

**MONOCLONAL ANTIBODY PRODUCTION FOR COILED-
COIL DOMAIN CONTAINING-124 (CCDC-124) AND ITS
MOLECULAR CHARACTERIZATION**

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THE DEGREE OF MASTER OF SCIENCE**

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AUGUST 2010**

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ABSTRACT

MONOCLONAL ANTIBODY PRODUCTION FOR COILED-COIL DOMAIN CONTAINING-124 (CCDC-124) AND ITS MOLECULAR CHARACTERIZATION

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Coiled-coil-domain-containing-124 (CCDC-124) is a novel protein of unknown function. The *Ccdc-124* gene is highly conserved among eukaryotic species and is predicted to encode a 223 amino acids long protein. Yeast-two-hybrid assays had shown that CCDC-124 interacts with RasGEF1B guanine exchange factor, which was previously identified as a specific guanine exchange factor for Rap2, a member of the Rap subfamily of Ras-like G-proteins. In order to reveal the cellular function of CCDC-124 protein, different biochemical experiments, including Western Blotting and Sub-cellular localization studies were performed. In those experiments, a polyclonal antibody against an N-terminal peptide of CCDC-124 was used. Nevertheless, due to its polyclonal nature this antibody exhibited non-specific binding. Although it was determined that the gene encodes a 33 kDa protein and that the protein has diffused cytoplasmic localization, the results were not precise. In order to detect the protein more accurately, monoclonal antibodies were generated against CCDC-124. Throughout the project, mice were injected with pure His-Tagged CCDC-124 protein. Via hybridoma technology antibodies were generated and selected for their recognition capacities. At the end, 3 positive hybridoma clones were produced: 7F7, 15C11 and 4B3. To characterize the produced monoclonal antibodies, Western Blot experiments were performed and their binding properties were compared to the polyclonal antibody. Among the three monoclonal antibodies, 4B3 gave the most promising results at 33 kDa, in Western Blotting. The antibodies will be used in the determination of the protein's sub-cellular localization and in the analysis of its response to extracellular signals. These in turn will aid further analyses related to the protein's role within the cell.

ÖZET

CCDC-124 PROTEİNİNE KARŞI MONO-KLONAL ANTİKOR ÜRETİMİ VE ANTİKORUN KARAKTERİZASYONU

İrem Gürbüz

Moleküler Biyoloji ve Genetik Yüksek Lisans Derecesi

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Sarılı-sarmal-bölge-içeren-124 (Coiled-coil domain containing-124; *CCDC-124*) henüz fonksiyonu belirlenmemiş olan yeni bir gendir. Bu gen ökaryotlar arasında korunmuştur ve genin 223 amino asitten oluşan bir protein kodladığı düşünülmektedir. Geçmişte Tazebay grup projeleri kapsamında yapılan maya ikili hibrid deneyleri, CCDC-124 proteinin RasGEF1B guanine değiştirme faktörü ile etkileşim halinde olduğunu göstermiştir. RasGEF1B'nin ise Rap2'ye özgü bir guanine değiştirme faktörü olduğu araştırmalarda gösterilmiştir. CCDC-124 proteinin hücre içindeki fonksiyonunu ortaya çıkarmak için, Western Blotlama ve hücre içindeki yer taramalarını da içeren çeşitli biyokimyasal deneyler yapılmıştır. Bu deneylerde, CCDC-124 proteininin N-uç bölgesini tanıyan, bir poliklonal antikor kullanılmıştır. Fakat, antikorun poliklonal doğası doğrultusunda, bir çok, spesifik olmayan bağlanma da görülmüştür. Yapılan deneylerde, genin 33 kDa'lık bir proteini kodladığı ve proteinin hücre içinde dağınık, sitoplazmik bir yerleşim izlediği gözlemlenmiştir. Fakat, spesifik olmayan bağlanmalar da göz önüne alındığında, bu sonuçlar kesin değildir. Proteinin daha hassas bir şekilde algılanabilmesi için, CCDC-124 proteinine karşı monoclonal antikorlar üretilmiştir. Bu proje süresince farelere His işaretli CCDC-124 proteini enjekte edilmiştir. Antikorlar hibridoma teknolojisi ile üretilmiş ve daha sonra, bağlanma kapasiteleri doğrultusunda seçilmişlerdir. Proje neticesinde 3 adet hibridoma klonu elde edilmiştir. Bunlar 7F7, 15C11 ve 4B3 klonlarıdır. Üretilen antikorları karakterize etmek amacıyla Western Blotlama deneyleri yapılmış ve antikorların bağlanma özellikleri poliklonal antikor ile karşılaştırılmıştır. 3 antikor içinde, 33 kDa'da, en net sonucu veren 4B3 klonu olmuştur. Üretilen antikorlar, ileride, proteinin hücre içindeki yerinin belirlenmesinde ve bu yerin dışarıdan gelen sinyaller doğrultusunda değişip değişmemesinin araştırılmasında kullanılacaktır. Bu tür deneyler, CCDC-124 proteininin hücre içindeki fonksiyonunun araştırılmasına ışık tutacaktır.

TO MY FAMILY

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1. INTRODUCTION

1.1 General Characteristics of CCDC-124

Coiled-coil domain containing protein 124, CCDC-124 (Human Genome Browser at University of California Santa Cruz; Kent. *et al.*, 2002) was first identified in a former study, during the analysis of the sodium/ iodide symporter (NIS) gene. In this study, evolutionary conserved non-coding genomic sequences were screened with the aim of revealing cis-acting elements regulating NIS expression (Alotaibi *et al.*, 2010). Through this former study, a conserved DNA sequence located at the 3' position of NIS gene was recognized, which was not regulating the transcription of the analyzed gene. Further studies showed that this region encoded a conserved eukaryotic gene of unknown function (*CCDC-124*; Hani Alotaibi, Serap Erkek and Uygur Tazebay, personal communication).

CCDC-124 is highly conserved among eukaryotes, and it is not present in prokaryotic species. Bioinformatics analyses show that this protein harbors 5 putative serine, 7 putative threonine and 1 putative tyrosine phosphorylation sites. Also in the Protein Knowledge Database UniProtKB (The UniProt Consortium, 2010), this protein is described as a phosphoprotein. Its conserved nature and its putative involvement in phosphorylation events point to a regulatory role of this protein within the eukaryotic cell (Serap Erkek, M.Sc. thesis, Bilkent University, 2008).

CCDC-124 gene is predicted to encode a 223 amino acid long protein in human. Immunological analyses, performed by using a polyclonal anti-N-terminus antibody generated in rabbit, show that the protein product of this gene is 33 kDa.

Although several bands were detected by western blot experiments, all of which disappeared after N-terminus peptide treatment; only the band corresponding to 33 kDa was stronger in *CCDC-124* transfected cells compared to non-transfected cells (Serap Erkek, M.Sc. thesis, Bilkent University, 2008).

According to the expressional analysis data, *CCDC-124* gene is expressed in different breast cancer cell lines and in every human tissue analyzed, such as brain, ovary, testes and liver, and is especially showing high expression profile in the skeletal muscle (Serap Erkek, M.Sc. thesis, Bilkent University, 2008).

Using the bioinformatics tools, it was predicted that CCDC-124 localizes in the nucleus with 84% probability. However immunofluorescence analysis and immunoblotting assays both performed by using the polyclonal anti-Ccdc-124 antibody revealed that the protein of interest exhibited a diffused cytoplasmic localization (Elif Yaman and Uygur Tazebay, personal communication).

In order to detect the interacting partners of CCDC-124, yeast-two-hybrid (Y2H) assays were performed by Hybrigenics S.A., Paris, France. Y2H assay is based on detecting the interaction between two proteins, which together make up a transcription factor, positively regulating the expression of a reporter gene (Fields *et al.*, 1989). Hybrigenics Company detected an interaction between CCDC-124 and Ras Guanine Exchange Factor 1B (RasGEF1B). Afterwards this interaction was validated by co-immunoprecipitation experiments (Elif Yaman and Uygur Tazebay, personal communication).

RasGEF1B is a specific guanine exchange factor for Rap2, a member of Rap family, close homolog of Ras G-proteins (Yaman *et al.*, 2009). RasGEF1B together with RasGEF1A only acts specifically on Rap2 and does not affect the nucleotide exchange of Rap1, a G-protein which differs from Rap2 by a single amino acid in the effector domain (Bos, 1997) and is about 70% identical to Rap2. Likewise,

RasGEF1s have no effect on nucleotide exchange of other members of the Ras subfamily, including H-Ras, R-Ras, M-Ras, TC21, RalB and Rheb. By site-directed mutagenesis it was identified that a single residue, Phe39, in the switch I region of Rap2 is responsible for the specificity of RasGEF1s (Yaman *et al.* 2009).

Although CCDC-124 was found to be in interaction with RasGEF1B, in vitro studies showed that it did not affect RasGEF1B's guanine exchange activity (Elif Yaman and Uygur Tazebay, personal communication).

1.2 Rap G-Proteins (Rap GTPases)

Ras-like GTPases superfamily has about 154 members in total, each belonging to five different families; Ras, Rab, Rho, Ran and Arf (Bos *et al.*, 2007). G-proteins are involved in several cellular processes, such as signaling pathways regulating cell differentiation, growth and proliferation, nuclear transport events, protein synthesis, lipid vesicle formation and cytoskeletal dynamics. The Ras family is the most intensely studied one among other Ras-like GTPases superfamily members since Ras oncoproteins have a high mutation rate in many human cancers (Wennenberg *et al.*, 2005). This family consists of three Ras proteins, five Rap proteins, two R-raslike proteins, two Ral proteins, and one Rheb protein (Bos, 1997).

The Rap subfamily of small Ras-like GTPases consists of two sub-groups, which are named Rap1 and Rap2. Rap1 is divided into two isoforms, Rap1a and Rap1b. Rap2 subgroup has three members, Rap2a, Rap2b and Rap2c. There is 95% sequence similarity between the two Rap1s and 90% similarity between the three Rap2s (Paganini *et al.*, 2006). Rap1 and Rap2 are close relatives; they only differ from each other in few aminoacid residues located outside of the effector region. Rap1 does not transform the cells, which is a known characteristic for Ras-related proteins. In fact Rap1 is identified as a tumor suppressor working in an opposing fashion towards Ras-induced transformation (Kitayama *et al.* 1989). Its close relative Rap2

however has no transformation suppression activity, although it also does not transform cells (Bos, 1997).

1.2.1 Action mechanisms of G-proteins, Guanine Exchange Factors and GTPase activating proteins

G-proteins cycle between inactive guanosine diphosphate (GDP) bound and active guanosine triphosphate (GTP) bound states (Figure 1.1). In their active form they regulate various downstream cellular processes. Thus these proteins have important impacts on the cell fate. It is therefore reasonable to think that they are tightly regulated. Although G-proteins are very similar to each other in their effector domain, Switch I region, their regulatory proteins have very different structures and show diverse action mechanisms (Vetter et al., 2001). Guanine Exchange Factors (GEFs) and GTPase activating proteins (GAPs) play roles in the control of G-protein activity. Related to their role in activating and inactivating G-proteins, GEFs and GAPs frequently lose their function in human tumors (Bos *et.al*, 2007).

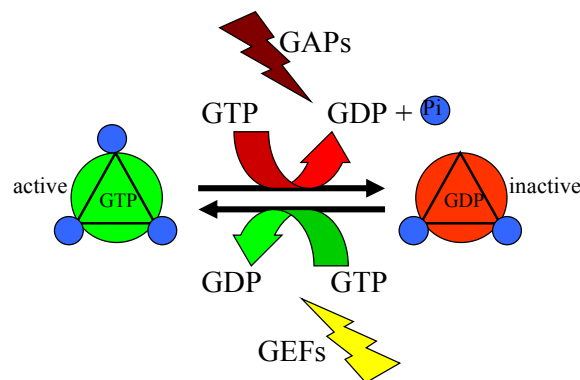


Figure 1. 1 Working principle of G-proteins (Adapted from Lundquist, 2006)

G-proteins have a high, and a similar, affinity for GDP and GTP nucleotides. Hence the dissociation of the nucleotide would take a long time, which is not the case in the cellular environment. GEFs facilitate the exchange of GDP to GTP by catalyzing the dissociation of GDP. These regulatory proteins modify the nucleotide-binding domain of G-proteins and decrease their activity. As a consequence the nucleotide is released. After the nucleotide is released, the task of GEF is finished, in other words it does not favor the binding of GTP nucleotides. However since GTPs are more abundant within the cell compared to GDPs, they easily replace G-protein bound GDP nucleotides (Bos *et al.*, 2007). Likewise a newly bound nucleotide, GTP in this case lowers the affinity of the G-protein for the GEF. So the new nucleotide becomes able to dissociate the regulatory protein and ensures its stay within the G-protein's effector site.

G-proteins work as GTP hydrolyzing proteins, that's why they are called GTPases. However under normal circumstances this hydrolysis reaction is very slow. At this point GAPs catalyze GTP hydrolysis and accelerate the process by several orders of magnitude (Bos *et al.*, 2007). Their action mechanism is based on the orienting a water molecule so that it attacks the γ -phosphate. While enabling the nucleophilic attack of the water molecule GAPs also ensure that the transition state is stabilized (Bos *et al.*, 2007).

1.2.2 Cellular functions of Rap2 G-protein

1.2.2.1 Rap2 in Wnt/ β -catenin signaling

Wnt proteins are secreted glycoproteins and act as ligands for serpentine frizzled receptors. When bound to their receptor, Wnt proteins activate dishevelled (Dsh) protein. In its activated state Dsh inhibits β -catenin destruction complex, which is composed of Axin, APC and GSK-3- β . This phenomenon leads to the release of β -catenin and enables that it changes its sub-cellular localization by entering from

cytoplasm to the nucleus. In the nucleus, together with TCF/LEF transcription factors β -catenin stimulates expression of the target genes (Choi *et al.*, 2005).

Wnt/ β -catenin signaling pathway is involved in various cellular events including pattern formation in the embryo; more specifically in the dorso-ventral axis specification (Wodarz *et al.*, 1998). Studies show that loss of β -catenin leads to problems in dorsal axis formation (Yost *et al.*, 1998). As a regulator of β -catenin levels within the cell, Dsh plays a role in this process by moving away from the vegetal pole and accumulating in the dorsal side of the embryo (Miller *et al.*, 1999). Following this fact, the enhanced β -catenin levels result in the establishment of the Spemann's organizer. These results indicate that the sub-cellular localization of Dsh is important in the development.

Going one step further, Choi *et al.* showed that *Xenopus* Rap2 protein, too, is required in the dorsal axis formation during early development. It is found that Rap2 is essential for the vesicular localization of Dsh, which in turn enables its phosphorylation and activation (Choi *et al.*, 2005). These results prove that XRap2 is involved in Wnt/ β -catenin signaling pathway as a modulator of the sub-cellular localization of Dsh protein and that it is important player in the embryonic development.

1.2.2.2 Rap2 in Integrin-mediated cell adhesion

Rap1 G-proteins control cell adhesion by being involved in integrin mediated cell adhesion and cadherin mediated cell junction formation (Bos, 2005). Previous studies show that Rap1 is involved in the receptor-induced integrin activation in T cells and myeloid cells although the action mechanism is unknown (Katagiri *et al.* in McLeod *et al.*, 2004). Similarly activated Rap2 can promote the activation of integrins in B cells. By increasing the expression of RapGAPII, a RapGAP which inhibits the activation of both Rap1 and Rap2, and by expressing constitutively active

Rap2, the scientists were able to demonstrate that integrin-dependent B cell adhesion is augmented (McLeod *et al.*, 2004). Cell adhesion is an important process for B cell migration and B cells have to move in order to enter lymphoid organs, to come across antigens and to receive help from T cells. Therefore Rap2 G proteins as well as Rap1s play an important role in B cell fate by regulating integrin mediated cell adhesion.

1.2.2.3 Rap2 in synaptic plasticity

Recent studies point out another function for Rap proteins in neuronal cells. Several research articles confirm the important role of Rap2 protein in synaptic plasticity.

Synaptic plasticity, the ability of synapses to change their strength, is an important research area in neurosciences due to the fact of its involvement in learning and memory (Bliss *et al.*, 1993). N-methyl-D-aspartate (NMDA) receptor opening, resulting calcium ion influx and transduction of electrical signals into the chemical ones; as well as α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-sensitive glutamate receptor trafficking (synaptic delivery and removal) at postsynaptic sites are known to affect synaptic strength (Stornetta *et al.*, 2010), hence neurological processes such as memory formation and learning. AMPA receptors consist of two major groups, based on their subunits; subunits with long cytoplasmic termini and subunits with short cytoplasmic termini.

Long-term synaptic potentiation (LTP) is a persistent and long-lasting enhancement in neuronal transmission, and can be quickly stimulated (Brown *et al.*, 1988). LTP is one of the mechanisms which can modify synaptic strength. LTP is induced by steering AMPA-Rs with long cytoplasmic termini into synapses. This phenomenon is enabled via the activation of MEK, ERK, PKB-Akt and PI3K pathways by certain stimuli and consequential phosphorylation of the indicated receptors (Stornetta *et al.*, 2010).

Long term depression (LTD) on the other hand is a long-lasting decrease in the efficacy of neuronal transmission (Bear *et al.*, 1996). In other words, it is the opposing process of LTP. Reduced number of postsynaptic receptors, as well as diminished presynaptic neurotransmitter release might result in LTD. Particular LTD inducing stimuli activates Rap1-p38-MAPK signaling pathway, which in turn causes phosphorylation of AMPA-Rs with short cytoplasmic termini. Then those receptors are removed from the synapses.

Another physiological mechanism underlying synaptic plasticity is depotentiation. Depotentiation is involved in the return of potentiated synapses to normal levels (Manahan-Vaughan *et al.*, 2003). Depotentiation is a form of depression; however it only occurs at recently potentiated synapses. The small G-protein Rap2 enters the play at this point. Rap2-JNK signaling pathway receives information from activated NMDA receptors and delivers information to downstream effectors which trigger dephosphorylation of AMPA-Rs with long cytoplasmic termini. Thus Rap2 is involved in the synaptic removal of these receptors during depotentiation (Zhu *et al.*, 2005). However the link between NMDA-Rs and Rap2-JNK is still unknown.

Furthermore Rap2 is involved in the retraction of neurites (axons and dendrites) in spiny neurons (Fu *et al.*, 2007). It is shown that RapGAPs are able to enhance the neuronal growth by inhibiting Rap function. In addition to that it is revealed that ubiquitination of Rap2A by E3 ubiquitin ligase, Nedd4-1 inhibits Rap2A function. This incident then reduces the activity JNK and promotes dendrite growth (Kawabe *et al.*, 2010).

In addition to learning and memory formation, several mental disorders, including Alzheimer's disease, Angelman Syndrome, Autism, Fragile X Syndrome, Neurofibromatosis Type 1 and Schizophrenia, are found to be linked to synaptic dysfunction (Stornetta *et al.*, 2010). According to the above mentioned data Rap proteins play an important role in synaptic plasticity, hence aberrant Rap signaling is

associated with mental disorders related to cognition and adaptive behaviors. Besides, on the contrary of LTP, which is the responsible mechanism underlying memory formation, depotentiation is thought to play a role in forgetting a memory (Manahan-Vaughan *et al.*, 2003). Additionally transgenic mice expressing constitutively active Rap2 in postnatal forebrain showed impaired loss of contextual fear (Ryu *et al.*, 2008).

1.3 Hybridoma Technology and Monoclonal Antibodies

An immunogenic agent induces an immune response within the body. In the scope of this immune response several antibodies are generated against the same antigen. These antibodies, each arising from a different B cell clone are able to recognize one specific epitope of the same antigen. Whereas these antibodies all together are polyclonal; an antibody originating from the same B clone, thus having a unique specificity is called monoclonal antibody (Nelson *et al.*, 2000).

Both polyclonal and monoclonal antibodies can be produced under laboratory conditions. The advantages of producing polyclonal antibodies include simplicity low cost and mass production. Additionally the fact that they recognize different epitopes of a single antigen might be useful in certain assays. However after the produced polyclonal antibody stock is finished one has to produce a new one, since there are no continuous reserves of polyclonal antibodies. As a consequence of producing a new batch, problems related to differences in reactivity and titer might arise (Nelson *et al.*, 2000). Monoclonal antibodies on the other hand are reproducible. A continuous culture of B cell hybridomas overcomes this problem. Although it is hard and time-consuming to generate monoclonal antibodies, they are target specific and provide an everlasting supply, thus enable standard and accurate immunological analyses.

Monoclonal antibody production consists of four major steps (Figure 1.2):

- 1) Immunization
- 2) Fusion and Selection
- 3) Screening
- 4) Expansion

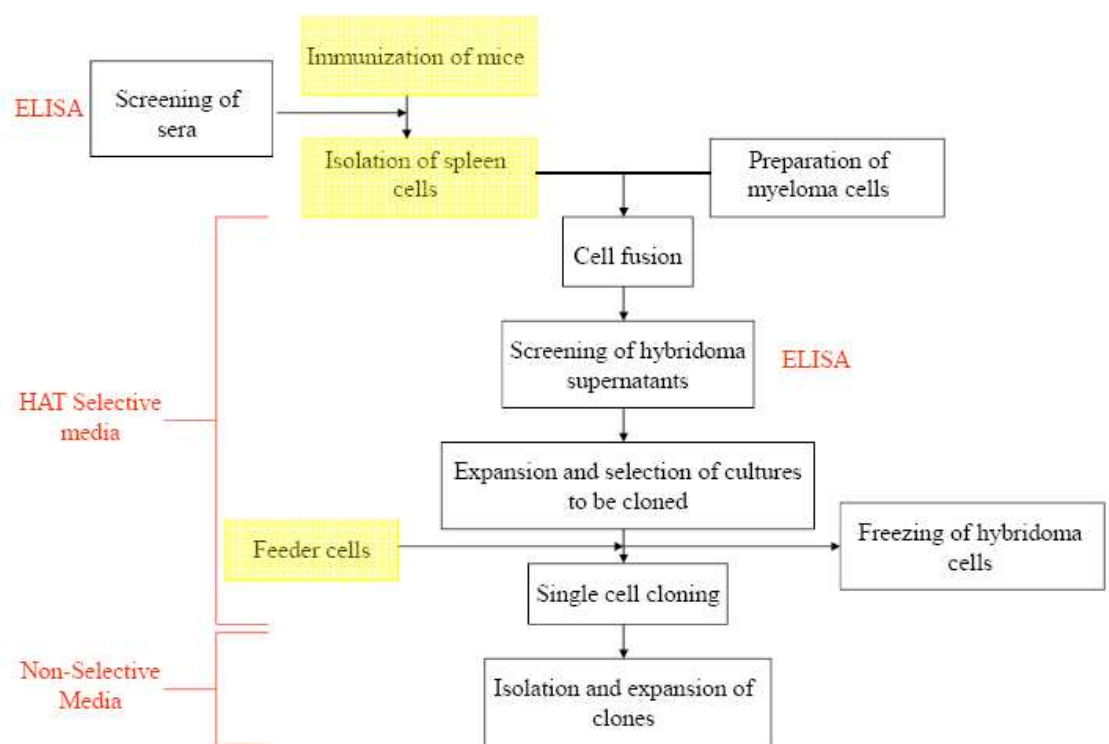


Figure 1. 2 Major stages of monoclonal antibody production procedure (Adapted from Committee on Methods of Producing Monoclonal Antibodies, 1999)

In order to immunize the animal, for instance Balb/c mouse, the antigen against which the antibody would be produced is used. The antigen of interest is given to mice together with an adjuvant, a substance which enhances the immune response (Nelson *et al.*, 2000).

B cells giving rise to specific monoclonal antibodies are mortal and do not survive in cell culture for a long time. In order to surmount this problem B cells are fused with immortal cancerous cells, mostly with myeloma cells, to generate hybrid cells. Generation of B cell hybridoma cell lines secreting specific antibodies was firstly proposed by Köhler and Milstein in 1975 (Köhler *et al.*, 1975). In this technique, spleen cells of a previously immunized animal serve as the source of antibody producing B cells and they are fused with myeloma cells of the same species (in this case mouse myeloma cells) with the help of polyethylene glycol (PEG). PEG is a chemical which causes lipid-based cell membranes to fuse (Committee on Methods of Producing Monoclonal Antibodies, 1999). SP2, NS1, NSO and X63Ag8 are myeloma cell candidates, which are all defective for an enzyme in purine metabolism, namely Hypoxanthine-guanine phosphoribosyltransferase (HGPRT) (Nelson *et al.*, 2000). In order to synthesize nucleic acids cells depend on two pathways, de novo pathway and alternative salvage pathway. Cells lacking HGPRT enzyme could not synthesize their nucleotides via salvage pathway. As a result myeloma cells can survive as long as they have an intact de novo synthesis pathway. To stop the growth of non-fused myeloma cells hypoxanthine-aminopterin-thymidine (HAT) selection medium is used. HAT medium contains an inhibitor called aminopterin, which blocks de novo nucleic acid synthesis pathway (Committee on Methods of Producing Monoclonal Antibodies, 1999). Consequently, neither myeloma cells which lack HGPRT enzyme nor the mortal splenic cells stay alive in HAT medium. Only immortalized hybrid cells are able to reverse the expression of

HGPRT and to grow under these conditions (Bakay et al., 1975). After about 20 days generated hybridoma cells can be transferred to HT (hypoxanthine-thymidine) medium, since no more selection, hence aminopterin is required (Nelson et al., 2000).

Hybridoma cells secrete the antibodies they produce into the culture medium. Therefore it is possible to screen the cell supernatant for the presence of antibodies. When the cells reach approximately three quarters confluence, one can perform the screening. One of the assay systems frequently used is Enzyme-linked immunosorbent assay (ELISA). In this assay ELISA plates are coated with antigen of interest. Then the antigen is treated with primary antibodies present in the hybridoma culture supernatant, enzyme conjugated secondary antibodies and chromogenic substrates respectively. At the end positive hybridomas are detected by the presence of a colored product (Nelson et al., 2000). Especially when the antigen is in a protein mixture an alternative screening method could be Sandwich ELISA in which the ELISA plate firstly is coated with capture antibodies to increase sensitivity.

At last, positive hybridoma cells are transferred from multi-well plates to larger cell culture flasks. This expansion step is required for the well-being of the cells, additionally enables that cell number increases. The more hybridoma cells are present the more antibody containing supernatant could be isolated. It is also possible to purify the antibodies by affinity based techniques. Purified antibodies are advantageous over supernatants since they occupy less space for storage and that way it would be possible to adjust the amount of required antibody for different assays.

2. OBJECTIVES AND RATIONALE

Coiled-coil-domain-containing-124 (CCDC-124) is a 33 kDa long protein of unknown function. Although previous studies performed by Tazebay group revealed important data related to the functional role of the protein, in order to uncover the function of CCDC-124 within the cell more precise results obtained by using antibodies with better specificity are needed. So far, the experiments, including Western Blots and sub-cellular localization analyses were carried out by using the polyclonal Ccdc-124 antibody generated against the N-terminus of the protein. This polyclonal antibody made possible to do research at protein level. Nevertheless, the polyclonal antibody was recognizing other bands than the band belonging to CCDC-124 protein. Hence, especially in sub-cellular localization studies the precise answers regarding to the localization of CCDC-124 were still missing. With the aim of eliminating the non-specific bands seen in Western Blot studies and in order to detect the localization of the protein more accurately monoclonal antibodies were generated against CCDC-124 in this thesis. Generated monoclonal antibodies can be used in further studies to reveal the protein's role within the cell.

3. MATERIALS AND METHODS

3.1 Ingredients of Cell Culture, Work Bench, Bacterial Culture Solutions and Buffers

Phosphate Buffered Saline, PBS (10X, 1L)

NaCl	80 g
KCl	2 g
KH ₂ PO ₄	2.4 g
Na ₂ HPO ₄	14.4

Tris Buffered Saline, TBS (10X, 1L, pH: 8.0)

12.1 g Tris Base
87.6 g NaCl

Tris Glycine Electrophoresis Buffer (5X, 1L)

15.1 g Tris Base
94 g Glycine
50 ml 10% SDS

Transfer Buffer (1L)

5.8 g Tris Base
2.9 g Glycine
0.37 g SDS
200 ml Methanol

Comassie Blue Staining Solution

0.2% Comassie Blue
7.5% Acetic Acid
50% Ethanol

Destaining Solution

40% Methanol
10% Acetic Acid

SDS-PAGE Gel Loading Dye (5X)

250 mM Tris-Cl (pH: 6.8)

10% SDS

0.1% Bromophenol Blue

50% Glycerol

Lysis Buffer (mammalian cells)

50 mM Tris-Cl (pH: 8.0)

250 mM NaCl

1% NP-40

1X Protease Inhibitor Cocktail (Roche)

DMEM Working Medium

DMEM (Dulbecco's Modified Eagle Medium) 1X (GIBCO, Invitrogen)

4.5 g/L (high), 1 g/L (low) Glucose

0.11 g/L Sodium Pyruvate

L-Glutamine

Fetal Bovine Serum (GIBCO, Invitrogen)

1% penicillin-streptomycin (Biochrom AG)

1% non-essential amino acid (GIBCO, Invitrogen)

LB Broth Medium (1L, pH: 7.5)

10 g Bacto-tryptone

5 g yeast extract

10 g NaCl

LB Agar Medium (1L, pH: 7.5)

10 g Bacto-tryptone

5 g yeast extract

10 g NaCl

15 g agar

Lysis buffer (bacteria)

50 mM Tris-Cl (pH: 7.5)

300 mM NaCl

3 mM β -Mercapto Ethanol

0.5% NP-40

0.5% Triton X 100

Wash Buffer 1

50 mM Tris-Cl (pH: 7.5)

100 mM NaCl

3 mM β -Mercapto Ethanol

Wash Buffer 2

50 mM Tris-Cl (pH: 7.5)
100 mM NaCl
3 mM β -Mercapto Ethanol
20 mM Imidazol

Elution Buffer 1

50 mM Tris-Cl (pH: 7.5)
100 mM NaCl
3 mM β -Mercapto Ethanol
200 mM Imidazol

Elution Buffer 2 (pH : 7.5)

50 mM Tris-Cl (pH: 7.5)
100 mM NaCl
3 mM β -Mercapto Ethanol
500 mM Imidazol

Phosphate Binding Buffer (20 Mm, 500ml, pH: 7.3)

28.25 ml 0.2 M Na-Phosphate Monobasic Stock (NaH_2PO_4)
21.75 ml 0.2 M Na-Phosphate Dibasic Stock (Na_2HPO_4)
450 ml ddH₂O

Citrate Buffer (pH: 3.0, 100 ml)

82 ml 0.1 M Citric Acid.anhydrous Stock ($\text{C}_6\text{H}_8\text{O}_7\cdot\text{H}_2\text{O}$)
18 ml 0.1 M Sodium Citrate Tribasico Stock ($\text{C}_6\text{H}_5\text{O}_7\text{Na}_3\cdot 5\frac{1}{2}\text{H}_2\text{O}$)

3.2 Production of Monoclonal Antibodies**3.2.1 Immunization of mice**

Within the scope of this project a total number of 11 Balb/c male mice were immunized. 11 mice were grouped into three:

Group 1: 4 mice

Group 2: 4 mice

Group 3: 3 mice

Mice were injected with purified CCDC-124 protein. In every injection the proteins purified by Dr. Yaman were used. In the first and second group always 60 μg proteins were used per injection. For the third group however, a variable and decreasing (between 60 μg and 30 μg) amount of protein was used. Proteins were

solved in 150µl Phosphate Buffered Saline (PBS). PBS-protein solution was mixed with 150µl Freund's adjuvant and was injected intraperitoneally. For the first injection only, complete Freund's adjuvant (Sigma-Aldrich) was used. For every following injection incomplete Freund's adjuvant (Sigma-Aldrich) was used. Between two regular injections there were about 20 days. Three days before the fusion one last boost injection was performed.

Prior to fusion blood was taken from mice via tail-veil sampling technique (Diehl *et al.*, 2001). Sera was isolated from blood samples according to the procedure stated below and stored at -20°C for a further ELISA test, in order to detect the presence of antibodies within the blood. If the antibody titer was high spleen of the animal was removed and fusion is performed.

Serum isolation protocol:

- *Wait approximately 1h at RT
- *Centrifuge at 13000 rpm for 5 min
- *Prepare 10 µl aliquots of the supernatant.
- *Discard the pellet and store the aliquots at -20°C

3.2.2 Generation of hybridoma cells-Fusion

For fusion mouse myeloma SP2 cells were used. SP2 cell were grown in DMEM working medium (20% FBS).

First of all an un-immunized mouse (preferentially a young female mouse) was euthanized and its spleen was taken, which serves as the source of feeder cells. After removed from the animal, the spleen was put into serum-free DMEM working medium, which was previously warmed up to 37°C. The spleen was swelled and dispersed with the help of a syringe (Cotton *et al.*, 1980). The cell suspension was centrifuged at 1400 rpm for 3 minutes at room temperature. Afterwards the

supernatant was discarded and the pellet was resuspended in serum-free DMEM working medium again. Centrifugation and serum-free medium washes were repeated once again and the final pellet was resuspended in 1X HAT supplement (GIBCO, Invitrogen) containing DMEM working medium (20% FBS, high glucose). Feeder spleen cells were cultured into 10 96-well cell culture plates (Corning, 3598) and stored in 37°C, 5% CO₂ cell culture incubator (Nuair AutoFlow IR direct heat CO₂ incubator).

After one day, the immunized animal was euthanized and its spleen is removed. The procedure for removing and dispersing the spleen was the same as for the feeder cells. This time, after the final centrifugation of the spleen cell pellet, cells were resuspended in serum-free DMEM and counted with a hemocytometer. SP2 myeloma cells were counted, too. For fusion the ratio of myeloma cells to spleen cells should be 1:4 (This ratio could be increased in order to augment the fusion efficiency). Later the required amount of SP2 and spleen cells were mixed and centrifuged together at 1400 rpm, for 3 minutes, at room temperature. After the centrifugation, the supernatant was discarded and 1ml PEG solution (50% w/v Polyethylene glycol, Sigma-Aldrich) was added on top of the pellet very slowly and by gentle mixing. Then serum-free DMEM was added slowly and the suspension was centrifuged at 1400 rpm, for 3 minutes, at room temperature. Afterwards the supernatant was discarded and the pellet is resuspended in HAT containing DMEM working medium (20% FBS, high glucose). The resultant hybridoma cells were cultured on top of the feeder spleen cells and the plates were stored in 37°C, 5% CO₂ cell culture incubator. After 10-15 days, cells were checked and grown up hybridoma cells were tested for the presence of antibodies of interest via ELISA.

3.2.3 Enzyme-linked immunosorbent assay (ELISA)

96 well ELISA plates (PolySorp Surface, Nunc-Immuno Plates) were coated with pure CCDC-124 protein. At the beginning proteins purified by Dr. Yaman were used. In order to test the hybridoma cells isolated from H8, CCDC-124 protein was purified again (see section 2.3). 110 ng-140 ng protein was used per well. Protein was solved in PBS and 100 µl of PBS-protein solution was added to every well. Plates were stored at 4°C overnight.

Coated plates were washed for three times with ddH₂O. After the washes wells were blocked with 5% (w/v) non-fat milk powder dissolved in PBS. 350 µl of blocking solution was added per well. Plates were stored for 30-60 minutes at room temperature.

After blocking plates were washed for three times with ddH₂O. 100 µl of the supernatant of grown up hybridoma cell was added in every well of the ELISA plate. 3% non-fat milk powder dissolved in PBS or DMEM working medium was used as negative control. For the positive control, serum isolated from the animal, whose spleen cells were used in fusion, was dissolved in 3% non-fat milk powder/PBS solution in 1:1000 ratio. 100 µl of positive and negative control solutions were added to the wells as duplicates. Plates were stored for 2 hours at room temperature.

After primary antibody incubation, plates were washed for six times with ddH₂O. Then Anti-Mouse IgG (Fc specific)-Alkaline Phosphatase secondary antibody (Sigma) dissolved in 3% non-fat milk powder/ PBS solution (1:1000) was added to wells (100 µl 2° Ab solution/ well). Plates were stored for 1hour at room temperature.

Afterwards plates were washed nine times with ddH₂O. Following the washes substrate was added to the wells. For the substrate incubation, firstly, SIGMA FAST

p-Nitrophenyl Phosphate Tablets (Sigma), 1.0 mg/ml pNPP and 0.2 M Tris Buffer, were dissolved in 20 ml ddH₂O. Then, 100 µl of the substrate solution was added to every well. Plates were stored in dark at room temperature. ELISA measurements were performed in 30 minutes-3 hours with KCJunior Program (Bio-Tek Instruments) under 405 nm wavelengths. Clones which were found to be positive (more than 3X of negative control), were selected and expanded.

3.2.4 Selection and Expansion of Positive Clones

Firstly spleen of an un-immunized mouse was taken according to the protocol explained in 2.2.2. These feeder spleen cells were distributed into 96 well cell culture plates and plates were stored in 37°C, 5% CO₂ cell culture incubator. The day after, the cells from the positive clone were added to HAT containing DMEM working medium (20% FBS, high glucose) and were counted with a hemocytometer. For sub-cloning 1-3 cells/ well were cultured on top of the feeder cells. The fewer cells are present in each well the more is the possibility that every well contains one single clone. Remaining cells were cultured on a different feeder spleen cell containing plate either by equal distribution or by limiting dilution method. In limiting dilution method, the number of cells decreases to the half from column 1 to column 12 and from row A to row H. Since relatively more cells are present in every well, these clones will serve as parental clones. After 10-15 days, cells were checked and grown up cells were tested for the presence of antibodies of interest via ELISA.

Parental clone cells which were identified as positives were transferred from 96-well plates to 24-well plates (Corning, 3527) and HAT containing DMEM working medium (20% FBS) was added. After the clone expands, this time, the cells were transferred from 24-well plates to 25 cm³ cell culture flasks (Corning, Canted Neck). The cells in flasks were grown in DMEM working medium (20%, without HAT) and splitted every 3-4 days. After approximately 3rd split, the cells were grown in 10% FBS containing DMEM. The same procedure was applied to the sub-clone cells.

3.3 His-tagged CCDC-124 protein isolation

3.3.1 Transformation of bacteria

DH5 α competent bacteria (200 μ l) were given to a transformation tube (Greiner bio-one PS-Tube, 14 ml) and 100 ng pQE81 Locus plasmids were added on top of bacteria. After 30 minutes incubation of bacteria on ice, heat shock was performed for 1.5 minutes in 42°C water bath. Then bacteria were put on ice again and 1 ml of LB medium was added into the transformation tube. Bacteria were incubated for 1 hour at 37°C shaker incubator. Later ~200 μ l bacteria were cultured on LB agar plates containing ampicillin selective reagent and plates were stored in 37°C incubator for 16 hours.

3.3.2 Growth of bacteria

After 16 hours colonies in LB plates were picked up and given in 1 ml LB medium. 1X ampicillin was added and bacteria were incubated in 37°C shaker incubator. Approximately after 2 hours OD of bacterial solution was measured (visible light, 600 nm fixed wavelength). As a blank sample, LB medium only was used. The measurements were repeated until the OD value is 0.5.

3.3.3 Induction

Induction was performed when the OD value is 0.5. Before the induction 1ml of bacteria was isolated from culture, which will later serve as un-induced control. 100 μ M IPTG was added into the bacterial culture. Both un-induced control sample and induced culture were incubated overnight at 23°C in shaker incubator.

3.3.4 Lysis and Sonication

After 24 hours OD values of un-induced and induced culture samples were measured with spectrophotometer (Beckman, DU 640). X μ l sample is taken, where $X = 500/\text{OD value}$ and centrifuged. The pellet was dissolved in 5% β -Mercapto Ethanol containing, 5X SDS Gel Loading dye (1/4), ddH₂O (3/4) mix. These samples were loaded to SDS-PAGE Gel as un-induced and induced controls.

IPTG induced bacteria were centrifuged at 4400 rpm, for 15 minutes, at 4°C. The supernatant was discarded and the pellet was dissolved in lysis buffer. 1 mM phenylmethylsulfonyl fluoride (PMSF) protease inhibitor was added into the resuspension.

The resuspension was sonicated at 4°C for 5 minutes (Pulse: 10/10, Amplitude: 50%) with Vibra-Cell ultrasonic processor (Sonics). After the sonication 10 μ l of sonicated sample was added to 20 μ l, 5% β -Mercapto Ethanol containing 5X SDS Gel Loading dye and the solution was stored in order to load to SDS-PAGE Gel as crude extract.

3.3.5 Ultra-Centrifugation

Following the sonication the solution was centrifuged at 25000 rpm, for 40 minutes, at 4°C. After the ultra-centrifuge, 10 μ l of supernatant sample was added to 20 μ l, 5% β -Mercapto Ethanol containing 5X SDS Gel Loading dye and the solution was stored in order to load to SDS-PAGE Gel as row extract.

Meanwhile Ni-NTA Magnetic Agarose Beads (Qiagen) were centrifuged at 2000 rpm, for 4-5 minutes, at room temperature. Supernatant was discarded and beads are washed in Wash Buffer 1. Centrifuge was repeated under the same conditions. The wash procedure was repeated.

3.3.6 Protein Trapping

The supernatant was added on top of the beads. This mix was rotated for 1-2 hours, at 4°C, at falcon rotator. Afterwards bead-supernatant mix was centrifuged at 2000 rpm, for 3 minutes, at 4°C. 10 µl of supernatant sample was added to 20 µl, 5% β-Mercapto Ethanol containing 5X SDS Gel Loading dye and the solution was stored in order to load to SDS-PAGE Gel as flow through sample. The supernatant, too, was stored (In case of unsuccessful protein binding to beads, rotation will be repeated with the same supernatant).

3.3.7 Washes

The beads (the pellet of the previous centrifuge) were washed for 5-6 times with Wash buffer 2 and centrifuged at 2000 rpm, for 3 minutes, at 4°C. 10 µl supernatant sample each of the first and the second washes were added to 20 µl, 5% β-Mercapto Ethanol containing 5X SDS Gel Loading dye and the solutions were stored in order to load to SDS-PAGE Gel as wash 1 and wash 2 samples respectively.

3.3.8 Elutions

After the washes were complete, the supernatant was discarded. Elution Buffer 1 was added on top of the beads. This mix was rotated for 30 minutes, at 4°C, at falcon rotator. Afterwards bead-supernatant mix was centrifuged at 2000 rpm, for 3 minutes, at 4°C. 10 µl of supernatant sample was added to 20 µl, 5% β-Mercapto Ethanol containing 5X SDS Gel Loading dye and the solution was stored in order to load to SDS-PAGE Gel as elution 1 sample.

Elution Buffer 2 was added on top of the beads (the pellet of the previous centrifuge). This mix was rotated for 30 minutes, at 4°C, at falcon rotator. Afterwards bead-supernatant mix was centrifuged at 2000 rpm, for 3 minutes, at 4°C.

10 μ l of supernatant sample was added to 20 μ l, 5% β -Mercapto Ethanol containing 5X SDS Gel Loading dye and the solution was stored in order to load to SDS-PAGE Gel as elution 2 sample.

3.3.9 Protein Isolation

All samples were loaded to SDS-PAGE gel (Un-induced control, induced control, flow through, wash1 and wash 2 samples: 10 μ l each; crude and row extracts: 7.5 μ l each; elution 1 and elution 2 samples: 20 μ l each).

SDS-PAGE and Comassie Staining experiments were performed (see section 2.8). If positive results were obtained, elution 1 and elution 2 samples were centrifuged together at 4000 G, for 5-20 minutes, at 4°C. This time 10K concentrator tubes (Millipore) were used for centrifugation. After the centrifuge, the concentration of proteins was measured by NanoDrop (Thermo Scientific). The protein solution was frozen with liquid nitrogen and stored at -80°C.

3.4 Mammalian cell transfection

One vial of frozen MCF-7N cells was taken from liquid nitrogen tank and placed in 37°C water bath until about 90% of cell solution is thawed. The cell solution was washed with DMEM working medium and centrifuged at 1200 rpm, for 3 minutes, at room temperature. The supernatant was discarded and the cell pellet was resuspended in DMEM working medium (10% FBS, low glucose) (thawing procedure for hybridoma cells is the same). MCF-7N cells were cultured in one six-well cell culture plate (Greiner Bio-one, Cellstar, 657 160), so that every well contains about 500.000 cells. When the cells were 80-90% confluent (approximately after one day) transfection was performed. As the transfection reagent FuGENE (Roche) was used. The transfection reagent (μ l) / plasmid (μ g) ratio was 3:1. Firstly, required amount of FuGENE was added to pure DMEM (without serum, antibiotics,

aminoacids). The mixture was shook rigorously and incubated for 5 minutes at room temperature. After 5 minutes, DNA was added and the mixture is shook rigorously. This time the complete transfection mix was incubated for 30 minutes at room temperature. Following the incubation the DMEM working medium of MCF-7N cells was changed to antibiotic free DMEM (10% FBS). Then the transfection mix was added slowly on top of the cells.

3.5 Protein extraction from cell lines

Cells were washed twice with PBS. Trypsin (Trypsin-EDTA, Invitrogen) was added on top of cells. Detached cells were collected and centrifuged at 1600 rpm, for 2 minutes, at 4°C. The supernatant was discarded and lysis buffer (3X of the volume of the pellet) was added on top of the pellet. The resuspension was stored on ice by vortexing every 5-10 minutes. After the ice incubation the resuspension was centrifuged at 13000 rpm, for 30 minutes, at 4°C. The pellet was discarded and the supernatant is stored at -20°C.

3.6 Cryopreservation of cell lines

Adherent MCF-7N cells were firstly washed twice with PBS. Trypsin was added on top of cells. Detached MCF-7N cells were collected and centrifuged at 1200 rpm, for 3 minutes, at room temperature. The supernatant was discarded and the pellet was resuspended in 90% FBS, 10% DMSO (Sigma) freezing medium. 1ml of this resuspension was given into cryotubes (Corning, 430289). The tubes were stored for 30 minutes at -20°C and transferred to -80°C. After overnight storage at -80°C, the tubes were moved to liquid nitrogen tank. The resuspension hybridoma cells were neither treated with trypsin nor with PBS. The remaining cryopreservation procedure was the same for hybridoma cells.

3.7 Sodium Dodecyl Sulfate -Polyacrylamide Gel Electrophoresis (SDS-PAGE)

10% SDS-PAGE resolving gel (Western Blot), 12% SDS-PAGE resolving gel (Comassie Staining) and 5% stacking gel were prepared according to shown data (Table 2.1, Table 2.2).

Table 3.1 Resolving gel components (10%)

Total volume (ml)	5	10	15	20	25	30	40	50
ddH ₂ O (ml)	1.9	4.0	5.9	7.9	9.9	11.9	15.9	19.8
30% Acrylamide mix (ml)	1.7	3.3	5.0	6.7	8.3	10.0	13.3	16.7
1.5 M Tris (pH: 8.8) (ml)	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
10% SDS (ml)	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
10% APS (ml)	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED (ml)	0.002	0.004	0.006	0.008	0.01	0.012	0.018	0.02

TEMED: Tetramethylethylenediamine, APS: Ammonium persulfate

Table 3.2 Stacking gel components (5%)

Total volume (ml)	1	2	3	4	5	6	8	10
ddH ₂ O (ml)	0.68	1.4	2.1	2.7	3.4	4.1	5.5	6.8
30% Acrylamide mix (ml)	0.17	0.33	0.5	0.67	0.83	1.0	1.3	1.7
1.0 M Tris (pH: 6.8) (ml)	0.13	0.25	0.38	0.5	0.63	0.75	1.0	1.25
10% SDS (ml)	0.01	0.02	0.03	0.04	0.05	0.06	0.08	0.1
10% APS (ml)	0.01	0.02	0.03	0.04	0.05	0.06	0.08	0.1
TEMED (ml)	0.001	0.002	0.003	0.004	0.005	0.006	0.008	0.01

First of all protein amounts were quantified by Bradford Assay. In the scope of this assay a standard curve was generated by measuring the absorbance of Bovine Serum Albumin (BSA) solution (BSA/ddH₂O) by spectrophotometer at 595 nm wavelength, visible light, in respect to concentration. Later absorbance of the protein samples dissolved in lysis buffer was measured and their concentration was calculated with the help of the BSA standard curve.

BioRad vertical gel system was set up according to the manufacturer's instructions. Loading samples were prepared. For this, protein samples (~5 µl protein for the cell lysate samples, ~40 ng for pure CCDC-124) were mixed with 1X Loading Dye and 1X dithiothreitol (DTT) reducing agent (Fermentas). The loading samples were fulfilled to 10 µl- 15 µl with ddH₂O. The samples were denatured at 100°C for 5 minutes and centrifuged at 13000 rpm for 1 minute. Later they were loaded onto gel. The electrophoresis took place at 250 V, 70 mA in 1X Tris Glycine electrophoresis buffer.

3.8 Comassie Staining

After the electrophoresis, the gel was put into comassie blue solution and incubated for 30 minutes on shaker. Later the comassie blue solution was removed and the gel was washed with either destaining solution or heated ddH₂O.

3.9 Western Blotting

An alternative way for visualizing proteins is the immuno-blotting procedure. First of all proteins were transferred from gel to PVDF transfer membrane (Immobilon-P, millipore) by Trans-Blot Semi Dry transfer cell apparatus (Bio-Rad) according to the manufacturer's instructions. For semi-dry transfer PVDF membrane has to be activated in methanol for 30 seconds. Besides Watmann papers and membrane should be wetted in transfer buffer.

After the transfer the membrane was blocked with 5% (w/v) non-fat milk powder dissolved in 0.5% Tween-20 containing TBS (TBS-T). Blocking lasts for 1 hour, at room temperature or overnight, at 4°C. Following the blocking step the membrane was incubated with primary antibodies (anti-Ccdc-124 (rabbit), anti-Flag (rabbit), monoclonal antibodies (mouse), again for 1 hour, at room temperature or overnight, at 4°C. After 3, 10 minute washes in TBS-T, the membrane was incubated with

Peroxidase conjugated secondary antibodies (anti-mouse or anti-rabbit, Sigma) for 1 hour. 3, 10 minute washes in TBS-T were repeated also after 2^o Ab treatment. Next, the membrane was exposed to SuperSignal West Dura Extended Duration Substrate kit (Thermo Scientific) for 5 minutes. The proteins were detected on X-Ray films (18x24, blue, KODAK) by exposing the membrane to the film for 10-30 seconds in dark.

3. 10 4B3 monoclonal anti-Ccdc-124 Antibody purification

4B3 clone cell supernatants are taken and pulled down in one bottle. pH value of the supernatant solution is measured and adjusted to 7.0 with 1M Tris Base (pH: 8.0). The supernatant solution is distributed in 50 ml aliquots for a later treatment.

For Antibody purification procedure Protein A Sepharose Beads (CL-4B, GE Healthcare) are used. Lyophilized beads are treated with ddH₂O. For 50 ml cell supernatant 250 µl bead medium (water treated beads) is used. The amount of required lyophilized beads is measured according to the manufacturer's instructions. Beads are washed with ddH₂O three times. Each time 50 ml water is added on top of beads and beads are centrifuged at 500 g for 15 minutes at 4°C. Following the wash step Phosphate Binding Buffer is added on top of beads (about 5X of the volume of the bead medium). Beads are washed twice with Phosphate Binding Buffer. Each time the solution is centrifuged at 500 g for 15 minutes at 4°C. (If the beads do not precipitate, the centrifuge steps might be repeated at 2000 rpm for 15 minutes at 4°C.) Finally the Phosphate Binding Buffer is removed from the beads and swollen beads are added in the supernatant solution aliquots (pH: ~7.0). This mix is rotated overnight, at 4°C, at falcon rotator.

Column (BIO-RAD) is washed three times with ddH₂O and later twice with Phosphate Binding Buffer. Then, supernatants incubated with beads overnight are passed through the column. The flow-through is stored.

Afterwards the column is washed three times with Phosphate Binding Buffer. The flow through is stored.

Following Phosphate Binding Buffer treatment the column is washed twice with Citrate buffer. The flow through is stored in a falcon tube, in which 2 ml Tris Base was present (pH: 9.0).

Elution sample is centrifuged at 2500 G, for 15 minutes, at 4°C. This time 10K concentrator tubes (Millipore) are used for centrifugation. Then the concentrator tubes are washed twice with sterile cold PBS and centrifuged. The purified antibodies are aliquoted and stored at -20 °C.

4. RESULTS

4.1 Production of Monoclonal Antibodies

Among 11 immunized mice only 7 were used in the production of hybridoma cells. The first injection began in early January, 2009 for the first group, in February, 2009 for the second group and in late July for the third group. As the injections proceeded, one animal from the first group and three animals from the second group died due to unidentified reasons. In the last group, the amount of protein used for injections was decreased to 30 µg; nevertheless the antibody titer within the blood was high enough. The presence of antibodies in serum samples was tested with ELISA assay and it was found that all three animals in the first group were successfully immunized since the assay gave positive results for serum concentrations ranging from 1/100 to 1/10000 (Figure 4.1). Serum-free samples as well as samples with serum isolated from un-immunized animal (H4) gave negative results. Polyclonal Ccdc-124 antibody produced in rabbit (α -Ccdc-124 in Figure 4.1), gave negative results, since in this ELISA, anti-mouse secondary antibody was used. Also H5 from the second group showed high antibody titer within its blood (Figure 4.2).

H1		H2		H3		H4 (Untreated)		α - Ccdc124		Serum-free		
*****	*****	*****	*****	3,944	*****	0,189	0,187	0,355	0,327	0,049	0,049	1/100
*****	*****	3,653	3,876	3,926	*****	0,117	0,116	0,123	0,128	0,049	0,048	1/500
0,105	0,11	3,661	3,462	3,291	3,327	0,117	0,112	0,128	0,125	0,048	0,05	1/1000
1,995	1,566	2,465	2,423	2,137	2,251	0,108	0,112	0,109	0,112	0,05	0,05	1/2500
0,524	0,499	1,913	1,637	1,707	1,711	0,109	0,108	0,113	0,122	0,046	0,046	1/5000
0,258	0,279	1,736	1,135	1,125	1,296	0,108	0,109	0,108	0,12	0,047	0,046	1/10000
0,094	0,099	0,106	0,11	0,109	0,101	0,104	0,108	0,114	0,124	0,042	0,046	PBS/BSA

Figure 4.1: ELISA showing antibody presence within serum isolated from three immunized mice belonging to the first group (H1, H2, H3). This assay was performed after the fourth injection. H4 sample, anti-Ccdc-124 antibody and serum free samples serve as negative controls. Different antibody concentrations were tested. Measurement: 30 minutes

(-)	1,382		0,071	0,084	0,081	0,074	0,086
(-)	1,509		0,067	0,066	0,083	0,076	0,073
(+)	0,063	1,985	0,072	0,081	0,069	0,068	0,075
(+)	0,08	2,592	0,12	0,104	0,095	0,114	0,088



H5 Serum

Figure 4.2: ELISA showing antibody presence within serum isolated from the immunized mouse belonging to the second group (H5). This assay was performed after the eighth injection. Serum sample from H1 was used as positive control. Serum was dissolved in PBS/ 5% non-fat milk powder solution in 1:1000 ratios. Measurement: 45 minutes. (-) and (+) symbolize the negative and the positive controls respectively (Section 3.2.3)

In case of high antibody titer, fusion experiment was performed. Firstly animals from the first group were sacrificed. And the fusion began with the animal giving the highest antibody titer. Since the ELISA results for H2 and H3 were similar, first fusion was performed on H2 after the fifth, boost injection. However no functional hybridomas were obtained from H2 as a result of contamination. Thus H2 hybridoma cells were discarded.

Before the spleen of H2 was isolated, it was observed that there was a fluid accumulation in the abdominal cavity of the animal. The fluid was sucked with a syringe and was stored. The presence of α -Cc dc-124 antibody in this ascites fluid was tested via ELISA assay (Figure 4.3). Since no clonal selection was performed, the ascites fluid was a candidate “poly”-clonal antibody. The ascites fluid was compared to the present polyclonal α -Cc dc-124 antibody.

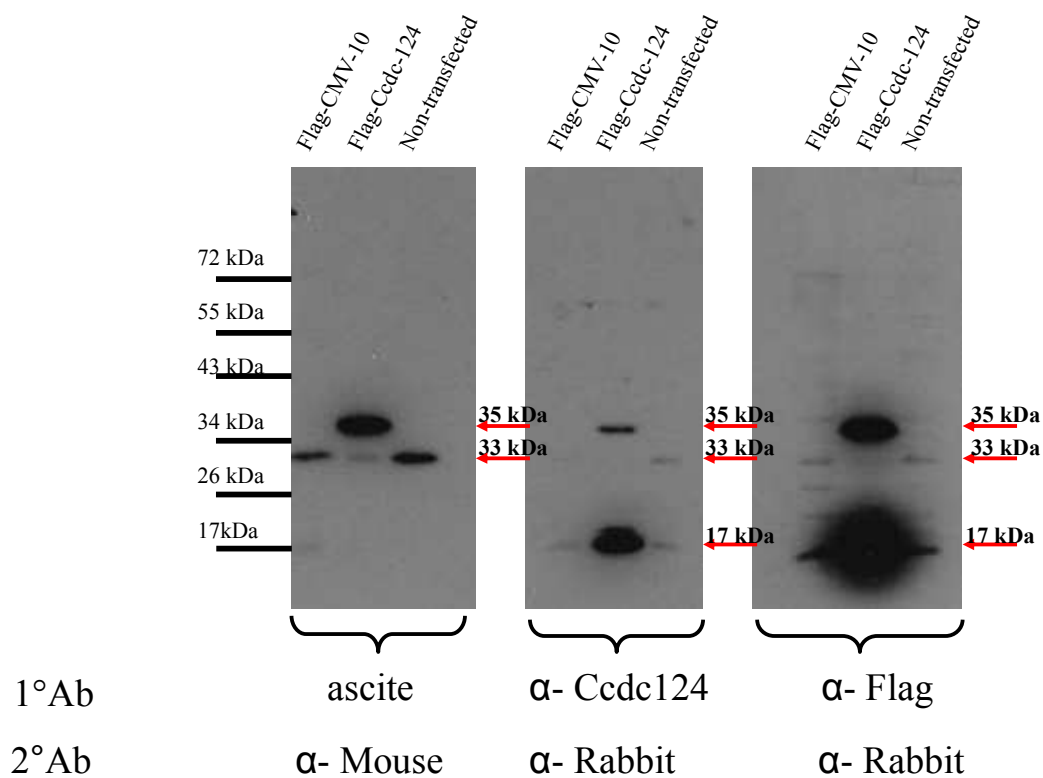


Figure 4.3: Western Blot showing antigen recognition capacity of ascites fluid (H2) as a candidate polyclonal antibody. The ascites fluid was tested as a candidate antibody source and its binding specificity was compared to the polyclonal α-Cdc124 antibody. The polyclonal Ab was dissolved in 5% non-fat milk powder/TBS-T solution in 1:500 ratio, the ascite in 1:1000 ratio and α-Flag An in 1:2000 ratio. Secondary antibodies were dissolved in 5% non-fat milk powder/TBS-T solution in 1:2000 ratios. 5 µg proteins were loaded for Flag-Cdc124-Transfected, CMV-Flag-CMV-10-Transfected and non-Transfected cell lysates. The α-Flag antibody was used as a control for testing the presence of transfected cell lysates. The images show 10 seconds exposure to X-ray film.

CCDC-124: 33kDa

Injections continued for the remaining mice of the first and the second group. After the sixth, boost injection H3 was euthanized and its spleen was isolated. Fusion was performed as the previous one. In one ELISA, 4 clones were identified as positives. Later positive clones were tested again and one of them was found to be still positive. Sub-cloning was performed at this step. However follow-up ELISA assays showed no positive results for the clones which were previously identified as positive. Consequently H3 hybridoma cells were discarded.

After the 9th, boost injection, H1 was euthanized and its spleen was isolated for fusion. In the ELISA some positive clones were found. However there was contamination problem. Hybridoma cells were washed with newly prepared HAT medium containing additional penicillin. However contamination problem could not be overcome so that H1 hybridoma cells were discarded.

Afterwards the mouse from the other group H5 was sacrificed. One of the generated hybridoma clones was identified twice as positive in ELISA. Sub-cloning was performed. Five among H5 single clones, emerging from the former positive clone were identified as positive and no other clones founded to be positive. Thus H5 single cell plates were discarded. Five positive clones were again sub-cloned via limiting dilution method. Some of these cells gave later positive results in ELISA tests. And they were expanded in 24 well plates. However after a while positive clones were lost. As a consequence also H5 hybridoma cells were discarded.

Subsequent to the lost of previous hybridoma clones the experiments continued with the last group of mice. The presence of antibodies in serum samples was tested with ELISA assay and it was seen that all three animals were successfully immunized (Figure 4.4).

H6:1,335	H7:2,141	0,067	1,411
H6:1,545	H8:0,869	0,067	0,992
H7:2,265	H8:0,961		

Figure 4.4: ELISA showing antibody presence within serum isolated from three immunized mice belonging to the third group (H6, H7, H8). This assay was performed after the eighth injection. Serum sample from H5 was used as positive control. Serum was dissolved in PBS/ 5% non-fat milk powder solution in 1:1000 ratios. Measurement: 30 min

Although H7 gave the highest antibody titer, spleen of H6 was isolated for the generation of hybridomas (it was a random choice). In ELISA tests one positive clone was detected (2E10). The cells of this clone were later sub-cloned and parental clones were expanded. In following assays 2 of the sub-clones were found to be positive. So these sub-clones together with parental 2E10 cells were cloned again. Although at the beginning 2E10 clone gave positive results, at the end no positive results were obtained, so H6 hybridoma cells were discarded.

Then hybridoma cells were generated by using the spleen of H7, the mouse with the highest serum antibody concentration. In the first ELISA test, 7F7 clone was identified as positive. Three other clones, 12F3, 11E8 and 15C11 were found to be positive in following ELISAs. Together with 7F7, the cells of four positive clones were sub-cloned. Parental cells were cultured on different plates via limiting dilution. In proceeding ELISA tests, 12F3 and 11E8 clone cells were shown to be negative. Therefore those plates were discarded. 7F7 cells which were again identified as positives were transferred firstly to 24 well plates, then to flasks and were expanded. Sub-clones of 7F7 and 15C11 were tested. Almost all of 7F7 sub-clones were positive, especially higher ones were expanded in 24 well plates and later stored at liquid nitrogen. However not every 15C11 sub-clone gave positive results. Only one of them was positive (15C11-3G3), which was later sub-cloned again. Positive one among 15C11-3G3 clones were expanded in 24 well plates and later in flasks. In

western blot experiments, the supernatants of cells arising from 7F7 parental clones and 15C11-3G3 clones were used (From now on 15C11-3G3 clone will be named as 15C11). Those clones were always positive in subsequent ELISA tests (Figure 4.6).

4.2 CCDC-124 protein isolation

Dr. Elif Yaman had previously isolated CCDC-124 protein in collaboration with Alfred Wittinghofer's group at the Max-Planck Institute, Dortmund, Germany. All injections and previous ELISAs were done by using this reagent prepared by Dr. Yaman during her Ph.D. studies. However, the reagent was not enough to carry out all required experiments. Therefore, new CCDC-124 isolation has been carried out in frame of this work in order to use it in ELISA tests for the hybridomas of H8. In order to detect whether the protein of interest (33 kDa) was purified successfully or not, comassie staining experiment was performed (Figure 4.5). Samples isolated at different steps of protein purification step were loaded to the gel and compared to each other.

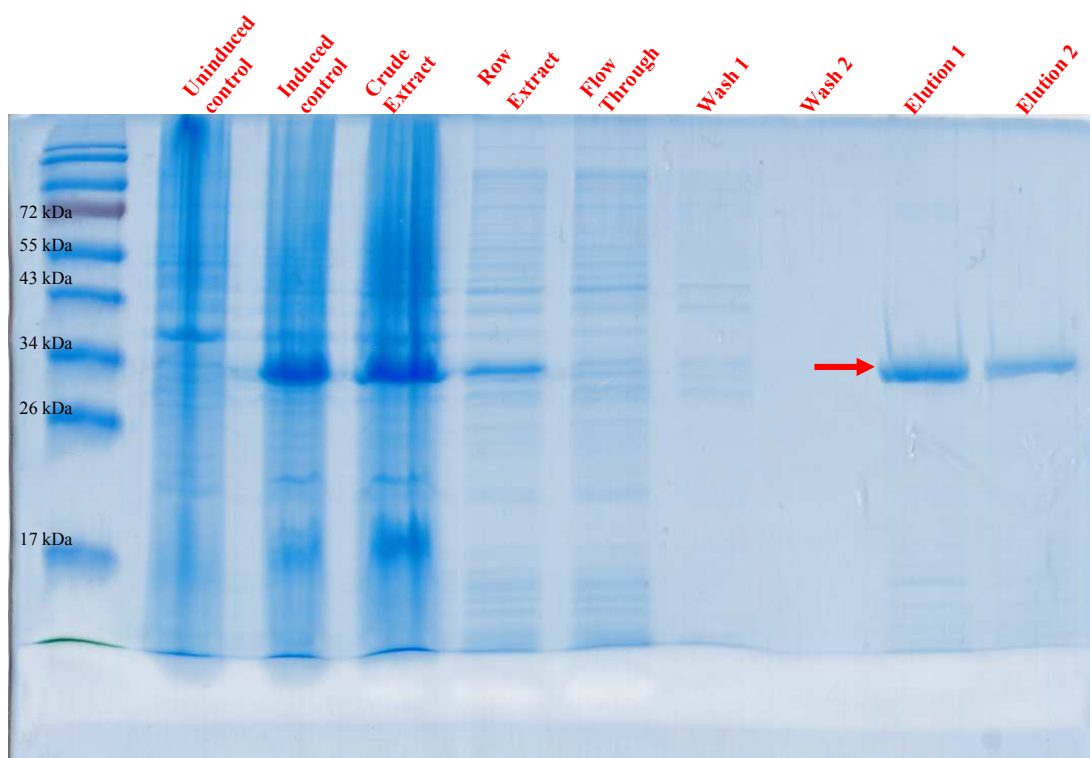


Figure 4.5: Coomassie Blue staining showing the isolated CCDC-124 protein (33kDa). DH5 α competent bacteria were transformed pQE81 Locus plasmid and the protein of interest was over-expressed. The protein was isolated according to the protocol mentioned in section 2.3. IPTG induced, un-induced samples are as shown in the figure. Crude extract: sample after sonication step, Row extract: sample after Ultra Centrifuge step, Flow Through: sample after the rotation with beads, Wash 1 & Wash 2: sample which remained after the beads were washed with wash buffers, Elution 1 & Elution 2: sample which remained after the protein of interest was detached from the beads with elution buffers.

The last animal left in the third group, H8, was already immunized and antibodies were present in its serum (Figure 4.3). So it was decided to continue to produce hybridoma cells. Hybridoma cells generated by fusing spleen cells of H8 and SP2 cells were tested and two high positive clones were identified (4B3 and 5D3, Figure 4.6).

4B3						15C11 and 7F7 cells					
0,091	0,074	0,114	0,069	0,074	0,075	0,095	0,118	0,09	0,081	0,883	0,162
0,078	0,077	0,085	0,086	0,08	0,068	0,069	0,07	0,103	0,106	0,844	0,574
0,08	0,072	0,088	0,085	0,073	0,074	0,066	0,066	0,078	0,075	1,205	0,09
0,098	0,065	0,107	0,074	0,098	0,087	2,48	0,07	0,08	0,084	1,014	0,631
0,077	0,086	0,124	0,082	0,067	0,065	0,064	0,064	0,078	0,072	0,453	0,091
0,073	0,069	0,192	0,086	0,084	0,087	0,085	0,063	0,069	0,086	0,073	0,109
0,089	0,077	0,088	0,081	0,099	0,087	0,079	0,067	0,068	0,748	0,069	1,213
0,1	0,096	0,133	0,116	0,101	0,112	0,104	0,069	0,066	0,799	0,07	1,444

Figure 4.6: ELISA showing the presence of antibody of interest in H8 clones. H8 Clones except 5D3 and 2B11 were tested for the first time. Expanded 15C11 and 7F7 clones (H7) were tested, too. Measurement: 2.5 hours. (-) and (+) symbolize the negative and the positive controls respectively (Section 3.2.3)

The ELISA on Figure 4.4 shows the presence of Ccdc-124 antibody in H8 clones. Among positive clones highlighted with yellow, 5D3 and 2B11 were previously found to be positive. However here it is shown that only 5D3 preserves its positive nature. 4B3 clone is tested for the first time and found to be positive, too.

According to these results two positive clones, 4B3 and 5D3 were sub-cloned. Their parental clones were transferred firstly into 24 well plates. The ELISA result in Figure 4.7 shows the antibody titer in parental cell supernatants.

4B3		5D3		
2,928	2,526	0,098	0,065	0,069
2,503	2,377	0,101	0,077	0,062
2,454	2,709	0,153	0,123	0,852
2,113	2,593	0,123	0,109	0,941

Figure 4.7: ELISA showing the presence of antibody of interest in 4B3 and 5D3 parental clones. Clones in 24 well plates were tested as duplicates. Measurement: 45 minutes. (-) and (+) symbolize the negative and the positive controls respectively (Section 3.2.3)

Since no positive results were obtained for 5D3 clones, parental clones and sub-clones were discarded. 4B3 clones on the other hand were transferred from 24 well plates to flasks and cells were expanded.

4B3-4F3				
0,237	0,315	0,205	0,883	(-)
0,327	0,262	1,787	0,45	(-)
0,201	0,477	0,182	1,462	(+)
0,521	0,392	0,309	1,495	(+)

Figure 4.8: ELISA showing the presence of antibody of interest in 4B3 sub-clones. Sub-clones were tested for the first time. Measurement: 90 minutes. (-) and (+) symbolize the negative and the positive controls respectively (Section 3.2.3)

Figure 4.8 shows that, one among the tested 4B3 sub-clones gives positive result (4B3-4F3). All the other sub-clones tested were negative. In a second ELISA other grown up hybridoma cells were tested together with 4B3-4F3 and parental 4B3 clones (Figure 4.9).

4B3						4B3-4F3		
2,227	2,108	1,702	1,441	0,588	2,001	1,776	0,078	(-)
2,205	2,035	1,59	0,071	1,536	1,739	1,781	0,06	(-)
1,777	1,777	1,737	1,624	1,165	2,165	1,892	1,121	(+)
1,163	1,491	1,471	1,269	1,364	1,498		1,039	(+)

Figure 4.9: ELISA showing the presence of antibody of interest in 4B3 sub-clones and 4B3 parental clones (4B3-P). Except 4B3-4F3, which was previously split into three, sub-clones were tested for the first time. Measurement: 45 minutes. (-) and (+) symbolize the negative and the positive controls respectively (Section 3.2.3)

Except the clone shown in the red box (Figure 4.9), every tested 4B3 sub-clone gave positive results. 4B3-4F3 clone, too, was found to be positive for the second time.

0,073	1,615	2,119	2,177	1,576	1,623	1,174	1,547	0,635	0,079	(-)
1,208	2,263	2,107	2,233	0,824	1,615	1,478	1,023	0,597	0,07	(-)
1,128	2,062	2,199	2,138	1,578	1,749	0,942	1,047	1,135		
1,4	1,822	0,067	1,91	1,851	0,976	1,395	0,078	1,136		
1,351	1,825	1,95	2,077	1,957	0,908	1,029	0,832	0,696		
1,102	2,024	1,702	1,969	1,031	0,9	0,562	1,36	0,915		
1,087	1,873	2,381	2,014	1,787	1,39	1,433	1,234	0,472	0,365	(+)
1,316	1,935	1,893	1,932	1,771	1,434	0,52	1,586	0,913	0,488	(+)

Figure 4.10: ELISA showing the presence of antibody of interest in 4B3 sub-clones. Some of 4B3 sub-clones were tested for the first time whereas some others, which were found to be positive, were re-tested. Measurement: 45 minutes. (-) and (+) symbolize the negative and the positive controls respectively (Section 3.2.3)

In Figure 4.10, the 4B3 sub-clone highlighted with red (the clones are arising from the same clone, which was split into two) was previously found to be negative. This Elisa assay proved that this clone does not give positive results.

Especially high positive values were obtained for five clones highlighted with yellow. Although there were other clones giving high positive results, only these five clones were split and later transferred to 24 well plates for expansion. Because, a microscopic examination showed that these five clones were hybridoma cells, in state of being fibroblasts. Additionally these were over-grown so that they had to split. However after a while cells in 24 well plates contaminated, hence they were discarded. Only 4B3 clones within flasks were stored.

4.3 Use of produced monoclonal Antibodies in Western Blotting and Comparison of these Antibodies with the polyclonal anti-Ccdc-124 Antibody

In order to perform Western Blot experiments with the produced monoclonal antibodies, first of all the supernatants of positive cells were tested again via ELISA (Figure 4.11). 4B3 clone, 7F7 clone and 15C11 clone were found to be positive, among which 15C11 gave the lowest positive and 7F7 gave the highest positive result.

Duplicates				
4B3	7F7	15C11	(-)	(+)
0,633	1,522	0,173	0,083	0,751
0,501	1,434	0,278	0,079	0,699

Figure 4.11: 1st ELISA showing the presence of antibody of interest in 4B3, 7F7 and 15C11 clones. Supernatants within flasks were tested. Measurement: 30 minutes. (-) and (+) symbolize the negative and the positive controls respectively (Section 3.2.3)

The Western Blot experiment, in which 4B3 supernatant is used (Figure 4.12) was performed after the ELISA assay shown in figure 4.11. The same supernatant was used in Western Blot and ELISA assays.

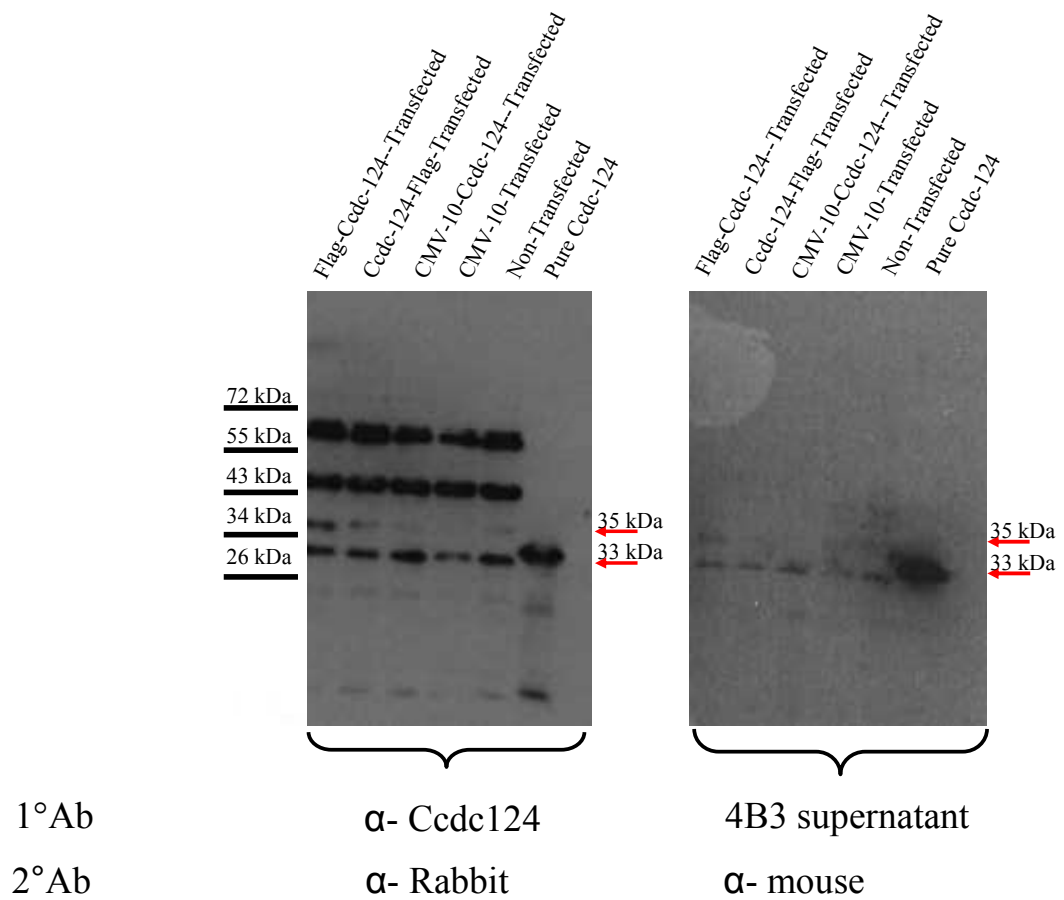


Figure 4.12: 1st Western Blot showing antigen recognition capacity of 4B3 monoclonal Antibody.

The monoclonal 4B3 antibody was compared to the polyclonal α-Ccdc-124 antibody. The cell supernatant was used for the monoclonal Ab; the polyclonal Ab was dissolved in 5% non-fat milk powder/TBS-T solution in 1:500 ratios. Secondary antibodies were dissolved in 5% non-fat milk powder/TBS-T solution in 1:2500 ratios. 5 µg proteins were loaded for Flag-Ccdc-124-Transfected, Ccdc-124-Flag-Transfected, CMV-10-Ccdc-124-Transfected, CMV-10-Transfected and non-Transfected cell lysates. 40 ng pure protein was loaded. The image on the left shows 10 second exposure of X-ray film, the image on the right is 20 seconds exposure. CCDC-124: 33kDa

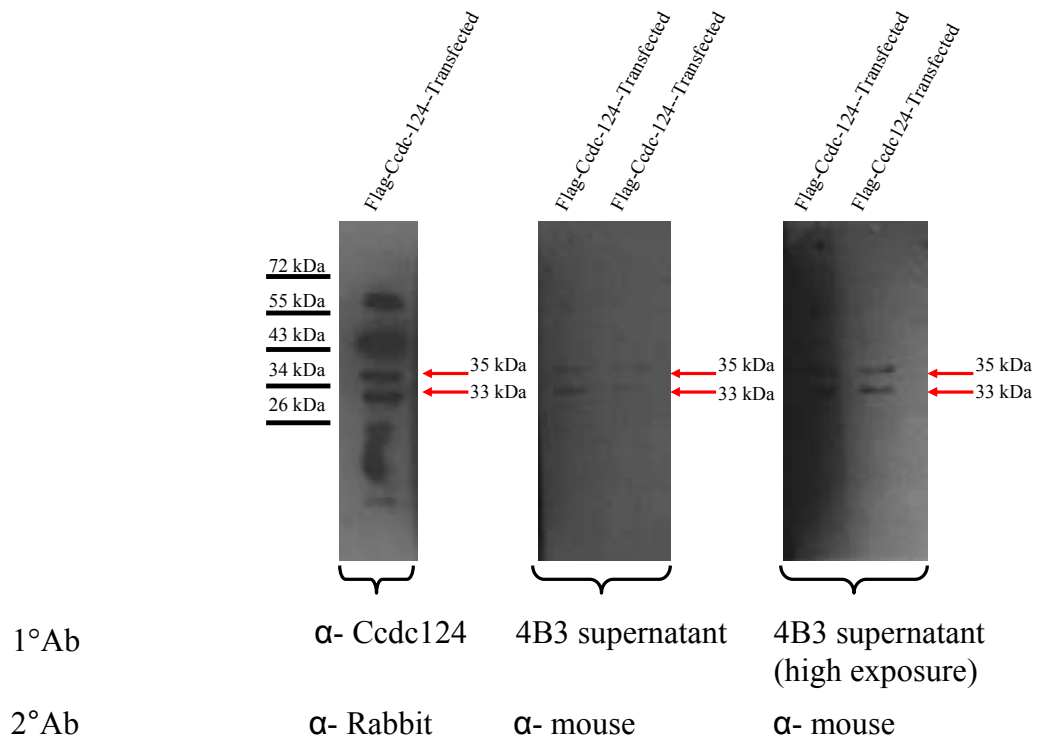


Figure 4.13: 2nd Western Blot showing antigen recognition capacity of 4B3 monoclonal Antibody. The monoclonal 4B3 antibody was compared to the polyclonal α -Ccdc-124 antibody. The cell supernatant was used for the monoclonal Ab; the polyclonal Ab was dissolved in 5% non-fat milk powder/TBS-T solution in 1:500 ratio. Secondary antibodies were dissolved in 5% non-fat milk powder/TBS-T solution in 1:2500 ratios. 5 μ g proteins were loaded for Flag-Ccdc-124-Transfected cell lysates. The image of polyclonal Ab was exposed for 10 seconds to X-ray film, the right and left images of 4B3 Abs were exposed for 10 seconds and 20 seconds to X-ray film respectively. CCDC-124: 33kDa

Duplicates				
4B3	7F7	15C11	(-)	(+)
0,916	0,662	0,311	0,06	0,648
1,231	1,003	0,222	0,062	0,566

Figure 4.14: 2nd ELISA showing the presence of antibody of interest in 4B3, 7F7 and 15C11 clones. Supernatants within flasks were tested. Measurement: 60 minutes. (-) and (+) symbolize the negative and the positive controls respectively (Section 3.2.3)

All of generated mono-clones; 4B3, 7F7 and 15C11 were found to be positive, among which 15C11 gives the lowest positive result (as in the previous ELISA, Figure: 4.11). This time 4B3 clone gave the highest positive result in state of 7F7. The Western Blot experiments, in which 7F7 (Figure 4.15) and 15C11 (Figure 4.16) supernatants were tested, were performed after the ELISA assay shown in figure 4.14, and the same supernatants were used in Western Blot and ELISA assays.

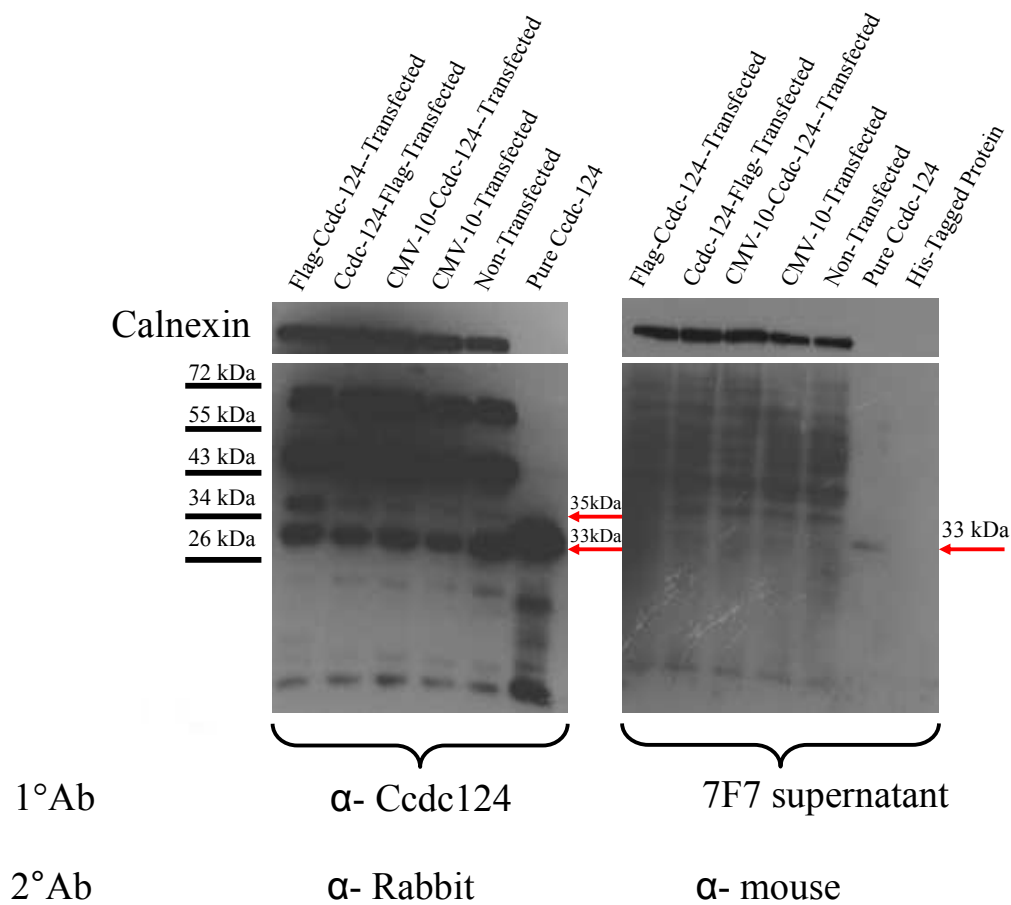


Figure 4.15: Western Blot showing antigen recognition capacity of 7F7 monoclonal Antibody.

The monoclonal 7F7 antibody was compared to the polyclonal α -Ccdc-124 antibody. The cell supernatant was used for the monoclonal Ab; the polyclonal Ab was dissolved in 5% non-fat milk powder/TBS-T solution in 1:500 ratio. Secondary antibodies were dissolved in 5% non-fat milk powder/TBS-T solution in 1:2500 ratios. 5 μ g proteins were loaded for Flag-Ccdc-124-Transfected, Ccdc-124-Flag-Transfected, CMV-10-Ccdc-124-Transfected, CMV-10-Transfected and non-Transfected cell lysates. 40 ng pure CCDC-124 protein and 50 ng pure unrelated His-tagged protein were loaded. The image on the left shows 10 second exposure of X-ray film, the image on the right is 20 seconds exposure. 1:10000 α -Calnexin antibody dissolved in 5% non-fat milk powder/TBS-T solution serves as loading control for cell lysate samples. CCDC-124: 33kDa

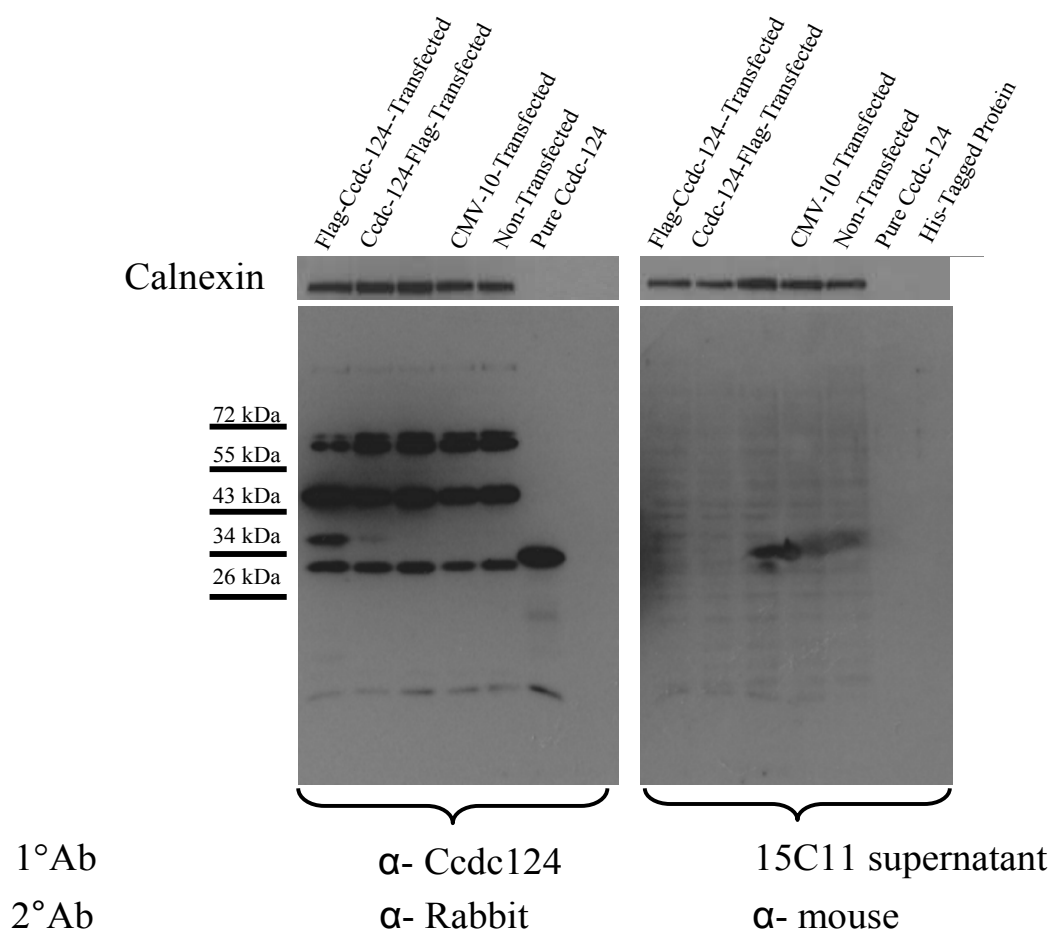


Figure 4.16: Western Blot showing antigen recognition capacity of 15C11 monoclonal Antibody.

The monoclonal 15C11 antibody was compared to the polyclonal α -Ccdc-124 antibody. The cell supernatant was used for the monoclonal Ab; the polyclonal Ab was dissolved in 5% non-fat milk powder/TBS-T solution in 1:500 ratios. Secondary antibodies were dissolved in 5% non-fat milk powder/TBS-T solution in 1:2500 ratios. 5 μ g proteins were loaded for Flag-Ccdc-124-Transfected, Ccdc-124-Flag-Transfected, CMV-10-Ccdc-124-Transfected, CMV-10-Transfected and non-Transfected cell lysates. 40 ng pure CCDC-124 protein and 50 ng pure unrelated His-tagged protein were loaded. The image on the left shows 10 second exposure of X-ray film, the image on the right is 20 seconds exposure. 1:10000 α -Calnexin antibody dissolved in 5% non-fat milk powder/TBS-T solution serves as loading control for cell lysate samples. CCDC-124: 33kDa

4.4 4B3 monoclonal anti-Ccdc-124 Antibody purification

Lastly, 4B3 clone, which previously gave the most promising results at 33 kDa, in Western Blot experiments, was purified (See Section 4.4). Before the purification, another ELISA was performed in order to see whether the 4B3 monoclonal antibody still gives positive results or not (Figure 4.17). It was observed that 4B3 monoclonal antibody was still able to recognize the antigen of interest.

4B3						(-)	(+)
1,87	2,429	1,971	1,454	1,141	1,203	0,093	0,443
2,213	2,208	1,776	0,99	0,973	1,972	0,069	0,291

Figure 4.17: ELISA showing the presence of antibody of interest in 4B3 clone. Supernatants within flasks were tested. Measurement: 60 minutes. (-) and (+) symbolize the negative and the positive controls respectively (Section 3.2.3)

After the purification, different antibody concentrations were tested via ELISA (Figure 4.18). Especially at high concentrations, 4B3 antibody was found to be positive.

1/10000	1/5000	1/2500	1/1000	1/500	1/250	(-)	(+)
0,086	0,122	0,198	0,514	0,77	1,498	0,084	0,927
0,113	0,149	0,175	0,484	0,66	1,178	0,095	0,953

Figure 4. 18: ELISA showing the antigen recognition capacity of purified 4B3 monoclonal antibody. Different antibody concentrations were tested Measurement: 60 minutes. (-) and (+) symbolize the negative and the positive controls respectively (Section 3.2.3)

Afterwards a Western Blot experiment was performed with the purified 4B3 antibody (Figure 4.19). The monoclonal antibody was compared to the polyclonal α -Ccdc-124 antibody.

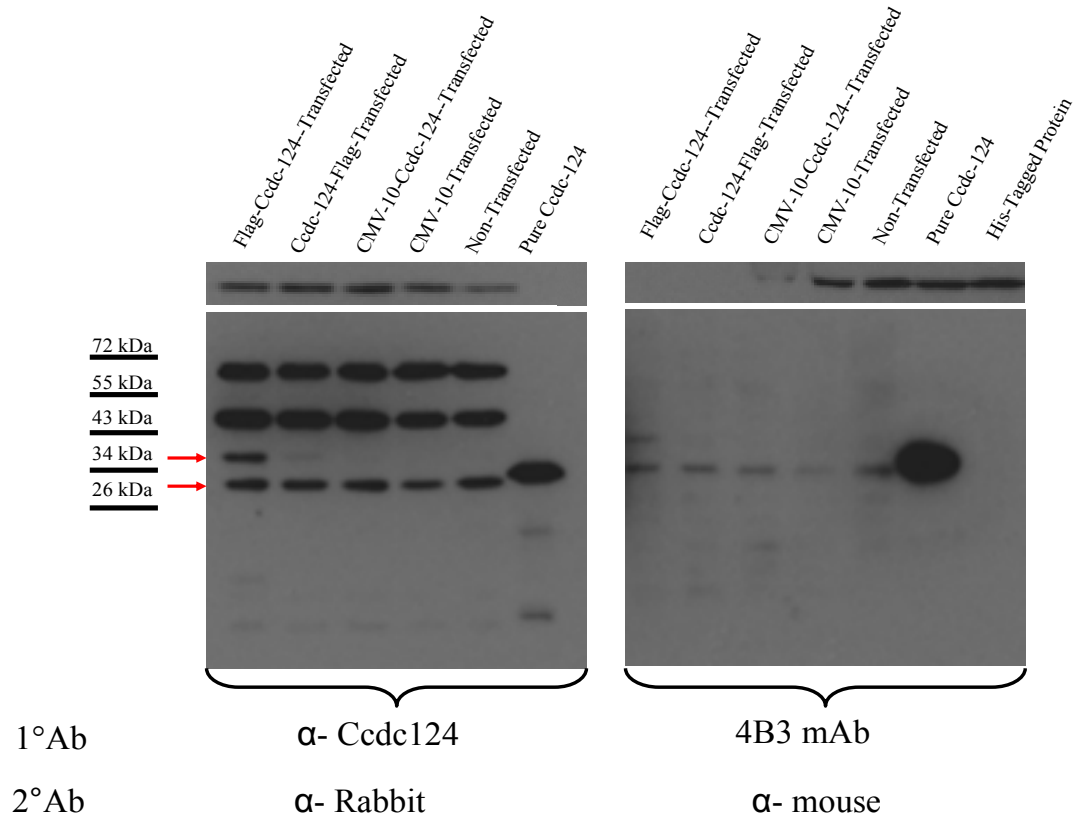


Figure 4.19: 3rd Western Blot showing antigen recognition capacity of 4B3 monoclonal Antibody. The monoclonal 4B3 antibody was compared to the polyclonal α -Ccdc-124 antibody. The purified 4B3 antibody dissolved in 5% non-fat milk powder/TBS-T solution in 1:500 ratios was used in this assay; the polyclonal Ab, too was dissolved in 5% non-fat milk powder/TBS-T solution in 1:500 ratios. Secondary antibodies were dissolved in 5% non-fat milk powder/TBS-T solution in 1:2500 ratios. 5 μ g proteins were loaded for Flag-Ccdc-124-Transfected, Ccdc-124-Flag-Transfected, CMV-10-Ccdc-124-Transfected, CMV-10-Transfected and non-Transfected cell lysates. 40 ng pure protein was loaded. The image on the left shows 10 seconds exposure of X-ray film, the image on the right is 60 seconds exposure. CCDC-124: 33kDa

5. DISCUSSION

5.1 Production of Monoclonal Antibodies

Throughout this project 11 mice were injected with the antigen of interest, purified CCDC-124 protein. 4 mice among 11 died as the injections proceeded and before their spleens were isolated. Although the exact reason for the death of animals is unknown, several treatments might result with in this outcome.

First of all, the site of injection and how the injection is performed are two important points. It is recommended that the antigen is injected sub-cutaneously or intra-peritoneally in state of intradermal, intramuscular, or foot pad injections. If no adjuvants are used, intravenous injection of soluble antigens might be another possibility (NHMRC, 2008). In this project injections were performed intra-peritoneally as recommended, however it is possible that the needle penetrated into the abdominal organs or muscles due to injection inaccuracies. This fact might result with poisoning and death of the animal.

Secondly, adjuvants triggering the immune response in mouse are irritants. Among these the Freund's adjuvant has a strong effect. When Freund's adjuvant is used, it is recommended not to use more than 100 μ l per injection (NHMRC, 2008). It is also stated that the complete Freund's adjuvant should be used only once in an individual mouse (NHMRC, 2008). Accordingly, in this project complete Freund's adjuvant was used only in the first injection. Facts related to adjuvant and antigen injection might lead to abscess accumulation. This was observed in some mice used in this project. Abscesses in the abdominal cavity might also result in the loss of the animal.

Another problem faced during the project was contamination of hybridoma cells. Although the cells were washed with media containing high amount of antibiotics, this problem could not be overcome. For the decontamination of hybridoma cells, another suggested method is *in vivo* cleaning. In this method the cells are given either to the abdominal cavity or subcutaneously to immunocompetent mice. After the immune system of the mouse cleans up the agents responsible for the contamination the hybridoma cells are recovered and stored in appropriate media (NHMRC, 2008).

In summary 4 animals were lost due to unknown reasons and hybridoma cells generated from the spleens of 2 mice were contaminated. Additionally, no positive clones could be obtained among the hybridoma cells produced from the spleens of 3 mice. Although at the beginning some of the clones gave positive results in ELISA tests, later no more positive results were obtained from the same clones. It is possible that those positive clones were dominated by other hybridoma clones which are not producing the appropriate antibody. This fact might be observed when single-cell cloning is unsuccessful and more than one clone is cultured in the same well. An alternative way to overcome this problem might be freezing of cells. Before the cells are over-grown and dominate others, one can freeze the cells for a later sub-cloning process (Committee on Methods of Producing Monoclonal Antibodies, 1999). Actually all 7 surviving animals were able to produce antibody against CCDC-124, which was proven by ELISA tests of blood samples. This information supports that cell culture experiments, including fusion and selection were responsible for the loss of positive clones in state of *in vivo* production of antibodies.

Since no positive results were obtained from the two groups of mice, experiments continued with a third group of mice. In fact the injections two group 3 began before all hybridoma cells of the first two groups were identified to be negative and discarded. The idea behind this was the long period of immunization of mice, which approximately takes 5 months. Thus when all clones of mice belonging to group 1 and 2 were discarded, the project continued without a lap phase.

Among the hybridoma cells generated from the spleens of mice belonging to group 3, 3 were found to be positive in every ELISA assay performed: 7F7, 15C11, 4B3. Whereas 7F7 and 15C11 were from the same mouse, 4B3 clone was generated from another mouse. In order to characterize those monoclonal antibodies Western Blot experiments were performed by using the hybridoma cell supernatants. Before the Western Blot experiments the supernatants were tested again via ELISA in order to see whether they preserve their positive nature or not. In two different ELISAs it is seen that 15C11 consistently was giving the least positive result. However in one of those ELISAs (Figure 4.11) 7F7 was the highest positive while in the second ELISA (Figure 4.14) 4B3 was the highest one. At this point one can comment that 4B3 cells were not grown enough while the first ELISA was performed, so that a low positive value was obtained. Another possibility to explain this discrepancy might be experimental errors faced during one of the ELISA tests.

5.2 Antigen recognition capacity of ascites fluid

Figure 4.3 shows a Western Blot in which the ascites fluid isolated from the abdominal cavity of H2 is used as a polyclonal antibody. The ascite recognizes the endogenous (33 kDa) and Flag-tagged (35 kDa) CCDC-124 proteins. α -Flag antibody is used as a control in order to see the Flag-tagged protein of interest. Interestingly although both polyclonal α -Ccde-124 antibody and α -Flag antibody detect a band at around 17 kDa, this band is not visible in the ascite sample. Since the polyclonal antibody is against an N-terminal peptide, this band probably corresponds to an N-terminal peptide. Additionally the Flag tag is at the N-terminus (Flag-Ccde-124 transfected sample) so that this peptide is also recognized by α -Flag antibody. Accordingly the ascites fluid contains antibodies which mainly recognize C-terminal sequences.

5.3 CCDC-124 protein isolation

Since the amount of the protein purified by Dr. Yaman was not enough to perform ELISA tests for H8 hybridoma cells, new CCDC-124 purification has been carried out. The comassie staining experiment (Figure 4.5) shows whether the protein of interest was purified successfully or not. Compared to un-induced control sample, CCDC-124 protein which has the molecular weight of 33 kDa is clearly seen in induced control sample. This fact shows that the protein over-expression in bacteria was successful.

Crude extract, the sample after sonication contains bacterial components in addition to proteins. Therefore beside the protein of interest there are many additional bands. After the centrifuge at high speed, only proteins which have a low molecular weight compared to other organic materials are left in the supernatant sample “row extract”. That’s why in row extract one is able to see subtle protein bands. The only dark band is at 33kDa and belongs to CCDC-124. This band disappears in flow through sample, which verifies a successful attachment of His-tagged CCDC-124 protein to beads. After rotation the beads were washed with wash buffers in order to eliminate non-specific binding. In wash samples there are no observable bands at 33 kDa, which shows that CCDC-124 proteins attached to beads efficiently. There are also some differences between Wash 1 and Wash 2 samples. Although there are some faint protein bands in Wash 1 sample there are no bands in Wash 2 sample. This result was expected since most of the proteins are washed out in the first wash. As a result in every wash the amount of protein within the sample decreases.

The successful purification of CCDC-124 is seen in Elution 1 and 2 samples (Figure 4.5). There are strong bands at 33 kDa which corresponds to the protein of interest. And in elution samples there are no other clear protein bands, which mean that only CCDC-124 was attached to the beads and that only this protein was detached from the beads with the help of elution buffers.

Furthermore, Western Blot experiments in which pure CDCD-124 protein is loaded to the gel show that the protein purification was successful. Although the polyclonal α -Ccdc-124 antibody recognizes non-specific protein bands in cell lysate samples, in pure protein samples it only recognizes one band at 33 kDa which corresponds to CDCD-124.

5.4 Use of produced monoclonal Antibodies in Western Blotting and Comparison of these Antibodies with the polyclonal anti-Ccdc-124 Antibody

Figure 4.12, Figure 4.13 and Figure 4.19 show the Western Blot experiments performed by using 4B3 monoclonal antibody. Whereas, in the first two Western Blots, as the source of antibodies, cell supernatants were used; in the last one, the membranes were incubated with purified antibodies. In the first experiment (Figure 4.12) 5 cell lysate samples and one pure CCDC-124 protein are loaded to the gel. Pure protein is recognized by both polyclonal and monoclonal antibodies. Endogenous CCDC-124 (33 kDa) band was observed in both illustrations (polyclonal Ab incubation and monoclonal Ab incubation). Flag-Ccdc-124-Transfected (Flag tag is at the N-terminus) and Ccdc-124-Flag-Transfected (Flag tag is at the C-terminus) cell lysate samples gave a band at 35 kDa, which corresponds to the over-expressed Flag-tagged CCDC-124. As expected CMV-10-Ccdc-124 vector transfected, CMV-10 vector (empty vector) transfected and non-transfected cell lysate samples gave no band at 35 kDa, since they have no Flag-tag. At this point it should be mentioned that the band corresponding to Ccdc-124-Flag-Transfected sample is more faint compared to the band corresponding to Flag-Ccdc-124-Transfected sample. The reason might be the unstable nature of CCDC-124 bearing a C-terminus Flag-tag (Serap Erkek, M.Sc. thesis, Bilkent University, 2008).

Additionally in Figure 4.12 it is seen that the polyclonal antibody recognizes at least two bands in pure protein sample. One of the strong bands is at about 33 kDa,

although it has a slightly larger molecular weight than the endogenous CCDC-124. 6 Histidine moieties tagged to the protein might be responsible for this observation. If one considers that the polyclonal α -Ccdc-124 antibody recognizes the N-terminal peptide of CCDC-124, the other strong band is probably showing an N-terminal degraded peptide.

The major difference between the blots showing poly- and monoclonal antibody incubation is the presence of strong bands at ~43 kDa and ~60 kDa. These proteins are only recognized by the polyclonal antibody. 4B3 however does not recognize these non-specific bands. Also in Figure 4.13 and Figure 4.19, only endogenous CCDC-124 bands (33 kDa) and Flag-tagged CCDC-124 bands (35 kDa) are visible in the figures showing the 4B3 incubation. The disappearance of the non-specific bands, which is a characteristic feature of using polyclonal antibodies makes 4B3 a strong candidate antibody which will make possible to obtain precise results in future experiments.

The antigen recognition capacity of 7F7 monoclonal antibody is also tested via Western Blotting (Figure 4.15). Unlike 4B3, 7F7 is not able to bind CCDC-124 protein present within the cell. There are no observable bands at 33 kDa (endogenous) or at 35 kDa (over-expressed, Flag-tagged) in Flag-Ccdc-124-Transfected, Ccdc-124-Flag-Transfected, CMV-10-Ccdc-124-Transfected, CMV-10-Transfected and non-Transfected cell lysate samples. In fact there are some weak non-specific bands, although not that clear as in the polyclonal antibody incubated blot. Nevertheless 7F7 antibody is able to recognize His-tagged pure CCDC-124 protein. The monoclonal antibody does not bind to another unrelated protein (~25 kDa) with His-Tag. It is possible that this antibody recognizes a certain sequence including the His-Tag (Figure 5.1). Consequently neither proteins carrying only the His-Tag in common with CCDC-124 nor the endogenous CCDC-124 proteins not tagged with His sequence are bound by 7F7.

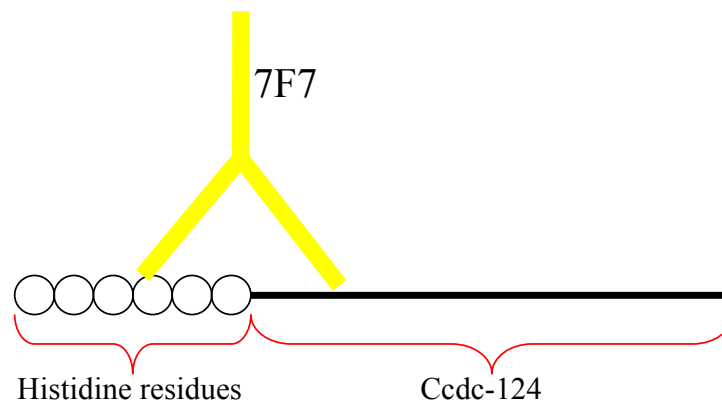


Figure 5.1 Schematic representation of 7F7's predicted antigen recognition property

The last positive clone, 15C11 is also characterized via Western Blotting (Figure 4.16). This Western Blot shows that 15C11 monoclonal antibody does not recognize anything including the pure His-Tagged CCDC-124 protein. The other unrelated protein (25 kDa) as well is not recognized by 15C11. The observed black spot on the membrane is most probably contamination. The polyclonal antibody exhibits the expected outcome by binding to CCDC-124 (33 kDa and 35 kDa) and to non-specific proteins. Hence, the Western Blot experiment itself was successful.

Since 15C11 clone always gave positive results in ELISA tests, this outcome was unexpected. It is possible that this antibody works in an assay restricted manner (Figure 5.2). When the target epitope is masked or denatured in a given assay type this phenomenon might be observed (Nelson *et al.*, 2000). The use of Sodium Dodecyl Sulfate (SDS) in Western Blotting might denature the protein so that the conformational epitope recognized by 15C11 is lost. Performing native Western Blotting in non-denaturing conditions (without detergents etc.) might overcome this problem in the future.

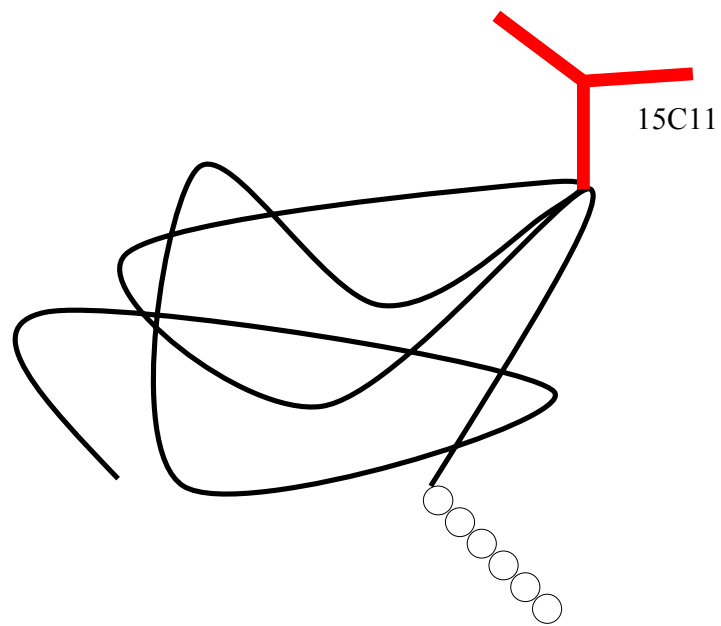


Figure 5. 2: Schematic representation of 15C11's predicted antigen recognition property

6. FUTURE PERSPECTIVES

Future studies are planned to detect the functional role of CCDC-124. Of first priority sub-cellular localization experiments should be carried-out by using monoclonal antibodies produced in frame of this study. The aim is to detect the exact localization of CCDC-124 within the cell by using organelle specific markers together with monoclonal anti-Ccdc-124 antibodies. Additionally the change of protein's localization in response to extra-cellular signals can be tracked. Among the produced antibodies (7F7, 15C11 and 4B3) 4B3 gave the clearest results in Western Blot experiments so it would be appropriate to use firstly this antibody in sub-cellular localization studies. Later 7F7 and 15C11 could be used to support the results obtained with 4B3 antibody. As mentioned in section 5.4, it is possible that 15C11 exhibits an assay restricted binding. Although it is functional in ELISA, no function is seen in Western Blot experiment. The activity of the mentioned monoclonal antibody should also tested in sub-cellular localization studies, for instance in immunofluorescence staining.

Another important goal is to knock-down *CCDC-124* gene by RNA silencing. First of all, cells where *CCDC-124* is down-regulated by siRNA strategy could serve as negative control samples in various experiments. Throughout this project, in Western Blot experiments non-transfected and empty-vector-transfected cell lysates were used as negative controls. However those samples still contain the endogenous CCDC-124 protein. So they only serve as negative controls for *CCDC-124* over-expressing cells. Consequently via siRNA knock-down technique the endogenous protein will be silenced and the cells which do not express *CCDC-124* at all could be used as negative control samples.

In the big picture, knocking-down *CCDC-124* could give clues about the functional role of the protein. As indicated in Section 1.1, *CCDC-124* does not change the guanine exchange activity of RasGEF1B *in vitro* assays. Nonetheless, the activity of RasGEF1B could change *in vivo*. This could be the case if there are other players within the cell, which modulate the impact of *CCDC-124* on RasGEF1B. It is also possible that post-translational modifications influence *CCDC-124*/RasGEF interaction. In the future RasGEF1B activity could be compared among *CCDC-124* knocked-down and normal cells.

In long term *CCDC-124* knock-out mice could be generated with the aim of observing the role of the protein at the organism level. Considering the conserved nature of the protein among eukaryotic species, *CCDC-124* might fulfill an important function within the cell so that knocking out the gene is lethal. In this case it would be more suitable to generate conditional knock-out mice.

7. REFERENCES

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