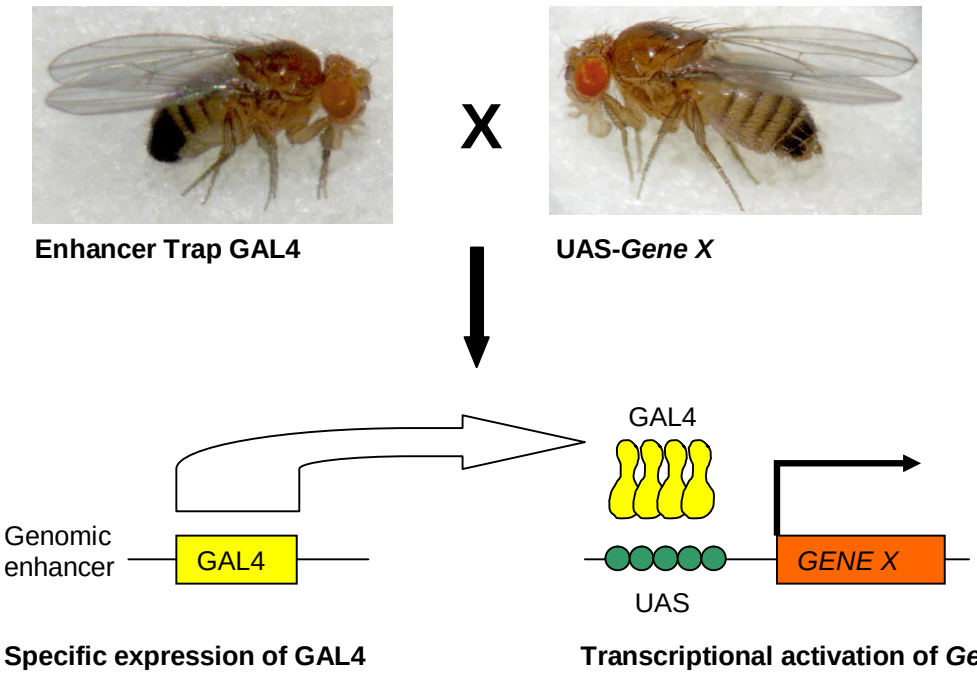

Figure 6. Transcriptional activation of the UAS-*Gene X* (figure modified from Brand & Perrimon 1993).

2.3.2 UAS-IR fly lines

Ribonucleic acid interference (RNAi) induced by double stranded RNA is a powerful tool for generating loss-of-function phenotypes. RNAi is used for silencing genes of interest. In *Drosophila* RNAi can be induced by injecting, feeding or expressing RNAi. (Leister & Herrmann 2007, 207-208.)

Transgenic UAS-IR flies have been genetically engineered to present short fragments of the target gene (300-400 bp) as inverted repeats (IR) in the antisense-sense orientation (figure 7). Before the inverted repeats is a UAS site, where the transcriptional activator protein GAL4 can bind to activate the expression of the hairpin RNA. These hairpin RNAs are processed by the ribonuclease endonuclease Dicer into siRNAs which attach to the sequence specific endogenous mRNA and promotes their degradation. (Dietzl et al. 2007.)

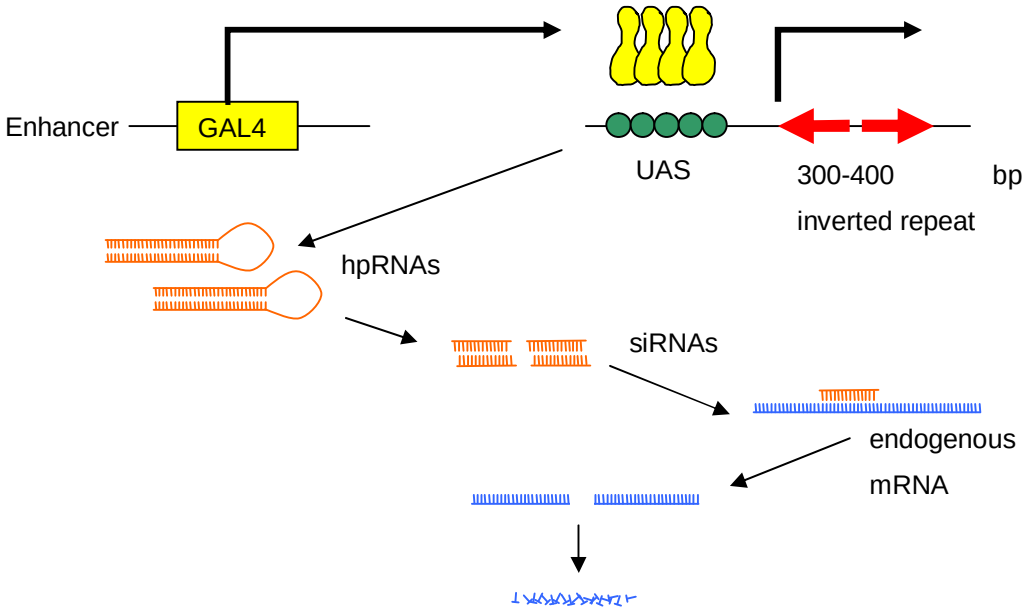

Figure 7. UAS-IR strategy in Drosophila melanogaster (figure modified from Leister & Herrmann 2007, 214).

2.3.3 NDI1 transgenic fly lines

Some organisms (for example plants, fungi, bacteria and archaea) have alternative enzymes that can bypass or replace the proton pumping complexes of the mitochondrial respiratory chain. These are for example the alternative oxidases (AOX) and the NADH dehydrogenases of the Ndi and Nde families. These enzymes provide an alternative respiratory chain which allows the respiratory chain to function when the normal function is limited by high ATP levels, the action of toxins or other physiological restraints. Ndi can bypass complex I and AOX bypasses complexes III and IV. (Yagi et al. 2006; Sanz et al. 2010.)

In mutant human cells lacking the essential mitochondrial DNA encoded subunit ND4 NADH quinone oxidoreductase of *Saccharomyces cerevisiae* (*NDI1*) can completely restore the NADH dehydrogenase activity (Bai et al. 2001). The NDI1 gene can protect against neurogeneration in a rotenone rat model of Parkinson's Disease (Marella et al. 2008.)

NDI1 transgenic flies carry insertions of yeast Ndi1 under control of a GAL4 dependent promoter. Expression of NDI1, driven by ubiquitous GAL4, is able to rescue the lethality of knockdown of either of two subunits of complex I: CG3683, homologue of human NDUFA8 and CG6020, homologue of human NDUFA9, indicating that NDI1 can compensate for a substantial deficiency of complex I in vivo. (Sanz et al. 2010.)

3 MATERIALS AND METHODS

3.1 Antibodies and peptides

Peptides (table 2) against proteins CG3683 and CG6020 were designed in coordination with the 21st Century Biochemicals company. *Drosophila* gene encoding CG3683 protein is homologue to human a NADH dehydrogenase 1 alpha subcomplex 8 (NDUFA8), which is 175 amino acids long and the protein is 19,8 kDa in *Drosophila*. The *Drosophila* gene encoding CG6020 is homologous to human NADH dehydrogenase 1 alpha subcomplex subunit 9 (NDUFA9), which is 416 amino acids long and the protein is 46,8 kDa in *Drosophila*. In appendix 1 there are the full protein sequences of the two proteins and the sequences of the designed peptides.

Table 2. Peptides and antibodies.

Peptide/Protein Name: 3683		
Peptide:	Preimmune bleed:	Test Bleeds:
1613 -3638, Lot: 09-8521-13930	P1613/r4259 (0)	P1613/r4259 (1)
	P1613/r4260 (0)	P1613/r4259 (2)
		P1613/r4259 (3)
		P1613/r4259 (4)
		P1613/r4260 (1)
		P1613/r4260 (2)
		P1613/r4260 (3)
		P1613/r4260 (4)
Peptide/Protein Name: 6020-pep1 / 6020-pep2		
Peptide:	Preimmune bleed:	Test Bleeds:
1614A -6020 –pep1, Lot: 09-8521-13931	P1614/r4261 (0)	P1614/r4261 (1)
1614B -6020 –pep2, Lot: 09-8521-13932	P1614/r4262 (0)	P1614/r4261 (2)
		P1614/r4261 (3)
		P1614/r4261 (4)
		P1614/r4262 (1)
		P1614/r4262 (2)
		P1614/r4262 (3)
		P1614/r4262 (4)

ATP synthase subunit alfa monoclonal antibody, ATPα, (MitoSciences cat. MS507, host mouse) was used in 1:80000 dilution as a loading control of the mitochondrial proteins. The secondary antibodies used were peroxidase labelled anti-Rabbit IgG made in goat (Vector Laboratories PI-1000) and

peroxidase labelled anti-Mouse IgG made in horse (Vector Laboratories PI-2000). Both secondary antibodies were used in 1:10000 dilution.

3.2 Dot blot assay

The dot blot apparatus were assembled and the nitrocellulose membrane (Hybond-C-Extra, 45 micron, cat. RPN303E, Amersham Biosciences) was pre-wetted in TBS (see appendix 2 for all dot blot solutions) and placed in the apparatus. The membrane was rehydrated with 100 µl of TBS per well to ensure the uniform binding of the antigen (peptide). Vacuum was applied to remove all of the TBS. 200 µl of antigen solution (1,0 µg of the peptide) was applied to wells. The entire sample was allowed to filter through the membrane by gravity flow.

After the antigen samples were completely drained from the membrane, 200 µl of the blocking solution was added to each well. Gravity flow was used to drain the blocking solution from each well. 200 µl of the washing solution was added to each well and vacuum was applied to remove all the liquid. The washing step was repeated. 100 µl of the primary antibody solution (1:250, 1:500, 1:1000 and 1:1500 dilutions) was added to each sample well. Gravity flow was used to drain the primary antibody solution from each well.

The sample wells were washed 3 times with 200 µl of the washing solution with vacuum to help the drainage of the sample wells. 100 µl of the secondary antibody solution were added to the each well. Gravity flow was used to drain the secondary antibody solution (1:10000 dilution, horseradish peroxidise conjugated antibody) from each well. The sample wells were washed 2 times with 200 µl of the washing solution with vacuum to help the drainage of the sample wells. The membrane was removed from the apparatus for chemiluminescent detection.

The membrane was place between two plastic sheets and 2 ml of chemiluminescent development solution was added on top of the membrane.

The membrane was developed for 2 minutes. Then it was removed from the sheets, excess developing solution was drained with paper and the membrane was placed between the clean sheets. The chemiluminescence on the membrane was detected with Molecular Imager ChemiDoc XRS from Bio-Rad, which was capable of detecting chemiluminescent signals.

3.3 *Drosophila melanogaster* stocks

Driver and RNAi lines were obtained from stock centers, Ndi1 transgenic flies were created in the lab. See table 3, which gives details of all genotypes. Flies were maintained on standard medium at 25°C with 12 hours light/dark cycle. The standard medium recipe is in the appendix 3.

Table 3. *Drosophila melanogaster* stocks used in the study, together with official database symbol designations and original references.

Stock	**Name used in main text**	**Official symbol /genotype in Flybase[#] or VDRC transformant ID**	**Original reference**
GAL4 driver line	*da-GAL4*	w^{*}; *P*{*GAL4-da.G32*}UH1	Wodarz et al., 1995
NDI1 transgenic line, insertion on chromosome 3	UAS-NDI1 ($NDI1^{A46}$ as genotype)	w^{1118}; *P*{*UAS-NDI1;* w^{+}}A46	Sanz et al., 2010
RNAi line targeted against complex I subunit	RNAi: CG3683	VDRC 46797	Dietzl et al., 2007
RNAi line targeted against complex I subunit	RNAi: CG6020	VDRC 13131	Dietzl et al., 2007

3.4 Isolation of mitochondria from *Drosophila melanogaster*

The isolation is carried out at +4 °C and the equipment should be chilled when the isolation is started. 100 flies were immobilized by chilling and then decanted into a chilled mortar. 1 ml of ice-cold isolation buffer (250 mM sucrose, 5 mM Tris-HCl, 2mM EGTA, 0.1 % BSA) was added to the flies. The flies were pressed gently with rotating movement.

The homogenate was poured with the help of brush, onto the net placed on the beaker. Another 1 ml of isolation buffer was added to the net. The filtering was finished by bundling and clamping carefully the net to collect the last drops of filtrate on the beaker. The filtrate was transferred from the beaker to a 2 ml tube. It was centrifuged at 200x g for 5 min at +4°C. The supernatant was collected to a new 2 ml tube and it was centrifuged at 9000x g for 10 min at +4°C. The supernatant was discarded and the fat was wiped away around the pellet with tissue. The pellet was resuspended in 50 µl of isolation buffer without albumin (250 mM sucrose, 5 mM Tris-HCl, 2mM EGTA). The mitochondria extractions were stored in -80°C. The protein concentrations were calculated by Bradford assay prior to western blotting.

3.5 Bradford assay

In a 96 well plate 300 µl of Bradford reagent (appendix 4) and 1 µl of the sample or standard were pipetted into each well. Bovine serum albumin (BSA) was used as a standard in 1-10 µg/µl concentrations. The absorbance of the samples and standards were measured at 595 nm with Chameleon plate reader (Hidex).

3.6 Western blotting

Isolated mitochondria samples were run with SDS-PAGE gel electrophoresis. Samples were diluted to 1 µg/µl with dH_2O and SB 4x loading buffer (see appendix 5 for all western blot solutions). Diluted samples were heated at +95 °C for 5 minutes. The precast SDS-Page gel (Criterion precast gel 4% stacking gel, 10-20 Tris-HCL separating gel, cat. 345-0042) was loaded into the electrophoresis chamber. The chamber was filled with running buffer. 35 µl of samples and 15 µl of the ladder (Bio-Rad broad range molecular weight, cat.161-0318) were loaded into the wells of the gel. Electrophoresis was run with 80 V until the proteins were concentrated. Proteins were then separated at 120 V for 2 hours, until the loading dye reached the bottom of the gel. The gel

was removed from the electrophoresis chamber, it was placed into the lid of the gel package and submerged under the blotting buffer.

The membrane (Hybond-C-Extra, 45 micron, cat. RPN303E, Amersham Biosciences), sponge and the filter papers were prewetted with blotting buffer for 10 minutes. The transfer cassette was packed at +4 °C starting with the black side down: sponge, filter paper, SDS-PAGE gel, membrane, filter paper and sponge. All parts were soaked with blotting buffer. Transfer electrophoresis was run at 200 mA for 2 hours. Transfer cassette was unpacked and the membrane was submerged in PBS-Tween.

The membrane was blocked with 5% milk in PBS-Tween for 3 hours on a shaker at room temperature. The primary antibody solution in 5% milk in PBS-Tween was added and the membrane was incubated overnight at +4 °C on a shaker. The membrane was washed with PBS-Tween three times for 15 minutes at room temperature. The secondary antibody (conjugated to HRP) solution in 5% milk in PBS-Tween was added and the membrane was incubated for 1 hour at room temperature on a shaker. The membrane was washed with PBS-Tween three times for 10 minutes at room temperature.

The membrane was place between two plastic sheets and 2 ml of chemiluminescent development solution was added on top of the membrane. The membrane was developed for 2 minutes. Then it was removed from the sheets, excess solution was drained with paper and membrane was placed between two clean plastic sheets. The chemiluminescence on the membrane was detected with exposing X-ray film (Kodak BioMax MS cat. 829 4985) and developing film in developing machine (Agfa Curix 60).

4 RESULTS AND CONLUSIONS

4.1 Preselection of the antibodies

The dot blot of the antibody for detecting protein CG3683 against the designed peptide showed that bleeds from rabbit r4259 gave stronger signal than bleeds from rabbit r4260 (figures 8 and 9). There was not much difference between bleeds 2 and 4. The preimmunisation bleeds (0) of both rabbits gave no significant signal. Since the binding to the peptide 1613 was strong, it was selected to generate the affinity purification column. The best signals were obtained with bleeds r4259 (2) and r4260 (2) which where then further compared in western blotting (at 1:1000 dilution).

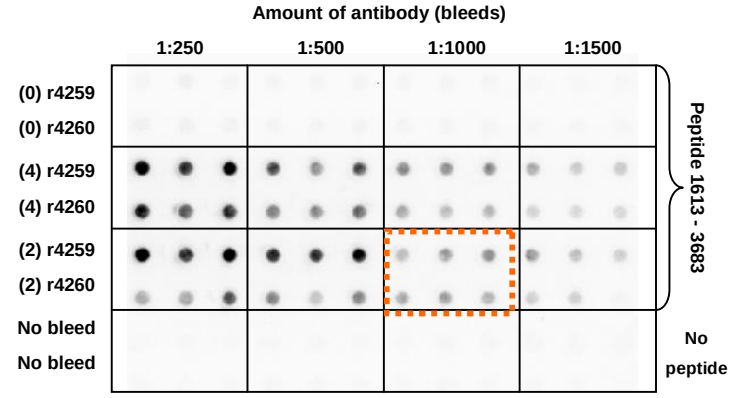

Figure 8. Dot blot for antibody CG3683.

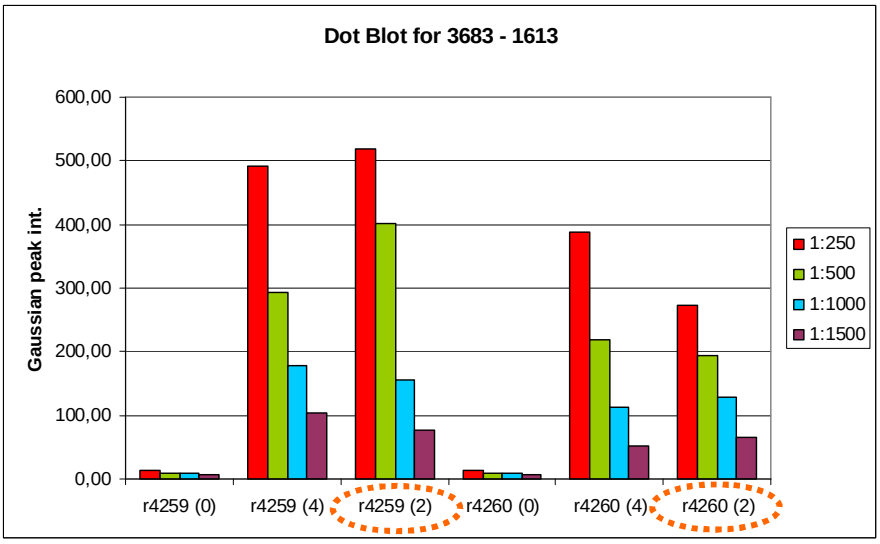

Figure 9.Quantification of the dot blot for CG3683.

The dot blot of the antibody for detecting protein CG6020 against the designed peptides (1614A and 1614B) showed that the binding of the peptide 1614B was four fold increased compared to peptide 1614A (figures 10 and 11). There was no significant difference between bleeds or rabbits. Since the binding with the peptide 1614B was stronger, it was selected to generate the affinity purification column. Bleeds r4261 (2) and r4262 (4) in 1:1000 dilution were chosen for western blotting.

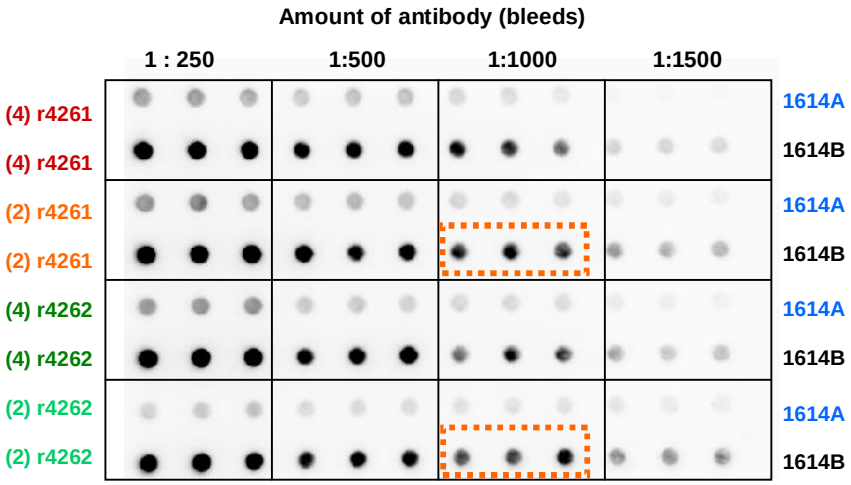

Figure 10. Comparison of the peptides 1614A and 1614B. Pre immune binding controls where presenting no significant signal (data not shown).

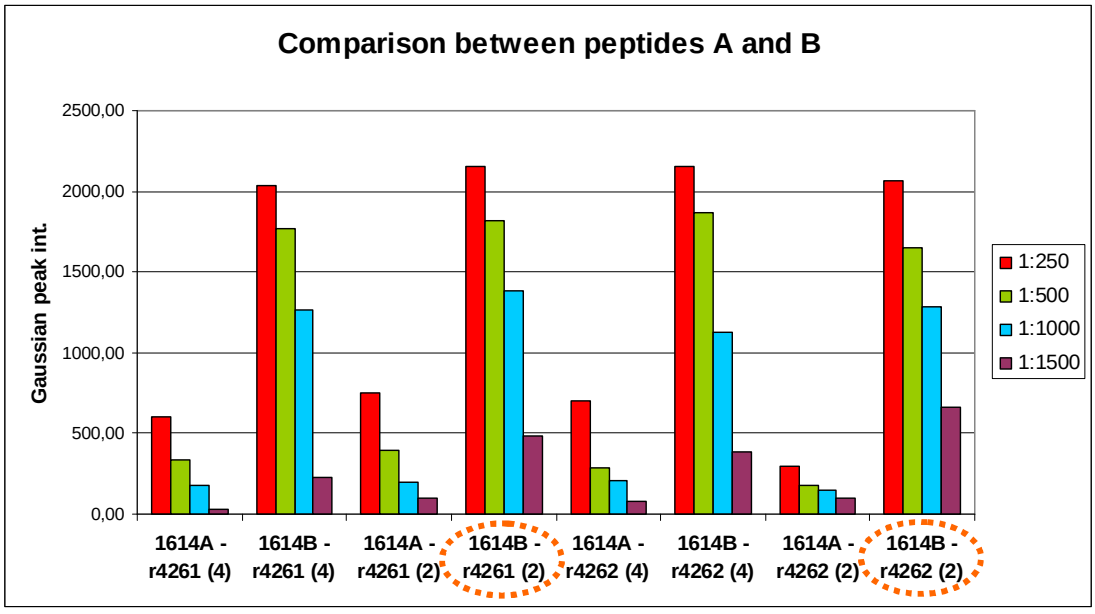

Figure 11. Quantification of comparison between peptides A and B.

4.2 Final selection of the antibodies

4.2.1 Choosing rabbits

Figure 12 A) showed that the antibody for CG3683 produced in rabbit r4259 gave less background than the one produced in rabbit r4260. For the final selection of the best bleed to use in affinity purification all the antibody (CG3683) bleeds from rabbit r4259 were tested in a final western blotting experiment. Since the signal was strong each bleed will be used at 1:2000 dilution.

Figure 12 B) showed that the antibody for CG6020 produced in the rabbit r4262 gave less background signal than the one from rabbit r4261. For further selection of the best antibody (CG6020) bleed rabbit r4262 was chosen. Since the signal was very strong bleeds will be used at 1:4000 dilution in further western blotting.

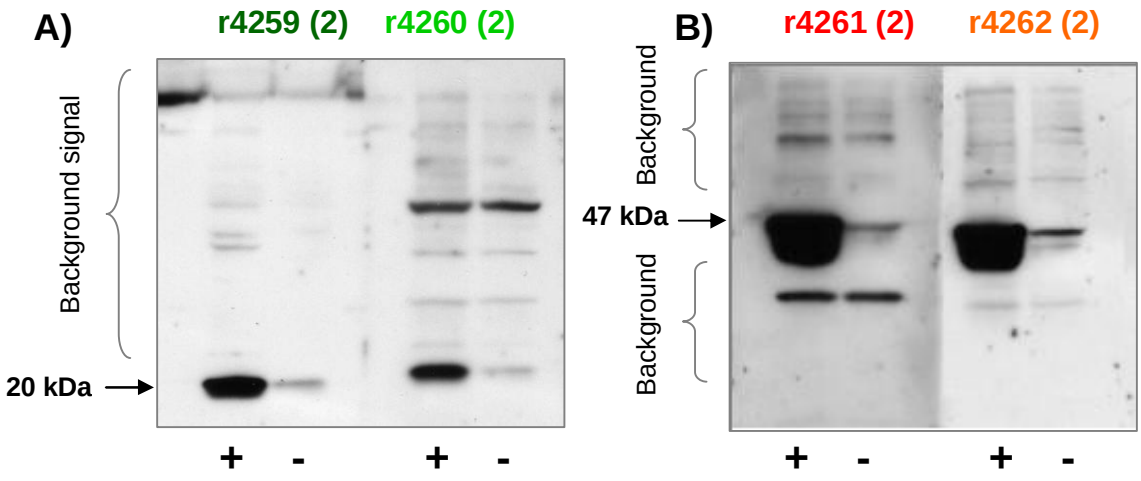

Figure 12. Western blot of the normal complex I (+) and knockdown of the subunit of the complex I (-) with antibodies A) against CG3683 in 1:1000 dilution B) against CG6020 in 1:1000 dilution.

4.2.2 Selection of the antibody bleed for protein CG3683

Further western blotting for antibody CG3683 showed (20 kDa, figure 13 and 14) that the bleed four of the rabbit r4259 showed lowest background signal and lowest unspecific binding. The loading control, ATP synthase subunit alfa in 1:80000 dilution, showed even loading of the proteins in all of the lanes (55 kDa, figure 14). On the basis of the western blot results the bleed four from rabbit r4259 is chosen for affinity purification.

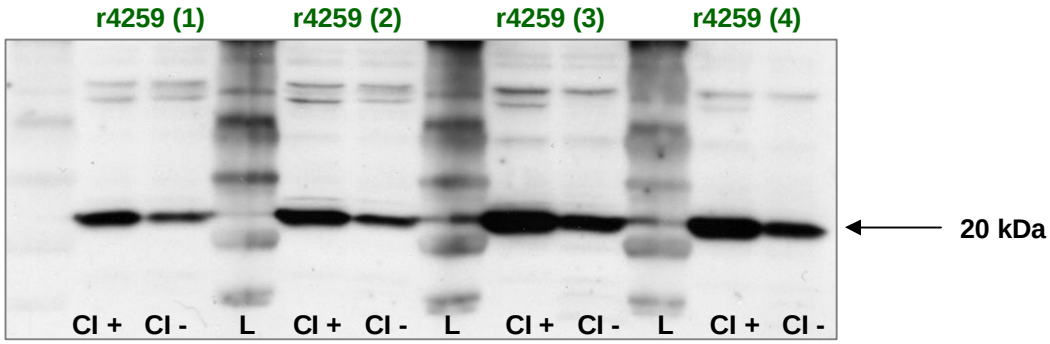

Figure 13. Western blot of the normal complex I (CI +) and knockdown of the subunit CG3683 of the complex I (CI -) with antibodies against CG3683 from rabbit r4259 all four bleeds. (L=Molecular weight marker).

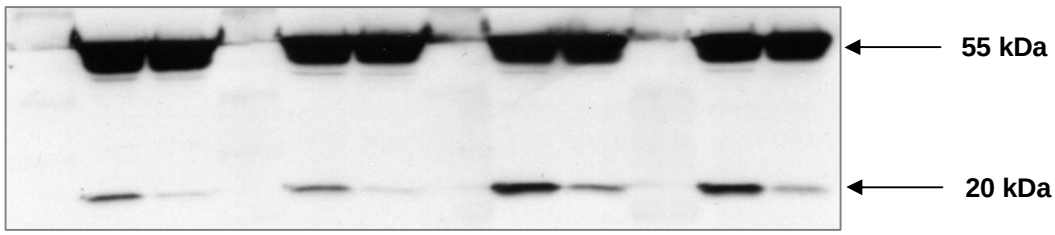

Figure 14. ATP synthase α (55 kDa) was used as a loading control of the mitochondrial proteins on the same blot as previously without stripping the membrane. Signal for GC3683 antibody can be seen as a 20 kDa band.

4.2.3 Selection of the antibody bleed for protein CG6020

Further western blotting for antibody CG6020 showed that the bleed four from rabbit r4261 showed lowest background signal and lowest unspecific binding (47 kDa, figure 15 and 16). The loading control, ATP synthase subunit alfa in 1:80000 dilution, showed even loading of the proteins in all of the lanes (55 kDa, figure 16). On the basis of the western blot results the bleed four from rabbit r4261 was chosen for affinity purification.

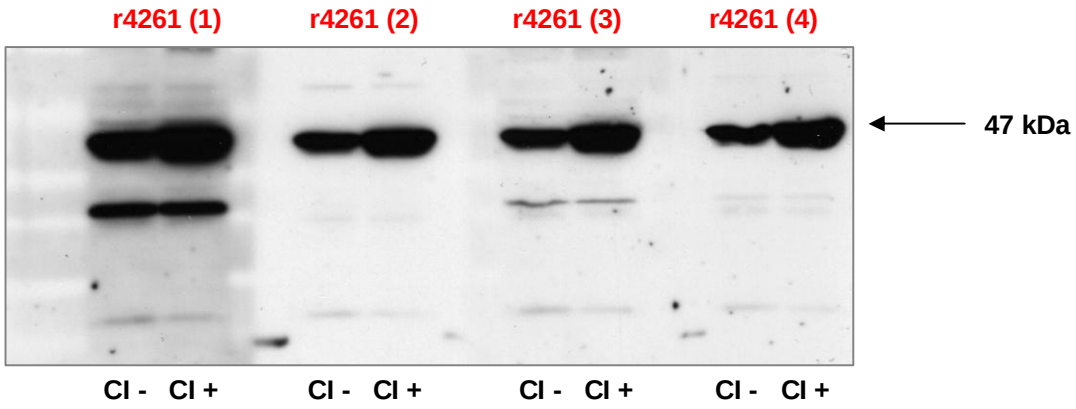

Figure 15. Western blot of the normal complex I (CI +) and knockdown of the subunit CG3683 of the complex I (CI -) with antibodies against CG6020 from rabbit r4261 all four bleeds.

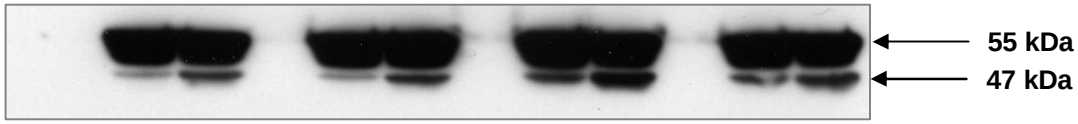

Figure 16. ATP synthase subunit alfa (55 kDa) was used as a loading control of the mitochondrial proteins on the same blot as previously without stripping the membrane. Antibody for GC6020 can be detected at the same time (47 kDa).

5 DISCUSSION

The lack of commercial antibodies against two subunits (CG3638 and CG6020) of the respiratory chain complex I has slowed our work with the RNAi knockdown flies. We need to quantify the level of the knockdown of these proteins in the RNAi fly lines as well as in the transgenic Ndi1 rescued flies. In this development project I tested two custom made antibodies made in rabbits. The polyclonal antiserum bleeds were used to test the specificity of the antibody.

For both antibodies very good bleeds were found during the testing and selected for affinity purification. Several experiments could have been performed to expand these results. The western blotting could have been repeated with cytosolic or total cell extract to confirm that the antibody does not cross react with other proteins. These antibodies must also be tested in immunofluorescence experiments. However the clear results obtained with mitochondrial fractions confirms that these antibodies suit our purpose. Further more such sensitive experiments would be better performed using the affinity purified antibodies.

Thus, once affinity purified, these custom made antibodies against CG3683 and CG6020 will be used in western blotting to quantify the RNAi knockdown of these subunits in fly mitochondrial extracts. We will determine the extent of knockdown in RNAi lines that can be rescued by Ndi1 from the whole flies mitochondria (both females and males) and from testes mitochondria of the adult males, which are sterile.

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APPENDIX 1 – Protein sequences of CG3683 and CG6020

Peptides against proteins CG3683 and CG6020 were designed by the 21st Century Biochemicals company. On the sequences of the proteins the red colour indicates a series of regularly spaced cysteines. Their presence in antigen sequence can (1) causes some issues with conjugation options particularly in the presence of lysine (K); and (2) indicates likely regions of secondary structure, which can impair in situ analysis. Grey areas could not be used for antigen due to poor sequence and/or unacceptable homology to other proteins. The location of the selected peptides is presented in green.

First protein – CG3683

MVITNNTTLPEESELNVQELNLSSAALRAGAFHLGKQCEQANNEFMLCRQELDDPRACLA
EGKAVTSCALDFFRKVKKTCHEEFTQYATCLDKSSGTMAFSHCRKTQGVFDKCIKDNFDW
DRPSYGYFSRAKVIQSAREAPKKEEKVSYPDATPGLPEDYPKPPAKYGSRFHWLE

Peptide 1613: CGLPEDYPKPPAKYGSRF-amide

Second protein - CG6020

MAAIVLTRNL QLAKHHGSGV VGVLCLRGYS AAAAPPEDGP RPLKTTNPAA MKRGTGGRSS FNGIVATVFG ATGFVGRYVC
NKLGKSGTQM ILPYRGDDSD VIRLKVTGDL GQVLFHFYNL EDPASIRDAV KHSNVVINLV GRDFETKNFK FKDVHVNGAE
RIARIAREAG VERLIHLSSL NVEANPKDLY VKGGSEWLKS KYEGELRVRD AFPNATIIRP ADIYGSEDRF LRYYAHIWRR
QFRSMPLWHK GEKTVKQPVY VSDVAQAIIN AAKDPDSAGR IYQAVGPKRY QLSELVDWFH RLMRKDQKRW GYMRYDMRWD
PTFLLKAKLN SFICPGTPIG GLHPARIERE AVTDKVLTGV PTLEDLGVTL TTMEQQVPWE LRPYRAALYY DAELGEFETP
SPPKCIEARD ELRLFA

Peptide 1614A: Acetyl-PEDGPRPLKTTNPAAMKRGC-amide

Peptide 1614B: Acetyl-FHRLMRKDQKRWGYMRYDMC-amide

APPENDIX 2 – Solutions for dot blot

Tris Buffered Saline, 1x TBS, 2 L

- 20 mM Tris-HCl, pH 7.5
- 500 mM NaCl

Dissolve 4,84 g Tris and 58,48 g NaCl in ~1,5 L dH_2O. Adjust to pH 7.5 with HCl. Adjust the volume to 2 L with dH_2O.

Tween-Tris Buffered Saline, 1x TTBS, 1 L

- 20 mM Tris, pH 7.5
- 500 mM NaCl
- 0,05% Tween 20

Add 0,5 ml Tween 20 to 1 L of TBS.

Blocking Solution, 100 ml

- 1% BSA-TBS

Add 1,0 g bovine serum albumin (BSA) to 100 ml TBS. Stir to dissolve.

Antibody Buffer, 200 ml

- 1% BSA-TTBS

Add 2 g BSA to 200 ml TTBS. Stir to dissolve.

Chemiluminescence Development Solution

1:1 mixture of Luminol/enhancer and peroxide buffer (Immun-Star™ HRP Chemiluminescent Kit).

APPENDIX 3 – Standard medium

Standard medium (fly food) used for the experiments.

Standard medium:	
1 % (w/v)	tayo agar
1,5 % (w/v)	sucrose
3 % (w/v)	glucose
3,5 % (w/v)	active dried yeast
1,5 % (w/v)	maize meal
1 % (w/v)	wheat germ
1 % (w/v)	soya flour
3 % (w/v)	treacle
0,5 % (w/v)	propionic acid
0,1 % (w/v)	Nipagin M

Simmer for 20-40 minutes in 1000 ml of water, cool below 70 °C and add:

Propionic acid	5 ml	(final volume 0.5 %)’
10 % nipagin M in EtOH	10 ml	(final volume 0.1 %)

Add warm water up to 1000 ml.

Pour to vials (7-8 ml or 3-4 ml) and let evaporate in the hood for 1-2 hours. Plug the vials and store at the 4 °C.

The 7-8 ml food vials will be used to grow the flies and the 3-4 ml food vials will be used to keep the flies before mating and for life span experiments.

APPENDIX 4 – Bradford reagent

Bradford reagent

- 100 mg Coomassie Brilliant Blue G250 = Serva Blue G
- 50 ml Ethanol 95 %
- 100 ml Phosphoric acid 85 %
- dH_2O to make 1 litre

Dissolve the stain in the ethanol. Add the phosphoric acid. Add water to make 1 litre. Filter through Whatman paper.

APPENDIX 5 – Solutions for western blotting

Sb4x loading buffer

- 2 ml glycerol (5,04 g)
- 0,8 g SDS
- 2,5 ml 1M Tris-HCL, pH 6.8
- 80 µl bromophenol blue slurry (5mg/ml in water, vortex well before using)
- H_2O to 8 ml

Add DTT to 20 % before using.

Running buffer

10x (stock solution)

- 0,25 M Trizma (Tris-HCl)
- 1,92 M Glycine
- 1 % SDS

No pH adjusting

1x (working solution)

1+9 (10x running buffer + dH_2O)

Blotting buffer

10x (stock solution)

- 0,25 M Trizma (Tris-HCl)
- 1,92 M Glycine

pH should be 8.3 (do not adjust)

1x (working solution)

1+2+7 (10x blotting buffer + methanol + dH_2O) store at +4°C

PBS-Tween (Washing solution)

- 1 tablet in 1 l of dH_2O

Blocking solution

- 5% Milk in PBS-Tween

Antibody solution

- 5% Milk in PBS-Tween

Chemiluminescence Development Solution

1:1 mixture of Luminol/enhancer and peroxide buffer (Immun-Star™ HRP Chemiluminescent Kit).