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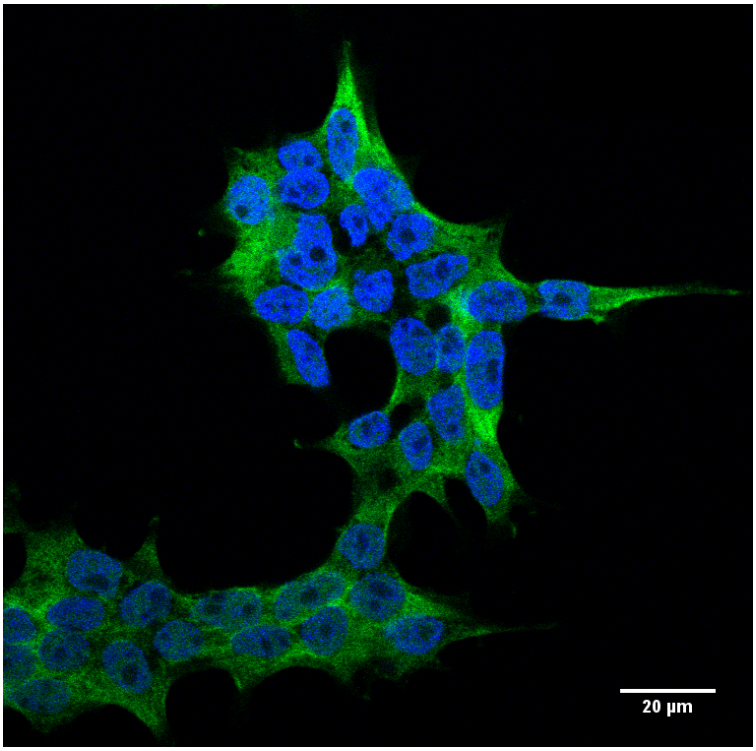

Probing new interaction partners for copper chaperones

A biophysical and immunochemical study with relevance in cancer

Master's thesis in Biotechnology

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Department of Biology and Biological Engineering
CHALMERS UNIVERSITY OF TECHNOLOGY
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Master's Thesis 2018
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Cover: Immunofluorescent cell staining of MEK1 in HEK293T cells with Alexa Fluor 488 and DAPI. Image taken with a confocal fluorescence microscope.

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Abstract

Copper (Cu) ions are cofactors found in many different proteins and are necessary for the proteins to be able to carry out their functions. However, Cu can also be harmful to cells as free Cu ions induce the formation reactive oxygen species, and Cu transfer is therefore closely regulated by special proteins called Cu chaperones. Cu is also connected to human disease as both Cu deficiency and Cu accumulation are harmful, and in recent years, Cu has emerged as a potential key factor in promotion of cancer as well. Since Cu chaperones are necessary for the distribution of Cu in cells, these proteins are also potential factors involved in Cu-dependent reactions in cancer. The purpose of this Master's thesis, was to investigate if the Cu chaperones Atox1 and CCS could interact with either of the proteins MEK1, S100A12, and the domain of CPEB4 containing its RNA recognition motifs (CPEB4-RRM). These proteins have previously been linked to cancer involvement, and if they interact with Cu chaperones, it could explain the connection between Cu and cancer. The protein-protein interactions were studied by combining three different techniques: far-western blot, co-immunoprecipitation and surface plasmon resonance. The results show that Atox1 is able to interact with all three proteins. The interaction to S100A12 is weak, and to CPEB4-RRM the interaction was detected only under specific conditions. MEK1 was able to interact with both Atox1 and CCS, with dissociation constants in micromolar range. Therefore, this thesis suggests that Atox1 and CCS are likely to deliver Cu to MEK1. This means both Atox1 and CCS are potential drug targets in treatments against cancer.

Keywords: Copper, Cu chaperone, cancer, protein-protein interaction, Atox1, CCS, CPEB4, MEK1, S100A12.

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Sebastian Valenzuela, Gothenburg, May 2018

Contents

List of Abbreviations

Atox1	Antixodant protein 1
CCS	Cu chaperone for SOD1
CD	Circular dichroism
Co-IP	Co-immunoprecipitation
CPE	Cytoplasmic polyadenylation element
CPEB4	Cytoplasmic polyadenylation element binding protein 4
CPEB4-RRM	Domain containing the two RRMs of CPEB4
Ctr1	Cu transporter protein 1
EDC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride
IF	Immunofluorescence
HEK293T	Human embryonic kidney cells 293T
HisAtox1	His-tagged Atox1
HisCCS	His-tagged CCS
MAPK	Mitogen-Activated Protein Kinase
MEK1	Mitogen-Activated Protein Kinase /Extracellular Signal-Regulated Kinase Kinase 1
NHS	N-hydroxysuccinimide
PAGE	Polyacrylamide gel electrophoresis
PVDF	Polyvinylidene difluoride
RRM	RNA recognition motif
RU	Resonance unit
SPR	Surface plasmon resonance
	Polyvinylidene fluoride

1
Introduction

In many enzymatic reactions, cofactors are necessary for the enzymes to be able to carry out their function. Among the several different kinds of cofactors, metals are one of the most commonly found in proteins. It has been estimated that around one third of all folded proteins contain a metal [1], making it an aspect of importance in the field of protein science.

One metal often coordinated in proteins is copper (Cu), where Cu ions acts as cofactors in enzymes involved in respiration, iron transport, antioxidative stress and pigmentation [2]. One of the main characteristics of Cu is the two oxidation states of its ions, Cu(I) and Cu(II), which enables Cu proteins to function as redox catalysts and electron carriers in biological systems. Interestingly, the two oxidation states also pose a threat to organisms, since changing between them may induce the formation of hydroxyl radicals, which can cause heavy damage to cells [2, 3]. To handle the issue of the toxicity of free Cu ions, organisms have developed a close regulation of Cu. Thus, free Cu ions are not present in cells but instead transported by certain proteins, called Cu chaperones [2, 3, 4]. This central role in Cu distribution makes the Cu chaperones very important proteins, since all enzymes which contain Cu in their mature form need to obtain the Cu from the Cu chaperones, either directly or indirectly.

In human cells, the uptake of Cu is controlled by Cu transporter protein 1 (Ctr1). Ctr1 transports Cu(I) ions through the membrane to the interior of the cell and delivers the Cu to one of three Cu chaperones: CCS, Cox17 and Atox1 [4, 5]. This creates three different pathways, where Cu is directed to specific targets. In the CCS pathway, Cu is delivered to cytoplasmic Cu/Zn superdioxide dismutase 1 (SOD1), while in the Cox17 pathway, the final target is the cytochrome c oxidase (COX), which is located in the mitochondria [2]. Atox1 transports Cu to two targets: the membrane bound proteins ATP7A and ATP7B located in the trans-Golgi network [2, 3]. Even though the Cu transporting is commonly accepted, there may still be additional functions of these Cu chaperones.

Cu is already known to be involved in human disease, where mutations in ATP7A and ATP7B causes Menkes syndrome and Wilson's disease respectively [2]. There have also been strong indications that Cu is involved in cancer, since Cu accumulation has been found in certain types of cancer tumors and several Cu-dependent proteins are vital for metastasis and angiogenesis in cancer progression [6]. Interestingly, reducing the influx of Cu to cancer cells has been proven to decrease tumor

growth [7]. Despite these findings, the actual role of Cu in cancer is not yet fully understood. However, since the Cu chaperones are essential for Cu transport in the cells, it is possible that these proteins are involved in cancer as well.

Aim

The aim of this project was to investigate the possible interactions between the Cu chaperones Atox1 and CCS and three proteins (CPEB4, MEK1 and S100A12), which all have connections to cancer progression. The purpose of this is to, potentially, identify new functions of the Cu chaperones, which may explain the role of Cu in cancer.

2
Background

2.1 Proteins

In this section, information is presented about the five proteins, which were of main interest in this study.

2.1.1 Atox1

Atox1 is a small protein, consisting of 68 amino acid residues, see figure 2.1 for the structure. After Cu is transported through the cell membrane by Ctr1, Atox1 binds Cu with two cysteines in a MXCXXC-motif (M=methionine, C=cysteine, X=any amino acid), and transports it through the cytoplasm to either of protein ATP7A or ATP7B [8]. The role of both these proteins is to transport Cu from the membrane into the lumen of the trans-Golgi network, where target proteins are loaded with Cu before they continue through the secretory pathway. Many important proteins such as clotting factors, tyrosinase and ceruloplasmin obtain Cu in the trans-Golgi network [2, 3], and thus indirectly depend on Atox1. Aside from being located in the cytoplasm, Atox1 has also been found in the nucleus, although its function is still not understood. In 2008 it was discovered that Atox1 can act as a transcription factor for *Ccnd1* (Cyclin D1) [9], but the published research concerning the function in the nucleus is limited. Nevertheless, this indication of Atox1 having additional functions means that the protein may have more undiscovered interaction partners. Identifying these possible binding partners can be the key to better understand the complete role of Atox1. Intriguingly, in 2015, several new interaction partners to Atox1 was identified using a yeast-2-hybrid screen. Many of the targets were DNA/RNA binding and/or related to cancer, which may mean that Atox1 is involved in transcriptional regulation and that it can be involved in pathology [10]. This finding goes along well with studies performed in embryonic development in mice. Disruption of the *Atox1* locus greatly affects the growth of the fetus and most of the mice progeny die after birth and/or has severe malformations [11]. Since, Atox1 seems to influence the development and survival of organisms, it is not unlikely for Atox1 to also be linked to diseases like cancer. In 2017, Atox1 was also proven to promote breast cancer cell migration [12], and Atox1 might therefore be a protein that connects Cu and cancer.

2.1.2 CCS

The copper/zink(Zn) superoxide dismutase 1 (SOD1) is a protein involved in the protection against oxidative stress by catalyzing the reaction of superoxide anions to hydrogen peroxide and oxygen. SOD1 is a homodimeric protein which requires the formation of a disulfide bond as well as the incorporation of both Cu and Zn for it to reach its mature form [13, 14]. The Cu chaperone for SOD1 (CCS) aids this process by directly interact with SOD1 and then deliver the Cu.

CCS contains three different domains, which all have different functions. Domain 1 is located in the N-terminal and its structure has a striking resemblance to Atox1 (see figure 2.1). Just like Atox1, Domain 1 also contains a MXCXXC-motif capable of binding Cu and domain 1 is therefore most likely responsible for carrying the Cu in CCS [14]. The second domain of CCS, domain 2, is homologous to SOD1, and the amino acid sequence of domain 2 is approximately 50 % identical to that of the SOD1 monomer [15]. Domain 2 is essential for CCS-SOD1 binding [14, 15], which is reasonable considering the similarity between domain 2 and SOD1 and the fact that SOD1 forms a dimer in its active form. Unlike the first two domains, the C-terminal domain 3 of CCS has no known resemblance to other proteins. It can bind Cu at a CXC-motif, but the affinity is not as strong as for the binding site in domain 1. The function of domain 3 has not been completely unraveled, however, domain 3 is not essential for Cu transfer, but appears to be involved in for the formation of the disulfide bond in SOD1 [14]. The crystal structure of CCS is shown in figure 2.2.

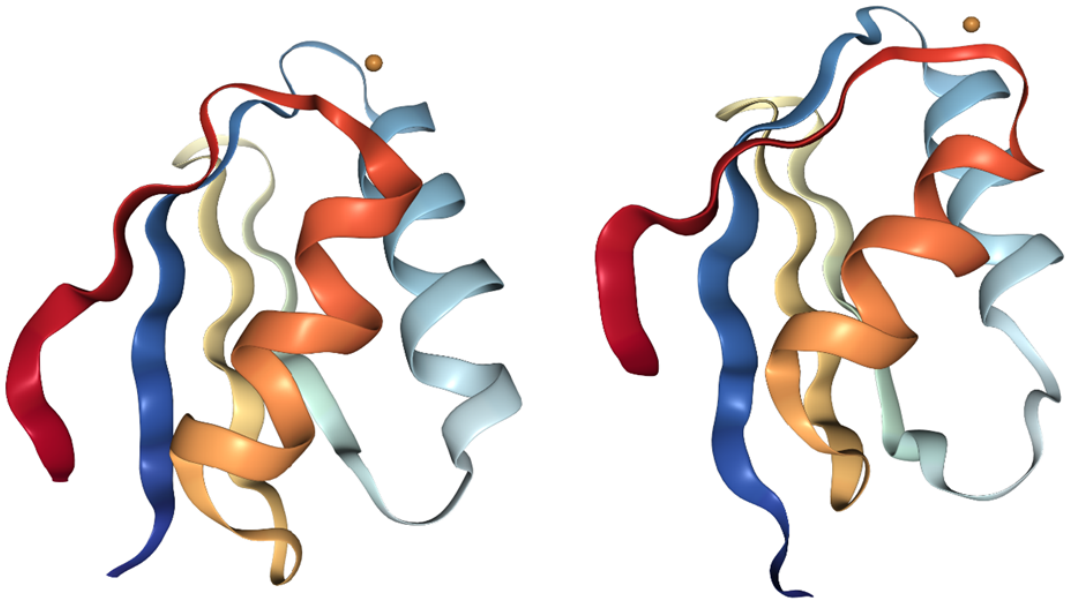

Figure 2.1: Solution structures of Cu-loaded Atox1 (left) and Cu-loaded domain 1 of CCS (right), which are strikingly similar. The Cu ion is located at the top of both structures. The structures (pdb ID: 1TL4 and 2RSQ) were obtained from the RCSB Protein Data Bank [16] and the NGL Viewer tool [17, 18] was used to visualize the structures.

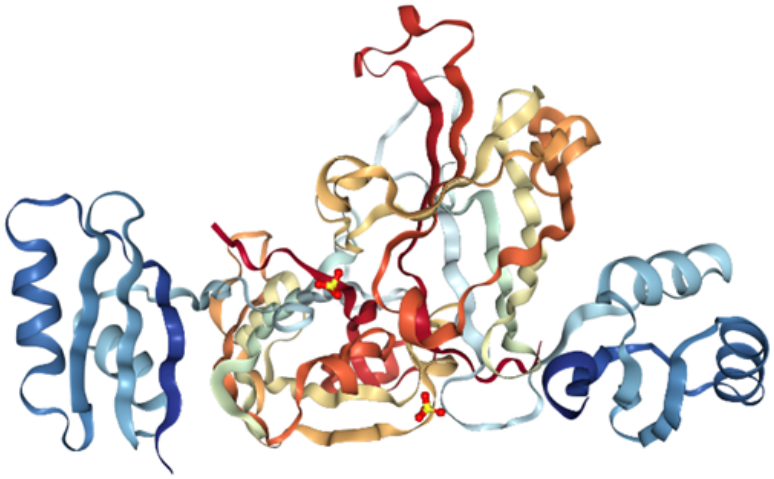

Figure 2.2: Crystal structure of CCS in homodimeric form. The blue sections consist of domain 1, and domain 2 and 3 are located in the center of the dimer. The structure (pdb ID: 1QUP) were obtained from the RCSB Protein Data Bank [16] and the NGL Viewer tool [17, 18] was used to visualize the structures.

Just like Atox1, CCS has been suggested to be involved in cancer. This is mainly because its target, SOD1, under recent years has come to the attention of cancer researchers. The rapid growth of cancer cells inadvertently leads to elevated levels of reactive oxygen species. To be able to handle this increased oxidative stress, proteins like SOD1 are necessary for the survival of the cancer cells [19]. This area of research is still relatively new, but the expression of SOD1 has been found to be increased in breast cancer cells [20]. As CCS is essential for the formation of mature SOD1, it indirectly has a role to play in cancer as well. Additional functions of CCS may extend this connection between CCS and cancer even further.

2.1.3 CPEB4

Cytoplasmic adenylation is a post-transcriptional regulation mechanism which affects mRNAs that contain a cytoplasmic polyadenylation element (CPE). In general, elongation of the poly(A) tail of the mRNAs activates the translation of the encoded protein. Cytoplasmic polyadenylation involves several different proteins, but for the process to start, a cytoplasmic polyadenylation element binding protein (CPEB) must first bind to the CPE [21]. The CPEB protein has two RNA recognition motifs (RRMs), which makes it possible for this binding to occur, see figure 2.3 for the structure of the RRMs. There are four known CPEB proteins (CPEB1-4), which all are paralogs and thus closely related [22]. CPEB4 was one of the proteins identified as a new interaction partner to Atox1 in the yeast-2-hybrid screen from 2015, and interestingly, the specific part of CPEB4 responsible for the binding to Atox1 was determined to be the RRM domain [10]. Considering that Atox1 seems to interact with the one part of CPEB4 which is important for its RNA binding capability,

it is not unlikely to assume that such an interaction could affect the translational regulation performed by CPEB4. CPEB4 has already been reported to activate the translation of mRNAs in pancreatic tumors, which are normally silenced in healthy cells, and thereby promotes tumor growth, cell migration and invasion [23, 24]. These findings make the possible interaction between the RRM domain of CPEB4 (CPEB4-RRM) and Atox1 highly interesting, and calls for further investigation.

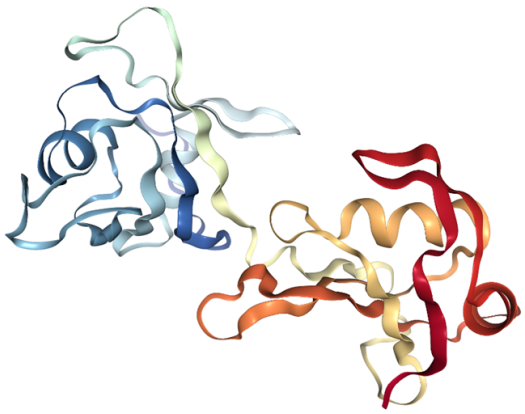

Figure 2.3: Crystal structure of the RRMs of CPEB4. The structure (pdb ID: 2MKJ) were obtained from the RCSB Protein Data Bank [16] and the NGL Viewer tool [17, 18] was used to visualize the structures.

2.1.4 MEK1

The Mitogen-Activated Protein Kinase (MAPK)/Extracellular Signal-Regulated Kinase (ERK) Kinase 1 (MEK1) is a copper binding protein involved in the MAPK signaling pathway, which controls several important cellular processes such as differentiation, proliferation and apoptosis. See figure 2.4 for the structure of MEK1. The function of MEK1 is to phosphorylate the MAPK, but first MEK1 itself needs to be activated through phosphorylation by the Raf protein [25], see figure 2.5. Increased intracellular uptake of Cu results in increased Cu-MEK1 mediated phosphorylation of MAPK, and thus also higher activity in the MAPK pathway [26]. The Cu binding property of MEK1 is a relatively new discovery and it is still not clear how MEK1 obtains Cu. The Cu chaperones Atox1 and CCS are strong candidates for delivering the Cu as they are located in the cytoplasm, just like MEK1.

Considering that up-regulation of the MAPK pathway is often found in tumors [27], the discovery that high levels of Cu increase the activity of the pathway becomes even more interesting. Inhibitors of the MAPK pathway protein B-Raf have already been approved for treatment of melanoma, and several of the other proteins in the pathway (MEK1 included) are now potential anti-cancer drug targets [28]. If Atox1 or CCS proves to deliver the Cu to MEK1, they will also become potential targets for drug treatment against cancer.

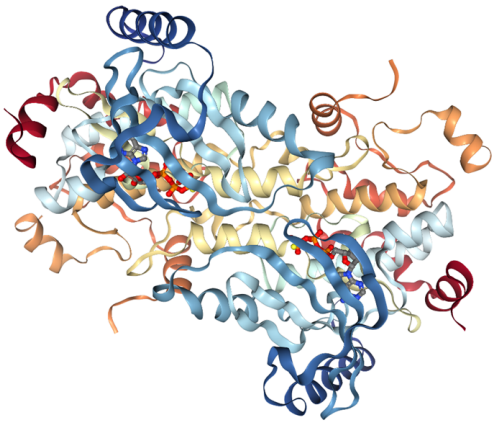

Figure 2.4: Crystal structure of MEK1 in homodimeric form. The structure (pdb ID: 3W8Q) were obtained from the RCSB Protein Data Bank [16] and the NGL Viewer tool [17, 18] was used to visualize the structures.

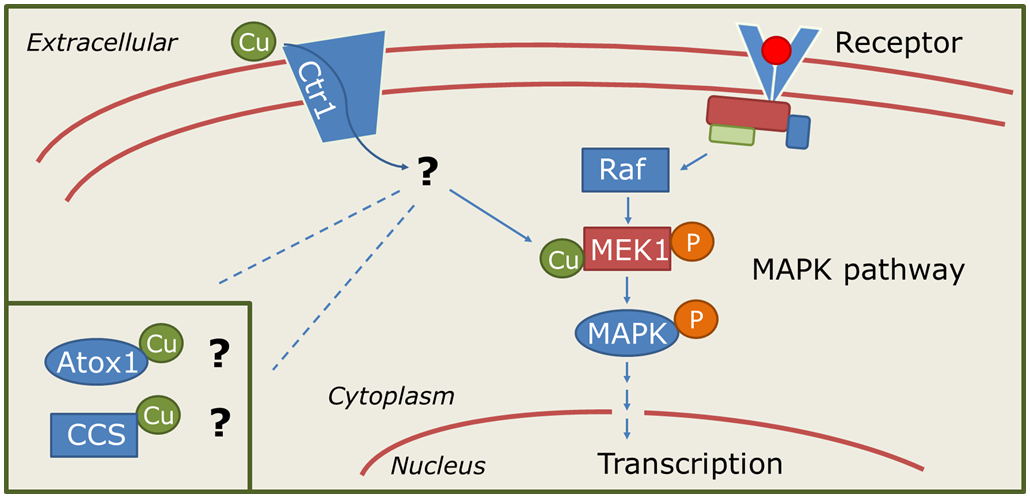

Figure 2.5: Cellular uptake of Cu is performed by Ctr1 and delivered to Cu chaperones, which transports Cu in the cytoplasm. MEK1 in the MAPK pathway can bind Cu, how it obtains Cu is unknown. Is Atox1 or CCS delivering Cu to MEK1?

2.1.5 S100A12

The S100A12 protein is a member of the S100 protein family, which consists of 25 calcium (Ca) binding proteins [29, 30]. See figure 2.6 for the structure of S100A12. The S100 proteins are involved in a number of intracellular activities such as proliferation, cell cycle regulation and cell migration. Due to the S100 proteins involvement in these key cellular processes, the S100 proteins have been suggested to play a role in cancer [30]. Apart from binding Ca, the S100A12 protein is also able to bind Cu and Zn [31], but whether these bindings affect the function of the protein is still to be investigated.

The S100A12 protein is mostly expressed in neutrophil granulocytes, and to some extent in monocytes, where the protein is involved in the innate immune response [32]. However, low expression of the protein have also been found to occur in cell lines derived from breast and lung tissue [33]. S100A12 is important for the Ca-

dependent activation of the granulocytes, although the responsible mechanism is not yet understood. However, S100A12 have been reported to be mainly expressed in the cytoplasm at low intracellular Ca concentrations, and relocated to the cytoskeleton and the cell membrane at elevated Ca levels [34]. S100A12 is also known to be extracellularly active, and is believed to be a ligand to the receptor for advanced glycation end-products (RAGE). When S100A12 binds to the RAGE it induces intracellular signal cascades, like the MAPK and the NF-κB pathways, and results in secretion of proinflammatory cytokines [29, 35].

S100A12 has been suggested to play a role in several different types of diseases, such as inflammatory, kidney and cardiovascular diseases [32]. Perhaps most interesting is that the expression of the S100A12 protein has been found the be increased in transitional cell carcinoma tissue [35]. If the Cu binding capability of S100A12 is involved with the cause of these diseases, how S100A12 obtains the Cu could be the key to effective treatments.

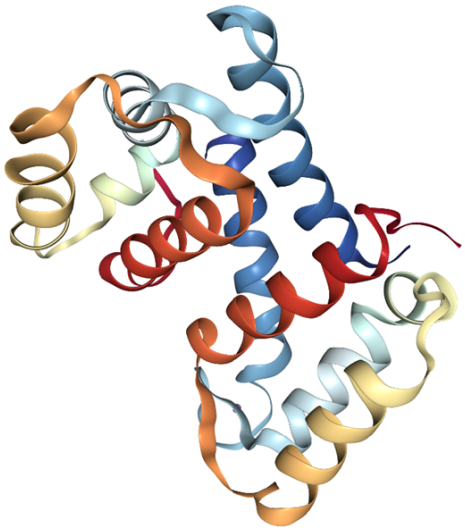

Figure 2.6: Crystal structure of S100A12 in homodimeric form without Cu. The structure (pdb ID: 2WCF) were obtained from the RCSB Protein Data Bank [16] and the NGL Viewer tool [17, 18] was used to visualize the structures.

2.2 Theory of methods

There are many different approaches when studying proteins-protein interactions. In this study, the experiments were mainly relying on biophysical methods, but also included cell studies. The theory of the different techniques are described in the subsections below.

2.2.1 Protein expression

The production of the proteins can be achieved by introducing a plasmid encoding the proteins into a bacterial host. The BL21(DE3) strain of *Escherichia coli* (*E. coli*) is a common host used for expressing proteins, due to its high capacity for protein production [36]. The BL21(DE3) strain contain the gene for T7 RNA polymerase, the expression vectors (e.g. pET-3a) used for protein production therefore often contain the T7 promoter (which requires T7 RNA polymerase for transcription). Because the T7 RNA polymerase is controlled by the lac promoter, it is possible to induce the expression of the proteins of interest by adding isopropyl β-D-1-thiogalactopyranoside (IPTG), after the cells have been cultured. The plasmid usually also contains an antibiotic resistance gene to make it possible to select for successfully transformed cells [37].

To simplify the purification of expressed proteins, the gene sequence encoding for six polyhistidines (the His-tag) is often introduced before or after the sequence for the protein of interest in the plasmid [38]. This approach was also be used in this project.

2.2.2 Protein purification of His-tagged proteins

For purification of the proteins, a series of chromatographic techniques can be used. If the protein is His-tagged (which was the case in this project), nickel-chelate-nitrilotriacetate (Ni-NTA) chromatography is first performed on the sample, since the His-tag binds strongly to the Ni-NTA chromatography column [38]. After the protein is attached to the column, it can then be eluted by adding imidazole, which competes with the protein binding to the column [39], and the protein is collected.

Following the Ni-NTA chromatography, an ion exchange chromatography can be performed to purify the protein further. Instead of binding the column by the His-Tag, the proteins will bind because of their charge, due to the column being either positively or negatively charged. By adding a gradient of counterions, the proteins can then be eluted in fractions, and the fractions containing the protein of interest can be collected [38].

Size exclusion chromatography, is usually the final purification step, where proteins are separated depending on size. The column consists of a matrix full of pores. Big proteins cannot enter the pores and will pass on the outside of them while smaller proteins can enter the pores and therefore take a longer path through the column. This leads to big proteins being eluted faster than smaller proteins and by collecting the elute in fractions, the protein of interest can be collected [38].

2.2.3 Absorption spectroscopy

In this project, it was important to determine the concentrations of the produced protein solutions and working solutions, to be able to perform any of the experiments

downstream in the process. In proteins, the aromatic side chains of tyrosine and tryptophan have a strong absorption around 280 nm. By illuminating a protein sample with light of that wavelength, and recording the absorbance, it is possible to calculate the concentration using Lambert-Beers law [38]:

$$A = \varepsilon * c * l \tag{2.1}$$

Where A is the absorbance, ε is the extinction coefficient ($M^{-1}cm^{-1}$), c the concentration (M) and l the length (cm) the light travels through the sample. The extinction coefficient is specific for each protein and has to be found in literature beforehand or predicted by the amino acid sequence of the protein [38].

2.2.3.1 Circular dichroism spectroscopy

Circular dichroism (CD) spectroscopy is a type of absorption spectroscopy, which is a useful technique when studying the secondary structure of proteins. The method relies on the use of circularly polarized light, which is light where the electric vector rotates around the direction of propagation, and how it can be polarized in both a left-handed and right-handed manner. If circularly polarized light is sent through a sample which consists of optically active components, e.g. a molecule with chiral centers, the left-handed and right-handed components of the light will be absorbed differently. This phenomenon is called circular dichroism, and the relationship between CD and the absorbance (A) of the two light components is expressed in the equation: $CD = A_{left} - A_{right}$. When circularly polarized light is sent through a sample of proteins, the chiral centers of the amino acids in the protein backbone will absorb the light in a characteristic way, depending on the secondary structure of the protein. This is due to that the ordered, or disordered, secondary structures induces a combined chirality. This makes CD spectroscopy a convenient method for identifying α-helices, β-sheets and random coils [38]. In this project, CD spectroscopy was used to determine if the produced proteins are folded and to study if the His-tag has an effect on the structure.

2.2.4 SDS-PAGE and native-PAGE

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE), is a common method for separating proteins according to size. The SDS is used to denature the proteins and also to negatively charge the proteins. One SDS molecule binds every two amino acids and therefore the charge per mass becomes approximately the same for all proteins. By adding the charged proteins to a gel made of polyacrylamide and then applying an electric field, the proteins will start to move towards the positive cathode. Since the polyacrylamide gel consists by a network formed by the polymeric structure, the proteins will travel through the gel at a different speed depending on size. Big proteins will be more retarded by the network while small proteins can travel faster. Due to the uniform mass-to-charge ratio created by the SDS solution, the proteins are separated solely depending on mass and not by electric charge. This way the proteins are separated, and by also including a

marker of known protein weights, the relative mass of the unknown proteins can be detected. To be able to detect the protein bands, the proteins need to be stained, for instance with Coomassie Brilliant Blue [38]. SDS-PAGE was used together with many of the other techniques in this project, mainly to verify that the protein size.

In a native-PAGE, the purpose is to keep the original structure of the proteins, and because SDS denatures the proteins, it can not be used to negatively charge the proteins. Instead, the proteins' native charge is used for separating them. However, the isoelectric point of the proteins have to be below the pH of the running buffer, otherwise the proteins will be positively charged and thus run in the wrong direction in the gel. Unlike for the SDS-PAGE, the mass-to-charge ratio of the proteins will not be uniform in a native-PAGE, and it is therefore not possible to use a marker to determine the size of the different proteins. To identify the separated proteins, other methods like western blot is necessary to perform after the native-PAGE [38].

2.2.5 Western blot

Gel electrophoresis can be used on its own to give information of the size of proteins but does not give any information about what specific proteins are present. In this study, it was important to both ensure that the right proteins were produced, which was done with western blot. After the gel elecrophoresis is performed, instead of staining the gel, the separated proteins are transferred from the gel to a membrane of nitrocellulose or polyvinylidene difluoride (PVDF). This is done by electro-blotting, where the gel and the membrane are mounted between two electrodes (between stacked paper soaked in buffer). An electric current is then applied and the negatively charged proteins are transferred to the membrane. When the proteins are on the membrane, specific antibodies can be used to detect the proteins. Generally, the blot is first incubated with a primary antibody which binds the protein of interest. The primary antibody is produced by exposing a host (often rabbit or mouse) to the protein of interest. By then using a secondary antibody, which binds specifically to the antibodies (in this case the primary antibodies) produced by a certain host, the bands of the proteins can be visualized. The secondary antibody usually has a conjugate which can be used for detection, for instance horseradish peroxidase that can catalyze a chemiluminescent reaction [38].

2.2.6 Far-western blot

An extension of western blot is far-western blot, which can be used to identify protein-protein interactions. After gel electrophoresis and transfer of the first protein (the prey) onto the membrane, the membrane is incubated with the predicted interaction partner (the bait). If interaction occurs, the bait will remain bound to the prey after washing and can be seen as a band at the location of the prey, after antibody detection of the bait. The location of the prey should be determined beforehand with a protein stain for membranes or a western blot run in parallel. Even if the far-western blot procedure is quite straightforward, some optimization may

be required to obtain a positive result. Since the prey protein is denatured during the SDS-PAGE, there may not be any interaction unless the protein is refolded. Renaturation of the protein generally occur when SDS is removed during the transfer step of the proteins from the gel to the membrane [40]. However, at least the interaction site of the prey needs to be refolded for the far-western blot to work, and this may not be the case even if the proteins are only partly refolded. If so, the gel must be run under non-denaturing conditions (native-PAGE) to ensure proper structure of the protein, or a renaturing step must be included. Another issue is if the interaction between the bait and the prey is very weak, which could result in the bait being washed away during the wash steps. To solve this problem, the bait can be covalently bound to the prey using a crosslinking agent [40]. An overview of far-western blot method is shown in figure 2.7.

In this project, the far-western blot was used to identify the protein-protein interactions to determine which partners that were of interest for further investigations.

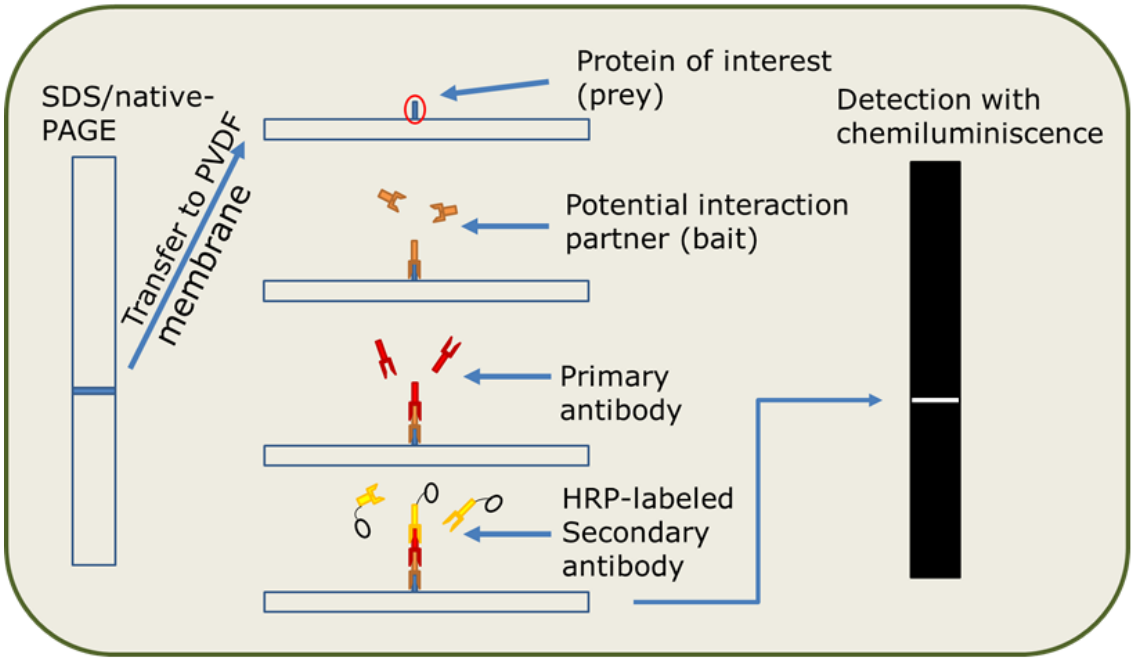

Figure 2.7: An overview of far-western blot, a method which can be used to identify new protein-protein interactions.

2.2.7 Co-immunoprecipitation

Immunoprecipitation (IP) is a technique used for isolating one protein from a solution containing a mix of proteins, e.g. cell lysate. Antibodies specific to the protein are crosslinked to either agarose or magnetic beads. When beads are incubated in the mix of proteins, the protein of interest will bind to the antibodies and thus also be attached to the beads. The beads can then be precipitated by either centrifugation (agarose beads) or by using a strong magnet (magnetic beads), and the supernatant can be removed. The beads are then washed a couple of times before the protein is eluted, for instance by using a low pH elution buffer. A western blot is usually

performed afterwards, to verify that the desired protein is in the elution. Protein complex immunoprecipitation (co-IP) is very similar to the original IP method. Co-IP is used to target a protein which forms a complex or strongly interact with other proteins. The interaction partners will thus also be immunoprecipitated along with the protein. If the possible interaction of a specific protein is of interest, western blot can be employed for the detection. However, if there are no predicted interaction partners, mass spectrometry can be used to identify new interaction partners. Co-IP is an effective method for detecting protein-protein interaction in cells. The main disadvantage is that the technique works mostly for strong interactions, and weaker interactions may not be detected [41]. However, weak interactions can be maintained by using a crosslinking agent prior to lysing the cells, but this may also generate false positives [42]. In this project, Co-IP was used to investigate if the protein-protein interactions occurs within cells.

2.2.8 Surface plasmon resonance

Surface plasmon resonance (SPR) is a method relying on a phenomenon which occurs when a light beam, of a certain wavelength and at a specific angle, is directed towards a thin metal surface. When the light hits the surface, the electrons in the metal will absorb some of the light, start to resonate and become so called surface plasmons. Since some of the light is absorbed, the reflected light will have a lower intensity and this makes it possible to detect at what angle the surface plasmons are formed by monitoring the reflected light beam with a detector. An important aspect of SPR is that the angle necessary to achieve the SPR effect is dependent on the refractive index of the surface. In a SPR instrument, this is used to study interactions between two molecules where one is immobilized to the surface (ligand) and one (analyte) is allowed to flow over the surface, see figure 2.8. The surface is usually on a sensor chip and made of a thin film of gold covered with dextran, to which the ligand is attached either transiently or permanently, through e.g. covalent binding. If the analyte interacts with the ligand while flowing over the surface, the mass on the surface will change. This increases the refractive index of the surface and thus changes the angle necessary to produce the SPR effect.The detector monitors the new angle where the intensity loss occurs, and the result is presented in a sensorgram by expressing the angle in so called resonance units (RU) over time. If the mass at the surface of the chip is increased by roughly 1 ng*mm^{-2}, it corresponds to about 1000 RU [38].

Attaching the ligand to the surface may be difficult, and in some cases it is necessary to covalently bind the ligand to the surface. This modification is not always optimal because it can affect the ligand-analyte interaction. However, SPR is still a very useful method since it does not require introduction of other factors, like a label on the molecule of interest, that can affect interactions [38]. Since both the association and dissociation is thoroughly monitored during SPR measurements, it is possible to obtain kinetic parameters like the association and dissociation rate constants. From these parameters, the binding affinity, in the form of the dissociation constant

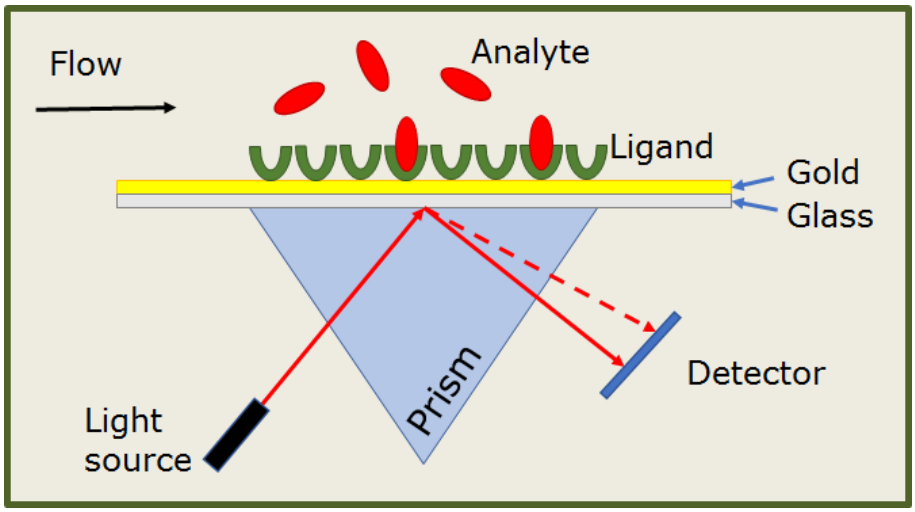

Figure 2.8: The SPR method can be used to study binding characteristics of protein-protein interactions. When analyte binds to the ligand, the refractive index of the surface changes and the refracted light is detected at a new angle.

(K_d), can also be obtained [38]. In this project, SPR was used for characterizing the interactions that were identified with far-western blot

2.2.9 Confocal fluorescence microscopy

Confocal fluorescence microscopy is a method commonly used to study the subcellular distribution of proteins in cells, by using fluorescence. However, due to that proteins are generally not fluorescent themselves, they first need to be labelled with a fluorescent probe to be able to be visualized. Since the technique relies on fluorescence, confocal microscopes are equipped with a laser, which excites the probes and makes them visible during emission. The laser only hits one point of the sample at a time and when the whole sample has been scanned and emission detected, all collected points are constructed into a full image. The main difference between a confocal microscope and other fluorescent microscope techniques, is a spatial filter in the form of a small pinhole, which is located in front of the detector. This filters out any emitted light from above and under a specific focal plane and makes it possible to examine cross sections of cells. This also makes it possible to visualize the cells in 3 dimensions, which is one of the main advantages of the technique [38].

Immunofluorescence (IF) cell staining is a commonly used method, prior to confocal fluorescence microscopy, for labelling the proteins with the fluorescent probes. Primary antibodies are used to bind specifically to the protein of interest, which is then followed by binding of secondary antibodies. The secondary antibodies are conjugated to a fluorescent probe, which can be detected with a confocal fluorescence microscope [38].

3
Methods

3.1 Overview

The project was roughly divided into three parts: protein production, studying protein-protein interactions and investigating the subcellular distribution of the proteins. Within each sub-project, one or several experimental techniques were used, thus creating three different experimental pathways. An overview of the whole process can be seen in figure 3.1.

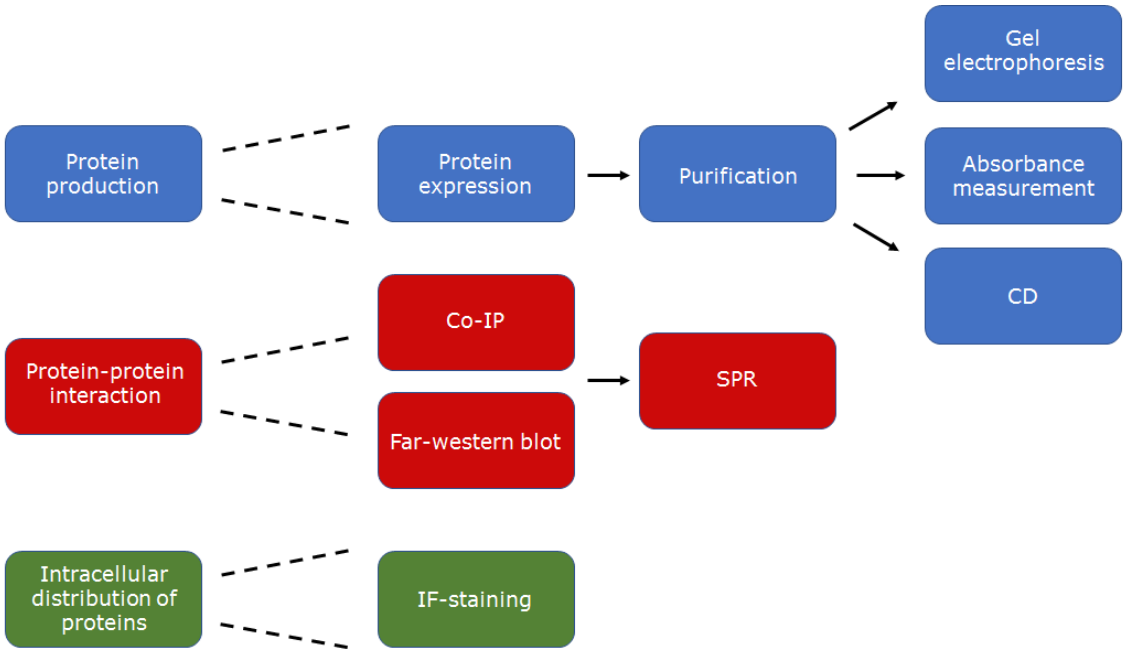

Figure 3.1: An overview of the three main sub-projects and the techniques used for the different parts.

3.2 Estimation of protein characteristic parameters

For the project, the following protein parameters (see table 3.1), were necessary to be able to perform some of the experiments. All of the values are calculated based on the amino acid sequences [43] with the ProtParam tool [44]. See appendix A.3 for the amino acid sequences. Some of the proteins also have been modified to have His-tags at the N-terminal. In this thesis, these proteins have "His" added as a prefix

to the name.

Table 3.1: Molecular weights (MW), isoelectric points (pI) and extinction coefficients (ε) of the proteins studied in this project.

	MW (kDa)	**pI**	ε **($M^{-1}cm^{-1}$)**
Atox1	7.4	6.70	2980
HisAtox1	9.0	6.22	2980
HisCCS	30.5	5.77	12490
CPEB4-RRM	29.1	5.45	25900
MEK1	43.4	6.18	24410
S100A12	10.5	5.81	2980

The values were estimated based on the amino acid sequences using the ProtParam tool[44].

3.3 Expression and purification of proteins

The BL21(DE3) *E. coli* strain was used for producing the protein domain containing the two RRMs of CPEB4 (CPEB4-RRM) as well as His-tagged Atox1 (HisAtox1). CPEB4-RRM was originally His-tagged, but it was cleaved off during the purification process. The expression vector (pET-3a), which contained a T7 promoter, a carbenicillin resistance gene and the inserts, was already transformed into the *E. coli* at the start of this project (see appendix A.1, A.2 and A.3 for plasmids, inserts and amino acid sequences). The *E. coli* cells were cultivated in lysogeny broth with carbenicillin (100 µg/mL), to ensure that the plasmids were maintained. When culture reached the exponential growth phase (OD_{600} = 0.6), IPTG was added to induce protein production. See appendix B.1 for a detailed protocol of the protein expression.

The other proteins used in this project (MEK1, HisCCS and S100A12) were either bought or provided as gifts from other research groups. Native Atox1, was produced in house, in a similar process as described in this section.

After expression of the proteins, the cell cultures were concentrated by centrifugation and a protease inhibition cocktail (Roche) was added to prevent protein degradation. The cells were lysed by sonication and nucleic acids were digested by addition of a universal nuclease (Pierce). The proteins were then purified in three steps with an ÄKTA Purifier (GE Healthcare). First, the proteins were purified with Ni-NTA chromatography, using a gradient of imidazole (5 mM - 1 M) in 20 fractions to elute the His-tagged proteins from a HisTrap FF (5 ml) Ni-NTA column (GE Healthcare). The fractions containing the proteins were collected and mixed. Afterwards, the His-tag of CPEB4-RRM was cleaved off with caspase 7. The second step was ion exchange chromatography, where the proteins were loaded onto a Q Sepharose Fast Flow (5 ml) column (GE Healthcare) and then eluted by using a gradient of NaCl (50 mM - 500 mM) in 20 fractions. The fractions containing the proteins were

collected and mixed. In the final step, the proteins were purified with size exclusion chromatography (SEC) on a HiLoad 16/600 Superdex 75 pg column (120 ml) (GE Healthcare), and the fractions containing the proteins were again collected and mixed. After each purification step, the size and purity of the purified samples were verified with gel electrophoresis (constant voltage, 200 V for 35 minutes). After the SEC, the samples were concentrated with 3 kDa (HisAtox1) and 10 kDa (CPEB4-RRM) Amicon Ultra Centrifugal filters (Sigma-Aldrich).

The purified proteins were confirmed to be the correct ones (HisAtox1 and CPEB4-RRM) with western blots, and HisAtox1 was also compared to native Atox1. Gels were run at constant voltage, 200 V for 35 minutes and the proteins transferred to a 0.2 µm PVDF membrane by using the Trans-Blot® Turbo™ RTA Mini PVDF Transfer Kit and the Trans-Blot® Turbo™ Blotting System according to the manufacturer's instructions. Primary antibodies for Atox1 (Abcam) and CPEB4 (Novus Biologicals) were used with the Thermo Scientific™ Pierce™ SuperSignal West Femto Fast Western Blot Kit (Thermo Fisher Scientific), according to the manufacturer's instructions, and a ChemiDoc MP (Bio-Rad) to detect the proteins. The concentration of the purified proteins were determined by measuring the absorption at 280 nm, with a Cary 50 bio UV-visible spectrophotometer, see section 3.4. The protein samples were also tested for DNA contamination by studying the A260/A280 ratio, where values below 1 were considered acceptable. The proteins were finally stored in 500 µL aliqouts at -80 °C, by flash freezing the samples with liquid nitrogen. See appendix B.2 for a detailed protocol of the purification.

3.4 Absorption spectroscopy

For the absorption spectroscopy, a Cary 50 bio UV-visible spectrophotometer was used at the medium setting to measure the absorbance between 200 - 300 nm. Baseline correction was performed before the measurements, where protein-free buffer was used for the baseline. A NanoDrop 1000 was also used for the absorption spectroscopy, using the Protein A280 setting and protein-free buffer for the baseline.

3.5 Circular dichroism

A Chirascan circular dichroism spectrometer (Applied Photophysics) was used to investigate if the proteins CPEB4-RRM and HisAtox1 were folded. HisAtox1 was also compared to native Atox1, to see if the secondary structure was affected by the His-tag. CD data was collected between 190-300 nm at a step interval of 1 nm. The experiment was performed at room temperature under constant flow of nitrogen, with time-per-point set to 1 s. Each sample was recorded 5 times and then averaged. The buffer for the proteins consisted of 20 mM Tris (pH 7.4), 50 mM NaCl and 1 mM TCEP, and the CD of the buffer alone was recorded and subtracted from the CD of the proteins. The data were then converted into mean residue ellipticity $[\theta]_{MRE}$ according to Kelly et al. (2005) [45], and plotted against the wavelength

between 195-300 nm.

3.6 Far-western blot

The far-western blots were carried out under both denaturing and non-denaturing conditions. The proteins used for the different conditions are summarized in table 3.2, and the procedures are described in the subsections below.

Table 3.2: Concentrations of the baits and the amount of prey loaded per well for the different experiments.

Condition	Bait	Concentration	Prey	Protein/well
Denaturing	Atox1	5.44 µM	CPEB4	0.5 µg
Native	Atox1	5.44 µM	CPEB4	0.5/1.0 µg
			MEK1	0.5 µg
			S100A12	0.33 µg
Native	CCS	15 µM	MEK1	0.5 µg

3.6.1 SDS-PAGE far-western blot

Far-western blots under denaturing condition was only performed with the CPEB4-RRM protein as prey and Atox1 as bait. The far-western blots were performed in three steps: SDS-PAGE, blotting to membrane and finally protein or antibody incubations. NuPAGE™ 4-12 % Bis-Tris protein gels (Thermo Fisher Scientific) were used for the SDS-PAGEs and run at constant voltage (200 V) for 35 minutes. The loading samples consisted of NuPAGE™ LDS Sample Buffer, protein samples and MilliQ water. 0.5 µg protein was added per well for CPEB4-RRM.

Electro-blotting of the proteins from the gel to a PVDF membrane was performed with a Trans-Blot® Turbo™ Blotting System (Bio-Rad) at 1.3A and 25 V for 7 minutes.

After the transfer, the membranes were processed under either of these different conditions:
1. Control, where the membrane was incubated in buffer (1mM DTT, 50 mM Tris pH 7.5) at 4°C overnight.
2. Incubation in Atox1 solution (5.44 µM Atox1 in buffer) at 4°C overnight.
3. Overnight incubation at 4°C in Atox1 solution (5.44 µM Atox1 in buffer), also containing $CuCl_2$ (5 µM).

Pierce protein staining for membranes (Thermo Fisher Scientific) was performed in parallel on an identical membrane to ensure proper transfer of proteins. After the incubation in Atox1 solution, all membranes were briefly washed in MilliQ water.

A 3 mM solution of crosslinking agent, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), was then added to some membranes and incubated at room temperature for 1 hour. The crosslinking was used in case the interaction between Atox1 and CPEB4-RRM was too weak to be maintained through all the necessary washing steps in far-western blotting.

To determine if the CPEB4-RRM on the membranes were binding Atox1, primary rabbit antibody solution of Atox1 in 1:1000 dilution (Abcam) was used for the immunodetection step of the far-western blots. This was performed with the Super-Signal West Femto Pierce Fast Western Blot Kit, Rabbit (Thermo Fisher Scientific), according to the manufacturer's instructions. A ChemiDoc MP (Bio-Rad) was used for obtaining the images. See appendix B.3 for a detailed protocol of the SDS-PAGE far-western blot.

3.6.2 Native-PAGE far-western blot

The far-western blots under native conditions were performed with CPEB4-RRM, MEK1 and S100A12 as prey and Atox1 as bait. CCS was also used as bait, but only with MEK1 as prey. The procedure was very similar to the SDS-PAGE far-western blot. However, the gels used were Novex™ 14 % Tris-Glycine Mini Gels and the running buffer were Tris-glycine (25 mM Tris and 192 mM glycine) at pH 8.3. The running conditions were also changed to a constant voltage of 60 V for 2 hours, and then increased to 70 V until the electrophoresis was complete (i.e when the loading dye reached the bottom of the gel). The amount of prey protein loaded on the gel is presented in table 3.2

The electro-blotting procedure was carried out in the same way as the SDS-PAGE far-western blot, except that the transfer buffer was exchanged (25 mM Tris and 200 mM glycine, pH 8.3). After the transfer, the membranes were treated in the same way as in the SDS-PAGE far-western blot. However, for the MEK1 membrane used with CCS as bait, the CCS concentration was increased to 15 µM. See appendix B.4 for a detailed protocol.

3.7 Cell culturing

Human embryonic kidney cells 293T (HEK293T cells) were cultured at 37°C and at 5 % CO_2 in a high glucose (4.5 g/L) Dulbecco's Modified Eagle Medium (Life technologies). The medium was supplemented with 10 % (v/v) fetal calf serum (FBS), 4 mM L-glutamine and 1 mM sodium pyruvate. Cells were split twice a week and diluted 1:20. At the time of splitting, the cells were approximately 80 % confluent.

3.8 Co-immunoprecipitation

The co-IPs were performed by crosslinking MEK1 antibodies (0.1 µg) to magnetic beads (Pierce Magnetic Protein A/G beads, Thermo Fisher Scientific) with bis(sulfosuccinimidyl)suberate (BS3) crosslinking agent. HEK293T cells were lysed (see appendix B.9) and the cell lysate was pre-cleared by incubation with fresh magnetic beads to remove the proteins that bind the beads non-specifically. A modified procedure was also used, where the HEK293T cells were treated with disuccinimidyl suberate (DSS) to crosslink interacting proteins before lysing the cells (see appendix B.9). The antibody-crosslinked beads were blocked with Roti-Block (Carl Roth) and then incubated with the pre-cleared lysate. Afterwards, the non-bound fraction of the lysate was collected and the beads washed. The proteins bound to the beads were then eluted with a low pH elution buffer. Both the washes and the eluate was collected.

To verify that MEK1 had been immunoprecipitated, western blots were performed (see appendix B.8), using primary antibodies againt MEK1 (Abcam) at 1:1000 dilution. Primary antibodies for Atox1 (Abcam) and CCS (Santa Cruz Biotechnology) were used at 1:1000 and 1:250 dilution, respectively, to potentially detect that Atox1 or CCS had been co-immunoprecipitated with the MEK1.

3.9 Surface plasmon resonance

Surface plasmon resonance was used to study the interaction between MEK1 and HisAtox1 or HisCCS. A Biacore X100 (GE Healthcare) was used for the experiment. The immobilization of MEK1 was performed with the amine coupling procedure using a mixture of NHS (N-hydroxysuccinimide, 0.05 M) and EDC (0.4 M) to activate the carboxyl groups on the surface. MEK1 was diluted to 20 µg/ml in sodium acetate buffer (pH 4.5) and immobilized at the surface of a CM5 sensor chip (GE Healthcare) with a final surface concentration of 2000 RU (approximately 2 ng/mm^2). Deactiviation of the carboxyl groups after immobilization was performed using ethanolamine.

The interaction between MEK1 and either of Atox1 and CCS was studied with single cycle kinetics, i.e the analyte concentration was increased in five steps with time for dissociation between each injection. The contact time for each injection was 180 s and the dissociation time was 600 s. Regeneration at the end of the experiment was performed with 50 mM NaOH with a contact time of 30 s. The protein concentration of Atox1 and CCS for the 5 injections was doubled each time (2.5 µM - 40 µM). DTT was used as reducing agent at a 5:1 ratio to protein concentration. The experiments were repeated with Cu ($CuCl_2$) added to the analyte solution at a 0.9:1 ratio to protein concentration. The running buffer, HBS-P+ (GE Healthcare), was used for diluting the samples.

After the experiments, the data was processed with the BIAevalutaion software. The data was fit to the 1:1 binding model to obtain the interaction characteristics

(rate constants, dissociation constant etc.) of the binding. Spike values (caused of the injections) were removed, and in some cases the first and second binding curves were excluded, to obtain better fitting. The binding characteristics of at least two separate experiments, were averaged.

3.10 Immunofluorescence cell staining

IF cell stainings were performed to study the subcellular location of the proteins in HEK293T cells. Atox1, CCS, CPEB4 and MEK1 were all stained in HEK293T cells. The cells were seeded on poly-L-lysine coated coverslips (18 mm) in 12-well plates (75 000 cells/well). The cells were fixed with 4 % paraformaldehyde and then permeabilized with 0.1 % Trition X-100 in PBS. The cells were blocked in 10 % goat serum and glycine (22.52 mg/mL) in PBST. The primary antibodies for Atox1 (Abcam), CCS (Santa Cruz Biotechnology), CPEB4 (Sigma-Aldrich) and MEK1 (Abcam) were diluted in PBST with 1 % goat serum at different dilutions, see table 3.3. The coverslips were incubated with primary antibodies overnight at 4°C, and then washed in three times in PBST. After the washes, the coverslips were incubated, in the dark, with the secondary antibody (Alexa Fluor 488, Thermo Fisher Scientific) diluted in PBST with 1 % goat serum (see table 3.3) for 1 hour at room temperature. The coverslips were then washed three times with PBST and then mounted on glass microscope slides with ProLong™ Diamond Antifade Mountant with DAPI (Invitrogen), before examination with confocal fluorescence microscopy, see section 3.11. Protocols for the coating of coverslips, seeding of cells and the IF-staining can be found in appendix B.5, B.6 and B.7.

Table 3.3: Dilutions of the primary and secondary antibodies used for the immunofluorescent cell stainings.

Antibody	**Atox1**	**CCS**	**CPEB4**	**MEK1**	**Alexa Fluor 488**
Dilution	1:1000	1:100	1:100	1:200	1:1000

3.11 Confocal fluorescence microscopy

To visualize the fluorescence-stained proteins, an Eclipse Ti inverted research microscope (Nikon) was used, equipped with a Nikon 60X/1.4 λS oil immersion objective. The Alexa Fluor 488 and the DAPI fluorophores were excited with a laser at 488 nm and 405 nm, respectively. The acquisition settings were identical for all samples, and two dimensional images were taken with a 1024 X 1024 pixel resolution. The image processing was carried out with the NIS Elements Advanced Research imaging software.

4

Results and Discussion

4.1 Expression and purification of proteins

The expression and purification was performed to obtain pure protein samples of HisAtox1 and CPEB4-RRM, which would then be used for the protein-protein interaction studies. The results from the final purification step (the SEC) for HisAtox1 and CPEB4-RRM are shown in figure 4.1. For both HisAtox1 and CPEB4-RRM, there is evidently only one band visible after the final purification step. The purified proteins also had approximate molecular weights close to the expected sizes (see table 3.1), as the proteins are slightly below 10 kDa (HisAtox1) and around 30 kDa (CPEB4-RRM).

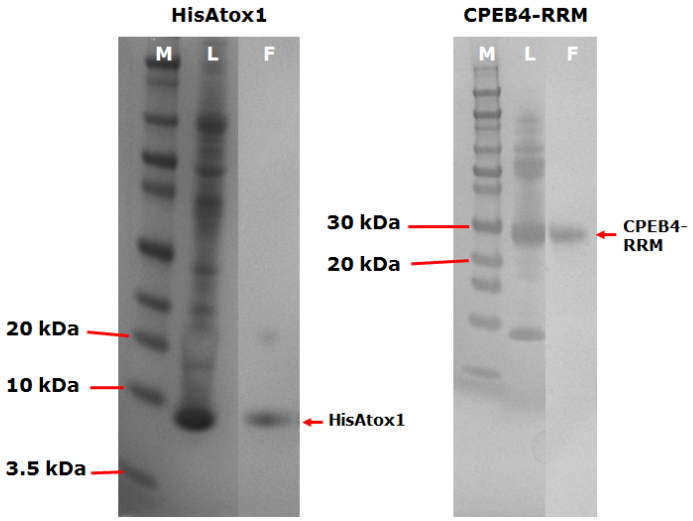

Figure 4.1: Gel electrophoresis after the final SEC. The marker (M) was used to confirm that the purified proteins were of the correct size. The load (L) was the sample before performing the SEC and the fraction (F) is one of the fractions containing the purified protein afterwards.

By comparing the loads with the purified fractions in figure 4.1, it is apparent that a lot of protein was lost in the purification process, which was not optimal. However, it is worth to emphasize the fact that even though two purification steps preceded the SEC, there were still a lot of impurities left (i.e. in the load). This justifies the necessity of using this extensive purification process. Despite the loss of proteins, the amount of proteins obtained (3 mg of HisAtox1 and 1 mg CPEB4-RRM from 2 L cell culture each) were sufficient for the protein-protein interaction experiments.

The purified proteins were investigated with western blot, see figure 4.2, to verify that the proteins were the correct ones. For Atox1 and HisAtox1, two strong bands are visible between 3.5 kDa and 10 kDa, which is where these proteins are expected to be located. The band for HisAtox1 is slightly above the band for Atox1, which is due to that the His-tag increases the size of the protein from 7.4 kDa to 9.0 kDa. Interestingly, a faint band is also visible between 15 kDa and 20 kDa in the HisAtox1 lane. Considering that dimerization of Atox1 have been reported before [46], this band is most likely a HisAtox1 dimer, which forms a 18 kDa complex.

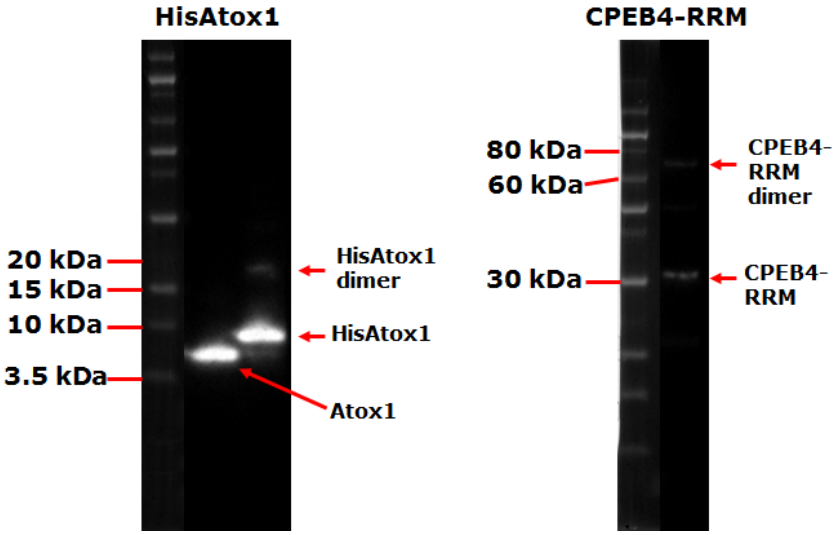

Figure 4.2: Western blots for Atox1, HisAtox1 and CPEB4-RRM. Atox1 was included for comparison with HisAtox1.

In the CPEB4-RRM western blot, it was also possible to detect two bands. One band was located at approximately 30 kDa, and this corresponds well with the expected size of 29 kDa for CPEB4-RRM. The faint band however, is located at around 70 kDa, which is slightly larger than the expected size of 60 kDa. Nevertheless, a dimer could be expected to behave unpredictably during gel electrophoresis, as it is not fully denatured by the SDS (if it was it would not form a dimer). This would explain why the dimer can be seen at 70 kDa, and not 60 kDa. Furthermore, since the CPEB4 specific antibody is able to bind to this suspected protein complex, it likely consists of two CPEB4-RRMs. Dimerization of the RRM containing domain has also been reported before [22], and it was therefore assumed that this was the case.

For the protein-protein interaction experiments, the dimers could possibly be problematic and affect the results. However, since the fraction dimer was relatively small compared to the monomer, the protein samples were considered acceptable. The most important thing was to prove that the purified proteins were in fact HisAtox1 and CPEB4-RRM.

4.2 Circular dichroism

The purpose of the CD experiments was to confirm that the produced proteins, HisAtox1 and CPEB4-RRM, were folded and to see if the His-tag on HisAtox1 affected the structure. The results are presented in two graphs, and these are shown as figure 4.3 and 4.4. In both figures, there is a positive CD signal at around 195 nm and a large negative CD signal that stretches from approximately 200 nm to 240 nm.

A negative peak at 200 nm is usually seen for proteins with unordered structure. A negative peak between wavelengths 210-225 nm however, indicates that the proteins contain either (or both) α-helices or anti-parallel β-sheets [45]. In both figure 4.3 and 4.4, the negative CD signals were not centered around 200 nm, but rather shifted to higher wavelengths. Furthermore, the negative peak at 208 nm with a shoulder at 222 nm in figure 4.3 indicates α-helical content in the CPEB4-RRM, while the negative peak in figure 4.4 indicates a mix of α-helices and anti-parallel β-sheets in Atox1. Therefore, both proteins were in a folded state and could be used for the protein-protein interaction experiments.

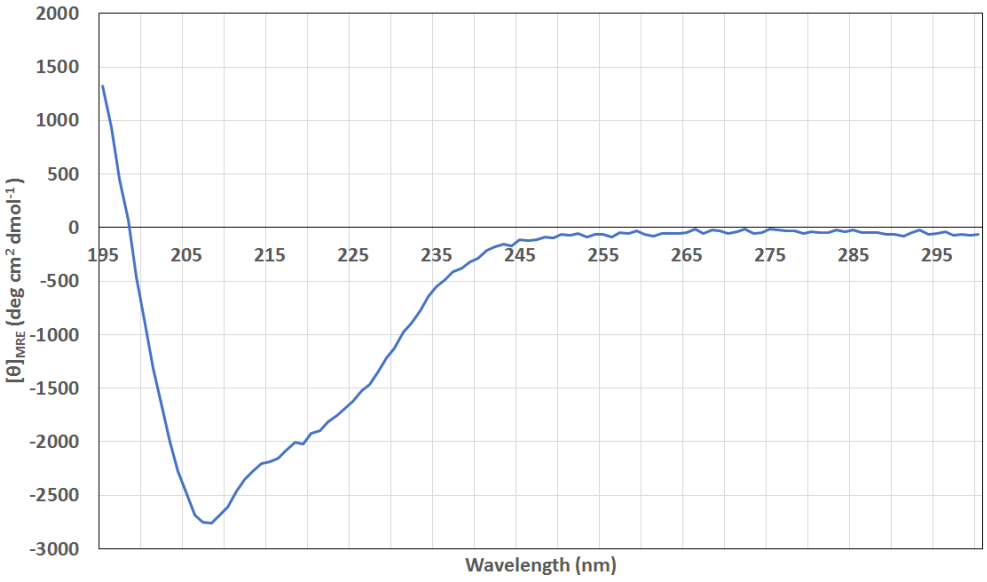

Figure 4.3: CD data, for the CPEB4-RRM sample, shown as mean residue ellipticity $[\theta]_{MRE}$.

The produced HisAtox1 was also compared to Atox1, and the plots indicate a very similar structure between the two (see figure 4.4). The main difference can be seen between 210-225 nm, where Atox1 has a longer negative CD signal than HisAtox1. However, the difference was very small and thus the His-tag does not a affect the Atox1 folded state.

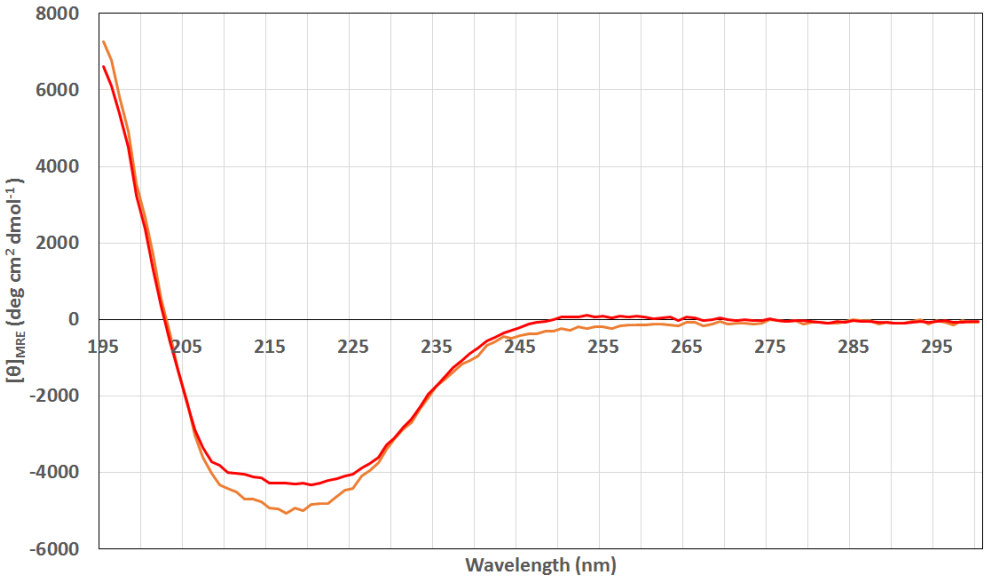

Figure 4.4: The CD spectra for Atox1 (orange line) and HisAtox1 (red line). The CD data is shown as mean residue ellipticity $[\theta]_{MRE}$.

4.3 Immunofluorescence cell staining

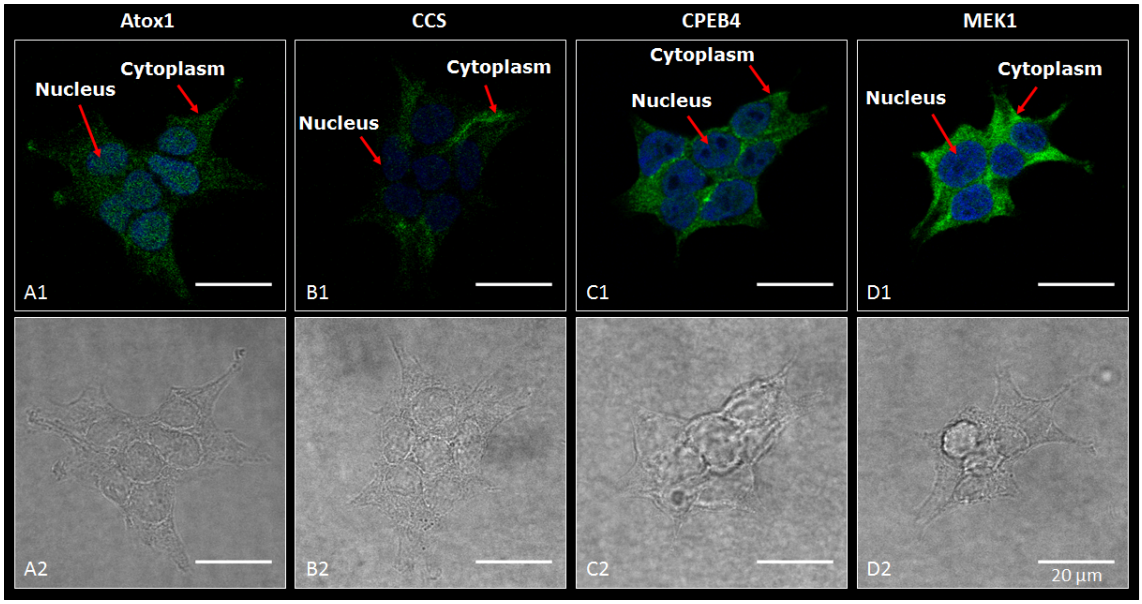

Figure 4.5: Confocal microscopy images of immunofluorescence stained HEK293T cells. Top images (A1-D1) show the fluorescence of the Alexa Fluor 488 labelled antibodies (green) against Atox1, CCS, CPEB4 and MEK1. The proteins were mostly located in the cytoplasm. DAPI (blue) was used to stain the DNA in the nucleus. The bottom panel (A2-D2) shows the bright field images.

The sole purpose of the IF-stainings was to study the intracellular distribution of the proteins Atox1, CCS, CPEB4 and MEK1, to determine if they could possibly encounter each other in the cells. The results are displayed in figure 4.5, and in all experiments, the proteins could clearly be seen in the cytoplasm of the cells. This corresponds well with the information found in literature [8, 23, 28], where all four

proteins are stated to function in the cytoplasm. To some extent, the proteins were also found in the nucleus (see top panel in figure 4.5). For Atox1, this is in line with earlier discoveries [9, 47], but for the rest of the proteins these results are somewhat surprising. However, this was not investigated further in this project, as the result was not very obvious.

Since all proteins could clearly be located in same subcellular compartment (cytoplasm), it is possible for Atox1 and CCS to encounter either of CPEB4 and MEK1. Thus, the interaction between Atox1 and CPEB4, as well as MEK1 and Atox1 or CCS, is likely to take place in the cytoplasm.

4.4 Far-western blot

The far-western blot with CPEB4-RRM and Atox1 was performed to confirm the interaction between the proteins, which had previously been reported in the yeast-2-hybrid experiment [10]. The results are presented in figure 4.6. In all images where Atox1 was used as bait, bands are visible although the bands are very faint for the conditions without crosslinking. The bands are stronger when crosslinking agent was used, which is expected since bound Atox1 would then not be washed away. Furthermore, Cu does not appear to affect the interaction. There is no visible band in the control, which means there are no non-specific binding of the primary or secondary antibodies to CPEB4-RRM, and thus no false positives. The fact that there are visible bands shows that some Atox1 was able to bind to the CPEB4-RRM, which confirms that these proteins interact. Furthermore, the interaction is probably weak, since crosslinking is required. However, it is important to note that this experiment was performed under denaturing conditions, and the CPEB4-RRM was perhaps not properly folded. It is possible that the interaction would be stronger if CPEB4-RRM was fully folded. Thus, one should repeat this experiment under non-denaturing conditions.

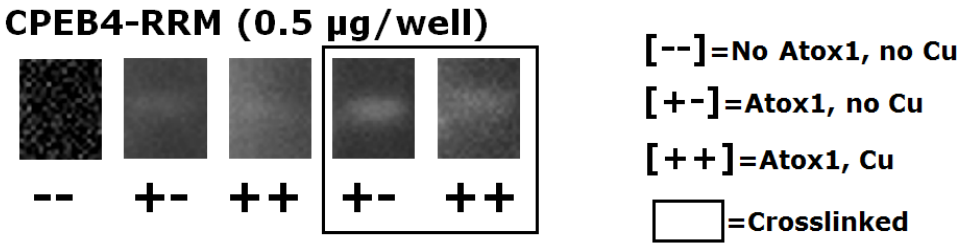

Figure 4.6: Far-western blot with CPEB4-RRM as prey and Atox1, with and without Cu, as bait under denaturing conditions. The location of the CPEB4-RRM (30 kDa) on the membrane has been zoomed in to show the bands. Two conditions were also treated with crosslinking agent, after incubation with bait solution.

Therefore, far-western blots with Atox1 as bait were performed under native conditions, and the proteins MEK1 and S100A12 were also included. The results of this far-western blot is shown in figure 4.7. In contrast to the results under denaturing conditions, surprisingly no bands could be detected in the lanes with CPEB4-RRM.

This indicates that Atox1 was only able to interact with unfolded (or partly folded) CPEB4-RRM, and not with its native form. However, since the CPEB4-RRM is only a part of the whole CPEB4 protein, one should be careful to implicate that this will also be the case for the RRMs in the full CPEB4 protein. The fold of the CPEB4-RRM domain, on its own, is not necessarily the same fold as it would be in full length CPEB4. In this project, the fold of CPEB4-RRM was not compared to the same domain in full length CPEB4, and it is therefore not accurate to draw definite conclusions about the interaction between Atox1 and the RRMs of full length CPEB4. However, from the far-western blot results it is possible to conclude that Atox1 can interact with the RRM domains under denaturing conditions. Furthermore, these results could have important implications for the yeast-2-hybrid screen [10], as it is possible the RRM-domain that was used in the yeast was in fact unfolded and therefore interacted with Atox1. To fully confirm whether Atox1 does interact with the RRMs of CPEB4 or not, further experiments would be necessary, for instance to compare the structure of the CPEB4-RRM to the CPEB4.

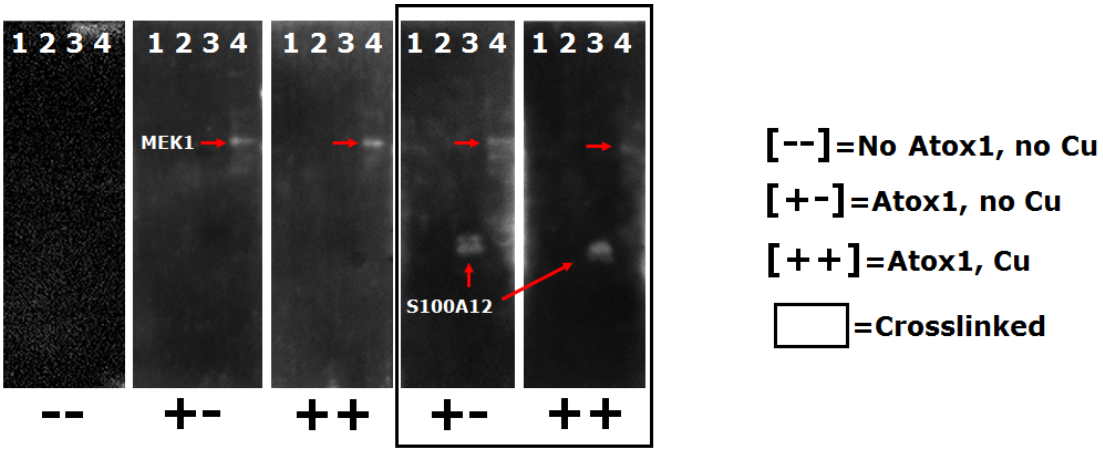

Figure 4.7: Native far-western blot, with CPEB4, MEK1 and S100A12 as prey and Atox1 as bait. Lane 1 and 2 contain CPEB4-RRM (0.5 µg and 1.0 µg per well). Lane 3 and 4 contain MEK1 (0.5 µg per well) and S100A12 (0.33 µg per well), respectively. Two conditions were also treated with crosslinking agent, after incubation with bait solution.

Interestingly, bands for the other two proteins could be detected, which is shown in figure 4.7. The Atox1-S100A12 interaction is only detected when crosslinking agent was used, and thus implicates a weak interaction. For MEK1 however, there are indications of a relatively strong interaction that withstands the multiple washing steps in the far-western blots, since bands could be seen in all conditions. MEK1 was therefore chosen to be investigated further as a potential interaction partner to Atox1, and we also tested another Cu chaperone, CCS. The S100A12-Atox1 interaction will be characterized in future studies. No band was detected in the control, which means that the results for the native-far western were accurate and not false positives.

Another set of native far-western blot experiment were performed to test if the results for MEK1 and Atox1 interaction were reproducible, and to investigate if

MEK1 also could interact with CCS. The results are shown in figure 4.8, and confirms that Atox1 and MEK1 are interacting with each other. The bands appear more intense when Cu is added, which could suggest that Cu increases the binding affinity. When CCS was used as the bait, no bands were visible and therefore, these proteins appeared not to interact. Nevertheless, the interaction between CCS and MEK1 could perhaps just be very weak and since the experiment was not performed with crosslinking, CCS was not yet excluded as a potential interaction partner to MEK1. Since the structure of Cu binding domain 1 of CCS is very similiar to that of Atox1 [48], it would not be surprising if this domain could interact with MEK1 like Atox1 does. It is however possible that the other domains of CCS are obstructing such an interaction from happening.

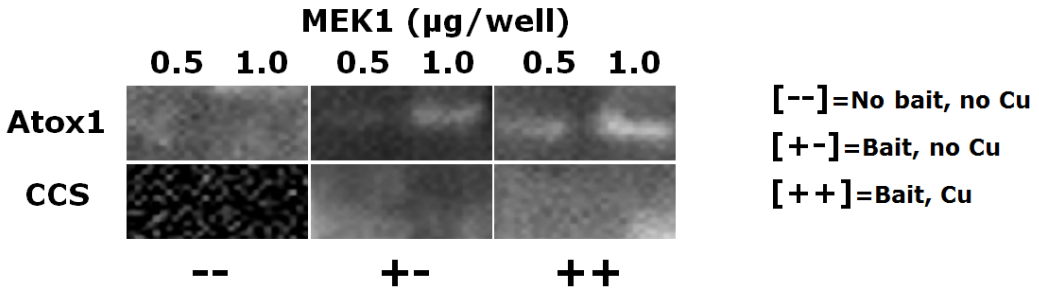

Figure 4.8: Native far-western blot with MEK1 as prey and Atox1 or CCS as bait. Two different amounts of MEK1 (0.5 µg or 1.0 µg per well) was used, and the legends to the right explains the different bait solutions used. Since crosslinking was not necessary to see the MEK1 interaction with Atox1, that condition was excluded in this experiment.

4.5 Co-immunoprecipitation

The co-ip was performed to probe if interaction between MEK1 and Atox1 or CCS could also be detected in cells. In figure 4.9, MEK1 is clearly be detected in the lysate, which means the cells had produced the protein. This corresponds well with the results for the IF-stainings, where MEK1 could be seen in the cytoplasm (see figure 4.5). In the eluate, a sharp band is visible, which confirms that the MEK1 was successfully immunoprecipitated from the cell lysate. There were also no visible bands in the control, which indicates that no non-specific binding to beads or antibodies occured during the immunoprecipitation.

Atox1 was also detected in the cell lysate and the unbound fraction, see figure 4.9. This proves that the cells have produced Atox1 protein. However, in the rest of the samples from the co-IP there are no bands at approximately 7.4 kDa where Atox1 would be expected. Thus, Atox1 was therefore not immunoprecipitated from the lysate together with MEK1. Together with the far western results, this indicates that MEK1 and Atox1 does not interact inside of the cells, but only *in vitro*. However, it is also possible that the potential interaction is too weak to be detected with a regular co-IP, or further optimization is needed for co-immunoprecipitating

of the complex. There were also some bands detected on the blot around 70 and 150 kDa. Considering that the total molecular weight of a heavy and light chain of an antibody is approximately 75 kDa, and thus 150 kDa for the full antibody [49], the bands were concluded to be antibodies which has detached from the magnetic beads during elution. The antibodies crosslinked to the beads and those used in the western blots should preferably have been produced in different hosts, to avoid problems such as this. However, in this project, other suitable antibodies were not available.

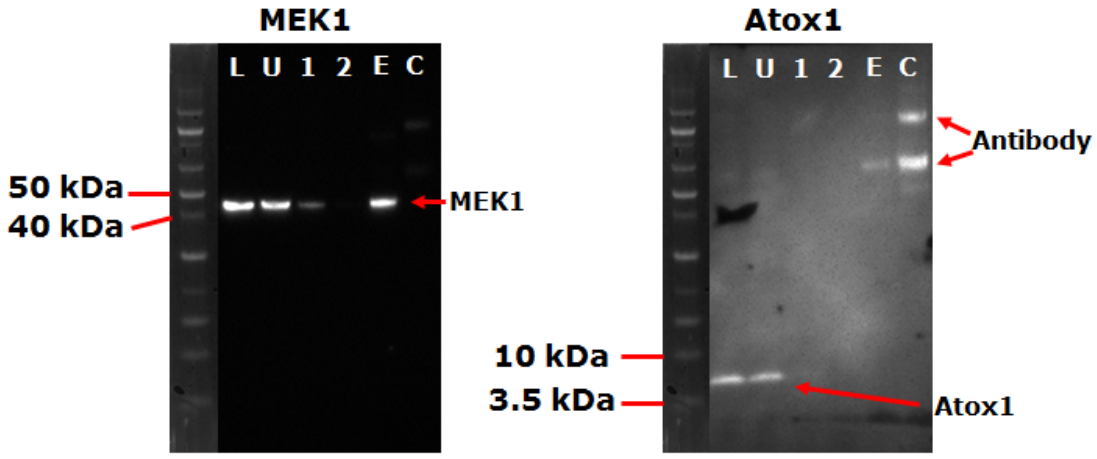

Figure 4.9: Immunoprecipitation of MEK1 from HEK293T cell lysate with the intention to also immunoprecipitate (MEK1 bound) Atox1. MEK1 (left) and Atox1 (right) was detected by western blot. The different samples in the images are: lysate (L), unbound fraction of lysate (U), first wash (1), second wash (2), eluate (E) and eluate of the control (C).

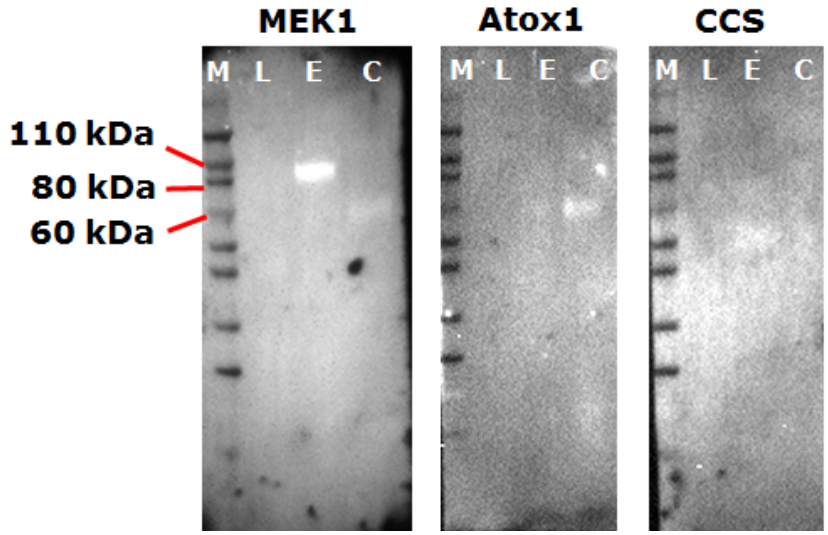

Figure 4.10: Co-IP where the lysate was first crosslinked, and then MEK1 was pulled out from cell lysate with the intention to also pull out (MEK1 bound) Atox1 and CCS. The different samples in the images are: lysate (L), eluate (E) and eluate of the control (C).

To investigate if the interaction was too weak to be maintained during the procedure, crosslinking of the proteins were performed before lysing the cells. However,

crosslinking the proteins of the cells proved to be difficult, as can be seen in figure 4.10. The experiment was not successful since no MEK1 can be seen in the blot, not even in the lysate. Consequently, there were no Atox1 and CCS detected either, and thus something had gone wrong during the experimental procedure. Still, there are bands visible in the eluate of MEK1 and in the control in both the MEK1 and Atox1 blots, but these are too large to be MEK1. It could possibly be MEK1 crosslinked to another protein, but since the lysate does not show anything, it is probably antibody fragments that have detached the beads. If the crosslinking would have been successful several bands should have appeared, since MEK1 has at least two known interaction partners in the MAPK pathway [28]. Therefore, all of these results were found unreliable, and should be repeated in the future.

Proving that two proteins interact *in vitro* only shows that an interaction is possible, but it does not prove that the interaction actually takes place in the cell. It would therefore have been an important discovery, for this project, if it could be proved that MEK1 interact with Atox1 or CCS in cells. Unfortunately, the co-IP experiments could not provide results to support this. However, we note that our collaborator in USA has been successful with co-IP of MEK1 and both Atox1 and CCS (unpublished).

4.6 Surface plasmon resonance

The SPR experiments were performed to characterize the identified interaction between MEK1 and Atox1, and a possible MEK1-CCS interaction was also investigated. In figure 4.11 and 4.12, the binding curves and the fittings to the 1:1 binding model are displayed for the MEK1-HisAtox1 experiments, and in table 4.1 the dissociation constants (K_d) are summarized. When comparing the two figures, Atox1 was found to bind MEK1, and it seems that Cu is not significantly affecting the binding. There was consequently no significant difference in K_d, with and without Cu, which further suggests that the Cu does not affect complex formation. The SPR results corresponds well with those obtained with far-western blot, both supporting that MEK1 and Atox1 are interaction partners.

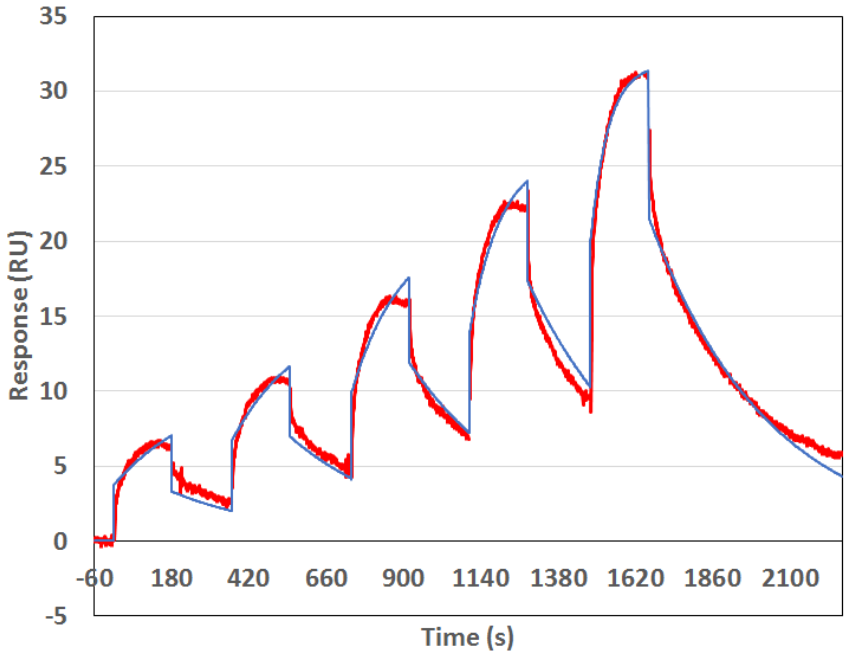

Figure 4.11: Binding curves obtained for the MEK1-HisAtox1 SPR experiment (red) and fitting to 1:1 binding model (blue). MEK1 was immobilized on the surface and HisAtox1 was the analyte, which was added in five cycles with doubled concentration each time (2.5 µM - 40 µM).

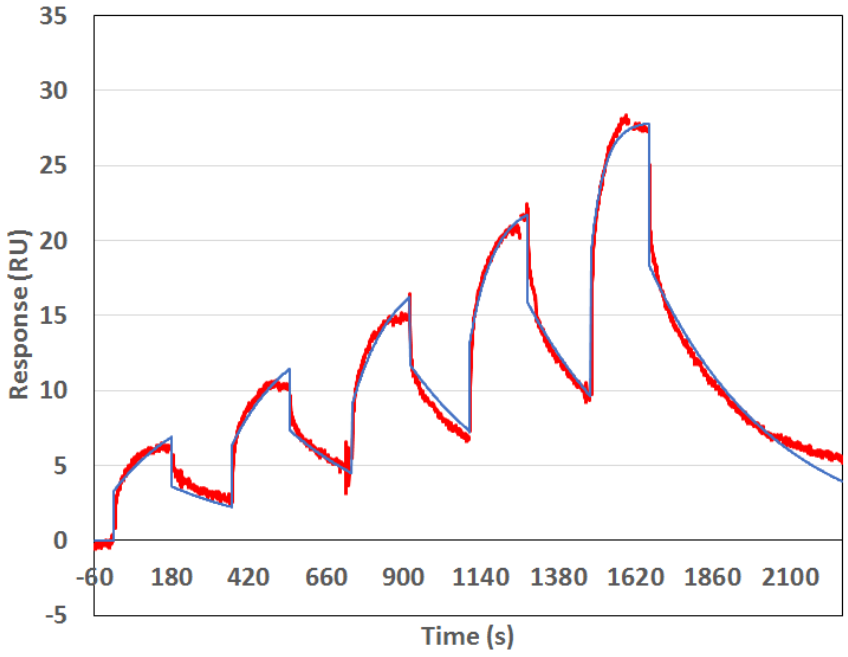

Figure 4.12: Binding curves obtained for the MEK1-HisAtox1 SPR experiment with Cu added (red) and fitting to 1:1 binding model (blue). MEK1 was immobilized on the surface and HisAtox1 was the analyte, which was added in five cycles with doubled concentration each time (2.5 µM - 40 µM).

In contrast to the far-western blot results, interaction was detected for MEK1-HisCCS with SPR, and this suggests that far-western blots are perhaps not reliable for detecting protein-protein interactions. The binding curves for the MEK1-HisCCS

interaction experiments are shown in figure 4.13 and 4.14. The overall shape of the binding curves are very similar when comparing HisCCS and HisCCS with Cu, and in both cases interaction is detected. However, the magnitude of the response differs a lot between the two, where the HisCCS signal is approximately doubled without Cu compared to when Cu is added. This means that less amount of HisCCS was able to bind to MEK1 during the experiment in the presence of Cu. Interestingly, since there was no significant difference between the dissociation constants (see table 4.1) for the two conditions, the difference may originate in binding/dissociation kinetics. With yeast CCS, it has been found that CCS without Cu is stable in monomeric form while Cu-loaded CCS has been found to dimerize, forming roughly equal concentrations of dimer and the monomer [50]. In the SPR experiments, assuming only the monomeric form of HisCCS can bind to MEK1, the signal was most likely lower for Cu-loaded HisCCS because of the formation of these dimers. Since the concentration of monomeric HisCCS was not as high as anticipated, due to the dimerization, the association rate constant k_a was probably underestimated during the data fitting. If the true value of k_a is larger than reported, the affinity of the interaction would also be stronger. Thus, the true value of K_d is most likely lower than the reported value found in table 4.1. This would mean that Cu-loaded HisCCS has a stronger affinity to MEK1 than HisCCS without Cu, which is reasonable considering that the suggested purpose of the interaction is to deliver Cu to MEK1.

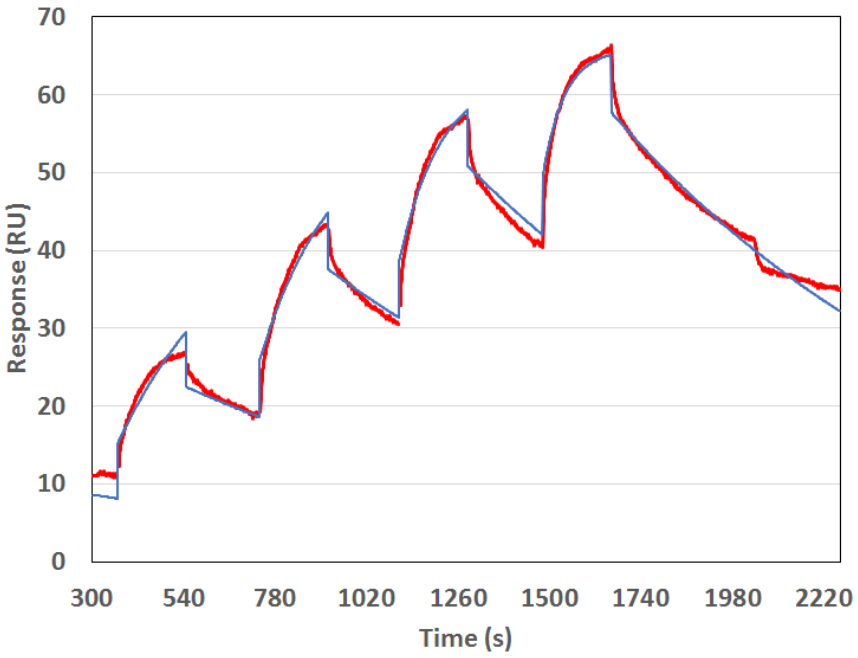

Figure 4.13: Binding curves obtained for the MEK1-HisCCS SPR experiment (red) and fitting to 1:1 binding model (blue). MEK1 was immobilized on the surface and HisCCS was the analyte, which was added in four cycles with doubled concentration each time (5 µM - 40 µM).

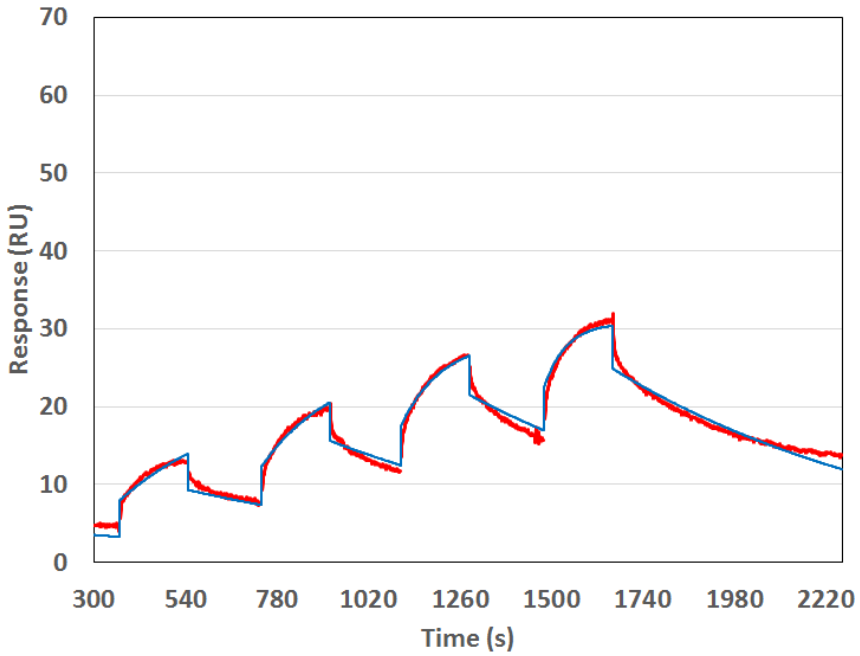

Figure 4.14: Binding curves obtained for the MEK1-HisCCS SPR experiment with Cu added (red) and fitting to 1:1 binding model (blue). MEK1 was immobilized on the surface and Cu-loaded HisCCS was the analyte, which was added in four cycles with doubled concentration each time (5 µM - 40 µM).

Table 4.1: Rate constants (k_a and k_d), the dissociation constants (K_d) and the approximated maximum signal (R_{max}) for the interactions between MEK1 and Atox1 or CCS, with or without Cu.

	k_a (1/Ms)	$k_d x10^3$ (1/s)	K_d (µM)	R_{max} (RU)
HisAtox1	309±109	3.07±0.55	10.9±5.65	28.67±3.92
HisAtox1+Cu	417±167	2.49±0.11	6.43±2.32	23.41±3.74
HisCCS	533±163	1.06±0.12	2.09±0.48	67.39±4.92
HisCCS+Cu	510±300	1.34±0.15	3.33±1.98	30.37±4.56

The SPR data was fitted with a 1:1 binding model. Presented values are the calculated mean values and standard deviations, based on 2-4 experiments.

Since both HisAtox1 and HisCCS interacted with MEK1, we can compare the results. In all cases, the obtained dissociation constants were similar, ranging between 2-11 µM (see table 4.1). These results suggest that the MEK1-HisCCS interaction is somewhat stronger than the MEK1-HisAtox1, but one should have in mind that the K_d for Cu-loaded MEK1-HisCCS may have been overestimated, and thus the difference may be larger. Nevertheless, the higher affinity of HisCCS to MEK1 indicates that CCS is more likely to be the main deliverer of Cu to MEK1, even though Atox1 also would be able to complete this task. Considering that the structure of Atox1 shows great resemblance to the Cu binding domain 1 of CCS [48], it is reasonable to

assume that it is domain 1 of CCS that interacts with MEK1. This would explain why the K_d for MEK1-HisAtox1 and MEK1-HisCCS interactions, are similar. However, this is mere speculations and can not be determined with the data presented in this thesis. A different approach would be required, such as testing interactions between each domain of CCS and MEK1, to be able to identify which part of CCS is responsible for the interaction with MEK1.

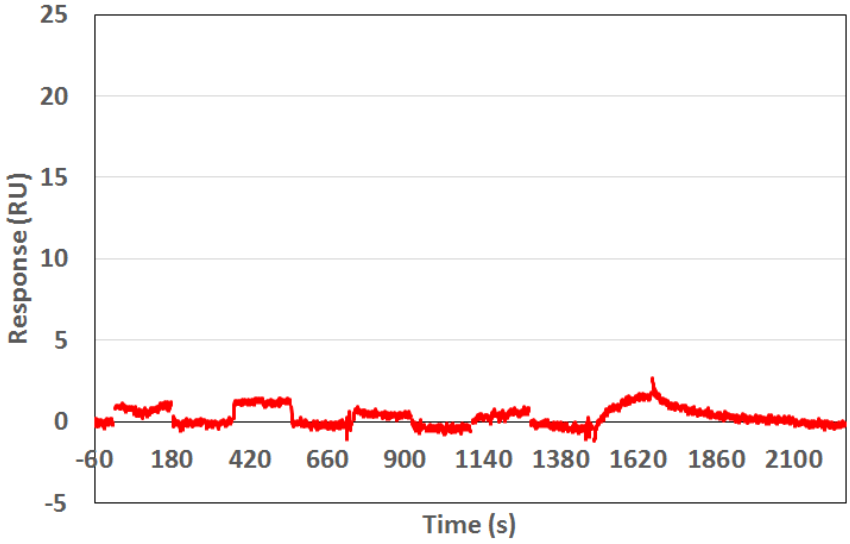

Figure 4.15: Negative control where MEK1 was immobilized on the surface and His-tagged Parvalbumin was used as the analyte. HisParvalbumin was added in five cycles with doubled concentration each time (2.5 µM - 40 µM), in the same way that the experiments with HisAtox1 and HisCCs were performed.

The result for the negative control is shown in figure 4.15. The negative control was crucial for the SPR experiments, since HisAtox1 and HisCCS was used, to prove that a His-tag on a protein alone would not be enough for it to be able to bind to MEK1. In figure 4.15 there was no signal change upon protein injections, meaning that there was no interaction between HisParvalbumin and MEK1. This proves that neither parvalbumin or the His-tag binds to MEK1, and it was therefore confirmed that the His-tag did not lead to false positive results.

5
Conclusion

Cu ions are often found in proteins where it acts as a cofactor to provide functionality. In recent years, Cu has emerged as a potential key factor in promotion of cancer. Since Cu distribution in cells is dependent on transfer performed by Cu chaperones, these proteins are likely to be involved in Cu-dependent reactions in cancer. The purpose of this Master's thesis, was to investigate if the Cu chaperones Atox1 and CCS could interact with either of three proteins: CPEB4-RRM, MEK1 and S100A12, where the latter two are Cu-binding proteins. All of these proteins have been connected to cancer, and their possible interactions with Cu chaperones could give insights to the link between Cu and cancer.

With far-western blot experiments, CPEB4-RRM was found to interact with Atox1 under denaturing conditions, but not when in native form. This implied that Atox1 would not bind to the full length CPEB4, despite the previous reports [10]. However, the reliability of the far-western blot method for detecting interactions can be questioned since CCS-MEK1 interaction could not be detected with far-western blot, but was still observed with SPR. Thus, other techniques for studying this protein-protein interaction should be introduced. The protein-protein interaction experiments should preferably be performed with full length CPEB4, to ensure the correct folding, before CPEB4 can be excluded as a potential *in vivo* interaction partner to Atox1.

The interactions between S100A12 and the Cu chaperones were not extensively investigated due to the time restraints of this project, but there were some promising results. S100A12 was shown to interact weakly with Atox1, with far-western blot. Even though the function of S100A12 binding Cu is still not known, the results presented in this thesis indicate that S100A12 can obtain the Cu from a Cu chaperone. Atox1 in particular appears to be involved, but CCS may also play an important role. This calls for further investigation of these interactions, and it would be important to study both binding and Cu transfer between the proteins, using purified proteins *in vitro*.

The results from MEK1 interaction studies were the most interesting in this project, because MEK1 interacted with both Cu chaperones. Furthermore, the dissociation constants of the MEK1-Atox1 and MEK1-CCS complexes were determined to be in micromolar range. Notably, although little is known about *in vivo* concentrations, it is thought that Atox1 is present in micromolar concentration in cells [51]. Nevertheless, the results in this thesis suggest that both Atox1 and CCS could potentially

deliver the Cu to MEK1. Given the already strong connection between Cu, MEK1 and cancer [7, 27], Cu chaperones could be potential drug targets for treatments against cancer. It will be important to now extend binding studies to Cu transfer experiments.

The experiments in this thesis show that the proteins can interact *in vitro*, but the cell studies (immunoprecipitation) need to be further optimized in order to detect potential interactions. In future studies, the focus should therefore be to show that the interactions also take place in cells. The IF-stainings proved that the proteins can encounter each other in the cells, as they were detected in the same subcellular compartment. Other methods than co-IP could also be tested, such as proximity ligation assays, to directly detect interactions in cells.

To summarize, the *in vitro* results presented in this Master's thesis provides evidence that Atox1 and CCS are capable of binding to cancer related proteins. These results are important for future studies and could contribute to a more comprehensive understanding of the involvement of Cu and Cu chaperones in cancer.

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A

Plasmids and Sequences

A.1 Plasmids - pET3a

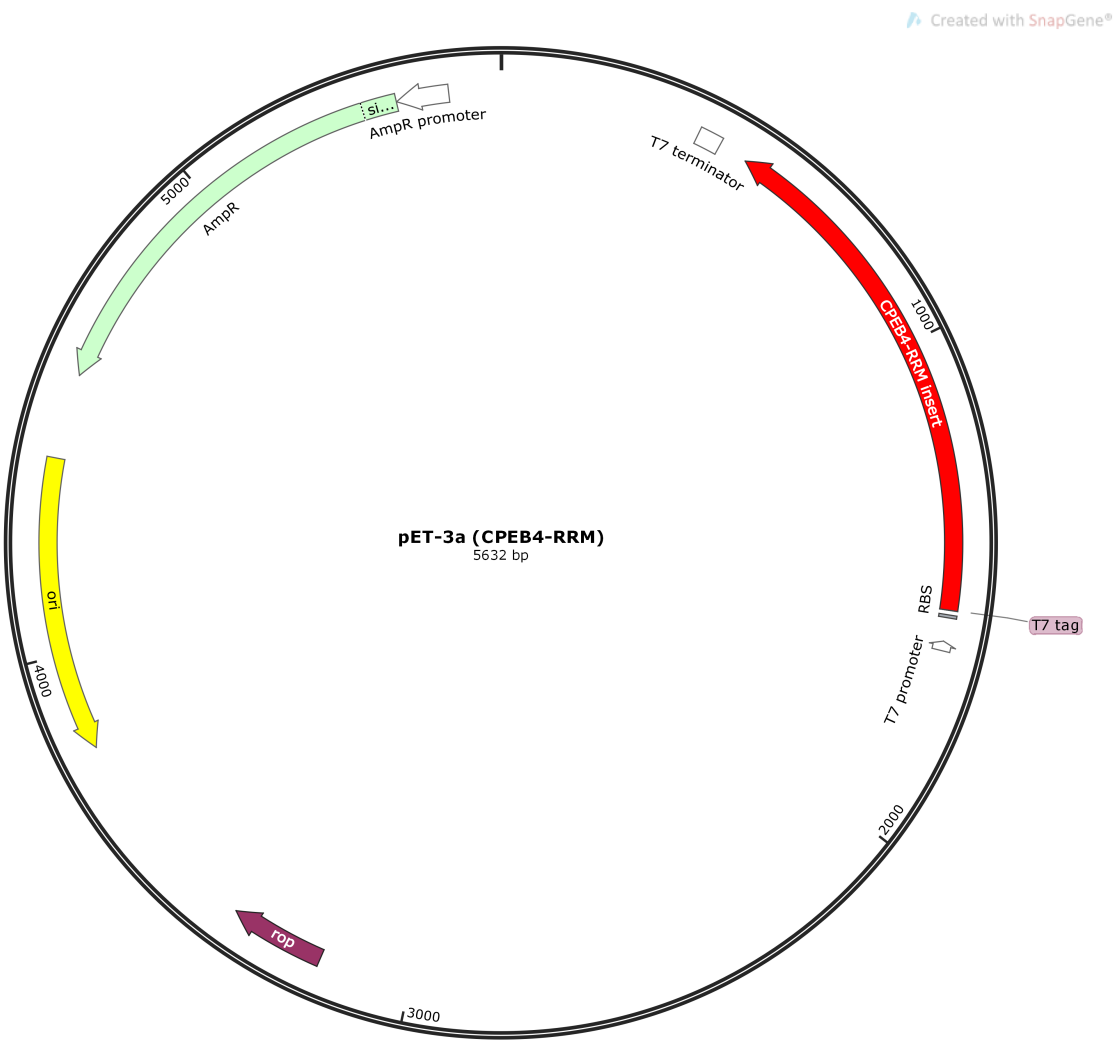

Figure A.1: The pET-3a plasmid with the CPEB4-RRM insert

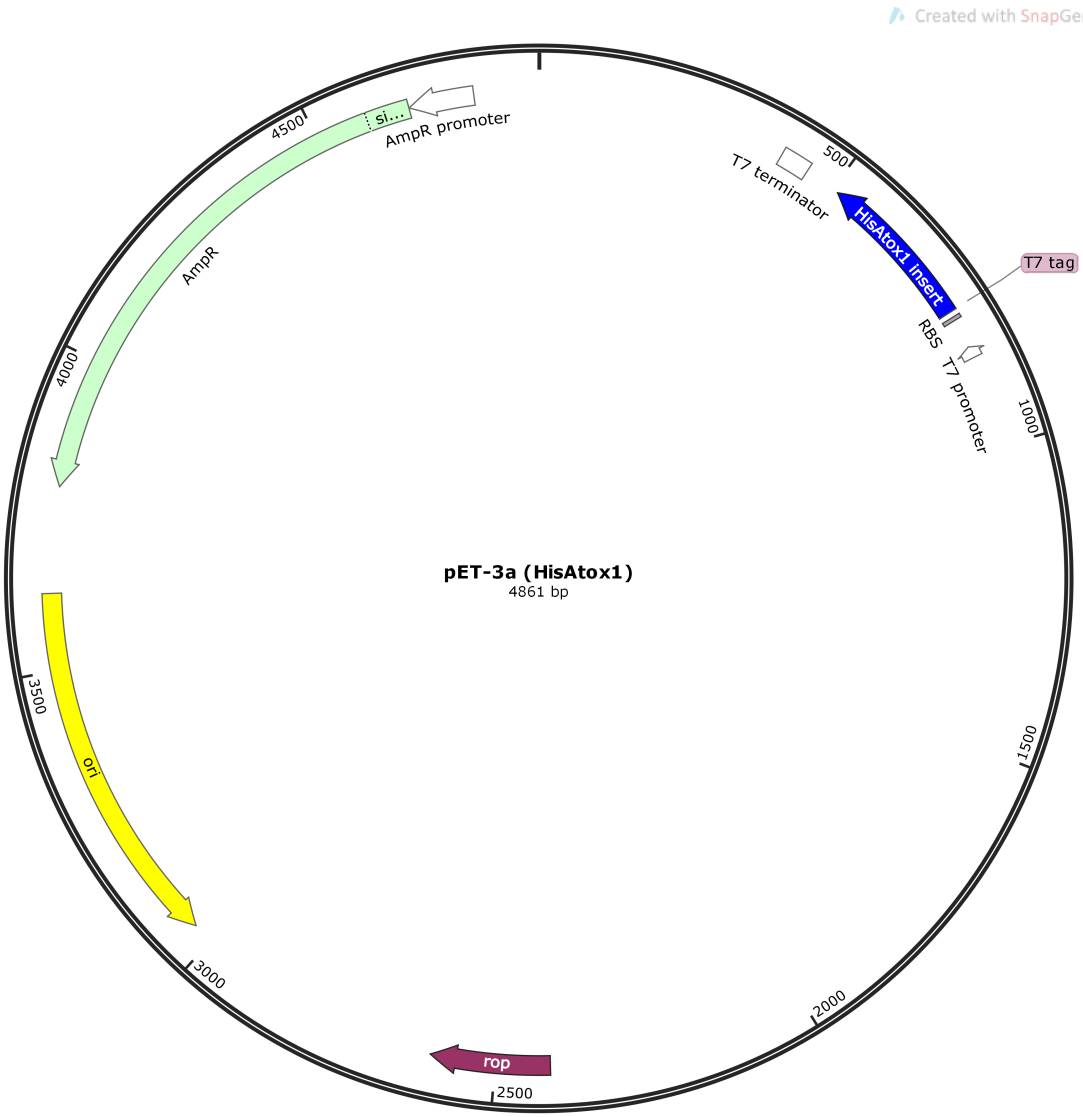

Figure A.2: The pET-3a plasmid with the HisAtox1 insert

A.2 Inserts sequences

A.2.1 CPEB4-RRM DNA sequence insert

```
   1 CATATGAGCA TTAGCAGCCG CGTGAAAAGC AAACGCATTC AGCTGGGCCT
  51 GAACCAGGCG GAACTGGCGC AGAAAGTGGG CACCACCCAG CAGAGCATTG
 101 AACAGCTGGA AAACGGCAAA ACCAAACGCC CGCGCTTTCT GCCGGAACTG
 151 GCGAGCGCGC TGGGCGTGAG CGTGGATTGG CTGCTGAACG GCAAACATCA
 201 TCATCATCAT CACATGGGCA GCAGCGATGA AGTGGATATG CTGATTAACG
 251 CGCGCACCTA TGGCCGCCGC CGCGGCCAGA GCAGCCTGTT TCCGATGGAA
 301 GATGGCTTTC TGGATGATGG CCGCGGCGAT CAGCCGCTGC ATAGCGGCCT
 351 GGGCAGCCCG CATTGCTTTA GCCATCAGAA CGGCGAACGC GTGGAACGCT
 401 ATAGCCGCAA AGTGTTTGTG GGCGGCCTGC CGCCGGATAT TGATGAAGAT
 451 GAAATTACCG CGAGCTTTCG CCGCTTTGGC CCGCTGATTG TGGATTGGCC
 501 GCATAAAGCG GAAAGCAAAA GCTATTTTCC GCCGAAAGGC TATGCGTTTC
 551 TGCTGTTTCA GGATGAAAGC AGCGTGCAGG CGCTGATTGA TGCGTGCATT
 601 GAAGAAGATG GCAAACTGTA TCTGTGCGTG AGCAGCCCGA CCATTAAAGA
 651 TAAACCGGTG CAGATTCGCC CGTGGAACCT GAGCGATAGC GATTTTGTGA
 701 TGGATGGCAG CCAGCCGCTG GATCCGCGCA AAACCATTTT TGTGGGCGGC
 751 GTGCCGCGCC CGCTGCGCGC GGTGGAACTG GCGATGATTA TGGATCGCCT
 801 GTATGGCGGC GTGTGCTATG CGGGCATTGA TACCGATCCG GAACTGAAAT
 851 ATCCGAAAGG CGCGGGCCGC GTGGCGTTTA GCAACCAGCA GAGCTATATT
 901 GCGGCGATTA GCGCGCGCTT TGTGCAGCTG CAGCATGGCG AAATTGATAA
 951 ACGCGTGGAA GTGAAACCGT ATGTGCTGGA TGATCAGCTG TGCGATGAAT
1001 GCCAGGGCGC GCGCTGCGGC GGCTAAGGAT CC
```

A.2.2 HisAtox1 DNA sequence insert

```
   1 CATATGCACC ACCACCACCA CCACATGGGT AGCAGCGACG AGGTGGATAT
  51 GCCGAAGCAC GAATTCAGCG TTGACATGAC CTGCGGTGGC TGCGCGGAGG
 101 CGGTGAGCCG TGTTCTGAAC AAGCTGGGTG GCGTGAAATA CGACATCGAT
 151 CTGCCGAACA AGAAAGTTTG CATTGAGAGC GAACACAGCA TGGATACCCT
 201 GCTGGCGACC CTGAAGAAAA CCGGTAAAAC CGTGAGCTAT CTGGGCCTGG
 251 AATAAGGATC C
```

A.3 Amino acid sequences

Known Cu binding sites are highlighted in orange.

A.3.1 CPEB4-RRM

```
  1 LINARTYGRR RGQSSLFPME DGFLDDGRGD QPLHSGLGSP HCFSHQNGER
 51 VERYSRKVFV GGLPPDIDED EITASFRRFG PLIVDWPHKA ESKSYFPPKG
101 YAFLLFQDES SVQALIDACI EEDGKLYLCV SSPTIKDKPV QIRPWNLSDS
151 DFVMDGSQPL DPRKTIFVGG VPRPLRAVEL AMIMDRLYGG VCYAGIDTDP
201 ELKYPKGAGR VAFSNQQSYI AAISARFVQL QHGEIDKRVE VKPYVLDDQL
251 CDECQGARCG G
```

A.3.2 HisAtox1

```
  1 HHHHHHMGSS DEVDMPKHEF SVDMTCGGCA EAVSRVLNKL GGVKYDIDLP
 51 NKKVCIESEH SMDTLLATLK KTGKTVSYLG LE
```

A.3.3 Atox1

```
  1 MPKHEFSVDM TCGGCAEAVS RVLNKLGGVK YDIDLPNKKV CIESEHSMDT
 51 LLATLKKTGK TVSYLGLE
```

A.3.4 CCS

```
  1 MASDSGNQGT LCTLEFAVQM TCQSCVDAVR KSLQGVAGVQ DVEVHLEDQM
 51 VLVHTTLPSQ EVQALLEGTG RQAVLKGMGS GQLQNLGAAV AILGGPGTVQ
101 GVVRFLQLTP ERCLIEGTID GLEPGLHGLH VHQYGDLTNN CNSCGNHFNP
151 DGASHGGPQD SDRHRGDLGN VRADADGRAI FRMEDEQLKV WDVIGRSLII
201 DEGEDDLGRG GHPLSKITGN SGERLACGII ARSAGLFQNP KQICSCDGLT
251 IWEERGRPIA GKGRKESAQP PAHL
```

A.3.5 MEK1

```
  1 MPKKKPTPIQ LNPAPDGSAV NGTSSAETNL EALQKKLEEL ELDEQQRKRL
 51 EAFLTQKQKV GELKDDDFEK ISELGAGNGG VVFKVSHKPS GLVMARKLIH
101 LEIKPAIRNQ IIRELQVLHE CNSPYIVGFY GAFYSDGEIS ICMEHMDGGS
151 LDQVLKKAGR IPEQILGKVS IAVIKGLTYL REKHKIMHRD VKPSNILVNS
201 RGEIKLCDFG VSGQLIDSMA NSFVGTRSYM SPERLQGTHY SVQSDIWSMG
251 LSLVEMAVGR YPIPPPDAKE LELMFGCQVE GDAAETPPRP RTPGRPLSSY
301 GMDSRPPMAI FELLDYIVNE PPPKLPSGVF SLEFQDFVNK CLIKNPAERA
351 DLKQLMVHAF IKRSDAEEVD FAGWLCSTIG LNQPSTPTHA AGV
```

A.3.6 S100A12

```
  1 MTKLEEHLEG IVNIFHQYSV RKGHFDTLSK GELKQLLTKE LANTIKNIKD
 51 KAVIDEIFQG LDANQDEQVD FQEFISLVAI ALKAAHYHTH KE
```

B

Protocols

B.1 Protein expression

Media

- Liquid media: Lysogeny broth (20 g/L) with carbenicillin (100 µg/mL).
- Agar plates: Lysogeny broth (20 g/L) with carbenicillin (100 µg/mL) and agar (16 g/L).

Day 1

- Streak *E. coli* (containing either of the vectors for CPEB4-RRM or HisAtox1) on one agar plate.
- Incubate overnight at 37°C.

Day 2

- Rinse the surface of the plate in 2 ml of lysogeny broth to suspend the colonies of *E. coli*, and add 500 μL to a falcon tube with 10 ml liquid media.
- Incubate for 2 hours at 37°C on a shaker.
- Add 5 ml of starter culture to 500 ml liquid media for the main culture.
- Incubate for 3 hours at 37°C on a shaker.
- Add IPTG to a final concentration of 1 mM.
- Incubate overnight at 25°C on a shaker.

B.2 Protein purification

Buffers

- Buffer A: 50 mM NaCl, 20 mM Tris (pH 8) and 1 mM TCEP. Filter with a 0.22 µm pore size filter.
- Buffer B1: 50 mM NaCl, 20 mM Tris (pH 8), 1 mM TCEP and 1 M imidazole. Filter with a 0.22 µm pore size filter.
- Buffer B2: 1 M NaCl, 20 mM Tris (pH 8) and 1 mM TCEP. Filter with a 0.22 µm pore size filter.
- Buffer C: 50 mM NaCl, 20 mM Tris (pH 7.4) and 1 mM TCEP. Filter with a 0.22 µm pore size filter.

Day 1

- Centrifuge the main culture at 4000 g for 30 minutes.

- Discard the supernatant and dissolve in buffer A.
- Aliquot the suspension and centrifuge at 8000 g for 5 minutes.
- Discard supernatant and disolve the pellet again.
- Collect all content in one tube and add a tablet of protease inhibition cocktail (Thermo Fisher Scientific) per 10 ml.
- Sonicate the solution for 15 minutes in cycles of on and off (5 seconds on, 10 seconds off).
- Add 0.1 µL nuclease solution (Thermo Fisher Scientific) per 1 ml of cell lysate and incubate for 15 min at 4°C.
- Centrifuge at 16000 g for 45 min, collect supernatant and filter through a 0.22 µm filter.
- Load the sample onto a HisTrap FF (5 ml) Ni-NTA column (GE Healthcare) with a peristaltic pump.
- Wash column with a 50 mM NaCl, 25 mM Tris (pH 8), 1 mM TCEP and 5 mM imidazole buffer, by mixing Buffer A and B1.
- Run the Ni-NTA chromatography with an ÄKTA Purifier in 20 fractions of 5 ml each at 1.5 ml/min. Use Buffer A and Buffer B1 to create a gradient from 5 mM to 1 M imidazole in the 20 fractions.
- Collect the fractions containing the protein.
- Verify that the collected fractions contain the protein by running gel electrophoresis on the samples. See appendix B.8 for SDS-PAGE.
- Cleavage of His-tag (for CPEB4-RRM). Mix protein solution and caspase 7 at approximately a 100:1 molar ratio (protein:caspase). Incubate overnight at 4°C with shaking.

Day 2

- Mix the collected fractions and load onto a 5 mL ion exchange Q Sepharose Fast Flow (GE Healthcare) with a peristaltic pump.
- Wash column with buffer A.
- Run the ion exchange chromatography with an ÄKTA Purifier in 20 fractions of 5 ml each. Use Buffer A and Buffer B2 to create a gradient from 50 mM to 500 mM NaCl in the 20 fractions at 1.5 ml/min.
- Do a second wash with only Buffer B2.
- Collect the fractions containing the protein.
- Verify that the collected fractions contain the protein by running gel electrophoresis on the samples. See appendix B.8 for SDS-PAGE.

Day 3

- Mix the collected fractions and run on a 120 mL size exclusion HiLoad 16/600 Superdex 75 pg column (GE Healthcare) with an ÄKTA Purifier. Run in 30 fractions of 4 ml each and at 1.5 ml/min. Use Buffer C as the mobile phase.
- Collect the fractions containing the protein.
- Verify that the collected fractions contain the protein by running gel electrophoresis on the samples. See appendix B.8 for SDS-PAGE.
- Measure the protein concentration.

- If necessary, concentrate the sample using protein concentrators (3 kDa and 10 kDa Amicon Ultra Centrifugal filters, Sigma-Aldrich) by centrifuging at 5000 g until the concentration is high enough for the intended use.

B.3 Far-western blot - SDS-PAGE

Day 1 - Gel electrophoresis, transfer to membrane and protein incubation
Gel electrophoresis and transfer to to membrane:

- Mix protein sample(s) (prey) with loading dye (NuPAGE™ LDS Sample Buffer). The amount of prey protein may need to be optimized. An appropriate starting point is 0.5 µg protein/well in the gel.
- Set up the electrophoresis apparatus with the gel (NuPAGE™ 4-12 % Bis Tris Gel).
- Pour on running buffer (NuPAGE™ MES SDS Running Buffer) and load the gel with the sample(s). Load at least two sets for each of the interested protein (one is sample, one is for control)
- Run the gel at constant voltage 200 V for 35 minutes.
- Use the Trans-Blot® Turbo™ RTA Mini PVDF Transfer Kit and the Trans-Blot® Turbo™ Blotting System (Bio-Rad) to transfer the protein(s) from the gel to a 0.2 µm PVDF membrane. Run according to the manufacturer's instructions (1.3 A and 25 V for 7 minutes).

Protein (bait) incubation:

- Add the bait protein to a solution of 50 mM Tris-HCl (pH 7.5) and 1 mM DTT. The amount of bait protein may need to be optimized. An appropriate starting point is to use a 5 µM protein solution. $CuCl_2$ (5 µM) may also be included.
- Also make a negative control solution, only containing 50 mM Tris-HCl (pH 7.5) and 1 mM DTT.
- Cut the membrane in two (or more depending how many conditions are tested). Incubate one part with the bait protein and the other with the negative control solution.
- Incubate at 4°C with agitation overnight.

Day 2

- Discard the bait solution and the negative control solution. Wash briefly in Milli Q water.
- ***Optional:** Crosslink with EDC solution. Incubate the membrane(s) in 3 mM EDC solution in room temperature for 1 hour with agitation. Wash three times with Milli Q water.
- Detect with chemiluminiscence by using the Pierce™ SuperSignal West Femto Fast Western Blot Kit (Thermo Fisher Scientific), according to the manufacturer's instructions, and the ChemiDoc MP (Bio-Rad).

B.4 Far-western blot - Native gel

Buffers and solutions

- Running buffer (pH 8.3): 192 mM glycine and 25 mM Tris base.
- Transfer buffer (pH 8.3): 200 mM glycine and 25 mM Tris base.
- Loading dye 4X: 248 mM Tris-HCl (pH 6.8), 0.04 % bromophenol blue and 40 % glycerol.
- 1 M Tris-HCl (pH 7.5)
- 1 M and 10 mM DTT
- 10 mM $CuCl_2$

Day 1 - Gel electrophoresis, transfer to membrane and protein incubation
Gel electrophoresis and transfer to to membrane:

- Mix protein sample(s) (prey) with loading dye (1X) and DTT (1 mM). The amount of prey protein may need to be optimized. An appropriate starting point is 0.5 µg protein/well in the gel.
- Set up the electrophoresis apparatus with the native gel (Novex™ 14 % Tris-Glycine Mini Gels, WedgeWell™ format, 15-well).
- Pour on pre-chilled running buffer and load the gel with the sample(s). Load at least two sets for each of the interested protein (one is sample, one is for control)
- Run the gel at constant voltage 60 V for 2 hours, then increase to 70 V until the electrophoresis is complete, i.e. when the loading dye reaches the bottom of the gel.
- Use the Trans-Blot® Turbo™ RTA Mini PVDF Transfer Kit and the Trans-Blot® Turbo™ Blotting System (Bio-Rad) to transfer the protein(s) from the gel to a 0.2 µm PVDF membrane. Run at 0.7 A and 25 V for 9 minutes. **Use the transfer buffer listed above**.

Protein (bait) incubation:

- Add the bait protein to a solution of 50 mM Tris-HCl (pH 7.5) and 1 mM DTT. The amount of bait protein may need to be optimized. An appropriate starting point is to use a 5 µM protein solution. $CuCl_2$ (5 µM) may also be included.
- Also make a negative control solution, only containing 50 mM Tris-HCl (pH 7.5) and 1 mM DTT.
- Cut the membrane in two (or more depending how many conditions are tested). Incubate one part with the bait protein and the other with the negative control solution.
- Incubate at 4°C with agitation overnight.

Day 2

- Discard the protein solution and the negative control solution. Wash briefly in Milli Q water.
- *Optional: Crosslink with EDC solution. Incubate the membrane(s) in 3 mM EDC solution in room temperature for 1 hour with agitation. Wash three times with Milli Q water.
- Detect with chemiluminiscence by using the Pierce™ SuperSignal West Femto Fast Western Blot Kit (Thermo Fisher Scientific), according to the manufacturer's instructions, and the ChemiDoc MP (Bio-Rad).

B.5 Poly-L-lysine coating of coverslips

- Clean coverslips in 1 M HCl at 55°C overnight.
- Remove HCl and wash with Milli Q water three times.
- Wash in 96 % ethanol and then Milli Q water three times.
- Dry the coverslips on paper in a laminar flow hood.
- Put the coverslips in a petri dish and add 250 µL of poly-L-lysine (0.01 %) to each coverslip and incubate at room temperature for 5 minutes.
- Wash coverslips in Milli Q water three times.
- Dry on paper in a fume hood and then move them to a petri dish.
- Seal the petri dish with parafilm and sterilize the coverslips with UV-light overnight.

B.6 Seeding and fixation of cells

- **Seeding:** Seed HEK293T cells on poly-L-lysin coated 18mm diameter coverslips in 12-well plates. Seed 75000 cells per well. Incubate overnight at 37°C and 5 % CO_2.
- **PFA fixation:** Remove growth media and wash once with pre-warmed (37°C) PBS.
- Discard the PBS and add pre-warmed 4 % PFA (EM-graded) to fix the cells to the coverslips. Incubate for 15 min at room temperature.
- Wash with PBS and store at 4°C or continue with immunofluorescence staining.

B.7 Immunofluorescence staining

Adapted from Abcam company

- **Permeabilization:** Incubate the samples for 10 min with PBS containing 0.1 % Triton X-100.
- Wash cells in PBS three times for 5 min on rotor.
- **Blocking:** Incubate cells with 10 % goat serum and 22.52 mg/ml glycine, in PBST (PBS + 0.1 % Tween 20) for 30 min.
- **Primary antibody incubation:** Incubate cells in the diluted antibody in 1 % goat serum in PBST in a humidified chamber overnight at 4°C.
- Wash the cells three times in PBST, 5 min for each wash on rotor.
- **Secondary antibody incubation:** Incubate cells with the secondary antibody, diluted in 1 % serum in PBST for 1 h at room temperature. Keep in the dark.
- Wash three times for 5 min each with PBST on rotor. Keep in the dark.
- Mount coverslips and store in dark at room temperature.

B.8 Western blot

Buffers and kit

- Trans-Blot® Turbo™ RTA Mini PVDF Transfer Kit (Bio-Rad).
- TBST: 20 mM Tris (pH 7.5), 150 mM NaCl and 0.1 % Tween 20.
- Blocking buffer: 5 % Skim milk in TBST.
- Antibody diluent: 3 % Bovine serum albumin (BSA) in TBST.

SDS-PAGE

- Mix protein sample(s) with loading dye (NuPAGE™ LDS Sample Buffer)
- Set up the electrophoresis apparatus with the native gel (NuPAGE™ 4-12 % Bis Tris Gel).
- Pour on running buffer (NuPAGE™ MES SDS Running Buffer) and load the gel with the sample(s).
- Run the gel at constant voltage 200 V for 35 minutes.
- Optional step: Stain with SimplyBlue™ SafeStain (Invitrogen) according to the manufactrurer's instructions. **Not for western blot**.

Electro-blotting and immunodetection

- Use the Trans-Blot® Turbo™ RTA Mini PVDF Transfer Kit and the Trans-Blot® Turbo™ Blotting System (Bio-Rad) to transfer the protein(s) from the gel to a 0.2 µm PVDF membrane.
- Block the membrane for 40 min at room temperature in blocking buffer.
- Incubate the membrane overnight at 4°C with primary antibody diluted in antibody diluent. Adjust dilution after manufacturer's recommendation.
- Wash 3 times for 5 min with blocking buffer at room temperature.
- Incubate the membrane with HRP conjugated secondary antibody.
- Wash 3 times for 5 min with TBST at room temperature.
- Use the Amersham ECL kit (GE Healthcare) to activate the HRP conjugated antibodies. Mix luminol and peroxide solutions 1:1 and incubate the membrane for 5 min.
- Image with Chemidoc MP.

B.9 Lysis of cells

General lysis of cells

- Remove culture medium from cells.
- Wash with ice-cold PBS.
- Add ice-cold lysis buffer (Pierce IP lysis buffer, Thermo Fisher Scientific)
- Add Protease inhibitor cocktail (Halt Protease Inhibitor Cocktail 100x, Thermo Fisher Scientific).
- Rock for 10 minutes at 4°C.
- Transfer to a tube and centrifuge at 13,000 x g for 10 minutes at 4°C.

- Transfer supernatant to a new tube.

Lysis of cells with crosslinking

- Suspend cells at 25 x 10^6 cells/mL in PBS.
- Wash cells three times with ice cold PBS.
- Prepare DSS in dimethyl sulfoxide (DMSO) at 10 mM, dilute with PBS to final concentration of 5 mM.
- Incubate for 30 min at room temperature with rotation.
- Add 1 M tris solution (ph 7.5) to a final concentration of 20 mM TRIS.
- Incubate for 15 min at room temperature with rotation.
- Add protease inhibitor cocktail (Halt Protease Inhibitor Cocktail 100x Thermo Fisher Scientific).
- Incubate for 5 min at 4°C with rotation.
- Lyse cells by adding 500 µL lysis buffer to 50 mg wet cell pellet (10:1 v/w)
- Centrifuge at 13 000 g for 10 min at 4°C.
- Transfer supernatant to a new tube.

B.10 Co-immunoprecipitation

Buffers and solutions

- Conjugation buffer (pH 7.4): 20 mM sodium phosphate and 150 mM NaCl.
- Quench buffer: 1 M Tris-HCl (pH 7.5)

Crosslinking antibodies to beads

- Add 25 µL beads per tube (Pierce Magnetic Protein A/G beads, Thermo Fisher Scientific)
- Wash beads twice with 500 µL conjugation buffer.
- Add 250 µL conjugation buffer with antibody against target protein. Always run a control in parallel by using a isotype control antibody. The recommended amount of antibody is often given by the manufacturer, but may have to be optimized. Same amount of isotype control antibody should be used.
- Incubate 15 min at room temperature. Vortex every 5 min.
- Wash beads 3 times with 250 µL conjugation buffer.
- Dissolve 2 mg BS3 crosslinking agent (Thermo Fisher Scientific) in 700 mL conjugation buffer. This gives a 5 mM solution.
- Add 250 µL BS3 to each tube.
- Incubate 2 hours at 4°C with rotation.
- Quench by adding 12.5 µL of Tris (1 M)
- Incubate 15 min at room temperature with rotation.
- Wash 3 times with 200 µL conjugation buffer.

Block beads crosslinked with antibodies

- *Run this step in parallel with the pre-clearing of lysate.*
- Add 500 µL Roti-Block (Carl Roth) per tube with the antibody-crosslinked beads.

- Incubate 30 min at 4 °C with rotation.

Pre-clear lysate

- Add 25 µL beads per tube (Pierce Magnetic Protein A/G beads, Thermo Fisher Scientific)
- Wash beads 3 times with 500 µL conjugation buffer.
- Add 500-750 µL cell lysate to each tube.
- Incubate 30 min at 4 °C with rotation.

Co-IP

- Add pre-cleared lysate to blocked beads.
- Incubate overnight at 4 °C with rotation.
- Collect non-bound fraction.
- Wash with conjugation buffer 2 times. Collect each wash.
- Elute by adding 100 µL elution buffer (Pierce IgG Elution buffer pH 2, Thermo fisher Scientific)
- Vortex and incubate for 10 min at room temperature with rotation.
- Collect eluate.
- Add 15 µL of 1 M sodium phosphate buffer (pH 8)
- Add Protease inhibitor cocktail (Halt Protease Inhibitor Cocktail 100x, Thermo Fisher Scientific).